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WITH SOUTHEAST EUROPE

Scientific results of the SEE-ERA.NET Pilot Joint Call

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Edited by **Jana Machačová, Katarina Rohsmann**

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Centre for Social Innovation (ZSI)
Linke Wienzeile 246, 1150 Vienna, Austria
Tel: +43 1 4950442 48; Fax: +43 1 4950442 40
machacova@zsi.at; rohsmann@zsi.at

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Foreword by the Commissioner Janez Potočnik

Since I took up office as European Commissioner for Science and Research I have made it one of my missions to bring all the countries of the Western Balkans region into the European Research Area (ERA) with full participation in the Framework Programme. The European Commission and the former Austrian Presidency of the EU jointly launched a 'steering platform' to strengthen research cooperation between the Western Balkan countries and the EU within the framework of ERA. In this context close cooperation with SEE-ERA-NET was established from the outset. The platform intensifies work already underway towards increased networking of national programmes and promoting cooperation between researchers from individual countries within the EU with researchers from countries in the South East of Europe.

The Research strategy for the Western Balkan countries, launched in Vienna in June 2006 together with the then Austrian Presidency, is progressing. It is far beyond my expectations that today all Western Balkan countries are already associated to FP7 and are therefore all fully part of the European research family.

Networks like SEE-ERA-NET have been at the forefront in laying the foundations and contributing actively to the achievements made. It is with great pleasure that I observe that not only the Framework Programme but also the coordination of national research programmes puts special emphasis on this region. The important point is that the funding of joint activities such as this Pilot Joint Call has been made possible by national contributions from all the participating countries. This engagement facilitates the sustainability of our collaboration in South East Europe. Through the first SEE-ERA-NET Pilot Joint Call, more than 160 research teams from all 14 participating countries[1] are now participating in 32 selected projects, funded to carry out trans-national research and networking. Hundreds of proposals were submitted, involving more than thousand research teams, including over 700 teams from the Western Balkan countries. This clearly shows the need but also the potential for more scientific cooperation with the Western Balkan countries.

Research and research cooperation brings benefits in its own right. At the same time, it is an important learning experience in a wider sense. Research cooperation breaks down frontiers, both physical and cultural, because researchers from all backgrounds, from all nationalities, understand and share the same hunger for knowledge and the same desire to improve society through science.

Janez Potočnik
European Commissioner for Science and Research
Brussels, May 2009

[1] Austria, Albania, Bulgaria, Bosnia and Herzegovina, Croatia, France, Germany, Greece, Hungary, the Former Yugoslav Republic of Macedonia, Montenegro, Romania, Serbia, and Slovenia

SEE-ERA.NET Pilot Joint Call, an experiment proved a success story

By this booklet we intend to highlight the scientific impact of the projects carried out in the frame of the SEE-ERA.NET Pilot Joint Call.

The SEE-ERA.NET has started as a regional ERA-NET, implemented by the national key stakeholders in fourteen European countries, including EU member states and all Western Balkan countries (WBC). The SEE-ERA.NET has created a strong commitment among the participating countries towards supporting multilateral research cooperation through a Pilot Joint Call (PJC) which was launched in 2006. All the 14 countries from the SEE-ERA.NET project participated with national contributions in the funding of the call and around 160 research teams from all participating countries in 31 selected projects have received funding for cross-national research and networking.

In the frame of this Pilot Joint Call, there have been submitted 321 eligible proposals involving 1437 research teams. It is to note that among them 739 teams have come from the Western Balkan countries. This clearly proves both the need for cooperation and the scientific potential of those countries to successfully participate in multilateral and international science and technology (S&T) cooperation.

It is therefore clear that there is a growing demand from the researchers in the Southeast Europe (SEE) and in particular the Western Balkan countries for regional multilateral and international S&T cooperation. The regional research communities are interested in the researchers' mobility initiatives, the establishment of scientific networks, the participation in multilateral international projects, and the access to S&T EU infrastructure. It could be said that the Pilot Joint Call has, to a certain extent, responded to their aspirations and acted as a catalyst to initiate and encourage the coordination and cooperation of researchers or research teams from different countries, both from WBC and EU member states.

It is also to note that the SEE-ERA.NET partner countries ministries (or/and funding bodies) have made clear their determination to support the Pilot Joint Call and have also cooperated in an absolutely efficient way in order to overcome barriers of several types met during the implementation of the projects. The oversubscription to this joint call has proved the need for regional initiatives of this kind, namely regional joint calls with increased national funding from the partner countries in order to correspond to the expectations of the research communities.

The next step of this effort in the region is the SEE-ERA.NET PLUS, started on April 1st, 2009. In the light of the success and the needs identified during the implementation of the previous SEE.ERA.NET, a consortium of 15 countries has been established to implement a joint regional call with the additional financial support of the European Commission.

Having in mind all the above, the aim in the near future is to establish in the SEE region a sustainable S&T programme with multiple activities addressing the real scientific and socio-economic needs of the region and it will be run and financially supported by the participating countries.

Professor Philippos G. Tsalidis
Secretary General for Research and Technology
Ministry of Development, Greece
Athens, April 2009

Chapter 1: SEE-ERA.NET – A New Approach to Structure RTD Cooperation between EU Member States, Candidate Countries as well as Western Balkan Countries

by **Marion Haberfellner,** SEE-ERA.NET Co-ordinator, Centre for Social Innovation, Austria

The Southeast European Era-Net SEE-ERA.NET is a networking project aimed at integrating EU Member States and Southeast European countries in the European Research Area (ERA) by linking research activities within existing national, bilateral and regional research and technological development (RTD) programmes.

SEE-ERA.NET is financed by the European Commission (EC) and managed by a consortium of 17 institutions from 14 European countries.

The objectives of SEE-ERA.NET are to:

- enhance research cooperation in Europe by fostering integration of Southeast Europe into the growing European Research Area;
- add value to existing bilateral S&T agreements through multilateral coordination;
- improve interregional research cooperation following the principles of the Stabilisation and Association Process in Southeast Europe; and to
- contribute to the EU-Balkan countries Action Plan in Science & Technology adopted at the Thessaloniki Ministerial Conference in 2003.

1.1 Project objective and state-of-the-art

SEE-ERA.NET aims at structuring and expanding the ERA to the Western Balkan countries by coordinating and supporting RTD activities conducted at bilateral level. Mainly, but not exclusively, bilateral intergovernmental programmes between the consortium partners are addressed. Through a well-targeted process of cooperation and coordination, added value is achieved for the research communities of all 14 participating countries. An important fringe benefit of SEE-ERA.NET is also the enhancement of the interregional RTD cooperation of the involved new Member States and candidate countries and to create a basis for a better involvement of these countries in RTD endeavours at European level.

The main objective of SEE-ERA.NET is to enhance RTD cooperation between already established and new EU Member States, candidate countries as well as Western Balkan countries. SEE-ERA.NET was designed to support a much better exploitation of existing bilateral RTD cooperation under a flexible network that enables

- a systematic exchange of information and best practice on bilateral RTD programmes and activities supported hereunder on the project level;
- a sound understanding of the state-of-art of the systems of research in the Western Balkan countries;

- a comprehensive needs analysis from the viewpoint of collaborating researchers – especially – from the Western Balkan countries as regards international RTD cooperation;
- awareness raising on important, yet sufficiently tackled, problems and opportunities, the identification of joint strategic activities and the development of practical policy recommendations;
- the implementation of joint instruments and initiatives under a variable geometry; and
- the conduct of multinational research by implementing a regional RTD programme with a pilot multilateral call for proposals.

SEE-ERA.NET contributed to the fulfilment of the EU-Balkan countries Action Plan adopted at the Ministerial Conference in Thessaloniki in 2003.[2]

As already stated, SEE-ERA.NET emphasises RTD cooperation between the European Union (EU), Southeast European candidate countries and Southeast European third countries. Starting point for our ERA.NET activity were bilateral RTD programmes. A special emphasis was put on bilateral intergovernmental RTD programmes. In fact, almost all Member States exercise different bilateral intergovernmental RTD programmes with other European and third countries encompassing a broad range of rather different programmatic approaches and designs with distinctive regional foci.

According to the INCOPOL study[3] commissioned by DG Research, however, little programmatic and operational coordination has been exercised between the EU Member States in performing their different bilateral RTD programmes with Central and East-European countries. It is not surprising that double-track actions, repetition of failures due to non-existing information and knowledge transfers and especially the lack of critical masses of the single isolated activities are to be observed. The scarce financing available under the bilateral activities, their unstructured relation (or non-relation) to each other as well as the target region's vast needs for assistance call for a coordinated effort!

It is important to stress that SEE-ERA.NET never aimed to substitute or hollow-out bilateral RTD initiatives between different European countries. It rather aims to complement existing isolated efforts by establishing synergies and adding critical mass and momentum to the yet scattered activities. By increasing RTD cooperation in Central and Southeast Europe, prevailing scientific and technological weaknesses of the participating countries will be gradually counterbalanced and existing strengths upgraded. Thus SEE-ERA.NET has actively contributed to the development of an enhanced European Research Area.

[2] http://cordis.europa.eu/greece/press45.htm
[3] http://ec.europa.eu/research/iscp/regions/oth-doc1.pdf

1.2 Fostering international RTD cooperation in the emerging European Research Area

From a genuine European perspective, the enhancement of activities targeting the RTD cooperation with Central and Southeast European countries is of utmost strategic importance for the future success of a truly European Research Area, because

- the participation of the new Member States and candidate countries in FP6 and FP5 was far from being satisfactory (see many statements from the "Conference on Acceding and Candidate Countries and the 6th Framework Programme", Bucharest, February 12–13, 2004 or for instance from the "Conference of Integration of Candidate Countries into the ERA", Brussels, March 8–9, 2001) and the potentials were by no means fully exploited yet. There is a rationale that regional RTD cooperation instruments are – at least for the time being – more appropriate to address the existing potentials of the new Member States and candidate countries then the "new instruments" of FP6. SEE-ERA.NET supported and continues to support the establishment of regional RTD markets by opening-up bilateral towards multilateral activities. SEE-ERA.NET has clustered research projects funded under bilateral RTD programmes, and has provided merging and training mechanisms for a future increased participation under the European Framework Programmes for RTD.
- In addition, the stepwise integration of the Western Balkan countries is at the political agenda for the next years after a lost decade of regional turmoil. Science and technology (S&T) cooperation always has been a pioneer in this respect. It has certainly contributed to accelerating the Community's effort for the development and stabilisation of the Balkan region.
- By integrating Serbia and Albania in 2006, SEE-ERA.NET became a project to involve all high level political decision makers in EU and Southeast European ministries and funding agencies that have an explicit interest in multilateral European and regional RTD collaboration on an equal footing. This created possibilities for RTD cooperation which simply have not existed to date and reinforce attempts on an EU level to promote peace and stability in the region.
- The benefits of SEE-ERA.NET can therefore be seen not only to further the concerns of the research community but also to contribute to social and political innovation (democracy building).

Based upon this fundamental sequence of the SEE-ERA.NET objectives, SEE-ERA.NET supports:

- Overcoming the fragmentation of – yet isolated – bilateral intergovernmental RTD programmes through knowledge exchange and strategic programmatic cooperation;
- Integration of alternative isolated bilateral RTD initiatives into multilateral, jointly agreed, activities with high synergetic impact;
- Contribution to standardised RTD governance processes in the target countries at programme level through exchange of know-how and training of responsible policy-delivery systems;
- Breaking-up the isolation of research communities in Southeast Europe through regional approaches (as requested for instance from the Stability Pact for South Eastern

Europe or DG Relex),
- Identification of RTD priorities (both instrumental and [inter]disciplinary) and improved response to these priorities through coordinated activities;
- Creation of a critical mass through pooling budgets and research networks;
- Improvement of the unsatisfactory low participation of research communities from the new Member States, candidate and Western Balkan countries in FP6 (and in the forthcoming European Framework Programme for RTD) by clustering, brokerage and training measures; and
- Contribution to the fulfilment of the EU-Balkan countries Action Plan adopted at the Ministerial Conference in Thessaloniki (June 26 – 27, 2003).

1.3 Achievements of the Project

One of the highlights of the project SEE-ERA.NET was certainly the single Pilot Joint Call (PJC) which was launched in 2006. This booklet presents the results of the funded projects and shows their scientific impact.

However, it is important to stress the impact of the projects is not only in terms of scientific output and achievement: SEE-ERA.NET provided new perspectives for scientists and already existing networks in Southeast Europe by opening up a European-wide perspective for scientific cooperation and funding. The need for a European perspective and collaboration in science and technology (S&T) cooperation was clearly shown by the huge interest in the call by scientists from the Western Balkan Countries and the resulting oversubscription of the call. More detailed information on this will follow in the forthcoming chapters.

Furthermore the project contributed to an awareness raising process as regards the needs of the Western Balkan region concerning science and technology. This led to a number of policy documents like the publication of the White Paper. Based on this and summarising the recommendations of the White Paper, a Joint Action Plan was created. According to the Joint Action Plan which is constantly updated within the frame of the project, priority should be giving to the following activities:

1. Strengthening strategic reform processes with regard to the national research and innovation systems in the Western Balkan countries and contributing to their sustainability through institution and capacity building.

2. Assuring a high level of participation of the Western Balkan countries in the 7th EU Framework Programme on Research and Technological Development and Demonstration activities.

3. Promoting the intensive use of existing cooperation instruments, or, where necessary, introducing new, coherent and complementary or joint cooperation instruments for those national and multinational institutions which exceed the scope of the 7th Framework Programme, allowing the full European integration of the Western Balkan science community.

Addressing these needs and building on the experiences of the "Pilot Joint Call" for proposals for S&T networks and projects, as launched by the SEE-ERA.NET consortium in November 2006, a "Regional Programme for Cooperation with South-East Europe" was proposed.

Consideration should also be given to complementary activities which aim at the coordination of measures fostering S&T infrastructure development, institution and capacity building human potential development.

The Joint Action Plan describes the general concept underlying these activities, with particular emphasis on those measures that will be further developed and implemented by the partners of the SEE-ERA.NET consortium through the expansion of previous and ongoing coordination activities within the successor project SEE-ERA.NET PLUS.

The next step for further cooperation in the region is the follow-up project SEE-ERA.NET PLUS, which started on April 1st, 2009. Following the identified needs and recommendations of the previous SEE-ERA.NET, a consortium of 15 countries has been established in order to implement a single joint call within the frame of FP7.

SEE-ERA.NET PLUS shall support the development and administration of the first major joint call for Joint European Research Projects (JERPs) in SEE, and therefore the implementation of the first pillar only of the Regional Programme for Cooperation with South-East Europe (ReP-SEE). The remaining three other pillars (Accompanying Measures in order to facilitate joint research, a Young Scientist Programme, an Innovation Programme with the aim of linking SMEs, technology/innovation centres and other innovation structures in the Western Balkan countries to existing thematic innovation networks/clusters in the EU partner countries) of ReP-SEE will be dealt with separately by the SEE-ERA.NET PLUS consortium.

1.4 USP – Unique Selling Points for SEE-ERA.NET

- SEE-ERA.NET was/is the main European Union networking project for the integration of the Southeast European countries into the European Research Area;
- SEE-ERA.NET was/is a network of 14 ministries and 3 agencies in 14 European countries that includes all Western Balkan countries and works directly on the level of policy makers;
- the SEE-ERA.NET partner countries provided funding for multinational research teams through Joint Calls for Proposals;
- SEE-ERA.NET put forward a joint reviewing system for research project proposals where the assessment procedures are transparent, fair and dedicated to scientific excellence;

- SEE-ERA.NET provided and provides new perspectives for scientists and already existing networks in Southeast Europe by opening up a European-wide perspective for scientific cooperation and funding;
- SEE-ERA.NET was and is linked to the "EU-Western Balkan Countries Steering Platform" by providing strategic and operational input;
- SEE-ERA.NET focused and focuses on an important target region of the 6th and 7th Framework Programme of the European Community;
- SEE-ERA.NET is a long term project of 5 years duration and will develop scenarios for sustainable cooperation beyond 2009.

Chapter 2: SEE-ERA-NET Pilot Joint Call for Research and Thematic Network Projects

by **Nikos Sidiropoulos,** WP5 leader, Hellenic Ministry of Development, General Secretariat for Research and Technology, International S&T Cooperation Directorate, Greece; and
by **Manfred Spiesberger,** external monitoring expert

2.1 General information

In order to establish a first pilot case of a Joint Call, the SEE-ERA.NET Steering Board had decided to create a new multilateral funding programme within the context of the SEE-ERA.NET network.

All 14 countries in the SEE-ERA-NET project had declared their participation with national financial contributions to this first pilot joint call: Albania, Austria, Bosnia and Herzegovina, Bulgaria, Croatia, France, Germany Greece, Hungary, the Former Yugoslav Republic of Macedonia, Montenegro, Romania, Slovenia, Serbia.

2.2 Topics, budget and instruments

The design of the call was based on the priorities and needs of the Western Balkan countries (WBC) with a view to their better integration into the European Research Area. Topics were chosen by taking into account thematic priorities of the EU's Framework Programme for Research and Development.

Due to the pilot character of the call, the topics of the call had been limited to three areas, which covered:

- Food, Agriculture and Biotechnology – Sustainable production and management of biological resources from land, forest and aquatic environments;
- Information and Communication Technologies – Applications research; and
- Environment – Environmental technologies.

The budget of the Pilot Joint Call (PJC) was rather modest and reached around 800,000 Euros. It is although very respectable that all countries involved in the consortium have indeed committed funds, including countries lagging economically behind. And it should also be taken into account that even modest financial means have higher impact in the Western Balkan countries due to differences in price levels.

In the frame of the PJC, applications for the following three instruments were possible:

- Research projects with the aim of supporting small scale research, feasibility studies, researcher exchange with a project duration of up to nine months and involving at

least three teams from three different countries among them at least one from the Western Balkan countries.
- Network projects with the aim of coordinating researchers and stimulating their cooperation via workshops or exchanges with project's duration of up to nine months and involving at least five teams from at least three different countries among them at least two from the WBC.
- Summer schools involving at least three different countries among them at least one from WBC.

2.3 Call implementation

The implementation of the Pilot Joint Call was divided into the following phases:
- Launch of the call, PR activities, submission of applications (30/11/2006–31/3/2007);
- Evaluation of applications (1/4/2007–15/6/2007);
- Contracting of selected projects (1/8/2007–30/9/2007); and
- Monitoring of running projects (1/9/2007–30/9/2008).

The PJC was implemented by the General Secretariat for Research and Technology (GSRT) of the Greek Ministry of Development with the support of a Central Administrative Body (CAB). For this function of CAB, the International Association for promotion of cooperation with Scientists of the New Independent States of the Former Soviet Union (INTAS) had been contracted. INTAS has a long standing experience in implementing calls for international R&D cooperation.

The PJC was supported in each consortium member state by a National Contact Point (NCP) for the PJC. The NCPs promoted the call and provided support to applicants to the PJC. Technical Support for the PJC was provided by the CAB. A helpline was providing support in case of technical problems with the electronic submission system.

2.4 PR activities for Pilot Joint Call

Public Relations activities for promoting the Pilot Joint Call embraced a wide variety of measures, such as e-mail information to scientists, distribution of printed material (project leaflets, etc.), placing of call information on websites, and thematic conferences.

Three thematic conferences had been held in February 2007, in Serbia (Belgrade), Montenegro (Przno) and in the Former Yugoslav Republic of Macedonia (Ohrid). Objectives of the conferences were to support project brokerage and clustering of researchers according to their specific scientific interests. More than 240 participants from 14 different countries attended the conferences. As a result of the conferences 73 project ideas had been developed in thematically specific workshops.

The importance of the networking aspect of thematic conferences should not be underestimated. It was certainly very important in the context of the WBC to enable meetings of scientists from different WBC and with scientists from EU member states. WBC scientists

can herewith rebuild WBC scientific networks and be connected and re-connected to a certain extent to scientific networks in EU countries, which were disrupted because of conflicts in the WBC. Effects of thematic conferences are well reflected in participation rates in the PJC: all countries, where conferences took place have a very high participation of their teams in applications to the PJC.

2.5 Pilot Joint Call results

A total of 345 applications were submitted to the SEE-ERA.NET Pilot Joint Call. Eligibility checks determined that 24 applications were ineligible, because they did not meet the published rules for participation. As a result, 321 applications involving 1437 teams were declared eligible and submitted for evaluation. Out of this group, 1420 teams were from SEE ERA-NET partner countries (681 were from European Union and 739 from Western Balkan Countries). 17 teams from European countries, not partners of the SEE ERA-NET, were also involved in applications. Each application was evaluated against the applicable criteria independently by at least two independent experts. Reviewers were primarily selected on the basis of the set of keywords provided by applicants to describe the scientific field of their application. 671 evaluations were received for the 321 eligible applications.

2.6 Response to instruments, evaluation and topics of the call

Project applications were usually evaluated remotely by two independent experts. In cases where the two experts differed strongly in their opinion on the project and scores showed high discrepancies, a third expert was asked to provide an evaluation. When looking at response per instrument, it can be observed that the biggest interest was by far in research projects (see Figure 1).

In terms of response to scientific topics, it can be observed that the most popular topics were environment, sustainable production and ICT.

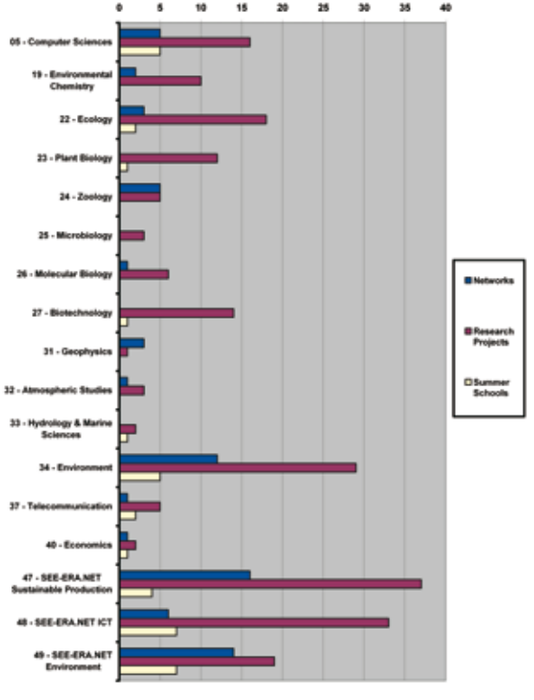

Figure 1: Instruments and topics of SEE-ERA.NET Pilot Joint Call (Source: INTAS, Statistical Analysis of SEE-ERA.NET Pilot Joint Call, 2007)

2.7 Country participation structure

The interest among the scientific community per country in this Pilot Joint Call can be well measured by checking the origin of the eligible teams participating in project applications.

In absolute figures, most teams have participated from the following countries:

- Serbia – 227;
- Greece – 192; and
- Croatia – 142.

But if the number of eligible teams per country is compared with the number of inhabitants per participating SEE-ERA.NET country[4], it is obvious that some smaller countries have in relation to their population size a higher participation: Montenegro, the Former Yugoslav Republic of Macedonia and Slovenia.[5] It should be noted that all WBC show a rather good participation, which is due to the fact that this PJC has been targeted specifically at their scientific communities and that PJC rules required the participation of at least one WBC team per application. It shows also that the WBC scientific communities took advantage of the PJC opportunity and that there is a tremendous interest in these countries to develop scientific cooperation with colleagues from the region and from EU member countries.

It should be noted that those countries, where stimulation measures (thematic conferences) have taken place are all in the top ranks of team participation:

- Former Yugoslav Republic of Macedonia;
- Montenegro; and
- Serbia.

On the side of the EU member states, Slovenia, Greece and Bulgaria have had the highest participation, while the bigger member states, such as France and Germany showed much less participation. Obviously Slovenia, Greece and Bulgaria are closer to the region, have stronger ties with WBC countries and scientists can rely on already established scientific networks. And probably low funding levels in the PJC have not been very attractive for high income countries, such as France and Germany.

Regarding patterns of institutions participating in the projects, for the Western Balkan countries it can be observed that it is concentrated on some important institutions, such as the University of Belgrade and Novi Sad in Serbia or the University of Zagreb in Croatia (e.g. around 50 % of teams from Croatia were from University of Zagreb).

[4] Data on inhabitants per country as accessed on October 20, 2007 at: https://www.cia.gov/library/publications/the-world-factbook/index.html

[5] There is of course no proportional relation between inhabitants of a country and its scientific potential or scientists; still the participation of smaller countries is remarkable.

Table 1: Origin of teams in SEE-ERA.NET applications (proposals) in comparison to inhabitants (Source: Table compiled based on data of SEE-ERA.NET Pilot Joint Call, 2007)

Country	No teams in eligible proposals	% of SEE eligible teams	SEE inhabitants in Mio	% of SEE inhabitants	Ratio: teams / Mio inhabitants
Albania	61	4,30 %	3,60	1,55 %	16,94
Austria	59	4,15 %	8,20	3,54 %	7,20
Bosnia & Herzegovina	83	5,85 %	4,55	1,96 %	18,24
Bulgaria	125	8,80 %	7,32	3,16 %	17,08
Croatia	142	10,00 %	4,49	1,94 %	31,63
France	44	3,10 %	63,71	27,49 %	0,69
FYR of Macedonia	118	8,31 %	2,05	0,88 %	57,56
Germany	73	5,14 %	82,04	35,40 %	0,89
Greece	192	13,52 %	10,71	4,62 %	17,93
Hungary	62	4,37 %	9,96	4,30 %	6,22
Montenegro	50	3,52 %	0,68	0,29 %	73,53
Romania	83	5,85 %	22,28	9,61 %	3,73
Serbia	227	15,99 %	10,15	4,38 %	22,36
Slovenia	101	7,11 %	2,01	0,87 %	50,25
SUM	1420	100,00 %	231,75	100,00 %	

2.8 Projects selected and the success rate

In the frame of the PJC, 31 projects have been selected for funding out of a total number of eligible applications of 321, which amounts to a success rate of 9,7 %. Mostly research projects have been funded in the PJC: 19 research projects, 7 networking projects and 5 summer schools.

Overall the success rate of the PJC was rather low, being for the different instruments either slightly below or slightly above 10 %. This demonstrates on one hand a huge interest in such a multinational funding instrument targeting the WBC, but on the other hand

it leads to frustration among applicants as a lot of well evaluated proposals cannot be funded.

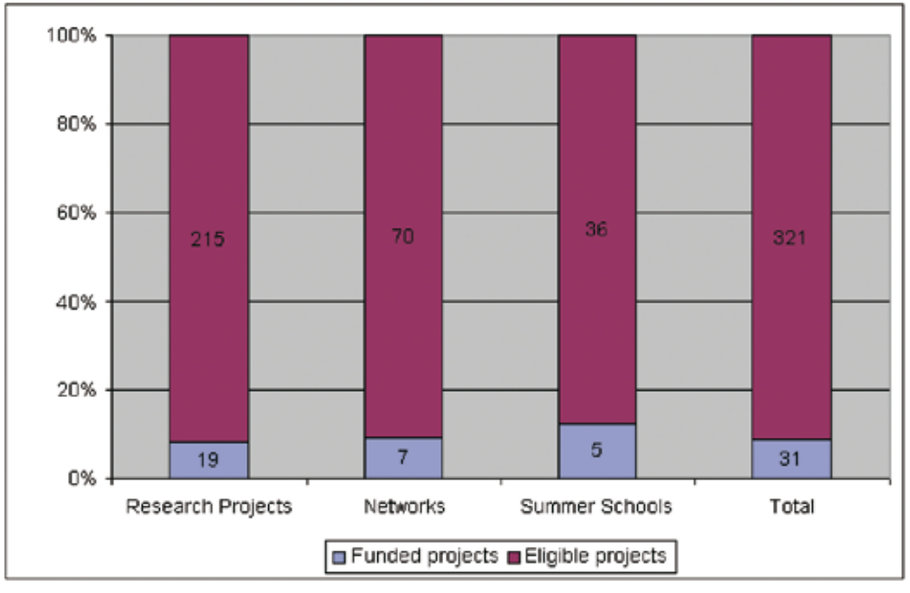

Figure 2: Success rate per instrument (Source: Table compiled based on data of SEE-ERA.NET Pilot Joint Call, 2007)

What concerns the success rate per country, Serbia and Greece had here with 18 teams in funded proposals in absolute figures most success. Smaller countries, such as the Former Yugoslav Republic of Macedonia or Slovenia, were in relation to their size also very successful. When checking teams in funded projects against teams in eligible projects, it turned out that France and Germany were quite successful. Serbia has in this comparison paid the price of the huge interest in the call; it had most eligible teams, but could fund only a limited number, as its budget in the virtual common pot scheme got exhausted.

Remarkably, out of 26 research and network projects only three (or slightly over 10 %) are coordinated by colleagues from the Western Balkan countries (one project coordinated by a Croatian team and two projects coordinated by Serbian teams). This is very limited; it is obviously due to a lack of experience in coordinating multinational projects on the side of the WBC.

Table 2: Success rates per country, in comparison to other countries and in comparison to eligible teams per country (Source: Table compiled based on data of SEE-ERA.NET Pilot Joint Call, 2007)

Country	No teams in funded proposals	Team success rate funded	No teams in eligible proposals	Team success rate eligible
Albania	8	5 %	61	13 %
Austria	8	5 %	59	14 %
Bosnia & Herzegovina	8	5 %	83	10 %
Bulgaria	13	8 %	125	10 %
Croatia	14	9 %	142	10 %
France	8	5 %	44	18 %
FYR of Macedonia	15	10 %	118	13 %
Germany	11	7 %	73	15 %
Greece	18	12 %	192	9 %
Hungary	9	6 %	62	15 %
Montenegro	6	4 %	50	12 %
Romania	6	4 %	83	7 %
Serbia	18	12 %	227	8 %
Slovenia	13	8 %	101	13 %
SUM	155		1420	11 %

Following figures 3 and 4 provide a comprehensive overview about selected research project (RP) and network project (NP) and summer schools within the SEE-ERA.NET Pilot Joint Call.

Table 3: Selected research projects (RP) and network projects (NP)

ID	Type	Title	Institution Co-ordinator	Country Co-ordinator
10724	NP	Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe. (Network PHYTO-PLASMA-EPIDEMIO)	Institut National de la Recherche Agronomique / Department of Plant Health and Environment (SPE) / UMR-1090 Génomique Diversité et Pouvoir Pathogène	France
9902	RP	Development of a non-toxic, ecologically compatible, natural-resource based insecticide from diatomaceous earth deposits of South Eastern Europe to control stored-product insect pests	Agricultural University of Athens / Department of Plant Science / Laboratory of Agricultural Zoology and Entomology	Greece
10105	RP	Understanding Ionic Liquids as Novel Solvent in Green Chemistry	Faculty of Science – University of Zagreb / Department of Chemistry / Laboratory of Analytical Chemistry	Croatia
10237	RP	Phenotyping and genotyping of cereal genetic resources to improve tolerance to abiotic and biotic stresses	Agricultural Research Institute / Department of Cereal Resistance Breeding	Hungary
10491	NP	Recreation of the BALKAN NET, a network of conservation bodies in countries sharing continuous large carnivore populations	ARCTUROS NGO / Scientific team	Greece
10503	RP	Building Language Resources and Translation Models for Machine Translation focused on South Slavic and Balkan Languages	Research Institute for Artificial Intelligence / Human Language Technology	Romania
10845	RP	Continuous water quality monitoring in surface waters at Montenegro and Serbia	European Economic Interest Grouping / Environmental Technologies	Greece
10369	RP	Development of Molecular Tools for FIG genetic resources characterization and preservation in West Balkan Countries	University of Primorska / Science and Research Centre of Koper (UP SRC) / Institute for Mediterranean Agriculture and Olive Growing	Slovenia
10506	RP	Definition of research needs on identification, prediction and surveillance of emerging and re-emerging zoonoses in West Balkan area.	Faculty of Medicine / University of Pristina / Kosovska Mitrovica / Preventive Medicine	Serbia
10326	RP	Distribution and initial molecular characterization of enterohaemorrhagic E. coli (EHEC) specific virulence factors of E. coli strains isolated from domestic animals and man: an assessment of zoonotic significance in the West Balkan	Veterinary Medical Research Institute / Enteric Bacteriology and Foodborne Zoonosis	Hungary
10859	RP	Immobilized Yeast Cells in Hydrogel Carriers for Bioproduction of Alcohols	Institute of Polymers / Polymer Chemistry / Polymerization Processes	Bulgaria
10004	NP	A Collaborative South East Europe Seismic Network (CoSEESNet): Towards Early Warning System and Real Time Seismic Monitoring in South East Europe	National Observatory of Athens / Institute of Geodynamics	Greece
10096	NP	Regional forest management support needs – comparative user requirements analysis with regional stakeholder groups in Balkan countries and middle and eastern European countries Re-FOR-MAN	Dresden University of Technology / Institute for Soil Science and Site Ecology / Forest- / Geo- and Hydrosciences	Germany
10023	RP	Use of Prescribed burning in Middle-East and South East Europe	University of West Hungary / Faculty of Forestry / Institute of Silviculture and Forest Protection / Department of Forest Protection	Hungary
10035	NP	The importance of chlamydia infections in birds for animal and human health in Southeastern Europe	University of Ljubljana, Veterinary faculty / Institute for Health Care of Poultry / Laboratory for diagnostics of infectious poultry diseases	Slovenia
9909	RP	Interactive Visual Analysis of Bio-signals	Jozef Stefan Institute / Department of Communication Systems	Slovenia
9608	RP	Landscape and regional context of insect agrobiodiversity in Southeastern Europe: a pilot survey of selected hemipteran pests, their parasitoids and predators, and bee pollinator diversity	Faculty of Biology / Department of Zoology / Laboratory for Entomology	Serbia
9633	RP	Phenotypic and genotypic characterization of Pasteurella multocida and Mannheimia haemolytica strains isolated from sheep and goats originated from Greece, Serbia, Bosnia and Herzegovina	Aristotle University of Thessaloniki / Faculty of Veterinary Medicine / Laboratory of Microbiology and Infectious Diseases	Greece
10648	NP	Multi-functional management of coppice forests. Contributions to rural development, maintenance of biodiversity, and climate change mitigation and adaptation in natural resource management	University of Natural Resources and Applied Life Sciences / Vienna / Department of Forest and Soil Science / Institute of Silviculture	Austria
10522	NP	Use of lactic acid bacteria in production ofhypoallergenic dairy products, and for the generation of mild antimicrobials	Institut National de La Recherche Agronomique / Unité Biopolymères Interactions Assemblages (BIA) / Fonctions et Interactions des Protéines Laitières (FIPL)	France
10193	RP	Exploring the molecular biodiversity of medicinal and aromatic plants	Mediterranean Agronomic Institute of Chania / Natural Products and Biotechnology	Greece
10627	RP	Population genetics of a highly invasive insect pest	Institut National de la Recherche Agronomique (INRA) – Centre d'Orléans / Département EFPA – Ecologie des forêts, prairies et milieux aquatiques / Unité de Zoologie Forestière	France
10374	RP	Development of Strategy and Methods for Monitoring of Electromagnetic Pollution in the Environment of the Western Balkans	Space Research Institute / Department of Aerospace management systems	Bulgaria
10561	RP	Use of SNPs and SSRs for genetic diversity assessment within cultivated olive germplast from Western Balkan Countries	Mediterranean Agronomic Institute at Chania / Horticultural Genetics & Biotechnology	Greece
10452	RP	An integrated strategy to assess and evaluate water quality of Lake Shkodra	University of Heidelberg / Department of Zoology / Sediment toxicity and integrated monitoring group	Germany
9527	RP	Major allergens in apple and olive fruits	Aristotle University of Thessaloniki / Biology / Botany	Greece

Table 4: Selected summer schools

ID	Institution Co-ordinator	Country Co-ordinator	Title
9945	University of Pannonia / Department of Limnology	Hungary	Toxic cyanobacteria in drinking water sources – problem and sanitation
10680	Groupe des Ecoles de Télécommunications / Institut National des Télécommunications / Foreign Languages and Social Sciences / GET eLearning	France	European Association for Technology-Enhanced Learning (EATEL) Summer School 2008
9898	Central Laboratory of General Ecology / Functional Ecology / Functioning of Terrestrial Ecosystems Research Group	Bulgaria	Summer school on "Biomonitoring methods for air quality in natural and man-made environment" (Biometh-Air)
10485	Technical University of Sofia / Department of Hydroaerodynamics	Bulgaria	Summer School on Build Environment
10207	Hungarian Meteorological Service / Climatological Department	Hungary	Summer school on Preparation of Climate Atlas, 10–14 September 2007

Chapter 3: SEE-ERA-NET Pilot Joint Call Accompanying Measures

by **Nikos Sidiropoulos,** WP5 leader, Hellenic Ministry of Development, General Secretariat for Research and Technology, International S&T Cooperation Directorate, Greece

3.1 General information

According to the focus of the overall pilot activity, the Pilot Joint Call (PJC) had been accompanied by a number of special measures for the strengthening of the capacities of the WBC as well as the contacts and networks between institutions from the participating SEE-ERA.NET partner countries.

These activities had been defined in a "top down" process by all SEE-ERA.NET partner countries without reference to applications from the scientific community:

- Management training for science administrators from the Western Balkan countries, implemented by International Bureau of the BMBF/BMBF, Germany;
- International Conference on R&D Evaluation and Benchmarking, organised by International Bureau of the BMBF/BMBF, Germany;
- International Conference on the Integration of SMEs into the SEE-ERA.NET "Joint Calls", organised by General Secretariat for Research and Technology, Hellenic Ministry of Development, Greece; and
- SEE-ERA.NET award for donations within the framework of RTDI collaboration with the Western Balkan countries, organised by Ministry of Higher Education, Science and Technology, Slovenia.

3.2 Management training for WBC science administrators

Bonn, Germany
December 3–7, 2007

The participants of the management training had been introduced into the national S&T system and S&T policy of the Federal Republic of Germany including relevant aspects of European and international cooperation. They received an overview on the responsibilities of the institutions and deepen their knowledge on the instruments and the administrative procedures for the implementation of the respective national and bilateral S&T funding programmes and the promotion of the participation in the European Framework Programme. The participants of the training had the opportunity to get an insight into the practical work in important areas of science administration and entered into an exchange of experiences.

3.3 Southeast European Conference on Innovation

Thessaloniki, Greece
November 29 – 30, 2007

3.3.1 Introduction

The "Southeast European Conference on Innovation" was organized under the auspices of the Hellenic Ministry of Development, General Secretariat for Research & Technology and was hosted by the Centre for Research and Technology Hellas (CERTH) in collaboration with EUROCONSULTANTS S.A.

The objective of the conference was to prepare the field for the participation of SMEs and other innovation related structures into the future planned SEE-ERA.NET innovation activities, aiming at:

- Raising awareness on the potential of joint innovation activities;
- Defining the scope of joint innovation activities, building on common interests of the innovation stakeholders by addressing their specific needs;
- Developing specific innovation related actions and tools; and
- Attracting the interest of possible international, national and regional funding bodies to participate in the joint innovation activities.

Existing innovation stakeholders from the SEE-ERA.NET countries, representatives of RTD and innovation related structures from countries outside SEE-ERA.NET and other European networks as well as other innovation related European and international funding had been invited to participate and discuss their innovation related policies and activities, their problems and needs, their expectations and proposals for future innovation related collaboration actions within SEE-ERA.NET.

The conference topics were focused on:

- Innovation policies and programmes;
- Activities and needs on RTDI support focusing on SMEs;
- Collaboration and networking activities and needs for supporting innovation; and
- Corresponding "good practice" examples.

The conference included the following sessions:

- Introductory plenary session (29/11/2007 – morning)
- Two working group sessions on the following specific topics, which were implemented in parallel (29/11/2007 – afternoon):
  - Support structures, services and financial support for innovation; and
  - Collaboration, networking and mobility for innovation.
- Reporting/discussion in plenary session, summarising the outcomes of each working group session, comparing and discussing the results and conclusions (30/11/2007 – half day)

3.3.2 Overview of introductory presentations

The innovation issues addressed during the conference introductory presentations included:

- Multi-lateral RTDI collaboration in Southeast Europe (SEE);
- Western Balkan Countries (WBC) and Southeast Europe (SEE): Innovation environment and infrastructure, absorption capacity and transnational cooperation needs.

Multi-lateral RTDI collaboration in SEE

The following RTDI collaboration issues were presented:

- SEE-ERA.NET
  - Integration of WBC into ERA;
  - Interesting pre-accession instrument for WBC;
  - Pilot Joint Call for RTDI actions, multi-lateral funding scheme, multinational RTDI teams, central administration system, evaluator's database, external monitoring and evaluation:
    - High demand for regional multilateral joint calls;
    - Facilitated coordination and collaboration of researchers;
    - Willingness to support multilateral programmes and overcome barriers; and
    - Increased national funding from national partners;
  - SEE-ERA.NET White Paper: political decision:
    - Strengthening strategic reform processes in WBC, institution and capacity building; and
    - Introducing new, coherent and complementary or joint cooperation instruments;
  - SEE-ERA.NET: Joint Action Plan.
- Regional Programme for cooperation within SEE
  - Built on SEE-ERA.NET experience;
  - Expressed willingness of partner countries for sustainable multilateral collaboration activities;
  - Widening SEE-ERA.NET activities;
  - Inviting additional stakeholders:
    - Network of funding bodies;
    - National Research Policy Makers; and
    - National Research programme owners;
  - Innovation pillar: multilateral activities in the field of integration of SMEs:
    - Participation of innovative SMEs;
    - Based on priorities and needs of SEE and WBC in particular;
    - Preparatory phase (Absorption capacity for RTDI; Potential innovation related policies and activities; Most appropriate SME innovation support actions and tools; International, national and regional funding bodies and other interested stakeholders outside SEE-ERA.NET); and
    - Next steps (Drafting Innovation Pillar; Building a new consortium; Drafting a proposal and submit in the frame of FP7 INCO Call; Launching SEE Innovation Programme, 2009).

Western Balkan countries (WBC) and Southeast Europe (SEE): Innovation environment and infrastructure, absorption capacity and transnational cooperation needs

The following issues were addressed, regarding innovation environment and infrastructure, absorption capacity and transnational cooperation needs in Southeast Europe, focusing on Western Balkan countries:

- WBC Innovation Environment and Infrastructure focused on:
  - General Environment for Setting Up Innovation Infrastructures;
  - Strategic documents;
  - Specific programmes and instruments;
  - Key government players;
  - Clusters; Technology and Innovation Centres; Technological and Science Parks; Business-Start-Up Centres/Technology Incubators; further related organizations; and
  - Main needs: Good policy framework, concrete action plans and adequate financial resources.
- RDTI absorption capacity identified among the following main SEE innovation stakeholders
  - Research, technological development and innovation centres;
  - Technology and business incubators;
  - Innovation support networks; and
  - High-tech SMEs, consulting companies and services;
- Main innovation activities of SEE innovation stakeholders include
  - Design and implementation of innovation policies and programmes;
  - Technology transfer;
  - Training or mentoring on entrepreneurship and innovation; and
  - Innovation promotion and dissemination;
- Main SEE innovation support and collaboration needs
  - Technology transfer, training and human resources development are the main innovation support needs, particularly in WBC;
  - Financial support needs are mainly towards the creation and development of innovative start-ups;
  - Collaboration and networking for innovation is the main need among SEE innovation stakeholders, for exchanging experience and know-how and innovation promotion;
  - Increased interest among SEE innovation stakeholders for transnational, interregional and science-industry collaboration; and
  - Increased interest among SEE innovation stakeholders for innovation networks, joint innovation activities and mobility.

3.3.3 Discussion and main conclusions

The final discussion and main conclusions were focused on the proposal for the Innovation Pillar of a new transnational collaboration programme in SEE, addressing the following issues and recommendations:

- Identification of needs, exchange of information, proposed joined activities;
- Eligible participants and key success factors;
  - Research or innovation programme managing authorities that fund corresponding national activities;
  - Focus on specific topics; and
  - Make it slim and simple;
- Preparatory activities: studies and tools suggested
  - A clear benchmark approach: do a benchmarking exercise, to enable the regional stakeholders to learn from the experiences of other regions;
  - Top priority is to understand better and study the innovation system: low performance of key innovation indicators, activities are locked in traditional sectors, funding is missing, small innovation systems, the larger the system the larger the possibility of finding appropriate cooperation;
  - Finding complementarities in innovation capabilities and combining skills;
  - Defining sectors from national strategies, then implementing technology and innovation audits or surveys of those specific sectors to identify problems in their innovation processes; and
  - Defining specific gaps either in research or in business, technology and innovation areas for development or problems to be solved – developing a chain where specific processes are supported;
- Preparatory activities: awareness creation and dissemination of information
  - Information campaign – awareness creation; and
  - Development of information web-tools;
- Pilot programmes and accompanying measures: examples of suggested activities
  - Training measures – summer schools or brief seminars to train entrepreneurial skills, starting from the beginning of the programmes, focusing on practical things, raw models or real cases;
  - Teaching entrepreneurship in students and young researchers;
  - Innovation is the most difficult area for SMEs to enter – training also SMEs and entrepreneurs to accept innovation, to make them aware or understand how they can benefit from innovations;
  - Development of networks for protecting, promoting and supporting researchers and inventors and their intellectual property;
  - Brokerage events to build up collaborations;
  - Enabling mechanisms such as pilot calls for proposals to support innovation as cooperation between research organizations, innovation intermediaries and SMEs, which can see the benefits of investing on innovation;
  - Pilot projects in creating and developing interregional clusters in the technology areas or needs identified, focusing on knowledge and technology transfer and innovation promotion; and
  - Transfer of knowledge (tacit knowledge) through mobility of human resources within research and business: mechanisms of reversing brain drain to brain circulations, enhancing mobility of researchers to other regions or to business or attract researchers to the region.

3.4 HERMAN POTOČNIK-NOORDUNG AWARD for donations within the framework of RTD collaboration with Western Balkan Countries

The Ministry of Higher Education, Science and Technology of Slovenia (MHEST) and the SEE-ERA.NET project partners have joined forces to organise an award for donations of scientific equipment within the area of Research Technology and Development (RTD) collaboration in the WBC. In this way, they are bringing the problem of infrastructural needs to the attention of the public and relevant stakeholders, such as science policy-makers and potential investors.

At the end of 2007, the SEE-ERA.NET project partners, Ministries of Science and Research from Albania, Bosnia and Herzegovina, the Former Yugoslav Republic of Macedonia, Montenegro and Serbia gathered information in order to prepare a list of the most urgent infrastructure needs in the WBC. Potential donors, such as research and technology organisations, universities, philanthropic organisations and development assistance organisations from EU and other developed European countries were approached and invited to provide their contributions.

Expected donations were either in the form of second hand scientific equipment or the financial means to purchase or develop the required infrastructure in the Western Balkan countries, with a value expected to be 20,000 Euros or above.

At the ad-hoc meeting on the results of the "SEE-ERA.NET Award for Donations within the Framework of the RTD Collaboration with Western Balkan Countries" (held in Paris, France on December 17, 2008), the members of the Noordung Potočnik Award Selection Commission decided to distribute the technical equipment, which has been offered from the European Commission - Joint Research Centre (the list of the JRC donated equipment is available on the website [6]).

The members of the Noordung Potočnik Award Selection Commission decided in case there is more than one research institution from WBC interested in the equipment donated by JRC, the criteria for the decision on the recipients are based on the level of investment in research in the respective WBC. Those countries, where the level of investments in science is relatively low, are given the priority.

MHEST received answers/interest from two WBC ministries: Ministry of Foreign Affairs of Bosnia and Herzegovina and Ministry of Education and Science of Montenegro. On the basis of Noordung Potočnik Award Selection Commission decision from December 17, 2008, Selection Commission decided as follows: 'research institutions from Bosnia and Herzegovina shall receive the equipment donated by JRC.'

[6] http://www.investsciencesee.info/news3.html

The selected institutions are the following:
- Faculty of Veterinary Medicine, University of Sarajevo; and
- Faculty of Pharmacy, University of Sarajevo.

The company Sava Kranj d.d., Slovenija (second donor) decided to donate their equipment to Faculty of Biotechnical Science, Bitola, the Former Yugoslav Republic of Macedonia (for donated equipment, please consult above mentioned website).

At the meeting in Paris, the members of the Noordung Potočnik Award Selection Commission decided to keep the website [7] still open to new donors and the principle of choosing whom to donate (in the case of more than one country is interested in the donation) will follow the data on investment in research, but other country from WBC will receive donation.

3.5 SEE-ERA.NET's International Conference on Evaluation and Benchmarking

Ohrid, Former Yugoslav Republic of Macedonia
October 8 – 9, 2008

The "International Conference on R&D Evaluation and Benchmarking" had contributed to the discussion of approaches and criteria for evaluation and benchmarking of R&D institutions by offering expertise and European standards for a dialogue between policy makers and the science community in WBC.

Against the background of fostering the integration of the WBC into the European Research Area and to contribute to the enlargement process, the overall aims of this conference were to:
- raise awareness on R&D evaluation and benchmarking within WBC;
- introduce European "best practice"; and to
- reach consensus on possible approaches to evaluation and benchmarking in the R&D system to be applied in WBC.

3.5.1 Rationale

The integration of the Southeast European countries (Albania, Bosnia and Herzegovina, Croatia, Former Yugoslav Republic of Macedonia, Montenegro and Serbia) in the European Research Area (ERA) is of high political and economic interest for the Southeast European region and the European Union. At the 2003 EU Summit in Thessaloniki, the region was confirmed the accession perspective by giving the Western Balkan countries (WBC) the status of Potential Candidate countries. Meanwhile, Croatia and the Former Yugoslav Republic of Macedonia have been granted the status of Candidate Countries.

[7] http://www.investsciencesee.info/infrastructure.html

Evaluation and benchmarking plays a significant role in strengthening R&D systems. Therefore, the "International Conference on Evaluation and Benchmarking" was organised to contribute to the discussion of approaches and criteria for evaluation and benchmarking of R&D institutions in the WBC by offering expertise and European standards for a dialogue between policy makers and the science community.

3.5.2 Summary and Conclusions

- The participants of the conference agreed that there is a need for a systematic approach to the different types of R&D evaluation in WBC.
- Improving and restructuring of the science landscape is needed in WBC evaluation of research institutes. Evaluation and benchmarking are key instruments to assess the scientific quality and the administrative and organisational structure of research institutes. A further developed science landscape can also reduce the risk of brain drain.
- During the discussions about evaluation and benchmarking, the differences of the approaches and implementation procedures of these two types of assessment of R&D institutes in the WBC became apparent. Accreditation issues were also discussed.
- As a precondition for a successful evaluation system, five basic principles were proposed: Regularity; Transparency; Independence; Acceptance; and Consistent implementation. The evidence based peer review was recommended as the most favourable evaluation method for R&D institutes.
- In some WBC, the issue of evaluation needs to be covered by the Science Law in order to initiate national evaluation procedures. In these cases, a political decision is necessary.
- It was agreed that international support would be beneficial for WBC in order to establish an appropriate framework for evaluation of R&D institutes.
- The participation of reviewers from the international science community in evaluation teams was stressed to assure independent evaluation procedures and to also avoid conflict of interests.
- Evaluation experts from EU member states offered their support to WBC including an invitation to policy makers and experts to participate in evaluation processes in their countries.
- A part of the discussion concerned the evaluation of project proposals. Coordination problems within cross border calls were mentioned. EU member states outlined their evaluation methods used for project proposals. Mutual exchange of information and support was considered to be helpful.
- The Western Balkan countries are invited to benefit from developments in research evaluation presented by ERAWATCH (http://cordis.europa.eu/erawatch/) in order to gain support and guidance in drafting an own national or regional process of evaluation. Participation in CREST meetings and Open Method of Coordination (OMC) working groups offers additional opportunities for discussion on R&D policy issues.
- The evaluation of research institutes in Croatia (which already started in 2007) was considered to be an example of best practice. The development of a common methodology for evaluation of R&D institutes in WBC by a regional working group could be based on this approach.
- Awareness raising at political level in the WBC is needed. The outcomes of the conference were, for example, presented at the meeting of the Steering Platform on Research for the Western Balkan countries, held in Paris, France on December 19, 2008.
- A high-level initiative on a regional R&D strategy including a ministerial conference with respect to the Multi-Beneficiary Instrument for Pre-Accession Assistance (IPA) 2009 is envisaged. It provides the appropriate framework for the inclusion of evaluation issues within the R&D policies in WBC.

3.5.3 Envisaged steps and measures for support

- Launching of national projects of evaluation of R&D institutes in WBC;
- Addressing EU member states for support (for instance in the framework of bilateral evaluation projects with the possibility of financial support);
- Setting-up the evaluation process as FP7 project or embedded in IPA;
- Considering invitations of EU member states to participate in the evaluation procedures;
- Setting-up of a regional working group to exchange information (seminars, workshops, etc.);
- Setting-up of joint peer-review teams for on-site evaluations of R&D institutes in WBC; and
- Presenting of results of evaluation issues at SEE-ERA.NET Steering Board meetings.

Individual Reports on the SEE-ERA.NET Pilot Joint Call

Projects are listed in alphabetical order per title of the project. The authors of the scientific reports are solely responsible for the content and English standards of their articles and they do not represent the opinion of the European Community or the editors of the reports.

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Building Language Resources and Translation Models for Machine Translation Focused on South Slavic and Balkan Languages

Dan Tufiş[1], Svetla Koeva[2], Tomaž Erjavec[3], Maria Gavrilidou[4], Cvetana Krstev[5]

[1] Research Institute for Artificial Intelligence (RACAI), Romanian Academy, 13, Calea 13 Septembrie, 050711, Bucharest, Romania; tufis@racai.ro
[2] Institute for Bulgarian Language (IBL), Bulgarian Academy of Sciences, 52, Shipchenski prohod, 1113, Sofia, Bulgaria; svetla@dcl.bas.bg
[3] Jožef Stefan Institute (JSI), Jamova cesta 39, 1000, Ljubljana, Slovenia; tomaz.erjavec@ijs.si
[4] Institute for Language and Speech Processing (ILSP), 6, Artemidos, 15125, Marousi, Greece; maria@ilsp.gr
[5] University of Belgrade (UB), 16, Studentski trg, 11000, Belgrade, Serbia; cvetana@poincare.matf.bg.ac.yu

Abstract

The aim of this short-term project was to investigate the feasibility of machine translation (MT) research and development for several South Slavic and Balkan languages, more precisely Romanian, Bulgarian, Slovene, Greek and Serbian. For these languages, MT systems are scarce and for some of them even non-existent. We provide a brief description of the project's major research tasks: Compilation of a multilingual parallel corpus for the concerned languages, the XML mark-up of the corpus (tokenisation, lemmatisation, tagging), the sentence and word alignment of the corpus and the building of the statistical translation models. Additionally, we conducted preliminary experiments on building prototype MT systems for Romanian-English, Greek-English and Slovene-English, based on the created resources and models. In the concluding section, we argue that by investing further efforts in extending the language resources we created as well as in the fine-tuning of the statistical parameters, the current machine-learning technologies can be successfully used for a quick development of acceptable MT prototypes, valuable starting points in implementing working systems.

Thematic Area / Type of the project

Information and Communication Technologies: Applications Research
Type of the project: research project

Keywords

Alignment; Corpus encoding; Extensible mark-up language (XML); Machine translation; Moses toolkit; Language model; Lemmatisation; Parallel corpora; Tagging; Tokenisation; Translation model

1 Introduction

Since the seminal work of the IBM group in statistical word-based translation [1], new methodologies (memory-based, phrased-based, syntax-based etc.) and techniques (reification, factorisation) emerged in multilingual data-driven approaches to machine translation. Yet, several studies underlined the idea that the quality of data to be fed into any machine learning system is of a crucial importance and cannot be compensated by using mass raw multilingual data. In spite of numerous attempts to construct MT systems entirely based on raw parallel data, the evaluations showed that although useful and encouraging results can be obtained in a short period of time, the translation quality can hardly be further improved by increasing the volume of data. The ongoing EuroMatrix project has started from this finding and adopted a very promising hybrid approach, combining the strength of rule-based and statistical machine translation and exploiting more and more linguistic knowledge. The Factored Translation Models [2] allow for exploiting, where available, different levels of linguistic pre-processing: lemmatisation, part-of-speech tagging, chunking, parsing, word-sense disambiguation, etc. For most of European languages, tools for ensuring the basic pre-processing steps required for a factored translation approach already exist. In fact, with current MT technologies [3], [4], [5] which, to a large extent, are language independent, the development of sufficiently large and high-quality training data became the critical part of an MT development project.

In this paper, we report on the main results of a small and short-term project[1]. The main project's objective was to provide the necessary linguistic and technological resources fostering machine translation RTD for the South Slavic and Balkan languages. The partners in the project were from Bulgaria, Greece, Romania, Serbia and Slovenia. Some partners harmonised the objective of this project with the objectives of other local or bilateral running projects and the project thus included Czech, French and German as additional languages.

2 Multilingual Data

The Acquis Communautaire is the total body of European Union (EU) law applicable in the EU Member States. This collection of legislative text changes continuously and currently comprises texts written between the 1950s and 2008 in all the languages of the EU Member States. Thus, the Acquis Communautaire is a collection of parallel texts in the following 22 languages: Bulgarian, Czech, Danish, German, Greek, English, Spanish, Estonian, Finnish, French, Hungarian, Italian, Lithuanian, Latvian, Maltese, Dutch, Polish, Portuguese, Romanian, Slovak, Slovene and Swedish. A significant part of these parallel texts have been compiled by the Language Technology group of the European Commission's Joint Research Centre at Ispra into an aligned parallel corpus, called JRC-Acquis [6], publicly released in May 2006. In November 2007, the European Commission's Directorate General for Translation (DGT) and the Joint Research Centre (JRC) have made available

[1] http://dcl.bas.bg/ssbc/home.html

a multilingual Translation Memory (DGT-TM) of the Acquis Communautaire in the above-mentioned official European Union languages. These unique language resources[2] are among the few available parallel corpora containing the languages we were interested in: Bulgarian, Greek, Romanian and Slovene plus Czech, English, French and German (called further SEE-ERA.net Administrative Corpus - SEnAC).

This resource does not yet exist for Serbian, and for that reason an additional resource, based on Jules Verne's novel "Around the world in 80 days" (called further SEE-ERA.net Literary Corpus - SEnLC) has been compiled.

2.1 SEnAC Corpus Construction and Encoding

From the entire JRC-Acquis, which uses the same identifiers (Celex numbers) for the same documents (trailed with the language code); we selected all the documents existing in all our target languages. This resulted in a list of 1204 files per language[3]. Since we have noticed several errors in the sentence alignments of the original JRC-Acquis corpus, we re-aligned the 1204 files for Bulgarian, Czech, French, Greek, German, Romanian and Slovene against the corresponding files in English, using RACAI's SVM sentence aligner [7]. From the XX-EN aligned sentences, we only retained the 1-1 alignment pairs (more than 99% on average of the total alignments) and each partner had the responsibility to check and correct, if necessary, the sentence alignment. We are not aware of any alignment error for the retained 1-1 XX-EN sentences. Finally, we merged the alignments into one XML document, containing 60,389 translation units, each containing one sentence translated in 8 languages, as exemplified in Figure 1.

```
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         Резултатите от консултациите и информацията , събрана
         съгласно членове 5,6 и 7 , трябва да се вземат предвид при
         процедурата по издаването на разрешението.</s></seg>
   <seg lang="cs">
      <s id="31985L0337.n.83.1">
         Informace shromážděné podle článků 5 , 6 a 7 musí být brány
         v úvahu v povolovacím řízení.</s></seg>
   <seg lang="de">
      <s id="31985L0337.n.85.1">
         Die gemäß den Artikeln 5 , 6 und 7 eingeholten Angaben
         sind im Rahmen des Genehmigungsverfahrens zu
         berücksichtigen.</s></seg>
   <seg lang="el">
      <s id="31985L0337.n.85.1">
```

[2] http://langtech.jrc.it/JRC-Acquis.html

[3] We used the JRC-Acquis version 2. In the last version of JRC-Acquis, the number of common files for the considered 9 languages is more than twice as large.

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      Οι πληροφορίες που συγκεντρώνονται δυνάμει των άρθρων 5 ,
      6 και 7 πρέπει να λαμβάνονται υπόψη στα πλαίσια της
      διαδικασίας για τη χορήγηση αδείας.</s></seg>
   <seg lang="en">
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      Information gathered pursuant to Articles 5 , 6 and 7 must
      be taken into consideration in the development consent
      procedure.</s></seg>
   <seg lang="fr">
      <s id="31985L0337.n.83.1">
      Les informations recueillies conformément aux articles 5 ,
      6 et 7 doivent être prises en considération dans le cadre
      de la procédure d'autorisation.</s></seg>
   <seg lang="ro">
      <s id="31985L0337.n.83.1">
      Informaţiile culese conform art. 5 , 6 şi 7 trebuie să fie
      luate în considerare în cadrul procedurii deautorizare.
      </s></seg>
   <seg lang="sl">
      <s id="31985L0337.n.83.1">
      Informacije , zbrane skladno s členi 5 , 6 in 7 , se morajo
      upoštevati v postopku za pridobitev soglasja za izvedbo.
      </s></seg>
</tu>
```

Figure 1: A translation unit from the 8-language SEnAC parallel corpus

2.2 SEnLC Corpus Construction and Encoding

One reason for chosing Jules Verne's novel is that this text is available in digital form for many of the languages that we were interested in. Moreover, for the majority of these languages, lexical resources exist in the same format, which enables comparable processing of the text in different languages. Translations of the novel in sixteen languages have been acquired, namely: French, English, German, Spanish, Portuguese, Italian, Romanian, Russian, Serbian, Croatian, Bulgarian, Macedonian, Polish, Slovenian, Hungarian and Greek. Not all of these texts have yet been aligned; alignment was done for the five Balkan languages, the French original and English.

In the preparatory phase, each translation was marked in accordance with the TEI-standard in XML, and the title (<head>), paragraph (<p>) and "sentence" (<seg>) were included as units of text logical layout. Before alignment, each text was transformed into the TEI-conformant format[4]. The XAlign system[5] was used for the alignment process. Starting with the French version, the goal of the alignment was to establish 1:1 relations on the segment level (<seg> tag) with all other languages. In order to achieve this goal, segments had to be further divided. So, the total number of segments in all texts is 4409, and the average number of words per language is about 60,000.

As this corpus is very small for MT studies (it is about 25 times smaller than SEnAC), it requires a significant extension[6]. This type of text alignment of bitexts required an intensive manual control of the output of the XAlign system. In this way, the missing segments or the inconsistencies between the source text and its translations were also identified.

```
<tu id="n569">
   <seg lang="fr">
      <s id="Verne80days.n569">
         Vous savez que cette formalité du visa est inutile, et que
         nous n'exigeons plus la présentation du passeport?
         </s></seg>
   <seg lang="sr">
      <s id="Verne80days.n569">
         Vi znate da je ova formalnost viziranja izlišna i da se
         više ne traži pokazivanje isprava?</s></seg>
   <seg lang="bg">
      <s id="Verne80days.n569">
         Знаете ли, че тази формалност с паспортите е безполезна и
         че ние вече не изискваме да представяте паспортите си?
         </s></seg>
   <seg lang="en">
      <s id=" Verne80days.n569">
         You know that a visa is useless, and that no passport is
         required?</s></seg>
   <seg lang="gr">
      <s id="Verne80days.n569">
         Ξέρετε ότι αυτή η τυπική διαδικασία της βίζας δεν είναι
         αναγκαία και δεν απαιτείται πλέον η εμφάνιση του
         διαβατηρίου;</s></seg>
   <seg lang="sl">
      <s id="Verne80days.n569">
         Ali vam je znano, da je ta formalnost vidiranja nepotrebna
         in da ne zahtevamo več predložitve potnega lista ?
         </s></seg>
   <seg lang="ro">
      <s id=" Verne80days.n569">
         Ştiţi cã formalitatea vizei e inutilă şi că noi nu mai
```

[4] http://www.tei-c.org/index.xml
[5] http://led.loria.fr/download/source/Xalign.zip

[6] The "1984" corpus, the encoding of which is available in exactly the same format, is available at the address: http://nl.ijs.si/ME. Although the average number of tokens in each language of the "1984" corpus is 110,000, the joined SEnLC and "1984" still makes a too small parallel corpus for MT experiments.

```
        cerem prezentarea pașaportului.</s></seg>
</tu>
```

Figure 2: A translation unit from the 7-language SEnLC parallel corpus

2.3 Sub-sentential annotation of the multilingual data

Each project partner took care about the tokenisation, morpho-syntactic tagging and lemmatisation of the texts in their own languages, using in-house or public-domain processing tools (adapted for the new languages). For instance, Romanian, English and French texts were processed based on the RACAI tools [8] integrated into the linguistic web-service platform available at http://nlp.racai.ro/webservices/. The German data was processed using Helmut Schmid's TreeTagger. This tagger has been successfully used for German, English, French, Italian, Dutch, Spanish, Bulgarian, Russian, Greek, Portuguese, Chinese and old French texts and it is available at http://www.ims.uni-stuttgart.de/projekte/corplex/TreeTagger/. The pre-processing of the Czech part of the corpus was kindly provided by Aleš Horák from the Faculty of Informatics at Masaryk University, Brno, Czech Republic.

After tokenisation, tagging and lemmatisation, this annotation has been added to the XML encoding of the parallel corpus. Depending on the available processing tools for different languages, additional information could be added to each language-specific segment of a translation unit. Figure 3 shows the representation of the Romanian segment of the translation unit displayed in Figure 1.

```
<tu id="3936">
...
   <seg lang="ro">
      <s id="31985L0337.n.83.1">
         <w lemma="informație" ana="Ncfpry">Informațiile</w>
         <w lemma="culege" ana="Vmp--pf">culese</w>
         <w lemma="conform" ana="Spsd">conform</w>
         <w lemma="art." ana="Yn">art.</w>
         <w lemma="5" ana="Mc">5</w>
         <c>,</c><w lemma="6" ana="Mc">6</w>
         <w lemma="și" ana="Crssp">și</w>
         <w lemma="7" ana="Mc">7</w>
         <w lemma="trebui" ana="Vmip3s">trebuie</w>
         <w lemma="să" ana="Qs">să</w>
         <w lemma="fi" ana="Vasp3">fie</w>
         <w lemma="lua" ana="Vmp--pf">luate</w>
         <w lemma="în" ana="Spsa">în</w>
         <w lemma="considerare" ana="Ncfsrn">considerare</w>
         <w lemma="în_cadrul" ana="Spcg">în_cadrul</w>
         <w lemma="procedură" ana="Ncfsoy">procedurii</w>
         <w lemma="de" ana="Spsa">de</w>
         <w lemma="autorizare" ana="Ncfsrn">autorizare</w>
         <c>.</c>
      </s></seg>
...
</tu>
```

Figure 3: Linguistically analysed sentence (Romanian) of a translation unit of the SEnAC parallel corpus

The tagsets used for all languages (except Bulgarian and German) were compliant with the MULTEXT specifications, for the most part with the MULTEXT-East specifications Version 3 [9] (for the details of the morpho-syntactic annotation, see http://nl.ijs.si/ME/V3/msd/). Table 1 shows some statistics concerning the result of the pre-processed corpus.

Table 1: Statistical data on the SEnAC parallel corpus

Language	No. of tokens	Average no. of tokens / sentence
BG	1436925	23.79
CS	1238981	20.51
DE	1314441	21.76
EL	1469642	24.33
EN	1466912	24.29
FR	1527241	25.29
RO	1422995	23.56
SL	1271011	21.04

The corpus SEnLC has been tokenised, lemmatised and tagged according to the same principles as used in the SEnAC encoding. The total number of tokens in this text in French is 71,793, while the total number of unique tokens (types) is 9,433 (ratio 7.6). The figures for other languages are different, e.g. for Serbian, the total number of tokens is 58,722, while the total number of types is 12,733 (ratio 4.6). For Bulgarian, the total number of tokens is 58,678, while the total number of types is 11,217 (ratio 5.2), while for Greek the total number tokens is 68,615, and the total number of types is 11,809 (ratio 5.8). Figure 4 shows the representation of the Serbian segment of the translation unit displayed in Figure 2:

```
<tu id="n569">
   <seg lang="sr">
```

```
      <s id="Verne80days.n569">
         <w lemma="vi" ana="Pp2-pn">Vi</w>
         <w lemma="znati" ana="Vm-p2p-an-n---p">znate</w>
         <w lemma="da" ana="C-s">da</w>
         <w lemma="jesam" ana="Va-p3s-an-y---p">je</w>
         <w lemma="ovaj" ana="Pd-fsn">ova</w>
         <w lemma="formalnost" ana="Ncfsn--n">formalnost</w>
         <w lemma="viziranje" ana="Ncnsg--n">viziranja</w>
         <w lemma="izlišan" ana="Afpfs1">izlišna</w>
         <w lemma="i" ana="C-s">i</w>
         <w lemma="da" ana="C-s">da</w>
         <w lemma="se" ana="Q-">se</w>
         <w lemma="više" ana="Rgp">više</w>
         <w lemma="ne" ana="Q-">ne</w>
         <w lemma="tražiti" ana="Vm-p3s-an-n---p">traži</w>
         <w lemma="pokazivanje" ana="Ncnsn--n">pokazivanje</w>
         <w lemma="isprava" ana="Ncfpg--n">isprava</w>
         <c>?</c>
      </s></seg>
</tu>
```

Figure 4: Linguistically analysed sentence (Serbian) of a translation unit of the SEnLC parallel corpus

2.4 Word Alignment of SEnAC

Based on the monolingual data from the SEnAC, we have built language models for each language. For Romanian, we have used the TTL tagging modeller [11], while for the other languages we used the METT tagging modeller [12]. Both systems, for language modelling and tagging, are able to perform tiered tagging [13], a morpho-syntactic disambiguation method that was designed specially to work with large (lexicon) tagsets.

In order to build the translation models from the linguistically analysed parallel corpus, we used GIZA++ [3] and constructed 8 unidirectional translation models (EN-RO, RO-EN, EN-BG, BG-EN, EN-SL, SL-EN, EN-GR, GR-EN). The processing unit considered in each language was not the word form, but the string formed by its lemma and the first two characters of the associated morphosyntactic tag (e.g. for the wordform "informațiile" we took the item "informație/Nc"). For each language, we used 20 iterations (5 for Model 1, 5 for HMM, 1 for THTo3, 4 for Model3, 1 for T2To4 and 4 for Model4). We did not include Model 5 nor Model 6 as we noticed a degradation of the perplexities. Given the formulaic language, used by the Acquis Communautaire documents, the perplexities of the resulting translation models were encouraging, and range from 13.07 (RO-EN) to 19.88 (EN-BG). Based on these models, we word-aligned the bitexts using the iterative high precision COWAL reified aligner [14]. The reified alignment method allows to combine different information sources to guide the identification of the most probable translation pairs in a bitext. In our alignment system, we used, among others, translation probabilities, string similarity scores, translation entropy, word positions, etc. Eight such information sources were combined by means of a linear interpolation formula into a scoring function used to build the most probable lexical alignment. As described in [14], the translation pairs prescribed by each unidirectional translation model were unconditionally included in the alignment skeleton. The rest of the links were established in the subsequent iterations of the aligner. The training corpora SEnAC, the alignments and the perplexities for each translation model are available on the internet[7]. An alignment viewer and editor (see Figure 5) is also available for visualisation and correction of the alignments with the purpose of further fine-tuning the translation models. Another useful tool available at the mentioned address, implemented by the Slovene partner, builds N way alignments from existing pair wise alignments, either sentence or word. More specifically, when given n pair wise alignments, such that one of the corpora in the alignment is the same for all of the alignments, and the other is different for all of the alignments, it produces n+1 way alignment. The corpus, which is included in all of the alignments, is called the hub corpus. The time complexity of the algorithm is O(S*A*C), where S is the number of sentences in the hub corpus, A is the number of input alignments used and C is the average number of corpora per alignment. In the standard scenario of using only pair wise alignments C = 2. The space complexity of the data structure for the mapping SL is O(C*S), where C is the total number of corpora in the result alignment and S is the average number of sentences per corpus.

This method, which exploits the translation equivalence transitivity, has many advantages versus the direct X1-X2 alignment:

- It allows a multilingual team to share the work so that different partners deal only with known pairs of languages (in our case EN-Xi); having a good command of a given language pair they can check and correct the alignments of a bitext extracted from a multilingual parallel text; the derived alignments Xi-Xj are usually of comparable accuracy as the alignment of PIVOT-Xi and PIVOT-Xj;
- The derived alignment is much faster than the direct alignment;
- The derived alignment is much cheaper in terms of human expertise, language resources, and computing power.

[7] http://www.racai.ro/ResearchActivity/WebServicesandResources/SEEERANETResources/tabid/131/Default.aspx

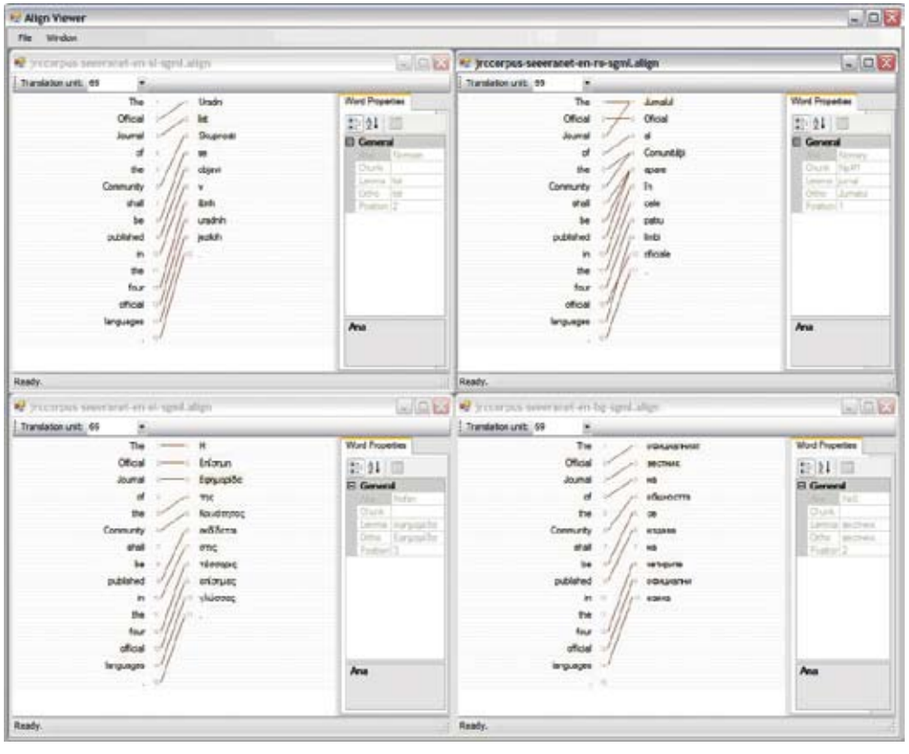

Figure 5: Snapshot of the alignment visualisation of a translation unit (no. 69) in four bitexts

3 Using MOSES Toolkit to Perform Machine Translation

The MOSES toolkit [5] is a public domain environment, which was developed in the ongoing European project EuroMatrix, and allows for rapid prototyping of Statistical Machine Translation systems. It assists a developer in constructing the language and translation models for the languages he/she is concerned with, and by its advanced factored decoder and control system, it ensures the solving of the fundamental equation of the Statistical Machine Translation in a noise-channel model:

$$Target^* = \text{argmax}_{Target}\ P(Source)*P(Target)$$

What is extremely useful is that the environment allows the developer to provide MOSES with externally-developed language and translation models, offering means to ensure the conversion of the necessary data structures into the expected format and to further improve them. Once the statistical models are in the prescribed format, the MT system developer may define his/her own factoring strategy. If the information is provided, MOSES can build various factored representations for each of the lexical items (be they word or phrases) to be used in deriving the best translation: occurrence form, lemmatized form, associated part-of-speech or morpho-syntactic tag. By dissociating the treatment of occurrence form of lexical items into distinct processes (translating lemma, translating morpho-syntactic properties of the current item and respectively generating the target inflected lexical item based on the translated lemma and the translated morphological input information), the system achieves a higher flexibility, a better generalisation of the linguistic facts and more reliable decisions in the quest for the optimal translation solution. Moreover, the system allows for integration of higher order information (shallow or even deep parsing information) in order to improve the output lexical items reordering. For further details on the MOSES Toolkit for Statistical Machine Translation and its tuning, the reader is directed to the EuroMatrix project web-page (http://www.euromatrix.net/) and to http://www.statmt.org/moses/.

seeera.net repsee

4 Conclusions / Outlook, next steps

The described language resources as well as the MOSES toolkit were used at the Research Institute for Artificial Intelligence (RACAI) to perform a series of very encouraging MT experiments for a part of the language pairs of the project, always using English as one of the source or target language. Due to the limited period of the project and of the human resources, as well as due to insufficient training data for Serbian, not all the language pairs of the project have been experimented. However, even the few experiments that were conducted proved that, whenever adequate language resources are available, the development of reasonably accurate SMT systems for the concerned languages is realistic and feasible within acceptable time limits. The extremely valuable language resources that were created during this project (they were carefully analysed and manually corrected where necessary) as well the publicly available tools offer the possibility to further experiment with new language pairs to all interested parties. All the partners in this project are working on research proposals or are already involved in ongoing projects that will definitely build on the results of the present project. Future work could address the more challenging tasks by extending the SEnLC literary corpus and building translation models and, provided that adequate data will be available, experiments with other Southeast and Balkan languages.

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CForSEE: Multifunctional Management of Coppice Forests

M.J. Lexer[1], M. Bobinac[3], S. Dekanic[4], T. Dubravac[4], G. Georgiev[2], M. Georgieva[2], E. Hochbichler[1], G. Ivanov[2], D. Jovic[3], E. Kastner[1], S. Krajter[4], L. Nestorovski[5], B. Nikolov[2], B. Stajic[3], A. Teischinger[1], P. Trajkov[5], H. Vacik[1], I. Velichkov[2], B. Wolfslehner[1], T. Zlatanov[2]

[1] University of Natural Resources and Applied Life Sciences Vienna, Austria; mj.lexer@boku.ac.at
[2] Forest Research Institute, Bulgarian Academy of Sciences (FRI-BAS), Sofia, Bulgaria
[3] Faculty of Forestry, Belgrade University (BU), Serbia
[4] Forest Research Institute, Croatia
[5] Forest Faculty, Ss. Cyril and Methodius University, Skopje, Former Yugoslav Republic of Macedonia

Abstract

Coppice forestry in general including all its variants is a silvicultural system that is still widespread over many European countries where it covers an area of about 23 million hectares. While in Central and Northern Europe, coppice forests comprise just a small share of total forest area, they make up major parts of the forest resource in South and South-Eastern Europe. CForSEE gathers institutions in countries with substantial shares of coppice forests and intensive research experiences in coppice forest management. The network of partners provides the unique opportunity to review the state of coppice forests and their management from first hand for substantial parts of the coppice area in South Eastern Europe. These activities are part of the SEE-ERA.NET Pilot Joint Call Project CForSEE: Multi-functional management of coppice forests. Contributions to rural development, maintenance of biodiversity, and climate change mitigation and adaptation in natural resource management are presented in the context of coppice forests.

Thematic Area / Type of the project

Environment
Type of the project: network project

Keywords

Coppice forestry; South Eastern Europe; Forest management; Multi-functionality; Ecology; Socio-economic conditions

1 Introduction

Forest management in Europe and globally is currently experiencing a paradigm shift from sustainable timber production towards a multi-dimensional understanding of sustainable forest management (SFM) [1], [2]. The challenge to forest resource management to

satisfy multiple societal demanded services and functions, however, is amplified by the potential adverse effects of a changing climate [3], [4]. Global change, whether generated by climate change, land use change, social or economic pressures increases the need to understand socio-ecological processes in forest resource management. For instance, the demand for bio-based products and the need to secure supply for bio-based industries and energy production [5] will increase in the future, which will increase pressure on forest ecosystems, which are a key component in maintaining biodiversity.

In existing forests in Europe the objective of increasing wood resource allocation to the production of green energy to combat climate change may be in conflict to the demand for secure timber supply for bio-based industries [6]. In a similar manner, EU-level directives relevant to forest resource management such as Natura 2000 and the EU Water Framework Directive (2000/60/EG) impose conflicting objectives between EU policies and with regard to an adaptive management approach to cope with adverse effects of a changing climate.

This clearly calls for a scientifically sound knowledge base for decision making at the internodes of energy and forest policy as well as in forest resource management. While this holds true for forest management in general, it is particularly relevant for coppice forests due to expected large potentials to increase biomass and value production in these forests.

Coppice forestry in general including all its variants is a silvicultural system that is still widespread over many European countries where it covers an area of about 23 million hectares [7].While in Central and Northern Europe coppice forests comprise just a small share of total forest area, in South and South-Eastern Europe coppice forests make up major parts of the forest resource.
Table 1 provides an overview on the shares of coppice forests in the countries represented by the consortium of this proposal.

Table 1: Forest area and share of coppice in the partner countries

Country	Total forest area [ha]	Coppice forests	
		Area [ha]	Share of total forest area [%]
Austria	3,992.000	70.000	2.0
Bulgaria	3,700.000	1,750.000	47.3
Croatia	2,387.387	530.000	22.2
FYR of Macedonia	947.653	557.392	58.8
Serbia	1,987.407	1,103.011	55.5

2 Experimental Techniques and Methods

The overall objective of "Coppice forests in South-Eastern Europe" (CForSEE) was to gather experts from South Eastern European countries to collect and synthesise available knowledge about coppice forests and forestry in SEE. Specific tasks were to:

- identify major ecological zones/ conditions with coppice forests in SEE;
- synthesise current coppice management practices in SEE countries;
- discuss stakeholder interests in coppice forests in SEE; and to
- propose possible future management approaches for coppice forests.

As a major communication means of CForSEE, a web-forum has been set up in project month 1. Figure 1 shows a screen shot of the main entry page of the web-forum.

Figure 1: Screenshot of the entry page of the web-forum [http://cforsee.boku.ac.at]

A user manual was prepared to encourage all partners using the tool (e.g., the online discussions, upload of documents). The discussions were structured according to the project proposal:
(1) Discussion Forum "State of coppice forest management in SEE and Central Europe"
(2) Discussion Forum "Stakeholder analysis for coppice forest management"
(3) Discussion Forum "Options for future management of coppice forests"
(4) Discussion Forum "Coppice forests and wood processing industries"
(5) Discussion Forum "Inventory, experimental design and planning tools in coppice forests"
Against this background, key activities within the project were as follows: literature review and collection of statistical data for comparative analysis of both ecological and socio-economic conditions in the partner countries.

The key activity within CForSEE was a workshop held in Razgrad, Bulgaria from May 19-21, 2008. The workshop has been prepared and convened by the Forest Research Institute (FRI), Sofia. A list of attendants can be found in the Annex. The intensive workshop program consisted of indoor sessions and field trips to coppice forests in the region of Razgrad. A press conference with the governor of the region of Razgrad covered by national TV and press was held on May 21, 2008.

3 Results and Discussion

Diversity of vegetation zones accompanied by the different socio-cultural backgrounds in different geographical regions has produced a wealth of diverse coppice forests and a number of traditional management practices. Some of these practices should be rediscovered and tested against ecological, economical and social sustainability indicators of sustainable forest management frameworks such as the Pan-European SFM guidelines of the Ministerial Conference for the Protection of Forests in Europe, [2], see [8], and updated with new, improved management concepts. While in the last 10-15 years, interest in coppice forest management has come to a halt, nowadays coppice forestry sees a revival which is mainly due to the increasing demand for biomass for energy production. This development was triggered by climate change mitigation policies in the wake of the Kyoto Protocol. This leads to increasing utilisation of coppice forest production. However, with increasing revenues from fuelwood ("biomass for energy"), coppice forest owners may be able to invest in improved management approaches and conversion programs, thus being detrimental with regard to the maintenance of traditional management schemes. Despite the long tradition of coppice forests, the potentials of coppice with standards systems to provide both, fuelwood and valuable timber are not yet fully explored [9]. Thus, knowledge gaps concerning improved coppice management systems need to be identified, and based on these findings, targeted research should be planned to improve the knowledge base for coppice forestry in the 21st century.

Partner institutions in CForSEE:

- University of Natural Resources and Applied Life Sciences, Vienna (Austria);
- Forest Research Institute, Sofia (Bulgaria);
- Faculty of Forestry at Ss. Cyril and Methodius University, Skopje (Former Yugoslav Republic of Macedonia);
- Forest Research Institute (Croatia); and
- Faculty of Forestry at Belgrade University (Serbia).

3.1 Ecological analysis

Biogeographically, the coppice forests in the partner countries cover a broad range of ecological conditions from shallow sites at steep mountain slopes in the temperate vegetation zone (e.g., Bulgaria, FYR of Macedonia, Serbia) to the Mediterranean (Croatia). Subsequently, they show a large gradient with regard to productivity. Due to the huge geographical area covered, the set of tree species in these forests is large: in major parts of Bulgaria's coppice forests, mixtures of oak species (Quercus petraea, Q. frainetto, Q. cerris, and Q. pubescens) prevail. Fagus silvatica occasionally occur with admixed hornbeam (Carpinus betulus) as well as forests with dominating lime (Tilia sp.) or Oriental hornbeam (Carpinus orientalis). In Croatia, the Mediterranean vegetation zone, the mountainous vegetation zone of colline, montane and subalpine forests, and the lowland forests are distinguished. Major tree species of Mediterranean coppices are Q. pubescens (23.8%) and Quercus ilex (15.2%). In other vegetation zones, Fagus sylvatica (21.2%), Carpinus betulus (12.0%) and Sessile oak (7.3%) are major coppice species [10]. Other tree species (Fraxinus sp., Castanea sp., Alnus sp., Tilia sp., Quercus sp. etc.) account for the remaining share of the Croatian coppice forest area. Most of the coppice forests of Serbia are situated in forest complexes of colline and mountainous regions: xero-termophyllic forests (Quercus cerris L., Quercus farnetto Ten., Quercus pubescens Willd., Tilia sp.), area of xero-mesophyllic forests (Quercus petraea Liebl., Carpinus betulus L.) and area of mesophyllic forests (Fagus moesiaca Cz.). Coppice forests in the FYR of Macedonia occur in the colline and submontane vegetation zones mainly from 40 up to 1300m a.s.l. Oak and beech dominated vegetation communities are most prevalent. In Austria, most coppice forests are located in the eastern parts of the country in the colline and sub-montane vegetation zone with prevailing oak-dominated vegetation communities.

3.2 Socio-economic analysis

The current ownership structure reflects the recent history in the partner countries. While in Austria, the majority of coppice forests is either community-owned or private, in Bulgaria, the FYR of Macedonia and Croatia, the share of state-owned or municipality-owned coppice forests ranges from ca. 50% (Croatia) to 88% (FYR of Macedonia). In Serbia, approximately 36% of the coppice area is in public ownership.

Despite the differences in ownership structure, some key characteristics regarding to management issues can be found: (i) substantial portions of the coppice forests suffer from a decreasing productivity due to over-aged stumps and a degrading shift to low-value softwoods, (ii) general drawback of many coppice systems is the very low percentage of sawn timber assortments in the overall cut, (iii) coppice forests frequently occupy sites where more valuable forests could be grown, and the conversion of coppices to high forests is therefore a recommended management practice, (vi) in most cases, attempts to improve the quality of produced timber assortments have not been successful in the past. The main reasons were a lack of systematic approaches because of high costs and the relatively large need for fuelwood. In most partner countries, a general classification of coppice forests acknowledges (a) coppice stands designated for transformation into high forests, (b) stands to be managed by means of a coppice system, and (c) degraded stands of low reproductive capacity scheduled for clearing and subsequent afforestation.

3.3 Terms and definitions

It was recognised that there is a variety of terms and definitions regarding coppice forestry which are due to different silvicultural practices and to diverging terms in the respective languages. Hence, one of the tasks of the project was to generate a common understanding and terminology with regard to coppice forests.
The following was agreed upon:

- Coppice stands are dominated by stool shoots (sprouting, root sucker) after the re-

generation phase, where vegetative and /or generative regenerated trees/shrubs are mixed. Some seed trees are needed also in coppice systems to renew the stands and sustain the reproduction capacity. These seed trees are removed after successful regeneration (after approx. ten years).

- Coppice with reserves is a coppice system where 20-30/ha trees are left not only as seed trees but also to produce higher diameters, e.g. fow sawn timber which leads to a two-aged system for the overstorey and the coppice layer.
- Coppice with standards is a combination of a coppice system and a high forest system revealing uneven-aged and multi-storeyed forest structuring. There are different age classes in the upper layer according to the rotation periods representing a multi-aged system.

In literature, there is a grey zone between coppice with standards and coppice with reserves. It was decided that the Croatian middle-forests are coppice with standards as there are trees of several rotation periods present.
Also, a variety of terms was identified for coppice and transforming operations. Figure 2 is to set the context for these activities in different coppice regimes.

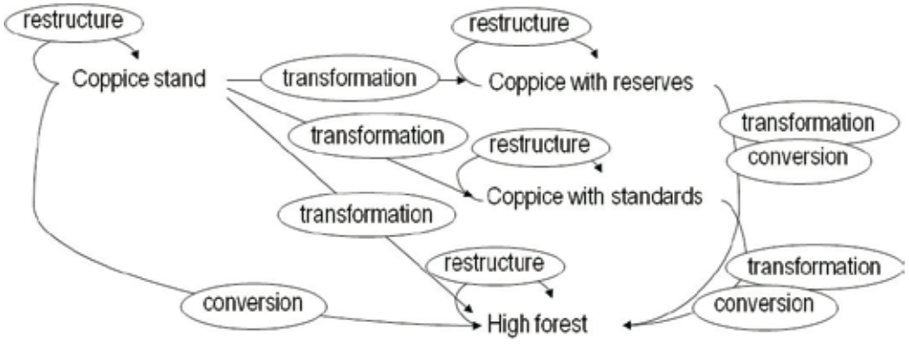

Figure 2: A common context for forestry operations and development of coppice forests

4 Summary and Outlook

The overall objective of CForSEE was to gather experts from South Eastern European countries to collect and synthesise the available knowledge about coppice forests and forestry in SEE.
This was supported substantially by the web-forum that has been maintained throughout the entire project life cycle and proved to be a very efficient means of collecting information and discussing open issues. It is planned to use the web forum further on as a platform for the exchange of communication.
From month 2 until just prior to the workshop in Bulgaria in month 7, there were 26 users registered within C4SEE. A total of 22 documents were uploaded. Altogether, 83 posts were counted (compare Figure 3).

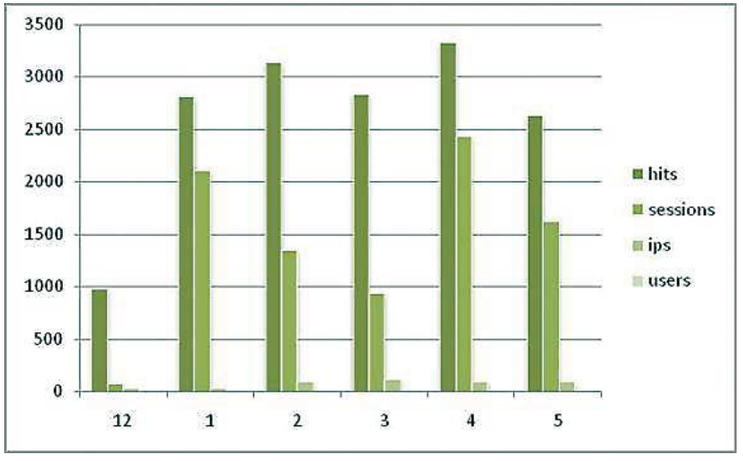

Figure 3: Selected key parameters of the web forum in CForSEE

Based on the data and information gathered during the web forum discussions and the workshop, it was decided to compile papers on coppice forests and forestry for a Special Issue of the forest sciences journal Silva Balcanica. Tzvetan Tzlatanov and Manfred J. Lexer will serve as guest editors. The production process of the Special Issue in Silva Balcanica will last longer than project lifetime and can thus be considered as post project activity. However, the partners agreed in the workshop in Razgrad to continue the cooperation after completion of C4SEE.

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Continuous Water Quality Monitoring of Surface Waters in Montenegro and Serbia

A. Koumoutsakos[1], S. Kapakli[1], G. Kaplanidis[1], P. Sotiropoulos[1], G. Sideris[1], R. Stojanovic[2], D. Djordjevic[3], G. Pilidis[4]

[1] Institute of Polymers, Bulgarian Academy of Sciences, 1113 Acad. G. Bonchev str. 103A, Sofia, Bulgaria; chtsvet@polymer.bas.bg
[2] Department of Technology and Biotechnology, University Ss. Cyril and Methodius, Rudjer Boskovic str. 16, 1000 Skopje, FYR of Macedonia; e-mail: eleonora@tmf.ukim.edu.mk
[3] Department of Polymer Chemistry and Material Science, Institute of Materials and Environmental Chemistry, Hungarian Academy of Sciences, 1525 Budapest, Pisztaszeri u. 59-67, PO Box 17, Hungary; bi@chemres.hu
[4] Department of Food Technology and Biochemistry, Faculty of Agriculture, University of Belgrade, Nemanjina str. 6, Post Box 127, 11081, Belgrade–Zemun, Serbia; vnedovic@agrifaculty.bg.ac.yu

Abstract

During this work, continuous water quality monitoring of 15 parameters of surface waters was performed in Montenegro and Serbia for the first time. Baseline data were collected for the water quality of the rivers Sava and Danube in Serbia. Data were also collected from Lake Skodra and Lake Biogradsko as well as from Tara River in Montenegro. This effort proved that monitoring the water quality in remote areas, using state-of-the-art technology, is feasible. The main advantages, as compared to traditional methods, are cost and effort reduction, while the information acquired is continuous. The variability of the parameters was observed among the locations, providing information about the condition of each area. Based on the limited extent of this work, it is emphasised that further investigation is required in order to acquire a more complete picture of the water quality throughout longer time periods. The ability to set alarms for parameters exceeding requirements by authorities makes this system a very useful tool in water quality management.

Thematic Area / Type of the project

Environmental Technologies
Type of the project: research project

Keywords

Alarm; Chemical; GIS; In-Situ; Lake; Physical; River; Real-time; Telemetry

1 Introduction

Montenegro has officially been an Ecological State since 1991 and announced that its development would be based on the principles of sustainability. International conventions and urban-development laws protect Montenegro's numerous national parks, which cover 28% of the country's territory. The city of Kotor and Kotor-Risan Bay were placed on UNESCO's World Cultural Heritage list in 1979. But in the current situation, the monitoring activities are inadequate, especially in urban areas. The monitoring programme currently relies primarily on random measurements. Some public institutions are monitoring the water quality in Montenegro. The approach is mainly traditional, involving manual water-sampling at remote sites and transporting the samples to a laboratory for chemical analysis. This approach, while easily repeatable, is also: (i) time-consuming, (ii) has a high labour cost, (iii) may be limited by weather conditions, (iv) may lead to inconsistent results and (v) does not allow continuous data collection. Due to the lack of financing, obsolete equipment and staff as well as a petty number of modern stations, these institutions could not provide a reliable picture of the water quality harmonised with relevant international standards. A similar financing situation exists in Serbia due to the civil war in Yugoslavia during the 1990s.

A consortium was developed with experts of neighbouring countries to collect data and initialise networking as well as transfer of knowhow. The partners have significant research potential and practical experiences in the field of water quality and resource management. The department of biological applications and technologies at the University of Ioannina has long experience in water quality technologies through projects in Greece and all over Europe. Recently, TerraMentor has developed an advanced multiparameter probe that has the ability to provide 15 parameters simultaneously. This state of the art piece of equipment is the main instrument for data collection and is well described in the next sections.

The main advantages of this system are:

- The monitoring system can record up to 15 physical-chemical parameters simultaneously.
- This multi-parameter sensor is accompanied by a modern software package for the operation/calibration of the ion selective electrodes, as well as the processing and transmission of data.
- This monitoring sensor can be submerged into wells of up to 200 m.
- There is a possibility of real-time data transfer.
- There is a possibility of immediate warning (alarm system) in case the concentrations of physical-chemical parameters exceed preset values (limits set by the EU).
- Connection to a GIS data base where all the information necessary for the overall water management is being registered.

2 Experimental Techniques and Methods

The multi-parametric probe for qualitative and quantitative surface and ground water monitoring in this project was the main system providing the information collected. It is mainly based on potential differences that a liquid system exhibits under different concentration conditions through selective electrodes.

A table that provides the measuring range, the uncertainty and the resolution of the physicochemical parameters can be found at [1]. The system has been successfully utilised in order to provide the sea water intrusion at the Kato Souli Basin [2].

The high accuracy and the resolution potential of the device allows using it for monitoring of underground waters in areas contaminated by toxic chemical matters coming from industrial, agricultural or household objects.

This device, as a monitoring concept, is the state-of-the-art in many aspects. There are several multi-parameter probes currently available on the market. With most multi-parameter probes, only one to four ions can be measured simultaneously with Ion Selective Electrodes (ISEs), whereas with the device that was developed, there is ability to measure up to seven ions.

3 Results and Discussion

A table that includes the average values of the measurements for the most important parameters is given below for each area under investigation.

Table 1: Average values of the water quality parameters measured with MTL

Location	Danube	Sava 1	Sava 2	Skodra 1	Skodra 2	Biogradsko	Tara
T, C	25	26	28.5	26	25.5	20.1	15
K, μS/cm	215	285	252	358	365	345	195
DO, mg/lt	20	17.5	18.7	13	16	17.6	22.5
Cl, mg/lt	18	25	21.6	5.9	5.4	1.6	2.45
F, mg/lt	0.09	0.08	0.07	0.045	0.04	0.03	0.027
Ca, mg/lt	39	41	45.7	50	49	36	36.7
Redox, mV	289	276	264	292	287	242	280
Na, mg/lt	12.5	14.7	10.5	4	4.5	1.56	2.4
NH4, mg/lt	0.15	0.14	0.1	0.05	0.06	0.02	0.04
pH	8.69	8.55	8.6	7.6	7.9	8.3	8.4
NO3, mg/lt	7.9	14	7.8	1.3	1.8	1.1	4.6

The magnitude of the average value can be considered as a crude indicator of the water quality at each site. We have observed that there is variability among the different places. Raw data for for 11 parameters is provided in Figures 1–11 below for the case of Danube River, in order to give a more detailed view of the dynamics during the measurements.

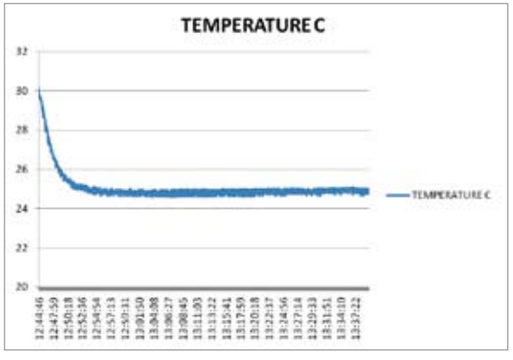

Figure 1: Sample of Temperature data acquired during measurements of the Danube River

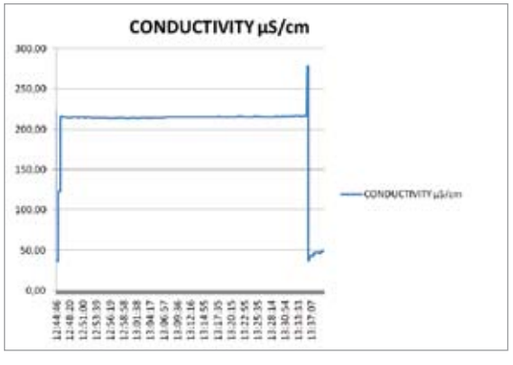

Figure 2: Sample of Conductivity data acquired during measurements of the Danube River

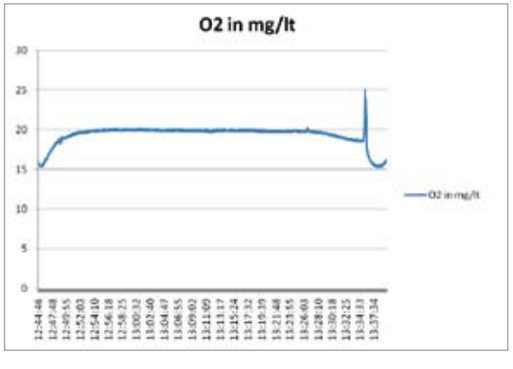

Figure 3: Sample of dissolved oxygen data acquired during measurements of the Danube River

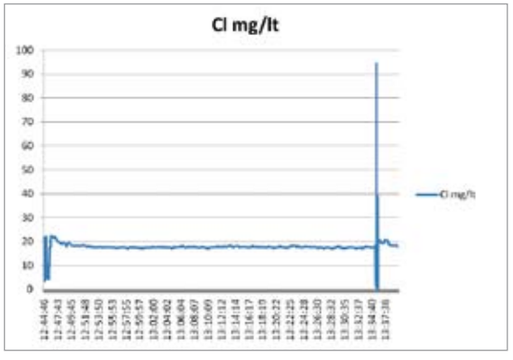

Figure 4: Sample of Cl ion concentration data acquired during measurements of the Danube River

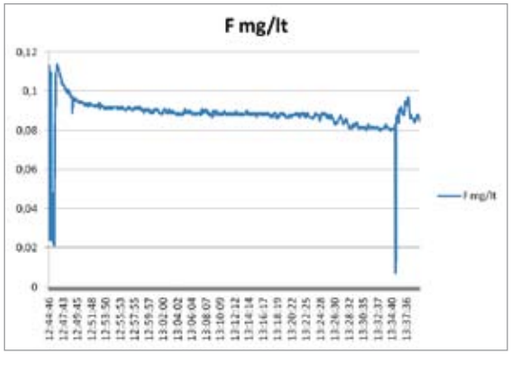

Figure 5: Sample of F ion concentration data acquired during measurements of the Danube River

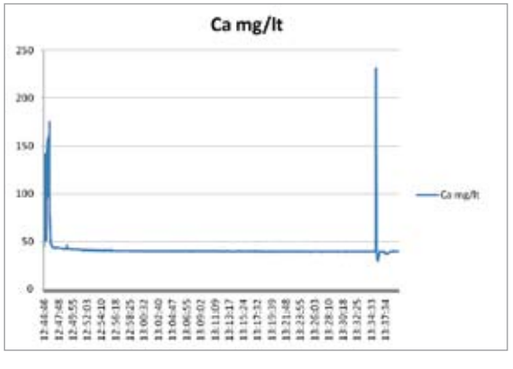

Figure 6: Sample of Ca ion concentration data acquired during measurements of the Danube River

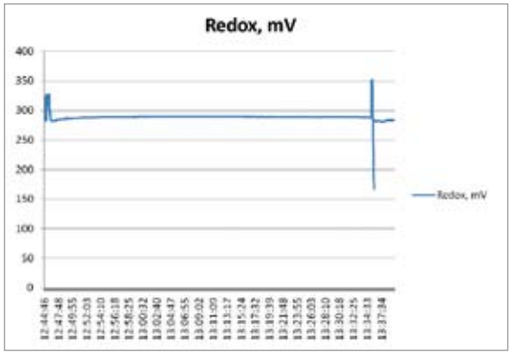

Figure 7: Sample of Redox data acquired during measurements of the Danube River

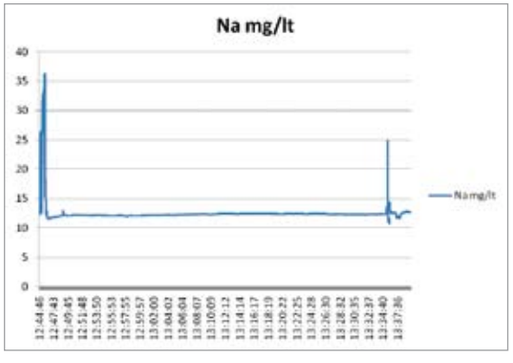

Figure 8: Sample of Na ion concentration data acquired during measurements of the Danube River

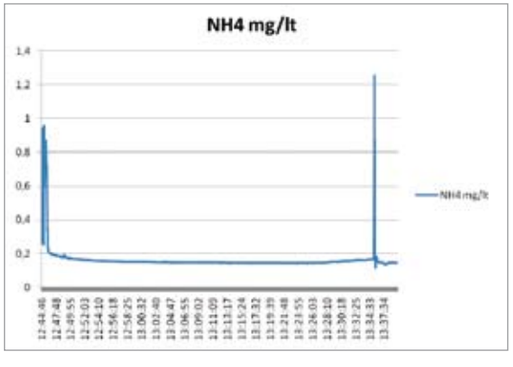

Figure 9: Sample of NH4 ion concentration data acquired during measurements of the Danube River

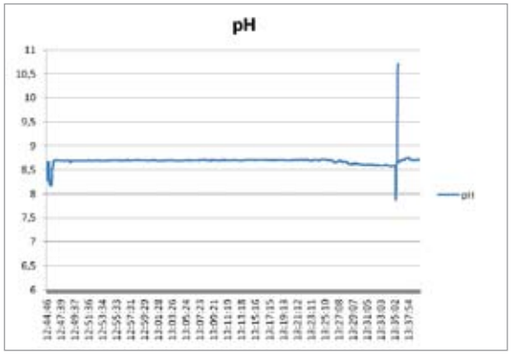

Figure 10: Sample of pH data acquired during measurements of the Danube River

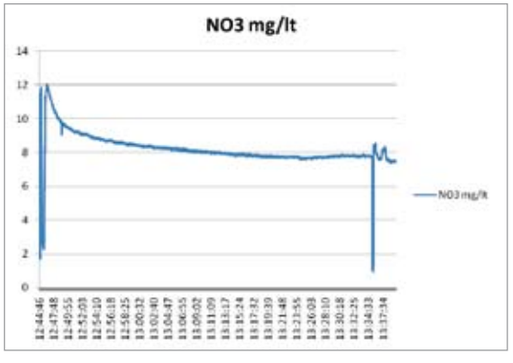

Figure 11: Sample of NO3 ion concentration data acquired during measurements of the Danube River

It has to be noted that in all situations measured, the parameters never exceeded the desired limits set by the EU and local directives. This is why we have never experienced the alarm feature that is implemented in the software of the system. Longer periods of measurements are expected to reveal the usefulness of this element.

We have observed a relative stability of the measured parameters within short time periods as is evident in figures 1–11. This is an expected behaviour when the measuring system is working well and the media is under equilibrium. Some disturbances occur simultaneously for all the parameters as can be seen comparing figures 1–11. There may be a dual explanation for this behaviour. Either the measuring system is affected by the conditions of the surrounding media in such a way that the reference state is disturbed, or there is a local disturbance of the condition of the measuring system that is evident throughout all the measurements, as they change abruptly through time at the same moment.
We must note here that theories explaining the data in such complicated situations as is

in nature – versus the usual conditions in the lab, where we can have control of the environment – can be extremely variable. It is expected that the more information we collect in situ, the harder it will be to give one single explanation for the nature of changes observed. Possible improvements in the system's hardware that will allow for sampling will give a better picture in the future, mostly in more static situations versus more dynamic ones (a lake versus a river).

4 Conclusions / Outlook, next steps

A series of measurements was successfully completed in Montenegro and Serbia with respect to water quality monitoring. The amount of information that was collected proved that continuous monitoring of water quality is possible for remote areas with limited but state of the art equipment. This is an inexpensive method to obtain a lot of information and allow development of better management of water resources. The magnitude of the variables measured offers a primary examination of the quality level of each variable. A more detailed study is expected to explain how these water quality parameters change through time, the time where the values of certain parameters exceeds acceptable limits and the actual development of response plans for emergency situations.

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CoSEESNet: A Collaborative South East Europe Seismic Network: Towards Early Warning System and Real Time Seismic Monitoring in South East Europe

Dr Nikolaos S. Melis[1]

[1] National Observatory of Athens, Institute of Geodynamics, P.O. Box 20048, 118 10 Athens, Greece; nmelis@gein.noa.gr

Abstract

The present pilot project aimed to bring together leading institutes in seismic monitoring in SE Europe. Most of the countries in the region were represented by their leading institutes. Moreover, neighbouring countries participated in the initiative as observers with their own funds. The project aimed to give all participants the chance to exchange ideas, define policies, express their needs and finally agree on a lasting collaboration for seismic multi-parametric monitoring of the region.
A network for seismic monitoring has started to be established. It includes bilateral and multilateral real time data exchange in order to facilitate transfer of valuable information during a seismic arrest or after a felt strong seismic event from individual institutes to their country. Moreover, a pilot joint seismic network has been agreed to start operating in the region, with parametric results to be used by all partners for strengthening their seismic monitoring operation.
Information also has been exchanged and a database of existing strong motion data is to be established. Experience from existing databases was used by the participants to establish a common database and common practise of data preparation and storing.
Information was exchanged and a database of existing GPS data measurements is to be established. Experience from existing databases was used to define rules for homogenising datasets, to prepare for distribution between partners, and eventually to make them available to the scientific community.

Thematic Area / Type of the project

Environment (including Climate Change): Environmental Technologies
Type of the project: network project

Keywords

Earth observation technologies & remote sensing; Applications for protection; Information theory & systems; Networks; Protocols; Seismic monitoring and observation networks

1 Introduction

The region of South East (SE) Europe is the most seismically active part of Europe and thus the region of the highest seismic hazard and risk in the continent. The level of earthquake monitoring in the area is below average compared to Central and Western European standards, despite the progress steps taken during the last period. Monitoring earthquake activity is the first step towards seismic hazard assessment, in order to finally optimise preparedness and response planning. Unfortunately, the limited cooperation between seismological institutions and the lack of data exchange between existing seismic networks lead to further limitations in seismic activity monitoring, especially within the border regions. Even though it is well known that political borders do not exist especially for natural hazards. For a complete assessment of the seismic hazard, all the available seismic information is needed, thus the present network-project tried to include collaborative monitoring from different networks: seismograph networks, accelerograph networks, macroseismic observation systems and GPS networks.

The main objective of the present network-project was to establish collaboration rules among the seismological community of SE Europe for homogenised multi-parametrical monitoring of earthquake activities, despite the different instrumentation, the different institutions involved and the different methods used, aiming at the development of common databases, easily updated, including all available seismic information. A comprehensive long-term collaboration plan for the standardisation of earthquake monitoring was established, concerning seismology and engineering seismology for the improvement of the earthquake preparedness especially in the border regions of South Eastern Europe.

The partners involved in this network project were:

- National Observatory of Athens, Institute of Geodynamics – Greece (Coordinating Partner)
- Institute of Seismology – Albania
- Seismological Survey of Serbia – Serbia
- Federal Meteorological Institute – Bosnia and Herzegovina
- Hydrometeorological Institute of Republic Srpska – Bosnia and Herzegovina
- National Institute for Earth Physics – Romania
- University of Zagreb – Croatia
- Seismological Observatory – Montenegro
- Geophysical Institute – Bulgaria
- Geodetic and Geophysical Research Institute – Hungary
- Institute of Geology and Seismology – Moldova
- Faculty of Natural Sciences and Mathematics – FYR of Macedonia
- Istituto Nazionale di Geofisica e Vulcanologia – Italy

Two main workshops and webpage, all dedicated to a new collaborative effort, are the main deliverables of this project. A full agreement of long-lasting collaboration is underway to be signed by all participants. It should be noted that neighbouring partners such as Italy, Moldova, and Slovenia, that have not entered the CoSEESNeT have agreed to sign and fully collaborate.

2 Pilot Project Network Activities

As it is mentioned above, the present network project involved mainly two workshops. These were the main tools to bring the partners together, exchange up to date information about their efforts for multi-parametric seismic monitoring and try to define rules of collaboration, that have so far not allowed the extensive data exchange that is needed in the region. In a second step, an agreement was established for long-term collaboration and a webpage was prepared to host and present results and future achievements, as well as to facilitate the data exchange activities between partners.

The two workshops were: (a) first in Athens June 5-6, 2008 hosted by the National Observatory of Athens and (b) second in Belgrade September 25-26, 2008 hosted by the Seismological Survey of Serbia. The CoSEESNeT project web page is part of web page of the new broadband seismic network operated by the National Observatory of Athens, Institute of Geodynamics, which is part of the new Unified National Seismic Network in Greece: http://bbnet.gein.noa.gr/CoSEESNet/welcome.html.

2.1 First Workshop (Athens, Greece, June 5–6, 2008)

The main objective of the first workshop organised by the coordinating partner NOA-IG in Athensduring on June 5-6, 2008, was to exchange information on the current state of seismic networks in SE Europe. Moreover, emphasis was given to the indication of acceptable ideas for bilateral or multilateral collaboration between partners. In addition to the seismic networks, accelerometric networks, GPS networks and macroseismic data collection and analysis systems were presented and discussed.

Although a more detailed analysis on the current state of networks in SE Europe follows, it should be noted that the main focus should be on homogenising data acquisition and establishing a common interface/platform allowing real-time data exchange between partners.

Round table discussions held at the first workshop supported the preparations of the second workshop. The latter was intended to follow and finalise this pilot network project, with general, practical and technical information needed to be available by then, in order to make a more concrete decision with secure recommendations for bilateral/multilateral collaborations that should then be initiated.

2.1.1 Real time Seismic Monitoring in SE Europe

Seismic networks were presented in the first workshop in Athens (Figure 1). Presentations as well as information on all partners and the networks they currently operate can be found on the dedicated webpage. They were found to have become denser in the SE Europe countries (i.e. Greece [1], Bulgaria [2, 3], Romania [4], Serbia [5] and Hungary [6]) during the last years. However, some of the areas still host almost no instrumentation

(i.e. Bosnia and Herzegovina [5]). Moreover, the seismic monitoring remains heterogeneous concerning the type of instrumentation and the methods used (maintenance of the instruments, processing of data, storage and databanks). A good number of stations are still analogue implying that routine processing is limited and time consuming, but could be used for education reasons and as a back-up system. Real-time systems require digital stations with real-time data transmission to the processing centre. Broad band seismic stations, implying the qualitative recording of far-field long period waves and the rise of datasets available in the region for the improvement of regional velocity models, are already used by some institutions.

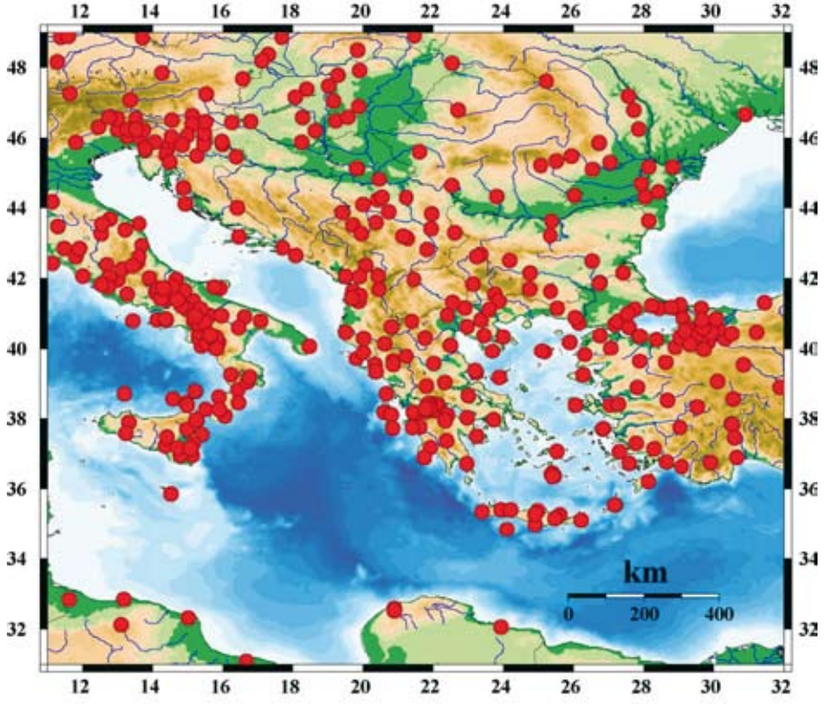

Figure 1: Seismic stations available in SE Europe and the adjacent region

The main objective for establishing collaboration was to find ways to agree on a platform that will allow real-time data exchange. It was decided to start trials of data exchange between partners using SeisComP shareware software and the appropriate SeedLink protocol, which is widely accepted in the European Seismological Community. However, major improvements and further computing support are needed to have spare computer servers to be used for such operations, even on a trial basis, and to plan the future establishment of such links. Furthermore, internet connections need to be improved in order to facilitate free connections and link between partners that will exchange data in real time mode

2.1.2 Accelerometric Networks in SE Europe

The accelerographic networks in SE Europe are not yet characterised by their density. One example of a dense local strong motion array is the one developed in the broader area of Athens, central Greece [7]. The majority of the instruments are digital but of different types. Usually, accelerographs are deployed in urban areas and they are portable units without immediate access during a seismic activity, when they are most useful for immediate evaluation and further results.

Telemetry connections only exist for some of the sites (leased lines, internet, satellite, GPRS). Different methods for data processing are used, mainly oriented at the instrument types. It is important to note that no appropriate databases have been built solely from accelerometric data. An example of a unified strong motion database was presented for Greece, which is named HEAD [7] and it is available by the National Observatory of Athens, Institute of Geodynamics, but reports earthquakes of the period 1973-1999 and includes mainly data from analogue instruments [7].

The main objective is to prepare further databases similar to HEAD for other urban areas. It is also important to establish new digital accelerometric networks in the region. This was seen by all partners and it was also shown that such efforts are on the way. Countries such as Romania, Greece and Bulgaria are trying to establish such networks.

2.1.3 GPS Monitoring activities in SE Europe

A limited amount of GPS stations exists in the region. Surveys have been executed from time to time and new approaches for differential GPS real time networks are in need. Attempts have been made lately by some institutions to include GPS stations in the same site where seismological observations in real time exist. Homogeneity of data recovering and databases still needs to be established.

Presentations of limited efforts were shown, however there are new efforts on the way in countries such as Montenegro, Croatia, Bulgaria, Greece and Romania. Some of them are just starting to establish a GPS network, others continue to expand and convert to real time continuous GPS networks.

2.1.4 Macroseismic collection and processing systems in SE Europe

Evaluation of the macroseismic observations is done using different intensity scales. Different methods of collecting macroseismic observations exist, but they are generally based on posted questionnaires and evaluation of the replies by expert scientists. The usefulness of the macroseismic observations is stated by the scientists and they are directly connected to the strong ground motion at a site and the attenuation models. Web sites for different languages and questionnaires available to the public via internet have yet to appear in the region. However, an attempt is made by Greece and the National Observatory of Athens, Institute of Geodynamics to develop such tools and to evaluate replies automatically via the internet.

2.2 Second Workshop (Belgrade, September 25–26, 2008)

The main objective of the second workshop organised by the Seismological Survey of Serbia in Belgrade, during a two day period (September 25-26, 2008), was to add information by partners missing at the first workshop and to re-evaluate the current state of the seismic networks in the region of SE Europe. More technical information was made available by all partners in order to find appropriate ways to start real-time exchange of seismic data and to plan other possible ways of exchanging GPS and accelerometric data. Moreover, emphasis was given to establish an agreement between partners towards a long-lasting collaboration in real-time data exchange through either bilateral or multilateral MoU as a well as an overall MoU agreement between all parties.

The establishment of a pilot seismic network facilitating access and exchange of data in real time between all partner institutions during a trial period was addressed. The establishment of troubleshooting, technical investigations for problem solving and recommendations towards specific problems raised during this operation was considered appropriate and useful. Partners from Greece, Italy and Romania agreed to make facilities at their institutions available and establish this common pilot seismic network consisting of real-time seismic stations, a few from each partner country. The data will be made available to the three institutions concerned through internet links and they will operate three different application systems available at their facilities. Results will be made available only to all CoSEESNet partner countries and they will recommend appropriate changes towards a homogeneous approach by all partners.

A common design for the accelerometric databases currently available was circulated. All parties agreed to take into consideration such a design and try to apply it for their individual cases. A common approach that can establish a common system for presentation to the public was also agreed upon for the collection of macroseismic data and their analysis.

2.3 Collaborative Network Dedicated Webpage

During the second workshop in Belgrade (September 25-26, 2008), the partner/coordinator NOA-IG presented a newly developed webpage hosted at: http://bbnet.gein.noa.gr/CoSEESNet/welcome.html. It was widely accepted by all partners and it was decided to construct further parts of the webpage and plan to establish it, as an eu or org dedicated node for future use. Material from the first workshop was integrated in the webpage and information by all partner Institutions was collected for future posting at this webpage. All activities by the collaborative parties will be introduced in this webpage, which will be supported by NOA-IG.

3 Results and Discussion

The present pilot project network brought together the majority of the countries involved in the SEE-ERA.NET scheme with their leading institutions towards a long-lasting collaboration in a common seismic multi-parametric monitoring network for the SE Europe region (see introduction for a full list of partner institutions). Neighbouring countries (i.e. Italy, Moldova) have also participated with their leading institutions in the earthquake monitoring field and provided their support and expertise in the present collaborative network. The institutions involved agreed to sign a Memorandum of Understanding (MoU) and using their own resources to establish collaboration useful for the entire region.

Discussions and exchange of experiences took place and helped to establish the present network agreement, which is under circulation for the official approval and signing by all parties. Ideas, experiences and problems faced in the past were taken into consideration, this was very useful and appropriate, and facilitated this MoU agreement.

It is important to note that such meetings were agreed to take part every year and thus, to keep this collaboration alive. It was also noted that the technical and responsible staff of the institutions, operating such monitoring networks in the region, will need to come to workshops or summer schools that will give them the opportunity to introduce themselves to new techniques and experiences. Such schools should be organised in the region.

Finally, SeiComP [8], a European developed freeware software (developed and supported at GFZ, Potsdam by the GEOFON Group) that is also supported by the leading institutions in Europe in this field, was chosen as a common platform for real time seismic data exchange.

4 Conclusions / Outlook, next steps

CoSEESNet has become a newly established collaborative network for SE Europe, thanks to the present pilot network project. It has brought to an agreement all major institutions in the region concerning earthquake multi-parametric monitoring. Future proposals will include presently involved partners in other opportunities for further development in the region. The cooperation will include yearly meetings to keep the present collaboration and possible summer schools to exchange knowhow and expertise in the field of multi-parametric earthquake monitoring in the region.

Finally, the pilot network in operation between the three major partners (Greece, Italy and Romania) will allow extensive troubleshooting on shareware dedicated software and will serve towards preferred cost-effective suggestions and solutions for the other partners. Summer schools will follow to serve the above mentioned needs.

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Definition of Research Needs on the Identification, Prediction and Surveillance of Emerging and Re-emerging Zoonoses in the West Balkan Area

Svetomir Samardzic[1], Tatjana Marinkovic[2], Bogdanka Andric[3], Aleksandra Milic[4], Dragan Marinkovic[5]

[1] Faculty of Medicine, University of Pristina/Kosovska Mitrovica, Anri Dinan Nr 1, 38220 Kosovska Mitrovica, Serbia; svelja@eunet.yu
[2] Sanitary-Medical School VISAN, Tosin bunar 7a, 11000 Belgrade, Serbia; tatjana.marinkovic@hotmail.com
[3] Faculty of Medicine, University of Montenegro, Dept. of Infective Diseases, Ljubljanska st, 81000 Podgorica, Montenegro; bogdanka@cg.yu
[4] Faculty of Medicine, University of Pristina/Kosovska Mitrovica, Anri Dinan Nr 1, 38220 Kosovska Mitrovica; Serbia
[5] Faculty of Medicine Foca/University of East Sarajevo, Studentska 5, 73300 Foca, Bosnia and Herzegovina; dragan.marinkovic@hotmail.com

Abstract

According to the World Health Organization (WHO), most of the emerging diseases around the world are zoonotic, diseases transmissible between animals and humans. Zoonotic diseases continue to occur in many countries, especially in the developing world, where they mostly affect the poorest segment of the human population and cause a serious amount of deaths and millions of affected people every year. All major zoonotic diseases prevent the efficient production of food of animal origin and create obstacles to international trade in animals and animal products. Thus they are an impediment to overall socioeconomic development. The aim of this study was to identify emerging and re-emerging zoonoses in the territory of Bosnia and Herzegovina, Montenegro and Serbia, thus defining the research needs on identification, prediction and surveillance of emerging and re-emerging zoonoses in West Balkan area. Results of the study pointed to several zoonoses that emerged during the analyzed time period of ten years, suggesting the West Balkan area to be a natural focus for several zoonoses including such of bacterial (i.e Lyme disease, Brucellosis, Q febris), viral (i.e HFRS, viral meningitis) and parasitic origin (i.e. Scabies, Trichinelosis). However, this study revealed a tremendous lack of systematic surveillance of zoonotic diseases in West Balkan area, particularly in the territories involved in war campaigns during the 1990s.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology
Type of the project: research project

Keywords

Chemometric analysis; Infrared spectroscopy; Ionic liquids; Quantum chemistry; Raman spectroscopy; Water

1 Introduction

Zoonoses are diseases transmissible between animals and humans. According to the World Health Organization (WHO), eleven of the last 12 emerging infections in the world with serious health consequences had probably arisen from animal sources. Many of zoonotic diseases are preventable and treatable, yet they continue to occur in many countries, especially in the developing world where they mostly affect the poorest segment of the human population. Beside being a serious risk to human health, zoonoses are leading to severe economic consequences in both developing and industrialised countries. All major zoonotic diseases prevent the efficient production of food of animal origin and create obstacles to international trade in animals and animal products, thus they are an impediment to overall socioeconomic development.

More than 200 zoonoses have been described and they involve all types of etiological agents: bacteria (lyme disease, tularemia, brucellosis), parasites (echinococcosis/hydatidosis, toxoplasmosis), ricketsia and rickettsia-like (typhus, Q fever), viruses (rabies, crimean-congo haemorrhagic fever, haemorrhagic fever with renal syndrome (HFRS), ebola, avian influenza) and unconventional agents (Bovine Spongiform Encephalopathy). Many of these diseases have the potential to spread by various means over long distances and to become global problems. A list of agents that can carry infectious organisms that may be zoonotic includes both domestic and wild animal such are ticks, lice, rodents, birds, but also cats, dogs, goats, cows, horses, etc

Bosnia and Herzegovina, Montenegro and Serbia are South East European countries with approximately 13 million inhabitants, positioned between Central and Eastern Europe on the Balkan Peninsula, close to Mediterranean See. Available data suggest this area to be a natural focus for several different zoonoses. For instance, hemorrhagic fever with renal syndrome (HFRS) seems to be endemic in this area [1-5]. The etiological agent for HFRS is the Hantaan virus that transmits to human through aerosols of excreta from small mammals, mainly rodents that have silent life-long infections [6]. In the Balkan Peninsula, HFRS appears both in form of sporadic cases and of larger outbreaks [1-5]. Most recently large HFRS outbreaks happened in this area during years 1989, 1995 and 2002 [5]. Disease outbreaks are considered to be in correlation with the weather and food abundance conditions that regulate the size of the rodent population in HFRS natural focus areas [5]. There is also an indication that Crimean-Congo hemorrhagic fever may emerge in the territory of the West Balkans. Several cases of this serious disease with a high lethality rate have been reported in Kosovo [7]. Although there is a significant chance for the Crimean-Congo hemorrhagic fever to spread across the Balkan Peninsula, till present no epidemiological analysis of the problem has been performed.

Recently, awareness of rickettsioses is increasing in the Balkan Peninsula. Several pathogenic Rickettsiae are described, including R. typhi (mooseri), the cause of murine typhus, transmitted by fleas; R. prowazekii, the agent of epidemic typhus, transmitted by lice; R. conorii, the agent of Mediterranean fever; R. rickettsii, the agent of Rocky Mountain spotted fever, as well as R. akari; R. astrakhan; R. izraeli; R. tsutsugamushi etc. [8-12]. Large epidemics of typhus occurred in the West Balkans during World War I and II, when the lethality rate of these epidemics was as high as 70% [13-16]. Nowadays, presence of Rickettsiae and rickettsioses is reported in Croatia [17-19], Montenegro (reported at a regional meeting by B. Andric), Bulgaria [20] and countries in the Mediterranean basin [21-25]. In addition, the presence of R. astrakhan in ticks was confirmed in a recent survey performed by the United Nation Forces stationed in the Kosovo region, [26]. The presence of an organism closely related to Rickettsiae, Ehrlichia chaffeensis, an agent of Human Monocytotropic Ehrlichiosis, is also reported in areas of the Balkan Peninsula [27, 28]. Sporadic cases of Lyme disease, a zoonoses caused by the tick-transmitting agent Borrelia burgdorferi, are constantly reported in the West Balkans [29], however, an extended epidemiological analysis of this issue was not conducted. Cases of brucellosis, leishmaniasis, trichinellosis and other zoonoses are constantly being reported in different areas of the West Balkans, further suggesting a need for a systematic investigation of zoonoses issue in this area.

Bosnia and Herzegovina, Montenegro and Serbia are South East European countries representing an important traffic point on the major route from Europe to the Middle East. Therefore, information regarding prevalence of infectious agents in this area exceeds local significance. Unfortunately, although WHO and Veterinary Public Health (VPH) have recognised the needs for surveillance and response to all zoonotic diseases which are or may emerge as public health threats, adequate programs are not established in this area yet.

The aim of this project was the identification of emerging and re-emerging zoonoses and the identification of their natural foci in the West Balkans to precede the project proposal for the control of these diseases.

2 Experimental Techniques and Methods

To identify the emerging and re-emerging zoonoses in the West Balkan areas, a cross-sectional study (prevalence study) was conducted. Data available from respective public health and veterinary health institutions in Serbia, Bosnia and Herzegovina and Montenegro were analysed. The analysis covered the territory of Serbia except Kosovo/UNMIK (88.361 km^2; 7.4 millions of inhabitants), Montenegro (13.812 km^2; 0.6 million of inhabitants), and the Republic of Srpska (an entity of Bosnia and Herzegovina that covers about 50% of the state territory of 51.209,2 km^2; 1.4 million of inhabitants out of 3.9 millions of inhabitants). A map of the region is presented in Figure 1.

The analysis included data from a period of ten years, from 1997–2007. Consortium members from Serbia, Bosnia and Herzegovina and Montenegro were responsible for the collection and statistical analysis of data from referent institutions in the respective countries.

Figure 1: Geographic region of South East Europe

Data were collected from the following governmental institutions:

- Institute of Public Health of Serbia, Dr Milan Jovanovic Batut, Serbia;
- Institute of Health, Montenegro;
- Ministry of Education and Science, Montenegro;
- Veterinary Directorate, Montenegro;
- Veterinary Directorate of the Republic of Srpska, Bosnia and Herzegovina;
- Institute of Public Health of the Republic of Srpska, Bosnia and Herzegovina; and
- Institute of Public Health of the Canton Sarajevo, Bosnia and Herzegovina.

In addition, consortium member visited and collected data from regional and local public and veterinary health institutions.

The data collection was followed by a workshop for the epidemiological analysis of data from referent institutions in consortium countries.

3 Results and Discussion

In the last 15 years, the West Balkan area went through several political crises that ended up with the formation of several independent states. Political disintegration led to disintegration in science. Therefore, all epidemiological studies made recently were prepared on local national levels excluding coordination with lately formed neighbouring states. In order to have a broader view to the magnitude of the zoonotic diseases problem in the West Balkan area, we attempted to collect information regarding zoonoses incidence in this area from official, governmental institutions in Serbia, Montenegro and Bosnia and Herzegovina. At this point, a first major obstacle of our study appeared – accurate data were not available for the complete West Balkan territory planned for the screening.

One of the major issues was the lack of information about the epidemiological status of the territory of Kosovo. Since the year 1999, Kosovo is under United Nations administration (UN Security Council Resolution 1244). Due to the unstable political situation and the ineffectiveness in medical services, a systematic follow-up of infectious disease was not conducted since. This represents a serious threat to public health, as data collected prior to war escalation in 1999 pointed to Kosovo/UNMIK as a natural focus for several different zoonotic diseases. There are clear evidences of constant appearance of HFRS [1-3] as well as the presence of rickettsioses [26, 30]. These facts suggest an urgent need for the establishment of a surveillance system for infectious diseases in Kosovo/UNMIK.

Similarly, due to the current political situation it was not possible to obtain complete data for the state of Bosnia and Herzegovina. This state is politically divided in two administrative entities and one district since the year 1996, when the four year long ethnic conflict was ended. Although one of the entities, the Republic of Srpska, has a functional central institute of public health, The other entity, the Federation of Bosnia and Herzegovina, has not succeeded in establishing a functional institution to survey infectious diseases and make data publicly available. Moreover, the territory of the Federation of Bosnia and Herzegovina entity is additionally administratively divided into nine cantons, each having an independent Cantonal Institute of Health. A central institution of public health responsible for the whole state of Bosnia and Herzegovina has not been established to date. Thus, we collected and presented data for the entity of the Republic of Srpska that represents around 49% of the area of Bosnia and Herzegovina and 1.4 millions of inhabitants out of a total population of 3.9 millions. Together with the data for Serbia (except Kosovo/UNMIK), covering 7.4 millions of inhabitants, and the data for Montenegro with 0.6 million of inhabitants, our study included just below 10 millions of people situated in the centre of the Balkan Peninsula. Geographically, the terrain of Serbia varies from the north which is rich fertile plains; to the east, limestone ranges and basins; to the southeast, ancient mountains and hills. Bosnia and Herzegovina is mostly characterised by mountains and valleys. The territory of Montenegro has highly indented coastline with a narrow coastal plain backed by rugged high limestone mountains and plateaus. The climate also varies from continental climate in most of Serbia and Bosnia to mediterranean climate in most of Montenegro and Herzegovina.

The analysis of publicly available data on zoonotic diseases in the area of Serbia, Montenegro and Bosnia and Herzegovina during time period from 1997 to 2007 revealed that zoonotic diseases were reported in more than 65 000 cases. The absolute number of cases for different zoonoses is presented in Table 1.

Results of the study pointed to several zoonoses that have arised during the last ten years time in the analysed territory, including such of bacterial (i.e. Lyme disease, Brucellosis, Q febris), viral (i.e. HFRS, viral meningitis) and parasitic origin (i.e. Scabies, Trichinelosis). Unfortunately, information the presented in Table 1 also reveals that some data regarding appearance of particular zoonoses in the West Balkan countries are often missing. Although, in case of some zoonoses with a low incidence rate, such as Congo-Crimean hemorrhagic fever (CCHF) or paratyphys, one can conclude that the nonappearance of

Table 1: The absolute number of zoonotic disease cases from 1997 to 2007 in the West Balkan area

	Salmonellosis	Scabies	Lyme disease	Trichinellosis	Enteroviral meningitis	Shigellosis	Pediculosis	Mycosis
Serbia	22488	8122	5652	3899	2547	2771	no data	no data
Montenegro	2578	6979	32	293	763	no data	1492	764
BiH	1271	no data	174	590	no data	no data	no data	no data
total	26337	15101	5858	4782	3310	2771	1492	764
	Q fever	Leptospirosis	Lamblia-sis	Amoebiasis	Toxoplasmosis	Brucellosis	Campylobacteriosis	HFRS
Serbia	213	330	663	498	464	379	371	210
Montenegro	5	2	no data	no data	5	7	no data	81
BiH	530	374	no data	no data	no data	no data	no data	60
total	748	706	663	498	469	386	371	351
	Echinococcosis	Tularemia	Tetanus	Malaria	Botulism	Ascariasis	Viral encephalitis ixodibus	Leishmaniasis
Serbia	291	295	176	97	85	no data	7	24
Montenegro	32	0	1	22	12	72	57	40
BiH	15	16	no data	no data	no data	no data	no data	no data
total	338	311	177	119	97	72	64	64
	Typhus abdominalis	Antrax	Psittacosis ornithosis	Listeriosis	Yersinia enterocolitica	CCHF	Paratyphus	Bartonellosis
Serbia	7	17	16	7	5	no data	1	no data
Montenegro	16	no data	no data	no data	no data	1	no data	1
BiH	no data	no data	no data	no data	no data	no data	no data	no data
total	23	17	16	7	5	1	1	1

registered cases is due to the fact that disease did not occur in this period. However, it is important to know that the nonappearance of cases may also reflect a flaw in the recognition and reporting of the diseases. We are aware that plenty of zoonotic diseases with mild clinical symptoms are often not properly diagnosed by local medical personal. Even if diagnosed, they are often not reported to the central public health institution. Having in mind that to a large extent, the medical service collapsed with the outbreak of civil war in former Yugoslavia in the early 1990s, this is not that surprising. Public health is not fully recovered in all areas of the West Balkans, and it is still greatly influenced by the unstable political situation.

Nevertheless, regarding the zoonoses problem, it seems that the Balkan area represents one undividable neighbourhood since vector borne diseases do not recognize official state borders. All Balkan countries share a similar climate, topography, and historical and cultural background. Furthermore, during the last conflict in ex-Yugoslavia, a large migration and redistribution of population occurred. It is still not clear what the epidemiological impact of this migration process is. Data presented in this and previous studies are suggesting the West Balkan area to be a natural focus for several zoonoses such as Lyme disease, HFRS, Q fever, brucellosis etc. We have compared the incidence rate for these diseases in Serbia, Montenegro and parts of Bosnia and Herzegovina for the time period of 1998 to 2005, since this was most reliable data period for all tree states, and we have observed similar trends in disease appearance (Figure 2).
Most of analysed diseases were constantly present in all tree countries, with larger outbreaks from time to time. The appearance of larger epidemics in one but not the other two countries could be explained by geographical and infectious agent-related specificities. However, nonappearance of brucellosis cases in humans in Bosnia and Herzegovina (Figure 2) could be questioned, as this disease is readily reported in animals by the Veterinary Directorate of Bosnia and Herzegovina (data not shown). The comparison of literature data with data available from official institutions for the control of infection diseases also shows significant discrepancies. For instance, while there is a report describing the appearance of Ehrlichioses in Serbia [31], this information is not presented in the reports of referent national institution. This is not an isolated case and, again, it points to flaws in the disease registration and reporting system.

4 Conclusion

Our data analysis suggests an urgent need for the establishment of a functional system for the zoonoses surveillance and control in the area of the West Balkans. It is necessary to define a precise map of natural foci for emerging zoonoses for Serbia, Bosnia and Herzegovina and Montenegro that would be a key reference for all further actions on zoonoses surveillance and control. However, mapping of zoonotic natural foci in West Balkan areas exceeds local significance, as this area re-appears as an important European traffic point since the calming of the Balkan conflicts. Thus, wider international collaboration is required, particularly with the neighbouring Balkan countries as such Croatia, Albania, the FYR of Macedonia and Bulgaria, but also with other states in the Mediterranean basin such as Greece, Italy, France and Spain.

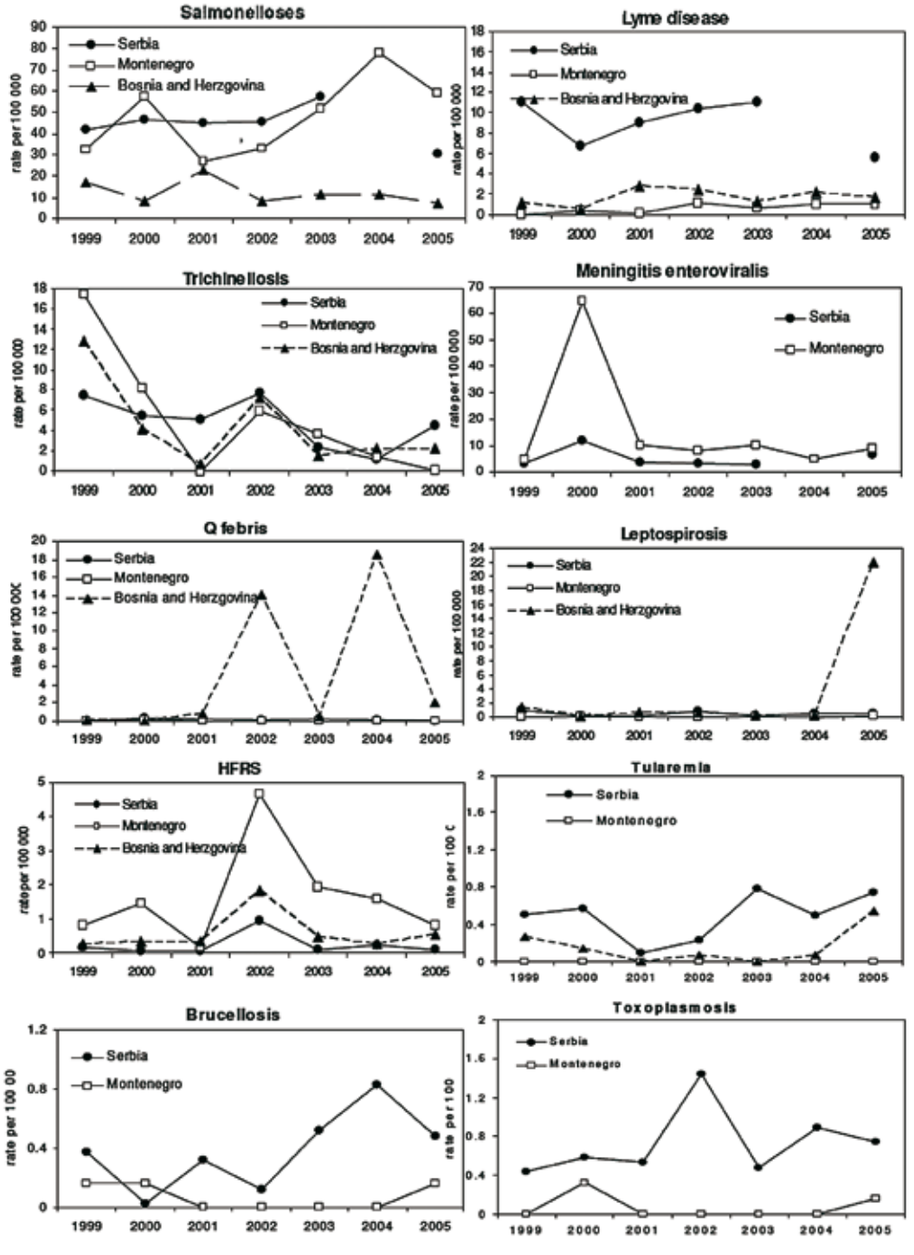

Figure 2: Trends in disease appearance

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Development of a Non-toxic, Ecologically Compatible, Natural-resource Based Insecticide from Diatomaceous Earth Deposits of South Eastern Europe to Control Stored-Grain Insect Pests

Christos Athanassiou[1], Nickolas Kavallieratos[2], Bill Vayias[3], Zeliko Tomanovic[4], Andjeljko Petrovic[5], Stanislav Trdan[6], Cornel Addler[7], Vlatka Rozman[8]

[1] Agricultural University of Athens, Department of Plant Science, Laboratory of Agricultural Zoology and Entomology, 75 Iera Odos, 11855 Athens, Greece; athanas@aua.gr
[2] Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 8 Stefanou Delta str., 14561 Kiffissia, Attica, Greece; nick_kaval@hotmail.com
[3] Agricultural University of Athens, Department of Plant Science, Laboratory of Agricultural Zoology and Entomology, 75 Iera Odos, 11855 Athens, Greece; bvayias@aua.gr
[4] Belgrade University, Faculty of Biology, Department of Zoology, 16, Studentski 11000, Belgrade, Serbia; ztoman@bf.bio.bg.ac.yu
[5] Belgrade University, Faculty of Biology, Department of Zoology, 16, Studentski 11000, Belgrade, Serbia
[6] University of Ljubljana, Biotechnical Faculty, Department of Agronomy,101 Jamnikarjeva, 1111, Slovenia; stanislav.trdan@bf.uni-lj.si
[7] Federal Biological Research Centre for Agriculture and Forestry, Institute for Stored Product Protection, 19 Königin-Luise-Straße, 14195, Berlin-Dahlem, Germany; c.adler@bba.de
[8] Department for Plant Protection, University of Josip Juraj Strossmayer in Osijek, 3 Trg Sv. Trojstva, 31100 Osijek, Croatia; vrozman@pfos.hr

Abstract

Diatomaceous earth (DE) deposits from regions of Southeastern Europe were evaluated as regards their insecticidal effect against several insect pests that infest stored products. Fifteen DE deposits were obtained from different geographical locations in Greece (Crete, Elassona), Serbia (Kolubara, Begora, Vranje), the Former Yugoslav Republic of Macedonia (FYR of Macedonia), Slovenia and Germany. Influence factors such as insect species, temperature, relative humidity, commodity, size of DE particles and exposure interval were assessed with regards to their impact on the efficacy of the local DEs. In a separate study, two different classes of wheat were treated with Silicosec under two different ways (dusting and spraying) and effectiveness of each application method was evaluated. Local DEs were overall less effective than the commercial formulation Silicosec which was used as positive control. Efficacy of the local DEs was enhanced at increased temperatures and longer exposure intervals while it was negatively related with increasing r.h levels.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology
Type of the project: research project

Keywords

Management of biological resources; Plant health & Crop protection; Sustainable production

1 Introduction

One of the most promising alternatives on the use of traditional pesticides in stored grains is the use of diatomaceous earths (DEs). DEs act in the insects' exoskeleton (cuticle) causing rapid desiccation resulting in death through water loss [1]. They are non-toxic to mammals (rat oral LD_{50} >5000 mg/kg of body weight), leave no toxic residues on the product and according to the US Environmental Protection Agency (EPA) they are classified in the category of GRAS (Generally Recognized As Safe) since they are used as food or feed additives [2]. DEs can be applied with the same application technology as traditional grain protectants, which mean that no specialised equipment is required [3]. Moreover, since they are inert (silicaceous) materials, no interaction with the environment occurs. DEs are composed by the fossil skeletons of phytoplanktons, also known as diatoms, which occur in fresh and salt water since the Eocene period and produce a soft sedimentary rock, which is composed mainly by amorphous silica (SiO_2 + H_2O). The DEs currently mined vary remarkably in their insecticidal activity, depending upon species composition, the geological and geographical origin as well as certain chemical characteristics, such as SiO_2 content, pH and tapped density [4, 5].

The search of newer, naturally-occurring DEs that are more effective in insect control is still in progress, especially in areas rich to silicaceous rocks. Korunic [4, 5] in an extensive screening of DEs from several parts of the world, found that local DEs from former Yugoslavia were very effective, and could be used with success against stored-grain pests. Similar results have been reported by Indic et al. [6] for certain DEs from Serbia. Despite their advantages, the use of DEs in stored-product protection remains rather limited, due to their main drawback: DE application reduces grain bulk density (volume/weight ratio). For a satisfactory level of efficacy, the commercially available DE formulations should be applied at doses between 400 and 1000 ppm [7]. Many researchers underline the need for using new DEs, which are effective at low dose rates. Some newer DEs have been already evaluated with promising results. For instance, Athanassiou et al. [8] noted that two new enhanced DEs were very effective at dose rates as low as 75 ppm.

Considering the increasing global focus on the safety of pesticides on human health and the environment, the development and assessment of suitable replacements of traditional contact insecticides, is of great importance. The development of a natural-resource-based integrated pest management (IPM) program to which new effective DEs are involved, would help adoption of a judicious control protocol in stored grains. However, the newer DEs – besides their effectiveness against stored product pests – should be also tested as regards their impact on grain properties. In our series of experiments, the efficacy of several DEs originating from different geographical locations of Southeastern Europe was assessed against serious stored product beetle pests such as the primary colonisers; rice weevil Sitophilus oryzae (L.) (Coleoptera: Curculionidae), lesser grain borer Rhyzopertha dominica (F.) (Coleptera: Bostrychidae) and the seconday colonisers; confused flour beetle Tribolium confusum (Duval) (Coleopetra: Tenebrionidae), red flour beetle T. castaneum (Herbst) and rusty grain beetle Cryptolestes ferrugineus (Steph.) (Coleoptera: Cucujiidae).

2 Experimental Techniques and Methods

2.1 Effect of temperature and r.h. on the efficacy of local DEs

Fifteen local DEs, which were collected during automn 2007, originating from Greece (Begora, Crete, Elassona 1, Elassona 2), Serbia (Kolubara 516, Kolubara 517, Kolubara 518, Kolubara middle, Upper Serbia, Lower Serbia, Vranje, Vranje 311207), Slovenia, the Former Yugoslav Republic of Macedonia and Germany, and one commercially used DE formulation, Silicosec (BioFa GmbH, Munich, Germany), which is a DE of fresh water origin and contains 91.2% SiO_2, were assessed as regards their effectiveness on hard wheat (var. Mexa), against adults (<21d old) of S. oryzae, R. dominica and T. confusum. Upon the beginning of the tests, all DE samples were dried out to about 6% moisture content, at 40° C for 24 h (Korunic, 1997). The wheat that was used for experimentation was untreated and had very little dockage (<0.8%). Before experimentation, the moisture content of the grain was determined by Dickey John moisture meter (Dickey-John Multigrain CAC II, Dickey-John Co, Lawrence, KS, USA) and ranged 11.1 - 12.0%. DEs were applied at 900 ppm on the tested commodity and their effectiveness was assessed at three temperature levels; 20 °C, 25 °C and 30 °C and two humidity levels; 55 ± 5% and 65 ± 5%. Mortality was assessed after 7d of exposure of the individuals on the treated wheat samples or control. The progeny production of the treated individuals was also assessed. Data were analysed separately for each species, according to the GLM procedure of SAS [9] with Abbott [10] mortality as the response variable and DE (local DEs and Silicosec), temperature and r.h. as the main effects. For the comparison of the means, the Tukey – Kramer (HSD) test was used [11]. In the case of progeny production data, Dunett's test was also used to compare progeny between treated and untreated wheat.

2.2 Effectiveness of the local DEs to different grain commodities

The DE deposits that were used in this series of experiments are described in the 2.1 paragraph. The tested grains were untreated hard wheat (var. Mexa), maize (var. Dias), whole (raw) barley (var. Persephone) and peeled rice (var. Thaibonnet). The moisture content of each grain was determined by a Dickey John moisture meter (Dickey-John Multigrain CAC II, Dickey-John Co, Lawrence, KS, USA) and ranged 10.8 - 12.2%. 1-Kg lots of each grain were treated with 500 ppm and 1000 ppm of DEs (local DEs and Silicosec) and their efficacy against adults of S. oryzae, R. dominica and T. confusum was assessed after 7d of exposure at 25 °C and 65% r.h. Also, progeny production of the exposed species on the

treated and untreated commodities was assessed. The analysis of data is similar to that described in paragraph 2.1.

2.3 Effect of particle size in the efficacy of the local DEs

This series of experiments was carried out by the Croatian and Greek team. The tested DEs are those described in paragraph 2.1, except for the FYR of Macedonia and Germany. All DE samples were dried out to about 6% moisture content and sieved (dry sieving) with the USA standard testing laboratory sieves with opening of 150 and 45 microns. Initially, for each DE sample 2 fractions of particles were obtained: >150 microns and 0-150 microns. Following, the fractions from 0-150 microns were again sieved to prepare an additional 2 fractions: from 45-150 microns and <45 microns. The particles with diameter above 150 microns were not used in bioassay because this fraction usually contains sands, rocks and only a few very large diatoms. The various fractions of DE at 600 ppm were added to hard wheat and efficacy of each fraction was evaluated against adults of C. ferrugineus, S. oryzae and R. dominica after 7d pf exposure at 30 °C and 70% r.h. Data were subjected to one-way analysis of variance (ANOVA) according to the GLM Procedure of SAS [9]. Means were separated using the Tukey - Kramer (HSD) test at P= 0.05 [11].

2.4 Effectiveness of applying DE as slurry and impact on grain bulk density

The DE that was tested here was the commercially available formulation, Silicosec, which was applied on hard red spring wheat and durum wheat by two different methods, dusting and spraying. The application rates for both methods were 300 ppm and 500 ppm. In order to prepare aqueous DE suspensions, 30 mg or 50 mg of DE were added to 1.5 ml of distilled water each time. After reconstruction, two 1-Kg lots of each wheat variety were sprayed with each DE suspension. By this way each wheat variety was sprayed with 300 and 500 ppm of DE respectively. Additionally, two 1-kg lots of each wheat variety were dusted with 300 ppm and 500 ppm of Silicosec. Also, 1 kg lot of each wheat variety was remained untreated and served as control. Efficacy of each application method was assessed against adults of S. oryzae and T. castaneum at 25 °C and 55 ± 5% r.h. Mortality was assessed after 5d, 7d and 14d for S. oryzae and after 14d and 28d for T. castaneum. Progeny was assessed only in the case of S. oryzae. In order to determine the impact of the above DE-application methods on bulk density (test weight) of grain, two 1-Kg lots of hard red spring wheat were sprayed separately with 10 mg (100 ppm) or 30 mg (300 ppm) of DE in a 2-ml aqueous suspension. Also, two 1-Kg lots of grain were dusted with 100 ppm and 300 ppm of DE respectively. The samples were held at 25 °C and 55% rh for 25d, and their test weight (Kg/Hl) as well as moisture content were determined 30 min, 6d and 25d post treatment. On the sixth and 25th day after treatment, the samples were shaken for 30 sec to initiate the unloading and loading of the grain. The moisture content of the samples was determined with a moisture, meter model 919 (Labtronics, Mb Canada). In both series of experiments, data were subjected to one-way ANOVA. Means were separated by the Tukey–Kramer (HSD) test at P= 0.05 [11].

2.5 Effectiveness of local DEs at surface treatment

The tested DEs were those described in paragraph 2.1 except for the FYR of Macedonia. The tested DEs were added in glass Petri dishes (5.5 cm in diameter) at three different application rates; 5 g/m^2, 10 g/m^2 and 20 g/m^2. Five Petri dishes were treated with each dose of each DE, whereas five Petri dishes remained untreated and served as controls. Then, 10 adults of T. confusum were inserted into each (treated or untreated) of the Petri dishes. To prevent insects from escaping, the treated (or untreated) dishes were covered with an empty glass Petri dish. Mortality of the exposed adults, was measured on a daily basis for a total period of 19d at 25 °C and 65% r.h. Additionally, in the case that DEs were applied at 10 g/m^2, progeny larvae from the parental individuals after 19d of exposure was also measured. To examine variations in mortality of T. confusum adults among the exposure periods, data were initially subjected to Repeated Measures Analysis [12]. Next, data were subjected to one-way ANOVA for each application rate and exposure combination. Means were separated by the Tukey–Kramer (HSD) test at P= 0.05 [11].

3 Results

3.1 Effect of temperature and r.h on the efficacy of the local DEs

Mean efficacy of the local DEs against adults of S. oryzae, R. dominica and T. confusum after 7d of exposure ranged 73.0%–83.3%, 68.6%–85.7% and 30.0%–72.2% respectively. Of the local DEs that were tested, the FYR of Macedonia, Elassona 1, Kolubara 517 and Kolubara 518 were the most effective against S. oryzae. The FYR of Macedonia, Kolubara 517 and Kolubara 518 were among the most effective DEs also in the case of R. dominica, while in the case of T. confusum, the most effective DEs were Upper Serbia and the FYR of Macedonia. On the other hand, Crete was the least effective DE in the case of S. oryzae and T. confusum whereas adults of R. dominica were shown to be less susceptible in Slovenian DE compared to the remainder DEs. Of the tested species, T. confusum was the less susceptible to the local DEs and Silicosec whereas S. oryzae was the most susceptible species. Silicosec was generally more effective than the local DEs against the tested species. Increase of rh overall decreased DE efficacy against all of the tested species. Nevertheless in the cases of (a) Begora and Elassona 1 against S. oryzae and (b) the FYR of Macedonia against R. dominica, significant differences in efficacy levels of the above DEs were not recorded between the tested r.h. Contrary to r.h., efficacy of the local DEs was increased with increasing temperature and this fact was noted to all of the tested species. At 20 °C, efficacy of the local DEs against adults of S. oryzae, ranged between 75.4% (the FYR of Macedonia) and 52.3% (Crete) while it was >93% at 30 °C for all local DEs. In the case that R. dominica adults were exposed for 7d on wheat treated with the local DEs, mortality ranged between 28.9 % (Slovenia) and 60.6 % (Kolubara 518) at 20 °C and didn't exceed 96.7% at 25 °C. At the same exposure interval, more than 93% of R. dominica adults were dead on wheat treated with the local DEs at 30 °C. In the case of T. confusum mortality didn't exceed 52.7% after 7d exposure of adults on wheat treated with the local DEs at 20 °C, but higher efficacy levels of the tested DEs were obtained with increasing temperature. Local DEs or Silicosec were not able to suppress progeny production of S. oryzae and R. dominica on wheat. However, progeny production of the above species was significantly lower to treated wheat than control. In the case of T. confusum, very few progeny (<1.6 adults / vial) were recorded to either control or treated commodity and thus, significant differences in progeny production of this species between control and treated wheat were not recorded.

3.2 Effectiveness of the local DEs to different grain commodities

Mortality of all species on the grain commodities treated with the local DEs or Silicosec, was generally increased with the increase of dose rate. Among the tested species T. confusum was the most tolerant while S. oryzae the most susceptible in local DEs or Silicosec regardless of dose rate, or commodity.

In the case of S. oryzae, more weevils were dead on treated barley or wheat in comparison with treated maize or rice. Generally, more weevils were dead after exposure on treated barley than wheat. However, significant differences in the performance of the local DEs were not always recorded between the tested commodities. DE performance against S. oryzae was lower in maize than rice and this fact was noted to the majority of the tested cases. In the case of maize treated with 500 ppm, efficacy of the tested DEs ranged 7.3% (Crete) – 29.6% (Kolubara 517) after 7d exposure while it ascended to 27.5% and 57.6% respectively with the increase of application rate. On the contrary, performance of the tested DEs was higher than 73% on wheat treated with 1000 ppm but did not exceed 32% at 500 ppm .

Similar trends among different grain commodities were drawn also in the case of R. dominica. Hence, the highest performance of the local DEs and Silicosec against adults of the lesser grain borer, was observed in treated barley, whereas the lowest performance was observed in treated maize. After 7d exposure of lesser grain borer on commodities treated with 500 ppm of the local DEs, adult mortality didn't exceed 71.6% (Upper Serbia), 53.8% (Begora), 18.2% (Germany), and 43.6% (Kolubara 518) in the case of treated barley, wheat, maize and rice respectively. With the increase of dose rate to 1000 ppm, a noticeable increase in mortality was observed in the treated commodities. Hence, after 7d exposure of adults of R. dominica on the commodities treated with 1000 ppm of the local DEs, mortality didn't exceed 88.9 % (FYR of Macedonia), 43.8% (FYR of Macedonia) and 69.1% (Vranje 3112107) on treated wheat, maize and rice respectively, whereas it ranged 88.0% (Begora) – 98.4 % (Kolubara middle) on treated barley.

The grain type also influenced the efficacy of the tested DEs against adults of T. confusum similarly as above described for the previous species. However, confused flour beetle appeared to be more tolerant than the previous species to all DEs. Thus, , low mortality levels were recorded with both dose rates of the local DEs after 7d exposure of beetles on the treated grains, with the exception of Upper Serbia and the FYR of Macedonia. In the latter case, efficacy of Serbia upper ranged 22.0%- 36.0% and 50.0 - 64.0% among the tested grains treated with 500 ppm and 1000 ppm respectively. The highest mortality of this species (85.6%) was observed to barley treated with 1000 ppm of Silicosec, whereas efficacy of the same dose of Silicosec on the remainder commodities ranged 58.2 % – 73.6%. Local DEs and Silicosec didn't suppress progeny production of S. oryzae or R. dominica from the treated commodities. However, in the majority of the tested cases, significantly more F1 adults were counted to the treated substrates in comparison with controls. In the case of T. confusum very few progeny were counted to either treated or untreated commodities, and thus, only in very few cases progeny production in treated substrates significantly varied to that was recorded in control groups.

3.3 Effect of particle size in the efficacy of the local DEs

Six days post treatment, with the exception of Begora, Kolubara 516, Lower Serbia and Vranje, which were significantly less effective than Silicosec against C. ferrugineus adults, all the remaining DEs were equally effective to Silicosec. DE fractions containing the smallest particles were overall more effective against this species compared to fractions with the largest particles, although significant differences between the fractions <45 microns and 0-150 microns were not always recorded (e.g Lower Serbia and Vranje). In the case of S. oryzae, effectiveness of DE fractions with small particles was significantly higher than that of fractions with the largest particles except for the fractions of Silicosec, which were 100% efficacious. The lowest mortality levels were noted in wheat treated with Begora, where less than 20% of weevils were killed, even with the fraction containing the smaller particles. Of the fractions containing the large particles (45-150 microns) the most effective belonged to Kolubara 518, where rice weevil mortality reached 89.6%. By contrast, the most effective of the non-commercial DEs with particles <45 microns was Kolubara middle (98.2%) followed by Kolubara 518 (97.6%) and as a result, significant differences between those DEs and Silicosec were not recorded. This was the only case that significant differences in efficacy of local DEs and Silicosec were not recorded against S. oryzae, since the remaining DEs were consistently less effective than Silicosec against this species. All tested local DEs were significantly less effective than Silicosec against adults of R. dominica and this was noted for all their fractions. Significantly more beetles were dead on wheat that was previously treated with the small-particles of a specific local DE compared to large particles of the same DE .After a 6d exposure, mortality of the lesser grain borer reached 63.8% on wheat treated with the smaller particles of Elassona 1, whereas it did not exceed 11% on wheat treated with the largest particles of Begora, Elassona 2, Kolubara 516, Kolubara 517, Crete, Lower Serbia or Vranje.

3.4 Effectiveness of applying DE as slurry and impact on grain bulk density

In the case of 500 ppm, dusting or spraying DE was of equal effectiveness against rice weevil after 5d exposure on hard red spring wheat. However, efficacy of Silicosec against rice weevil 14d post exposure was higher with dust application compared to slurry application on hard red spring wheat treated with 300 ppm. In the case of durum wheat, dusting or spraying was of equal effectiveness against adults of S. oryzae and this was noted for both dose rates. DE efficacy against rice weevils was generally higher on durum wheat than hard red spring wheat and this fact was noted for both dose rates as well as application methods. However, DE efficacy of both methods was increased with the increase of exposure interval and ascended to 100% on durum wheat irrespective of application method. At the same exposure period, weevil mortality was 86.5% on hard red spring wheat sprayed with 300 ppm. In addition, dusting hard red spring wheat with 500 ppm of DE completely suppressed progeny production, whereas this was not the case with spraying method, where a significant number of F1 individuals were recorded.

Mortality of red flour beetle after 14d exposure on hard red spring wheat treated (dusted or sprayed) with 300 ppm of DE was very low and did not exceed 4%. Fourteen days later, mortality on grain dusted with 300 ppm was 88.3% and was significantly higher than that recorded on grain sprayed with the same dose. DE efficacy on durum wheat was gener-

ally higher than that recorded on hard red spring wheat against T. castaneum. However, effectiveness of both application methods was increased with the increase of exposure interval. Hence, after 28d of exposure, efficacy of both dose rates of DE on durum wheat against T. castaneum was >99% regardless of the application method and thus, significant differences in the efficacy of the application methods were not recorded.
Spraying or dusting wheat with a DE significantly reduced bulk density of grain in comparison with untreated grain. Between spraying and dusting, the former method significantly less reduced bulk density than the later method and this was the case for all post treatment periods.

3.5 Effectiveness of local DEs at surface treatment

Efficacy of the tested DEs was significantly increased with the increase of exposure interval ($F_{18,487}$ = 2847.2; P<0.001). More adults were dead after exposure on Silicosec than on the local DEs. In the case of Silicosec, almost complete (>95%) mortality of T. confusum adults was observed at less than 6d exposure. Of the local Des, the most effective was Slovenia followed by Elassona 1 and Begora. Higher than 6d exposures were required for an effective control of T. confusum adults with the remainder of the local DEs. The least effective of the local DEs was Germany, since satisfactory control of T. confusum was achieved at exposure intervals longer than 13d. Ending, except for the case of Silicosec where no progeny larvae were found, T. confusum adults successfully oviposited on the local DEs. However, the majority of the progeny larvae were dead.

4 Summary

Factors such as relative humidity, temperature and grain type, which overall affect DE efficacy, also affected the efficacy of the local DEs. DEs are not effective under humid conditions and therefore, DE efficacy is declining with increasing r.h. [5, 13, 14]. It is generally accepted that insects can moderate internal water loss in humid conditions and thus, desiccation through the action of DE particles on the insects' cuticle is reduced [7]. It is also possible for the DE particles to absorb humidity from the air and gradually become inactive [13, 15]. Contrary to rh, increasing temperatures are positively related to DE efficacy [3, 14, 16] although Aldryhim [17] noted that efficacy of the DE formulation Dryacide against T. confusum was higher at 20° C than 30° C. On the other hand, Arthur [16] noted that efficacy of the DE formulation Protect-It against T. confusum and T. casteneum is progressively increased with the increase of temperature. This finding was also confirmed by newer studies [3, 14] for which the effectiveness of Silicosec against adults and larvae of T. confusum was evaluated. The results of the present study stand in accordance with the above findings, since increasing of temperature from 20° C to 30° C considerably enhanced the performance of the local DEs against the tested species. It seems that the increasing temperature enhances water loss, which is positively related with DE efficacy. Also, at warmer conditions insects become more agile so the possibility of getting in contact with DE particles is increased [3, 7, 13]. In light of the results of the present study, mortality of all of the tested species after exposure on wheat treated with 900 ppm of the local DEs was very high (>88%) at 30 ° C. The fact that increasing temperatures were positively related to efficacy of the local DEs is very promising, given that heat and local

DEs can be successfully combined in an IPM strategy. Heat is not always feasible to be involved in an IPM strategy, since it can impair the performance of other control methods, such as chemical control. For instance, the temperature may negatively affect some pyrethroids although the reverse has been noted for organophosphates [18-20]. Also, the fact that the performance of local DEs varied among different grains should be seriously taken into account and each case should be handled separately, avoiding generalisations on the labeling rates of DEs. Arthur [21], proposed that combining DEs with other IPM compatible methods is a possible alternative to using DEs at high dose rates. DE efficacy is affected by the type of grain it is applied to. Athanassiou et al [22] found that among grain commodities, Silicosec was more effective in wheat rather than maize or rice against S. oryzae. Also, Aldryhim [17] reported significant variations in the efficacy of Dryacid among different wheat classes against adults of lesser grain borer. In our experiments, DE effectiveness varied considerably among the tested grains and as a result, performance of the local DEs was greater on barley and wheat than rice or maize. Hence, higher DE doses or exposure intervals are required for an effective control of maize or rice compared to barley or wheat and therefore, generalisations on the application rate of DEs among different grain commodities should be avoided.

DE is prepared for commercial use by quarrying, drying and milling. Hence, practically, the only change to DE during this process is the reduction in the moisture content and mean aggregate particle size. The result of this process is a fine, talc-like dust, with the mean particle size distribution from 0 to more than 100 microns with the majority from 10 to 50 microns. Korunic [5] indicated that the particle size distribution in a certain range (median particle size from about 0 to 30 microns) may be a very important DE property affecting insecticidal activity. Our results confirm the above statement also for the local DEs. In light of our findings, fractions of 0-45 microns were more effective than that of 45-150 microns for a specific local DE. This suggests that reducing moisture to 6% as proposed by Korunic [4], producing small particles with majority <45 microns by milling and separating process and removing particles >150 microns could improve effectiveness of the local DEs. However, the above process may be not cost effective, so in light of our findings, local DEs such as Crete, Begora, Elassona 2, Varnje or Kolubara 516 should be avoided because of their lower performance compared to others. On the other hand, Kolubara 518 and Elassona 1 contained medium-sized particles (45-150 microns) which were more effective even than the smallest particles (<45 microns) of other DEs such as Begora, Elassona 2 or Crete. According to our results, Kolubara 518 and Elassona 1 were shown to be very effective DEs though their effectiveness should be confirmed under field trials too.

Besides dusting, DE can be successfully applied as a slurry mainly for surface treatment in empty storage facilities [13]. However, this practice may reduce DE efficacy, although water in the slurry evaporates within one hour after the application, leaving a dust film that is toxic to insects [13]. In our experiments, we examined the application of Silicosec as a slurry on two classes of wheat. According to our results, it is possible to control rice weevil on wheat by applying Silicosec as a slurry at 500 ppm even 5d post exposure. However, this was not always the case with T. castaneum, since a satisfactory control level was achieved only in the case of durum wheat. Another parameter that was tested here

was the effect of slurry to the physical properties of grain. According to our results, bulk density (test weight) of grain was significantly less reduced with spraying in comparison to dusting. This is an important finding, since the main drawback of the use of DEs is their negative impact on the physical properties of the grain [4, 5, 13]. In light of our results, applying DEs as a slurry has some significant advantages over dusting such as reduced friction among the kernels (because of lower effect on test weight), better flowability of grain and less airborne dust during the transportation. Although encouraging, these results correspond to specific conditions, doses, formulations and grains. Before adopting such a method, slurry application of DE on grain commodities needs further evaluation in terms of efficacy, application apparatus and impact on physical properties of grain and thus, generalisations should be avoided.

As a second step, the most effective of the DEs should be selected for further evaluation either alone or as a mixture against stored product insects. These DEs and their mixtures should be applied as slurries or dusts on several commodities or surfaces and their combination with other IPM compatible methods should be also assessed.

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Development of a Strategy and Methods for Monitoring of Electromagnetic Pollution in the Environment of the Western Balkans

Petar Getsov[1], Dimitar Teodossiev[1], Eugenia Roumenina[1], Michel Israel[2], Garo Mardirossian[1], Georgi Sotirov[1], Stoyan Velkoski[5], Peter Gajesek[4], Dina Simunic[3], Kamen Iliev[6]

[1] Space Research Institute – Bulgarian Academy of Sciences, 6 Moskovska St., 1000 Sofia, P.O.Box 799, Bulgaria; director@space.bas.bg, roumenina@space.bas.bg, dteod@space.bas.bg
[2] National Centre of Public Health Protection, 15 Ivan Ev. Geshov Bl., 1431 Sofia, Bulgaria; m.israel@ncphp.government.bg
[3] Faculty of Electrical Engineering and Computing, University of Zagreb, 14 Trg maršala Tita, 10000 Zagreb, Croatia; dina.simunic@fer.hr
[4] Institute of Non-Ionizing Radiation (INIS), Pohorskega bataljona 215 a, 1000 Ljubljana, Slovenia; Peter.Gajsek@inis.si
[5] Institute for Geobiology, Archaeology, Ground Water and Ecology (IGAPE), 10/6 Dimche Mirchev St., 1000 Skopje, Former Yugoslav Republic of Macedonia; contact@igape.edu.mk
[6] EUROSENSE Bulgaria Ltd., 83 Gioeshevo St., Business Center Serdika, 1330 Sofia, Bulgaria; kamen.iliev@eurosense.bg

Abstract

The project's aim was to create a strategy for integrated GIS-based monitoring of EMP, using data from ground based, aerial and space monitoring and data available in most of the countries from the Western Balkans region and neighbouring countries.

Developing a strategy for unifying methods for measurement and exposure assessment of EMF was another task which was implemented within the project. This part of the project was designed to investigate the real exposure to EMF radiated by different EMF sources. As results, adequate methods for measurement of EMF around EMF sources were developed and regions with high exposure levels were determined. Special attention was paid to mobile communication, radio and TV broadcasting, etc.

Another outcome of the project was the development and validation of methods for measurement, exposure assessment and dosimetry around sources of EMF: power lines, radio and TV stations, base stations for mobile communication, mobile telephones.

Finally, a strategy for the development of regional databases for EMF sources was proposed. This part of the project is connected to the development of a strategy for collecting data and developing a database for EMF sources and their distribution over regions of Central and South-East Europe.

Thematic Area / Type of the project

Environment (including Climate Change): Environmental Technologies.
Type of the project: research project

Keywords

Electromagnetic field; Electromagnetic Pollution; Environment; Exploiting equipment; Geographic Information System; Healthcare; Remote sensing; Spatial Data Base Management Systems

1 Introduction

In order to obtain a clear picture of the EMF levels in living environments in the countries of the Western Balkan region, there is a need of improving links between research institutes in this field;developing joint action plans in order to solve the problem; and creating monitoring regions with a less developed research profile by more experienced partners through mutual exchange of experience and best practices.

Only a common EMF platform, harmonised methods, appropriate and unified equipment, adequate control of EMF and good collaboration among the research organisations, universities, enterprises and national authorities could ensure adequate information and protection of the general public in the region.

2 Experimental Techniques and Methods

2.1 Ground measurement

The data from electromagnetic measurements were collected in order to characterise, evaluate and analyse electromagnetic pollution sources in defined territories in the participating countries.
Technical specification of a ground complex for measurement parameters of natural and anthropogenic electromagnetic field consists of three subtasks:

- Equipment composition
  Selection of equipment for measurement parameters of electromagnetic fields according to effective EU standards and norms for measurement parameters, measurement units, dynamic range, measurement accuracy etc.
- Number and configuration of measurement stations
  Configuration of structure ground measurement complex (GMC), in principle consisting of peripheral measurement posts (PMP) and a central post (CP), such as number and arrangement in the investigated area.
- Algorithms of measurements
  Synthesis of optimal algorithms and cyclograms for the measurement and registration of data, guaranteeing the highest possible efficiency.

2.2 Aerial based measurement complex (Aerial segment – AS)

Aerial segment (AS) is used for the measurement of EMF components. It aims to provide parallel supporting measurements, offering information mainly for the saturation of separate frequency bands of the electromagnetic spectrum with electromagnetic radiation. During the flight of the measurement complex over defined points of territory in built-up areas and outside, there is an integral effect on ground-based sources of electromagnetic radiations and changes of their intensity. The main task of AS is the measurement and control of electromagnetic fields with the aid of aerial based means like airplanes, helicopters, balloons and dirigibles. The use of these means provided overcoming of the bigger part of limitations by application of traditional ground based technologies for ecological control. Their advantages are defined as follows:

- Wider area of monitoring territory;
- Possibility of monitoring defined regions and objects with determined regularity in different bands of the electromagnetic spectrum at day and night in different meteorological conditions;
- Possibility of receiving images - maps with high resolution in information capabilities in operational plan; and
- Connection of obtained EMF data to geographic coordinates.

2.3 Space based measurement complex (Space segment – SS)

The role of space segment (SS) involves measurement of EMF components from several satellites flying in ionosphere orbits (with heights to ~1000 km). The main objective of SS is the observation of ionosphere interferences caused by human activity (anthropogenic impact). Such kinds of interferences can exercise influence on environment, weather and climate. It is foreseen to use of data from acting orbital satellites with suitable equipment for the study and evaluation of next effects as:

- Evaluation importance of artificial electromagnetic waves;
- Geographical map of intensive radiations;
- Correlation of interferences with industrial areas;
- Geographical map of thunderstorm activity;
- Correlation with industrial areas;
- Influence upon global warming;
- Evaluation of interferences in charged particles as a result of artificial influences; and
- Relation to influence of thunderstorms.

Space segment (SS) can also play an essential role in receiving key information to study the influence of human activity on the ionosphere. It is planned to evaluate the artificial emission of the ionosphere caused by powerful ground very low frequency (VLF) transmitters and radiation from high voltage power supply nets, to clarify how they influence the natural balance of the ionosphere and also to be evaluate how thunders warm the ionosphere. However, it is planned to determine location in height so called "height thunders" or Red Sprite and Blue Jet. It is interesting to clarify if global warming can bring the number of thunders in atmosphere to a catastrophic growth. The diagram in Figure 1 demonstrates data of components of the electromagnetic fields of natural and anthropogenic origin

from DEMETER satellite. This data is given in accordance with the possibility of establishing a monitoring of the electromagnetic field distribution and its changing in time and space.

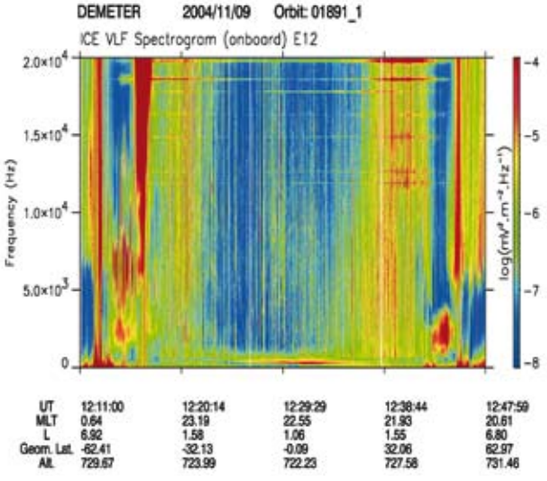

Figure 1: Spectrogramme of the electric components obtained during trespassing of DEMETER satellite over ground based VLF transmitters

2.4 Creation of geodatabase

The natural condition and exploration level of natural resources has determined the ecological problem in the territory in which the electromagnetic pollution has been evaluated. The project has provided a complex application of information received from the ground measurement complex, airspace segment, reserved and institutional databases and integrated in a united geodatabase. It ensures the integration and overlaying of all kind of data, as the only condition is their geospatial character. That allows for regular objective controls and the evaluation of sources and level of electromagnetic pollution in a given territory. The creation of the geodatabase has been necessary for the integrated management of the received attributive information as well as for the visualisation, statistical processing and analysis which are accessible only in GIS environment. By that means, the following achievements were accomplished:

- Regular cartographic expert analysis of the potential and real threat created by the various sources of electromagnetic pollution;
- Model studies, related to the development of criteria and recommendations for reducing the risk of electromagnetic pollution and for the territory's sustainable development;
- Express and unbiased control of the electromagnetic pollution reduction measures, applied by the local bodies and non-government organisations.

The proposed geodatabase structure is depicted in the following Figure 2.

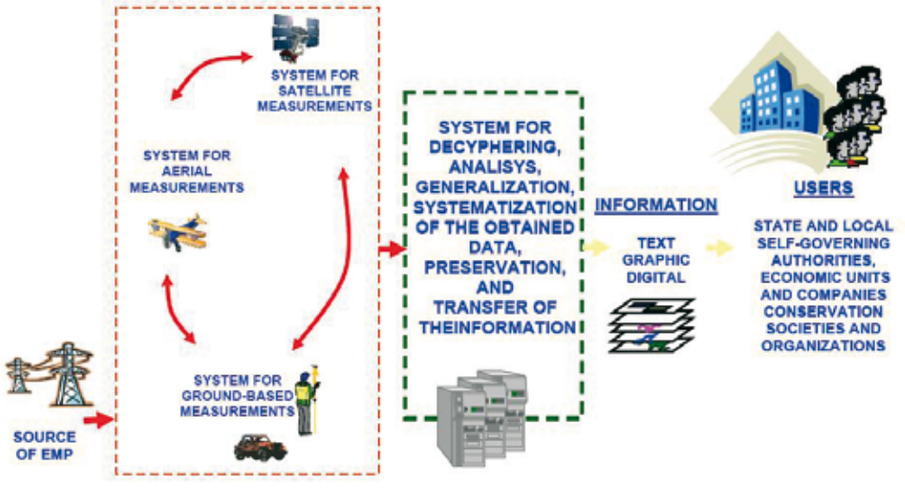

Figure 2: Model of Geodatabase organisation

3 Results and Discussion

Numerous results were delivered by the project. The most significant ones are as follows:

- Proposal for the creation of the Balkan Electromagnetic Fields Association (BEmFA) based on the Foundation "Faraday" - Non-ionizing Radiation and Electricity foundation acting in the Republic of Bulgaria.This non-governmental organization (NGO) was the main body of the Bulgarian National Programme Committee for the international project „Electromagnetic fields", which was responsible for the organisation of the international workshop on implementation of the model legislation (WHO) in the field of EMF human exposure, quality assurance in measurements. As a result of this workshop, the proposal to establish BEmFA was made.
- Reports, articles and other documents were presented at scientific conferences such as SENS2007, FSR 2008 [1, 2, 3, and 4].
- Efforts' synchronisation of countries participating in a project for the realisation of the policy of the European Union, World Health Organisation (WHO) and other international organisations concerning the harmonisation of standards, introduction of new legislation, investigation of risks deriving from EMP, such as applying principles of warning approach.
- Conductance of pilot measurements of EMP in the territory of the capitals of the participating countries. Two test sites were chosen next to the capitals of Bulgaria, Sofia, and the capital city of the Former Yugoslav Republic of Macedonia, Skopje.
- Comparison of received preliminary experimental results using the developed software programmes:
- With the aid of such software, localisations of correspondent sources of electromagnetic radiation as well as distribution curves of radiation connected to electromag-

netic pollution were visualised. One software product for this utilisation is AldeMap, developed by the Italian company Aldena. This software contains a GIS - core for the localisation of sources of electromagnetic radiation. During the project implementation, the preliminary results were derived using standard GIS packages using spatial analysis functions.

- A scientific report about the current status of the available geodatabases for monitoring electromagnetic pollution was prepared. This provided for improving the study's methods in modelling the geodatabases, based on international experience. The practical use of this experience provided opportunities for joint work with various centres (national, European or international), using GIS to monitor electromagnetic pollution.
- Design of a model of a geodatabase for monitoring of electromagnetic pollution. The geodatabase comprises of two main modules: information module and analysis module. Both modules have different implementation and purpose. The information module is used for data storage, retrieval and manipulation, while the analysis module is used for storing and analysing the data from the information module. The real design and implementation of the model of the geodatabase for electromagnetic pollution was done in ArcGIS9.2/ArcInfo license shareware application environment.
- Development of a strategy for integrated GIS based monitoring of EMP. The strategy requirements were outlined in a way that the responsible authority figures of each country are able to take them into consideration in advance in case of a joint EMP policy in the Western Balkans [5].

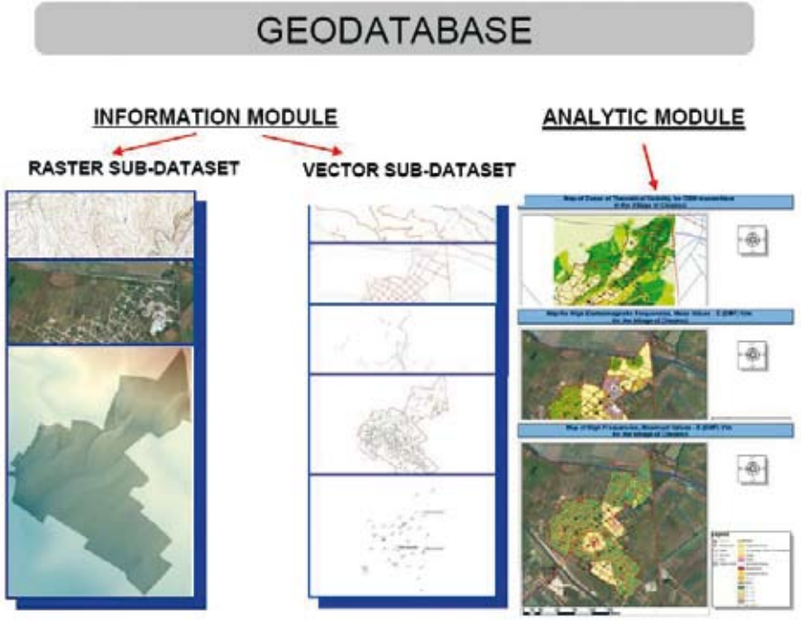

Figure 3: Geodatabase structure

4 Outlook, next steps

- In future, there would be room for cooperation in the following fields:
- Harmonisation of standards in all Balkan countries based both on European requirements and ensuring good protection of the population.
- Organisation of measurements and exposure assessment of EMR in all regions where transboundary "pollution" of EMR is possible.
- Creation and support of an international database for the EMR "pollution" in dangerous regions and development of a special map of the environmental EMR "pollution" including all Balkan countries.
- Organisation of meetings, workshops and symposia on different topics connected to the EMR human exposure problems in the Balkans.
- Accreditation of a qualified laboratory is a fundamental need for repeatable and accurate measurement and calculations.
- Preparation of a project proposal for participation in FP7 involving other Western and South-Eastern Balkan countries and the EU.

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Development of Molecular Tools for Fig Genetic Resources Characterisation and Preservation in the Western Balkan Countries

Dunja Bandelj[1,2], Maja Podgornik[1,2], Irma Tomažič[1,2], Jernej Jakše[1,3], Branka Javornik[3], Irena Vrhovnik[4], Frane Strikić[5], Katja Zanić[5], Slavko Perica[5], Biljana Lazović[6], Miroslav Čizmović[6], Mirjana Adakalić[6], Ranko Popović[6]

[1] Science and Research Centre Koper, University of Primorska, Garibaldijeva 1, 6000 Koper, Slovenia; dunja.bandelj@zrs.upr.si
[2] Faculty of Mathematics, Natural Sciences and Information Technologies, University of Primorska, Glagoljaška 8, 6000 Koper, Slovenia; dunja.bandelj@zrs.upr.si
[3] Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia
[4] Camber of Agriculture and Forest of Slovenia, Agricultural and Forestry Institute Nova Gorica, Agriculture Consultancy Koper, Ulica 15. maja, 6000 Koper, Slovenia
[5] Institute for Adriatic Crops and Karst Reclamation, Put Duilova 11, 21000 Split, Croatia; slavko.perica@krs.hr
[6] Biotechnical Institute, Center for Subtropical Cultures, Topolica bb, 85000 Bar, Montenegro; miroslaw@cg.yu

Abstract

In the Western Balkan countries, the common fig is an underutilised fruit species spread along the eastern Adriatic coast and islands. Its cultivation occupies special niches at local levels and is maintained by cultural preference and traditional practices. In order to enhance fig investigation as an important constituent of a sustainable agricultural system and contributor to agrobiodiversity, a field survey of fig genetic resources in Slovenia, Croatia and Montenegro was performed. Collected plant material was evaluated using traditional and molecular approaches. Morphological characterisation showed the presence of similar varieties in three countries, while genotyping analysis with microsatellite markers confirmed the complexity of fig variety denominations. Beside synonyms, many homonyms of varieties were found and their controversial denominations caused some difficulties in comparing reference varieties among the Western Balkan countries. A developed uniform and accurate identification system of fig varieties based on microsatellites enabled the establishment of national databases of genotypes of reference varieties. The results of the investigation will contribute to the preparation of a preservation strategy for the Adriatic fig genetic resources, to a better knowledge of their value and diversity, and to the promotion of the fig as an important Mediterranean fruit species.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest, and aquatic environments
Type of the project: research project

Keywords

Biodiversity; Eastern Adriatic coast; Ficus carica; Genotyping analysis; Molecular markers; Morphological evaluation; Underutilized plant; Sustainable agriculture

1 Introduction

The common fig (Ficus carica L.) is a typical Mediterranean fruit species and was probably the first intentionally cultivated plant during the Neolithic revolution [1]. Figs are widely cultivated in North African and Middle Eastern countries, where it represents a significant source of agricultural branch income. According to the FAO [2], figs are harvested on approximately 415.000 ha, and the leading countries in production are Turkey, Egypt, Algeria, Iran, Morocco and Syria, which produce more than 71 % of the world's annual harvest. In the Western Balkan countries, fig cultivation is extended along the Adriatic coast and nearby islands. In some regions, cultivation is also extended into the hinterland, where the impact of the Mediterranean climate spreads in river valleys, particularly of the Neretva, Moraca and Zeta. In Slovenia, Croatia and Montenegro, figs are planted on 2577 ha [2]. The fig tree was introduced into the Adriatic growing region in the age of the Illyrians and Greek colonisation.

In the Western Balkan countries, the economic potential of fig cultivation has been poorly addressed, and the fig tree is considered an underutilised plant, the production of which can not compete the one of olives, grapes and citrus, the leading crops of Mediterranean agriculture. Along the Eastern Adriatic coast, fig growing occupies special niches at local levels and is maintained by cultural preference and traditional practices. In Slovenia, Croatia and Montenegro fig growing is especially important to local communities, providing a nutritionally rich diet and the development of typical products (jams, cakes, liquors...). As a functional food, figs are inadequately characterised and neglected by research and conservation.

Increasing attention is being paid and some promotion has been taking place over the last few years towards underutilised species; motivations behind such interest originate from a variety of concerns and expectations, ranging from ethical and social to economic. Reemergence of underutilised crops could lead to greater agricultural diversification, a greater use of marginal lands, greater food security, more balanced diets, maintenance of our agro-biodiversity heritage, and to better preservation of cultural identities and traditions [3].

To preserve and encourage fig growing in western Balkan countries, research into the inventory of fig genetic resources and variety structure is required. Germplasm management and evaluation will facilitate and improve European production and contribute to greater promotion of fig fruit as a typical and healthy Mediterranean product. Characterisation of fig genetic resources is also essential for appropriate utilisation, conservation and management of germplasm, as well as for identification of genotypes of commercial value, adapted to sustainable cultivation.

The exchange of planting material between countries of the Mediterranean basin and the presence of synonyms and homonyms contributed to the confusion of fig varietal denomination, and suggests the need to establish referential national collections characterised by standardised identification criteria. Traditionally, fig genetic resources have been evaluated by morphological markers [4], [5]. By development of fig microsatellite markers [6], [7], [8], new approaches, based on molecular characterisation, are available. These markers are especially suitable for DNA fingerprinting analysis, since they generate non complex banding patterns revealing high polymorphism are co-dominant and, genotyping results of analysis are comparable and exchangeable among different laboratories.

The study of genetic variability of the common fig in the Western Balkan countries is therefore a first necessary step for optimidation and acceleration of fig growing in the WBC or in any other Mediterranean region. The choice of a variety is very important, since the quality parameters are genetically determined. Information about fig varieties from the Western Balkan region is limited and molecular characterisation has not yet been carried out. With this research project, we aimed to:

- inventory and identify the main fig varieties and local ecotypes grown in Western Balkan countries;
- establish a uniform and accurate identification system for fig varieties based on molecular markers; and to
- establish the first genotype database of figs grown in Slovenia, Croatia and Montenegro.

2 Experimental Techniques and Methods

2.1 Morphological evaluation of fig genetic resources

Data that have been collected from the field surveys included plants growing in various locations from Slovenia, Croatia and Montenegro. Plant material was collected in September 2007 and June 2008. The morphological characterisation of fig varieties was performed according to the guidelines provided by the International Plant Genetic Resources Institute (IPGRI), now Bioversity International [9]. The descriptor list includes over 70 characters to be recorded in this study; only the main characters important for commercial value of fruits are presented (fruit weight, shape, skin and pulp colour, maturity). Fully mature fruits of breba and main crop and leaves were collected during vegetation, then measured and morphologically described. Fruits were measured by using a vernier caliper and scales while the leaves were photographed and measured by using Image Tool software [10] (data are not presented). The study was carried out on 37 local varieties from three countries, but only the 24 varieties that are included in national fruit catalogues are presented here.

2.2 Molecular characterisation of fig genetic resources

2.2.1 Plant material

The genotyping analysis with microsatellites included 20 varieties and three wild figs from Slovene (SI), Croatian (CRO) and Montenegrin (MNE) cultivation areas. From one to three fig trees were sampled per variety. Fig leaves were collected in September 2007 from in-

dividual trees at different locations. The names of genotyped fig varieties and locations of sampled plant material are listed in Table 1.

2.2.2 Fig DNA isolation and amplification of microsatellites

Fig DNA was extracted from leaves by a modified CTAB method, and amplification of microsatellites with fluorescence-based detection was performed as previously reported [8]. Four primer pairs for fig microsatellite loci (FCUP008-2, FCUP038-6, FCUP068-1, and FCUP070-2) were used in the genotyping analysis. The amplification products were separated on a 7.5% polyacrylamide denaturing gel, containing 7 M urea. Electrophoresis was performed on an automated ALFexpressII sequencer (Amersham Biosciences), and the length of alleles was determined with the help of an external standard (50-500 bp, GE Healthcare), and an internal standard using Allele Locator 1.03 software.

3 Results and Discussion

Despite the fact that the fig tree is an underutilised agricultural plant, its cultivation is relevant in the Western Balkan countries because of its adaptability to specific, Mediterranean climatic conditions and to poor soils, and very low demand for fertilisation and pest control. Thus, the fig might contribute significantly towards obtaining sustainable fruit production. In the market, there is an increasing demand for fresh fig fruits; therefore production with different varieties and at different latitudes and altitudes could provide the opportunity to extend the marketing season. Advanced handling, packing and transportation facilities could also enable the establishment a strong and long lasting chain between the producer and the consumer. A number of fig varieties have been developed under different environmental conditions in the the Eastern Adriatic area, which retained the properties of mother plants or parent trees through vegetative propagation. The most important varieties from the Western Balkan countries included in national fruit catalogues are:

- in Slovenia: 'Miljska figa', 'Zelenka', 'Flazana', 'Pinčica', 'Laščica', 'Sivka' (one-crop varieties), and 'Bela Petrovka', 'Zuccherina' (two-crop varieties);
- in Croatia: 'Sušioka', Bružetka Bjela', 'Šaraguja', 'Zimica' (one-crop varieties) and 'Petrovača Bjela', 'Petrovača Crna', 'Bjelica', 'Fico della Madonna' (two-crop varieties); and
- in Montenegro: 'Sušilica', 'Rezavica', 'Crna Patlidžanka', 'Zimnica' (one-crop varieties), and 'Crna Sultanija', 'Bijela Petrovača', 'Crna Petrovača', 'Trojka' (two-crop varieties).

In Slovenia, there are more than 20 varieties under cultivation, but only the listed varieties are of commercial value. The interest in fig growing is increasing among growers and figs are frequently planted in home gardens along with olives and grapes. In Croatia, 11 fig varieties have been defined as autochthonous or domesticated, but the origin of some varieties has not been determined with reliability. The largest number of fig varieties was noted in Montenegro, where 58 different phenotypes were observed after the Second World War (26 two-crop and 32 one-crop varieties) [11]. A significant decrease in tree number occurred over the last decades of the previous century, due to fig mosaic disease.

Table 1: List of fig varieties included in the genotyping analysis, number of analysed samples and location of sampling

Variety name	Number of samples	Sampling location
'Bela Petrovka', 'Petrovača bijela'	2	Slovene Istria, Neretva valley
'Belica', 'Bjelica'	2	Slovene Istria, Neretva valley
'Bružetka Bjela'	1	Neretva valley
'Bružetka Črna'	1	Slovene Istria
'Crna Carigradska'	1	Montenegro
'Crna Padližanka'	1	Montenegro
'Crna Sultanja'	1	Montenegro
'Črna Petrovka'	1	Slovene Istria
Wild fig	3	Slovene Istria, Croatia, Montenegro
'Lipovka'	1	Montenegro
'Miljska figa'	1	Slovene Istria
'Rezavica'	2	Mljet (Croatia), Montenegro
'Sušilica'	2	Montenegro, Bosnia & Herzegovina
'Sušioka'	1	Croatia
'Šaraguja'	3	Split (Croatia), Mljet (Croatia), Montenegro
'Termanjača'	2	Split (Croatia), Montenegro
'Trojka'	1	Montenegro
'Vodenjača'	1	Slovene Istria
'Zamorčica'	1	Croatia
'Zelenka'	1	Slovene Istria
'Zimica'	2	Split (Croatia), Montenegro
Sum of samples	31	

The lack of interest towards this fruit species in the past has caused the loss of several fig genotypes and confusion over their identification. With the aim to preserve fig germplasm and to prevent the additional loss of these unique genotypes, a fig gene bank in each country has been established. Fig varieties in collections have been surveyed on the basis of generally known denominations and some morphological traits. Slovenian collection was established in 2007 and includes 19 varieties. In Croatia, there are two national collections of figs; at the Institute for Agriculture and Tourism, Poreč, and at the Institute for Adriatic Crops and Karst Reclamation, Split. Collection in Split was established in 2007 and contains 14 different fig varieties and three wild types. With urbanisation and reparation of agricultural land to former owners, all experimental orchards and collections were destroyed in Montenegro. Settled fig orchards are still present in Sutomore, where figs are planted on around 6 ha of land. In 2004, the Centre for Subtropical Cultures in Bar (Montenegro) reestablished a collection of 10 fig varieties. Establishment of new fig collections in three countries shows an increasing responsibility for genetic resources preservation.

In order to provide some basic information on the diversity of Adriatic figs, the project consortium started the investigation of fig genetic resources using traditional and molecular approaches. The inventory of plants based on morphological descriptions enable the first insight into the extent of phenotypic diversity and is of great importance for planning a genetic resources preservation strategy. The basic fruit characteristics measured and observed during the project are presented in Table 2.

Pomological descriptions, denominations, synonyms and photographs of sampled varieties are also available online at http://www.zrs-kp.si/SL/Projekti/See_era/sl/index.html. The inventory of fig genetic resources using morphological descriptors showed the presence of some similar varieties in the three countries. Synonyms and homonyms are frequently reported in vegetative propagated fruit species and are in most cases the result of an exchange of plant material among the growers and regions, modification of names due to language translation or the introduction of a new variety name in some cultivation areas. Controversial denominations of varieties make the management of plant genetic resources complex. A reliable identification system is very important in the production of certified planting material in nurseries and available data of reference varieties is crucial for planning a varietal structure in orchards in particular growing regions. The development of DNA markers has brought up new approaches in varietal investigation. Identification procedures based on DNA profiling enable the identification of a variety by generating genotype-specific DNA profile or fingerprinting.

In order to establish a uniform and accurate identification system for fig varieties from the Adriatic coast, molecular markers were included in the genotyping analysis. For the DNA genotyping analysis, four previously developed microsatellite loci by the Slovenian team (FCUP008-2, FCUP038-6, FCUP068-1, and FCUP070-2) were chosen. These markers were selected on the basis of their high polymorphic information content (PIC value), low probability of identity (PI value) and high number of observed genotypes in analysis of nineteen cultivated fig trees in Slovenia [8]. The microsatellite markers were successfully amplified in all 31 samples with four primer pairs used. Altogether, 29 alleles were amplified at four

Table 2: Some basic fruit characteristics of the Western Balkan's most important figs varieties

Name	Growing region	Breba crop					Main Crop				
		Weight (g)	Shape	Skin Colour	Pulp Colour	Maturity	Weight (g)	Shape	Skin Colour	Pulp Colour	Maturity
'Miljska figa'	Slovene Istria	-	-	-	-	-	47	ovoid	purple	dark red	middle
'Zelenka'	Slovene Istria	-	-	-	-	-	57	ovoid	green	dark red	middle
'Flazana'	Goriška Brda	-	-	-	-	-	48	pyriform	purple green	red	middle
'Pinčica'	Slovene Istria	-	-	-	-	-	44	ovoid	green	red	middle
'Laščica'	Slovene Istria	-	-	-	-	-	29	ovoid	yellow green	red	middle
'Sivka'	Slovene Istria	-	-	-	-	-	40	ovoid	brown green	dark red	middle
'Bela Petrovka'	Slovene Istria	82	ovoid	green	pink, amber	late	59	ovoid	green	pink, red	middle
'Zuccherina'	Slovene Istria	67	ovoid	green	yellow	middle	45	ovoid	yellow green	pink	early
'Sušioka'	Croatian coast	-	-	-	-	-	36	ovoid	yellow	red	middle
'Bružetka Bjela'	Croatian coast	-	-	-	-	-	31	ovoid	green	red	middle
'Šaraguja'	south Dalmatia	-	-	-	-	-	51	ovoid	green	red	middle
'Zimica'	Croatian coast	-	-	-	-	-	22	ovid	green	dark red	late
'Petrovača Bjela'	Dalmatia	80	ovoid	green	pink, amber	late	50	ovoid	green	pink	middle
'Petrovača Crna'	Croatian coast	80	pyriform	brown green	pink	late	60	ovoid	brown green	purple	middle
'Bjelica'	South, central Dalmatia	67	ovoid	yellow green	pink, yellow	middle	39	ovoid	yellow green	amber	early
'Fico della Madona'	Dalmatia	129	pyriform	green	pink	late	69	pyriform	green	red	middle
'Sušilica'	Boka Kotorska	-	-	-	-	-	35	pyriform	yellow green	pink	early
'Rezavica'	Monte-negro	-	-	-	-	-	45	oblong	green yellow	dark red	early
'Crna Patlidžanka'	Bar region	-	-	-	-	-	45	oblong	purple black	red	middle
'Zimnica'	Monte-negro	-	-	-	-	-	38	round	green	red	late
'Crna Sultanija'	Bar region	70	oblong	dark violet	light pink	late	25	round	dark violet	pink	middle
'Petrovača bijela'	Monte-negro	80	round	light green	yellow	late	30	round	light green	yellow	middle
'Petrovača crna'	Monte-negro	90	round	purple black	yellow	late	40	round	purple black	yellow	middle
'Trojka'	Monte-negro	80	pyriform	green yellow, purple	red	very late	32	round	green yellow	red	middle

Legend: - one crop accession

loci in 31 fig samples. Eight alleles were observed at locus FCUP008-2, ten alleles were amplified at locus FCUP038-6, 6 at locus FCUP068-1, and five alleles were found at locus FCUP070-2. Samples of 'Bela Petrovka' (SI) and 'Petrovača bjela' (CRO) showed identical DNA profiles on all four microsatellite loci, indicating that the variety is well defined in both countries. Identical DNA profiles were also observed in samples 'Crna Carigradska' and 'Lipovka'. Varieties with the denomination 'Rezavica', 'Sušilica', 'Termenjača', 'Zimica' and 'Šaraguja' sampled in different growing regions in Croatia and Montenegro, differed greatly at a majority of genotyped loci. These results indicate that the same denomination is assigned to different fig genotypes. Genotyping analysis also confirmed that 'Belica' and 'Bjelica' are not synonyms of the same genotype (Slovenian-Croatian synonym). Our study revealed 6 unique alleles characteristic for the varieties 'Črna Petrovka' (SI), Sušioka (CRO), Zimica (MNE), 'Crna Sultanja' and for a wild fig sample from Slovenia. These four varieties could be immediately identified by genotyping loci FCUP008-2, FCUP038-6 and FC UP068-1. Out of 46 observed genotypes, 16 were unique to some varieties.

A higher confusion than expected in denomination of fig varieties from the Adriatic region was confirmed by data of genotyping analysis with microsatellite markers. Beside synonyms, many homonyms of varieties were found and their controversial denominations caused some difficulties in comparing the reference varieties among the Western Balkan countries. These results suggest that in each country, all surveyed varieties in national collections should be genotyped by molecular markers and thus defined as reference varieties. Once the reference DNA profiles are known, the identification of trees with similar morphological characters originating from different cultivation areas is easier. The high confusion in fig variety denominations from the Adriatic region is probably a result of uncontrolled transfer of plant material between the countries and ignorance of investigation into fig genetic resources.
Field survey and morphological evaluation of the Adriatic fig genetic resources also showed high infection of figs with mosaic disease, presumably caused by fig mosaic virus. The situation is alarming in Montenegro, where almost all analysed fig samples displayed the symptoms of mosaic. In Slovenia, affected young fig plants were also observed in nurseries. Uncontrolled vegetative propagation by cuttings obtained from affected mother plants and the lack of sanitary selection are probably the most important factors that have contributed to the spread of the disease. These results suggest the urgent need to establish protocols for virus elimination, to introduce micropropagation protocols into a production scheme of planting material, to develop methods for virus detection, and to investigate vectors and transmission paths of the disease.

4 Conclusions / Outlook, next steps

The pilot investigation into Adriatic figs confirmed the complexity in varietal denomination, the presence of synonyms, homonyms or intra-varietal variability complicate the management of fig genetic resources. As a result, the direct comparison of reference fig varieties among the Western Balkan countries is difficult and unreliable. During the project, uniform protocols of molecular analysis and an accurate identification system based on highly informative microsatellite markers were developed, which will allow collaborating countries to establish their own national database of fig genotypes of reference varieties, and to generate results comparable among the project partners. Distribution of genetic variability of cultivated fig forms and their possible relationships with natural fig populations will be the subjects of further collaboration with the aim to provide basic knowledge on the development of genetic structure of Adriatic figs. By the inclusion of new partners into the project consortium, the study will be geographically broadened to the west of the Mediterranean basin. Eastern and western Mediterranean gene pools will be investigated by comparison of wild and cultivated forms in order to clarify the domestication process in local areas, to determine the extent and distribution of genetic diversity and possible genetic relationships between two geographically distinct gene pools.

The fig mosaic disease was observed in all three countries, suggesting that the collaboration of the partners in the future should also be focused on investigation into mosaic causers, virus detection and transmission of the disease.

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Exploring Molecular Biodiversity of Medicinal and Aromatic Plants

Antonios M. Makris[1], Svetlana Kulevanova[2], Johannes Novak[3]

[1]Mediterranean Agronomic Institute of Chania, Department of Natural Products, Makedonias 2, Alsylio agrokepiou, Chania 73100, Greece; Current address: INA/ Center for Research & Technology, Hellas (CERTH), 6km Charilaou-Thermi, Thermi, P.O.Box 60361, Thessaloniki 57001, Greece; antoniosmakris@yahoo.gr, makris@certh.gr
[2]Faculty of Pharmacy, University Ss. Cyril and Methodius - Skopje, Inst. of Pharmacognosy, Vodnjanska 17, Skopje 1000, FYR of Macedonia; Svetlana.Kulevanova@ff.ukim.edu.mk
[3]University of Veterinary Medicine, Institute of Applied Botany, Veterinaerplatz 1, 1210 Vienna, Austria; Johannes.Novak@vu-wien.ac.at

Abstract

The current project aimed to initiate the exploration of molecular biodiversity of terpene synthases, the enzymes responsible for the production of essential oils in sage species and exchange the developed expertise between the partners.
Plant populations of two species growing in the wild in Crete, Salvia fruticosa and Salvia pomifera, and Salvia officinalis from the Skopje region were identified in representative locations and cuttings were collected and used to propagate the selected individuals clonally. An analysis of the essential oils of selected plants was performed using Gas Chromatography/Mass Spectrometry (GC/MS). One gene of the genetic material (cDNA) of a Salvia pomifera plant, resembling in sequence a terpene synthase, was fully sequenced and expressed in yeast. The terpenes produced in-vivo by yeast cells were analyzed by SPME-GC/MS. The products confirmed that the new Sp P20 gene was a sesquiterpene synthase yielding multiple products including α-cubebene, α-copaene and trans-β- caryophyllene.
During the enzyme characterisation procedure, in the context of a short-term scientific visit, all the partners had the opportunity to interact and exchange information on optimising analytical procedures.
The molecular approach to plant biodiversity has the potential to utilise, in an environmentally friendly manner, the wealth of chemical diversity to develop novel products for agriculture, industry and the pharmaceutical sector.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest, and aquatic environments
Type of the project: research project

Keywords
Agricultural Biotechnology; Biochemistry; Essential oils; Genetics of plants; Medicinal and Aromatic plants; Plant Breeding; Terpenoid Biosynthesis

1 Introduction

Plants produce an enormous variety of low molecular weight organic compounds called secondary metabolites through various biosynthetic pathways. Terpenoids and isoprenoids contribute more than 50,000 compounds to this chemical diversity. Many of them have attracted commercial interest as flavour and fragrance additives in the food and cosmetic industry, and for their medicinal properties. Among them taxol, a diterpene from yew has successfully been established in the clinic as a major antineoplastic agent and artemisinin a sesquiterpene compound used against malaria[1-3].
All terpenoids are biosynthesized from two C5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). All terpenoids are biosynthesized from two C5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [4]. Two distinct and independent biosynthetic routes to IPP formation exist. Many plant species, including Salvia sp., produce and store large amounts of terpenoid-rich resins and essential oils in differentiated tissues such as glandular trichomes found on the surface of plant aerial parts [5].
Salvia officinalis (common sage) is a very common herb with strongly aromatic leaves used as flavouring in cooked foods. Sage has a long history of effective medicinal use and it is an important domestic herbal remedy for disorders of the digestive system. The plant produces a broad range of acyclic, monocyclic and bicyclic monoterpenes and was the first sage plant used for the characterisation of terpene synthases. Salvia fruticosa Mill and Salvia pomifera L. are Eastern Mediterranean species of sage. S. fruticosa, often referred to as Greek sage, is one of the most commercially utilised sage plants. A long list of compounds have been described with the predominant being 1,8-cineole, camphor, α and β-thujone. Previous studies from our group identified a high degree of variability in quantity and composition of the essential oils from different populations and clones of S. fruticosa. These differences in essential oils were maintained stably when the clones were grown under controlled conditions and correlated closely to the genetic relatedness of the plants examined [6]. Salvia pomifera also known as “bitter sage” is morphologically differentiated by the leaf and calyx shape. The essential oil is characterised by high levels of α and/or β-thujone, whereas 1,8-cineole and camphor are found in low amounts.
In the context of an EU-FP5 funded program, new genes involved in terpenoid biosynthesis from S. fruticosa and S. pomifera were identified and some of them characterised for their enzymatic activity. This was achieved using a large scale approach by constructing cDNA libraries from specialised terpene producing tissues, the glandular trichomes [7]. Data pointed to the existence of high molecular diversity among terpene synthases to enable the swift chemo-adaptation of plants to altered environments. In the project, we explored aspects of the molecular diversity of terpene synthases that exist in the wild from populations of S. officinalis, S. fruticosa and S. pomifera. The collection area spanned three different Balkan countries from the extreme North to the most Southern point. These plants were used as primary sources of genetic material. Through scientific exchange visits, information and expertise on the analysis and in-vivo enzymatic characterisation approaches of novel terpenoid synthase enzymes were disseminated to all partners.

2 Experimental Techniques and Methods

2.1 Collection of plant material
Cuttings of Salvia plants, both S. fruticosa and S. pomifera, were collected from sites throughout Crete, predominantly in Western Crete but extending also to Southern Crete (East of Lerapetra) and, in the case of S. pomifera, to Northeastern Crete, cuttings from S. offininalis were collected around Skopje and from some locations in Austria. The sites were selected mostly on the basis of previous evidence of diversity of volatile content and composition. Only one of the species was growing in most sites. Attention was given to a few sites in which were found in close proximity. The cuttings collected from the wild were used for clonal propagation in a rooting chamber and, once established, were transferred into pots. When mature, they will be planted in the field.

2.2.1 Cloning procedure and analysis
Primers were designed to amplify the open reading frame of the Sp P20 putative terpene synthase. The designed primers contained two restriction sites. EcoRI prior to the ATG initiation of translation sequence and a SalI after the translational termination sequence. The ORF was amplified in a PCR reaction using a high fidelity Platinum Taq polymerase and the purified PCR amplicon was cloned into a pCRII TOPO TA vector according to the manufacturer’s instructions. A plasmid containing the insert was identified by plasmid extraction, followed by restriction digest to verify the presence of the PCR product. Subsequently, it was sequenced to verify that no mutations occurred during the amplification process. The insert was excised using EcoRI and SalI, purified and ligated to a yeast expression vector pJG4-6 digested with EcoRI-XhoI. The composite construct pJG4-6/Sp P20 shown in figure 3 will express the Sp P20 gene as a fusion with a short N-terminal HA tag in an inducible manner in yeast. In this way, expression of the protein can be monitored at all times. Identification of Terpenes by Solid Phase Microextraction (SPME) method was performed by extracting volatile compounds from the head-space of the sample on 7 mm bonded polydimethylsiloxane (PDMS) fibers (Supelco, Bellefonte, PA). 20 ml of samples in enzymatic reaction buffer (100mM MOPS, 20mM MgCl2, 2mM MnCl2, 50% Glycerol, 10mM DTT) were exposed to a SPME fiber for 30 min at 30°C while mixing the sample with a small magnet. After extraction, the analytes were thermally desorbed at 230°C from SPME fiber into the injector of a QP2010 Shimadzu gas chromatograph equipped with QP2010 mass selective detector, and a ZB5 (0,25mm x 30m, 0,25μm film thickness) column, in the splitless mode. The products were identified by comparing retention times and mass spectra with authentic reference compounds.

3 Results and Discussion

Representative leaves from the three Salvia species were analysed for their essential oil content (Table 1).

Table 1: Terpene composition of Salvia species upper leaves

Compound	% of total		
	S. fruticosa	*S. officinalis*	*S. pomifera*
α-thujene	0.30 – 1.04	0.18 – 0.48	0.24 – 0.84
α-pinene	2.58 – 6.26	1.26 – 4.62	0.42 – 2.40
camphene	0.17 – 1.05	0.95 – 7.96	0.51 – 4.42
sabinene	0.31 – 0.69	0.13 – 0.43	1.00 – 2.65
β-pinene	8.92 – 18.8	1.92 – 5.67	0.40 – 1.85
β-myrcene	1.47 – 5.03	0.65 – 1.47	0.48 – 1.38
limonene	0.06 – 1.28	0.6 – 1.94	0.27 – 0.59
1,8-cineole	22.7 – 49.2	5.81 – 9.16	0.09 – 0.20
γ-terpinene	0.38 – 0.80	0.32 – 0.53	0.20 – 0.49
α-thujone	0.30 – 3.53	5.42 – 43.5	9.80 – 47.4
β-thujone	0.22 – 1.72	3.56 – 8.33	17.8 – 27.7
camphor	0.13 – 1.72	4.72 – 27.7	0.34 – 1.18
borneol	0.51 – 1.34	0.53 – 4.22	0.14 – 0.93
α-cubebene	0.03 – 0.19	0.00 – 1.63	1.40 – 4.68
β-caryophyllene	4.64 – 12.75	1.53 – 14.0	7.82 – 22.8
α-humulene	2.18 – 4.13	6.00 – 11.2	0.43 – 1.49
curcumene	0.03 – 0.18	0.00 – 0.03	0.12 – 5.16
δ-cadinene	0.03 – 0.70	0.00 – 0.05	2.29 – 8.00

In S. pomifera, the main monoterpenes were α-thujone (9.8 – 47.4 % of total mono- and sesquiterpenes), β-thujone (17.8 – 27.8 %) and sabinene (1.0 – 2.7 %), accompanied by small amounts of camphene (0.5 – 4.4 %), α-pinene (0.4 – 2.4 %), β-pinene (0.4 – 1.9 %), and camphor (0.3 – 1.2 %) and the main sesquiterpenes were α-cubebene (1.4 – 4.6 %), β-caryophyllene (7.82 – 22.8 %) and δ-cadinene (2.2 – 8 %), (Table 1).

The cuttings collected from the wild were used for clonal propagation in a rooting chamber and, once established, were transferred into pots. When mature, they will be planted in the field.

We screened a number of Expressed Sequence Tags (ESTs) of two medicinal and aromatic plants of choice (Salvia fruticosa and Salvia pomifera) from the recently constructed cDNA libraries, to identify cDNAs that share homology to terpene synthases from other plant species. Several sequences were identified that appeared to encode partial or full length genes. In the context of the current cooperation project, we chose one full length cDNA clone named P20 that was isolated from the Salvia pomifera glandular trichome library. The full length of the cDNA was read by DNA sequencing using a series of designed gene internal primers that enabled us to cover the full length of the cDNA several times. The full length cDNA is 1,850 bp and appears to encode a 555 amino acid protein that does not have a transit N-terminal sequence and shares higher homology to characterised sesquiterpene synthase enzymes (Figure 1).

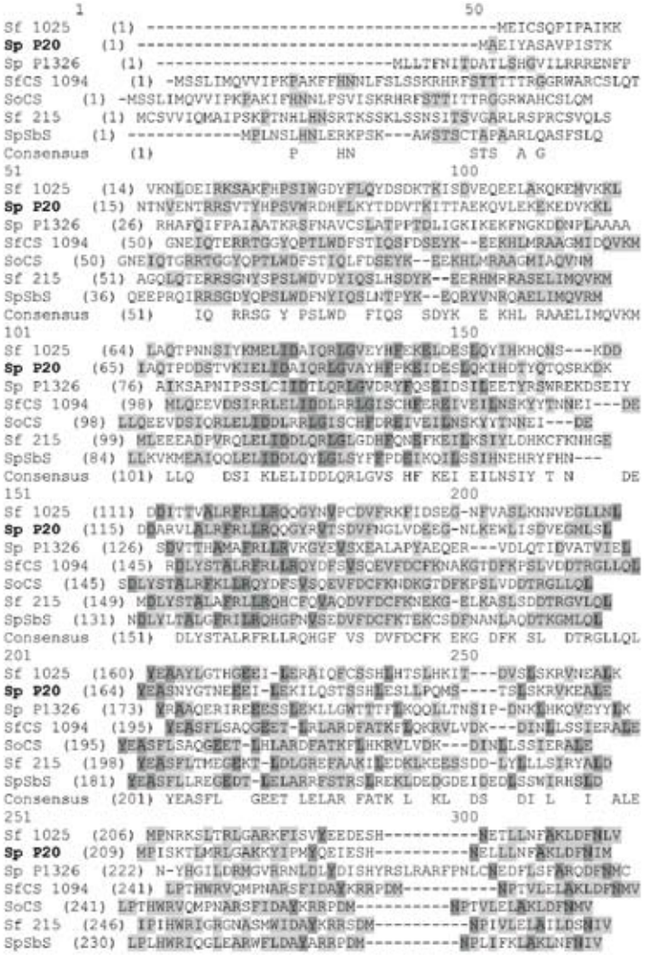
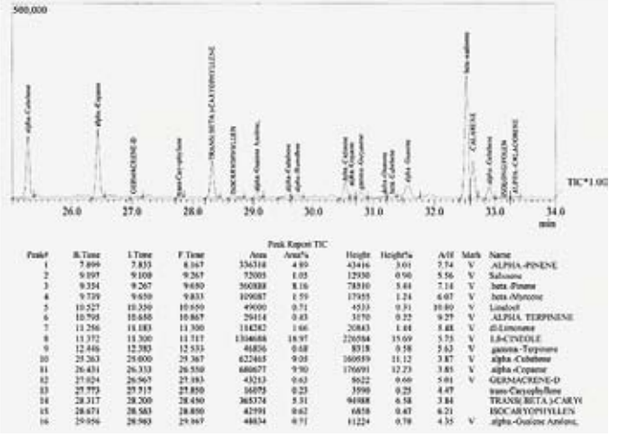


Figure 1: Amino acid sequence alignment of terpene synthases isolated from Salvia fruticosa and Salvia pomifera, Sf 1025, a novel sesquiterpene synthase from Salvia fruticosa, Sp P20, Sp 1326, a putative sesquiterpene synthase from Salvia pomifera, SfCS 1094, a cineole synthase from Salvia fruticosa (the orthologue of the previously isolated cineole synthase from Salvia officinalis), SoCS cineole from Salvia officinalis, Sf 215, a second novel cineole synthase from Salvia fruticosa, SpSbs, a sabinene synthase from Salvia fruticosa.

Prior to the gene isolation, we had developed a novel yeast system for the facile characterisation of terpene synthases. This is based in an optimised yeast strain that harboured an additional mutant copy (K6R mutation) of the critical control gene HMG2 which conferred increased stability to the protein, as well as high inducible expression of the internal GPP-FPP synthase ERG20. All these changes lead to increased production of the enzymatic substrates (Geranyl Pyrophosphate – GPP) of Farnesyl Pyrophosphate (FPP) with end result a threefold to fourfold increase in terpenes produced by the expressed heterologous plant terpene synthases. This enables a quick "in-vivo" characterisation of the new putative terpene synthases.
Subsequently, the Sp P20 gene was introduced into the special yeast strain and cells were induced to express the protein. The induced cells were transferred to a buffer medium and were sampled the 2nd day for their terpene content by SPME/GC-MS (Figure 2).



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	7.899	7.833	8.167	336318	4.89	43416	3.01	7.74	V	ALPHA-PINENE
2	9.197	9.100	9.267	72005	1.05	12930	0.90	5.56	V	Sabinene
3	9.354	9.267	9.650	560888	8.16	78810	5.48	7.14	V	beta -Pinene
4	9.739	9.650	9.833	109087	1.59	17955	1.24	6.07	V	beta -Myrcene
5	10.527	10.350	10.650	49000	0.71	4533	0.31	10.80	V	Linalool
6	10.795	10.650	10.867	29414	0.43	3170	0.22	9.27	V	ALPHA. TERPINENE
7	11.256	11.183	11.300	114282	1.66	20843	1.44	5.48	V	dl-Limonene
8	11.372	11.300	11.517	1304688	18.97	226084	15.69	5.75	V	1,8-CINEOLE
9	12.446	12.383	12.533	91856	0.68	8518	0.58	5.63	V	gamma-Terpinene
10	25.263	25.000	25.367	622465	9.05	160589	11.12	3.87	V	alpha-Cubebene
11	26.431	26.333	26.550	680627	9.90	176691	12.23	3.85	V	alpha-Copaene
12	27.024	26.967	27.183	43213	0.63	9622	0.66	5.01	V	GERMACRENE-D
13	27.775	27.717	27.850	36078	0.25	3590	0.25	4.47	V	trans-Caryophyllene
14	28.317	28.200	28.450	365374	5.31	94988	6.58	3.84		TRANS(BETA.)CARYO
15	28.671	28.583	28.850	42991	0.62	6858	0.47	6.21		ISOCARYOPHYLLEN
16	29.056	28.983	29.367	48834	0.71	11224	0.78	4.35	V	alpha.-Guaiene Azulene,

Figure 2. Profile of volatile sesquiterpenes produced from the expression of the Sp P20 in the optimised yeast strain. The ambient environment of an induced culture was sampled with Solid Phase Microextraction (SPME) and the sample was analysed in a GC/MS.

The Sp P20 enzyme was confirmed to be a terpene synthase producing several sesquiterpenes which included α-cubebene, α-copaene, trans-β-caryophyllene, β-cadinene and traces of germacrene D and isocaryophyllene. Surprisingly a fair amount of monoterpenes are also produced with most prominent being cineole. This may be due to increased availability of both GPP and FPP in the cells, as the gene ERG20 is considered a bifunctional enzyme. Among the identified components, α-cubebene, trans-β-caryophyllene and, to a lesser extent, germacrene D have been regularly identified in Salvia pomifera, which indicates that the newly characterised enzymes are responsible for a substantial proportion of the sesquiterpene compounds produced in pomifera. Firmenich, an international flavour company, has patented cubebol, the sesquiterpene alcohol as a refreshing agent in various products ranging from chewing gum to sorbets, drinks, toothpaste and gelatin-based confectionaries in 2001 (U.S. patent 6,214,788).

4 Outlook

Considering the recent realisation that terpene synthases are extremely plastic enzymes – that means that small changes in the protein sequence can lead to large alterations in product composition and yield – and the extensive gene duplication that occurs in Salvia plants as assessed by sequencing of genome fragments by our group, we anticipate the presence of a wealth of genetic information for terpene synthases producing terpenes that are either found in Salvia in trace quantities or not found at all. To this end, the availability of molecular tools generated will enable us to further identify and characterise new biosynthetic enzymes. Information generated from the genomics data will provide new insights on how to manipulate the enzymes so as to produce novel valuable terpenoids. In addition, work will focus in further improving the yeast in-vivo production system to increase yields and become a commercially feasible "green chemistry" approach for the production of high-added value compounds.

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Genetic Study of an Invasive Forest Insect and its Parasitoid in the Balkan Region

Carlos Lopez-Vaamonde[1], Sylvie Augustin[1], Alain Roques[1], Ejup Cota[2], Endrit Kullaj[2], Nikolaos Avtzis[3], Rumen Tomov[4] and Sterja Naceski[5], Irena Papazova[5]

[1] INRA, UR0633 Zoologie Forestière, 45075 Orléans, France; carlos.lopez-vaamonde@orleans.ina.fr, sylvie.augustin@orleans.inra.fr, alain.roques@orleans.inra.fr
[2] Plant Protection Department and Department of Horticulture, Faculty of Agriculture and Environment, Agriculture University of Tirana, Albania; endritkullaj@yahoo.com; cotaentom59@yahoo.com
[3] School of Agricultural Technology, Drama, Greece; avtzisn@yahoo.gr
[4] Forestry University, Sofia, Bulgaria; rtomov@yahoo.com
[5] Ss. Cyril and Methodius University, Skopje, Former Yugoslav Republic of Macedonia; naceskisterja@yahoo.com, ipapazova@sf.ukim.edu.mk

Abstract

The invasion of species outside their native range is increasingly frequent. It can have a serious impact on biodiversity and cause health risks and economical damage to agriculture and forestry. The rapid spread of these alien invasive species has mainly been investigated from an ecological point of view. Little is known about the evolutionary aspects of invasions.
Invasions are rapid evolutionary events in which populations are unusually subjected to founder effect during colonisation event followed by a rapid expansion. Thus newly arrived populations are likely to be less variable than the original population from which they derived. This represents a paradox since successful invading species are likely to be genetically depauperate compared to their source population. In this project, we have studied this paradox using Cameraria ohridella, an invasive microlepidoptera, whose origin is unknown as a model system. Since its discovery in the Former Yugoslav Republic of Macedonia twenty years ago, this insect has progressively colonised all Western and Central European countries, including nearly the whole of France.

In the SEE-ERA.NET Pilot Joint Call project, both molecular markers were used to study the genetic variability of C. ohridella across its geographical distribution to assess whether C. ohridella shows a loss of genetic diversity associated with the process of invasion and for the presence of "invasive" haplotypes. The main objectives of this project are (i) to study the genetic structure of both the area of origin and invaded areas of C. ohridella and (ii) to see whether the invasion process and associated demographic expansion is due to changes in the genetic structure of the populations.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest, and aquatic environments
Type of the project: research project

Keywords

Aesculus hippocastanum; Balkans; Cameraria ohridella; DNA barcode; Invasion; Leaf miner; Phylogeography

1 Introduction

More and more insects are introduced deliberately or unintentionally outside their natural habitats due to the increasing rate of trade and travel in the world. Some of these insects have the ability to establish themselves in these new environments, develop dense populations and out-compete native species. Determining the area of origin of these invasive insects is a key step in the development of control strategies, particularly with biological control.

The horse-chestnut leafminer, Cameraria ohridella Deschka and Dimic (Lep.: Gracillariidae) is a micromoth (Figure 1) which was first found in the Former Yugoslav Republic of Macedonia in the early 1980s (Deschka and Dimic, 1986). Since then, it has spread over most of Europe, and its distribution now covers an area from England to the Ukraine and from Sweden to Spain, Italy and Greece (Figure 2). It took over four years to invade the whole of France at an unusually high speed of 60 km per year (Guichard & Augustin, 2002; Augustin et al., 2004; Gilbert et al., 2005). Its main host, the common horse-chestnut (Aesculus hippocastanum) is a major urban tree in most of Europe. In most regions where the pest occurs, horse-chestnut trees are severely defoliated year after year (Figure 3). The trees are not killed, but the aesthetic damage is so severe that municipalities are already replacing this highly valuable tree by other species. The horse-chestnut is endemic to the Balkans where the few remaining natural stands are also attacked, causing concern for the survival of this rare tree species. Furthermore, the moth can also attack and develop on maple species (Acer pseudoplatanus and A. pseudoplatanoides) and, considering the pressure on the moth to find new suitable host plants after having totally defoliated horse-chestnut trees, it is likely that the damage sustained by maples will increase in the near future.

For the moment, control methods are limited to the aerial spraying of diflubenzuron and bifanthrine, and the removal of dead leaves, in which the moth overwinters. However, these methods are expensive and practically difficult to conduct over large areas. The only method that may provide a long-term and sustainable solution to the problem is classical biological control, through the release of natural enemies in particular parasitoids from the region of origin of the moth (Kenis et al 2005).

Despite numerous ecological studies and a European project (CONTROCAM, finished in 2004) focused on the control of C. ohridella, the region of origin is not yet known (Kenis et al. 2006). Preliminary molecular analyses show very little genetic variability in European populations (Lakatos et al., 2006). However, these results were based on populations collected in plantations in Central Europe only, and the natural distribution area of A. hippocastanum was excluded. In our project, we propose to study the genetic diversity of Cameraria populations in natural forests of A. hippocastanum in the Balkans. However, if Cameraria has a balkanic origin, why does it only spread now, and so rapidly? This clear example of rapid expansion provides an important opportunity to study the relative importance of genetic and ecological processes in generating this spread.

There are three main evolutionary scenarios that may have been involved in the expansion of C. ohridella:

1. The rapid spread of one or a small number of pre-adapted populations has been responsible for the expansion of the range, with little contact with other populations. If this hypothesis is correct, we would expect to find significantly higher genetic diversity in populations in natural stands of the host plant than in new populations from parks and gardens in Central Europe. If the Balkans are the area of origin of C. ohridella, this species should show populations at equilibrium in this area, and show a significant loss of genetic diversity in the recently invaded areas.
2. New or existing mutations have spread from a low frequency in a single marginal population to many newly-founded populations at the expanding range. Thus, the invasion of Central and Western Europe by both species has been done by a few "invasive" haplotypes.
3. New mutations arose independently in several established populations, and spread through existing parts of the range to increase the frequency of generalist genotypes at the range margin. In this case the alleles underlying adaptation will differ in different parts of the newly-established range, so we will find many „invasive" haplotypes.

Finally, the parasitoid complex of the moth is well studied across Central and Western Europe and less well-known in the Balkan region. A total of 30 parasitoids have been recorded attacking C. ohridella, mostly eulophids (Grabenweger, 2003; Toth & Lukas, 2005). However, their impact is very limited (Freise et al., 2002; Grabenweger, 2003) although there is some sign of a slow adaptation of some parasitoids to C. ohridella (Toth & Lukas, 2005). The major difference between the Balkans and the rest of Europe is the prevalence of the eulophid pupal parasitoid Pediobius saulius, the dominant species in artificial and natural stand in the Balkans. In Central and Western Europe, it is a common parasitoid of other leaf miners, but rarely attacks C. ohridella. This parasitoid has a broad range of hosts, however no study has ever addressed the possibility of cryptic species within this alleged polyphagous parasitoid species. In this project, we have used molecular markers to test whether the P. saulius is in fact a complex of cryptic species, some of which may be specific to C. ohridella and in turn could be used as biocontrol agents.

The serious damage caused by the larvae of this moth and the speed of its invasion of Europe has attracted the attention of many researchers. Indeed, the European multidisciplinary project CONTROCAM (http://www.cameraria.de/index.php) was implemented by a consortium of eight institutions, namely INRA (France), TUMUC (Germany), CSIOCB (Czech Republic), CABI (Switzerland), UBW (Austria), UBERN (Switzerland), UTRS (Italy) and TEIK (Greece), aimed at developing a sustainable method of control of this invasive species. All these efforts have caused C. ohridella to become one of the best known microlepidoptera. However, despite all these research efforts, the area of origin of C. ohridella remains unknown, very little is known about its population genetics and no potential biocontrol agent has been found yet. The main reason for the collaboration was to gather genetic data from C. ohridella populations and its parasitoid Pediobius saulius to fill this gap of knowledge.

This project has generated genetic data across the distribution range of C. ohridella, in particular from the natural forests of A. hippocastanum in the Balkans.
This newly generated data has helped to:

- understand whether the invasion process and associated demographic expansion is due to changes in the genetic structure of the populations;
- evaluate the changes in population structure and effective population size that have been involved in the range expansion; and to
- study the phylogeography and demogenetics of a highly invasive microlepidoptera associated with major urban trees which represented the base for a comparative approach to the study of the causes of invasion.

The implemented research is multidisciplinary as it seeks to employ a range of research methodologies and skills and to integrate their results to provide a major boost to the field of biological invasions. An exciting combination of population genetics and field collections has provided the basis for testing several key hypotheses. The research has fostered collaboration with members of Augustin's and Lopez-Vaamonde's research group and with international researchers who have assisted in the planning and execution of fieldwork. Indeed, the present project includes one partner from each country where natural stands of horse chestnut exist: Albania (Ejup Cota and Endrit Kullaj); Greece (Nikolaos Avtzis); Bulgaria (Rumen Tomov) and the FYR of Macedonia (Sterja Naceski). In addition, we have used specimens already collected in the frame of the European project CONTROCAM, and we have received specimens collected across a European field site network of the european project ALARM (Assessing Large scale Risks for biodiversity with tested Methods), (http://www.alarmproject.net/alarm/fsn.php) and SCOPES.

2 Experimental Techniques and Methods

The project's work was divided into two workpackages:
Workpackage 1: The genetic diversity of Cameraria populations from natural and artificial stands of A. hippocastanum in the Balkans and Central Europe was studied to determine the origin of the moth.

Workpackage 2: The tests whether Pediobius saulius could be a complex of cryptic species, some of which may be specific to C. ohridella, we carried out.

2.1 Methodology Workpackage 1

We have used specimens already collected in the frame of the European project CONTROCAM and specimens collected across a european field site network of the European project ALARM (Assessing Large scale Risks for biodiversity with tested Methods). In addition, our partners in Albania (Ejup Cotra), Bulgaria (Rumen Tomov), Greece (Nikos Avtzis) and the FYR of Macedonia (Sterja Naceski) have collected Cameraria ohridella at different localities of natural stands of horse chestnut across the Balkans and several localities of artificial Aesculus plantations across Central Europe and the Balkans.
We have extracted DNA using standard protocols for genotyping and sequencing in our ABI 3100 capillary sequencer at INRA Orléans.

We used cytochrome oxidase I sequences (DNA barcode fragment) and a set of six polymorphic microsatellites developed at INRA Orleans (Mari Mena et al 2008) to assess the genetic variability of C. ohridella populations and to test the hypothesis that C. ohridella comes from the southern Balkans (Albania, FYR of Macedonia and Greece). Sample size: 486 individuals sampled across 87 populations of natural and artificial horse chestnut stands. In addition, 212 individuals from 7 populations from the Southern Balkans were typed for 6 polymorphic microsatellite loci.
Finally, basic parameters of population genetics such as the number of variable sites, haplotype diversity, nucleotide diversity, the average number of differences among haplotypes and Tajima's D have been calculated. Analyses of molecular variance (AMOVA) and test for a possible correlation between genetic and geographical distance and estimates of F_{ST} have been carried out. The genetic differences between localities taken by pairs have been calculated.

2.2 Methodology Workpackage 2

We sampled leaves on A. hippocastanum in localities of natural and artificial stands. Infested leaves were collected on the different stands when the majority of the moths were in the pupal stage. Leaves were collected randomly from the lower branches of the trees or on the ground. Mines were dissected and mines containing parasitoids were isolated and parasitoids were reared in a climatic chamber at 20°C under long-day conditions. In addition, the leaves which were not foreseen to be dissected were kept in emergence boxes for mass rearing. After emergence, all adult parasitoids were killed in alcohol 100° for genetic analysis. In addition, we have used specimens already collected across Europe within the frame of the projects ALARM (Assessing Large scale Risks for biodiversity with tested Methods) and SCOPES.

We used standard procedures to sequence their DNA. A branching rate model was used to identify genetic clusters of very closely related individuals separated from other such clusters, representing distinct populations or species by longer internal branches. Sample size: 150 individuals sampled across natural and artificial horse chestnut plantations have been sequenced.

3 Results and Discussion

This study exposed the first comprehensive population molecular data set of a highly invasive leaf mining moth. It shows a clear reduction in genetic diversity from the Southern Balkans to Western and Central Europe. This high genetic diversity found in the Southern Balkans and the presence of a high number of unique haplotypes and private microsatellite alleles in natural stands of A. hippocastaneum supports the hypothesis that the Southern Balkans is likely to be the area of origin of C. ohridella.

DNA-based methods have mostly been advocated as an accurate way of identifying non-native invasive species. In particular, the use of a fragment of the cox1 gene as a DNA barcode sequence has been shown to be an effective way to identify invasive species of insects. Indeed, this gene region shows a low intraspecific nucleotide variation, but a substantial interspecific variation, which allows to assign unknown specimens to species.
By accumulating COI sequences within species, the ongoing DNA barcoding campaigns (ie. http://www.lepbarcoding.org) represent a potential valuable source for documenting intraspecific variations and answering questions about phylogeography and invasion genetics. Thus, this gene region has successfully been used to determine whether invasive earthworm species are established via single vs. multiple introduction events and to identify the area of origin of invasive thrips.
Our study shows that the high number of haplotypes found in the Southern Balkans yet the small number of mutations between these haplotypes makes DNA barcodes a useful region to identify the source populations of C. ohridella. Regarding the pupal parasitoid Pediobius saulius, our preliminary results show that this species is not a complex of cryptic species.

4 Conclusions / Outlook, next steps

During this project we carried out field work across all southern Balkans. The Albanian colleague Dr. Endrit Kullaj visited INRA Orleans in two occasions in 2008 (for a total of 4 weeks). He was trained in the following molecular techniques: DNA extraction and PCR using microsatellites as molecular markers to study the population genetics of C. ohridella.

High haplotype and low nucleotide diversities and Tajima's D indicate that C. ohridella has experienced rapid population expansion during its dispersal across Europe. Both mtDNA and microsatellites show a reduction in genetic diversity of C. ohridella populations sampled from parks across Europe compared to C. ohridella sampled in natural stands of horse chestnuts in the Southern Balkans. These findings suggest that European populations of C. ohridella may indeed derive from the Southern Balkans.

C. ohridella is known to attack maple species in invaded areas, in particular when maple trees are in the vicinity of heavily attacked horse-chestnut trees. The fact that a high percentage of individuals with haplotype B are found on Acer may suggest that there could be some degree of host associated molecular divergence. Further sampling of moths on maple trees will help to test this hypothesis.

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Some of the parasitoid wasps known to attack C. ohridella may be more specific than previously thought and could be used as agents in a biological control program. Studies on the population dynamics and mortality factors in natural horse-chestnut stands should be carried out, in order to better understand the extraordinary invasion success of the moth and, possibly, develop new management strategies. We hope to be able to carry out our collaborative work by studying the ecology and genetics of other natural enemies of C. ohridella in the natural stands of Aesculus in the Southern Balkans.

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Global Epidemiology of Phytoplasma Diseases of Economic Importance in Southeast Europe

Xavier Foissac[1], Sylvie Malembic-Maher[1], Michael Maixner[2], Wolfgang Jarausch[3], Nicolas Sauvion[4], Florin Oancea[5]

[1] UMR-1090 INRA and University Bordeaux 2, BP81, 71 avenue E. Bourlaux, 33883, Villenave d'Ornon, France; foissac@bordeaux.inra.fr
[2] Julius Kuehn Institute (JKI), Federal Research Centre for Cultivated Plants. Institute for Plant Protection in Fruit Crops and Viticulture, Brüningstraße 84, 54470 Bernkastel-Kues, Germany; Michael.Maixner@jki.bund.de
[3] AlPlanta - Institute for Plant Research, RLP AgroScience GmbH, Breitenweg 71, 67435 Neustadt/W., Germany; wolfgang.jarausch@agroscience.rlp.de
[4] INRA, UMR BGPI, CIRAD TA A 54/K, Campus de Baillarguet, 34398 Montpellier Cedex 5, France; sauvion@supagro.inra.fr
[5] Department Phytopathology, Institute Research Institute for Plant Protection, Academy of Agricultural Sciences and Forestry, 8 Ion Ionescu de la Brad Nr 8, 013813, Bucharest, Romania; florino@ping.ro

Abstract

The network 'Phytoplasma epidemiology' allowed thirteen different laboratories to experience genotyping of phytoplasma strains and insect vectors, a strategy promoted to better trace the routes of phytoplasma diseases spread. After a first meeting in Bologna during which working groups were organised, an inventory of infected plant and insect DNA collections was made. Working group 1 (four teams) genotyped the quarantine grapevine pathogen Flavescence Dorée (FD) phytoplasma and related phytoplasmas inhabiting alders in order to assess their possible role as an epidemic reservoir. The second working group (nine teams) did a multilocus genotyping study of stolbur phytoplasma responsible of bois noir (BN) disease of grapevine and yellows in solanaceous crops, which led to the first description of more than 22 strain genotypes some of which are specific to the Balkan area. The third working group submitted fruit tree phytoplasmas to genotyping for four genetic loci and described the genetic diversity of pome and stone fruit phytoplasmas. Four laboratories started to collaborate on insect vector population genetics as a fourth working group. All laboratories agreed on a database format which was established by the Romanian team as a website. Four bilateral visits between teams helped to exchange biological materials and methods and 24 participants attended three days of practical training held in Bordeaux, followed by two days of scientific presentations, reporting and discussions on future collaboration and common applications.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest and aquatic environments
Type of the project: network project

Keywords

Crop protection; Diagnosis; Environmental Microbiology; Epidemiology; Genotyping; Insect vectors; Phytoplasmas

1 Introduction

Among the phloem-restricted bacteria, phytoplasmas are the most damaging plant pathogens with an important economical impact on perennial as well as corn and vegetable crop [1]. They are naturally transmitted by leafhoppers, plant hoppers and psyllids which are hemipteran phloem feeders [2], but also spread through nurseries when infected planting material is propagated. Disease control relies on prophylactic sanitary measures consisting in the destruction of infected crop, the chemical control of insects and protection of nurseries.

A potential driving force for phytoplasma evolution and epidemiology is the necessity to adapt to new plant or insect hosts after an invasion of new ecological niches. This can happen when new insect vectors or new plants are introduced. However, phytoplasmas like the other members of the bacterial class Mollicutes have limited genome, with size ranging from 530 kbp to 1,350 kbp. The first two phytoplasma genomes have recently been sequenced and many others are currently sequenced ([2]; [3]; [4]). This genomic information permits the fine genetic identification of phytoplasma strains through Multi Locus Sequence Typing strategies [5].

This project has coordinated the efforts of plant pathologists and entomologists of Southeast European countries to better monitor phytoplasma strains incidence and propagation at the European scale both in plants and insects using updated molecular typing tools. It is also aimed to improve the certification of plant material by using updated real-time PCR detection technology, to develop a common phytoplasma typing technology and to initiate the development of mitochondrial gene markers for the description of hemipteran vectors at species and ecotype level to promote phylogeographic studies.

Three different groups of phytoplasmas with important impact on Southeast European agriculture will be targeted. The quarantine Flavescence dorée of grapevine phytoplasma (FD, group 16SrV) transmitted by a leafhopper of North American origin, has been recently reported in the Balkans and the related alder phytoplasmas could constitute a wild reservoir of FD according to recent findings. The stolbur phytoplasma (group 16SrXII-A) is a European endemic phytoplasma of wild compartment origin and is transmitted from bindweed, nettle and lavender to grapevine, maize, solanaceous crop (tomato, pepper, potato, eggplant), strawberry and sugar beet by polyphagous planthoppers of the Cixiidae family. Some stolbur phytoplasma strains recently appeared to be associated with specific insect vector ecotype which could indicate some strains specialisation [6]. Finally, European stone fruit, pear decline and apple proliferation phytoplasmas (group 16SrX) are affecting South European orchards and are transmitted by psyllids, some of which are doing part of their ecological cycle on wild hosts.

2 Experimental Techniques and Methods

2.1 Constitution of the list of samples

2.1.1 Working group 1, group 16SrV phytoplasmas

In September 2008, the following call was sent to all the participants in order to constitute the list of DNA samples. For grapevine and/or S. titanus samples: countries where FD is recorded (Serbia and France) should compile infected samples representative of the main focal points on their territory. Twenty to 30 samples by country should be enough. Countries were FD is not recorded could possibly compile other grapevine yellows phytoplasma isolates already identified as 16SrV. For alder samples: countries having FD should collect symptomatic alder samples in a vine area where FD outbreaks have been identified, in a vine area known free of FD and in a vine free area. Countries without FD, if interested, could collect symptomatic alders in one area (with or without vines). Ten samples from each area should be sufficient. Before the end of December 2007, the final lists of 132 isolates were collected: 57 from alder (AldYp), 50 from grapevine (FDp and PGYp), 18 from O. alni (AldYp) and seven from S. titanus (FDp).

2.1.2 Working group 2, group 16SrXII-A phytoplasmas

Nine partners participated in the setting up of the collection of DNA isolates namely DBUZ-IPPAF (Croatia), INRA-Bordeaux (France), BBA-Bks-UnivMainz (Germany), LPP-UTH (Greece), PPDL-Experta (Hungary), PSI-ISC (Macedonia), IPPE (Serbia), PERI-Belgrade (Serbia), UTH (Greece). Altogether, 393 plants samples including 256 grapevine samples as well as 20 bindweed and stinging nettle samples were proposed to be included in the genotyping study.

For the insect vectors, 161 DNA extracts were proposed including 157 Hyalesthes obsoletus from Germany, Croatia and Serbia.

2.1.3 Working group 3, group 16SrX fruit tree phytoplasmas

A set 74 of Prunus samples infected with Ca P. prunorum (ESFY), 25 Malus samples infected by Ca. P. mali (AP) and 35 Pyrus samples infected with Ca. P. pyri (PD) were collected in Germany, France, Serbia, Hungary, Greece and Croatia. This list was completed by a set of isolates coming from Spain, Italy, Azerbaijan and Turkey.

Psyllids DNA were also extracted from 64 insects positive for ESFY (42), AP (25) and PD (3).

2.2 Detection of the phytoplasmas in the samples

Detection of phytoplasmas in the samples had already been done by the different teams by single or nested-PCR with group specific primers. For group V and group XII-A phytoplasmas, a practical session entitled "Detection of Flavescence dorée and Bois noir phytoplasmas in grapevine by a triplex real-time PCR" was organised during the workshop held in Bordeaux in June 2008.

2.3 Typing of the phytoplasmas and insects

- For group V phytoplasmas, as a first step, the typing of every isolate was performed by sequencing of the gene map as previously described [7]. In a second step, some isolates were selected for the sequence typing with a second marker uvrB-degV. IPPE team realised the typing of their own isolates and INRA Bordeaux team characterised the rest of the isolates.
- For group XII-A phytoplasmas, the three markers tuf, secY and vmp1 were used by PCR-RFLP or sequencing.
- For group X, four genes were sequenced namely aceF, pnp, secY and imp according to previously published procedures [8, 9)].
- For insects, DNA extracts from the established list were tested during the workshop with two pair of primers :
  - COI primers: LCO-1490 / HCO-2198 [10].
  - ITS primers: FCM / BrD [11]. At the beginning of the project (first meeting Bologna), it was decided to test a range of samples of each species from different localities (that is, from the largest possible number) rather than many individuals of few localities.

2.4 Rationale for a website design

The website of the project is indented to reach two aims:

- Communicate the importance of the ERANET project regarding the "Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe" to the stakeholders; and
- Exchange results between scientists who are members of the network.

The web site was expected to achieve the following major objectives:

- The website had to be dynamic, by incorporating a database, to allow for simple and secure communication of results between the Phytoplasma epidemio project members.
- The website had to be easily updatable by the project leader / activity leaders until completion of the scientific project.
- The website had to be informative, by containing a section which would provide relevant information about project.
- The website had to be accessible, also considering the needs of those with low bandwidth connections and older technologies so that they can use, interact, understand, navigate and fully perceive the website.

The main characteristics of the web site of the projects were considered to be: (i) fast-loading; (ii) flexible; (iii) functional and (iv) accessible. Special attention was paid to the way of presenting the information. This was effectively presented in a front-loading manner, called the inverted-pyramid. This style ensures that the paragraphs are kept short, presenting one main point at a time.

For the database, a workflow decided for the curation of new genotypes to be described in the database is shown in Figure 1.

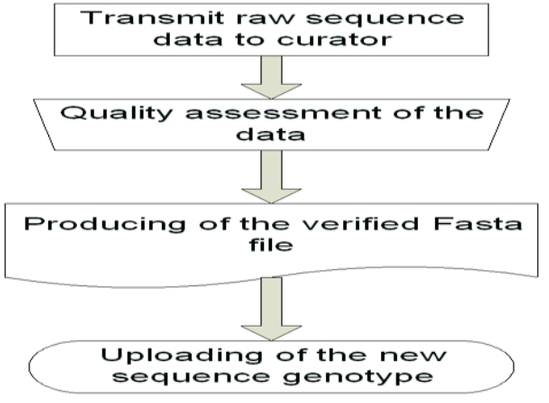


Figure 1: Procedure for the description of a new sequence genotype agreed with the members of the Phytoplasma epidemio network

3 Results and Discussion

3.1 Working group 1, group 16SrV phytoplasmas

Chromatograms of the gene map for 116 isolates were fully exploitable on 674 bp. Some isolates' chromatograms revealed sequences ambiguities (superposition of nucleotide pics) which were detected for one up to ten positions. This indicates a mix of different phytoplasma populations in the same sample. "Mixed" infections were detected, in 1/18 O. alni and in 30/56 alders but were not detected in grapevine and S. titanus. For the mixed infections, the map gene was re-amplified by single PCR with a taq proof-reading enzyme (30 cycles with the FD9F6 and MAPR2 primers), cloned in the pGEMt Easy plasmid and four clones were sequenced (named A, B, C and D). The cloning was performed at INRA Bordeaux during the bilateral visit of Jelena Jovic from IPPE team-Serbia (from May 25-31). She was trained to the cloning techniques and we were able to clone eight French and three German alder isolates. Phylogenetic analyses were performed on a total of 117 sequences plus 19 sequences of reference strains [7]. A phylogenetic tree is presented Fig. 2. Forty-six different genotypes were identified: Three in S. titanus, seven in O. alni, 11 in grapevine and 37 in alder. Analyses confirmed that grapevine and alder phytoplasmas have a common phylogenetic origin. Twelve genetic clusters could be described. Three clusters containing FDp isolates were named FD1, FD2 and FD3. FD1 was strictly French, FD2 was French and Italian and FD3 was Serbian and Italian. No German isolates were found in these clusters. French AldYp isolates with exactly the same genotype as FD isolates were found in the FD1 cluster. French and Serbian AldYp isolates presented three and four SNPs respectively with the FD isolates from the cluster FD2. There were no AldYp isolates genetically close to the FD3 cluster. Nine genetic clusters containing AldYp and PGYp isolates were named AldY 1 to 9. They contained German, Hungarian, Serbian and French isolates.

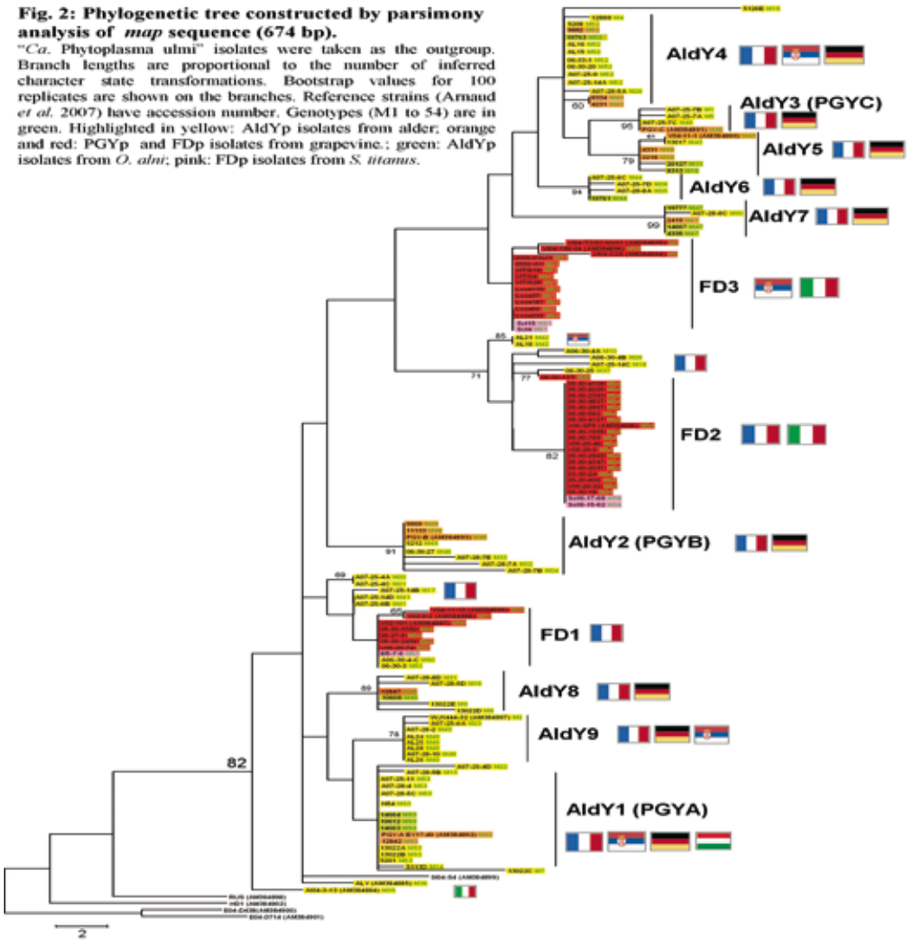


Fig. 2: Phylogenetic tree constructed by parsimony analysis of *map* sequence (674 bp).
"*Ca.* Phytoplasma ulmi" isolates were taken as the outgroup. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values for 100 replicates are shown on the branches. Reference strains (Arnaud *et al.* 2007) have accession number. Genotypes (M1 to 54) are in green. Highlighted in yellow: AldYp isolates from alder; orange and red: PGYp and FDp isolates from grapevine.; green: AldYp isolates from *O. alni*; pink: FDp isolates from *S. titanus*.

The uvrB-degV gene was amplified and sequenced for eight grapevine isolates (five from Germany and three from Serbia). Two different genotypes were identified in Germany and two different ones in Serbia. This is the first time that different genotypes are identified in grapevines from Serbia.

3.2 Working group 2, group 16SrXII-A (stolbur) phytoplasmas

127 samples were typed by HpaII-RFLP of tuf-PCR: 79 samples were of tufII genotypes while 43 samples were of tufI and five of tufIII genotypes. This may indicate that in our collection two third of the isolate were coming from a bindweed reservoir. In the Balkan countries, genotype tufII was largely dominating, while in France and Germany the ratio between the two genotypes was balanced.

According to vmp1 RsaI-PCR-RFLP, 141 samples could be examined. Fourteen different RFLP patterns could be evidenced. Pattern V1 (39 isolates) and pattern V4 (33 isolates) were predominating. The V1 genotype appeared specific of France and Germany, while the genotype V4 was present in all countries except Hungary. Patterns V2 and V3 accounted for 18 isolates each but was absent from Germany, V18 and V14 represented 13 and 11 isolates respectively and were specifically abundant in the Balkan countries. Genotypes V6, V7, V8, V12 and V19 were only detected in one or two French grapevine samples, whereas genotype V13 and V15 were only detected in one infected grapevine in Croatia and Germany respectively.

SecY sequence genotypes were obtained for 138 isolates and seven different genotypes were distinguished (see Figure 3). Most of the stolbur genotypes belonged to the S6 (56 isolates), the S1 (44 samples) and the S4 (26 samples) genotypes. Four genotypes, namely S18 (four samples from Serbia, FYRM, Hungary), S7 (three samples from Croatia), S20 (one sample from Greece) and S21 (one sample from Greece) were only detected in samples from Balkan.

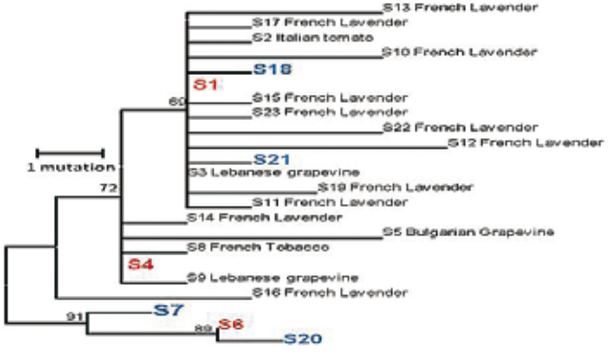
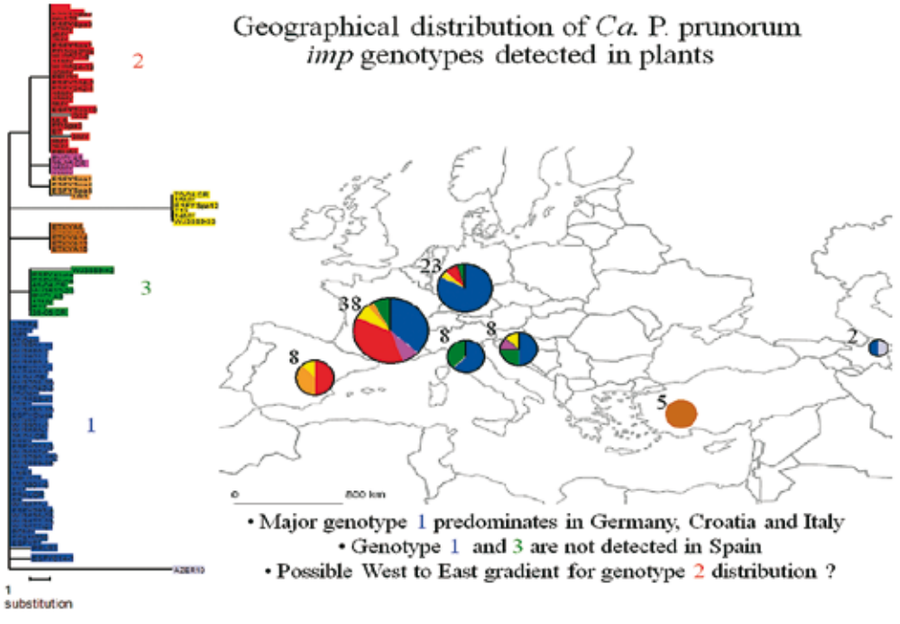


Figure 3: Phylogenetic analysis of secY genotypes detected in plant samples. Genotypes in red are the most abundant genotypes. Genotypes in blue colour represent minor genotypes detected in the Balkans.

191 insects were typed by HpaII-RFLP of tuf-PCR: 96 samples were of tufII genotypes while 89 samples were of tufI and six of tufIII genotypes. The genotype tufII was the only detected in Serbian insects.
According to vmp1 RsaI-PCR-RFLP, 44 samples could be examined. Eight different RFLP patterns could be evidenced. Patterns V1 (15 insects) and pattern V2 (ten insects) were predominating. Patterns V4 was detected in seven insects whereas pattern V14 was detected in four Serbian insects. Four other patterns, namely V3, V12, V14 and V16 were only detected in one or two insect samples.
SecY sequence genotypes were obtained for 30 insect isolates and only the three main genotypes were detected, namely S6 (12 insects), the S1 (eight samples) and the S4 (six insects) genotypes. Four mixed infections between S1 and S4 genotypes were also evidenced.

3.3 Working group 3, group 16SrX fruit tree phytoplasmas

Most of the samples could be amplified for the four gene markers and sequenced. Each type sequence was given a genotype number according to the following nomenclature: first letter of the marker and the number of the genotype. For example: A1 is the first genotype for the aceF marker. The most diverse situation was found for the marker imp, for which 28 different genotypes were found. An example of the analyses which are going to be performed is shown in the figure below.



3.4 Working group 4, insect vector of phytoplasmas

The majority of the C. melanoneura gave positive results with ITS primers, but negative results with COI primers, independently of the origin of psyllids. C. picta gave good results with ITS primers, and COI primers, except for insects from Czech Republic with COI.
Disappointingly, typing of C. pruni gave negative results (except for positive control!) with ITS and COI, except for individuals from Germany with COI. We cannot exclude a problem because of the DNA extraction with the Zygem protocol for the Czech samples. New extractions from these localities will be done with the classical (CTAB) protocol to test again the ITS and COI primers. The other psyllid species (C. pyri, C. pyricola, and C. pyrisuga) gave negative results for almost all samples. Bad DNA extraction or conservation of the extracts could explain these results.
ITS and COI primers used in this study seem to be good tools for Scaphoideus titanus. Except for some individuals, all samples gave positive results. On the contrary, almost all the Hyalesthes obsoletus samples are negatives, particularly those which were extracted with the Zygem protocol. New extractions should be done with the classical (CTAB) protocol, testing the ITS and COI primers again before coming to any definitive conclusion concerning the interest of the used primers.
The results described show the difficulty of finding the adequate primers. To by-pass this problem, two strategies could be followed: (1) use degenerated primers at one or more sites (see Simon et al 2006 for a large list of primers); (2) refine primers by sequencing the region surrounding the primer for representatives of the desired taxa (by using bracketing primer pairs), and reducing or eliminating degeneracy where possible. These two strategies will be tempted at INRA-Montpellier, at least in a first step on the psyllids.

- Apart from the technical problems met during this study, a major conclusion is that it was very difficult to collect insects from the various partner countries of the project. This proves a real and crucial problem in finding people for trapping insect in the field.
- All the samples from which we have had positive results will be sequenced.

3.5 Databases

The web page is presented in fig. 4. The design of the web page was chosen by the Phytoplasma epidemio project members among 29 different website designs. On the homepage, there is a header with links to the 9 pages of the web site. This page also includes a summary description of the Phytoplasma epidemio project, a link to the objectives, a link to the events pages and a link to the results pages (which includes the link to internal section of the web site - database).

On the team page, there is a list of research entities participating in the Phytoplasma epidemio project, with la ink to the webpage of each entity. On the participants' page, there is a list of the research entities with secured links to the participants' e-mail address.

Figure 4: Website of the "Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe" – network Phytoplasma epidemio

The page "results" provides short presentations of the reports on each activity, with links to the complete reports as (locked) pdfs. It also contains the entrance to the database (secured link to the database, based on a password). On the "activities" page, there are presentations of project activities objectives and task leaders (with a secured e-mail link, opening an outlook form).

In order to create a database of the project Phytoplasma epidemio, the first step was to chose the DBMS (Database Management System), i.e. the software that defines a database, stores the data, supports a query language, produces reports, and create data entry screens. The EU public funding of this network requires the use of open-source software applications PHP, MYSQL and Apache. PHP: HyperText Preprocessor, stands for Personal Home Page tools and is a scripting language designed to be embedded in HTML code (Valade 2002). When a user requests to see a HTML static website, the web server sends the HTML code as it is, which is then read by the web browser. In contrast, code containing PHP on a dynamic site is processed by PHP software such as Apache, before it is returned to the web browser. It is this processing which allows for complicated tasks such as data entry on a dynamic site. The processed data can be stored with MySQL, a small, efficient, easy-to-use and secure open source database popular with Web developers.

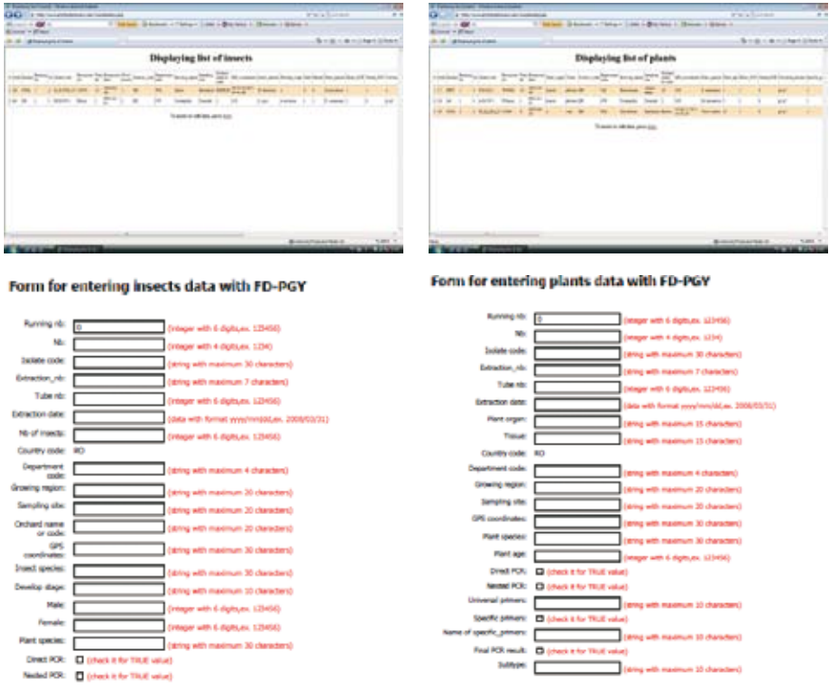


Figure 5: Aspect of pages of the web database and form for entering data for insect phytoplasma and plant phytoplasma. a) insects; b) plant.

The phytoplasma isolate properties to be described for the isolates are listed as columns.

The links for database are the following:

- http://www.phytoplasma.eu/view/vizualizareins.php (for insect phytoplasma database on the Phytoplasma epidemio network); and
- http://www.phytoplasma.eu/view/vizualizarepl.php (for the plant phytoplasma database).

The aspect of pages of the web database and form for entering data for insect phytoplasma and plant phytoplasma are presented in Figure 5.

Databases are necessary for a proper analysis of the results of the molecular typing tools, and a dedicated website is a useful tool, both for communicating the importance of Phytoplasma epidemio network research and co-coordinating the activities of the participants to Phytoplasma epidemio network.

3.6 Workshop and final meeting

The practical courses and final network meeting was held in Bordeaux on June 23-27, 2008 at the UMR-1090 (INRA and University of Bordeaux 2). Twenty four participants from 9 countries attended the meeting.

4 Conclusions / Outlook, next steps

During the nine months of this network, a motivated group of European laboratories set up an active collaboration for using genotyping tool for epidemiological survey and ecology of phytoplasmosis and their insect vectors. Partners are now sharing common protocols for phytoplasma strains genotyping. First common congress communication and publications ([7]; [9]; [12]) were produced and first descriptions of phytoplasma genetic diversity have been produced. A database describing genotypes as well as geographical and ecological properties of phytoplasma strains now just need to be filled by the participants. Additional partners from Spain, Italy, Slovenia, Czech Republic, Bulgaria, Turkey, Israel, Egypt and Lebanon and have informal interactions with the group. This constitutes a network of laboratories with common aims and practices in phytoplasma molecular epidemiology at the scale of the Euro-Mediterranean basin. Most of the partners participating in the SEE-ERANET network showed interest to renew such networks in the frame of a European call for application.

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Immobilised Yeast Cells in Hydrogel Carriers for the Bioproduction of Alcohols

Christo B. Tsvetanov[1], Eleonora Winkelhausen[2], Bela Ivan[3], and Viktor Nedovic[4]

[1] Institute of Polymers, Bulgarian Academy of Sciences, 1113 Acad. G. Bonchev str. 103A, Sofia, Bulgaria; chtsvet@polymer.bas.bg
[2] Department of Technology and Biotechnology, University Ss. Cyril and Methodius, Rudjer Boskovic str. Nr 16, 1000, Skopje, FYR of Macedonia; e-mail: eleonora@tmf.ukim.edu.mk
[3] Department of Polymer Chemistry and Material Science, Institute of Materials and Environmental Chemistry, Hungarian Academy of Sciences, 1525 Budapest, Pisztaszeri u. 59-67, PO Box 17, Hungary; bi@chemres.hu
[4] Department of Food Technology and Biochemistry, Faculty of Agriculture, University of Belgrade, Nemanjina str. Nr 6, Post Box 127, 11081, Belgrade-Zemun, Serbia; vnedovic@agrifaculty.bg.ac.yu

Abstract

An approach for fabrication of double-layered hydrogels systems comprising a macroporous hydrogel core with immobilised yeast cells and an outer poly(ethylene oxide) (PEO) hydrogel layer was developed. Macroporous hydrogels of various hydrophilic and temperature-responsive polymers were synthesized via both UV irradiation of moderately frozen systems and phase separation radical polymerisation. Well-defined PEO outer layers were obtained by UV-induced crosslinking for extremely short time, which makes the preparation of double-layered hydrogels systems a very facile and convenient procedure. Yeast cells of various concentrations can be immobilised into the macroporous core. The outer layer of PEO hydrogel encapsulates the core containing cells, thus reducing the cells leakage. The conversion of glucose to ethanol by Saccharomyces cerevisiae and xylitol by Candida boidinii was determined.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology
Type of the project: research project

Keywords

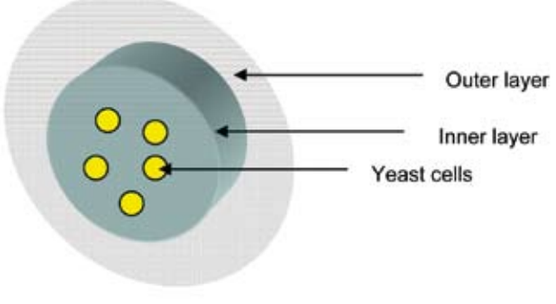
Bioethanol; Candida boidinii; Cryogels; Immobilization; Macroporous hydrogels; Saccharomyces cerevisiae; Xylitol

1 Introduction

The use of immobilised cell systems is an attractive and rapidly expanding research area because of the technical and economical advantages compared to the free cell systems.

The selection of a proper support matrix is very important for the successful performance of an immobilised cell system. It is desirable that the cell carrier possesses a large surface area, permeability, chemical, mechanical, and thermal stability, insolubility and suitable shape. In the last few years, a new round of interest in biomass and bioenergy has been initiated. The depletion of the oil reserve is going much faster than previously predicted and the environmental deterioration resulting from the over-consumption of petroleum-derived products is threatening the sustainability of human society. Ethanol, both renewable and environmentally friendly, is believed to be one of the best alternatives and its production is continuously increasing. For such a vast production, any improvement in its fermentation technologies will be economically important [1, 2]. Attempts are being made to bring such improvement by using immobilised yeast cells. Possessing lower energy value than that of sucrose, xylitol became more interesting for the consumers. When supplied in the diet, this naturally occurring sweetener limits the tendency to obesity, and therefore attracts individuals concerned with their body weight and health. The anticarcinogenic properties of xylitol have important commercial application. The most recent finding concerning xylitol application was reported by King et al. [3], who discovered the usefulness of xylitol as an ingredient in functional foods for appetite control. Granström et al., [4] reported on the use of xylitol as a raw material for production of L-lyxose, L-xylose and L-arabinose. Xylitol is routinely produced chemically, but this process requires several purification steps, which lead to increased production cost. An alternative to the chemical process is the use of microorganisms, mainly yeasts, which directly convert xylose to xylitol. One of the strategies aiming at improving the performance of the microbial process is the use of immobilised cells. In that respect, we tried to synthesise and investigate synthetic polymer carriers for cell immobilization and to explore these systems for alcohol production, particularly ethanol and xylitol as alcohols that currently, for quite different reasons, attract the attention of the society.

The project aims at the preparation and investigation of double-layered hydrogels systems, where the inner hydrogel contains yeast cells and the outer layer ensures the mechanical strength and acts as a barrier membrane against cells leakage. Two techniques are tested for processing porous inner polymer scaffolds: via UV irradiation of moderately frozen systems and via phase separation radical polymerization. The production of ethanol and xylitol by yeast cells immobilised in the double-layered hydrogels systems is evaluated.



2 Experimental Techniques and Methods

2.1 Formation of porous inner matrixes

2.1.1 Super-macroporous polymer hydrogels via UV irradiation (BG team)

An appropriate amount of each polymer/monomer was dissolved in distilled water under stirring to obtain homogeneous aqueous solution. Given amounts of initiator (and cross-linking agent) were added under stirring at room temperature. The resulting homogeneous solution was then kept in a freezer at minus 20 ° C for 2 h. The frozen system was irradiated with full spectrum UV-vis light with a "Dymax 5000-EC" UV curing equipment with 400 W metal halide flood lamp for 2, 5 or 10 min (irradiation dose rate = 5.7 J/cm^2. min; input power = 93 mW/cm^2).

2.1.2 Macroporous hydrogels via phase-separation method (H team)

Monomer mixtures were poured into cylindrical shape holders and filled with double processed tissue cultured water and ammonium persulfate solution. The cross-linker was adding to the solution by press-lock microsyringe, and then the solution was purged with argon for approximately 5–10 min to remove oxygen. Then, tetrametylethylenediamine was injected by microsyringe into the reaction mixture while the argon bubbled through the solution. The sample holders were kept in an oven at 50 °C for three days. After the reaction, the resulting hydrogels were cut into disks and immersed in double processed tissue cultured water to extract the unreacted materials.

2.2 Preparation of cell cultures

2.2.1 Microorganisms (team from the FYR of Macedonia)

Saccharomyces cerevisiae, a commercial grade baker's yeast, with 32 % dry mass and Candida boidinii with 29 % dry mass were used in the investigations. The yeast strain of C. boidinii was kindly provided by Dr. C.P. Kurtzman of the USDA, ARS Culture Collection, USA.

2.2.2 Culture media (teams from the FYR of Macedonia and Serbia)

Nutrient medium used for the reincubation of the immobilized Saccharomyces cerevisiae, had the following composition: 0.1 %w/w KH2PO4, 1 g 0.7 %w/w MgSO4.7H2O, 0.1 %w/w (NH4)2SO4, 0.4 %w/w yeast extract and 10 %w/w glucose. The media were autoclaved at 120°C for 15 min.

Nutrient medium for inoculum preparation and fermentation with C. boidinii had the following composition (per liter): 1.7 g yeast nitrogen base without amino acids and ammonium sulfate (Difco), 5g casamino acids (Difco), 5 g urea and 50 g xylose.

2.3 Immobilisation of yeast cells

2.3.1 Addition of yeast cells before cross-linking (teams from Bulgaria and the FYR of Macedonia)

The polymer aqueous solutions were first mixed with yeast cells suspension to a 10% concentration (w/v) followed by addition of photoinitiator, cooling to –20 ° C and irradiation with UV.

2.3.2 Soaking of yeast cell culture into already prepared cryogel (teams from Bulgaria and the FYR of Macedonia)
Yeast cells were immobilised into the macroporous core simply by soaking. First, the polymer cryogel was prepared, freeze dried and, then, immersed in the cells suspension.

2.4 Outer layer formation (teams from Bulgaria and the FYR of Macedonia)
Cryogels containing yeast cells were, first frozen and covered with polymer solution containing photoinitiator and crosslinking agent. Then, the samples were irradiated with full spectrum UV-vis light with a "Dymax 5000-EC" UV curing equipment with 400 W metal halide flood lamp for 2 minutes.

2.5 Batch and repeated batch fermentations (teams from the FYR of Macedonia and Serbia)
Double-layered gels with immobilized S. cerevisiae were incubated in 100 mL nutrient medium placed in 250-mL Erlenmeyer flask on a rotary shaker at 28°C. Three successive batch runs with recycling of the immobilised cells were carried out. At the end of each cycle, the fermented broth was replaced with fresh medium. Each batch lasted 3 days. Afterwards the samples were kept for 6 months in distilled water at 4 °C and tested again for ethanol production in seven successive batch runs in duration of two days.

The performance of the immobilised cells was investigated by carrying out batch and repeated batch fermentations. Immobilized cells of C. boidinii in the gel matrices were incubated in 100 mL nutrient medium placed in 250-mL Erlenmeyer flask on a rotary shaker at 30°C. Seven successive batch runs with recycling of the immobilised cells were carried out. At the end of each cycle, the broth was unloaded, the gel with immobilized cells was washed with distilled water, and the flask was refilled with fresh medium. Each cycle (except one) lasted 7 days.

2.6 Scanning electron microscopy formation (teams from Bulgaria and the FYR of Macedonia)
Micrographs of cross-sections and the interior of macroporous hydrogels were obtained by using a JEOL JSM-5510 scanning electron microscope (SEM), operating at 10 kV. The specimens were first dried, then quenched in liquid nitrogen, fixed on a glass substrate and coated with gold for 60 s.

2.7 Analytical methods (teams from the FYR of Macedonia and Serbia)
The cell concentration was estimated by UV.-VIS spectrometer. Xylose, xylitol and glycerol were determined by high-performance liquid chromatograph, equipped with a refractive index detector. Ethanol was analysed by gas chromatography using a Varian CP 3800 with a capillary column.

3 Results and Discussion

3.1 Synthesis of macroporous hydrogels (cryogels) via UV irradiation
Biocompatible cryogels were selected to build up the core of double-layered hydrogels

systems because the cryogels are macroporous materials with an open porous structure. The process of cryogel formation involves a moderate freezing of the system, a reaction of cross-linking and a subsequent thawing. In this process, most of the water is frozen and forms ice crystals while the non-freezable water and the soluble substances (polymer or monomer, photoinitiator, etc.) are accumulated into a non-frozen liquid microphase (NFLMP). The gel formation occurs in this liquid microphase and the ice crystals perform as porogens.

Macroporous hydrogels of various hydrophilic and temperature-responsive polymers were synthesized via UV irradiation of moderately frozen systems. Firstly, hydroxyethylcellulose (Figure 1) and hydroxypropylcellulose cryogels (Table 1) were prepared by UV irradiation of 1 wt.% moderately frozen systems for 2 min at an irradiation dose rate of 5.7 J/cm^{2}min using a water soluble photoinitiator, (4-benzoylbenzyl)trimethylammonium chloride, and subsequent thawing. At these experimental conditions gels of good quality and high gel-fraction yield (> 95 %) were prepared. In order to ensure regular cryostructuration, the semidilute aqueous solutions of polymer and photoinitiator were at first kept at minus 20 °C for 2 h and then irradiated with UV light. Macroporous temperature-responsive poly(N-isopropylacrylamide) and poly(N-vinylcaprolactame) hydrogels (Table 1) were synthesized via UV irradiation of 10 wt.% moderately frozen systems of the corresponding monomer, poly(ethylene glycol) diacrylate (10 wt.% to the monomer) and H_2O_2 for 10 min. H_2O_2 initiator can be successfully substituted for the benzophenone derivative thus suppressing the existence of by-products in the material.

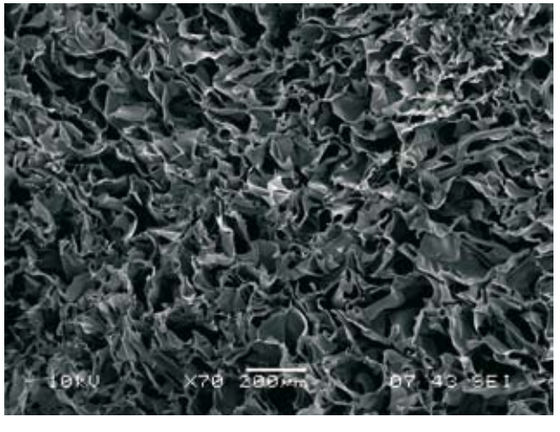


Figure 1: SEM micrograph of hydroxyethylcellulose cryogel

Generally, two types of water co-exist in the polymer cryogel, water bound to the polymer via hydrogen bonds and capillary-bound water that fills the space of macropores. Since the capillary-bound water is 65 – 70 % of the total amount, the diffusion coefficient of low-molecular weight species within the cryogel is increased significantly compared to the conventional hydrogels.

Table 1: Gel-fraction yield and degree of swelling of polymer cryogels

Gel	Gel-fraction [%]	Degree of swelling at 20 °C	Degree of swelling at 50 °C
Hydroxyethyl-cellulose	96	15	-
Hydroxypropyl-cellulose	96	26	16
Poly(N-isopropyl-acrylamide)	93	13	5
Poly(N-vinylcapro-lactame)	68	23	16

3.2 Synthesis of macroporous hydrogels via phase-separation method

Two series of macroporous networks, as potential cell immobilisers, were synthesized from 2-hydroxyethyl methacrylate and N,N-dimethylacrylamide. The macroporous network series were successfully synthesized by phase separation polymerisation in an aqueous solution. The low molecular weight and soluble hydrophilic components of the polymer networks were removed by extraction in double processed tissue cultured water. Depending on the experimental conditions hydrogels with different swelling properties can be obtained. The cross-section of the samples, analyzed by Scanning Electron Microscopy, however showed that only poly(hydroxyethyl methacrylate) hydrogels are porous (Figure 2).

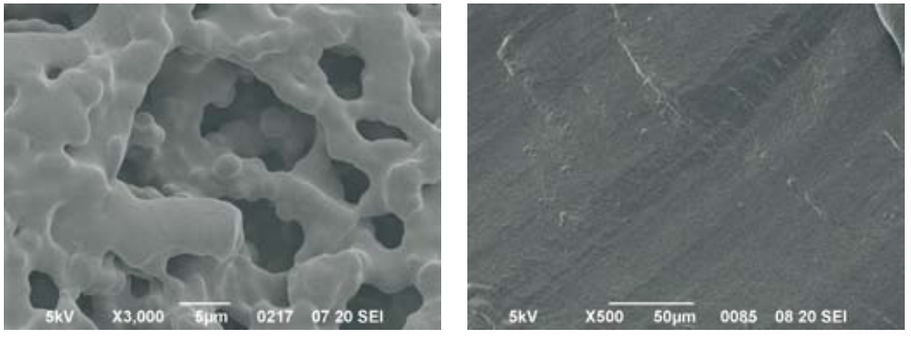


Figure 2: Poly(hydroxyethyl methacrylate) based hydrogels (a) and polydimethylacrylamide based hydrogels (b)

In this case, poly(hydroxyethyl methacrylate) networks with high solvent volume fractions have larger pores which originate from the phase separation polymerization in an aqueous solution. The solution (water) fills the space during the polymerization and larger pores are

generated. The increase of the polymer mass in the network cause smaller cavities, and the polymer matrix dominates the hydrogel system in swollen state.

3.3 Immobilisation of yeast cells

Two different approaches for the immobilisation of yeast cells in cryogels were tested. The first one includes mixing of the cells suspension and polymer solution followed by freezing and subsequent crosslinking. Due to the cryostructuration effect, this method leads to incorporation of cells into the polymer matrix which, however, at higher cells content (> 300 wt% with respect to the polymer weight) decreases the crosslinking efficiency and, consequently, the quality of the cryogel.

The second approach is more flexible and allows immobilisation of much higher amount of yeast cells into the cryogel. In this case, the polymer cryogel is prepared, freeze dried and, then, immersed in the cells suspension. As mentioned above, the cryogels are macroporous, spongy-like materials. So, by freeze drying one maintains the morphology of material and ones immersed in the suspension, the liquid fills the channels (interconnected pores) of the cryogel. This method allows immobilisation of up to 2000 wt.% yeast cells with respect to the polymer weight.

For porous poly(hydroxyethyl methacrylate) samples, cell adsorption on the polymers was the only immobilisation technique that could be employed. The disks were immersed in cell suspension for 2 weeks. The degree of equilibrium swelling of the sample was 3 after the sorption was over, the polymer gel was slightly swollen, its consistency resembled a jelly-gum, and the color turned white. Additionally, there were no visible signs of cell adsorption on the surface.

3.4 Synthesis of outer layer of hydrophilic hydrogel

The outer layer has to encapsulate the macroporous gel containing yeast cells. Importantly, it has to ensure both the mechanical strength of the system and the diffusion of nutrient media and alcohols, as well as to prevent or, at least, to reduce the cells leakage. A suitable outer layer was obtained from high molecular weight poly(ethylene oxide) (HMW PEO) and crosslinking agent. It forms dense network for 2 min UV irradiation leading to a well-defined outer layer. In addition, the viscosity of the semidilute PEO solution is very high, which prevents penetration into the pores of the core (in case of partial ice melting). Altering the PEO:crosslinking agent ratio one may tune the crosslinking density and the mechanical properties of the outer layer. As a rule, the increase of crosslinking agent content increases both the rigidity and density of polymer network.

3.5 Ethanol production by immobilised Saccharomyces cerevisiae

The suitability of the hydroxyethylcellulose cryogels as immobilisation matrices for entrapment of cells of Saccharomyces cerevisiae was tested in a batch ethanol fermentation performed with gels containing initial cell mass concentration (dry weight) of 17.6 g/L and 29.4 g/L, which corresponds to 15 and 25%. At the beginning, the fermentation was carried out in three batches of 72 hours. Throughout the fermentation, in all batches, the substrate was totally consumed at the end of each cycle. The average volumetric productivity

was 0.72g/Lh and 1.02g/Lh for the initial cell concentration of 15 and 25%, respectively. Therefore, in the further experiments, the fermentation time was shortened to 48 hours. Important characteristics of the immobilised cell system are the stability of the system during the operation and its stability during the storage. The operation stability of the system can be tested in either continuous process or by repeated batch fermentation. We opted for the latter one. The immobilised cells were stored in distilled water at 4°C for 6 months. After this period they were used in 7 repeated batches, each of 48 hours. The cell concentration inside the gels was maintained almost constant during the cycles. In course of the first cycle the cells adapted and revived their fermentative activity. The product yield was between 70–95% of the theoretical yield for the gels with 15% cells, and between 87–95% for the gels with 25% cells. It should be emphasised that the stability of the cryogels during the storage was notable. All through this time the gels kept their mechanical stability and showed no signs of degradation.

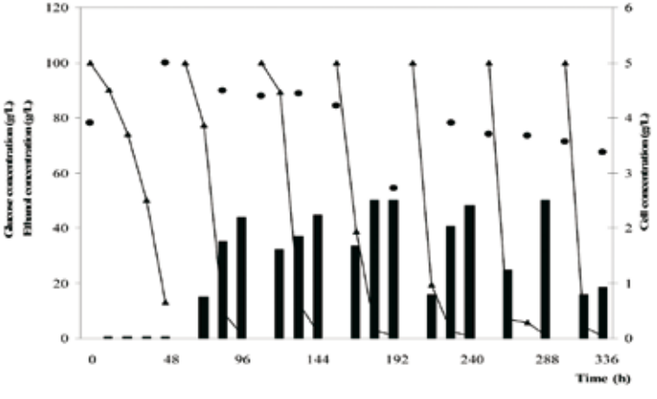


Figure 3: Repeated batch cultivation of immobilised system with 25% cells of S. cerevisiae in hydroxyethylcellulose cryogel; biomass (●), glucose (▲) and ethanol (■)

The performance of double-layered careers of Saccharomyces cerevisiae was tested at a larger scale in a stirred tank bioreactor, convenient to test mechanical stability and resistance to abrasion of the biocatalyst and in a fluidized bed bioreactor. The results of the fermentations studies showed that hydroxyethylcellulose cryogel coated with the layer of poly(ethylene oxide) hydrogel is a suitable matrix for yeast cell immobilisation aimed for the process of glucose conversion to ethanol. After the immobilisation step, yeast cells were able to propagate and kept the activity to catalyse the fermentation process. Except viability of cells, another important characteristic in immobilised cell systems is the stability of the biocatalyst during a long-term operation process. However, the materials were not able to withstand high shear stresses caused by non-uniform turbulent flow in a tank with rapid mechanical mixing. In case of the fluidized bed reactor, there were no notable changes in a shape of gel peaces. In large scale industrial applications, mechanical stirring is envisaged, therefore, the mechanical strength of the hybrid system needs to be improved.

3.6 Xylitol production by immobilised Candida boidinii

Preliminary tests of xylose consumption and xylitol formation during the repeated-batch culture of immobilised C. boidinii were carried out. In the first cycle, 51.8% of the xylose was consumed, while in the subsequent three cycles, the consumption increased up to 73.5%. This progressive improvement of the xylose consumption stopped in the fifth cycle when the consumption was lowered to 42.7%. Even though the time in the next (sixth) cycle was increased to 13 days, xylose consumption did not surpass 50%. The cell leakage of this system was minor, however, the experimental condition still has to be optimized.

4 Conclusions / Outlook, next steps

Novel double-layered polymeric hydrogels were synthesised and tested as carriers of different cells. The results from the fermentations studies showed that hydroxyethylcellulose cryogel coated with thin layer of poly(ethylene oxide) hydrogel is a suitable matrix for yeast cell immobilisation aimed at the process of glucose conversion to ethanol. From a technological point of view, this is a fast, convenient and effective method to design and prepare hybrid hydrogel system. The future experiments should focus on the mechanical stability of the outer layer and the continuous fermentation for production of alcohols with double-layered hydrogels under different operating conditions.

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6 Appendices

The results were published in two papers:

Winkelhausen E., Kuzmanova S., Jovanovic-Malinovska R., Cvetkovska M., Tsvetanov Ch. Hydrogels based on u.v.-crosslinked poly(ethylene oxide)-matrices for immobilization of Candida boidinii cells for xylitol production. World J Microbiol Biotechnol (2008)DOI10.1007/s11274-008-9707-5

Petrov, P; Petrova, E; Tsvetanov, Ch B. UV-assisted synthesis of super-macroporous polymer hydrogels. Polymer, doi:10.1016/j.polymer.2008.12.039

The results were presented at the following conferences:
Velickova E., Kuzmanova S., Winkelhausen E., Cvetkovska M., Tsvetanov Ch. New immobilization technique of cell entrapment applied for ethanol production, 4th Central European Congress on Food, Cavtat, Croatia, May 2008.
Velickova E., Petrov P., Fodor C., Manojlovic V., Nedovic V., Ivan B., Csaba, Winkelhausen E., Tsvetanov Ch. New Carriers for cell immobilization, "Sixth International Conference of the Chemical Societies of the South-Eastern European Countries", Sofia, September 2008.

IVAB: Interactive Visual Analysis of Bio-Signals

Roman Trobec[1], Krešimir Matković[2], Karolj Skala[3]

[1] Jožef Stefan Institute (JSI), Jamova 39, 1000 Ljubljana, Slovenia; roman.trobec@ijs.si
[2] VRVis Research Centre (VRVis), Vienna, Austria; matkovic@vrvis.at
[3] Ruđer Bosković Institute (RBI), Zagreb, Croatia; skala@irb.hr

Abstract

In the present article, research background, methodology and theoretical and applied results obtained within the IVAB project are presented. Our report is a mutual document assembled from contributions by each partner according to his responsibility, with some common actions like publications, talks and meetings and other kinds of dissemination of research results.

The research project "Interactive Visual Analysis of Bio-signals" was focussing on the methodology for the analysis of measured bio-signals and automatic diagnosis of the current state of the patient. It was planned to combine existing expertise in visualisation and conventional approaches to the medical data analysis in order to generate synergetic results. Within the project, we consolidated a core group, which has shown that the visual analysis of bio-signals is a prospective discipline with a great potential for future work. The project implementation plan has been based on the parallel work of all partners. Each partner contributed resources from his research areas. RBI offered background on the computing infrastructure, multivariate statistics, data bases and internet services; VRVis contributed implemented software tools for visual analysis and wide knowledge in the area of data mining; Jožef Stefan Institute (JSI) offered parallel computing abilities, cooperation with medical personnel and suggested some concrete problems in the medical field to be solved.

Knowledge and know-how was exchanged among all groups, which contribute to the synergy and new ideas. Some of these ideas have been published or are planned to be published at conferences and in journal papers. Younger researchers have had the opportunity to exchange ideas and to practice with the scientific work in the field of visualisation.

Thematic Area / Type of the project

Information and Communication Technologies
Type of the project: research project

Keywords

Bio-signals; Body Sensors; ECG; Interactive Visual Analysis; Image Processing; Visualisation

1 Introduction

Electro-cardiac signals (ECG) are essential indicators in the diagnostic and clinical work. A personal ECG acquisition module should be small, virtually wireless, be able to work in real-time and simple to manage. Data should be partially analysed locally and transmitted on demand or in suspicious cases. Incorporating technologies such as bluetooth, GPRS, GSM or Wi-Fi to these systems allows wireless transmission to health or control centers [1, 2]. Data should be interpreted and visualised in different standard forms. Several publications, e.g. [3], describe the possible applications of mobile personal ECG data, with test measurements from standard data bases [4], some of them reported on the development the ECG module [5] and wireless communication [6, 7]. Other works [8, 9] have proposed techniques for signal processing via software to reduce noise or classify heart pathologies.

The standard 12-lead ECG is not appropriate for mobile applications because redundant data are transmitted through a limited bandwidth capacity. Six pre-cordial electrodes V1-V6 and three arm and leg electrodes I-III plus one additional reference electrode are needed [10]. Additionally, the distribution of electrodes requires long wires, which could be inconvenient or even unacceptable for the person under care. Cardiac experts are usually able to make detailed diagnoses utilising information from the standard 12-lead ECG rather than holter recordings. Therefore, the 12-lead ECG should be derived from modified placement of recording electrodes for some applications,.

Theoretically, three orthogonal leads (four electrodes - three independent potentials) contain all information from the ECG. The remaining leads should be generated as a combination of the mentioned tree potentials. In some earlier attempts, such a transformation was made in analogue technique, simply by a proper combination of resistors [11]. Later, some authors reported the use of neural networks to develop a personalised transformation from modified ECG to the standard 12-lead ECG [12]. Even by four electrodes, the wires remain quite long. Promising results are expected from using the differential ECG measurements, which consist of a set of measurements from two electrodes, placed in a distance less than 5 centimeters. Differential leads, are easy to design with short wires, represent an independent module which is able to produce a local look on the heart activity, however, minor work has been done in the direction of generating the standard ECG from differential leads. On the other hand, multi-channel electrocardiography (MECG) is used in many clinical experiments to enable more accurate diagnostics [13, 14]. The MECG device and all implemented algorithms were tested on more than 300 patients and volunteers.

All those measurements, together with patients' data, create a large, complex data set. The analysis of such a complex data is far from trivial. Interactive visual analysis [15, 16, 17] or interactive visualisation is an active field of research that provides new methods of data analysis, which can be used together with conventional data mining, analysis and statistics. The main idea is to support the analyser with interactive graphics. In doing so, the key advantages of human over computer, intuition, imagination, experience, and superior visual

system are being supported in order to get insight into data. The goal of interactive visual analysis is not presentation, but data exploration and analysis. Similar techniques have been applied to a wide range of problems, having a similar data model, containing scalar values as well as function graphs [18]. In the project, interactive visualisation was merged with standard data analysis methods based on statistics, mathematical models and data mining. We expected innovative solutions in the visualisation and analysis of medical data sets, particularly in the area of discovering redundant data and hidden relations.

2 Experimental Techniques and Methods

The project implementation plan has been based on the parallel work of all partners with the following work packages:

- WP1 – Presentation of the equipment and software tools (JSI);
- WP2 – Project web portal (RBI);
- WP3 – Optimization (JSI);
- WP4 – Interactive visualization and optimization (VRVis);
- WP5 – MECG measurements (JSI);
- WP6 – Database web access (RBI); and
- WP7 – Dissemination of results (JSI).

In order to implement the project goals, we used the existing technologies embedded into a multichannel ECG measuring device (MECG) and a software tool for interactive visual analysis – ComVis.

2.1 MECG device

Body surface potential mapping is an extension of conventional electrocardiography aimed at refining the non-invasive characterisation of cardiac generated potentials. Improved characterisation is accomplished by increased spatial sampling of body-surface electrocardiographic information. Much information can be read out of appropriately drawn body surface maps that can show the distribution of the potential or other characteristics of measured signals. It is also possible to compute the potential distribution at the heart surface, especially when individual torso models are used. This is the solution of the so-called inverse problem in electrocardiography. Here, we present a multichannel ECG system that can measure signals and show the gathered information in several different ways. The system has been designed for clinical research.

The front-end of the measurement system is a battery-powered unit, which is connected to the computer by optical fibres (see Figure 1). The basic front-end unit has 32 channels, which can be extended up to 64 in the same box of 220x125x110 mm. Another extension of up to 128 channels is possible in a larger box. The signals from the chest electrodes are referenced to the Wilson central terminal (WCT). Each signal is first amplified and filtered, then converted into digital form (ADC) and put into the optical fibre as a serial stream of short light pulses. On the other side of the optical cable, the serial data are first transformed into parallel form and then transferred to the computer memory. The complete front-end unit is controlled through another optical fibre. This fibre is also used for switch-

ing of the whole unit on and off. In the basic unit, the frequency response of the filters is from 0.05 Hz to 100 Hz, which can accurately follow slow varying portions of the signals such as ST segments.

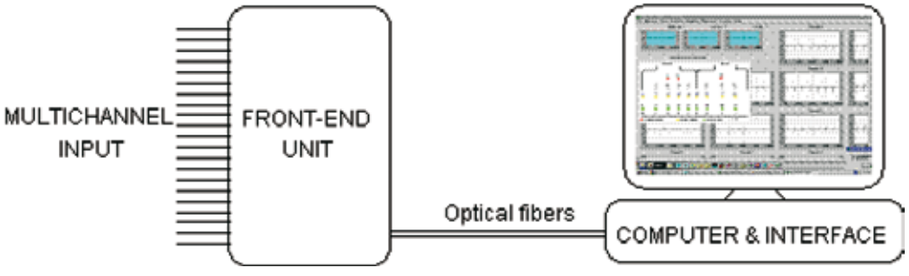

Figure 1: Components of the MECG measurement system

The complete software package is implemented in a National Instruments LabWindows environment. The recording time (64 channels, 1000 Hz sample rate) varies from 100 seconds to 5 hours, or even more, if enough system memory is present. We developed a small computer program for the transmission of MECG measurements to the ComVis software tool.

An example of 31-channel measurement with the corresponding ST isointegral map is shown in Figure 2. The 20μV step between contours demonstrates that the resolution of this map is very high. The map can be saved in order to be compared with a map obtained under different conditions.

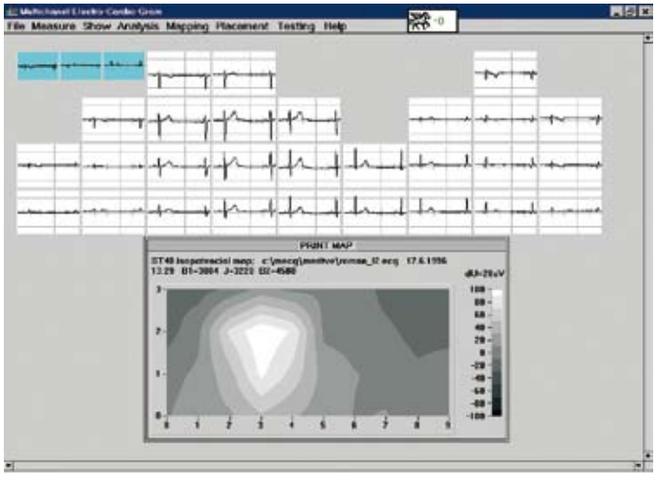

Figure 2: Isointegral map of the ST segment (40 ms integral)

2.2 Interactive Analysis with ComVis

ComVis is an interactive visualisation tool based on the coordinated multiple views principle. It supports well-known views such as scatter plot, parallel coordinates and histogram, as well as a a special curve view used for displaying families of function graphs. The combination of views makes it possible to analyse a wide variety of data sets. The main idea of coordinated multiple views is to display more views simultaneously. Every view can be of any supported type. ComVis pays great attention to interaction. Linked views enable user to easily select a subset of data points in any view and all corresponding items in other views will be highlighted. This offers significant advantage compared to static views.

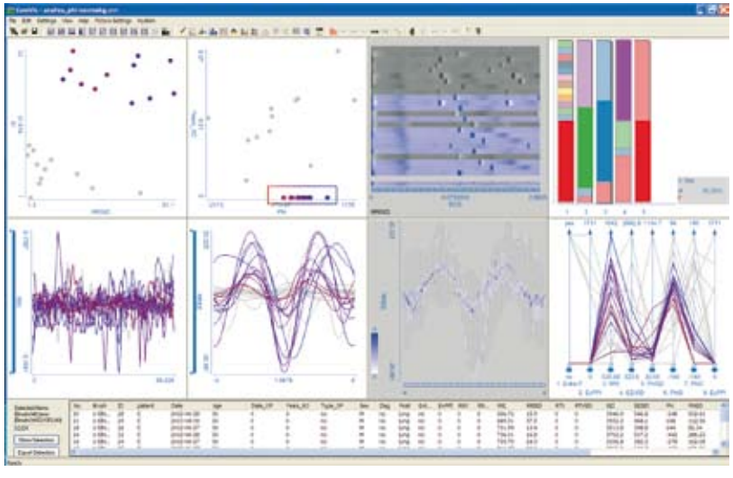

Figure 3: A screenshot of ComVis depicting various parameters of signal data sets

We have used the curve view and segmented curve view for time series data and scatterplots, histograms and parallel coordinates for scalar parameters. The curve view depicts all curves simultaneously. It can successfully depict tens of thousands of curves, using transparency and alpha blending in order to see main trends. ComVis offers advanced interaction as well. The main idea is to select some items in one view and to see corresponding items highlighted in all other, linked views. The user can intuitively select a subset of curves, e.g. by simply drawing a line across some of the curves. Besides simple brushing, ComVis supports composite brushing in an iterative manner as well. This means that the user selects a current operation (AND, OR, or SUB) and draws a brush. The previous brushing state is then combined with the new brush. The new state is computed and the new state is used when user draws a further brush. This way, the user immediately gets visual feedback, and can very easily broaden his selection (using OR), or can further restrict selection (using AND or SUB).

3 Results and Discussion

3.1 Optimisation of the number of electrodes

The determination of the minimal set of electrodes with their positions, which enable a reliable reproduction of the standard 12-channel ECG, was one of our main goals within the IVAB project. We have analysed the method of electrode selection based on optimisation through genetic algorithms. The simplest form of selection would work as an optimisation of set of electrodes $e=<e_1, e_2, ..e_n>$, where e_i is an electrode from a multi-channel ECG measurement. One or more optimisation criteria could be chosen (minimum distance between selected electrodes, maximum fidelity of the results).

We have investigated the potentials of the Principal Component Analysis (PCA) in process of determining a minimal set of electrodes (less than 10) that can be used to generate standard 12-leads ECG. For that purpose, we have used multiple 31-leads (or 35-leads) multichannel ECG (MECG) of the same person as data source. The methodology for the identification of best electrodes is personalised, in a sense that a different set of electrodes is identified for each person.

First estimations show a satisfying agreement between all pairs of approximated and real waves for one person. All waves have the same orientation, amplitudes, and durations (see [19] for details). The results of approximation can be seen in Figure 4.

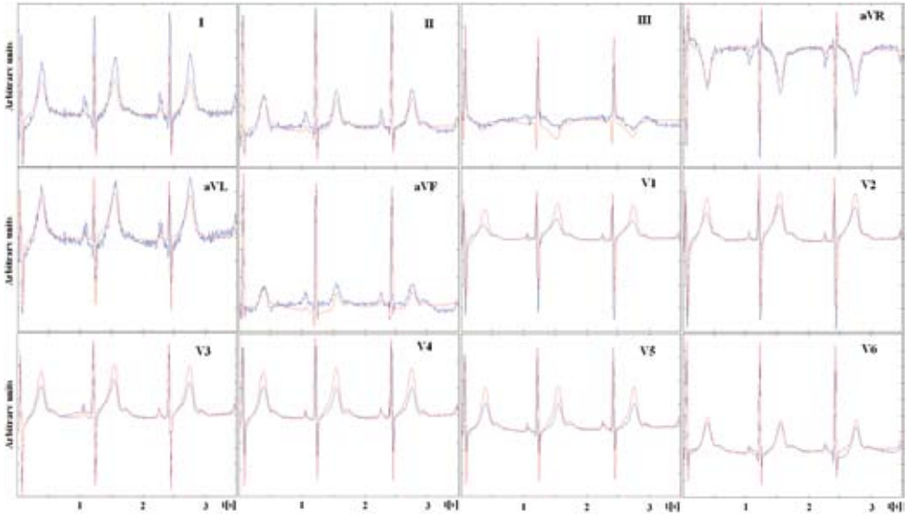

Figure 4: Target and approximated 12-leads ECG (the blue line is the target and the red line is the approximation)

3.2 Visual diagnostic of MECG measurements

We have applied known visualisation techniques to the ECG data sets of patients and healthy persons for comparisons. Each data set incorporates ECG, respiratory and blood pressure measurements with a lot of scalar parameters. We have used ComVis to analyse various data sets. The most interesting findings were made with transplanted heart data. In this case, we have explored various parameters of patients with heart transplants as well as those of control persons. The interactive visual analysis helped us to understand the problem and has lead to new findings, namely about the way how the heart gets reinervated as time after the operation passes by. We have used advanced interaction and coordinated multiple views to analyse the data. Figure 5 illustrates a part of the analysis process. Some solutions have been proposed and described in our conference paper [20]. All further details about the project IVAB can be found on project web page http://www.ivab.irb.hr/.

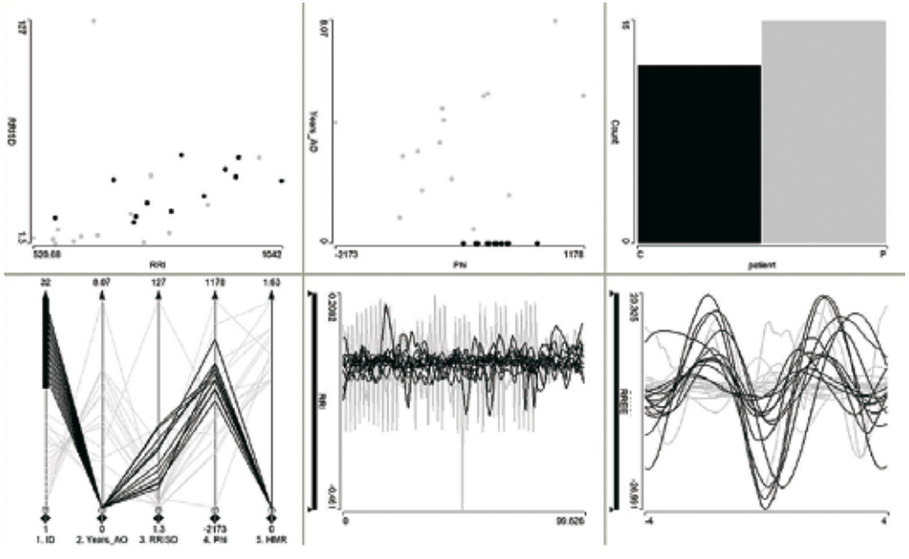

Figure 5: Multiple coordinated views used for data analysis. User selected all controls. Note the correlation between RRI and RRISD (upper left view) and coherent shapes of RREE curves (lower right view).

4 Conclusions / Outlook, next steps

Following the needs and possibilities of the proposed project, we have implemented a new approach in interaction between users and diagnostic experts (medical doctors) through visual diagnostics. We have used the conventional methods whenever they have a clear advantage and interactive visual diagnosis when user's intuition, experience and vision can improve the diagnostic outcomes.

We have elaborated and developed a conceptual pilot demonstration system for remote cardiologic diagnostics with advanced visualisation options. We have created a first data base of the multichannel ECG measurements that was opened for the public use. With the existence of highly integrated intelligent sensors and data gathering systems, the emphasis of the new generation of mobile remote diagnostics relies on expert systems based on a great quantity of data and sophisticated multi-dimensional visualisation. The research results could save cost for the community and increase the quality of life for the monitored persons by enabling them to stay at home and attend work and leisure activities.

The created consortium will support the multidisciplinary co-operation, whose ultimate goal is the further development and establishment of the new visual analytics in medical diagnostics as an upgrade to the existing data mining in bioinformatics models and procedures.

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Landscape and Regional Context of Insect Agrobiodiversity in Southeastern Europe: A Pilot Survey of Selected Hemipteran Pests, their Parasitoids and Predators, and Bee Pollinator Diversity

Željko Tomanović[1], Nickolas Kavallieratos[2], Slobodan Krnjajić[3], Gabrijel Seljak[4], Snježana Hrnčić[5], Teja Tscharntke[6], Olivera Petrović-Obradović[7], Ljubiša Stanisavljević[1], Aleksandar Ćetković[1], Slobodan Jovanović[1], Jasmina Šinžar-Sekulić[1], Gordana Tomović[1], Dejan Radović[1], Milan Plećaš[1], Andjeljko Petrović[1], Vesna Gagić[1], Branka Čubrilović[1], Christos Athanassiou[2], Jelena Jović[3], Milana Mitrović Desančić[3], Tatjana Cvrković[3], Ivan Žežlina[4], Sanja Radonjić[5], Andrea Holzsschuh[6], Alexandra Klein[6], Christoph Scherber[6], Carsten Thies[6]

[1] Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia; ztoman@ bio.bg.ac.rs
[2] Benaki Phytopathological Institute, Stefanou Delta str. 8, 14561 Athens, Greece; nick_kaval@hotmail.com
[3] Institute for Plant Protection and Environment, Banatska 33, 11080 Belgrade-Zemun, Serbia; krnjajic@eunet.yu
[4] Agriculture and Forestry Institute of Nova Gorica, Pri hrastu 18, 5000 Nova Gorica, Slovenia; gabrijel.seljak@kvz-ng.si
[5] Biotechnical Institute, University of Montenegro, Kralja Nikole bb, 81000 Podgorica, Montenegro; hrncic@cg.yu
[6] Department of Agroecology, Georg-August University of Göttingen, Waldweg 26, 37073 Göttingen, Germany; ttschar@gwdg.de
[7] Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade-Zemun, Serbia; petrovic@agrifaculty.bg.ac.yu

Abstract

Within the broader concept of agrobiodiversity research and promotion of sustainable use and management of biological resources, our pilot project was designed to introduce the landscape and regional scale approach and some sophisticated laboratory techniques, and to establish the regional network of scientist in order to facilitate further research and capacity building in agricultural biodiversity science in the context of Southeastern Europe. The project was structured to cover the following topics: diversity of cereal aphids and their natural enemies, pollinator bee diversity and abundance in agricultural landscapes, diversity and vector status of Auchenorrhyncha in corn fields, and refinement of detecting and monitoring systems for invasive hemipteran species. Through cooperation of six research teams from seven institutions in five countries (Serbia, Greece, Slovenia, Montenegro, Germany), we initiated several comprehensive and long-term research programmes, and set the conceptual and methodological basis for future integrative studies in the region. In this phase, direct results and outcomes from the field surveys and laboratory analyses are mainly preliminary, but highly relevant for the selected topics and the intended goals.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest, and aquatic environments
Type of the project: research project

Keywords

Agrobiodiversity; Hemipteran pests; Natural enemies; Pollinators; Southeastern Europe

1 Introduction

Biodiversity loss in agricultural landscapes does no only affect the production of food and other direct goods, but also a range of ecological services supporting water supplies, habitats for wild species, etc. Among the key research foci recently set up in the Agrobiodiversity Science Plan and Implementation Strategy [3], we tried to address the primary one, the assessment of biodiversity in agricultural landscapes and the anthropogenic drivers of biodiversity change. In the context of Southeastern Europe, it is essential to develop and introduce new approaches, methods and models for the assessment of agrobiodiversity at the landscape scale, and for determining strategic issues that affect the sustainable use and conservation of biodiversity in agriculture [9,10]. These goals are closely tied with the establishment of international networks that could promote research and capacity building among researchers involved in biodiversity science in agricultural landscapes.
Mediterranean and Pannonian ecosystems and landscapes, representing the most important agricultural areas of Southeastern Europe, are under great pressure from intensification. The agricultural practice in Serbia and Montenegro is in a transitional phase, facing many problems, including the lack of scientific support for new approaches – e.g. the landscape perspective in the application of general agrobiodiversity conservation strategies, monitoring, etc. A cooperation and network partnership with prominent EU-based institutions was initiated to contribute to the development and dissemination of necessary knowledge, standards and good practise, and to improve the capacity of local (SEE) institutions and researchers to deal with these urgent issues. In this pilot survey, the focus was on the status of selected beneficial and pest/invasive insect species in relation to the habitat fragmentation and agricultural intensification. Hence, the principal research goals of the project was to establish the general scientific (conceptual, methodological, etc.) framework and to gain the necessary experience, skills and baseline information from the field, that should enable the development of a comprehensive research programme in the area. The project was structured to cover the following topics: diversity of cereal aphids and their natural enemies, pollinator bee diversity and abundance in agricultural landscapes, diversity and vector status of Auchenorrhyncha in corn fields, and refinement of detecting and monitoring systems for invasive hemipteran species. Cooperation involved six research teams from seven institutions in five countries (Serbia, Greece, Slovenia, Montenegro and Germany).

2 Experimental Techniques and Methods

Four tasks (divided below) were designed to cover the structure of the listed topics, related to different target organisms and methodological approaches. Accordingly, the methodology was presented in the separate respective subsections.

2.1 Diversity of Cereal Aphids and their natural enemies

Aphids and their selected natural enemies were sampled in two principal regions: the area of the northern Belgrade lowlands in Serbia (representing continental Southeastern Europe) and the Mediterranean region of the Balkans in Greece. The target crops were the cereal agroecosystems of winter wheat. In the vicinity of Belgrade, Serbia – the area of mesic/hygrophilous flat lowlands dominated by more or less intensive agricultural practice – we selected three series of landscape sectors representing three different types of landscape structure: (A) simple landscapes with predominantly large crop fields and some water feature within it; (B) somewhat more complex landscapes which are predominantly composed of small fields and some non-crop habitats; and (C) complex landscapes with mainly small fields and a large proportion of non-crop habitats. Landscape complexity was assessed by field surveys of major vegetation classes, in combination with official digital maps and satellite images (principally from Google Earth). The two fields in the Mediterranean area were in the coastal mainland Greece, in the vicinity of Athens (simple landscape structure) and Volos (complex landscape structure), respectively.

Aphids and parasitoids were sampled by the 100 wheat stem method. Samples were taken during wheat flowering in May and ripening in June. In addition to parasitoids, we sampled the predators from the beetle family Carabidae (only in the Serbian area). The percentage of parasitism was calculated as the ratio of the number of mummies to total aphid number. The parasitoid abundance and diversity was also analysed by collecting aphid mummies during a one-hour search per field, and in some sample plots, also in the wild/weed vegetation along the field margin [7]

2.2 The landscape context of the diversity and abundance of bees

We selected two series of landscape sectors representing two different types of landscape structure (simple landscapes with predominantly large crop fields and complex landscapes with mainly small fields and a larger proportion of non-crop habitats), in the close vicinity of Belgrade, Serbia (the same area and sectors used for task 1). A preliminary survey of the representative landscape types was performed in the southern periphery of Belgrade, where more hilly terrains are dominant. In addition, we selected two landscape plots in the wider area north of Belgrade, on the hilly terrains of Fruska Gora Mt., both characterised by a similar structural complexity and agro-technical regime, but with a different intensity of chemical pollution (one site is situated close to the large cement factory). Landscape complexity was assessed by field surveys of major vegetation classes, in combination with official digital maps and satellite images.

The principal method for collecting and monitoring the bees in all areas and habitats was hand netting, in combination with the visual recording (the lather mainly for bumble bees).

Trapping has been conducted principally on the Fruska Gora sites (yellow, blue and white pan traps and reed nest traps), following the design developed within the project ALARM [1]. The similar design is prepared to be applied in localities around Belgrade during the next season.

2.3 Auchenorrhyncha in corn crops – diversity and vector status

From June to July 2008, the collection of Auchenorrhyncha species was preformed every 15 days on three sites in the Serbian district of Braničevo, three sites in the South Banat district, one site in the Belgrade district and on one site each in Montenegro and Slovenia. The specimens collected during sampling were deposited in 96% ethanol for subsequent molecular analysis in order to determine their phytoplasma infection status. The samples that had been collected in corn fields in Serbia were PCR analysed. The samples from Slovenia and Montenegro were sent to the laboratory for molecular analyses of the Institute for Plant Protection and Environment, Serbia, and specimens of all abundant species will be analyzed for phytoplasma presence. The sampling of other cicadas on all sites was continued until the beginning of September, and further analyses will be preformed.

2.4 Monitoring of invasive hemipteran insects

In order to determine the spreading of Metcalfa pruinosa, an invasive cicada that was firstly recorded as present in Serbia in 2006, we inspected woody plants in the surroundings of Belgrade. During the season of 2008, we surveyed cicadas and aphids in vineyards in Montenegro by using yellow sticky traps and other usual techniques. In the same area, we sampled grapevine symptomatic for Grapevine Yellow too.

3 Results and Discussion

In accordance with the structure of the project tasks, the results are presented in the same subsections.

3.1 Diversity of Cereal Aphids and their natural enemies

The aim of this task was to establish and compare the faunistic composition and population dynamics of the cereal aphid parasitoids and selected predators in continental and Mediterranean cereal crops with respect to climatic and landscape factors, and with particular emphasis on the type of agricultural practice. Recently, we reviewed this topic from a more general perspective [8], and now we intend to introduce the landscape scale approach.

In total, more than 200 aphid/parasitoid samples were collected and reared to adult parasitoids, and this material is currently being processed. The rearing yielded more than 500 primary and about 200 secondary parasitoid specimens, representing approximately 14 species. When all the samples will be identified, we will analyse the effects of host-plant density, fertiliser use, the proportion of arable land and specific vegetation composition and structure on aphid densities, aphid parasitism and parasitoid and predator diversity. Differences in trophic interactions between cereal aphids and their parasitoids and predators between areas with cereal crop and non-crop habitat and between the regions will be analysed. The impacts of regional and landscape features, as well as agricultural practice, will be further analysed with appropriate GIS techniques and procedures.

3.2 The landscape context of the diversity and abundance of bees

We planned to set the conditions for the baseline assessment and long-term monitoring of bees in agroecosystem-dominated landscapes. The ultimate goal of these future studies is to analyse the impact of the landscape structure (with respect to the included habitat types) and the character of the agricultural practice on the trends in abundance and diversity of social and solitary bees, as the key pollinators of cultivated and wild plants [2,4]. The samples from the traps are still being processed. On the basis of the hand netting and observation surveys in 2008, supplemented by the material and other data from the previous period, we have preliminary established the composition of the bee fauna of the study area. We could confirm the presence of at least 29 genera, comprising more than 140 species. These results represent only about 50% of the expected bee fauna, but probably include most of the common, abundant and widespread representatives, those that are carrying the largest share of pollinating services. As expected, we may preliminary confirm that the faunistic composition of bee assemblages from different localities, habitat types and landscapes was markedly different.

Regarding the extent of the preparatory work needed to establish the comprehensive set of methods for the assessment and monitoring (including the selection and testing of suitable sites/landscapes), it is obvious that more than one season is necessary to reach the reliable conclusions and schemes. Among other issues, the effects of selective efficiency of different methods for assessing diverse bee taxa have to be evaluated and accounted for. Local and regional taxonomic diversity of the group and the usual impediments related to the unavailability of the taxonomic expertise for many genera require careful selection of the target taxa for future studies and monitoring programmes. Activities within the project and the related communication with other interested specialists and working groups from all over Europe have helped us to build the preliminary network of experts interested in future collaboration on diverse pollination projects and initiatives.

3.3 Auchenorrhyncha in corn crops – diversity and vector status

We recorded diversity of different cicadas but only cixiid Reptalus panzeri, the known vector of Maize Redness disease was an extremely abundant species in corn fields. The presence of Reptalus panzeri was recorded in corn fields of Branicevo and the South Banat districts. In other corn fields, higher abundance was recorded for some Auchenorrhyncha species: Laodelphax striatellus, Zyginidia pullula and Psammotettix alienus, but large number of specimens of these species was also found on surrounding weeds, which indicates that they are not strictly connected to corn fields like R. panzeri. The presence of wheat fields next to corn field is very important for presence of dense population of Reptalus panzeri in corn fields.

The results showed a high percentage of Stolbur infected newly emerged R. panzeri adults at all three sites in Branicevo and all three sites in the South Banat district. From every site 50 R. panzeri specimens were analysed in pulls of 3. The average rate of Stolbur infection inside R. panzeri population was 12-18%. Also specimens of P. alienus, the second most

abundant species in corn fields, were analysed for phytoplasma presence, and the presence of Aster Yellows phytoplasma (16S rRNA I-c group) was detected in them.

3.4 Monitoring of invasive hemipteran insects

During the vegetation season 2008, we found out that Metcalfa pruinosa is expending its host range in the Serbian district of Belgrade, as it happened in all other countries were this pest is present. Specimens of M. pruinosa were recorded on different woody plants: grapevine, Juglans regia, Fraxinus ornus, Rubus fruticosus, Rubus idaeus, Prunus domestic and Prunus persica.

The sampling of crop hemipterans in Montenegro resulted in recording (among other species) the north-American species Scaphoideus titanus in Montenegro for the first time. Further monitoring of the abundance and distribution of this allochtonous species is very important, regarding its vector status for the destructive vineyard phytoplasma Flavescence dorée. In order to determine the causal agent of Grapevine Yellows symptoms in Montenegro, we sampled and analysed symptomatic vine plants and detected the presence of Stolbur phytoplasma which is causing a disease of grapevine known as Bois noir [6]. The incidence of up to 20% symptomatic plants in some vineyards indicates a need for adequate phytosanitary measures. The known vector is the cixiid species Hyalesthes obsoletus [5], so further research on insect vector(s) and alternative host plants of Stolbur phytoplasma in Montenegro is needed to determine the phytoplasma epidemiological cycle(s).

Also, during field trials we discovered new invasive aphid species Aphis illinoissensis on grapes in Montenegro. It seems that this species become established in the whole Mediterranean part of Southeastern Europe. We expect the expansion of this species on the whole Adriatic cost (Croatia and Slovenia) in near future.

4 Conclusions / Outlook, next steps

The principal research and organisational goals of the project were to establish the general scientific (conceptual, methodological, etc.) framework and to gain the necessary experience, skills and relevant baseline information in the field, which was expected to enable us to develop and conduct comprehensive, large-scale agrobiodiversity-related research programmes in the future. The structure of the project topics tackled some highly relevant subject and issues in agrobiodiversity studies: the status and interspecific patterns of selected insect pests and their natural enemies (parasitoids and predators) as well as status and trends of native bee pollinator guilds in semi-natural and anthropogenic environments – both from the perspective of multiscale analyses (from landscape to regional); spatial patterns and population dynamics of selected Auchenorrhyncha guilds associated with corn crops, with particular emphasis on the detection of phytoplasmic diseases by novel techniques; detection and monitoring of invasive hemipteran species in the region and their effects on crop production and native biodiversity. These field-based studies generally require longer a duration for sampling and data analyses, so the results are mainly preliminary, but yet very relevant for the subject area.

Modern comprehensive biodiversity studies should encompass a larger geographical extent, which require a coordinated and spatiotemporally distributed effort of several research groups organised around similar research foci and methodology. Our pilot survey of listed subjects, although preliminary with respect to the direct outcomes, contributed largely to the important exchange of practical scientific experience between the involved teams (particularly in the application of advanced techniques and concepts), that should enable and facilitate further region-wide cooperation and an integrative approach in agrobiodiversity research and practice.

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Major Allergens in Apples and Olive Fruits

Vlachonasios K. E.[1], Sfakiotakis E.[1], Chatzisavva K.[1], Kaldis A.[1], Papadopoulou E.[1], Laimer, M.[2], Marzban, G.[2], Heard A.[2], Iska P.[3] and Thomai T.[3]

[1] Aristotle University of Thessaloniki, School of Biology, Department of Botany, 54124 Thessaloniki, Greece; kvlachon@bio.auth.gr
[2] University of Natural Resources and Applied Life Sciences, Institute of Applied Microbiology, Department of Biotechnology, Plant Biotechnology Unit, Muthgasse 18, 1190 Vienna, Austria; m.laimer@iam.boku.ac.at
[3] Agricultural University of Tirana, Department of Horticulture, 21222 Tirana, Albania; ttho mai@yahoo.com

Abstract

The importance of fruits in human diet is unquestionable; therefore fruits containing allergenic proteins present an important and interesting field of research with major impacts and applications to plant biotechnology, agronomy and human health.
The objectives of this project were to detect and evaluate the presence of fruit allergens in apple cultivars grown in Austria, Greece and Albania, and to isolate, detect, localise and characterise putative fruit allergens from table olives grown in Greece and Albania.

The results showed valuable information about potential genotypic differences in the expression level of different allergens. If production data are recorded carefully, there might be valuable information about the influence of agronomic parameters influencing the amount of allergens in similar cultivars.
In this collaboration, young researchers (one from each country) were trained in a partner's laboratory.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology
Type of the project: research project

Keywords

Apple allergens; Fruit allergens; Mal D1; Olive pollen allergens; Olive fruit

1 Introduction

The importance of apple and olive allergens for real food allergy in in Central and Southern European countries has been demonstrated repeatedly [2]. Mal d 1 is the major apple allergen for patients from Austria, the Netherlands and Northern Italy. More than 85% of

the patients from these countries are already sensitised to Mal d 1.
Allergies to olive pollen and fruit are widespread in the southern parts of Europe; however, little is known about their significance in Greece and Albania.

In the frame of SEE-ERA.NET Project 9527, efforts were performed to win a first insight into this area of research by screening different apple cultivars cultivated in Greece and Albania and Austrian apples which have been collected from orchards in the Vienna area. Greek apples have been collected from Mt. Pelion (Central) and Panagitsa (Northern). Albanian apples were collected in Korsita and areas near Tirana. All collected apples were send to Austria for screening of Mal d 1, a major apple allergen, and immuno-tissue-printing for the detection and localisation of different apple allergens. Further, analogous to apples, Greek olives of different cultivars were collected and investigated for homologous allergenic proteins available also in apples (Mal d 3 and Profilin).

2 Experimental Techniques and Methods

2.1 Fruit material

Apples have been collected from orchards near Vienna and two different places in Greece: Pelion-Zagora and Panagitsa (Table 1).

Table 1: Cultivars used and their geographical region

Greek cultivars collected in two different areas	Austrian cultivars collected in the Vienna area
Firiki, Red Delicious (Starking), Golden Delicious, Starking, Fuji, Jonagold, Granny Smith	Gloster, Champagner Renette, Fuji, Welschbrunner, Topaz, Golden Delicious, Granny Smith, London Pepping, Kronprinz Rudolf, Canada Renette, Steirischer Maschanzker, Schweizer Orangenapfel, Elstar, Delbard Estival, Gravensteiner, Rubens, Gala, Roter Berlepsch, Cox Orange

2.2 Allergen extraction from apples

Ripe apples of commercially valuable cultivars were washed extensively with hot water before preparation. Peel and pulp were extracted separately. Apple material was homogenised to powder under liquid nitrogen in a mortar. Apples were harvested at cultivar specific dates, and stored at 4°C before extraction. To assure a valid sampling, five apples per cultivar were peeled (5 mm) with a paring machine, shock frozen and powdered in liquid nitrogen. Frozen powder was mixed in a blender with extraction buffer (10 mM K_2HPO_4,1 0 mM KH_2PO_4, 0.27 mM EDTA, 13.3 mM DIECA, 2% PVPP, pH7) in a ratio of 1:2 w/v and extracted by stirring at 4°C. The mixture was filtered through two layers of Miracloth (Calbiochem, USA), centrifuged at 10,000 g for 30 min at 4°C and the supernatant immediately frozen at minus 80°C [1].

2.3 Allergen extraction from olive pollen

0,1 g of pollen material was frozen in liquid nitrogen. The ground powder was shaken on ice for 60 minutes in 1 ml aqua dest. After 10 minutes of centrifugation at 4°C at 10000 rpm, the supernatant was aliquoted in 2 ml tubes and stored at –20°C.

2.4 SDS-PAGE (SDS-polyacrylamide gel electrophoresis)

Sample extracts were diluted in sample buffer, β-mercaptoethanol was added to the mixture and denaturated for 4 min at 95°C. Samples loaded onto hand-made gels (4% stacking gel and 13% resolving gel). Electrophoresis and electroblotting were carried out using a BIORAD apparatus. Proteins were transferred onto a nitrocellulose membrane by electroblotting. The membranes were incubated for 1h at RT with the first antibody: either a polyclonal rabbit anti- Mal d 1 (1:20.000) or an anti- Mal d 3 (1:2.000) (one for each membrane) in dilution buffer (PBS+0.05% Tween20+ 1% defatted milk). After 3 wash cycles (5min each) with washing buffer (PBS+0.05%Tween20), the membranes were incubated for 1h at RT with the second anti-rabbit goat (anti-IgG) antibody labeled with alkaline phosphatase (1:10.000) in dilution buffer. The blots were to be washed 3 times as described before and then incubated for 5 min in staining buffer for alkaline phosphatase (100mM Tris-HCl, pH9,5+100mM NaCl+5mM $MgCl_2$). The membranes were developed in 20ml substrate buffer supplemented with 24µl BCIP (5-bromo-4-chloro-3-inodyl-phosphatase) and 24µl NBT (nitroblue-tetrazolium) at RT. The reaction was stopped with destilled water and the membranes were air-dried. As a molecular weight marker, prestained Precision Plus Protein Standards (Fermentas) was used.

2.5 Polyclonal antibodies

Polyclonal rabbit antibodies directed to Mal d 1, Mal d 2 and Mal d 3 were produced in house by the Plant Biotechnology Unit, IAM, Vienna, Austria. An anti-profilin antibody was kindly provided by Drs. H. Breiteneder and I. Swoboda.

2.6 ITP (Immuno-Tissue Printing)

ITP was used to localise the allergens in the Greek apples. 0.2 µm Nitrocellulose membranes were soaked in 100mM ascorbic acid (to prevent oxidation) and air-dried prior to printing. After cutting the fruits in two parts, they were placed on the pre-treated membranes and pressed. The membrane was air-dried and stored at room temperature (RT). The prints were developed, then incubating in Vienna by soaking in blocking buffer (Na_2HPO_4 1,15g, KCl 0,2g, KH_2PO_4 0,2g, NaCl 8g and 1000ml Aqua dest.)+ 0.05% Tween20 + 5% defatted dried milk for 2h in RT. The prints were incubated for 1h at RT with the first antibody, a polyclonal rabbit(IgE) anti-Mal d 1 (1:20.000), anti-Mal d 2 (1:1.000) and anti-Mal d 3 (1:2.000) (one for each membrane) in dilution buffer (PBS+0.05% Tween20+ 1% defatted milk) diluted in dilution buffer (PBS+0.05% Tween20+ 1% defatted milk). After 3 washing cycles (5min each) with washing buffer, the ITPs were incubated for 1h in RT with the second antibody, an anti-rabbit goat(IgG) antibody labeled with alkaline phosphatase, diluted 1:10.000 in dilution buffer. The prints were washed 3 times and then incubated in staining buffer for alkaline phosphatase (100nM Tris-HCl, pH9.5+100mM NaCl+5mM $MgCl_2$). Membranes were developed in 20ml substrate buffer supplemented with 24µl BCIP (5-bromo-4-chloro-3-inodyl-phosphatase) and 24µl NBT (nitroblue-tetrazolium) until

the desired intensity appeared on the shaker at RT. Then the reaction was stopped with dest. H_2O and the membranes were air-dried [2].

2.7 Mal d 1 ELISA

Microtiter plates (Nunc-Immuno Plate MaxiSorp Surface) were coated with 100 ml/well polyclonal rabbit anti-Mal d 1 antibody diluted 1:1000 in coating buffer (8.4 g/l $NaHCO_3$ + 4.0 g/l Na_2CO_3, pH 9.6) at +4 °C over night. The following consecutive incubation steps (100 ml/well) with three washes (300 ml TBS + 0.05% Tween20) between each step were: (1) apple extracts diluted 1:5 to 1:40 in dual steps (37 8C and 1 h) and purified recombinant Mal d 1 was added as a reference (500–7.8 ng/ml); (2) mouse mAb 4C3C10 1:500 (37 8C and 1 h); (3) alkaline phosphatase labelled goat anti-mouse IgG (Fab specific; Sigma A1682) 1:2000 (37 8C and 1 h); (4) p-nitrophenylphosphate (1 mg/ml) in coating buffer supplemented with 5 mM $MgCl_2$ (at room temperature and 90 min in the dark). Samples and antibodies were diluted in TBS + 2% PVP + 0.5% BSA + 0.1% Tween20. Photometric measurement was performed at 405 nm (reference wavelength 620 nm) with a microplate reader. Absorbance values were expressed as ng Mal d 1 per ml extract by interpolation with the reference curve (DeltaSoft from BioMetallics). All determinations were run in duplicates and Mal d 1 amounts were converted to microgram Mal d 1 per gram fresh weight [2].

3 Results and Discussion

3.1 Detection and localisation of fruit allergens of apple cultivars grown in Austria and Greece

In order to detect homologous allergens in different Greek apples, three different antibodies directed to the most important apple allergens were applied. ITP and Western blotting were performed to detect Mal d 1, Mal d 2 and Mal d 3 in three different Greek apple cultivars. To ensure the results of ITP, a negative control was performed in parallel in the absence of detecting antibody.

A strong reaction with the anti-Mal d 1 could be detected in all three cultivars analysed by the ITP (Figure 1A, 1B and 1C) and in the Western blot (Figure 2A). A slight reaction with anti-Mal d 2 was observed in the ITPs (Figure 1A, 1B and 1C) for all three cultivars. ITPs did not show the expected accumulation of Mal d 3 in the peel of Greek apples, however in Western blots (Figure 2B) a clear reaction of the pAb against Mal d 3 can be detected in the peel. Firiki showed the strongest reactivity followed by Red Delicious and Golden Delicious. Mal d 3 was not detected in the pulp extracts (Figure 2B).

3.2 Determination of Mal d 1 content in Greek and Austrian apples by ELISA

Different regional cultivars with commercial value were collected at particular cultivar-dependent time window. The Mal d 1 content of each apple cultivar was tested using Mal d 1 specific ELISA. The results obtained allowed a first insight into the Mal d 1 content of apple cultivars produced in the Central and Southern regions of Europe. To compare the influence of geographical differences on the Mal d 1 expression in apples, similar cultivars were selected from two different places in Greece and Austria (Table 1). The Mal d 1

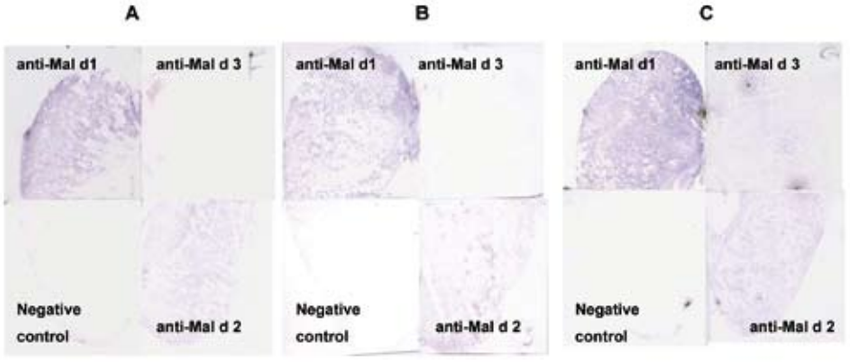

Figure 1: Immuno-tissue Printings of three apple cultivars (A: Firiki, B: Red Delicious (Starking) and C: Golden Delicious). The ITPs were cut in four pieces and developed with three antibodies plus the negative control.

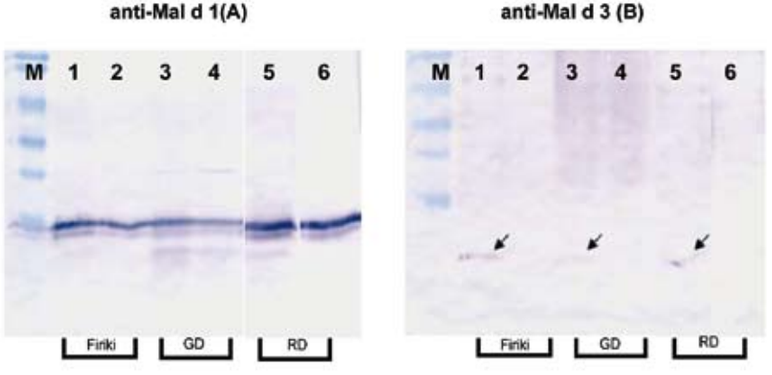

Figure 2: Western Blots of apple cultivars (Firiki, Red Delicious [Starking] and Golden Delicious) with (1, 3, 5) and without peel (2, 4, 6). A Membrane was developed with anti-Mal d 1 and B with anti-Mal d 3.

content was determined in Austria of Austrian and Greek apples in different local cultivars (Table 2 and 3). The Mal d 1 content of Austrian apples ranged from 28.6 (cv Golden Delicious) to 159.8 (cv Cox) µg/g fresh weight and of Greek apples from 19.8 (cv Fuji) to 1579.1 (cv Golden Delicious) µg/g fresh weight. In general, Greek apples contained much higher concentrations of Mal d 1. The comparison of two different cultivation areas in Greece showed further significant differences in the range of 0.5-3 fold between identical cultivars like Golden Delicious and Firiki. Cultivar Starking contained similar amounts of Mal d 1 in both areas. Further, the differences in Mal d 1 content in Austrian and Greek cultivars were significantly higher (0.6 fold for cv Granny Smith and 56 fold for cv Golden Delicious). Only the Austrian Fuji showed a 0.5 fold higher Mal d 1 content than Greek Fuji. The differences might be due to geographical and cultivation modes that influence the PR-10 protein expression in different apples.

Table 2: Mal d 1 content of Austrian apple cultivars (Vienna area)

Austrian cultivars (cultivated in the Vienna area)	Mal d 1 µg/g fresh weight (peel + pulp)	Austrian cultivars (cultivated in the Vienna area)	Mal d 1 µg/g fresh weight (peel + pulp)
Gloster	54.5	Steir. Maschanzker	123.3
Ch. Renette	108.6	Sch. Orangenapfel	86.8
Fuji	35.2	Elstar	104.9
Welschbrunner	119.28	Delbard Estival	32.9
Topaz	36.1	Gravensteiner	54.8
Golden Delicious	28.6	Rubens	159.6
Granny Smith	160.9	Gala	35.36
London Pepping	52.9	Roter Berlepsch	25.2
Kronprinz Rudolf	50.5	Cox	159.8
Canada Renette	84.1		

Table 3: Mal d 1 content in Greek apples cultivars in two different areas

Greek cultivars (cultivated in the Pelion Zagora area)	Mal d 1 µg/g fresh weight (peel + pulp)	Greek cultivars (cultivated in Panagitsa)	Mal d 1 µg/g fresh weight (peel + pulp)
Golden Delicious	525.1	Golden Delicious	1579.1
Firiki	351.8	Friki	233.7
Starking	185.1	Starking	171.1
		Fuji	19.8
		Jonagold	249.4
		Granny Smith	244.6

3.3 Allergen detection of Greek olive pollen and fruit extracts

Due to the missing availability of Greek olives, it was decided to work with olive pollen in order to validate the test system for the detection of homologous proteins in olives. Four different Greek olive pollens were collected in Greece, transferred to Austria, extracted and analysed using Western blotting and polyclonal antibodies directed to Mal d 3, apple LTP and Mal d 4, a profilin. Processed olive fruits were obtained from a local market (cv. Kalamata and Chalkidikis) and analysed for LTP and profilin.

Polyclonal antibodies directed to Profilin and Mal d 3 (LTP) were used to visualise homologous allergens in olive pollen and fruits of different Greek cultivars. LTP homologous protein could not be detected, neither in pollen (Figure 3) nor in fruit extracts (Figure 4). Profilin homologues, however, were clearly visualised in three of four pollen samples and weakly in processed olives cv. Chalkidikis, but not in Kalamata. Also, in cultivar Kalamon, no bands reacted an with anti-profilin antibody, which can be due to very low expression of this protein. The lack of LTP bands are more likely caused by masking effects of pollen tissue as shown in previous reports [3]. Therefore, in future investigations, a olive-specific LTP enrichment protocol should be developed.

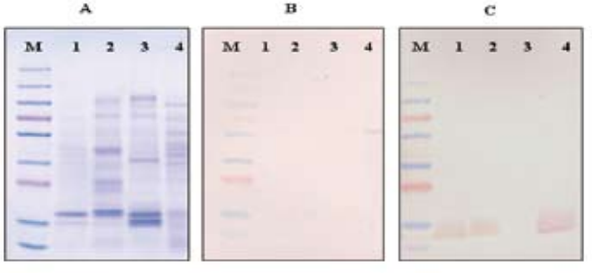

Figure 3: Different olive pollen analysed by SDS-PAGE and Western blotting. The samples, M) Molecular weight marker, 1) cultivar Amfissis, Area of Lamia 2) Mastoides, Crete, 3) Kalamon, Kilkis, 4) Koroneiki, Crete were separated using SDS-PAGE and stained by (A) Coomassie Blue and (B) Western blotted using polyclonal antibodies against apple Mal d 3, a lipid transfer protein and (C) a polyclonal antibody against profiling.

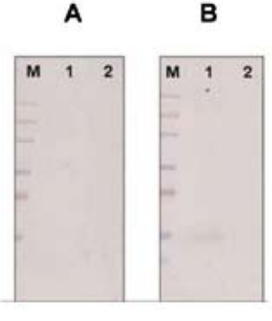

Figure 4: Two different table olive cultivars, Chalkidikis (1) Kalamata (2), have been extracted and Western blotted using anti-Mal d 3 (A) and anti-Mal d 4 (B) polyclonal antibodies.

4 Conclusions / Outlook, next steps

During this research project, the presence of the fruit allergens in Greek, Austrian and Albanian apple cultivars was investigated. All the major apple allergens were observed in all varieties tested. Greek varieties showed an increased amount of Mal D1. In addition, mal d3 and profilin was detected in Greek olive pollen and fruit cultivars.

In order to characterise the influence of storage conditions, ripening, ethylene signaling and superficial scald on the expression of Mal d 1 major apple allergen, two cultivars will be selected, which are cultivated in all contributing countries, e.g. Golden Delicious and Granny Smith. The study design will be performed by Dr. Vlachonasios and Dr. Sfakiotakis (Greece) for all measurements to be made, including starch and internal ethylene concentration.

Further investigations envisage the influence of food processing on the content of olive allergens (comparison of Spanish and Greek traditional olive processing). For this purpose, analyses will be performed on firmness, soluble solids, colour, and antioxidants and at protein and RNA level as well. The main focus will be the content of LTPs in native and processed olive fruits.

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Phenotypic and Genotypic Characterisation of Pasteurella Multocida and Mannheimia Haemolytica Strains Isolated from Sheep and Goats Originated from Greece, Serbia, Bosnia and Herzegovina

Evanthia Petridou[1]

[1] Department of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, Aristotle University, University Campus, 54 124 Thessaloniki, Greece; epetri@vet.auth.gr

Abstract

Slaughterhouses located in different areas in Greece, Serbia and Bosnia and Herzegovina were selected. Nasal swabs along with tissues (lung and tonsils) from healthy and diseased sheep and goats were collected. All samples were forwarded to the regional or national laboratory of microbiology, respectively for further microbiological examination. Strain isolation was followed by strain characterization. All P.multocida isolates were classified by a multiplex capsular polymerase chain reaction (PCR) typing system which was used as a rapid assay for the definitive classification of P.multocida capsular types A,B,D,E and F, while all Mannheimia haemolytica isolates were classified according to biotype A and its serovars. These serovars were determined by a passive haemagglutination test. All collected data were recorded on a PC, while a software package was designed. A code number was given to every isolate. Finally, statistical analysis of the results revealed the proportions of serotype recovery for both bacterial species.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology
Type of the project: research project

Keywords

Bacteriology; Clinical microbiology; Mannheimia haemolytica; Pasteurella multocida; Sheep; Goats

1 Introduction

The animal production sector of all participating countries relies on the extensive farming of sheep and goats mainly for milk and dairy products as well as for meat production. There is a large proportion of sheep and goat herd movement and mixing occurring among neighboring Balkan countries especially during Christian Easter when a great demand of lambkins and lamb in the market is recorded.
This project was an effort to strengthen the link between those three Balkan countries, along with their mutual goal to identify the serotypes responsible for the pasteurellosis

incidence in their regions and to provide information on the most appropriate serotypes to be used for successful vaccination against M.haemolytica and P.multocida, while technological advancement was promoted by using modern molecular techniques and the know-how transfer.

Furthermore, the correlation of serotypes isolated from healthy and diseased animals provided information for the selection of the most potential vaccine, while information about the efficacy of the antimicrobial therapy that is used was also extracted by testing several antimicrobial substances against the M.haemolytica and P.multocida isolated strains leading to a more effective therapeutic scheme.

2 Experimental Techniques and Methods

All isolates resembling to M.haemolytica or P.multocida were further identified according to biochemical properties. All identified strains were stored in –70°C freezer for serotype analysis. Multiplex PCR for the molecular identification was performed in all isolates (from all countries) at the Laboratory of Microbiology and Infectious Diseases of Veterinary Faculty in Thessaloniki, Greece.

Passive Indirect Haemagglutination assay for the serological classification was performed in all isolates (from all countries) at Department of Bacteriology, Moredun Research Institute, Edinburgh, Scotland, United Kingdom by Prof. W. Donachie, who kindly offered his support.

2.1 Sample collection – Bacterial characterization

Swabs from nasal cavity, tissue from tonsils and lungs of both healthy and diseased sheep and goats of varying ages from different slaughterhouses located in different areas were collected. All swabs and tissue samples were cultured by inoculation onto 5% blood agar plate containing vancomycin and incubated overnight aerobically for 16hours at 37°C. Subsequent to incubation, the plates were visually examined for M.haemolytica and P.multocida colonies. The former tends to create small, circular, clear colonies whereas colonies of the latter are grayish in colour, mucoid and irregular in shape. A single colony of each type was selected and subcultured to ensure the purity of the isolate. Isolates with a similar morphology to the bacterium of interest and which were tested positive for catalase and oxidase were stored in a representative medium (with glycerol) at –70°C for further biochemical and serological identification.

2.1.1 Molecular identification of all Pasteurella multocida strains

All P.multocida isolates were classified by a multiplex capsular polymerase chain reaction (PCR) typing system, which was used as a rapid assay for the definitive classification of P.multocida capsular types A,B,D,E and F. DNA was extracted from whole bacterial cells. A loopful of the desired strains was taken from the agar plates and resuspended in nuclease-free water, and incubated at 99°C for ten min to lyse bacterial cells. Bacterial lysates were cooled and centrifuged to produce a distinct genomic DNA. An aliquot of DNA was resuspended in a PCR amplification mixture which consisted of multiple primers for all capsular serogroups (Table 1). The primer sequences were based on the coding strand of various serogroup cap genes that all had their own Genbank accession numbers.

Table 1: Coding strand of various serogroup cap genes

Serogroup	Gene	Amplimer size (bp)
All *P.multocida* capsular types	KTM1	460
A	*hyaD-hyaC*	1.044
B	*bcbD*	760
D	*dcbF*	657
E	*ecbJ*	511
F	*fcbD*	851

2.1.2 Serological classification of all isolated Mannheimia haemolytica strains

All M.haemolytica isolates were classified according to biotype A and its serovars. Biotype A was determined biochemically by the fermentation of arabinose, to differentiate biotype A from biotype T. The serovars of the M.haemolytica were determined by a passive haemagglutination test. M.haemolytica comprise serotypes 1, 2, 5, 6, 7, 8, 9, 12, 13, 14, 16 and 17, while some isolates were found to be untypable.

Passive Indirect Haemagglutination assay for the serological classification was performed in all isolates (from all countries) at the Department of Bacteriology, Moredun Research Institute, Edinburgh, Scotland, UK by Prof. W. Donachie, who kindly offered his support.

3 Results and Discussion

The Greek isolates were 50 Pasteurella multocida strains and 103 Mannheimia haemolytica ones. The Serbian isolates were 15 P.multocida and no M.haemolytica strains, while the Bosnian-Herzegovinian isolates were 3 out of 10 suspected P. multocida and none Mannheimia haemolytica strains.

The result analysis revealed that Pasteurella multocida seems to be an important animal pathogen in all three participating countries, while the dominating biotype was found to be A and secondarily D. This fact is in contrast to the data recorded in countries located in Central Europe [1]. Moreover, the antibiotic susceptibility test that was performed in all Greek strains revealed resistance to tetracycline [2]. All 50 isolated P.multocida strains revealed M.I.C.s higher than 1228μg/ml. PCR analysis of chromosomal DNA identified Tet genes of class H only in one case, while PCR analysis of the plasmid DNA identified Tet genes of class B, Tet(B) in four strains, Tet genes of class H and Tet(H) in two strains but

not Tet genes of class M Tet(M). No correlation between the Tet genes and the MIC of tetracycline was observed.

All M.haemolytica strains were analysed at Moredun Research Institute in Edinburgh, Scotland U.K (since the funds were originated from a national research project). According to the serotyping the dominating in Greece serotype was A2 (49/103) 47.5% and secondarily A5 (8/103) 7.76% .
The results revealed an important difference between the countries of Central Europe and Greece. Prof. W. Donachie visited Greece and gave lectures on the subject, not only to the participating teams in the SEE-ERA.NET Pilot Joint Call project but also to the students of Veterinary Faculty of Aristotle University, taking into account our results and highlighting the difference.

More details is provided in the scientific booklet "Sampling, Identification and Typing of the Species Pasteurella multocida and Mannheimia haemolytica" (2008) edited by E. Petridou, L. Ekateriniadou, N. Giadinis, A. Zdragas, Ch. Vougidou and S. Kritas.

4 Summary / Conclusions / Outlook, next steps

The major aim of the project was to determine the incidence of P.multocida and M.haemolytica serotypes in sheep and goats populations in Greece, Serbia and Bosnia and Herzegovina. The work plan was initially to collect the samples and proceed to the investigated bacteria isolation. The phenotypic and genotypic characterisation was performed in all isolated strains.

A small workshop was performed during the project. All participants had the opportunity to meet and get know each other. Moreover, all scientific teams introduced themselves to the others and had discussions about the incidence of Pastereullosis in their countries and how they usually handle the cases. Prof. W. Donachie Director of Bacteriology at the Moredun Research Institute, Edinburgh, Scotland, UK, was invited as a speaker. The coordinating team distributed a scientific booklet with SOPs (Standard Operating Procedures) concerning sampling, isolation and identification of the above bacteria to all the participants.

During the project, one scientist from Bosnia and Herzegovina and two scientist from Serbia were trained for one week at the coordinating teams' laboratory in Greece. The scientist had the opportunity to visit industrial slaughterhouses and to become familiar with the sampling procedure. The next days, they performed microbiological examination of the samples while they were trained in molecular techniques.

At the end of the project, all P.multocida strains were collected and analysed in Greece, while all the Mannheimia haemolytica strains were analysed at the Moredun Research Institute. The result analysis revealed an important difference in the incidence of P.multocida and M.haemolytica serotypes in the sheep and goats population between the Balkan area and Central Europe.

The preliminary results of our small scale research can only lead to the hypothesis that the dominating biotypes of Pasteurella multocida and serotypes of Mannheimia haemolytica in the West Balkan area are significantly different from those that are dominating in Central Europe, maybe due to the different climate and/or due to the different breeds.

In conclusion, in the frame of the SEE-ERA.NET, a small-scale research project was performed with three Balkan countries participating. A scientific network was established and functioned well. The preliminary results of the research mainly underlined the fact that data about animal pathogens that have been generated in countries of Central Europe can not be adopted and accepted as a fact in the Balkan Countries. For this reason, research needs to be conducted in order to obtain well-documented data for these countries as well.

A scientific network has been well established on the basis of good and fruitful collaboration.
Our research needs to be continued in the future on a larger scale, probably with more partners from the West Balkan Countries in order to better document our hypothesis that iron- regulated protein vaccines seem to be effective against M.haemolytica in sheep, but little is known about their effect in goats. Moreover, it must be underlined that the recovery of the predisposing factors remains crucial for the effective control of the disease.

The participating scientific teams share the same enthusiasm to continue the collaboration. Large vaccine producers showed a great interest in the results of our study. One of them sponsored the travel and accommodation expenses of Prof. W. Donachie in Greece, who also showed a great interest in our results and kindly offered to analyse the isolated strains.

Proposed next steps:

- The same research project should be performed on a larger scale, maybe with more participants in order to obtain better documented results from a statistical point of view; and
- Sustain the already existing network.

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Phenotyping and Genotyping of Cereal Genetic Resources to Improve Tolerance to Abiotic and Biotic Stresses

Veisz, O.[1], Karsai, I.[1], Vida G.[1], Petcu, E.[2], Kobiljski, B.[3], Ivanovska, S.[4]

[1] Agricultural Research Institute of the Hungarian Academy of Sciences, Brunszvik u. 2, 2462 Martonvásár, Hungary; veiszo@mail.mgki.hu
[2] National Agricultural Research and Development Institute, Titulescu str. 1, 8264 Fundulea, Romania; petcue@ricic.ro
[3] Institute of Field and Vegetable Crops, Maksima Gorkog Nr 30, 21000 Novi Sad, Serbia; kobboris@ifvcns.ns.ac.yu
[4] Faculty for Agricultural Sciences and Food, Ss. Cyril and Method University in Skopje, Aleksander Makedonski BB, 1000 Skopje, Former Yugoslav Republic of Macedonia; s.ivanovska@zf.ukim.edu.mk

Abstract

The aim of the joint research activities was to initiate the exploitation of the genetic diversity existing in active small grain cereal breeding programs in Central and Eastern Europe and the West Balkan region with the ultimate purpose of improving abiotic and biotic stress tolerance and developing new breeding materials with better adaptability to changing environments. For this purpose, a collection of small grain cereal genotypes was set up, consisting of 117 winter wheat, 18 winter durum wheat, 25 triticale, 21 winter and 17 spring barley, and 13 spring oat genotypes from four breeding teams located in and around the Carpathian Basin. The collection was used in genotypic and phenotypic experiments. The deviations within and between the variety groups and the distribution patterns of the molecular markers and agronomic traits underlined that there were significant differences between the genotypes available to each breeding team, making it possible to utilise this diversity both for breeding purposes and for genetic studies.

Thematic Area / Type of the project

Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest, and aquatic environments
Type of the project: research project

Keywords

Abiotic; Biotic stress tolerance; Genetic diversity; Small grain cereals

1 Introduction

Climate variability has a fundamental influence on natural and artificial agro-ecosystems. The weather in recent years seems to confirm earlier predictions that the frequency of

extreme weather conditions would increase. The weather extremes experienced most frequently in Central Europe and the West Balkan countries and having the greatest influence on artificial ecosystems are low or high temperature and a deficiency or excess of rainfall. Responses to changes in climatic factors are greatly influenced by the adaptability of crops and by their tolerance to abiotic stress factors. Climate change scenarios forecast significant decreases in cereal productivity, surpassing even 20%, for a substantial part of Europe. The productivity loss will be coupled with a general decline in ecosystem stability in agriculture. Climate change raises new demands such as adaptability to stress, which will gain priority over the quantitative aspects of yield. This demands new approaches both in plant breeding and in the strategy and policies of biodiversity conservation.

The continual breeding of new varieties is essential if the damage caused by climate change is to be mitigated and reliable food supplies are to be ensured. If plant breeders are to rise to this challenge, there will be a need for extensive basic research, the investigation of abiotic stress factors, and technical developments designed to facilitate breeding. Wide-ranging international cooperation will be required to extend the genetic variability of breeding stocks and to improve adaptability.

Thus the long-term aim of the joint research activities is to initiate the exploitation of the genetic diversity existing in active small grain cereal breeding programs in the Central and Eastern European as well as the West Balkan regions, with the ultimate purpose of improving abiotic and biotic stress tolerance and developing new breeding materials with better adaptability to changing environments.

The exploitation of a germplasm collection with a wide genetic basis may make a substantial contribution to the future tasks of breeders, which will involve reducing the risks faced by cereal production due to both the effects of extreme weather events and the application of agrochemicals to cereals. To achieve this goal, cooperation was established between four participants within the framework of this pilot joint proposal, comprising specialists in the fields of plant breeding, genetics, molecular biology and plant pathology from Central and Eastern Europe and from the West Balkan region. These participants were:

- (1) Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary (MV);
- (2) National Agricultural Research and Development Institute, Fundulea, Romania (FU);
- (3) Institute of Field and Vegetable Crops, Novi Sad, Serbia (NS); and
- (4) Institute of Agriculture, Skopje, Former Yugoslav Republic of Macedonia (MC).

The major objectives of the cooperation were to assess (1) the biotic and the abiotic stress tolerance of the small grain cereal germplasm collection under diverse ecological conditions, (2) to assess germplasm diversity based on pedigree information, multivariate analysis of phenotypic traits, and molecular markers, and (3) to build up a core collection of small grain cereals with the highest phenotypic and genotypic information contents.

2 Experimental Techniques and Methods

The experimental fields of the four breeding teams are located in and around the Carpathian Basin, where the weather is very variable, due to the random interactions of three climatic zones: the Atlantic, the Continental and the Mediterranean zones (Table 1).

Table 1: Geographical information and meteorological data of the experimental sites in the period from October 2007 to the end of June 2008

	Martonvásár (MV)	Fundulea (FU)	Novi Sad (NS)	Skopje (MC)
Latitude (°)	47.2 N	44.3 N	45.3 N	42.0 N
Longitude (°)	18.5 E	26.3 E	19.8 E	21.4 E
Altitude (m)	150	55	84	240
No. of days below 0 °C	42	51	33	21
No. of days with rain	66	87	111	84
Rainfall in the vegetation period (mm)	299	340	488	362
No of days over 25 °C	4	7	8	13

2.1 Plant materials

In order to assess the level of genetic diversity present in various breeding programs in Central and South-Eastern Europe, a collection of small grain cereal genotypes was set up, consisting of 117 winter wheat, 18 winter durum wheat, 25 triticale, 21 winter and 17 spring barley, and 13 spring oat genotypes. The same collection was then used in a series of genotypic and phenotypic examinations, some of which were carried out by all the teams, while some were team-specific. The results collected for the 117 winter wheat genotypes are discussed in detail.

2.2 Genotypic characterisation

Two marker technologies were applied: (1) AFLP, which identifies a higher number of marker loci randomly distributed over the whole genome, and (2) SSR, which identifies a higher number of alleles within a marker locus with known chromosomal location. For AFLP analysis, the methodology of [1] was applied. Three AFLP reactions were used for the whole small grain genotype collection, which are designated with the abbreviations of the four selective nucleotides (TCAT, TCGA, TCTC). For SSR analysis, four primer pairs

were tested on the whole germplasm collections: gwm46, gwm95, gwm262 and gwm680, each identifying a single marker locus on chromosomes 7B, 2A, 2D, and 6B, respectively [2].

Each fragment generated by one primer pair was considered to be the allelic version of the same marker locus for the SSR method, while for AFLP each polymorphic fragment was considered to represent a separate marker locus. Thus, the total number of alleles (SSR) and loci (AFLP) was observed, together with a number of rare and single alleles (SSR) and loci (AFLP). An allele (SSR) or marker locus (AFLP) was considered to be rare if its frequency was lower than 0.15 in the wheat genotypes. The polymorphic information content (PIC) value was calculated for each locus (SSR) and each selective primer pair (AFLP) [3] (Anderson et al. 1993): $PIC=1-\sum_{i=1}^{n} p^2 i$, where p_i is the frequency of the ith allele or locus. The hierarchical clustering protocol was carried out using the UPGMA grouping of the SPSS 16.0 software package on the matrix of Jaccard's distance values.

2.3 Phenotypic characterization

To assess the phenotypic variability of the varieties under diverse ecological conditions, field experiments were carried out in the multiple environments of the four participating research teams, applying the same experimental design. The genotypes were sown in head rows with two replications; the sowing dates at the various locations were between October 10 (FU) and October 18 (NS) 2007. Heading date, plant height, leaf diseases, and yield components were scored by the teams, as listed in Table 2. The leaf diseases (powdery mildew and leaf rust) were scored when the symptoms of the disease were at their highest level, by evaluating the ratio of the diseased leaf area to the whole plant area as a % [4]. The following categories were used: 0–5% tolerant, 5–20% moderately tolerant, 20–40% moderately sensitive, and 40–80% sensitive. The data were processed using the Windows Excel and Statistica 6 for Windows programmes.

3 Results and Discussion

3.1 Experimental location effects

The weather conditions in the experimental period showed variations between the four locations (Table 1). The winter was the coldest in FU, followed by MV, while the spring and early summer period was the warmest in MC, followed by NS. The average monthly minimum temperature was the lowest in January with a value of –5.3°C in FU, which was not enough to differentiate the winter hardiness of the wheat genotypes. The amount of rainfall during the growing season was the highest in NS and the lowest in MV. The distribution of the precipitation showed similar tendencies at all four locations; January and February were the driest, while the highest amount of precipitation was measured in October and November. Of the four locations, spring was the driest and warmest in MC, but even there, there was 84 mm rain in April and May, preventing the evaluation of drought tolerance under natural field conditions.

The location and genotype main effects were highly significant for each trait measured in at least two locations (Table 2). The variance in heading date and plant height was mostly derived from the location and genotype main effects. Together they explained 98.1% of the total variance for heading date, and 89.3% for plant height. For both traits, the location effect contributed the largest portion, underlining that environmental differences influenced the wheat genotypes similarly. The pair-wise correlation coefficients between the individual locations were in the range of 0.57 – 0.87 for heading date, and in the range of 0.52 – 0.74 for plant height. The correlation was the strongest between MV and FU for both traits. The location × genotype interaction had a more significant role in determining the leaf rust and powdery mildew tolerance of the genotypes, which was probably due to the different heading dates and to the differences in the prevailing pathogen race compositions at each individual location. Accordingly, the correlations between locations were lower, in the range of 0.41 (NS-FU) – 0.53 (MV-NS) for powdery mildew, and 0.01 (FU-MC) – 0.41 (NS-FU) for leaf rust tolerance.

Table 2: Location effects in the variance analysis of traits measured in at least two locations, and the location averages

	Location SS (%)	Genotype SS (%)	MV	FU	NS	MC
Heading date (days)	93.1***	5.0***	219	224	206	185
Plant height (cm)	58.2***	31.1***	81	92	-	67
No. of kernels / spike	18.1***	57.8***	-	-	62	55
1000 kernel weight (g)	35.5***	52.7***	-	-	47	41
Powdery mildew (%)	36.3***	36.2***	38	25	9	0
Leaf rust (%)	25.0***	34.3***	23	30	11	2

3.2 Genetic diversity assessment in the wheat collection

One of the major aims of the research was to assess the level of genetic diversity in the germplasm collections of wheat varieties bred by the collaborating teams and/or grown in

the Central and South-Eastern European region. In addition to the overall genetic diversity, the diversity within the variety groups of each team was examined and compared.

The three AFLP reactions resulted in 90 polymorphic fragments (markers) in the combined group of 117 wheat genotypes, 10% of which proved to be rare (Table 3). Thus, the PIC values of each reaction were high, giving an average value of 0.77. As the average r value between the individual marker pairs was 0.004, with a deviation of 0.178, these 90 polymorphic fragments were considered to represent 90 possible marker loci. When the separate variety groups were examined, a slight decrease in the level of polymorphism was apparent in the case of FU and MC varieties. No marker loci, characteristic of only one of the variety groups were identified with AFLP, but there were significant differences between the groups for the ratio of complete absence or presence of a marker locus and for the ratio of rare loci. All these values were the highest in the MC varieties, followed by the FU varieties.

The four SSR primers made it possible to examine the level of polymorphism within four loci. On average, 10.2 alleles per marker locus could be detected in the 117 wheat genotypes, 85.4% of which proved to be rare. Thus, the average PIC value of the SSR was 0.50, with substantial variation between the individual loci. There was also considerable variation in the PIC contents of the SSR primers within the individual variety groups. The most frequent allele was the same for all four wheat groups with three of the four SSR primers. Eighteen of the 35 rare alleles were characteristic of only one of the variety groups; the occurrence of such rare alleles was the highest in the NS group (9), followed by MV (4), FU (3) and MC (2).

Table 3: Polymorphic information contents achieved using various molecular marker techniques for the winter wheat varieties contributed by four breeding teams

Primers	117 wheat varieties			MV varieties			FU varieties			NS varieties			MC varieties		
	No. of loci / alleles	PIC value	No. of rare loci / alleles	No. of loci / alleles	PIC value	No. of rare loci / alleles	No. of loci / alleles	PIC value	No. of rare loci / alleles	No. of loci / alleles	PIC value	No. of rare loci / alleles	No. of loci / alleles	PIC value	No. of rare loci / alleles
TCAT	25	0.803	3	25	0.815	4	22	0.686	8	25	0.803	3	20	0.707	7
TCGA	34	0.765	4	34	0.720	3	30	0.621	6	34	0.789	9	24	0.710	11
TCTC	31	0.743	2	31	0.741	5	24	0.620	5	30	0.752	7	23	0.676	6
gwm46	12	0.655	10	7	0.632	4	5	0.507	3	7	0.647	5	3	0.542	1
gwm95	7	0.546	5	5	0.465	3	4	0.414	3	3	0.537	1	4	0.514	3
gwm261	12	0.491	11	3	0.215	2	5	0.443	4	11	0.664	10	3	0.542	1
gwm680	10	0.312	9	4	0.167	3	4	0.222	3	6	0.519	5	2	0.153	1

The data matrix of the AFLP and SSR markers was used to establish the genetic diversity between the 117 wheat varieties (Figure 1) and between the various breeding teams (Table 4). The varieties were placed in 8 clusters. The two largest clusters were Clusters 3 and 2, which contained 36% and 27% of the wheat varieties, respectively, but even within these clusters the variance was relatively high.

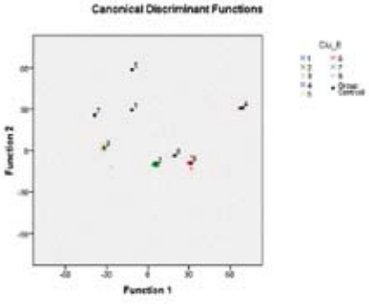

Figure 1: Genetic distribution of 117 wheat varieties based on the data matrix of AFLP and SSR markers

Table 4: Genetic distribution patterns of the winter wheat varieties contributed by four breeding teams as a result of discriminant analysis carried out on the data matrix of molecular markers

	MV	FU	NS	MC	Total number
Cluster 1	1	0	0	0	1
Cluster 2	22	5	3	1	31
Cluster 3	10	28	4	0	42
Cluster 4	2	0	4	3	9
Cluster 5	1	0	0	0	1
Cluster 6	0	0	14	3	17
Cluster 7	0	0	4	0	4
Cluster 8	0	0	7	5	12

The varieties contributed by the individual breeding teams differed from each other in their distribution over the 8 clusters, including the numbers of clusters and the most prevalent cluster. Most of the MV varieties were grouped in Cluster 2, the FU varieties in Cluster 3, the NS varieties in Cluster 6, and the MC varieties in Cluster 8. Based on the clustering patterns, the MV and FU varieties showed a closer relationship, while the NS and MC varieties were grouped together more frequently.

3.3 Phenotypic diversity assessment in the wheat collection

Based on the results of the one-season field test, there were no significant differences in the average values of the agronomic traits (heading date, plant height and yield components) between the four variety groups (Table 5). With respect to the heading date, the ratio of medium early varieties was the highest for all the breeding teams. The widest interval was characteristic of the MV and FU varieties; both the earliest and the latest heading genotypes belonged to these two breeding teams. In the case of plant height, number of kernels per spike and 1000-kernel weight the MV and NS varieties showed greater variation.

There was a considerable in the biotic stress tolerance, which was apparent not only in the group average values, but also in the distribution patterns. For powdery mildew, the majority of the 117 wheat varieties (54.7%) proved to be moderately sensitive. On average, the level of tolerance in the MV and FU varieties was higher and the ten most tolerant varieties also belonged to these two groups (five from each). Not only were there no tolerant varieties in the NS and MC groups, the ratio of moderately sensitive varieties was also the highest for these groups (69.4 and 66.7, respectively). In the case of leaf rust, there was a more even distribution of the varieties over the tolerance categories; 23.1% of the 117 varieties proved to be tolerant, 29.9% moderately tolerant, 30.8% moderately sensitive and 16.2% sensitive. Of the groups, the average level of tolerance was the highest in the NS varieties followed by the MV and FU varieties. Of the 27 tolerant varieties, 59.2% were contributed by NS, 25.9% by MV and 14.8% by FU. The disease tolerance level of the MC varieties was the lowest for both foliar diseases, which may reflect the fact that during the examination period, the overall disease pressure was the lowest in the Former Yugoslav Republic of Macedonia, thus making selection for tolerance unnecessary.

The larger deviations within the varieties of each breeding team and the distribution patterns of the agronomic traits underlined that there were significant differences between the genotypes, making it possible to utilise this diversity both for breeding purposes and for genetic studies.

4 Summary

The major aim of this pilot research was to establish the level of genetic diversity apparent in the small grain cereals contributed by various breeding teams in Central and South-East Europe with the dual purpose of setting up core collections covering the extent of genetic variance present in the region and identifying special genotypes for improving biotic and abiotic stress tolerance. Genotypic and phenotypic datasets were collected during the nine-month period and they revealed basic information and tendencies, based on which it will be possible to chart the course of further research and to strengthen the cooperation between the research teams. The methodologies applied by the teams to predict genetic diversity were appropriate to establish the basic relationships within and between the variety groups. This research revealed significant genetic variability in the small grain cereals, proving that these collections may be successfully utilised in further genetic studies, during which high-throughput marker technologies and candidate gene approaches

Table 5: Averages and distribution patterns of various agronomic traits measured in at least two environments in the group of winter wheat varieties contributed by four breeding teams

Traits	MV varieties			FU varieties			NS varieties			MC varieties		
	Ave.	Interval	Dev.	Ave.	Interval	Dev.	Ave.	Interval	Dev.	Ave.	Interval	dev
Heading date (days)	209	201 – 217	3.9	209	201 – 215	4.0	207	204 – 214	2.4	207	205 – 210	1.6
Plant height (cm)	80	62 – 126	10.0	81	71 – 96	6.7	80	63 – 92	6.1	79	70 – 85	4.9
No. of kernels / spike	59	41 – 75	8.2	59	46 – 70	6.1	57	49 – 81	6.7	58	47 – 72	7.5
1000 kernel weight (g)	42	34 – 52	4.5	45	39 – 54	3.1	45	36 – 56	3.8	43	36 – 50	4.3
Powdery mildew (%)	21	1 – 45	12.0	18	1 – 38	8.8	29	7 – 49	9.2	37	20 – 55	10.5
Leaf rust (%)	24	0 – 57	17.1	24	2 – 55	16.2	12	0 – 43	12.2	35	10 – 58	15.0

can be used for establishing a more detailed genome comparison of the varieties. The phenotypic characterisations in the field-sown experiments were subject to the prevailing weather conditions during the growing season, which only allowed the evaluation of certain agronomic characteristics and disease resistance during the period of the project, but prevented the measurement of abiotic stress tolerance. It is thus important to widen the phenotypic characterisation of the small grain cereal collections both in repeated field experiments over years and in controlled environmental tests. These preliminary phenotypic data, however, have already made it possible to examine location effects and to identify special genotypes and biotic resistance sources, which can be used directly in breeding. In addition, the detailed evaluation of the data collected by the teams and the preparation of scientific publications for publishing in peer-reviewed journals is now in progress.

In summary, the establishment of the core collection and the recording of preliminary results were necessary, fundamental prerequisites for association mapping and allele mining for candidate genes and for the comparative examination of inter- and intra-species variation in the magnitude of tolerance present in the various species. This may help to dissect and explain the genomic basis of adaptive functional diversity in stress tolerance and of species distribution, and provide information on the possible performance of field crops. The expected results will permit the greater utilisation of resources for variety development, gene discovery and marker-assisted selection.

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Recreation of the BALKAN NET, A Network of Conservation Bodies in Countries Sharing Continuous Large Carnivore Populations

Lazaros Georgiadis[1], Alexandros Karamanlidis[1], Giorgos Giannatos[1], Magda Sindicic[2]

[1] ARCTUROS, Rogoti 3, 54624, Thessaloniki, Greece; lgeorgiadis@arcturos.gr
[2] Faculty of Veterinary Medicine of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia; magda.sindicic@gmail.com

Abstract

The project supported within the SEE-ERA.NET Pilot Joint Call is related to the natural environment and the application of research on the conservation of biodiversity, with emphasis on large carnivores and their habitat in trans-boundary areas in the Balkan Peninsula. Based on the current conservation needs of the species targeted, the project aimed at improving communication and cooperation between local stakeholders involved in the conservation of large carnivores.

The main goal of this project was to recreate "BALKAN NET", a network of conservation bodies in countries sharing continuous large carnivore populations. The project included three distinct phases. An initial meeting was held in order to exchange scientific know-how and better manage the project. During the main phase of the project and depending on the state of knowledge on the status of large carnivores and the available resources, rapid countrywide surveys were carried out in each partner country. The final phase of the project included a final workshop during which the data collected and ideas for the development of joint future activities were presented. The main achievements of the project include the improvement of trans-boundary communication on a scientific and management level, as well as the improvement of capacity building in the countries participating in the project. Furthermore, an overview of the current status of large carnivore in SE Europe was prepared and common research and management methods were outlined and improved. The joint Action Plan defined outlines immediate and future common research and management activities required for the most effective wildlife and nature conservation.

Thematic Area / Type of the project

Environment (including Climate Change): Environmental Technologies
Type of the project: network project

Keywords

Balkans; Conservation; Ecosystem Management; Environmental Monitoring; Large Carnivores (LC); Terrestrial Ecology; Transborder Cooperation

1 Introduction

The recent rapid political and economic changes in south Eastern Europe are expected to trigger the expansion of infrastructure, industry and tourism in the region. Such a development will most likely increase the already existing stress and pose a new threat to local biodiversity. Wide ranging species, such as rare, large carnivores are especially vulnerable to such changes – the brown bear (Ursus arctos), grey wolf (Canis lupus) and Eurasian lynx (Lynx lynx) are nowadays endangered throughout Western Europe. Conserving viable populations of large carnivores in the modern European landscape is a big challenge. The status of large carnivores (LC) is a complex issue that requires systematic and long-term research. Currently, in most of the countries in South Eastern Europe, general knowledge on the status of large carnivores, which is required for their effective conservation and management, is still rudimentary. Moreover, management, research and conservation of large carnivores cannot be restricted within and limited by the boundaries of each respective country. Thus, a high level of trans-boundary cooperation is required for the in-depth research of all the factors that influence the status of carnivore populations and for their effective management.

One of the foremost priorities in the conservation of large carnivores in South Eastern Europe is the development, on a population level, of common management strategies, especially along trans-border areas. In order, however, to prepare, adopt and implement such management strategies, a solid knowledge baseline is required. This knowledge baseline does not exist in many South Eastern European countries; there is an acute lack of up-to-date field data on the status of large carnivores and their habitat. For example, the Dinaric-Pindos brown bear population is one of the most important in Europe. In contrast to the northernmost part of the population (in Slovenia and Croatia) that has been the focus of numerous ecological studies, such studies (except for Greece) have been limited further south. This area is also home to the last remains of the most endangered European lynx population; the one of the indigenous Balkan lynx population, whose ecology, size and distribution are virtually unknown. Ongoing advances in modern technology create nowadays new opportunities for common trans-border research and expertise exchange. Such actions, that until now have not been implemented consistently enough, can help stem the loss of precious biodiversity.

In the early 1990s a trans-border initiative named "BALKAN NET" that aimed at preserving biodiversity in the region, was coordinateded by ARCTUROS. The successful cooperation of approximately twenty organisations produced several important publications and the first overview of the status of large carnivores in the late 90s [1, 2, 3, 4]. The goal of the current project was to recreate the "BALKAN NET" scheme (i.e. recreate the dynamic network of conservation bodies in countries sharing continuous large carnivore populations) and achieve the following:

- Promoting trans-border cooperation on a scientific and management level and facilitate transfer of current scientific knowledge;
- Improving capacity building in target countries;
- Providing an overview of the current status of large carnivores in SE Europe;
- Outlining common research and management methods; and
- Preparing a joint Action Plan for immediate and future common research and management actions that will promote the more effective protection of wildlife.

The stakeholders/partners that participated in the project are listed below:

- ARCTUROS NGO, Greece
- Faculty of Veterinary Medicine (FVM), University of Zagreb, Croatia
- Faculty of Forestry, University of Sarajevo, Bosnia and Herzegovina
- Transborder Wildlife, Albania
- Wildlife Conservation Society MUSTELA, Republic of Serbia
- Bulgarian Biodiversity Preservation Society – SEMPERVIVA, Bulgaria
- MOLIKA NGO, Former Yugoslav Republic of Macedonia

The non-governmental organisations (NGOs) of MUSTELA and MOLIKA participated in the project on a voluntary basis.

2 Experimental Techniques and Methods

The project was implemented throughout three different phases:

- Phase 1: An initial meeting was held in order to coordinate the project, exchange information, and outline suitable, common methodologies for studying large carnivores in the field.
- Phase 2: During the main period of the project a rapid countrywide carnivore survey was carried out in each country.
- Phase 3: A final workshop was held, where the data collected and ideas for the development of a joint research Action plan were presented and future wider research projects were prepared.

The responsibilities among the partners of the project were shared as following: the team of ARCTUROS led the overall management of the project, including the organising of the kick-off meeting, the team of the Faculty of Veterinary Medicine of the University of Zagreb (FVM) coordinated the field survey and the team of MUSTELA organised the final workshop.

2.1 Initial meeting

The initial (kick-off) meeting was held on October 19 – 21, 2007 at the Environmental Centre of ARCTUROS, in the Region of Western Macedonia in Greece. The meeting was attended by all project partners, except of the team of the Faculty of Forestry of the University of Sarajevo (Bosnia and Herzegovina) due to bureaucratic problems in obtaining a travel visa

The meeting lasted for two days and consisted of two separate sections. The first section of the meeting included short presentations of each partner organisations' activities as well as their future activity plans. During the second section, an open discussion followed, focusing on appropriate research methodologies for countrywide surveys in each partner

country. A common methodology was proposed by each partner, taking into account the state of knowledge on the status of large carnivores and the difficulties in each respective country, as well as the financial limitations of the project. Emphasis was given to the collection of data related to the population of brown bears, especially in trans-border areas. A decision agreed upon by all attending members of the project was the design of a common protocol for collecting data regarding the species and the production of a scientific compendium on brown bears in the Balkan Peninsula, that would update the initial compendium produced by ARCTUROS in 1997, within the "BALKAN NET" initiative [1]. Among several research methods that were considered as potential research methodologies for monitoring the status of bears in the area, there was also the analysis of genetic data. It was decided to expand the "Hellenic Bear Register", a monitoring system that was developed for the genetic tracking of brown bears in Greece, into Albania and the Former Yugoslav Republic of Macedonia. The methodology applied within this monitoring system is based on the marking behaviour of bears and uses signs and genetic material (i.e. hair) found on power (electricity) poles to document the presence of the species in a given area [5].

2.2 Field survey

The general framework of the field survey was organised by the team of the Faculty of Veterinary Medicine (FVM), University of Zagreb, Croatia, but each partner had the responsibility for completing his own survey in his own country. The common methodology and the specific research priorities were outlined during the kick-off meeting, while a special protocol for data recording was designed by the teams of FVM Croatia and ARCTUROS. As mentioned previously, main research activities focused on the brown bear, as it is the most important and threatened species in the mountainous trans-border areas of the Balkan Peninsula. During this phase of the project, information on the following topics was collected:

- Species distribution;
- Population size and trend;
- Mortality;
- Reproductive status of the species in the country;
- Legal status of the species in the country;
- Management;
- Human – bear conflicts;
- Threats;
- Conservation actions carried out within the country;
- Judgment of the status of the population within the country & most urgent actions needed; and
- Conservation projects.

The partners of the project were in constant communication during the implementation of this task, in order to share experiences and ideas and adjust the common research methodology in face of unpredicted difficulties in the field. This task lasted six months, and included five months of data collection (field work, collection of information from other sources) and one month of writing the final report.

2.3 Final workshop

The final workshop was held at the Tara National Park in Serbia on June 1-2, 2008 and was organized by MUSTELA. The meeting was attended by scientists of each partner who presented the current status of large carnivores, with an emphasis on brown bears in their country (i.e. presentation of data gathered during the second phase) and recommendations for their most effective protection. A special round table was held where members from countries sharing common borders outlined the most urgent common actions to be undertaken. The last day of the workshop included a field trip to Tara National Park, where the participants had the opportunity to appreciate the biodiversity of the area and receive first hand information on the management of the local brown bear population. The meeting had the honour to host as a special guest, Dr John Beecham, biologist and bear conservation expert from USA and the ex-president of the International Bear Association (IBA).

3 Results and Discussion

The most important result of the project, both on a scientific and research and conservation level, was the drafting of a Action Plan that outlined common activities, for both, the short and long term trans-border protection of the brown bear.

The main points of interest of this Action Plan are:

- The production of an updated edition (Compendium) on the brown bear in the Balkan Peninsula and the publication of a relevant scientific article in the scientific journal of the IBA.
- The decision to apply the most modern technology and methods in scientific research related to the brown bear. The widespread and extensive use of genetic analysis in combination with other modern technologies, such as Geographical Information Systems, stable isotope analysis and satellite telemetry is one the main targets for the future.
- The first efforts on data collection in countries with no previous research such as Albania and the Former Yugoslav Republic of Macedonia.
- The expansion of the 'Hellenic Bear Register" into neighbouring countries. The project supported within the SEE-ERA.NET Pilot Joint Call, in combination with other complementary projects and funds (ARCTUROS, IBA, ALERTIS, World Society for the Protection of Animals, HELLENIC AID) provided the opportunity for the expansion of the project into neighbouring Albania and the Former Yugoslav Republic of Macedonia (i.e. and the creation "Southwest Balkan Bear Register") and the establishment of a permanent bear monitoring system. One of the main goals of the Action Plan is to find the logistic resources to keep this initiative running.

4 Conclusions / Outlook, next steps

The project activities provided an excellent opportunity to strengthen the working relationships between the members of the BALKAN NET, exchange knowledge, know-how and experience and design common, future conservation activities. Within the framework

of the project, it became clear to all the participants that:

- All of the countries in the Balkan Peninsula are currently undergoing a procedure of economical development; within this development several threats emerge for their unique natural environment and wildlife.
- The economic situation in combination with the political relationships amongst countries in the region increases the difficulties for the development of trans-boundary cooperation. A characteristic of this situation is the fact that due to visa problems, the partners from Bosnia – Herzegovina and Albania were not able to participate in the first and second meeting respectively.

Despite the aforementioned difficulties, the project pointed out the vivid interest existing in the cooperation and development of trans-boundary activities in the conservation and management of large carnivores. Building upon the dynamic network established during the "BALKAN NET", the partners of the project are now in constant contact between them in order to plan and implement new conservation and research activities.

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REFORMAN: Regional Forest Management Support Needs – Comparative User Requirements Analysis with Regional Stakeholder Groups in Balkan Countries and Middle and Eastern European Countries

Christine Fürst[1], Franz Makeschin[1], Carsten Lorz[1], Nenad Potocic[2], Dijana Vuletic[2], Silvija Krajter[2], Ivan Seletkovic[2], Harald Vacik[3], Dragan Matijasic[4], Andrej Breznikar[4], Matjaz Zupanic[4], Primoz Simoncic[5], Andrej Verlic[5], Zoran Galic[6], Vilem Podrazsky[7], Tarik Trestic[8]

[1] Institute for Soil Science and Site Ecology, Dresden University of Technology, Pienner Rd. 19, 01737 Tharandt, Germany; fuerst@forst.tu-dresden.de, Makeschin@t-online.de, carsten.lorz@tu-dresden.de

[2] Forest Research Institute Jastrebarsko, Cvjetno naselje 41, p.p. 40, 10 450 Jastrebarsko, Croatia; nenadp@sumins.hr, dijanav@sumins.hr, silvijak@sumins.hr, ivans@sumins.hr

[3] Institute of Silviculture, University of Natural Resources and Applied Life Sciences, Peter Jordan-Straße 82, 1190 Vienna, Austria; harald.vacik@boku.ac.at

[4] Slovenian Forestry Service, Central unit Ljubljana, Večna pot 2, 1000 Ljubljana, Slovenia; dragan.matijasic@zgs.gov.si, Andrej.Breznikar@zgs.gov.si, Matjaz.Zupanic@zgs.gov.si

[5] Department Forest Ecology, Slovenian Forestry Institute, Večna pot 2, 1000 Ljubljana, Slovenia; primoz.simoncic@gozdis.si, andrej.verlic@gozdis.si

[6] Institute of Lowland Forestry and Environment, Antona Čehova 13, 21000 Novi Sad, Serbia; galicz@uns.ns.ac.yu

[7] Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Kamýcká 129, 165 21 Prague 6 – Suchdol, Czech Republic; podrazsky@fle.czu.cz

[8] Forest Faculty, University of Sarajevo, Zagrebačka 20, 71000 Sarajevo, Bosnia and Herzegovina; mavdibegovic@hotmail.com

Abstract

The aim of REFORMAN was to bridge the gap between practical research needs and scientific research options and to identify generaliseable and differing needs for research & development corridors in the field of forest management. In a series of workshops, the frame conditions and planning and management support needs of regional end-users in forestry (forest managers) were inquired. The focus was laid on an in-depth analysis of the natural and societal frame conditions and management support needs in exemplary Western Balkan partner countries (Bosnia and Herzegovina, Croatia, Serbia) and in some Middle and (South) Eastern European countries (Austria, Czech Republic, Germany, Slovenia).

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest, and aquatic environments; Information and Commu-

nication Technologies: Applications Research; Environment (including Climate Change): Environmental Technologies.
Type of the project: research project

Keywords

Environmental technologies & build environment; Forest land-use management decision support; Increased sustainability of forestry; Management support needs; Natural frame conditions; Socio-economic frame conditions

1 Introduction

Forest management affects various levels of the ecosystems from the gene to the landscape [1]. Additionally, environmental changes influence landscapes and forest ecosystems in an intensive and sustainable way. Due to increasing human use, there is an ongoing trend to a shift from natural ecosystems to systems developed and cultivated by man [2]. In most European forest ecosystems, changes of nutrient, water and energy cycles, genetic diversity, species composition, habitats and landscape structures can be observed. Forests are damaged by wildlife and grazing, insects, diseases and pollution. There is also a linkage between acid deposition and forest decline. Forest trees are influenced directly and indirectly through numerous effects like loss of soil bases, aluminium toxicity, interactions with air pollutants such as ozone and sulphur dioxide and interaction with natural phenomena such as drought and deficiencies of soil nutrients [3]. Losses of forest area to urban and transport infrastructure are compensated by afforestation of agricultural and other land, by about one million ha/year in EU average, which all in all leads to slowly expanding forests in EU. Forests are a key resource serving a multitude of functions: providing industries with timber and communities with drinking water, protecting infrastructure in mountain regions against natural hazards, creating and managing habitat for wildlife species, maintaining biodiversity and aesthetic values, sequestering carbon and others. The growing need to consider so many different kinds of values has posed considerable challenges for management of natural resources, which now must additionally consider multiple, and often conflicting, ecological and non-timber objectives.

Considering the complexity of decision problems in natural resource management with many site and stand attributes, different stakeholder perspectives and various temporal and spatial scales in management planning neither intuitive nor schematic solutions are appropriate planning approaches. For such problems, the use of tools and techniques which support decision analysis is strongly recommended. In this context, management and decision support systems provide support by (a) structuring the decision problem, (b) assessing the impacts of each possible solution, (c) determining the preferences of the decision maker and (d) comparing the decision alternatives ([4]; [7]). Management and Decision Support Systems have proved to be most useful for complex, strategic problems, i.e. problems that cannot be completely supported by algorithms and analytical solutions [10].
According to a systems analysis view proposed by the European Environmental Agency,

the improved understanding of the relations between environmental and human system can be supported in an indicator-based assessment approach. Social and economic developments are identified as Driving Forces (D), which exert Pressures (P) on the environment. As a consequence, the State (S) of the environment changes, such as the conditions for health, availability and quality of resources or maintenance of biodiversity. Finally, these changes lead to Impacts (I) on human health, ecosystems and materials that may elicit a societal Response (R) that feeds back on the Driving Forces, or on the state or impacts directly, through adaptation or curative action ([6], [5]). REFORMAN followed this scheme for the analysis of regional basic parameters.

2 Experimental Techniques and Methods

REFORMAN addressed planning and management support needs of regional forest managers in Austria, Bosnia and Herzegovina, the Czech Republic, Croatia, Germany, Slovenia and Serbia. Figure 1 shows the respective target area of REFORMAN, which was structured in three sub-areas according to their natural and socio-economic frame conditions and as background for the analysis of needs and preferences in supporting forest management.

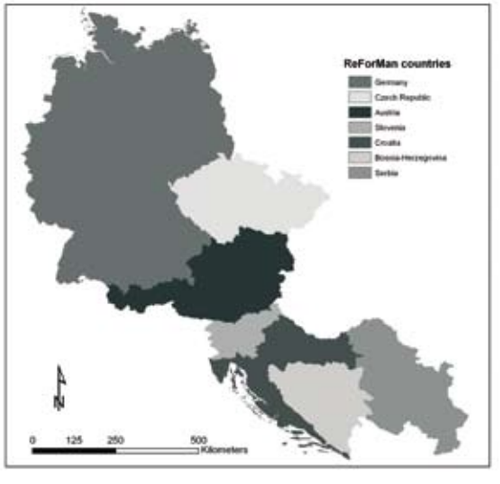

Figure 1: Target area of REFORMAN: Central European countries (Germany, Czech Republic), Alpine countries (Austria, Slovenia) and Submediterranean countries (Bosnia and Herzegovina, Croatia, Serbia).

The natural frame condition analysis was done on the basis of existing and freely available digital information on national and EU level (digital maps) and available reports on national / EU level. The analysis of the socio-economic frame conditions was based on similarly structured national contributions from the partner countries Germany for the region Central Europe, Austria and Slovenia for the Alpine region and Croatia and Serbia for the Submediterranean part of the REFORMAN countries. Possible conflict areas with and areas of interest for supporting forest management were analysed in a discursive

process in some regional workshops. The workshop participants came from national forest administrations or state forest enterprises and some from administrations and other organisations dealing with forestry and environmental management.

3 Results and Discussion

3.1 Results

Forest growth in the Alpine partner countries, Austria and Slovenia, is driven by the altitude and the resulting regional climate conditions. These characterised by high mean annual precipitation and low annual temperatures. Typical soils are shallow (leptic) and in steep terrain, only in lowlands, intra-mountainous basins and valleys soils with greater thickness occur. The climatic conditions of the Central European countries, the Czech Republic and Germany, are in contrast moderate, although the eastern parts of Germany and the Czech Republic have low mean annual precipitation. Temperatures depend very much on altitude. The edaphic conditions are in general moderate. The Submediterranean partner countries, Bosnia and Herzegovina, Croatia and Serbia, are characterised by a high contrast in the climatic conditions. While the costal areas underlay distinct maritime influence, the inland areas have pontic characteristics (high temperatures and low precipitation). The soil properties are similarly unevenly distributed. The coastal regions have leptic and calcic soils, whereas the eastern parts have moderate to steppe-like soils.

The land-use pattern of the REFORMAN area is characterised by a heterogeneous mosaic between and within the regions as it is typical for Europe. The forest cover supports a subdivision into countries with a high percentage of forest (> 40-60 %), such as Austria and Slovenia as Alpine countries and Bosnia and Herzegovina as Submediterranean country, and a lower share of forest (<35 %), such as the Central European countries (the Czech Republic and Germany) and the Submediterranean countries (Croatia and Serbia). While the Central European countries, Croatia and Serbia have forest covers below average, for the other countries much higher forest covers are to notice. The average forest cover of the study area is 34 %.

The quota of forests dominated by conifers and deciduous forests is differing according to the above described frame conditions. In the northern part of the REFORMAN area, the share of conifers is above 50 %. In some of the South-eastern European countries the share of coniferous trees is around 50 %, while in Serbia and Croatia the share of broadleaves is > 80 %. The dominating conifers are Norway spruce (Picea abies) and Scots pine (Pinus sylvestris). A smaller share has the silver fir (Abies alba). Within the group of broadleaves various oak species and common beech (Fagus sylvatica) are of highest importance. To notice is the frequent occurrence of hornbeam and of other unspecified trees (shares <1 %).
The most important abiotic damage in the partner countries is wind throw. In Austria, a foehn storm caused a loss of 5.6 million m^3 wood in 2002. In the Czech Republic, wind-related savage felling was dominant for 2002–2004 (2005: 66 % of all felling, 1.5 to 6.1 million m^3). In the Czech Republic and Germany, a huge amount of wood and savage felling is expected as consequence of the Kyrill storm in 2007. The areas of high to very high risk are located primarily in countries with a high share of conifers and shallow soils, such as the Alpine countries Austria and Slovenia, and the Central European country (the Czech Republic). Here, the percentage of areas with high to very high risk ranges between 5-10 % of the total area and between 20-37 % of all coniferous areas. For the other countries, the percentage of high-risk areas is low (< 2 %), even if the percentage of coniferous areas is between 10-19 %. Although, in these countries high-risk areas do exist on a regional level, such as in the mountainous and alpine parts of Germany.

The second most important abiotic damage risk is drought. The effect of the 2003 drought showed that water shortage is not just a change in primary productivity, but may also compromise tree health and survive. Based on the climatic period 1961-1990, large parts of eastern Germany, northern Serbia and smaller parts of Austria and the Czech Republic have negative water balances. In general, water balances with minimal or no water deficit occur in the REFORMAN area (~34 % area with forests). A distinct water surplus is to be noticed for countries with higher altitudes, such as in the Alpine countries Austria and Slovenia, while a distinct water shortage is to notice for the Submediterranean countries, especially along the coastline. The region with the highest risk of drought is the Vojvodina in Serbia.

Fire poses a major threat in the dry region of the Submediterranean region of Croatia. In Croatia, 300 major fires occurred during the last 15 years, which burned 15,000 ha thereof 4,600 ha forest, causing a damage of 2.7 Million Euros. For REFORMAN, the fire risk was estimated from the average climatic water balance. Areas with a climatic water deficit of over 250 mm were defined as high fire risk areas. Hot spots from 2008 and burnt areas (> 50 ha) for the period 2004-2007 mapped by the European Forest Fire System were included in the analysis. For the Alpine countries Austria and Slovenia, the risk areas are small. Most of the other countries have a share of fire risk areas below 10 %. Croatia and Serbia have higher shares of 19 % and 75 % with a high fire risk respectively, because of their much more continental climatic conditions. Most of the hot spots and burnt areas – 35 out of 45 – are within areas of high fire risk. Assuming a higher potential evapotranspiration due to climate change, the resulting higher climatic fire risks were calculated. The climatic fire risk for the total area increases by three times. Only the countries with high altitudes still have a low fire risk. For all the other countries, a dramatic increase of fire risk areas is to be noticed.

In some parts, air pollution is still a stressor for forests. For the Czech Republic, ozone is reported as a major pollutant, while SO2 and NOx are not considered a problem anymore. Air concentrations of ground level ozone is generally high throughout the REFORMAN area. The concentrations range between 24 and 45 ppb. Sub-regions with higher concentrations (>30 kg ha-1 a-1) are southern Croatia, southern Serbia, southern Germany, and south-western Austria. The development of nitrogen immissions causes a general concern of long-term damage by eutrophication. A reduction of nitrogen emission has so far not taken place. For oxidized nitrogen, the focus of deposition (>7 kg ha-1 a-1) is on urban-industrial regions such as in western Germany, southern Slovenia, south-western Croatia, the Vienna region, and northern Bohemia. For the investigation area, the deposition of re-

duced nitrogen is >10 kg ha-1 a-1 for north-western and southern Germany and southern Slovenia as well as south-western Croatia. For most of the remaining areas, the nitrogen deposition is still high, with the exception of the most south-eastern regions (<5 kg ha-1 a-1, fig. 17).

The most important biotic threat is the infestation by different bark beetle species. In Croatia, especially the mountainous fir and partially also beech forests are threatened by bark beetles. In the Czech Republic, savage fellings due to bark beetle damage amounted up to 40,000 to 80,000 m^3/ year, and e.g. 846,000 m^3 must have been harvested in 2005. In Austria, the highest ever recorded loss through bark beetle damage was in 2003. For Bosnia and Herzegovina, bark beetle risk is especially present in former war zones and in combination with drought and fire. In Germany and the Czech Republic, bark beetle damage was prevented by the wet summer of 2007.

In the alpine country Austria, the proportion made up of public forest is 18 % of the total forest area. 80 % of the Austrian forests are in private ownership. Austria is characterised by mainly private forest owners who own about four fifths of the Austrian forest. The forestry sector's contribution to gross domestic product growth was about 2.1 % in 2003 according to the most recent calculations. Therefore, 0.4 % was accounted for by forest management, 0.9 % by wood processing and 0.8 % by paper and cardboard production and processing. In absolute terms, the gross value added by the forestry sector at cost price amounted to € 4.8 billions (forestry: € 1.0 billion, wood processing: € 2.0 billions, paper and cardboard production: € 1.8 billions). The total value of wood exports amounts up to 2.4 billion € in 2004. Taking into account the export value of all forestry and wood products, including paper and cardboard (2004: 7.7 billions), the forest and wood-based sector is the second largest source of foreign currency after the tourism sector, with an export surplus of approximately 3.3 billions Euro [8].

In Slovenia, the ownership comprises private forests owned by individuals (71%), private forests owned by legal persons (1%), national forests (26%) and municipal forests (2%). It is expected that only about 20% of all forests will remain state-owned after denationalisation, which puts Slovenia among the European countries with the lowest share of national forests. The Slovenian wood and furniture industry, in terms of its number of employees, is one of the largest industry branches. In comparison to other industries, Slovenian wood industry has a 6.2% share by gross value added, while its share in the EU reaches only 3.1%. This share is somewhat lower in comparison with neighbour Austria. Its productivity is 2.2 times lower than the productivity in the EU in the primary segment of the industry, and 2.0 times lower in the furniture segment, , but it records a considerably higher average growth. In the primary segment of the industry, the ratio between the growth of productivity in Slovenia and the growth of productivity in the EU is 5.0%:1.8%, and in the furniture segment 4.4%:1.0 %.

In Germany as a Central European country, forestry is the second most important land-use type after agriculture. Private persons, corporate entities and the Federal states own woodlands. According to current surveys, the forest and timber industry, including processing and paper as well as printing and publishing, offers nearly 1 million jobs with an annual turnover of over 120 billion €. The turnover of the forest industry amounts to 2.3 billion €. While Germany is generally a country poor in raw materials, it disposes of the largest total growing stock of timber in Europe at ca. 3.4 billion m^3. In view of the short supply of fossil resources from crisis-ridden regions of the world, forest utilisation in Germany makes an important contribution to securing the future of the country.

Forests in Croatia as a Submediterranean country are divided into state owned forests, managed by Croatian forests Ltd. Co. (75%), state owned managed by other economic subjects in the country (3%) and privately owned (22%). In the year 2006, the contribution of the forest industry sector to the GDP was 1.0%, with the furniture industry, pulp and paper and wood and wood products. It has been estimated that the contribution of forests to the GDP is additional 0.5% [9]. The forestry sector is a significant source of employment, especially in rural areas. In total, there are approximately 49,000 employees, out of which 9.200 people work in a company for forest management, 6000 employees work as entrepreneurs for forestry works, 4000 people are seasonal forestry workers, 11.500 people work in the primary wood processing industry, 11.600 work in the furniture industry and 6.250 work in the pulpwood and paper industry. Exports account for 315,000 m^3 out of the total production of 580,000 m^3 of sawn wood. Total imports of sawn wood amounted to 180,000 m^3, of which 150,000 m^3 is coniferous wood. Forests and forest industry products, including wooden furniture, accounted for 7% of the total exports in 2001, while imports represented 3.8%. The total forest area of Serbia accounts for about 23.9 % of the total land area. Forests with state ownership, administration and management amount up to 39.8%, while 52.2 % are privately owned. The majority of the private forests is very small in size. The actual total recorded wood production in the year 2002 amounted to 2.45 million m^3, of which just over 1 million m^3 was industrial round wood and the remaining 1.45 million m^3 was fuel wood. If another estimated 1.5 million m^3 of unrecorded and illegal production is included, the total production could reach to about 3.95 million m^3, which is still more than 1 million m^3 short of the current annual increment. The wood processing industry in Serbia is oriented towards saw milling, and the manufacturing of plywood, veneer, furniture elements, furniture, wood pallet and parquet. According to the Republic Statistics Office, the direct economic contribution of forestry, in 2001, was a modest 0.32 % of the GDP, and with the inclusion of forest industry the contribution went up to 2.59 %.

The identified user needs to support forest management in the future were clustered to a number of major application fields for present and future and local to regional as well as national to EU-wide. On the time scale, at present, forest management planning is the most important application field. Traditional instruments, such as forest management plans or written documents are in use and considered as sufficient for the actual situation. Other application fields, such as land-use planning, integrated/interdisciplinary planning, etc. experience subsequently a decreasing importance. Comparing present and future application areas, a development trend from preferred support in sectoral planning expressed by the high importance of forest management planning at present towards an integrated and participatory planning emerges. The participants gave the highest impor-

tance in future to the support of integrated, i.e. interdisciplinary planning. The application field forest management planning is believed to loose of importance because of increasing complexity of planning in interaction with multiple actors. When switching to the spatial scale and asking for application areas at local/regional level, integrated/interdisciplinary planning was ranked very close with land-use planning and management, participatory planning and support of the implementation of laws and new regulations/directives. Forest management planning, education and training were considered to be of minor importance. On the national and EU wide level, support of the implementation of laws and new regulations/directives even gained major importance. Other application areas, such as support on operational and strategic level/policy support etc. were ranked lower. The extreme importance of the support to the implementation of laws and new regulations / directives on national and EU level refers mainly to planning questions related to nature protection with a high uncertainty of their impact on management planning. Compared to the sector "future application areas", the sector "application areas at national/EU wide level" is even further away from the findings than the sector "application areas at local/regional level". The reason given by the participants is that uncertainty in this case comes from two directions, from the difficulty to look into the future in general and from the problem to foresee the necessities on a larger scale level in particular.

A consensus on some of the most important attributes of an optimal management support tool was achieved, which includes: broad accessibility for users at any time and any place and the possibility to integrate iteratively experience from case studies, regional experts and scientific results in terms of a learning-system. A self-explanatory user interface is expected.

Considering the application of management support tools themselves, the participants showed a preference to use them in the planning phase and in the preparatory phase. Regarding the technological preferences, free software download from internet or free online services were the clear favourites compared to products and services liable to pay costs or and were also preferred against written documents.

The analysis of the willingness to pay for management support tools revealed that such willingness nearly does not exist. Around 90 % of the participants pointed out that they would prefer not to pay for a management tool. If participants are forced to pay for software licenses or services, they prefer to pay maximally once. The willingness to pay a lump sum for software was higher than for online services: participants were willing to pay up to 1.000 Euros for software and up to 100 Euros for an online service. The maximally acceptable sum for a written document amounted to 50 Euros.

3.2 Discussion

The presented study was done to identify differences and common aspects between of the participating partner countries considering natural, socio-economic frame conditions and their impact on research and development needs in management support tools and possible future research fields. Especially the natural frame conditions supported a clustering of the participating countries into three main areas, the Alpine, the Central and the

Submediterranean part of the study region. Though differences in the frame conditions could be identified, the demands for supporting forest management in a world of change are very comparable. REFORMAN tried to describe such demands as a basis for future common research projects, which are thought to provide operational concepts and tools for better supporting such an economically important business area as forestry. The ownership shares and the legal status of the nationally responsible authorities for managing and administrating the forests still vary, but there is a clear tendency that multifunctionality of forests with a little dominance of the production function is a broadly accepted management goal in all the participating countries. This finding together with the fact the an increasing number of groups impact the discussion on the role and the target-setting of forests on national and EU-level, make it understandable that the participants of the study identified an increasing need to support forest management with tools supporting the handling of complex decision situations. Here e.g., the importance of management support tools for the implementation of laws and new regulations/directives on national and EU level was highlightened. The participants showed a preference for computer-based tools, which support interaction and communication in planning processes probably better than paper-based tools, such as the usual forest management plans. The free access to support instruments was a consistently repeated at the workshops. As well, the preference of cost-free solutions and the unwillingness to pay for support unify the participants.

4 Outlook

The project carried out within the frame of the SEE-ERA.NET Pilot Joint Call provided a unique possibility to compare the situation between countries along a very broad climatic and also socio-economic gradient from "old" EU countries to new members and up to those, which will join the EU in the next future. The focus on the Balkan region opened the window to one of the most dynamic hot spots for economic and political development on the socio-economic side and climate change impact on the natural frame condition side. However, the frame conditions for common projects have been changed. The follow-up programme had already been launched before the intended input from this pre-study could be provided. Furthermore, Germany does not participate in the follow-up phases. As a consequence, future projects will focus on the partners in South-Eastern Europe and Austria and the presented results can be involved as a basis.

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Summer School: Built Environment

Institution / Coordinator country

Centre for Research and Design in Human Comfort, Energy and Environment (CERDECEN), Technical University of Sofia, Bulgaria
Director of the Summer School: Prof. Peter Stankov, PhD, DSc

Related website

http://www.cfdc.tu-sofia.bg/seminars/era_net/Anons.htm; http://www.cfdc.tu-sofia.bg

Field

Environment

Place and number of participants

Pamporovo, Bulgaria
June 7 – 12, 2008
33 participants

Short summary

The Summer School on Built Environment was based on the existing long-term research and training collaboration between the partners in the project:
CERDECEN, Technical University of Sofia, Bulgaria;
LSTM, Friedrich-Alexander University, Erlangen-Nuernberg, Germany;
St. Cyril and Methodius University, Skopje, Former Yugoslav Republic of Macedonia; and University of Niš, Serbia

The main outcomes of the Summer School could be summarised as follows:

- 26 young researchers from the FYR of Macedonia, Serbia, Montenegro and Bulgaria attended the Summer School.
- Seven lecturers from the FYR of Macedonia, Serbia, Germany and Bulgaria gave lectures and participated in the workshops.
- 30 lectures and two workshops were attended by the participants.
- Three cultural events – country presentations of the FYR of Macedonia, Serbia, and Bulgaria were organised to facilitate the cross-cultural communication between the participants.
- A book of Proceedings of the Summer School was published and spread among all participants before the event.
- An e-version of the Book of Proceedings was made available.

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Summer School: Preparation of Climate Atlas

Institution / Coordinator country
Hungarian Meteorological Service/Hungary

Related website
http://www.met.hu/pages/seminars/seeera/index.htm

Field
Environment

Place and number of participants
Sitke, Castle-Inn (see homepage for more details)
September 10 – 14, 2007
In total 12 participants from seven SEE states, among others four West Balkan Countries with six participants (50 %).

Short summary
The main aim of the project was to organise a summer school where the theoretical elements of preparation of climate atlases are surveyed. The result is a set-up of a common knowledge platform on the individual steps of preparation of a climate atlas. This platform is a good starting point for common developments in any field of preparation of the climate atlas both theoretically and/or practically. Theoretical co-operation could mean methodological development taking into account specific national features, practical could mean developments in the field of observations. Possible collaborations can be on a bilateral basis or for a preparation of a transnational climate atlas according to the decision of the participants by preparation of common project proposals.

Lecturers were invited from University Rovira i Virgili de Tarragona (Spain), Central Institute of Meteorology and Geodynamics (Austria), World Meteorological Organisation, German Weather Service (DWD), Hungarian Meteorological Service (OMSZ), Environmental Agency of Republic of Slovenia (EARS), EUMETNET/ECSN and met.no. The programme and presentations are available on the homepage http://www.met.hu/pages/seminars/seeera/index.htm.

Outcomes:
- The lecturers opened a wider view for the region (Manager of the European Climate Support Network, project coordinator of the Drought Management Centre of South East Europe – DMCSEE, the World Meteorological Organisation – WMO, etc.);
- The participants received an overview on the whole data quality process from the metadata issue to the interpolation and applications;
- Free software for homogenisation and interpolation was presented to the summer school's participants (downloadable from the summer school's website);
- Practical applications of interpolation were suggested like drought events (presentation of the coordinator of DMCSEE);

- The participants were trained on the use of homogenisation and interpolation software;
- The participants and lecturers built up narrow co-operations for the future (follow up actions, among others project proposals for SEE Transnational Programme call, project proposal to the European Parliament, etc.); and
- The participants' presentations gave good basis for the further collaboration.

EUMETNET, Central Institute of Meteorology and Geodynamics (Austria) and the Hungarian Meteorological Service gave additional support for the event.

Summer School: Technology Enhanced Learning (TEL) & Knowledge Management 2008

Institution / Coordinator country

Coordinator of SEE-ERA.NET funding and General Co-Chair: Katherine Maillet, Associate Professor, Institut National des Télécommunications, France
Local Organisation Chair: Klime Poposki, Ph.D, Faculty of Tourism and Hospitality, Ohrid, FYR of Macedonia

Related website

http://www.prolearn-academy.org/Events/summer-school-2008

Field

Information and Communication Technologies (ICT)

Place and number of participants

Ohrid, Former Yugoslav Republic of Macedonia
June 15 – 21, 2008
70 participants

Short summary

The Summer School on Technology Enhanced Learning (TEL) & Knowledge Management 2008 was the fourth in a series of summer schools initiated by the EU funded PROLEARN Network of Excellence in TEL. Our ambition was to foster cross-domain training and collaboration opportunities among researchers in Europe and beyond, working in the disparate fields of expertise which promote the advancement of TEL, more especially at the workplace. The programme included lectures and working sessions by leading professors, tutoring, mentoring and joint research opportunities. Additionally, the school offers practical sessions in TEL research methodology. Advanced PhD students were identified to present their research at the annual European Conference on Technology Enhanced Learning.

In 2008, the summer school benefited from cutting-edge scientific expertise being explored in several EU funded projects:

- PALLETTE, which aims to facilitate and augment individual and organisational learning in Communities of Practice;
- MATURE, which is studying knowledge maturing processes in order to build tools and services for companies and employees to mutually learn and develop their competencies;
- APOSDLE, which is developing a software platform and tools to support learning@work on demand;
- GRAPPLE, which is developing a generic, responsive, adaptive, and personalized learning environment;

- ICAMP, which created an infrastructure for collaboration and networking across systems, countries, and disciplines in HEIs, based on constructivist learning theories, self-organised learning, social networking, and the changing roles of educators.

The scientific programme was complemented with summer sports activities and cultural visits in and around Ohrid, including the St. Naum Monastery and Vevcani Mountain village. ICT was implemented for organising pre-summer school videoconferencing sessions, recording lectures and supporting documents, and creating virtual dialogue among students and lecturers throughout the week. Examples of these can be seen at the summer school website.

The Summer School on Technology Enhanced Learning & Knowledge Management 2008 extended constantly expanding networking opportunities, fostered during previous summer schools for academics working across Europe in the disparate fields of TEL. As a measure of success for the event in 2008, twelve R&D projects have already committed to support the 2009 Joint European Summer School on Technology Enhanced Learning (http://JTELSummerSchool.eu).

Summer School: Toxic Cyanobacteria in Drinking Water Sources – Problem and Sanitation

Institution / Coordinator country
University of Pannonia/Hungary

Related website
http://www.limnologia.hu/cyanotraining/

Field
Environment

Place and number of participants
Veszprém, Hungary
July 7 – 12, 2008
10 participants

Short summary
Man-made eutrophication of freshwater is one of the most relevant causes of water quality deterioration. Eutrophication often results in proliferation of cyanobacteria, many species of them are producing toxins. Human health hazards from toxic cyanobacteria in freshwaters have been suspected since the 1930s and are still considered a topical issue. Climate change will most possibly increase the risk posed by cyanobacteria, both in freshwater and coastal ecosystems. The main objective of the project was to provide up-to-date information necessary for the management of this problem, for selected participants from West Balkan Countries and Bulgaria.

Completed topics and lecturers:
- "Eutrophication, cyanobacteria, lab practice: identification" (Prof Judit Padisák, University of Pannonia). Lab practice "Identification of cyanobacteria".
- "Toxins, lab practice: toxicology" (Assoc. Prof. Nora Kováts, University of Pannonia). Lab practice "Determination of cyanobacterial toxicity".
- "Toxins, lab practice: analytical measurements" (Assoc. Prof. Rita Földényi, University of Pannonia). Lab practice "HPLC analysis of cyanobacterial toxins".
- "Technologies, tap water treatment" (Ms Ramóna Lugosi, Regional Waterworks of Transdanubia).

A course excursion was organised to study the reconstructed wetland "Kis-Balaton" that serves as a protecting reservoir for Europe's largest shallow lake, Balaton. A 150 page book was written and made available for participants (Padisák, J. & N. Kováts, eds., 2008, Toxic cyanobacteria in drinking water sources: Problem and sanitation: 1 – 13, University of Pannonia University Press, Veszprém, ISBN 978 963 9696 49 5).

Understanding Ionic Liquids as Novel Solvent in Green Chemistry

Snežana Miljanić[1], Tomislav Biljan[1], Zlatko Meić[1], Vasile Chiş[2], Bernhard Lendl[3], Nicolae Leopold[3]

[1] Laboratory of Analytical Chemistry, Department of Chemistry, Faculty of Science, University of Zagreb, Horvatovac 102a, 10000 Zagreb, Croatia; miljanic@chem.pmf.hr
[2] Faculty of Physics, Babeş-Bolyai University, Kogălniceanu 1, 400084, Cluj-Napoca, Romania; vchis@phys.ubbcluj.ro
[3] Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164, 1060 Vienna, Austria; bernhard.lendl@tuwien.ac.at

Abstract

- Ionic liquids as solvents in green chemistry
  - Ionic liquid-water mixtures: Effect of water on physical and chemical properties of ILs
- Study of the mixtures containing ionic liquids, 1-ethyl-3-methylimidazolium tetrafluoroborate, 1-buthyl-3-methylimidazolium tetrafluoroborate, 1-buthyl-3-methylimidazolium hexafluorophosphate, and water
  - FT-Raman spectroscopy of ionic liquids during on line dilution with water
  - Chemometric analysis of the Raman spectra of ionic liquid-water mixtures
    - Two-Dimensional Correlation Spectroscopy
    - Multivariate Curve Resolution
  - Quantum chemical calculations of ionic liquid-water electronic structures
    - Density Functional Theory calculations.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest, and aquatic environments
Type of the project: research project

Keywords

Chemometric analysis; Infrared spectroscopy; Ionic liquids; Quantum chemistry; Raman spectroscopy; Water

1 Introduction

To prevent or reduce pollution at its source, approaches encouraging the design of products and processes that reduce or eliminate the use of hazardous substances have been

established. Alternative solvent concepts in particular are of interest as the organic solvents used to date have come under some considerable criticism due to their health risks and threats to the environment. Based on their non-volatility and non-flammability as well as on recycling potential, ionic liquids (ILs) have been recognized as solvents for "green chemistry" [1].

Ionic liquids are salts composed of organic cations and inorganic or organic anions that are liquid near room temperature. They have many properties of conventional organic solvents, such as excellent solvation ability and good thermal stability, and due to their ionic composition show good electrical conductivity. ILs have been described as designer solvents, since their properties can be adjusted to requirements of a particular process. By simple changes in the structure of the building ions, properties such as melting temperature, viscosity or hydrophobicity can be varied. For all of these reasons, in the last few years, ILs have attracted much attention as media for chemical reactions [2].
Several studies indicate that the addition of water can strongly affect the physical and chemical properties of ILs, such as viscosity, electrical conductivity, reactivity and solvation properties. Hence attention has been paid to the study of water dissolved in room-temperature ILs. It has been shown that the water content in various ionic liquids may be strongly influenced by the nature of the cation and the anion [3-8]. Molecular dynamics simulations [9] and spectroscopic studies in the near [7] and middle [3] infrared regions stated that in alkylimidazolium ionic liquids water molecules tend to be isolated from each other due to the strong interaction between the water molecules and the anions of the ILs. Moreover, self-association of water forming dimers was found to occur in the studied hydrophilic ionic liquids but not in the hydrophobic one [10].

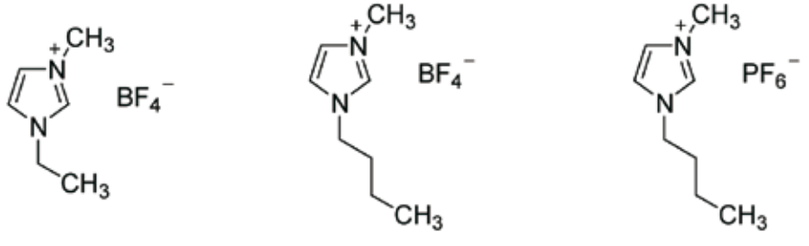

Figure 1: Molecular structure of 1-ethyl-3-methylimidazolium tetrafluoroborate ($emimBF_4$), 1-buthyl-3-methylimidazolium tetrafluoroborate ($bmimBF_4$) and 1-buthyl-3-methylimidazolium hexafluorophosphate ($bmimPF_6$)

The goal of this work was to study water dissolved in several 1-alkyl-3-methylimidazolium-based ionic liquids using Raman spectroscopy (Figure 1). Presence of different associations of molecules in ionic liquid-water mixtures was proposed by means of two-dimensional correlation spectroscopy (2DCoS). Multivariate curve resolution (MCR) was used to recover pure spectra and concentration profiles of the different species. Quantum chemistry calculations were applied to study the interactions at the molecular level between the water molecule and the ionic liquids. A reliable assignment of vibrational bands in spectra was performed using density functional theory (DFT) methods.

2 Experimental Techniques and Methods

2.1 FT-Raman Measurements

Ionic liquids, 1-ethyl-3-methylimidazolium tetrafluoroborate ($emimBF_4$), 1-buthyl-3-methylimidazolium tetrafluoroborate ($bmimBF_4$) and 1-buthyl-3-methylimidazolium hexafluorophosphate ($bmimPF_6$) were purchased from Solvent Innovation. FT-Raman spectra were taken on a Bruker Equinox 55 interferometer equipped with FRA 106/S Raman module using Nd-YAG laser excitation at 1064 nm of a 500 mW laser power. Spectra were measured in the spectral range between 4000 cm^{-1} and 100 cm^{-1} at a spectral resolution of 4 cm^{-1}. For on line dilution measurements a flow cell with a quartz window and a Gilson Evolution Minipuls peristaltic pump of a 50 µl min^{-1} flow rate were used. Raman spectra were measured every 50 seconds for a total period of 5000 seconds.

2.2 Chemometric Analysis

2DCoS analyses, including slice spectra and moving window plots, were performed using the 2D Shige software [11].
MCR analysis was performed in MATLAB, using the MCR-ALS graphical interface developed by Romà Tauler and Anna de Juan [12]. The number of components involved in a particular experiment was elucidated using singular value decomposition (SVD) or principle component analysis (PCA), both of which were performed in MATLAB, along with the MCR analysis.

2.3 Computational Methods

The molecular geometry optimisations and vibrational frequencies calculations were performed with the Gaussian 03W software package [13] by using DFT approaches. B3LYP hybrid exchange-correlation functional was generally used due to its ability for reproducing a large variety of molecular properties. The split-valence basis sets 6-31G(d) and 6-311+G(d,p) of the Pople's group were used for the expansion of molecular orbitals.
The geometries were fully optimised without any constraint with the help of analytical gradient procedure implemented within Gaussian 03W programme. The calculated stabilisation energies were corrected for basis set superposition error by using the Counterpoise method proposed by Boys and Bernardi [14].

3 Results and Discussion

The Raman spectra of three ionic liquids $emimBF_4$, $bmimBF_4$ and $bmimPF_6$ are shown in Figure 2.a. Comparison of these spectra assisted in assigning Raman bands to each of the ionic components: $bmim^+$, $emim^+$, BF_4^- and PF_6^-.

To study the IL-water mixtures the pure ILs were diluted with water during the Raman spectra measurement. The band intensity in the spectra of $emimBF_4$ and $bmimBF_4$ was high at the beginning followed by the significant intensity decrease and, finally, negligible change in intensity (Figure 2.b). According to the water content calculated relative to the calibration curves, the intensity started to diminish for mixtures containing 15 % water with both ILs. Unlike hydrophilic ILs, the intensity of the Raman bands of the hydrophobic

bmimPF$_6$ remained constant during the experiment indicating that water content in the samples was to low to produce a significant change in the Raman signal.

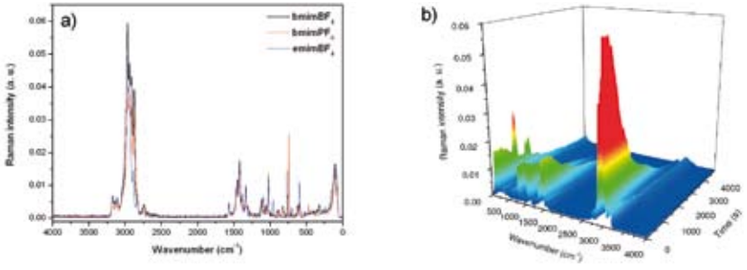

Figure 2: Raman spectra of emimBF$_4$, bmimBF$_4$, bmimPF$_6$ (a) and of bmimBF$_4$ during on line dilution with water (b)

2DCoS analysis was used to assess the rate of change in intensity of a Raman band, relative to others in a spectrum. For the spectra analysed here, the Raman spectrum has been split into low and high wavenumber regions, and the 2D correlation analysis performed on each section individually. In a normal 2D synchronous plot, the diagonal highlighted all the bands in the spectrum that were increasing or decreasing in intensity. For simplifying the visualisation of the order of intensity changes, moving window plots were used as well. This plot displayed the wavenumber of the Raman bands that were changing in intensity, and associated this intensity change with the perturbation applied, in this case the water content.

The 2D correlation and moving window analysis suggested that there were two stages to the intensity changes observed for bmimBF$_4$ (Figure 3). The first stage was indicated by the bands recorded at ~764, 1018, 1334, 1387, 1417 and 1488 cm^{-1} that were all changing in intensity during the time corresponding to water contents of 0–55 %. The second stage, corresponding to a water content of 60 to 65 %, was much less intense and involved less Raman bands, mainly those at ~1018 and 1417 cm^{1}. The bands at high wavenumber reflected one stage of change that corresponded to a water content of 0 to 55 %. The main period of intensity change, shown by the close contours in the moving window plot, was around 750 seconds and corresponded to a water content of approximately 15 %.

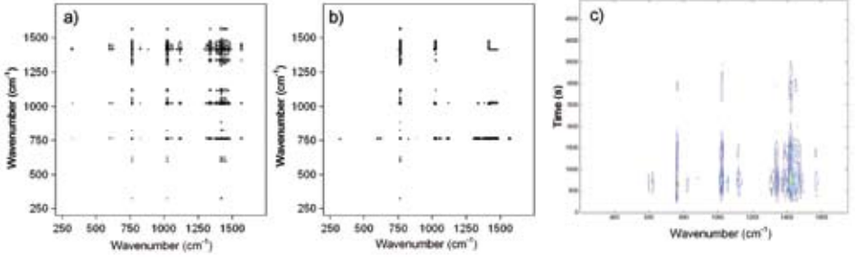

Figure 3: The synchronous plot (a), the asynchronous plot (b) and the moving window plot (c) for the low wave number region of the bmimBF$_4$ Raman spectra

Unlike for bmimBF$_4$, two stages of intensity change for emimBF$_4$ were not obtained. The most intense changes were associated with bands at ~597, 765, 1022, 1337 and 1422 cm^{-1} and the bands observed at ~ 958, 1092, 1387, 1455 and 1568 cm^{-1} corresponding to a water content of 0 to 70 % and 0 to 40 %, respectively. The majority of the changes at higher wavenumber were confined between 200 and 1500 seconds implying water content of between 0 and 65 %.

The singular value decomposition (SVD) and principle component analysis (PCA) indicated that there were four components that can be used to describe the bmimBF$_4$ experiments (Figure 4). The first component identified by the MCR analysis has a close spectral profile to that of the spectrum recorded at 0 seconds. The spectral profile of the second component is similar to that of the raw spectrum but with a an additional band at ~2744 cm^{-1} and greater relative contribution of the Raman bands at ~343, 652 and 1086 cm^{-1} to the overall spectrum. Component three differ from the raw spectrum in a greater background contribution, especially below 650 cm^{-1} and in the higher wavenumber region. The spectral profile of the fourth component contains the majority of the bands observed for the other components and the raw data, but also a much higher background signal and two additional, broad bands around ~1640 and 3400 cm^{-1}. These bands are usually associated with O–H bending and stretching and are likely to reflect contributions to the spectrum from water.

The three experimental components identified by MCR for the emimBF$_4$ experiments were very similar to components 1, 3 and 4 identified for the bmimBF$_4$ experiments.

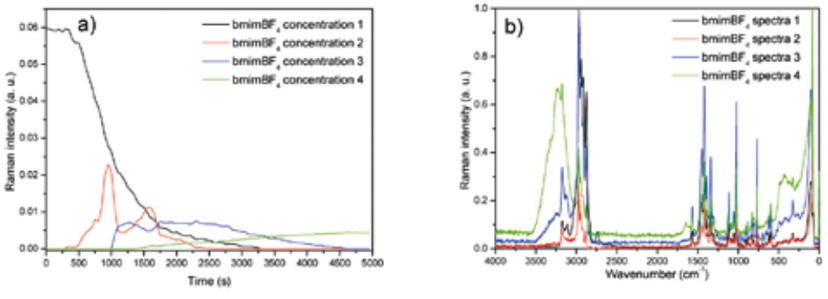

Figure 4: Concentration profiles (a) and spectral profiles (b) for the four MCR components identified for the bmimBF$_4$ experiments

Using quantum chemical calculations different models were investigated with emphasis on the interaction between water molecules and BF_4^- and PF_6^- ions. Calculated interaction energies in anion-water, cation-water, water-water and cation-anion systems at B3LYP/6-31G(d) level of theory are given in Table 1. The energies were corrected for basis set superposition error by using the Counterpoise method proposed by Boys and Bernardi [14].

As seen in the table, the stabilisation energy is higher for water-BF_4^- than for water-cations and lower for water-PF_6 system than for water-cations or water-BF_4^-. On the other hand,

the water–water interaction energy is significantly lower than the interaction energies corresponding to water-anions or water-cations systems. This fact suggest that the water molecules will bind directly to the anions and they will not be in a self-assembled structure as already evidenced by Cammarata et al. [15]. Moreover, due to the large calculated values for the interaction energies between anion-cation systems, the anion-cation interactions can hardly be disturbed by the water molecules adsorbed into the liquid anions.

Table 1: B3LYP/6-31G(d) calculated interaction energies between different components in an ionic liquid

System	DE (Kcal / mol)	DE corrected (Kcal / mol)	DE corrected (KJ / mol)
BF_4^--H_2O	−18.92	−12.48	−52.23
PF_6^--H_2O	−14.41	−9.48	−39.66
$bmim^+$-H_2O	−13.35	−11.50	−48.11
$emim^+$-H_2O	−13.56	−11.71	−49.00
H_2O-H_2O	−7.80	−3.94	−16.48
$emim^+$-BF_4^-	−95.81	−85.06	−355.89
$bmim^+$-BF_4^-	−95.10	−84.19	−352.27
$bmim^+$-PF_6^-	−88.37	−76.59	−320.45

IR and Raman spectra were calculated on the optimized geometries of the investigated systems and all the vibrational bands were assigned to normal modes. In some cases (water monomer, water dimer and BF_4^- and PF_6^- anions) anharmonic wave numbers were also obtained. However, no significant differences were noted between harmonic and anharmonic frequencies.
Particular attention has been paid to the librational bands and hindered translations of water molecules in the investigated models. Thus, the librational bands corresponding to rocking and wagging modes of the water molecule in BF_4^- based ionic liquids the B3LYP/6-31G(d) calculated wavenumbers are 483 and 508 cm^{-1} in BF_4^--water-BF_4^- system (Figure 5.a), 506 and 510 cm^{-1} in BF_4^--water-water-BF_4^- system with a linear arrangement of the water molecules and 484 cm^{-1} in the bmim-BF_4^--water complex. All these values are in perfect agreement with the experimental FIR values at 460 cm^{-1}.

The same vibrations appear quite higher in energy, in the 506–599 cm^{-1} range for the rhombic arrangement of the $2BF_4^-$-2 water system. It is worth mentioning that the simpler BF4--water complex is not able to reproduce the experimental findings, the calculated values for the ρ(H_2O) and τ(H_2O) vibrations being in this case 308 and 604 cm^{-1}, respectively. This is in line with the results obtained by Cammarata and co-workers [15] who concluded

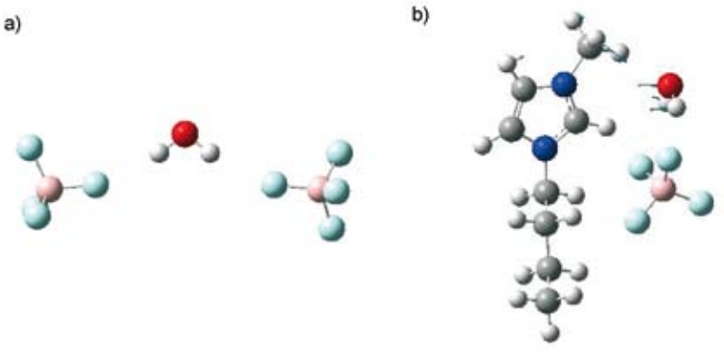

Figure 5: B3LYP/6-31G(d) optimized geometry of the $[BF_4]^-$-water-$[BF_4]^-$ complex (a) and a sketch of the hindered translation in bmim-BF_4^--water complex (b)

that at low concentration of water in ILs, the water molecules are present in symmetric 1:2 H-bonded complexes anion-water-anion. The calculated v_s and v_{as} wavenumbers of water for the complex presented in Figure 5.a (unscaled values) are 3667 and 3778 cm^{-1} in good agreement with the corresponding experimental values reported by Cammarata et al. [15]. For the $2BF_4^-$-2 water complex in linear arrangement the two calculated bands appear at lower wave numbers: 3616 cm^{-1} v_s H-bonded, 3702 cm^{-1} v_s not H-bonded and 3743 and 3745 cm^{-1} v_{as}.
As pointed out by Lendl et al. [16], for the hydrophobic ionic liquid like bmim-PF_6^- the librational band is shifted to 388 cm^{-1} this fact suggesting a less hindered rotation of the single water molecules.
According to calculations performed on PF_6^--water-PF_6^- complex, the rocking and wagging vibrations of the water molecule have the calculated wavenumbers of 374 and 397 cm^{-1}, respectively, in perfect agreement with experimental value.

The hindered translation motions give rise in the experimental spectrum of BF_4^- based IL the band at 148 cm^{-1}. The corresponding calculated values are 196 cm^{-1} for BF_4^--water complex and at 198 cm^{-1} for $2BF_4^-$-2 water linear complex. However, much better agreement between experiment and theory is obtained when the cation is also considered. Thus, for bmim-BF_4^--water complex the calculated wavenumber is 163 cm^{-1} (Figure 5.b). Also, a better agreement is obtained for the cation ($bmim^+$) – water complex, with the calculated wavenumber of 150 cm^{-1}. As expected, no significant difference is observed between $bmim^+$ and $emim^+$ cations for this motion, the calculated wavenumber in the last case being 151 cm^{-1}.

4 Summary / Conclusions

FT-Raman spectra of three ionic liquids 1-ethyl-3-methylimidazolium tetrafluoroborate ($emimBF_4$), 1-buthyl-3-methylimidazolium tetrafluoroborate ($bmimBF_4$) and 1-buthyl-3-methylimidazolium hexafluorophosphate ($bmimPF_6$) were obtained during on line dilu-

tion with water. Using chemometric analysis (2DCoS and MCR) of the Raman spectra of hydrophilic ILs-water mixtures, three and four components were detected for the emimBF_4 and the bmimBF_4, respectively, indicating presence of different ionic liquid-water species. Water content in the samples with the hydrophobic bmimPF_6 was too low to produce a change in the Raman signal which could be analysed.

Interaction energies were calculated for anion-water, cation-water, water-water and cation-anion systems. Calculated stabilization energy for the water-water interaction is significantly lower than the interaction energies corresponding to water-anions or water-cations systems indicating that the water molecules will bind directly to the anions. Among the investigated cation-anion systems, bmim-PF_6 was less stable than emim-BF_4 or bmim-BF_4 system and the interaction energy in cation-anion systems was almost one order of magnitude greater than for ion-water systems. For the calculation of the water librational bands and theoretical hindered translations of water molecules, different models were used: anion-water, anion-2 water, anion-water-anion and anion-water-water-anion systems, the last two being considered in a linear or a rhombic arrangement. The linear arrangement was found to be more stable than the rhombic one. The best agreement with experiment is done by the cation-anion-water system for which the hindered translation of the water molecule was calculated at 163 cm^{-1}. Theoretical IR and Raman spectra calculated at B3LYP/6-31G(d) level of theory were in perfect agreement with the experimental ones.

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Use of Lactic Acid Bacteria in the Production of Hypoallergenic Dairy Products and for the Generation of Mild Antimicrobials

Jean-Marc Chobert[1]

[1] Institut National de la Recherche Agronomique, UR 1268 Biopolymères Interactions Assemblages, Equipe Fonctions et Interactions des Protéines Laitières (INRA, BIA-FIPL), Rue de la Géraudière, BP 71627, 44316 Nantes Cedex 3, France

Abstract

The project supported within the SEE-ERA.NET Pilot Joint Call aimed to start the identification and characterisation of novel and original antimicrobial lactic acid bacteria (LAB) substances allowing to improve food safety standards by the inhibition of the growth of emerging pathogens and of spoilage microbial floras. These LAB were also used in the fermentation of milk proteins in order to reduce their allergenicity.

Due to the short duration of this project (9 months), the main goal in the support of such a new consortium of seven countries (Austria, Bosnia-Herzegovina, Bulgaria, France, Former Yugoslav Republic of Macedonia, Greece and Serbia) was to create the conditions necessary to give the students the opportunity to participate in research activities with LAB, to give the different teams the opportunity to share their knowledge in particular areas and mainly to prepare a more vast project in the scope of FP7.

The participants met at the general meeting in Vienna, Austria in December 2007. This meeting was dedicated to formulate a vast project including some of the objectives already depicted in this project. An FP7 project was been elaborated (TradFoodBlackSea) and proposed in February 2008. Even though the project was well evaluated, it was not funded since funding was limited to one single project.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest, and aquatic environments
Type of the project: network project

Keywords

Allergenicity; Bacteriology; Biologically Active Compounds; Environmental Microbiology; Milk proteins; Proteins; Enzyme Function

1 Introduction

New challenges for modern biotechnology and biosciences are emerging for different reasons: (i) The dimension of the phenomenon of the multi-resistance of pathogenic agents against conventional antibiotics; (ii) The emergence of new infectious agents such as Salmonella typhimurium DT104, Escherichia coli O157:H7, Enterobacter sakazakii, Listeria monocytogenes; (iii) The reinforced requirements in food supply microbiological safety, discovery of new techniques of food bio preservation, new antibiotics, new targets and the development of the food vectors of probiotic bacteria. All these elements constitute the background, as scientific and technologic dimensions cannot be dissociated from important economic and social issues.

The interest of consumers for many fermented food products such as dairy products, fermented vegetables and meats has increased considerably in the recent years, thanks to the positive perception of impact on consumer health considered generally as beneficial. Severe agricultural and economic crises in the recent years (mad cow, foot and mouth disease, avian influenza) indicate how important is the sanitary status of the consumed food products and how deep and economically devastating may become outbreaks of food borne infections and resulting consumer fears and panics.
Hence, an evident need for search of novel ways and new food preservation agents (preferably of natural origins and produced by relatively well-known and widely accepted organisms) arises. As far as this is concerned, the lactic acid bacteria (LAB) possessing GRAS status (Generally Recognised as Safe) are very good candidates.

It is also claimed that the onset of food allergies is on the significant rise, afflicting especially the infant part of the European Community's population. According to French epidemiological surveys, an increase of approximately 10% of the number of children allergic to different foods is registered every year. In some cases, very acute allergic reactions to consumed foods and to dairy proteins in particular, are leading to severe anaphylaxis.

From this perspective, it may be expected that the well-described and well-studied influence of fermentative processing of foods and in particular on the dairy food systems would contribute to the decrease of adverse allergenic reactions because of the difference in allergen presentation or simply because of the pre-prandial proteolysis happening in fermented dairy systems in this particular case. As in the case of antimicrobial actions of LAB, it may be expected that the well-managed transformation of fermented cheeses, milks, kefirs and yoghurts using appropriate LAB strains could create multiple hypoallergenic products if not for all, then at least for some classes of allergic patients.
The consortium also expected to be able to identify novel antimicrobial agents the interest of which would exceed unique Food Safety issues but would possibly present some potential in the fight against other pathogens, not necessarily linked with Food Security but also with medical/sanitary countermeasures, as well.

This possibility has stimulated the interest of the consortium of the "Use of lactic acid bacteria in the production of hypoallergenic dairy products and for the generation of mild antimicrobials" project to address these issues driven in the first place by consumer health needs and Food Safety concerns, and in second place by general social and market demands.
The diverse origins of the consortium members can help in the very efficient solution of certain problems linked with public health and food security issues in their countries. The involvement of some of the participants in the formulation of national food and health protection legislation and policies guaranties well-targeted and efficient dissemination of the most relevant results.

The attention has been turned also to the particular composition of our consortium, embracing partners from diverse South Western Balkan countries in a common quest for the advancement of the health status of the populations of their societies protecting them against food borne pathogens. It is clear that this mobilisation is already very pertinent for the SEE-ERA.NET project, setting up productive links between close and more distant neighbours and linking them additionally to countries such as Austria, Bulgaria and France. Moreover, the engagement of all the partners in an intense, useful and successful project can contribute to create an excellent working atmosphere and to promote the understanding and communication between the university and research elites from participating countries and engaging the young generation (Master and PhD students and young researchers) into this process.

It should be highlighted that recently, the interest of consumers for many fermented food products such as dairy products, fermented vegetables and meat has increased considerably thanks to the positive perception of their impact on consumer health generally considered as beneficial. Particular public attention is paid to the capacity of certain selected strains of LAB to exert a beneficial influence on health. It is due to a variety of factors such as antimicrobials, inhibiting the growth of competing micro-organisms including pathogenic and food spoilage bacteria, and in the case of specific protease inhibitors inhibiting viruses. Sometimes, the claims of the positive impact of secondary metabolites of LAB on the stimulation of the human immune system are also formulated. The antimicrobial peptides constitute an important source of molecules of bio-medical and agro-alimentary interest.

Several authors suggest that some of these peptides and small proteins could: (i) Exert powerful albeit selective, antimicrobial and antifungal/antimycotic effects; (ii) Be co responsible for probiotic effects of probiotic strains contributing to maintenance or re-establishment of beneficial gut microflora; (iii) Constitute potential novel therapeutic agents used in human and veterinary medicine; (iv) Constitute a class of potential new food preservatives.

Some of these substances are already used in the above-mentioned domains of application. However, despite all these positive facts, exact knowledge about the biologically active substances of dairy products Bifidus milk, milk drinks fermented with certain strains of lactobacilli and bifidobacteria (such as Lactimel®) and traditional products such as cheeses, yoghurts, kefirs or koumisses is limited. Knowledge about the correlations between

structure/spectrum of activity/efficiency of antimicrobial peptides/proteins is insufficient for the understanding of the mechanism of actions and the capacity of target specifically certain "adverse" bacterial species. The lack of correlation between efficacy of these peptides and the complexity of targeted bacterial cellular walls is one of examples of the deficiencies in our understanding of these systems. The knowledge about immuno-stimulatory and allergy reducing influences of LAB fermentations and fermentation products is also very limited. Consequently, the study of these bacterial systems and of all their diversity would contribute greatly to the domain of biosciences and biotechnology, biodiversity food security, public and individual health and consumer and citizen protection. Finally, any additional end-users would also improve the sanitary and allergenic status of their products.

2 Experimental Techniques and Methods

2.1 Isolation and selection of antimicrobial-producing strains

2.1.1 Objectives

Screening antimicrobial producing strains and strain identification. The cultures of LAB composing the microflora used in the manufacture of traditional dairy products have been collected from different regions in South-East Europe (Bulgaria, Serbia).

2.1.2 Methodology

The traditional screening approach of otherwise unknown original and potentially very interesting microbial collections and of specific symbiotic biota and microbial populations has been accompanied by the most modern analytic molecular biology techniques. The possibility of overproduction and over-expression of the most interesting and promising antimicrobials would allow to launch a study about their use in real animal and human foods increasing the conservation times (shelf-life) and biosecurity of humans and animals in the participating countries as well as all over Europe and the world.

The detailed characterisation of bacterial strains in the collection is essential: knowledge of the species to which they belong, their physiological, metabolic and technological properties must be determined by all the partners following the same protocol. The polyphasic taxonomy approach combining morphological, biochemical and physiological characteristics with molecular based genomic techniques (such as DNA-DNA hybridization, RAPD-PCR, amplified ribosomal DNA restriction analysis (ARDRA), rybotyping PCR with species specific primers, 16S rRNA sequencing) has been applied in identification studies of active LAB strains.

The strains have been screened according to their inhibitory activity against pathogenic micro organisms, such as Escherichia coli, Staphylococcus aureus, Candida ssp, and Listeria ssp. and food spoiling moulds.

2.1.3 Task Input

Many labs are specialised in microbiology for purification of LAB strains from their countries: Bulgaria, Greece, Former Yugoslav Republic of Macedonia, Serbia.

For the determination of antimicrobial substances producing strains, a combination of molecular genetics techniques like pulse-field electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified ribosomal DNA restriction analysis (ARDRA), restriction fragment length polymorphism (RFLP), repetitive extragenic palindromic sequence analysis (REP) and denaturing gradient gel electrophoresis (DGGE) can be used. The capability of antimicrobial compounds synthesis by isolated LAB has been analysed by growing them under different culture conditions.

2.2 Production and purification of antimicrobials

2.2.1 Objectives

In the case of strains producing bacteriocins, they have been collected from large scale cultures (1 to 5 L) and prepared for physicochemical analysis and the first steps of purification process have been performed.

2.2.2 Methodology

Filtration and chromatography techniques (ion-exchange, gel filtration, reversed-phase, affinity) have been used. After determinating the amino acid composition, the primary structure was assayed by Edman degradation and mass spectrometry. However, because of the short duration of this project, such a task can only be be continued later.

2.2.3 Task Input

Task Isolation and selection of antimicrobial producing strains was performed with the necessary equipment, which exist in different labs in France, Serbia an Austria. The main equipment used was: HPLC, FPLC, fluorimeter, dichrograph, mass spectrometry, MS/MS et MALDI-TOF, electrophoresis (1D and 2D).

2.3 Use of antimicrobial producing LAB strains in production of hypoallergenic products

2.3.1 Objectives

During the fermentation process, milk proteins were acidified by the production of lactic acid and were hydrolysed by proteases and peptidases from bacteria. This proteolysis was followed by a reduction of the number of epitopes and consequently by a decrease in allergenicity of hydrolysed proteins.

2.3.2 Methodology

Strains selected for their hydrolytic properties were used to hydrolyse the main allergenic proteins and to reduce their allergenicity. Serums of allergic patients to dairy products were collected in France. The testing of isolated lactic acid bacteria (LAB) hydrolytic properties was performed to analyse the pattern of the obtained peptides. The LAB used in the testing were isolated from home made fermented milk products manufactured in specific ecological niches. For this purpose, the enzymatic assays have been done as follows. LAB were grown on MCA plates for 48 h at 37°C prior to cells collection. Collected fresh cells were mixed with substrate (alphaS1-, beta-, or kappa-casein fractions of milk protein) and hydrolytic properties have been tested by SDS-PAGE. Special attention has been paid to the LAB which has the capability to hydrolyse caseins without production of allergenic

peptides. Obtained peptides have been tested against serums of patients by using the adapted technique of micro arrays (available in the French laboratory).

2.3.3 Task Input

The task depended on Task Isolation and the selection of antimicrobial-producing strains. Contacts with allergology departments were necessary in order to obtain sera from patients allergenic to dairy products.

3 Results and Discussion

This project received a total amount of 20.000 Euros, which were essentially used for a meeting in one participating country (Vienna, Austria), which was near the geographic centre of the consortium countries. This meeting allowed the different participants to present their domain of research and the aspects in which they could act.

France had already been engaged in bilateral collaboration with different countries: Azerbaijan, Armenia and Russia. Consequently, these countries, as well as Georgia, have been integrated to the project consortium for the redaction of an FP7 proposal, coordinated by the French partner. The project named "Biologically active compounds from traditional fermented food products improving consumer health (TradFoodBlackSea)" was prepared in the scope of the call KBBE-2008-2-2-02: Bioactive compounds in traditional food products - SICA (Black Sea Region).

The consortium was composed of the following partners:

- Institut National de la Recherche Agronomique (INRA) France;
- Institute of Biotechnology CJSC, Yerevan State University, Armenia;
- Baku State University, Azerbaijan;
- Eliava Institute of Bacteriophage, Microbiology and Virology (Eliava IBMV), Tbilisi, Georgia;
- Institute of Nutrition, Russian Academy of Medical Sciences, Russia;
- Department of Food Science & Technology, University of Natural Resources and Applied Life Sciences, Vienna, Austria;
- Faculty of Biology, Sofia University, Bulgaria;
- Department of Microbial Genetics, Institute of Microbiology, Bulgarian Academy of Sciences, Bulgaria;
- Department of Microbiology and Infectious Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece;
- Laboratory of Food Technology, Department of Hygiene and Technology of Food of Animal Origin., School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece;
- St Kliment Ohridski-Bitola University, Former Yugoslav Republic of Macedonia;
- Laboratory of Dairy Science, Faculty of Agriculture and Food Sciences, University of Sarajevo, Bosnia-Herzegovina;
- Laboratory for Molecular Genetics of Industrial Microorganisms, Institute of Molecular Genetics and Genetic Engineering (IMGGE), Serbia;
- Institute for Genetic Engineering and Biotechnology, Bosnia-Herzegovina; and
- three end-users:
  - SIBIO-93, Bulgaria;
  - Farmbioal, Russia;
  - SANTE GMT Products, LLC, Georgia.

The goal of this project was to study and characterise LAB microflora originating from typical artisanal made traditional fermented food products from the countries of the Black Sea region. The aim was to assess precisely their beneficial nature, their safety and their effect on the consumers' health and on their potential in the development of new functional foods. While well evaluated by the European Commission, this project has not been funded, as only one project was selected for funding.

However, independently of this absence of financial help, bilateral collaborations in which France had already been engaged have been pursued and students from Bulgaria, Armenia, Azerbaijan and Serbia have had the opportunity to stay in the French lab for several months in order to purify bacteriocins from selected lactic acid bacteria (LAB), and to use these LAB for their proteolytic activity to decrease allergenicity of dairy proteins.

4 Summary

In the frame of the SEE-ERA.NET Pilot Joint Call, the established consortium of seven countries was enlarged to other countries, and was able to propose a project in the scope of FP7 call, involving eleven countries and three end-users. Unfortunately, due to the absence of financial support, only the previously established collaborative projects have been developed. This allowed students of these countries to stay at the French laboratory for a while. Several publications have been performed and are listed below. We hope that other calls in areas related to the proposed consortium will be published in the future.

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Use of SNPs and SSRs for the Genetic Diversity Assessment within Cultivated Olive Germplasm from the Western Balkan Countries

Christos Bazakos[1], Stelios Spaniolas[1], Aida Dervishi[2], Arjola Baku[2], Jernej Jakse[3], Dunja Bandelj[4], Branka Javornik[3], Panagiotis Kalaitzis[1]

[1] Department of Horticultural Genetics and Biotechnology, Mediterranean Agronomic Institute at Chania, Alsyllion Agrokipiou, PO BOX 85, 73100 Chania, Greece; panagiot@maich.gr
[2] Biological Research Institute, Department of Molecular Biology, Sami Frasheri St. 5, 1534 Tirana, Albania; a_bacu@yahoo.com
[3] University of Ljubljana, Biotechnical Faculty, Jamnikarjeva St. 101, 1000 Ljubljana, Slovenia; branka.javornik@bf.uni-lj.si
[4] University of Primorska, Science and Research Centre of Koper, 6000 Koper, Slovenia

Abstract

SNPs and SSRs were used to characterise a significant number of Western Balkan olive cultivars in order to assess the genetic diversity among them. A significant number of selected, morphologically characterised Albanian and Slovenian cultivars were included in the samples. The genetic relationships among the cultivars were established using cluster analysis while the genotyping data of 10 commercial Greek cultivars were included in the dataset analysis to improve the genetic diversity assessment. Nine cultivars out of the 30 could be discriminated with various combinations of SNPs, while 12 cultivars could be discriminated with SSRs out of the 62. Two large clusters could be clearly defined by Neighbour joining cluster analysis based on SNPs, both comprising cultivars from the three Western Balkan countries indicating movement of olive germplasm among these countries and no sign of geographic isolation. However, UPGMA cluster analysis based on SSRs revealed a tight clustering of Albanian cultivars. Conclusively, these preliminary results show a distinguished genetic variability requiring further detailed studies. This study has enriched a pre-existing olive SNP and SSR database of Greek and Slovenian cultivars to be used for cultivar discrimination and also for the identification of the varietal origin of olive oil extracted from these cultivars.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Sustainable production and management of biological resources from land, forest, and aquatic environments
Type of the project: research project

Keywords

Fingerprinting; Genetic diversity; Molecular markers; Olea europaea; Olive germplasm; SNPs; SSRs

1 Introduction

Olive tree (Olea europaea L.) is the most important crop species of the Mediterranean basin, while the beneficial effects of olive tree products (oils and table olives) on human health are well characterised. The genus of Olea includes 30 different species, one of which is the Olea europea L. euromediterranea sativa (diploid), which comprises a large number of improved asexually propagated varieties [1].

A large effort was initiated on cultivar fingerprinting using molecular markers such as RAPDs, AFLPs, SSRs and recently SNPs (Single Nucleotide Polymorphisms) several years ago, which resulted in the construction of olive genetic maps. The most widely used marker systems are RAPDs, SSRs and AFLPs, while SNPs have been widely used only recently due to certain advantages and mainly due to the lower cost of generating them. Nikoloudakis et al. [2] and Owen et al. [3] have observed a high degree of genetic diversity among the major Greek olive cultivars studied using RAPD and AFLP markers,. It was also observed that the Greek olive germplasm is genetically distinct from the Turkish germplasm, while a high degree of genetic variability was reported within the cultivated germplasm from the Eastern Mediterranean region [2]. However, there is a lack of data on the genetic diversity of olive germplasm in the Western Balkan countries supporting conservation strategies and breeding programmes.

In addition, SSR markers [4, 5, 6, 7], SCAR markers [8] and ISSR markers [9] were also extensively used to genotype a large number of varieties and assess genetic distances. SSRs have been efficiently applied in genetic diversity studies, population analysis, and genotyping and fingerprinting of individuals, genetic mapping and marker-assisted selection in many plant species.

Recently, Reale et al. [10] have reported the use of SNPs in combination with CAPS (cleaved amplified polymorphic sequence) and SCARS (sequence characterised amplified regions) in order to genotype 65 olive cultivars from Europe and Australia. The set of predominantly SNP-based markers was able to clearly discriminate 77% of the cultivars and also to resolve nomenclature issues indicating the significant potential of these markers for genotyping. SNPs provide several advantages compared to other molecular markers: they are the most abundant markers in the genome; they are stably inherited, bi-allelic in most cases, codominant and amenable to high throughput genotyping and automation due to the existence of several technology platforms such as pyrosequencing, capilary electrophoresis and real time PCR. Although the identification of SNPs is capital and labour intensive, their use for genotyping studies is simple, although it requires sequencing systems, and has the potential for allele frequency estimations.

Up to now, neither the combination of SNP and SSR markers for discrimination of olive cultivars, nor structural details of genetic diversity within cultivated germplasm from the Western Balkans have been reported. In addition, the genetic fingerprinting of Albanian olive germplasm in combination with the neighbouring Greek and Slovenian one has never been investigated. Therefore, the use of SNP and SSR markers for the fingerprinting of Albanian, Greek and Slovenian olive germplasm was studied and a first assessment of the genetic diversity within the Western Balkan region was also discussed.

2 Experimental Techniques and Methods

2.1 DNA Extraction

Genomic DNA was extracted from young olive tree leaves by using a standard cetyltrimethylammonium bromide-based protocol. The leaf DNA was used for SSR analysis and for target amplification and further sequencing in order to discover SNPs among varieties. Moreover, it also served as a control in order to design PCR and single-base extension primers.

2.2 SNP discovery

The SNP discovery work was mainly based on sequencing of 16 Albanian and 4 Slovenian cultivars. Sequencing itself included fragment from lupeol synthase. Sequencing traces obtained were then aligned against each other and SNP candidates were located by using the Staden software package.

2.2.1 Primers design

The PCR primers used throughout this study were designed with PrimerExpress® 2.0 software (Applied Biosystems). After the designing procedure, a BLASTn [11] search was carried out to ensure the uniqueness of the complementary DNA region against newly designed primers. SNaPshot™ primers were designed to give an annealing temperature of approximately 50 °C and were blasted against the amplicon target to ensure that they do not anneal on any extra sites.

2.2.2 PCR chemicals and conditions

Fifty microliters of PCR reaction volume were set up containing 5.0 µl 10x AmpliTaq Gold®, 25 mmol/L $MgCl_2$, 200 µmol/L for each dNTP (Promega), 300 µmol/L for each primer, 0.5 units of AmpliTaq Gold® Polymerase, 2.0 µl of DNA solution and nuclease free water. PCR conditions were 95 °C for 10 min, 35 cycles (40 cycles for SNaPshot™ assay) of 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 1 min and 1 cycle at 72 °C for 10 min. All PCR reactions were carried out in a PTC-200 thermocycler (MJ Research). Amplicons were initially purified with StrataPrep® DNA Gel extraction kit (STRATAGENE) and the samples were sequenced using a BigDye Version 3 dideoxyterminator sequencing kit (PE Biosystems) from both orientations in a PERKIN ELMER, ABI Prism™ 310 Genetic Analyser (PE Biosystems).

2.3 SNP exploitation

Some of the SNPs were further confirmed by single base extension analysis by using the SNaPshot™ kit according to the manufacturer's instructions.

2.3.1 Single-base extension (SNaPshot™) analysis

PCR amplification of target DNA was carried out as described above. Five microliters of the PCR product was then mixed with 2 µL ExoSAP-IT® (USB Corporation) and incubated

at 37 °C for 45 min and then at 80 °C for 15 min. For the primer extension reaction, a mixture containing 5 µL of SNaPshot Reaction Mix, 3 µL of previously cleaned up PCR product, 1 µL of 2 pM SNP primer and 1 µL water was incubated for 25 cycles at 95 °C for 10 sec, 50 °C for 5 sec and 60 °C for 30 sec and then incubated at 4 °C for further treatment. The whole primer extension product (10 µL) was mixed with 1 µL shrimp alkaline phosphatase (USB Corporation) and incubated at 37 °C for 60 min and then at 75 °C for 15 min. The mixture was then stored at –20 °C until further use. After this post-extension step, 0.5 µL of the sample was mixed with 0.5 µL of GeneScan™ -120 LIZ™ size standard and 9 µL of Hi-Di™ formamide and then incubated at 95 °C for 1 min and put on ice before being loaded into the ABI PRISM® 310 Genetic Analyser (Applied Biosystems).

2.4 SSR analysis

Sixty-eight Albanian olive samples were included in the SSR analysis with four Greek, six Slovenian, 11 Italian and three Spanish varieties as reference varieties for SSR genotyping. Two olive specific microsatellite primer pairs (ssrOeUA-DCA3 and ssrOeUA-DCA16 described by Sefc et al. [12] were used for genotyping. They were chosen on the basis of the results of Bandelj and Javornik [13], where their suitability for identification of olive planting material in olive nurseries has been confirmed. The universal labelling approach was used [14], where forward primer of the species specific primer pair is elongated for the M13(-21) sequence to introduce a common sequence for PCR product labelling. The third universal primer, labelled with CY5 dye at 5'end, incorporates a fluorescent tag during PCR amplification and permits fluorescent detection of amplicons. Amplification reactions were carried out as described by Bandelj and Javornik (2007). Briefly, the amplification volume was 10 ul, containing 20 ng of olive genomic DNA, 1x supplied PCR buffer with 1.5 mM $mgCl_2$, 0.2 mM of each dNTP, 0.25U of Taq DNA polymerase and 0.5 uM of olive specific primers and 0.075 uM of fluorescent M13(-21) sequence specific primer. Amplifications were performed in a thermal cycler with described conditions (Bandelj and Javornik, 2007). PCR reactions were diluted with an equal volume of formamide loading dye, heated and resolved in 7.5% polyacrilamide-urea gels (19:1, 7M urea) assembled in short gel cassette of AlfexpressII sequencer. Limiting power for electrophoresis was 15W at temperature 55°C. External (50-550 bp ladder) and internal (in house amplified PCR product of defined length) were used for proper allele size calling in Allele Locator 1.03 software (Amersham Biosciences). The relationships among the analysed olive varieties were assessed by a distance-based clustering method using the NTSYS computer package.

2.5 Data analysis

The SNP data were scored as homozygous or heterozygous for each cultivar and each SNP was designated with alphabetical letters; AA for homozygous and AB for heterozygous. Allele frequencies and expected and observed heterozygosity estimations were conducted using the PowerMarker software [15]. The same software was used to calculate genetic distance according to Nei (1983) [16] and the cultivars were clustered using the neighbour-joining tree.

3 Results and Discussion

The aim of this project was to use Single Nucleotide Polymorphism (SNP) and Simple Sequence Repeat (SSR) molecular markers for the discrimination of olive cultivars from the Western Balkan countries and for a preliminary assessment of the genetic diversity among these cultivars. The plant material used for SNP analysis throughout this study was young healthy leaves from 16 morphologically discriminated Albanian olive cultivars and four of the most important Slovenian varieties. Sequencing was carried out directly on PCR amplicons rather than on cloned fragments. This choice was based on the fact that homozygous as well as heterozygous SNPs could be monitored through direct sequencing, whereas through cloning would have required a lot of sequenced colonies from each variety in order to uncover heterozygocity. The SNP discovery was based on a DNA fragment of approximately 500 bp that corresponds to the gene lupeol synthase. Sequencing regions with a relatively higher signal-to-noise ratio were excluded from the actual analysis and nine candidate SNP positions were found. The nine SNPs showed higher observed heterozygosity than expected, while the Polymorphism information content (PIC) values for these markers were adequate, in order to be considered polymorphic enough for genetic diversity studies. The high observed heterozygosity can be easily explained by the fact that olive is a cross-pollinated species.

In addition, we have used genotyping data for 10 Greek cultivars obtained by the same set of nine SNPs to better assess the genetic diversity among Western Balkan cultivars. To this end, a cluster analysis was employed using the Neighbor joining tree (Figure 1).

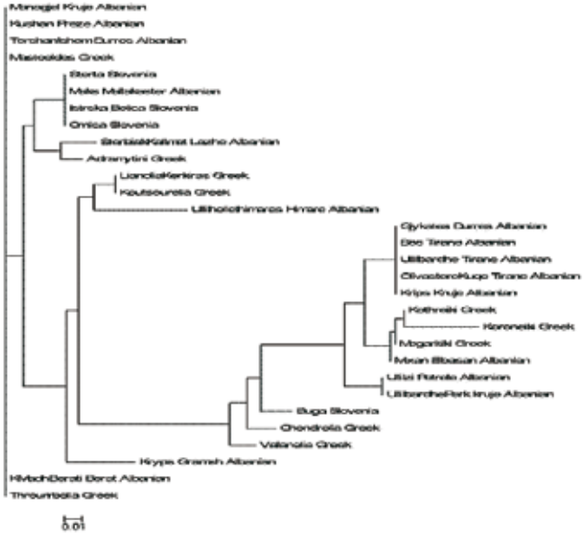

Figure 1: Cluster analysis of the 30 Albanian, Slovenian and Greek olive cultivars using nine SNP markets using the Neighbor joining tree

Two large clusters could be clearly defined, both comprising cultivars from the three Western Balkan countries. The Slovenian cultivar Buga is highly divergent compared to the other three Slovenian cultivars which closely cluster into one subgroup. Eight Albanian cultivars cluster into two subgroups comprising three and five members respectively, while the rest is genetically more similar to Greek and Slovenian cultivars than to Albanian cultivars. We can assume that the two large, distinct clusters evolved separately from ancestral times, although a much higher number of SNPs is required for a better assessment of the genetic diversity among them.

An appropriate SNP combination was chosen to discriminate several of the selected cultivars based on the 9 SNPs found. Only four Albanian cultivars (Sterbiak, Kryps, Mixan, and Ulli i holle i himares) and one Slovenian (Buga) were efficiently discriminated by a combination of 4 SNP positions (SNP1, SNP2, SNP3, SNP7). Furthermore, an additional Albanian variety, Maks, can be discriminated among the Albanian germplasm but comprises the same SNP positions as three of the Slovenian Varieties (Storta, Crnica and Istrska Belica). Moreover, three groups of Albanian varieties cannot be discriminated (Figure 2). The first group includes Kushan, Managjel, Tershan and K Madh Berati, the second group includes Krips, Boc, Olivaster e kuqe, Ulli I Bardhe (Tirane), Mixan and Gjykates, and the third group includes Ulli I bardhe (Perk) and Ulli I zi.

In addition, single-base extension (SBE) analysis was used for 4 SNPs in 4 different cultivars, confirming the initial results obtained by sequencing traces. The principles of the SBE assay rely on the amplification of target DNA that includes the SNP under detection and the annealing of a primer adjacent to the 3' – end of the SNP. The length of the PCR target can be just as long as it is required for a successful annealing between amplicon and the SNP primer. In practice, that could be smaller than 100 bp.

The discrimination of olive cultivars from the Western Balkan countries by SSR markers was performed by using two highly polymorphic SSR markers, DCA3 and DCA 16 and for a preliminary assessment of the genetic diversity among these cultivars. On a set of 62 olive samples both SSR markers amplified in total 23 different alleles, 9 alleles by DCA 3 and 14 alleles by DCA16. Some of the Albanian samples gave similar to reference varieties genotypic formulas and were not distinguished by these two SSR markers. It was somehow expected that some of the samples would give the same allelic profile due to clonal nature of the samples, homonyms or synonyms. Among 36 Albanian sample 12 gave a unique genotypes and the same SSR profiles were found three times in four and two samples and twice in three samples. To further differentiate analyzed higher number of SSR must be applied.

The assessment of the genetic relationship among analysed samples is best presented by a dendrogram constructed on the data of genetic distances. Our dendrogram (Figure 2) shows a high variability among the analysed olive samples and tight clustering of the Albanian samples in comparison to the reference samples suggesting distinguished germplasm which might be characteristic for the Albanian olive varieties and landraces.

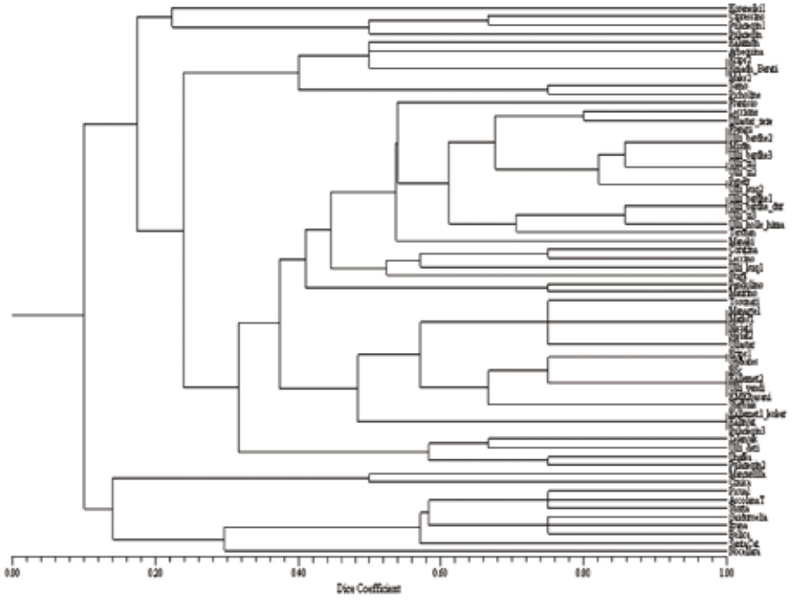

Figure 2: UPGMA dendrogram obtained from SSR data for 62 olive samples showing their genetic relationship

4 Conclusions / Outlook, next steps

Previously identified SNPs were used for the genotyping of 20 selected olive varieties from Albania and Slovenia, creating the first SNP database of Albanian and Slovenian cultivars. The genotyping data of additional ten Greek cultivars were used for a better assessment of genetic diversity. The cluster analysis revealed the existence of two large clusters comprising cultivars from all the three countries, indicating movement of olive germplasm among the Balkan countries and no sign of geographic isolation. Various combinations of the SNPs can discriminate several of these cultivars, providing a tool for their certification and the preservation of germplasm collections in these countries. This database can be further enriched with more SNPs in the future and used for genetic diversity studies of the Western Balkan cultivars. In addition, two highly polymorphic SSR markers were employed to genotype 36 Albanian cultivars out of 62 cultivars comprising Greek, Slovenian and Italian cultivars, which were used for the clustering analysis. This analysis revealed a tight clustering of the Albanian cultivars and high variability. This database can be used in marker-assisted breeding and construction of genetic maps to further assist olive breeding programmes. These preliminary results of genotyping olive varieties and assessment of genetic relationship show distinguished genetic variability among the olives from Western Balkan and justify further, detailed studies on the structure of olive germplasm diversity, which is important for its effective use through applications of association genetics and olive breeding.

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The Importance of Chlamydia Infections in Birds for Animal and Human Health in Southeastern Europe

Alenka Dovc[1], Konrad Sachse[2], Karine Laroucau[3], Estella Prukner-Radovčić[4], Emina Rešidbegovič[5], Vlatko Ilieski[6]

[1] University of Ljubljana, Veterinary Faculty, Institute for Health Care of Poultry, Gerbičeva 60, Ljubljana, Slovenia; alenka.dovc@vf.uni-lj.si
[2] Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health), Institute of Molecular Pathogenesis, Naumburger Str. 96a, 07743 Jena, Germany; konrad.sachse@fli.bund.de
[3] Agence Française de Sécurité Sanitaire des Aliments (French Food Safety Agency), Laboratoire d'Études et de Recherches en Pathologie Animale et Zoonoses, 23, Avenue du General de Gaulle, 94706 Maisons-Alfort, France; k.laroucau@afssa.fr
[4] Faculty of Veterinary Medicine, University of Zagreb, Department of Poultry Diseases with Clinic, Heinzelova 55, 10000 Zagreb, Croatia; prukner@vef.hr
[5] University of Sarajevo, Veterinary Faculty, Department of Health Care of Poultry, Zmaja od Bosne 90, 71000 Sarajevo, Bosnia and Herzegovina; emina.residbegovic@vfs.unsa.ba
[6] Ss. Cyril and Methodius University, Skopje, Faculty of Veterinary Medicine, Institute for Veterinary Biomedicine, Lazar Pop-Trajkov 5/7, 1000 Skopje, FYR of Macedonia; vilieski@fvm.ukim.edu.mk

Abstract

The present chlamydia research project was based on various topics of molecular biology. DNA-based methods for rapid detection and identification of chlamydiae, such as conventional PCR, RFLP-PCR, real-time PCR, MLVA typing and DNA microarray technology were used. DNA microarray assays for routine detection and multilocus genotyping of chlamydiae are the most advanced tests for the diagnosis of chlamydial infections. Research activities were placed in the context of characterising the occurrence, dissemination and host range, as well as the importance and zoonotic potential of chlamydial agents.

The Institute for Health Care of Poultry at the Veterinary Faculty of the University of Ljubljana, Slovenia, as the coordinator and main performer of the project, supervised all activities of the other collaborators. Participation of partners from Croatia, Bosnia and Herzegovina, the FYR of Macedonia, Germany and France was successful and contributed to the generally efficient work on the project.

In a preliminary study of 531 samples of free-living birds, domestic poultry and pet birds collected and tested for the presence of Chlamydophila (Cp.) psittaci, 18 (3.39%) were found positive: One free-living bird (wild duck) in Slovenia, thirteen birds (five parrots, three free-living pigeons, two seagulls, one canary, laying hen and turkey) in Croatia, three birds (two wild ducks and turkey) in Bosnia and Herzegovina and one free-living pigeon in the FYR of Macedonia.

Thematic Area / Type of the project

Food, Agriculture and Biotechnology: Veterinary Sciences: Applications Research: Genetics of Microorganisms
Type of the project: network project

Keywords

Bacteriology; Birds; Chlamydiae; Zoonoses

1 Introduction

Chlamydiae are widely distributed throughout the world, causing various forms of disease in animals and humans. Several species, particularly Chlamydophila (Cp.) psittaci, are known to be transmissible from birds to humans, causing significant zoonotic infections. The unique biphasic lifestyle of these obligately intracellular bacterias, which includes an infective extracellular and a parasitic intracellular phase, renders the respective diseases difficult to control. This is compounded by the special growth conditions for the organisms and the lack of a genetic based system for the transformation of chlamydiae, both of which have hampered research on these pathogens. To escape the host immune response, these bacteria are capable of transforming into persistent stages of development characterised by a distinct antigenic profile. Cp. psittaci is a pathogen responsible for Avian Chlamydiosis and is the most important member of the family Chlamydiaceae, having a zoonotic potential. This bacteria primarily infects birds, but it can be transmitted from birds to humans. More than 467 different species of birds can be carriers of the pathogen [1]. The severity of the disease ranges from inapparent to systemic illness with severe pneumonia. Avian Chlamydiosis is a very important infectious disease in parrots and pigeons and is also known as Psittacosis, Parrot fever or Ornithosis. It was and still is an important disease in the intensive breeding of poultry (turkey, geese and ducks). Disease causes typical influenza-like symptoms in people and can lead to severe pneumonia and non-respiratory health problems. Breeders of pet birds, mostly parrots, breeders of pigeons and persons in specific occupations (employees in poultry slaughters, veterinarians, veterinary technicians, laboratory workers, workers in avian quarantine stations, farmers, wildlife rehabilitators, zoo workers and employees in shops for pet birds) are more often exposed.

Cp. psittaci strains are similar in virulence, grow readily in cell culture, have 16S-rRNA genes that differ by < 0.8%, and belong to nine known serovars. All of them should be considered to be transmissible to humans. Chlamydial infections in birds are important as they represent a biological hazard to human health (major risk groups are veterinarians, breeders and animal shop keepers), as well as economic loss to the poultry industry. Apparently healthy birds shedding chlamydiae can infect other birds or humans through contact. Infectious chlamydiae in respiratory secretions or faeces may remain viable for several months. Transmission of disease is mainly through aerosols of faecal or feather dust, but oral infection is an alternate route. Transmission through eggs has been shown in chickens, ducks, seagulls and psittacine birds. In the nest, parent birds may infect their young, which may carry the infectious agent for many years. Young birds are more susceptible to infection than older birds, and some species seem to be more susceptible than others. Often, disease carriers may be identified by the transmission of disease to other susceptible birds or by the sudden death of young nestlings with apparently healthy parents. Although outbreaks of Cp. psittaci infection in feral birds appear uncommon, it is possible that wild birds may act as natural reservoirs of the agent and introduce chlamydiae into farmed bird populations such as turkeys and ducks, with potentially devastating consequences. Wild and racing pigeons, the psittacine bird trade, and migrations of wild birds such as seagulls, finches, sparrows and waterfowl may all contribute to the dissemination of chlamydiae throughout the avian population [2], [3].

According to the literature data [4], [5], Cp. psittaci serovar A is endemic among psittacine birds and has caused sporadic zoonotic disease in humans, other mammals and also tortoises. Infection is often systemic in birds and can be inapparent, severe, acute or chronic with intermittent shedding. Serovar B is endemic among pigeons and has been isolated from turkeys, and has also been identified as the cause of abortion in dairy herds. Serovars C and D are occupational hazards for slaughterhouse workers and for people in contact with birds. Serovar E isolates have been obtained from a variety of avian hosts worldwide and, although they were associated with the 1920s–1930s outbreaks in humans, a specific reservoir for serovar E has not been identified. The M56 and WC serovars were isolated during outbreaks in mammals. Several Cp. psittaci strains have an extrachromosomal plasmid. Many Cp. psittaci strains are susceptible to bacteriophages.

The aim of this collaboration was to exchange experience in new and advanced diagnostic procedures among laboratories in the Balkan region and to establish collaboration with specific reference laboratories for Psittacosis in Germany and France. They were providing the diagnostic facilities for the examination of samples from the Balkan region. These laboratories performed testing for chlamydia by real-time PCR and identification by DNA microarray assay, as well as genotyping of Cp. psittaci by a MLVA typing and DNA microarray test [6], [7], [8], [9], [10], [11], [12], [13].

2 Experimental Techniques and Methods

Sampling was done in autumn 2007. In this time, 531 samples of free-living birds, domestic poultry and pet birds have been collected (Slovenia 142, Croatia 190, Bosnia and Herzegovina 118, and FYR of Macedonia 81 samples). Ethical norms and conformity with legislation and international conventions were considered, as well as the European Community Council Directive 86/609/EEC. Besides faeces or cloacal swabs, samples from conjunctiva, pharynx, and different organs or excrements from dead birds have been collected for further investigations.

In our research, samples of 35 different bird species were included:

- 26 free-living birds (passerines, wild ducks and wild geese, swans, seagulls, cormorants, free-living pigeons);
- Seven poultry (laying hens, turkeys, grey partridges, peacocks, quails, guinea fowls,

ostriches); and
- Two pet birds (parrots, canaries).

The following preliminary tests [4] for the identification of the Cp. psittaci prior molecular testing were used:
- Direct immunofluorescent test;
- Enzyme Linked ImmunoSorbent Assay;
- Commercial fast Enzyme Immuno Assay;
- Histochemical staining;
  - Giemsa
  - Gimenez
- Isolation in specific pathogen-free eggs; and
- Conventional PCR.

The research procedures and protocols provided by the laboratories from Germany (Friedrich-Loeffler-Institut, Jena) and France (Agence Française de Sécurité Sanitaire des Aliments/ French Food Safety Agency, Paris) were:
- Conventional PCR;
- RFLP-PCR;
- Real-time PCR;
- MLVA typing;
- Identification by DNA microarray assay; and
- Genotyping of Cp. psittaci by a new DNA microarray test.

3 Results and Discussion

Wild birds act as natural reservoirs of chlamydiae and represent a possible danger for human health. They are also potentionally hazardous for poultry production, with economical consequences. Wild and urban pigeons and migrations of wild birds, such as seagulls, finches, sparrows and waterfowl, may contribute to the dissemination of chlamydiae throughout avian population.
Previous investigations [14], [15], [16], [17], [18] conducted in different European and Non-European countries showed that pigeons and other free-living birds, because of their specific migrations route, represent a potential source of infection for humans and poultry in suburban areas.

In our study, 35 different bird species were tested (Table 1). None of the captured free-living birds showed any clinical signs of disease.

Very little is known about genotypes in wild birds. This study was the first investigation ever performed in the Balkan region. Avian chlamydiosis has been continuously present, but genotyping or serotyping of Cp. psittaci has not been conducted yet.

Table 1: Species of birds, number of samples and results of molecular tests (/ Samples not collected)

Species	No. of tested birds / No. of positive (% of positive)				No. of positive (% of positive)
	Slovenia	Croatia	Bosnia and Herzegovina	FYR of Macedonia	
Mallard *Anas platyrhynchos*	52 / 1 (1.92)	/	40 / 2 (5.00)	11	3 (2.91)
Reed Warbler *Acrocephalus scirpaceus*	1	/	/	/	0
Goldfinch *Carduelis carduelis*	2	/	/	/	0
Greenfinch *Carduelis chloris*	6	/	/	/	0
Siskin *Carduelis spinus*	6	/	/	/	0
Robin *Erithacus rubecula*	27	/	/	/	0
Great tit *Parus major*	1	/	/	/	0
Tree sparrow *Passer montanus*	5	/	/	/	0
Red start *Phoenicurus Phoenicurus*	1	/	/	/	0
Dunnock *Prunella modularis*	8	/	/	/	0
Serin *Serinus serious*	1	/	/	/	0
Starling *Sturnus vulgaris*	2	/	/	/	0
Blackcap *Sylvia atricapilla*	19	/	/	/	0
Garden warbler *Sylvia borin*	1	/	/	/	0
Whitethroat *Sylvia communis*	1	/	/	/	0
Blackbird *Turdus merula*	6	/	/	/	0
Song thrush *Turdus philomelos*	3	/	/	/	0

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Black capped chickadee *Parus atricapillus*	/	8	/	/	/
Magpie *Pica pica*	/	2	/	/	0
Seagull *Larus ridibundus*	/	35 / 2 (5.71)	/	/	2 (5.71)
Mute swan *Cygnus olor*	/	11	/	/	0
Wild pheasant *Phasianus colchicus*	/	11	27	/	0
Free living pigeon *Columba livia domestica*	/	32 / 3 (9.38)	/	48 / 1 (2.08)	4 (5.00)
Turkey (breeding flock) *Meleagris gallopavo*	/	36 / 1 (2.78)	23 / 1 (4.35)	1	2 (3.39)
Grey partridge *Perdix perdix*	/	10	/	/	0
Laying hen *Gallus gallus*	/	1 / 1 (100.00)	/	/	1 (100.00)
Parrot *Psittacidae*	/	34 / 5 (14.71)	/	3	5 (100.00)
Canary *Serinus canaria*	/	10 / 1 (10.00)	/	10	1 (5.00)
Peacock *Pavo cristatus*	/	/	14	6	0
Quail *Coturnix coturnix*	/	/	2	/	0
Wild goose *Anser anser*	/	/	7	/	0
Cormorant *Phalacrocorax carbo*	/	/	2	/	0
Raven *Corvus corax*	/	/	1	/	0
Ostrich *Struthio camelus*	/	/	2	/	0
Guineafowl *Numida meleagris*	/	/	/	2	0
TOTAL	142 / 1 (0.70)	190 / 13 (6.84)	118 / 3 (2.54)	81 / 1 (1.23)	53118 (3.39)

The first results of genotyping show the presence of various important genotypes of Cp. psittaci prevailing in this region. Genotypes A and B were confirmed in one mallard and one parrot, two were of unknown genotype and four of them have not been determined yet. The continuous presence of Avian Chlamydiosis in the Balkan area was confirmed, but the prevalence of Cp. psittaci infection in the bird population from Southeast Europe has to be determined in future work. An assessment of potential zoonotic risks for contact persons can be given on the basis of more genotyping results collected in the future. Most laboratories do not have the special equipment required for molecular diagnostic testing. The complete set of diagnostic procedures for Avian Chlamydiosis is performed only by few laboratories because of technical difficulties and safety concerns. This is an additional difficulty in diagnosis of avian chlamydiosis.

4 Conclusions / Outlook, next steps

Results have shown the presence of Cp. psittaci in populations of live birds in all collaborating countries. Some of the strains identified appeared to be specific for the region, so we intend to continue our work if we can get funding for future activities.
One of the most important conclusions is the fact that in further epizootiological investigations on waterfowl, urban pigeons and yard poultry have to be included. These groups of birds are in close contact with people and intensive poultry production. Infection to humans can be easily spread from domestic and companion birds, as well as from other free-living birds. Our future research should be directed especially towards waterfowl, urban pigeons and yard poultry.
Avian chlamydiosis is an important zoonosis transmitted not only from diseased birds but also from clinically healthy birds, which are carriers of the pathogen.

The collaboration between the specialised host laboratories and the southeast European partners produced an intense and productive exchange of experience and contributed to an important increase in knowledge about the epidemiological status of Southeast European bird populations.

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Appendix – Photo Gallery

ID 10485 – The participants of the Summer School on Build Environment Summer School on Build Environment, Pamporovo, Bulgaria

ID 10485 – During an afternoon session, Summer School on Build Environment, Pamporovo, Bulgaria

ID 10724 – Practical courses and final network meeting held in Bordeaux, France on June 23–27, 2008 at the UMR-1090 (INRA and University of Bordeaux 2)

ID 10491 – Visiting the Wolf Information Center at ARCTUROS' Environmental Centre in Agrapidia, Florina, Region of West Macedonia, Greece

ID 10491 – The systematic collection of hair from power poles can provide a valuable tool in the conservation of the local Brown Bear population

ID 10491 – Field Visit to the Tara National Park, Republic of Serbia

Centre for Social Innovation (ZSI)
Southeast European Era-Net
Coordination Office
Linke Wienzeile 246
1150 Wien
AUSTRIA
Tel +43 1 495 04 42
Fax +43 1 495 04 42-40
see-era.net@zsi.at
www.see-era.net

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