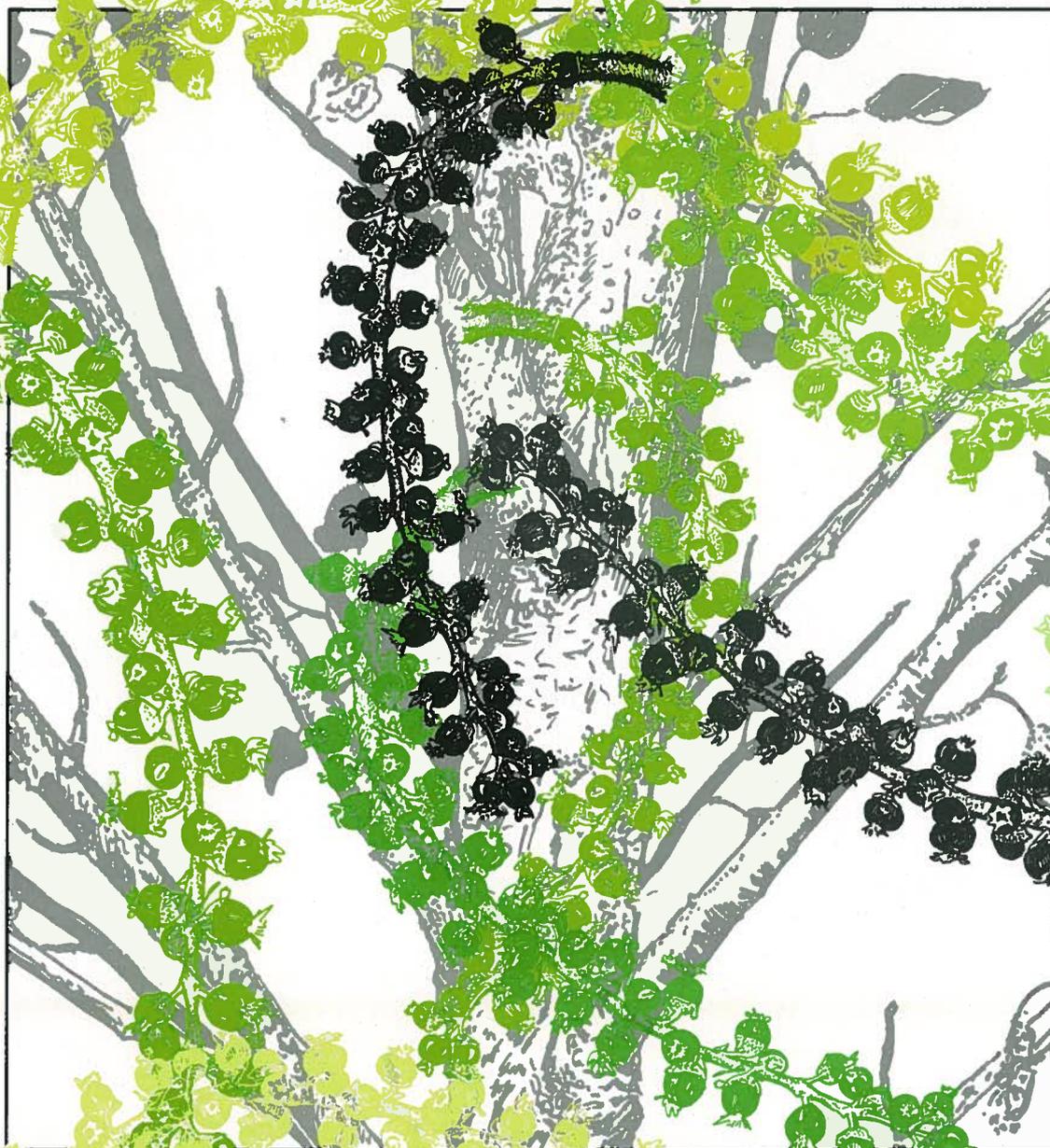
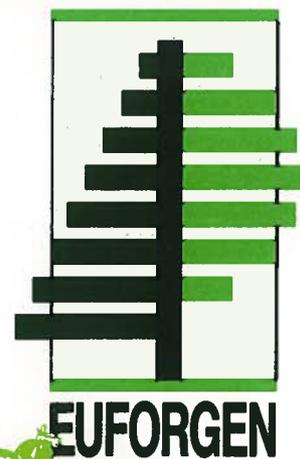


EUROPEAN FOREST GENETIC RESOURCES PROGRAMME (EUFORGEN)

# **Populus nigra Network**

*Report of the fourth meeting  
3-5 October 1997  
Geraardsbergen, Belgium*

**J. Turok, F. Lefèvre, S. de Vries, N. Alba, B. Heinze**  
*and J. Van Slycken, compilers*



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The European Forest Genetic Resources Programme (EUFORGEN) is a collaborative programme among European countries aimed at ensuring the effective conservation and the sustainable utilization of forest genetic resources in Europe. It was established to implement Resolution 2 of the Strasbourg Ministerial Conference on the Protection of Forests in Europe. EUFORGEN is financed by participating countries and is coordinated by IPGRI, in collaboration with the Forestry Department of FAO. It facilitates the dissemination of information and various collaborative initiatives. The Programme operates through networks in which forest geneticists and other forestry specialists work together to analyze needs, exchange experiences and develop conservation objectives and methods for selected species. The networks also contribute to the development of appropriate conservation strategies for the ecosystems to which these species belong. Network members and other scientists and forest managers from participating countries carry out an agreed workplan with their own resources as inputs in kind to the Programme. EUFORGEN is overseen by a Steering Committee composed of National Coordinators nominated by the participating countries.

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## Summary of the meeting<sup>1</sup>

### Introduction

Participants from 15 countries attended the meeting (see List of Participants). They were welcomed by J. Van Slycken, Director of Instituut voor Bosbouw en Wildbeheer (Institute for Forestry and Game Management) in Geraardsbergen. J. Turok, EUFORGEN Coordinator, thanked the host of the meeting and wished the participants a fruitful meeting. He mentioned the current activities in other Networks and described briefly the recent international developments in the area of forest genetic resources.

F. Lefèvre, Chair of the Network, then opened the meeting. He suggested that the most important objectives of the meeting were: (i) to evaluate outputs from the previous joint tasks, (ii) to closely involve the participants from all newly attending countries, and (iii) to initiate the development of *in situ* conservation strategies which should also result in practical work with shared responsibilities. The agenda of the meeting was approved (see Agenda).

### Joint research

Common research needs were discussed during previous Network meetings. The most important research needs for the development of overall conservation strategies, and to accomplish Network tasks, were listed (see Reports of previous meetings). The research topics had previously not been prioritized. Research had been emphasized as a keystone of genetic conservation activities in EU member as well as non-member countries. Collaboration in this area would be beneficial for all.

A project focusing on research activities in black poplar was submitted for funding to the European Commission during the last call in March 1997. The Project was approved for funding and coordination undertaken by Dr B.C. van Dam from the Institute for Forestry and Nature Research (IBN-DLO) in Wageningen, the Netherlands.

B. van Dam was invited to present the Project EUROPOP to the members of the Network. She kindly accepted the invitation and gave an overview of the objectives and the work content. She also concentrated on the possible interactions and benefits resulting from collaboration with the EUFORGEN *P. nigra* Network.

EUROPOP Project members and the EUFORGEN *P. nigra* Network are working together with specialists in the field of molecular genetics to achieve common goals. It was stated that the collaboration between EUFORGEN and EUROPOP will ensure a broad dissemination of the results of the Project and will ensure the implementation of strategies and guidelines for practical use. She acknowledged that the comments given by the Network members on the project proposal were very useful in preparing its submission to the EU. In her opinion, this was one of the reasons why the revised version was highly appreciated in the scientific evaluation. The management structure of the project proposal was improved during a meeting held in Wageningen earlier this year.

The objectives of the EU/FAIR Project "Genetic diversity in river populations of European Black Poplar for evaluation of biodiversity, conservation strategies, nature development and genetic improvement (EUROPOP)" are:

- to develop strategies for the conservation of *P. nigra* and its restoration to riparian ecosystems based on the measurements of the genetic diversity in wild populations
- to describe the genetic diversity within *ex situ* collections in order to evaluate the current state of conservation in Europe
- to study key parameters of stand dynamics for *in situ* management and re-introduction strategies

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<sup>1</sup> This summary was adopted and distributed at the meeting.

- to maintain populations with a broad genetic diversity to ensure adaptation to changing environmental conditions and that breeding programmes are provided with new genotypes.

To achieve these objectives, eight EU member countries (Austria, Belgium, France, Germany, Italy, Spain, the Netherlands and United Kingdom) prepared a list of tasks which the following river systems: Danube, Drome, Loire, Rhine, Elbe, Ticino, Ebro, Rhine/Waal, Maas, Usk and Dee. The following tasks will be undertaken:

1. Standardization of methods
2. Adaptation of molecular technology
3. Determination of cytotypes with chloroplast DNA analysis
4. Isolation of microsatellites
5. Assessment of diversity within and between natural river populations
6. Verification of existing genebanks
7. Life history traits of black poplar stands
8. Evaluation of data
9. Synthesis.

The project will start on 1 March 1998 for a period of 3 years. The total budget is estimated at ECU 2 100 000 (with the contribution of the EU amounting to ECU 1 260 000).

Possibilities of joint meetings, publications and a linked Internet home page with the Network will be explored, to provide maximum benefits of the results achieved for black poplar conservation in European countries. It was agreed that progress made by the EUROPOP Project will be briefly reported at each Network meeting. S. de Vries kindly offered to ensure the information flow with the Network.

### Country reports and updating of information

Brief updates on the progress made in each country during the period since the last meeting were presented. It was agreed that updating information on the national activities play an important part of the tasks of the Network. They should be regularly published and distributed in Reports of Network meetings (see this volume). The updates should be no longer than one printed page and may include tables/figures to document the information given. The updates will be sent (preferably in English) to J. Turok for compilation **before 1 November 1997**. Introductory country reports on *P. nigra* genetic resources and conservation from Poland (J. Figaj), the Ukraine (R. Volosyanchuk) and FR Yugoslavia (S. Orlovic) will be sent to J. Turok **before 15 November 1997**.

### *In situ* conservation strategies

Paul Tabbush presented an overview of the existing knowledge and ongoing research projects on the dynamic processes in riparian ecosystems. The implications of these processes for conservation strategies in *P. nigra* were discussed. A strategy for *in situ* conservation of the species needs to be based on good knowledge of genetic diversity, its structure and underlying processes in populations.

It was agreed that long-term conservation of the genetic diversity of *P. nigra* across its distribution range is the main objective, which should be achieved through both dynamic and static, *in situ* and *ex situ*, methods. These methods are listed below and numbered in order of priority for the conservation of genetic diversity in populations.

Method	<i>In situ</i>	<i>Ex situ</i>
Dynamic	1. Natural stands	2. Restoration schemes; breeding populations
Static	3. Relict populations	4. Genetic collections

The methods are closely linked with each other, e.g. restoration schemes often become *in situ* conservation stands.

The main responsibility for *in situ* conservation of *P. nigra* is with the national, regional or local authorities concerned. An international network will be built up on the basis of stands designated and managed at national level.

The suggested network of *in situ* populations (stands) should cover the natural distribution range of the species, with regard to latitude, longitude, altitude, biophysical and environmental factors. Common criteria for choosing a site which should be included in the network are:

- protection status; ownership; management
- potential for natural regeneration
- large size.

To promote the establishment of a network of *in situ* populations, the *P. nigra* Network will:

1. Conduct an inventory and produce a synthesis of existing knowledge on the effects of ecosystem management on dynamic processes in *P. nigra* populations – S. de Vries and I.I. Popivshchy.
2. On the basis of a literature survey, prepare draft recommendations (including research needs) for the restoration of riparian ecosystems (use of genetic material for planting, etc.) – B. Heinze and M. Dubský.
3. Review possible indicators for monitoring the evolution of diversity in riparian ecosystem – F. Lefèvre and D. Kajba.

These three tasks will be developed for the next Network meeting; background documents on each of the tasks will be circulated one month **before the next meeting** (to be held in September 1998). The participants will send literature and written inputs to the coordinators of the new tasks of the Workplan **by the end of the year 1997**.

#### **Standardized minimum list of descriptors for inventories of *P. nigra* stands**

The draft list of descriptors developed and circulated by N. Alba was reviewed. All participants will send additional comments and suggestions to N. Alba **by 1 November 1997**. N. Alba will send the final version for inclusion in the Report of the meeting to J. Turok **by 1 December 1997**. Missing diagrams and environmental descriptors will be prepared jointly by N. Alba, J. Van Slycken and P. Tabbush (in collaboration with existing relevant projects).

Participants from countries with ongoing *in situ* conservation programmes will bring a sample list of stands already designated, following the new list of descriptors, for discussion during the next meeting of the Network. Optional (voluntary) descriptors, in addition to the common minimum list, are encouraged and should cover specific needs and conditions in each country.

#### **Synthesis of *in situ* conservation measures and activities**

The synthesis was prepared and presented by S. de Vries. An update will be produced, including as many countries as possible from the distribution range. All participants will check the information on their respective country and send modifications to S. de Vries **by 1 November 1997**. S. de Vries will send the new version/table for the Report of the meeting to J. Turok **by 1 December 1997**. J. Turok will contact countries not participating in the Network (southern and eastern parts of the distribution range), to inform them about ongoing activities and ask for input **by 15 October 1997**.

### EUFORGEN core collection

S. Bisoffi presented the status of the collection (see Reports of previous Network meetings). The multiplication of the collection has been delayed because of technical problems with cuttings received at the host institute in Casale Monferrato (see report of Italy in this volume). It was proposed to employ intensive multiplication techniques and duplicate the collection in a cooler climate at IBN-DLO in Wageningen (S. de Vries). The collection will also be made available for EUROPOP research.

Certain clones need to be re-sent. S. Bisoffi and J. Turok will contact participants in countries which should re-send clones or send new material (from countries in the distribution range not participating in the Network). A reminder/invitation letter will be distributed **by 1 December 1997**. All material should be sent to both Casale Monferrato and Wageningen. **Instructions for shipment should be followed carefully. Passport data according to the Network's list must accompany all clones.**

### European database of clones

The database contains 1960 entries (as of October 1997; see report of Italy in this volume). Passport data for the clones in the core collection have been included, but some relevant information is still missing for individual clones. S. Bisoffi distributed sheets with these missing data directly to Network members, asked them to update the information on the core collection and send it back to him **by 1 November 1997**. He illustrated the importance of the missing data with examples of conflicting data for clones held at different institutions, and to detect possible duplicates. Belgium proposed to test such clones for which there is confusion about identity with genetic fingerprints. On the technical side, some database fields have been enlarged, and a new field added at the end (presence in the EUFORGEN core collection). S. Bisoffi stressed the importance of including the geographical coordinates with some examples.

All participants received the data file on diskette (DOS/Windows, Excel 5, compressed with Pkzip). J. Turok will ensure that the updated file replaces the older version on the Internet home page of the Network, **by 1 November 1997**. New data as well as regular updates, including the supply of previously missing data, the correction of wrong data, or the deletion of clones no longer held in collection, should be done by the Network members and sent to S. Bisoffi on diskette by mail, or directly by e-mail **by the end of the year 1997** and regularly afterwards. The next uploading on the Internet will be made **earlier than 1 June 1998**.

### Characterization of *P. nigra* with molecular methods

A review presentation was given by B. Heinze on biochemical and molecular methods available for investigating introgression, genetic variation and clone differentiation in *P. nigra*. Chromatography of leaf and bud phenolic compounds, isoenzymes and DNA techniques were covered. Studies in *P. nigra* and other *Populus* species were compared and some thoughts for the use of suitable markers for different problems given. A working draft was distributed. Participants will supply further information and references to B. Heinze **by 1 November 1997**. The draft will be updated and sent by B. Heinze to J. Turok for inclusion in the Report of the meeting **by 1 December 1997**.

A questionnaire was also distributed with a request for information on laboratories concerned with the application of molecular techniques to *Populus* species. Participants will contact such laboratories in their countries and send the questionnaire back to B. Heinze **by 1 November 1997**. The information received will be collated and supplemented with the list of EUROPOP participants, included in the Report of the meeting (to be sent **by 1 December 1997**). Contacts and possibly Internet home page links will be established with the Poplar Molecular Network based at the University of Washington, USA (B. Heinze and J. Turok).

### Public awareness activities of the Network

The Network's collection of slides was reviewed. S. de Vries mentioned the importance of receiving slides from all countries. The following areas are still not covered and should be supplemented before the next Network meeting:

- wood patterns/burls
- fodder for animals
- pollen
- mixed-species riparian stands
- particular landscape: North Africa
- drawings from the Identification Sheet.

It was confirmed that a CD-ROM would be produced by IPGRI at a later stage.

It was noted that the Report of the previous meeting (800 copies) had been widely distributed according to the mailing lists provided by the Network members. Network members are encouraged to send instructions/addresses for further distribution in their countries to J. Turok **by the end of the year 1997**.

### Miscellaneous

- Literature review (addendum for 1997) was provided and participants will send additional references to F. Lefèvre **by 1 November 1997** in the format specified earlier; see Reports of the previous Network meetings).
- Exchange of reference clones: requests for cuttings should be addressed to J. Van Slycken **before the end of the year**.
- Leaf morphology: D. Kajba presented results of a recent study in Croatia (see this volume) and will prepare an overview for the next Network meeting.

### Conclusions

Following a short discussion among the participants, it was agreed to slightly broaden the scope of the Network and include *Populus alba* on the agenda in the future. This decision was made in light of the increasing efforts of the Network towards *in situ* conservation strategies in riparian ecosystems.

The Network expressed its strong concern about the continuing uncertain situation of the Istituto di Sperimentazione per la Pioppicoltura (Poplar Research Institute) in Casale Monferrato, Italy and recommended that every effort be made to ensure the maintenance of the unique collections and the scientific experience available at this Institute.

It was suggested to hold the next, fifth Network meeting in conjunction with the International Union of Forestry Research Organizations (IUFRO) Poplar and Willow Breeding and Genetic Resources Working Group, in September 1998, in Orléans, France.<sup>2</sup> A tentative schedule suggests that Network meeting participants join the IUFRO excursions planned for 16-17 September 1998, as well as indoor sessions on 18 November 1998, and then continue for two days (19-20 September) with the actual Network meeting. This arrangement would enable Network members to attend both meetings, inform IUFRO Working Group members about the Network activities and jointly discuss several aspects of poplar conservation and genetic resources management.

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<sup>2</sup> This meeting was later postponed. The dates and venue of the next EUFORGEN *Populus nigra* Network meeting will be announced in due time.

## Reports on the progress of activities in countries

It was agreed that exchanging and updating information on the activities in countries play an important part of the Network's tasks. The progress reports from countries, briefly discussed during the meeting, are presented below in alphabetical order. They describe the period between the two Network meetings (October 1996-October 1997). The information provided typically includes mention of research, field inventories, practical implementation and public awareness activities.

In addition, the report from Moldova summarizes the results of a study conducted within a project on geographic variability of black poplar in the former USSR. An interesting contribution from Malta, included for the first time in this Network, suggests that white poplar (*P. alba*) be given more attention in the future activities.

Poland, Ukraine and FR Yugoslavia (Serbia and Montenegro) participated for the first time in a Network meeting as well and their introductory country reports are included at the end of this section. They bring the total number of countries which contributed information about the status and the national gene conservation activities on *P. nigra* to 20 countries (see Reports of previous meetings).

## Austria

*Berthold Heinze*

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### Research

Investigations in our own laboratory focused on markers for chloroplast and nuclear genes for the investigation of introgression of *P. nigra* with *P. × euramericana*. Chloroplast DNA can be readily distinguished with PCR tests by analyzing appropriate regions on the chloroplast genome (Heinze 1998). One nuclear DNA marker has been investigated that is also amenable to quick screenings of plants (Heinze 1997). By combining both tests, and applying further nuclear gene markers more recently developed, some introgression was found that was unnoticed phenotypically, in plants between 2-10 years of age. The magnitude of this introgression was roughly between 2 and 10%, depending on the population.

More data were collected on residual populations and single trees. The Austrian participant for the common research project EUROPOP, the Austrian Research Centre Seibersdorf (K. Burg, S. Fluch) is ready for the start of the project.

### Public awareness

A meeting on international collaboration matters was held in Vienna in April 1997 which served as an introduction of the Networks to Austrian decision-makers (mainly in administration) and tried to clarify the relationships between the different conventions and international agreements concerning biodiversity and conservation. The World Wide Fund for Nature (WWF) organized a meeting on rare and endangered tree species which included *P. nigra*. A number of articles were published in local forestry-related journals.

### Implementation of genetic conservation

The National Park of the Danube Floodplains east of Vienna which was opened last October brought with it a discussion on management plans which now address the black poplar vs. hybrid poplar problem. Local foresters within the National Park started *P. nigra* propagation from seeds in the nursery. The WWF, one of the landowners within the National Park, started a floodplain restoration programme by opening up sidearms and waterways for frequent flooding. Experience with this project will be especially interesting in the light of the great flooding that occurred in eastern Austria in July 1997.

There is very active support of the Network from the Salzburg and Styria regional governments with active help in surveying trips and an interest in black poplar trees as bird sanctuaries. The certification of the first Austrian *P. nigra* seed stand is pending which would allow the trading of seed and seedlings for forestry purposes without case-by-case permissions.

Collecting of clones was carried out by the Institute of Forest Genetics in the areas of Styria, Lower Austria, parts of Salzburg and Upper Austria. Around 300 clones were collected, 200-250 of which successfully rooted and tested positive for 'purity' in laboratory. This lot, supplemented with a few more clones already collected and a few seedlings from previous years, will form the future Austrian core collection of *P. nigra*.

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## Belgium

*Jos Van Slycken and An Vanden Broeck*

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### ***In situ* conservation**

There are no young stands or natural regeneration of *Populus nigra* occurring in Belgium so far. *Populus nigra* is only found as relict trees, almost exclusively on private properties in the neighbourhood of farms. The trees were planted and propagated several decades ago around meadows and farms for the production of firewood. They were mostly kept, together with willows, as pollarded trees. This makes the *in situ* conservation of black poplar very difficult, as it depends on the goodwill of the farmers.

*In situ* conservation, however, is possible by local authorities through legal protection as a nature monument. This procedure has been used until now by one municipality (Oosterzele).

Many of the trees are old and show damage from cattle or lightning, as well as by the increase in the scale of farming activities. This makes the *ex situ* conservation of *P. nigra* in Belgium very important.

### ***Ex situ* conservation activities**

During the last year further surveying of relict trees has been undertaken. The work aims at locating the relict trees, evaluating their vitality (phytosanitary state, dimensions, etc.), enhancing and rejuvenating the *ex situ* collection, and updating the database. The location of each tree is registered by differential GPS (Global Positioning System). Trees already included in the database and conserved in nature, as well as new individuals, were collected. Owners, mostly farmers, were informed as much as possible about the rarity of black poplar in Belgium and about the current conservation programme.

The activities undertaken in 1996 resulted in 23 new accessions in 11 different locations. For the first time, relict trees of *P. nigra* were found along the Meuse, the largest river in Belgium (Leten, Maasmechelen). Three new locations of *P. nigra* were found in the Dender Valley (Elst, Gages, Castiau), seven in the Yzer Valley (five of them were found in the same village of Woesten, two in Oost-Vleteren) and one in the Schelde Valley (Antwerpen). This brings the national *ex situ* collection to a total of about 130 trees in 40 different locations. We expect that this collection contains a certain number of genetically identical individuals due to vegetative multiplication by farmers which was, and still is, a common way to propagate poplars. For the screening of the genetic diversity of the *ex situ* collection, fresh young leaves from each individual of the *ex situ* collection were taken and are kept at  $-80^{\circ}\text{C}$ . This material will be used in the future for extraction of DNA and genetic diversity studies at the DNA level.

Duplicates of the newly collected material were exchanged with the Forest Research Institute at Gembloux (Walloon Region), so that in the near future the *ex situ* genebank will be maintained in two places in Belgium.

Apart from collecting new material of *P. nigra*, the regeneration programme of the existing genebank, i.e. *ex situ* plantations of parents and offspring from intraspecific crossings made in the past, was given high priority. This programme started in 1994.

### Actions planned in the future

Surveying of the relict population will continue. More attention will be paid to the morphological and genetic diversity of the *ex situ* collection. From each individual, a DNA fingerprint analysis will be made using the AFLP technique (Vos *et al.* 1995) and a database of the fingerprints will be set up. This will make the exclusion of identical genotypes possible and will facilitate the management of the *ex situ* collection.

The study of the genetic diversity in the collection and among the different populations (Dender Valley, Yzer Valley, Schelde and Meuse Valley) is planned for the near future. The relationship between relict trees in Belgium and adjacent countries will also be studied. This will offer important information for nature development projects where re-introduction of black poplar along the main river courses is considered. These efforts are made possible through the participation of the Institute for Forestry and Game Management in the EU-funded EUROPOP Project.

Furthermore, our Institute will study the genetic diversity of the Hungarian *ex situ* collection in the context of a Flemish-Hungarian cooperation project funded by the Flemish Community.

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## Croatia

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During 1997, selection of black poplar trees in the territory of the Republic of Croatia and the adjacent Republic of Bosnia and Herzegovina was continued. In the area of the upper river Sava, 18 trees in total were selected and propagated. The success of autovegetative propagation ranged between 15 and 67%, depending upon the clone. In the central part of river Sava, 12 trees in total were selected, and 10 were successfully propagated vegetatively. The success of rooting per clone ranged from 4 to 96%. With the assistance of colleagues from the Faculty of Forestry in Sarajevo (Bosnia and Herzegovina), in the areas of Sarajevo, Kakanj and Zenica, 54 trees were selected and 50 trees were propagated. Rooting success of cuttings of these clones varied from 4 to 60%, with their very marked plagiothropic growth.

The poplar clonal archive established in spring 1995 by the Mura river (Čakovec, Podturen) was completed this year with 27 new clones of black poplar, bringing the number to 61 clones. In spring of 1998 it is planned to complete the clonal archives with 28 additional new clones. In the Osijek area, by the Drava river, the establishment of another clonal archives is anticipated. So far, in the tree nursery Višnjevac, near Osijek, 50 clones have been propagated for the purpose of establishing this new clonal archive.

For the coming year we also plan to make a selection of the black poplar trees in the area of Eastern Slavonia and Baranja, along the Danube river. As a UNTAES zone, this area has been inaccessible until now, but its reintegration is just taking place. In this area, the best conserved specimens of native black poplar in Croatia can be found.

## Czech Republic

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There are two research institutes involved in the conservation of *Populus nigra* genetic resources in the Czech Republic, the Research Institute of Ornamental Gardening (with three research projects concerning conservation of genetic resources, genetic analyses and selection of new clones with good yield properties) and the Research Institute of Forestry and Game Management (with one research project concerning conservation of genetic resources and the central clonal archive maintained as a stool-bed). Since the last Network meeting we have continued the activities which were mentioned in the introductory country report last year.

### Plus trees of *Populus nigra*

We described 12 new plus trees in the Central Labe Basin, 6 of which were vegetatively propagated. In spring 1998 we plan to describe an additional 12 plus trees in the Vltava Valley and 21 trees from extreme sites (uplands with altitude 400-600 m), which have already been vegetatively propagated (Table 1). Surveying and designating of plus trees will also continue in the valleys in South Moravia and in the Odra Valley in North Moravia.

By surveying plus trees we discovered areas with natural regeneration. Altogether eight areas were registered, but only one is in natural conditions at a site which is sporadically flooded. Others are linked with human activity due to earthmoving works.

**Table 1. Plus trees of *P. nigra***

Area/period	Plus trees	Females	Males	Clones in clonal archives
Since 1997	204	92	112	65
Labe Basin 1997	12	4	8	6
<b>Total</b>	<b>216</b>	<b>96</b>	<b>120</b>	<b>71</b>
Plan for				
Vltava Valley	12			
Uplands	21			21

### Planting adult tree plots

In 1997 a stand of 0.5 ha was established with seedlings from controlled crossings as well as clones in the river Morava Basin. Next year we plan to establish a 1.5 ha stand in the Vltava Valley with seedlings from controlled crossings carried out in 1996 (Table 2).

This year we carried out controlled crossings with two objectives: one is to conserve genetic resources of regional populations, the other is to obtain clones with good yield properties. For this purpose, we used plus trees from various areas from lowlands to uplands, where we expect to find genetic variability due to different selection pressure. The seedlings will be used for planting additional research plots.

**Table 2. Controlled crossings of *P. nigra* plus trees**

Year	Parents		Objective	Combinations		Seedlings	
	f	m		Total	Successful	Total	Successful
1996	8	6	conservation	38	21	–	1500
1997	9	14	conservation	21	14	800	600
1997	9	14	selection	32	20	2400	1700

### Genetic analyses

Enzyme systems for easy identification *P. × euramericana* (6-PGD, LAP, PGI), *P. × berolinensis* (ACO) and *P. trichocarpa* (PER) were chosen. These enzyme systems were also tested for identification of introgression between *P. nigra* and *P. × euramericana* and *P. nigra* and *P. × berolinensis* from controlled crossings. The knowledge obtained was used for analyses of young populations of *P. nigra* (two populations from areas with natural regeneration, three populations from open-pollinated progenies). In these populations 0-10% of introgressive hybrids were detected.

## France

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### Conservation tasks

The National Commission for the Conservation of Forest Genetic Resources has prepared a 'charter' to be signed by all partners involved in the conservation of forest tree genetic resources. This document defines the general scope of the programme and operational organization. The conservation of *P. nigra* is included in that national programme.

The collection of clones, initially set up by INRA, has been transferred to the administrative nursery of Guémené Penfao and a new stool-bed of 251 accessions was planted according to Network's guidelines (de Vries 1996). A new *populetum* (conservation adult tree plots) was also planted in Bourret, with 215 accessions.

The collection is now split into (1) active collection, and (2) complementary collection. The active collection is conserved both in stool-beds and as adult tree plots, submitted to evaluation process in the nursery and through laboratory techniques. It presently contains 262 clones, and it will be increased to 500 clones in the coming years. The sampling strategy allows 1 to 5 clones per collecting site, according to the original population size. The active collection will be maintained at 500 accessions, but adjustment will be allowed either to include new origins or to improve the representation of some origins according to new information on the organization of genetic diversity. The complementary collection is only conserved as adult tree plots, and may be used as a 'reservoir' of genetic resources in the future.

### Research activities

Cultivated hybrid poplars can interact with wild *P. nigra* in different ways. One is the introgression process, another interaction possibly involves parasites. *Melampsora* rust populations are evolving rapidly, following the evolution of the set of cultivated clones with race-specific resistance (Pinon and Frey 1997). From another point of view, the natural co-evolution processes with *P. nigra* led to different *Melampsora* rust populations in the wild riparian sites. In a collaboration between pathologists and geneticists, we studied the diversity of both *P. nigra* and *Melampsora* in eight natural stands along four rivers. The rust populations differed in terms of species or pathotype content. However, the poplar populations were not different, neither at isoenzyme markers nor for genetic components of partial resistance to different rust races. Quantitative components of partial resistance were under genetic control, and quantitative clone-race interaction was significant. It was concluded that differences among rust populations in wild riparian sites could not be attributed to genetic differences of the host populations, although rust can represent a significant selection pressure at within-stand level, due to the high genetic variation for resistance and the quantitative clone-race interactions (Louveau 1997).

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## Hungary

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Surveying of *Populus nigra* occurrences continued, consisting of the selection and registration of additional individuals and smaller populations in northwestern Hungary (areas under the influence of the Danube) and in eastern Hungary (mainly in the alluvial forests of the river Tisza).

Field description of *P. nigra* stands or subcompartments registered in the national forest inventory has been continued, but at a less than necessary rate because of the lack of financial means. The conclusion drawn so far is that only a small part of *P. nigra* stands registered in the management plans are true *P. nigra* occurrences.

The Ministry of Agriculture founded a Council for Plant Genetic Resources. Its tasks are as follows:

- organizing the genebank works according to international standards
- development of guidelines for the management of gene reserves in Hungary. In the frame of the above-mentioned Council, a forestry taskforce is aiming at forest gene conservation including that of *P. nigra*. To also promote the *ex situ* conservation of forest genetic resources, a central collection is being established at the Sárvár Research Station of the Forest Research Institute (ERTI) in western Hungary.

The establishment of the collection with selected *P. nigra* clones was started in the form of a stool-bed. A database of the clones was established. Maintenance of the existing clone collection is evidently a continuous task.

To comply with the request from the Network's core collection of clones (Casale Monferrato), cuttings from two clones were repeatedly sent to the host Poplar Research Institute in Italy.

The National Institute for Agricultural Quality Control in Hungary prepared for the investigations of samples taken from *P. nigra* genotypes in order to identify black poplar individuals. The RFLP technique was applied in collaboration with Dr B. Heinze, FBVA, Vienna, Austria, but due to insufficient financial means, only in limited quantity.

*In situ* gene conservation of *P. nigra* is promoted by the recent Act on Nature Conservation adopted in Hungary in 1996 (see Report of the previous meeting).

The establishment of a central *ex situ* clone collection is hindered by financial problems. The National Institute for Agricultural Quality Control put an application to the National Environment Protection Fund for a nationwide gene conservation programme. A special subprogramme deals with the *P. nigra* gene conservation. It includes the establishment of an *ex situ* collection and propagation of genetic material.

The data collection has not yet been totally finished due to financial difficulties. Colour slides have been made for the completion of the Network's archives.

## Italy

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In 1997 the activity of ISP included the maintenance of the *P. nigra* collections, as well as updating the Network's *P. nigra* database.

### ***Ex situ* collections: genebanks as stool-beds**

#### ***ISP collection***

Almost all the clones included in the ISP collection (532 genotypes) were propagated in a stool-bed in the Mezzi farm in Casale: about 30 cuttings per clone were planted.

#### ***EUFORGEN core collection***

All the cuttings obtained from the material received last year (from Austria, Belgium, Bulgaria, Croatia, Czech Republic, Germany, Hungary, Italy, the Netherlands, Slovakia, Spain, Turkey, United Kingdom and FR Yugoslavia) for the constitution of the 'core collection' were propagated in a new stool-bed in 1997 together with the cuttings obtained from the reference clones. About 800 cuttings were planted altogether.

Also the cuttings received this year from Romania and Ukraine and those re-sent from Austria, Czech Republic and the Netherlands (about 200 cuttings) were included in the same stool-bed.

The stool-beds established in 1996 were maintained so that it will be possible to collect cuttings planted in both years in 1998.

### ***Ex situ* collections: genebanks as adult trees plots**

A collection was planted with 179 clones to complete the duplication of the ISP collection as adult tree plots in another locality (near Rome).

Considering the great number of genotypes, no more than four plants per clone were planted with a spacing of 4 × 3 m; two trees per clone will be removed after several years so as to widen the spacing.

#### ***Experimental plantation***

An experimental plantation was established at Roaschia, a locality near Cuneo in the Piedmont Region, using some of the genotypes selected from the nursery tests conducted several years ago, where certain genotypes were used as parents in the artificial crossings (50 genotypes altogether). 'J. Pourtet', the only *P. nigra* clone registered in Italy for commercial use, was used as reference clone.

### **Artificial crossings**

*Populus nigra* genotypes were used in two different kinds of crossings during 1996 and 1997.

Some of the genotypes selected as best parents from progeny tests and some of the genotypes selected from nursery tests were used as parents in intraspecific crossings planned in the *P. × euramericana* breeding project. Five different families with more than 100 seedlings were obtained.

Some artificial crossings were carried out with genotypes highly resistant and highly susceptible to *Marssonina*, *Melampsora* and *Phloeomyzus passerinii*, to evaluate the behaviour of progenies towards diseases and insects both in the field and in the laboratory (with molecular markers). Six different families with over 200 seedlings were propagated in the field.

### European *P. nigra* database

In addition to the information about the *ex situ* collections in France, Italy, the Netherlands, Spain and Turkey, data regarding the clones maintained in Austria, Belgium, Croatia, Hungary, United Kingdom and FR Yugoslavia were included in the *P. nigra* database during 1997 (1960 entries in October 1997).

Similarly the information regarding the clones sent to ISP for the constitution of the core collection was included in the database.

Although for most of the clones in the database a lot of the information (passport data) is available, for some genotypes only the name and the country of origin are known.

### Environmental restoration

Three genotypes of *P. nigra* of Piedmont origin (about 50 plants) and some genotypes of *P. alba* (about 120 plants) were planted together with some other riparian species for the re-establishment of a riparian forest within the Park of the river Po.

### Legislation

No specific legislation about *P. nigra* was proposed, but some recent regulations regarding rivers and poplar cultivation might be relevant to *P. nigra* conservation.

According to the national Law n.37 approved in 1994 (Regulation for the environmental protection of rivers, torrents, lakes and other State waters), the right of pre-emption on the use of land abandoned by rivers as a consequence of river dynamics was shifted from the owners of the adjacent land to public authorities or consortia provided they use the land for nature conservation, environmental restoration or other nature protection purposes. Land use for other purposes (including poplar cultivation) is subject to the approval by a provincial commission.

A plan regarding fluvial areas was approved in 1996 by the Po river Authority. This plan classifies fluvial areas into three zones on the basis of the risk of flooding. As for the first zone, i.e. the area next to the river and subject to regular flooding, a proposal is at present under discussion which would put severe restrictions on poplar cultivation along several tributaries of the river Po.

## Russian Federation

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### Current distribution of black poplar in the European part of Russia

As a tree of lowlands, black poplar grows in the European part of Russia between the rivers Severnaya Dvina in the north and Terek in the south, and Seversky Donets in the west and the Urals in the east. Its northernmost border at 63°N latitude is of particular interest (according to V.H. Sukachev, see Lositsky 1955). In Siberia black poplar reaches 64° latitude in the lowlands of the Enisei river. The northern border of the black poplar range in Europe recently has been defined more precisely by Skvortsov and Gadirca (1986). The northernmost black poplar occurrence has been discovered on one of the islands on the Severnaya Dvina river. It seems strange that in the most inclement climate, black poplar can almost reach the White Sea, but in the west, it sharply deviates to the south. It is assumed that the northern border of this species' range from northeast to southwest coincides with the contour of the so-called Wurm glacial cover.

### Modern tendencies in black poplar stands development

In spite of many useful properties and incidentally undertaken protection measures, there has never been a special gene conservation programme for black poplar in Russia (see Report of the previous meeting). Generally, some unfavourable tendencies are currently recognized: areas under black poplar stands are reducing; the stands are threatened by pests and diseases; the productivity is declining.

In 1955 Prof. K.B. Lositsky wrote that black poplar was one of the most disease-free and pest-resistant species. In the area of the middle Volga river, the mean growing stock of black poplar mature stands reached 824 m<sup>3</sup>/ha (Table 1), an annual increment of nearly 20 m<sup>3</sup>/ha. Black poplar was considered to grow from 1.5 to 2 times faster than aspen.

**Table 1. Some characteristics of black poplar stands in the middle Volga river area (Samara-Saratov-Volgograd regions)**

Characteristics	1950s	1990s
Area (ha)	92 100	36 537
Growing stock of mature stands (m <sup>3</sup> /ha)	400-824	172-240
Mean stand density of stocking	0.74	0.56
Prevailing site quality classes	1-1a	3-4
Phytosanitary conditions	Healthy, disease-free stands	Healthy stands only in younger age classes

The situation has worsened since the end of the 1960s (Kargov 1968). Black poplar stands have been increasingly declining. Their areas, stock, mean stand density stocking, prevailing site quality classes and overall phytosanitary condition were lowered (Shatalov *et al.* 1984).

One of the main causes of black poplar decline is the regulation of river flow (see Table 2). Many poplar stands existed only because of additive flooding. The regulation of flooding led to an increase of natural aridity. On the other hand, many poplar stands disappeared owing to man-made hydroelectric power station dams. For example, the Republic of Mari-El lost all poplar stands for this reason. Because of river flow regulation, the period of winter flooding in the zones of low-level black poplar stands is doubling, having a negative impact on the stands (Nevidomov 1993).

**Table 2. The main causes of the current black poplar decline in Russia**

1. Changed flood dynamics due to hydroelectric power stations
2. Increase of the total aridity because of flow regulation
3. Decrease of sediment particles in the water (due to their accumulation in dam ponds), decline of alluvial process
4. Doubling of winter flooding period in the zone of low-level black poplar stands as a result of flow regulation
5. Air pollution
6. Global climate changes.

Other reasons for black poplar decline are air pollution and climate change. As a result of all these factors, according to our latest data, black poplar stands in 14 regions of European Russia have been completely lost, and in others are greatly reduced (Table 3).

**Table 3. Changes in the area (in ha) of natural black poplar stands in Russia, 1955-97**

Region	1955	1984	1997
Astrakhan	45700	13520	~7000
Bashkortostan	15500	–	588
Tatarstan	6500	–	800
Samara	21100	10560	190
Saratov	35700	–	7683
Volgograd	35300	18420	28854 <sup>†</sup>
Uljanovsk	11300	–	flooded
Voronezh	–	1985	1552
Rostov	–	3913	2139
Orenburg	–	25092	27400
Krasnodar (Kuban)	–	760	5337
Adigeya	–	–	3326
Chuvash reg.	–	–	704
Belgorod	–	–	21
Kursk	–	–	6
Chelyabinsk	–	–	23
Lipetsk	–	–	41
Ryazan, Vladimir, Nizhny Novgorod	–	3156	lost

<sup>†</sup> Black, white and hybrid poplars.

### Current tasks of black poplar gene conservation

Inventories of natural resources of black poplar would be the first step for providing an insight of the level of threat to the species. Such an inventory must be planned for some regions and subregions at the level of local administration, national parks, reserves, forestry departments and divisions. The distribution of black poplar in the plains of European Russia offers an ideal possibility to study and preserve its diversity along the geographic gradient (clinal patterns) from north to south.

It is necessary to maintain populations of the species with considerable morphological variation (burl forms, flushing, etc.) in the areas of Volga, Don, Terek and other rivers. Methodical training and instruction of collaborators for field surveying and identification of black poplar stands are very important. The exploration of introgression and its consequences are also of great importance. Black poplar was actively used in Russia and the former USSR in breeding programmes as a parent of some hybrid varieties, such as 'Pioneer', 'Russky', 'Michurinets', selected by acad. A.S. Yablokov and others. Many experimental plantations and genebanks have been established in breeding institutions (stool-beds, adult tree plots and others). A revision of the plantations and genebanks network as *ex situ* conservation units is needed.

A particular problem is the regeneration of old collections. It may be necessary to establish protected areas, especially for black poplar, and take legal and technical measures to restrict the plantation of other poplar species and hybrids in the vicinity of such protected areas. The scientific programme of black poplar conservation must include studies of its population genetic structure, geneflow, vegetative *versus* sexual reproduction of native stands in different environments, and others. However, all of the above measures require corresponding funding.

An example to illustrate the scattered distribution and the difficulties faced can be given from the Volgograd region, where black poplar grows in 33 forestry enterprises. Every enterprise includes from 10 to 15 divisions. Each of these forestry divisions comprises several hundred compartments, where black poplar is represented by 1 to 10 units. The area of one compartment ranges from 0.1 to nearly 50 ha, depending on relief, landscape and other local peculiarities. For example, in one such forestry division belonging to the Sredne-Ahtubinsky forestry enterprise, black poplar grows in an area of 706 ha in 301 compartments.

A general programme of black poplar conservation is lacking in Russia. The breeding institutions fulfil their specific breeding tasks. Therefore, at a national level, we first need to coordinate funding, inventories and research on the amount and structure of genetic diversity of the species. The minimum task is to identify and preserve an adequate sample of this diversity.

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## Slovakia

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The project on the conservation and reproduction of black poplar was continued. During the year, 27 plus trees were selected in the lower part of the river Morava, in the central Váh region and in the East Slovakian lowland. Reproductive materials taken from the plus trees were rooted in the poplar nursery.

A subpopulation from intraspecific hybridization *P. nigra* '5' × *P. nigra* '74' was evaluated for qualitative and quantitative parameters. The results will be published in 1998.

Inventory of an experimental plot with high representation of *P. nigra* (0.5 ha) was conducted which is part of a larger gene reserve. The possibilities for natural regeneration were investigated in the lower part of the river Hron. A central stool-bed in Gabčíkovo was inventoried.

## Spain

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### Survey

During the last year our activities with regard to surveying of new materials focused on the Ebro Valley in the autonomous province of Navarra where there are approximately 170 ha of Natural Reserves of riparian forest type. *Populus nigra* grows within these Reserves.

Surveying in the autonomous provinces of Castilla-Leon, and particularly in the Duero Valley, has shown a very different scope. Throughout this area, the riverbank forests are practically disappearing because of the prolonged human activities. Nowadays, these areas have been replanted with new clones and very often *P. nigra* grows mixed with other cultivated poplars. We catalogued a few reduced stands with *P. nigra* in spite of the scarce surface of riverbank forest stands and the difficulties in identifying the clones.

### *Ex situ* conservation: clone collection

We continued with the collecting. Twenty-three clones from the newly surveyed area in Duero Valley were collected.

At the SIA-DGA nursery, the activities aimed at *ex situ* conservation were continued. During the last year, a stool-bed was established with 42 clones from the existing collection that maintains a total of 110 clones. The remaining clones will also be used for the establishment of stool-beds later.

In collaboration with a LIFE Project to restore riparian areas, some clones were propagated in a nursery to be used in the re-establishment of the riparian vegetation.

### *In situ* conservation

There is no conservation *in situ* in the strict sense of the word. At the most there are Natural Protected Areas, which protect the wildlife and particularly the birds; however, any management is excluded. Some trees are catalogued as singular individuals, but no special regulation for their protection has been applied.

## United Kingdom

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### Research

Clone collections established at three sites in East England and Wales contain a total of 76 clones. In the winter of 1997, efforts will be made to ensure that all the clones are established at all the sites. As soon as sufficient material is available, a stool-bed collection will be established at Alice Holt. It is clear that there is much duplication in this collection and, as soon as sufficient information is available from DNA studies, the collection will be rationalized to maximize the number of distinct genotypes.

Cottrell *et al.* (1997) used RAPD markers to study 36 accessions from this collection and found only 17 distinct genotypes. Genotypes were local in their distribution and genetic diversity was low. These authors also concluded that there had been so much interference by humans that there are unlikely to be distinct Eastern and Western types. In a more concentrated study of black poplar in the Upper Severn area, Winfield *et al.* (1998) used AFLP analysis to examine genetic diversity in 146 individuals and 3 individuals considered to be non-*betulifolia* poplars. Genetic diversity was low, confirming the results of Cottrell *et al.* (1997). There was a general correlation between geographic proximity and genetic similarity. They concluded that it was possible to identify a small number of individuals showing maximum diversity for inclusion in a replanting/conservation programme.

Of the 36 trees sampled by Cottrell *et al.* (1997), only 6 were female, and DNA analysis of these revealed that there were only two distinct genotypes, despite the fact that they were sampled from a wide geographic range.

The ERMAS I programme was established in 1992 to increase our understanding of the processes controlling the structure and function of European river margin ecosystems through a Europe-wide network. ERMAS II focuses on the role of biodiversity in determining the sensitivity of river margin ecosystems to environmental conditions, particularly temperature and hydrology (see Tabbush this volume).

### Implementation

A group of researchers and interested parties from local authorities meets once per year to consider the national strategy and to coordinate the research. A national database of *in situ* preserved trees has been established and is being maintained through the Botanical Society of the British Isles (BSBI). Trees are selected on morphological characteristics by a BSBI referee. Large, old trees are most likely to be selected as true black poplars.

Since many of these old trees are in imminent danger of collapse, the main conservation measure is to take cuttings, to preserve the genotype. However, this programme is not well resourced, and is often only recorded at local level. There is a danger that knowledge of the existing distribution and variability of these trees will be lost.

### Awareness

The initial press campaign resulted in much local interest and this has enabled the collection of data about the distribution of the existing trees. There is a high level of awareness about the importance of the old trees, at local and national levels, but as yet this has not been translated into funding for a concerted campaign to study and conserve the genetic diversity of the remaining resource.

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## Moldova: Ecogeographic variability of *Populus nigra* L. in Moldova and the former USSR

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### Introduction

Black poplar grows rapidly and mature stands reach the productivity of about 28-32 m<sup>3</sup>/ha annually. Mature stands are generally considered to be 20-25 years old. From this point of view, the economic importance of black poplar is obvious.

Black poplar extends over a very broad geographic area. We investigated different populations from the Arctic Ocean to the Caucasus. We presumed that there is an ecogeographic variability correlated with climatic conditions in this area. This contribution summarizes the results of a study conducted within a comprehensive project on geographic variability of black poplar in the former USSR.

### Materials and methods

The study used bibliographical data and different herbaria from Moscow, St. Petersburg, Kiev, Arkhangelsk, etc. In accordance with data studied, the first information about black poplar in the eastern limit of its area is from the middle of the 17th century, from the end of the 19th and first part of the 20th century. We studied black poplar in the following geographic regions:

- Arkhangelsk, Severnaya Dvina river
- Republic of Komi, Vyceгда
- Ulianovsk, Volga
- Volgograd, Volga
- Moldova, the river Nistru
- North Caucasus, the river Terek
- Transcarpathians, Uj and Tisa rivers.

A comparative trial with the different provenances was created at the Great Botanical Garden (Moscow). Over a period of 3 years we studied the adaptation of black poplar in these conditions.

### Results of the study

Only the populations which grow in the conditions of inundation were studied. We observed that the natural regeneration from seeds had occurred only in one case, on the bank of the Hoper. Generally, natural regeneration of poplar is very rare but represents a special interest because the forests which grow from seeds always have better development.

We carried out a special investigation of black poplar in the northeastern part of the distribution area, in the Arkhangelsk region and the Republic of Komi. The northern limit in which black poplar grows was defined more precisely. At the same time we observed that black poplar grows satisfactorily in northern conditions, even at 62-64° latitude.

The geographic variability was studied by analyzing the comparative trial established in homogeneous conditions at the Great Botanical Garden.

***The first experiment***

In 1983 we planted material from four geographic regions. Better growth was reached with the plants collected from the centre of distribution (Ulianovsk) as seen in Table 1. The levels of variation in all cases are relatively uniform.

**Table 1. Growth characteristics of 3-year-old trees (1983-85) – first experiment**

Origin	Height (cm)			Diameter (mm)		
	min.	avg.	max.	min.	avg.	max.
Caucasus	162	226	292	14.0	20.2	24.8
Moldova	167	260	348	14.2	22.3	38.9
Volgograd	141	211	289	10.3	16.3	21.2

***The second experiment***

In 1984 we planted materials from all geographic regions. In general, the second experiment confirms the results of the first experiment (see Table 2).

**Table 2. Growth characteristics of 2-year-old trees (1984-85) – second experiment**

Origin	Height (cm)			Diameter (mm)		
	min.	avg.	max.	min.	avg.	max.
Caucasus	117	191	223	14.0	21.4	24.8
Transcarpathians	163	213	270	16.4	25.2	54.5
Volgograd	115	149	187	8.3	13.6	14.0
Ulianovsk	212	278	306	17.2	22.9	27.8
Komi	47	74	119	8.8	11.4	17.7
Arkhangelsk	49	71	93	7.6	10.1	12.5

During our investigations it was observed that the plants collected from the south were partially damaged by frost. Therefore, we carried out a supplementary study of the structure of vegetative buds of plants which developed from cuttings and from seeds.

A special study was dedicated to sexual dimorphism, but no differences between female and male plants in relation with the vegetative traits were observed.

**Main conclusions**

Black poplar was studied in a vast area of about 2300 km wide with rather uniform geomorphological conditions. This gave an ideal opportunity to study the geographic variability correlated with the changes of climate. The investigations led to the following conclusions:

1. The plants collected from the south, being introduced to central regions and hence moved to the north, were damaged by frost. The plants from the north finish their vegetative cycle earlier and have a slow growth; the best growth performance was shown by the local genotypes.
2. The variation observed has a normal distribution.
3. The vegetative buds of plants which grow from cuttings are more sensitive to unfavourable winter conditions than buds of plants grown from seeds.
4. Black poplar grows satisfactorily under extreme conditions. This may be confirmed with the state of some natural populations from the northeastern margins of the distribution area.
5. In accordance with our investigations it was impossible to determine sex in relation to the vegetative traits.

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## Malta: The case for extending the scope of the Network to include *Populus alba* L.

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### White poplar (*Populus alba* L.)

It is believed that the ancient Maltese dedicated this tree to Hercules, but after their conversion to Christianity in AD 60, poplar (*luq* in Maltese) was looked upon as a sign of gaiety and happiness. In fact, historians of the Middle Ages describe how on Mnarja – the farmers' festival held annually in Buskett woodland – the menfolk used to pluck branches of poplar from this woodland to take away with them. Mnarja, broken down from the Italian word "luminaria" or "lume" (in Latin) meaning *illumination* was so-called because of the number of bonfires the revellers made for cooking and for lighting the night festivities. The populace was allowed in this woodland only once a year on the eve of St. John's feast, which falls on 29 June, by the ruling Order of St. John, better known as the Knights of Malta.

### The tenacious poplar

There are no natural stands of *Populus nigra* in Malta, although a few specimens were imported and cultivated. However, there are a few small stands of *P. alba* growing in the valleys as true natives. The largest colony is at Buskett in a valley carrying the name of the poplar in the vernacular, i.e. *wied il-luq*. As is expected, poplars are found in areas where the soil is rather deep (as in the case of silt and soil washed down into the valley from higher ground, due to soil erosion) and where water is available for most of the year. The ability of the white poplar to sucker freely has enabled it to service our heavily populated country (1037 persons/km<sup>2</sup> excluding the annual 1 million tourist arrivals). Moreover, its ability to sucker freely after a fire has ensured its survival on an island, situated in the middle of the Mediterranean, from Phoenician times to this century. Invaders, conqueror pirates, slave traders and mercenaries all have two things in common, i.e. the sword and fire. Burning away what could not be pilfered had its effect on Malta's woodlands. Grazing of goats, the poor man's cow, also took its toll on poplars, but since the Buskett woodland was "out of bounds" to the population for several hundred years, the ribbon stands of poplar along the valley embankment thrived well.

### Urban and rural landscaping with *P. alba* as a component

Seedlings of poplar raised from cuttings, and from suckers taken from these stands in winter over the last 30 years or so by the Department of Agriculture for propagation purposes, have extended the poplar population to various streets, housing estates and other urban plantations, where tall, fast-growing trees were needed to screen or scale down tall buildings. This species is also included in afforestation schemes involving valleys. Its qualities of bright, shimmering leaves in summer, bark colour and fast growth are being exploited more and more by the landscape gardener. Lack of knowledge of the capabilities of this species and particularly its vigorous growth sometimes leads to problems with services, e.g. water pipes, sewage, etc. Attention is paid to avoid planting stands close to agricultural land because of the suckers which may intrude into the fields, thus drawing complaints from farmers.

### Legal protection

The Structure Plan for the Maltese Islands (1990) followed by the Environment Protection Act of 1991, offer a basis of protection for, among other things, the poplar stands growing in the wild.

Moreover, the proposed "Trees and Woodlands (Protection) Regulations", awaiting endorsement through Parliament, have listed *P. alba* and *P. balsamifera* under Schedule I for strictly protected trees.

### Proposal

Malta proposes that this Network extend its work to cover *P. alba*. Much work has already been carried out on this species by various countries in the 1950s. However, there is scope for streamlining and updating this work as has been done for *in situ* and *ex situ* conservation of *P. nigra*. The economic value of this species is well known, but one must also see to the preservation of the genetic diversity within the existing stands. The tissue culture facilities available in the countries participating in the Network could be included in conservation work in favour of this species.

## Black poplar (*Populus nigra* L.) in Poland

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The black poplar, an Eurasian species with a very broad range of distribution, occurs in almost all parts of Poland. The northern limit of its range passes across Poland. Black poplar can be found in many places beyond this limit, but its natural stands are distributed in southern and central parts of Poland along major rivers such as the Vistula, Bug, Odra, Warta and their tributaries, but not eastern and western Pomerania. It grows along river banks, very often on flooded alluvial terraces together with willow, creating the characteristic forest association *Saliceto-Populetum*. Up to now, smaller and larger areas of a poplar-willow forest are preserved in groups and as single trees. In the Vistula valley, especially in its central part where the river is still almost wild, *P. nigra* is better preserved than on the river Odra, which is a more regulated river.

Along the Vistula valley, old clumps of the species meet old riverside forests on backwaters and islands. Small and large clumps occur near Ostomecko, "Tokarska" near Plock. From these natural stands the black poplar trees spread onto neighbouring land where they are cultivated, sometimes mixed with other poplar species and hybrids on meadows, banks, along roads and in villages and towns. The distribution of black poplar in Poland is described in many floristic papers, but primarily in the "Atlas of distribution of trees and shrubs in Poland" Part 8 "*P. nigra*" by K. Borowicz and M. Gostyńska-Jakuszczyńska (1969).

Ecotopes of poplar stands are continuously under transformation by natural processes (changes of courses of unregulated rivers) and civilization pressure (control of rivers, expansion by farming and forest plantations, urbanization, river transport, etc.). These transformations in Poland, however, are at a lower level compared with western Europe, mainly due to economic problems and opposition to the regulation of rivers by nature conservation movement. Many plans for regulating the Vistula, such as building reservoirs, dams, polders and cascade systems, have not yet been carried out, but are planned to prevent flooding and to keep water to the correct deficit in the future.

It is necessary to preserve black poplar, in its natural ecotopes. Up to now there are no special collections of *P. nigra* as representatives of populations. Towards the end of the 1970s, the surveying, selection and registration of trees in natural stands was conducted by the the author. Many of them are still preserved to this day.

## Conservation and breeding of black poplar (*Populus nigra* L.) in the Ukraine

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Of the fast-growing tree species, black poplar (*Populus nigra* L.) and its hybrids are the promising source of pulpwood and other timber products. In the Ukraine, only 14% of which is covered by forests, this species has been used in breeding programmes for a long time.

*Populus nigra* occurs as scattered individual trees or small groups of trees over the whole territory of the Ukraine, excluding mountain regions. There are pure and mixed stands of black poplar located along the rivers on plains.

Over the last 10-15 years, systematic surveys of poplar stands have been made by collaborators of our Institute. Over 8200 ha of poplar stands including more than 3500 ha of *P. nigra* stands were surveyed. Unfortunately, pure natural *P. nigra* stands were not found. Most of the poplar stands are artificial plantations; the origin of the rest is unknown. The stands are on average 25-35 years old. They were planted during the campaign which was carried out in the 1960s. These plantations belong to the commercial forests, and thus most of them have already been felled. The rest of the stands are, on the whole, in a poor state. Most of the trees show pronounced damage and there are many epicormic shoots on the trunks.

At the same time, about 30 plus trees were chosen within the stands. These trees were propagated vegetatively to the clonal archives and some of them were propagated by seeds in the collection plantations.

Much prominence was given to obtain the productive resistant individual trees, which were used for planting. This large programme was carried out in the 1950s and 1960s under the supervision of Prof. N. Starova. In 1959 the breeding programme, composed of 10 breeding centres and 17 variety-testing points, was organized (Starova 1962, 1980). This system covered almost all the climatic zones of the Ukraine. Its tasks were:

1. to find, select, propagate and test native productive and resistant forms of poplar species and their spontaneous hybrids
2. to obtain, test and propagate productive artificial hybrids.

According to the first task, about 250 plus trees were chosen including more than 40 trees of *P. nigra*.

The main activity concentrated around the second task because interspecific hybrids often have more valuable features than their parents. During a 10-year period, over 460 crossings were made and more than 600 000 hybrids were obtained, from which about 900 plus trees were chosen. These trees were tested in preliminary trials and over 40 varieties were selected, more than 10 with *P. nigra* as one of the parents. A part of these clones has been included in the Register of certified varieties of plants of the Ukraine. The rest are being tested.

Simultaneously, studies of flowering and fructification were carried out. Forming of flower buds, stamens, pistils and ovules, micro- and megasporogenesis, male and female gametophytes, pollination, fertilization and fructification were investigated, as well as inheritance and early diagnostics of sex. As one of the outputs of these studies, the sexual dimorphism of seeds and seedlings was recognized. The male plants grow from light-pink seeds (colour is estimated using a binocular), female plants grow from light-yellow seeds. The analogous pattern is also shown for colour of hypocotyles. Similarly regular

patterns were recognized for *Populus tremula*, *P. alba*, *P. canadensis* and *P. pyramidalis* (Vasylenko 1970, 1971).

The Ukraine has no specific legislation for the protection of poplar stands. A part of poplar stands is included in protected forest communities (water-protective forests etc.). The majority of poplar stands are in managed forests. Natural regeneration of poplar forests is common practice.

At the same time, a substantial genepool of plus trees is stored in clonal archives. Such plantations were created at each breeding centre or at each testing point. Unfortunately, financial support for these units is now drastically decreased. Thus, we risk the loss of these units owing to the financial constraints, the selection of new plus trees is also reduced. Now the main task of our activity is to maintain these units with minimal losses.

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## Conservation of black poplar (*Populus nigra* L.) in Yugoslavia

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The group of 'European black poplars' in Yugoslavia includes several described species: *Populus nigra* L., *P. pubescens* (Parl.) Jov. et Tuc., *P. pannonica* Kit. et Bess., *P. metohiensis* Tuc., and occurrences of spontaneous, simple and complex hybrids also have been recorded. All these species occur in Yugoslavia naturally, the former three species in the north (Vojvodina Province), and the fourth in the south. The area of distribution of domestic black poplars is smaller and narrower than that of aspens and white poplars. Black poplars build pure stands (*Populetum nigrae*) and they also form part of the Slavonian oak, white poplar and white willow forest types, as well as of a large number of shrub willows in the pioneer stage of development of these communities (Herpka 1963; Jovanovic and Tucovic 1965). The distribution of black poplar is connected with the distribution of sandy alluvial soils on which poplars have no competition. Pure and mixed young stands usually inhabit the higher reaches of sandbanks, and only rarely the lowest parts of alluvial deposits, where young growth of black poplar occurs in a mixture with white poplar and willows (Herpka 1963). In Yugoslavia, only a small portion of European black poplars has been preserved. There were better and more handsome specimens in stands that existed up to the 1970s. Today, the stands of domestic black poplars have been greatly reduced in favour of Euramerican poplar, or else they have been destroyed in the course of the river regulation works, or during the development of water storage facilities. In addition, European black poplar is slowly disappearing because of its susceptibility to bark diseases (*Dotichiza populea*) and leaf diseases (*Melampsora* spp. and *Marssonina brunnea*). Under natural conditions, they mostly reproduce by seeds, which results in frequent occurrences of spontaneous hybrids.

The works on the conservation and development of the genepool of European black poplar (*P. nigra*) in Yugoslavia were parallel to the programme of poplar breeding, namely the creation of new poplar cultivars, carried out by the Poplar Research Institute in Novi Sad. In this programme, European black poplar genotypes were used as male partners in controlled crossings, mainly with the genotypes of *Populus deltoides*.

The main focus of the programme is *ex situ* preservation. Within this goal, 60 trees have been selected since 1993. The cuttings were planted in lines. Thirty-nine genotypes were successfully propagated and they have been maintained in genetic collections (Table 1). These genotypes were examined for their susceptibility to leaf diseases (*Melampsora* spp. and *Marssonina brunnea*). The results showed great variability in this respect, i.e. that the collections include genotypes ranging from very susceptible to resistant. The research performed by Orlovic (1996) shows that European black poplar genotypes have a stable behaviour pattern regarding several anatomical, physiological and morphological characters in various sites, which has an enormous significance for further breeding.

It has been planned to establish plantations from these genotypes with a significant potential for seed production, i.e. the source of new combinations.

In Yugoslavia the total area of poplar plantations is about 31 000 ha. About 400 ha are natural areas of European black poplar and white willow.

Table 1. Number of selected trees of black poplar included in clonal archive

Year of collecting	No. of selected genotypes	Included in clonal archive
1995	10	2
1996	10	2
1997	40	35

The largest areas of European black poplar suitable for conservation *in situ* occur in the northern part of Yugoslavia (river Danube, region of Apatin). This is an area of about 200 ha covered by natural populations of this species. Activities have been started to protect this region completely and to exclude it from regular commercial management.

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## Presentations

### Dynamic processes in riparian ecosystems – implications for *P. nigra* gene conservation strategies

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#### **Summary**

This paper reviews existing research on floodplain ecosystems and considers the research needs of genetic conservation, contrasting the need for studies of genetic diversity for *ex situ* conservation with the need for studies of genetic adaptation in relation to ecosystem dynamics to guide *in situ* conservation strategies.

#### **Introduction**

In setting about the genetic conservation of forest trees, it is perhaps surprising to find that black poplar has been seen as a good place to start. Reasons for seeking to conserve it are:

- its natural habitat – the floodplain forest – has been lost to agriculture over large areas of Europe, and is still being eroded
- it is easy to propagate by cuttings
- it is one parent of the commercially important hybrid *Populus* × *canadensis* (syn. *P. euramericana*) (*P. deltoides* × *P. nigra*)
- it is highly resistant to bacterial canker (*Xanthomonas populi*) and shows some resistance to other important diseases of poplar
- on a European scale, poplar is highly significant economically.

Existing populations therefore certainly contain genes of commercial value, but there is also a wealth of genetic variation of which we are unaware, and which may be used one day by tree breeders to counter adverse environmental changes, or new pests and diseases.

Relict populations which no longer reproduce sexually will contain less genetic wealth than populations which continue to evolve naturally and continue to be subject to selective pressure. The natural habitat of poplar is in the highly dynamic riparian ecosystem in which successful genotypes will have developed strategies to deal with fluctuating water tables, silting and flooding. If such systems could be conserved, they would offer the best place to look for adaptive traits as a source for tree breeding.

#### **Conservation strategies**

Relict populations remain threatened by competition for land use (White 1993). The introduction of cultivars and hybrids, over almost two centuries, means that young specimens may contain genes which are foreign to the local type. Therefore, one strategy is to take cuttings from older individuals to keep in *ex situ* genebanks either as stool-beds, or to grow into mature trees.

Where actively breeding populations still exist, relatively free from genetic pollution from imported stock, it may be possible to establish an *in situ* reserve of much greater value, in that new successful combinations of genes through natural selection and new mutations are still emerging.

In the absence of clear information about the genetic make-up of a given population, and about the origins of these genes, it is difficult to be clear about whether the

population is "native" or not. Using analysis of chloroplast DNA, Ferris *et al.* (1997) were able to distinguish an East Anglian population of pedunculate oak from the many introduced specimens, and also found a marker which distinguished eastern and western European populations of both species of oak. This also showed that a number of very old oaks in Britain were in fact introductions from eastern Europe. Thus it is difficult to define the boundaries of a block of genetic material which is to be conserved, unless we can be clear about its taxonomic boundaries based upon molecular genetic information.

### Relevance of riparian systems and processes

Riparian systems are characterized by enormous ecological gradients along their length, and by rapid and dramatic changes in environment caused by flooding or by changes in the course of the river, by snow melts and by debris carried down by the river. This is the habitat to which poplars are specifically adapted, regenerating on elevated shingle-bars and mudflats in the braided streams of meandering rivers. The intense selection pressures of this environment have moulded the genetic structures of the poplars which ultimately the river leaves behind in the relatively stable environment of the floodplain forest.

An excellent description of this progression is given in Peterken and Hughes (1995). These authors subdivide floodplain woodlands into four types:

1. Pioneer stands of fast-growing poplar and willow on recently deposited sand and shingle.
2. Alder-dominated mixtures in peaty depressions in extinct channels and back-swamps.
3. Mixed elm, oak, ash and alder with scattered poplar and willow on well-drained mineral soil, sometimes flooded in winter.
4. Oak, hornbeam and lime woodland on the floodplain margins.

Evolution of diseases, disease resistance and biocontrol agents, and adaptations to site and climate make these systems a rich source of genetic material for biological study or as a source for tree breeding.

"River margin ecosystems offer a dynamic interface between terrestrial and aquatic systems where biological processes and biodiversity tend to be maximised. Recent evidence suggests that river margin ecosystems are highly sensitive to global, regional and local environmental changes" (introduction to ERMAS II). If we learn how to monitor dynamic processes in riparian ecosystems, this might give us a sensitive barometer with which to assess the effects of climate change.

The priority for conservation will be to conserve actively reproducing populations, in a habitat which is as 'natural' as possible, that is an *in situ* population subject to the selection pressures of an uncontrolled river system. In the following table, value to genetic conservation declines from types 1 to 4:

	<i>In situ</i>	<i>Ex situ</i>
<b>Dynamic</b>	1. Natural stands	2. Restoration schemes
<b>Static</b>	3. Relict populations	4. Genetic collections

### Current understanding

We begin with a relatively poor understanding of the genetic diversity of black poplar. The origins of the taxon are unclear, and we have little understanding of how the subspecies have differentiated, and hence where to look for the extreme genotypes within the species. Did populations of black poplar become isolated during the last ice age? Is subspecies *betulifolia* in Britain distinct from the same subspecies on the continent? To what extent is *betulifolia* distinct from subspecies *typica*?

The dynamics of poplar populations has been considered by Robert Farmer in Chapter 2 of "The Biology of *Populus*" (Stettler *et al.* 1996). He contrasts opposing processes of genetic drift and genetic 'communication' over long distances. Poplar is specialized for rapid genetic adaptation because of the following traits:

- dioecious sexual system
- small, effectively wind-distributed seed/pollen borne in high crowns
- early sexual maturity.

It exhibits strong clinal patterns of variation for photoperiodism with latitude, for frost hardiness and for drought tolerance, for example. Farmer concludes:

- geneflow has been sufficient to prevent genetic drift – geographic variation is not usually the result of geographic isolation
- adaptations are rapid and large
- studies such as that showing the adaptation of aspen to damaging low-level ozone (Berrang *et al.* 1986, 1989) point to dynamic processes that could not be elucidated simply by assessing variation in genebanks or by molecular genetic surveys. It is necessary to study dynamic processes in action in sexually reproducing populations.

### Existing research

#### ***Molecular genetic studies***

Modern molecular methods for analyzing genetic material now offer means for examining genetic diversity and defining relationships between individual taxa. Legionnet (1997) used isoenzymes to examine genetic diversity and population biology in *P. nigra* growing in France and found that there was more genetic diversity within rather than between stands. As a consequence, it would be more efficient to conserve more individuals from a small number of stands than *vice versa*. Cottrell *et al.* (1997) used RAPD markers to study 36 accessions of black poplar broadly sampled within Great Britain and found only 17 distinct genotypes. Genotypes were local in their distribution and genetic diversity was low. These authors also concluded that there had been so much interference by humans that there are unlikely to be distinct eastern and western types. In a more concentrated study of black poplar in the Upper Severn area, Winfield *et al.* (in prep.) used AFLP analysis to examine genetic diversity in 146 individuals and 3 individuals considered to be non-*betulifolia* poplars. Genetic diversity was low, confirming the results of Cottrell *et al.* (1997). There was a general correlation between geographic proximity and genetic similarity. They concluded that it was possible to identify a small number of individuals exhibiting maximum diversity for inclusion in a replanting/conservation programme.

Of the 36 trees sampled by Cottrell *et al.* (1997), only 6 were female and DNA analysis of these revealed that there were only two distinct genotypes, despite the fact that they were sampled from a wide geographic range.

**ERMAS II**

The ERMAS I programme was established in 1992 to increase our understanding of the processes controlling the structure and function of European river margin ecosystems through a Europe-wide network. ERMAS II focuses on the role of biodiversity in determining the sensitivity of river margin ecosystems to environmental conditions, particularly temperature and hydrology. There are three main tasks:

- to understand the role of biodiversity in maintaining the structure, function and stability of ecosystems
- to analyze ecosystem processes with particular reference to the organic matter cycle
- to determine and compare interactions and links between ecosystem processes and physical processes in contrasting situations, defined on two scales: climatic region and patch.

The study sites range from 64°N to 43°N, with a climatic gradient from subpolar to maritime temperate, Mediterranean and temperate continental. Partnership details for this and the following EU shared-cost project can be obtained from the Internet via CORDIS (on-line information service about the activities of the European Community concerning research and development).

***Floodplain biodiversity and restoration***

The full title of this project is "Floodplain biodiversity and restoration: hydrological and geomorphological mechanisms influencing floodplain diversity and their application to the restoration of European floodplains." The main objectives are:

- to contribute to the development of a scientific methodology for determining the flow needs of riparian plant communities on selected European floodplains
- to create effective links between the scientific understanding of the functioning of riparian ecosystems and the institutional mechanisms by which river management for conservation and restoration occur.

The main activities are:

1. To identify and quantify hydrological and sedimentological conditions favoured by riparian species for their establishment and growth. Using black poplar in the UK and France, and alder and willow in Sweden, field and laboratory experiments are investigating species' response, e.g. to waterlogging and drought.
2. To link contemporary floodplain patterns to our understanding of past climatic and land-use changes at a catchment scale and over a range of time scales. Archival studies of river flows and management practices will be linked to studies of catchment-scale riparian vegetation patterns and land-use practices.
3. To investigate the institutional framework within which river restoration projects take place and the degree to which knowledge of the functioning of floodplain ecosystems influences their implementation. An inventory of restoration projects across the European Community will be made, and the institutional frameworks involved and their knowledge of the functioning of floodplain ecosystems will be studied in the UK, Sweden and France.

**Research needs**

Against this background, it is possible to identify two broad areas of research need:

1. Studies of genetic diversity and genetic origins of black poplar, designed to guide *ex situ* conservation strategies, and in particular to increase the efficiency of those strategies by identifying individuals to conserve.
2. Studies of adaptation and ecological dynamics in *in situ* populations, concentrating on breeding populations subject to the selection pressures in dynamic river

systems. For instance, it will be important to decide how large such populations need to be to conserve the ecological and genetic processes which give rise to valuable new genetic combinations.

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## Standardized minimum list of descriptors for inventories of *P. nigra* stands

*Nuria Alba*

Area de Selvicultura y Mejora Genética, INIA, 28080 Madrid, Spain

### 1. Registration number

A code to identify the stand starting with the acronym of the Institute.

### 2. Institution responsible for the description

### 3. Name of the Recorder

### 4. Institution responsible for the database

### 5. Date

In the format YY/MM/DD

### 6. Ownership

6.1 Public: Y/N

6.2 Private: Y/N

### 7. Designation

7.1 Protected area: Y/N

7.2 Black poplar conservation area: Y/N

### 8. Clones collected: Y/N

### 9. Geographic location of site

9.1 Country (ISO code)

9.2 1st administrative subdivision of the country

9.3 2nd administrative subdivision of the country

9.4 3rd administrative subdivision of the country

9.5 4th administrative subdivision of the country

9.6 Location of site

Specify the nearest town/village

9.7 Local name

9.8 Map

9.9 Coordinates of the centre of the stand

9.9.1 Latitude

Degrees and minutes followed by N (North) or S (South)

(e.g. 40°07' N: 04507N)

9.9.2 Longitude

degrees and minutes followed by E (East) or W (West)

(e.g. 4°17' W: 0417W)

9.9.3 Elevation (m)

9.10 River

9.10.1 Main river

9.10.2 Last level tributary

**10. Population structure**

- 10.1 Forest type
  - 10.1.1 Scattered trees: pure/mixed stand
  - 10.1.2 Linear (width <50 m): pure/mixed stand
  - 10.1.3 Riparian forest: pure/mixed stand
    - Surface area (ha):
  - 10.1.4 Description of the mixed stand
    - Percentage of the surface area covered by *P. nigra*
    - Other trees and shrubs species
- 10.2 Type and number of trees
  - 10.2.1 Number of flowering trees
    - 0 0
    - 1 <10
    - 2 10-100
    - 3 >100
  - 10.2.2 Size classes
    - 10.2.2.1 Irregular: Y/N
    - 10.2.2.2 Number of even-aged cohorts
    - 10.2.2.3 Approximate size of dominant trees (h/dbh)
  - 10.2.3 Presence of remarkable trees: Y/N
  - 10.2.4 Sex ratio
    - 10.2.4.1 Females: Y/N
    - 10.2.4.2 Males: Y/N
    - 10.2.4.3 Sex ratio:
  - 10.2.5 Occurrence of natural regeneration
    - 10.2.5.1 Generative: Y/N
    - 10.2.5.2 Vegetative: Y/N

**11. State of health and disturbances**

- 11.1 Significant damage: Y/N
  - 11.1.1 Discolouration: Y/N
  - 11.1.2 Defoliation: Y/N
  - 11.1.3 Other:
  - 11.1.4 Possible damaging agent
- 11.2 Visible cultivated poplars: none/many/occasional

**12. Environment**

- 12.1 Soil type
- 12.2 Soil texture (upper 15 cm)
  - 12.2.1 Clay: Y/N
  - 12.2.2 Silt: Y/N
  - 12.2.3 Sand: Y/N
  - 12.2.4 Gravel: Y/N
  - 12.2.5 Rock fragments: Y/N
- 12.3 Soil pH (upper 15 cm)/ mixed sample)
  - 1 Acidic (pH <6)
  - 2 Neutral (pH 6-7)
  - 3 Basic (pH >7)
- 12.4 Occurrence of suitable conditions for regeneration
- 12.5 Climate zone
  - 12.5.1 FAO classification
  - 12.5.2 Average annual sum of the temperature >5°

- 12.5.3 Highest monthly mean temperature
- 12.5.4 Lowest monthly mean temperature
- 12.5.5 Total annual precipitation
- 12.5.6 Length of the dry period  
Days during which rainfall (mm) is less than double the daily mean temperature ( $^{\circ}\text{C}$ ) ( $P \text{ mm} < 2T^{\circ}$ )
- 12.6 Characterization of the water regime
  - 12.6.1 Floodings
    - 1 Annual. Winter /spring /summer/ fall
    - 2 Rare. Winter /spring /summer/ fall
    - 3 Exceptional:Y/N
  - 12.6.2 Control of the river:Y/N
- 12.7 Potentially dominant native tree species in the area beyond the river influence (maximum 2)

### 13. Ongoing management of the forest

- 13.1 Exploitation of wood: Y/N
- 13.2 Artificial plantations: Y/N

## Biochemical and molecular genetic methods available for the characterization of *Populus nigra* L.

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### Introduction

The conservation of genetic resources requires a detailed knowledge of the underlying genetic variation that causes the diversity we observe in a species. We need to know, for example, whether populations that we find in the field are very diverged from each other or not, or whether individuals within one population are very similar to each other or not. Practical measures of conservation can be based on this information, e.g. which clones to include in collections of *P. nigra*, how to restore close-to-nature poplar stands, and so on. While the assessment of visible traits is often straightforward, we do not always know about their inheritance. Genetic markers as revealed by biochemical techniques, on the other hand, allow the direct study of genes of the species. An overview on DNA marker technologies has recently been given by Karp *et al.* (1997), and in more detail by Westman and Kresovich (1997). Hayward and Sackville Hamilton's (1997) chapter deals with the interaction of population structure, as measured by molecular markers, and conservation matters. In this short overview, laboratory tests for inherited traits that (i) distinguish between *P. nigra* and other *Populus* species and hybrids (introgression), (ii) allow an assessment of genetic diversity within *P. nigra*, and (iii) distinguish different clones, will be discussed.

Genes and their alleles – encoded in chromosomal DNA – influence phenotypic traits by directing the production of RNA and proteins within the cells. Besides the chromosomes, mitochondria and chloroplasts in plants also contain a small amount of DNA. Flavell and Moore (1996) have recently given a very useful introduction to the organization of plant DNA and the components of plant genomes. Genes are strings of on average a few thousand DNA building blocks (nucleotides). There are four different nucleotides. To be translated into protein sequence, each triplet of nucleotides of the 'coding' parts of a gene codes for a specific amino acid (or a signal is given to the translation machinery of the cell), so that the DNA sequence determines the protein structure by way of determining the exact amino acid sequence of that protein. Allelic variants of a given gene differ by a small number of changes in nucleotide sequence that sometimes cause a different amino acid to be incorporated into the protein. This subtle change may alter the protein's physiological behaviour, or its metabolic properties, or simply its mobility in an electrophoresis gel. As there is redundancy in the coding of amino acids by nucleotide triplets (different triplets code for the same amino acid - there are 64 possible triplets, but only 20 different amino acids), the variation may only be present in the DNA, but not in the protein.

To analyze which genetic variants are present in an individual, one could in principle look at (i) the metabolites produced by the proteins encoded by a given gene, (ii) the proteins, or (iii) the chromosomal DNA, (iv) the chromosomes. Below, examples of such analyses in *Populus*, especially *P. nigra*, and their respective merits and problems will be mentioned.

Chromosomal DNA inheritance follows Mendel's laws. This implies that alleles of both poplar parent species will be present in first generation hybrids, but segregation will occur in later generations and backcrosses. In contrast, chloroplasts and mitochondria seem to be inherited from the female mother tree in most angiosperms.

In this paper, 'biochemical markers' will refer to metabolites of the cell other than DNA, RNA and proteins, while the term 'molecular' will refer to the latter three classes.

### Biochemical markers

Böriz (1962) analyzed leaf juice of pure *Populus* species and hybrids on large paper chromatograms. He was able to differentiate sections and species, but there was insufficient resolution in the *P. × euramericana* clones. His observations were semi-quantitative for several compounds, which makes their application in introgression studies more complicated. He also introduced two-dimensional chromatograms for enhanced resolution. A number of papers report on hybrid classification using paper chromatography. For instance, Eckenwalder (1982) established the hybrid contribution of *P. nigra* to some spontaneous trees in North America using similar methods. Malvolti *et al.* (1991) were able to differentiate between species and some groups of hybrids, but not between clones, in a study of controlled crosses of *P. deltoides* × *P. nigra*. Again, two-dimensional chromatograms were involved. Greenaway *et al.* (1991) have published extensively on the use of gas chromatography-mass spectrometry for *Populus* hybrid identification. A list of references on analyses of leaf and bud exudates is available from the author.

Summarizing these studies, it was often possible to differentiate hybrids and pure species, although it was not always possible to find the correct parents for a given hybrid. While there was some variation within species, hybrids with identical parent species often could not be distinguished from each other on the basis of the chromatograms. Moreover, none of the authors attempted to assign single phenolic compounds to the action of particular genes. However, the methods are quick and convenient, and no sophisticated instrumentation is needed for paper and thin layer chromatography.

### Isoenzymes

Isoenzyme analysis has been applied in a number of *Populus* studies (Table 1). A number of staining protocols are available that have permitted the analysis of up to 30 loci in some studies. They served for studying clone distinction, species distinction, introgression and genetic variation within a species. However, different purposes may require the application of different enzyme systems (Table 1).

Comparing results from different laboratories reveals important discrepancies in some cases – for instance introgression analysis with *P. deltoides* (Table 2). In several cases, authors directly contradict each other – GOT-4/AAT-B, 6PGDH, PGM and MDH are examples. Sometimes this may be due to limited numbers of individuals of one species available. In most cases, laboratories in North America have access only to a limited number of *P. nigra* clones (Rajora 1989a), while European laboratories are not able to sample *P. deltoides* extensively. Consequently, rare variants in one of the species may be missed, and wrong conclusions about mutually exclusive alleles may be drawn. This dilemma, in principle, applies to any marker type (see Discussion). Nevertheless, from Table 2 it appears that authors agree on the usefulness of locus LAP-A(1) for introgression analysis. At this locus, Janßen (pers. comm.) has recently detected additional alleles, including a null-allele. Other loci that have not been tested as widely include ACO-2(b) and DIA-a(2). For locus PGM-1(A) it appears that gel resolution in Rajora (1989a) was insufficient while mutually exclusive allelic bands at this locus were resolved by Janßen (1997) on starch and by Malvolti *et al.* (1991) on polyacrylamide gels. There is no consensus on the use of the 6PGDH enzyme system. Pospíšková *et al.* (1997) have also addressed the issue of introgression of the male ornamental hybrid 'Berolinensis' into *P. nigra* with isoenzymes.

Janßen (pers. comm.) has analyzed offspring of controlled crosses among *P. nigra* and *P. × euramericana* for segregation of the variants in introgression constellations.

Frequencies in accordance with Mendel's law were obtained. This means that with a limited number (4) of markers available, a small proportion of offspring (6.25%) will show *P. nigra* markers only, although derived from a *P. nigra* with *P. × euramericana* mating. Legionnet and Lefèvre (1996) raised the possibility of selection against introgressed seedlings in later years, so that gene frequencies may shift away from theoretical predicted ones over time.

Analyses of genetic variation within a single species, and the use of isoenzymes to differentiate between clones, are less controversial. A number of enzyme systems and loci have been found useful (Table 1). For instance, Rajora and Zsuffa (1989) found identical genotypes for 31 loci for the two clones, 'Ostia' and 'Canada Blanc'. For the origin of 'Canada Blanc', they cite "selected in Spain". 'Ostia' provoked some doubt in the extensive morphological analyses conducted by R. Müller before 1962 in Germany (Müller and Sauer 1957-1961). It was concluded that 'Ostia' was indistinguishable from the Spanish clone 'Pinseque'. From this it appears that indeed 'Canada Blanc' was a selection from the original 'Pinseque' clone in the same way as 'Ostia', which was brought to Germany by a forester, on pilgrimage in Rome, visiting Ostia, in the Holy Year of 1925 (Müller and Sauer 1957-1961).

Rajora (1990a) and Müller-Starck (1992) tested for linkage among isoenzyme loci in controlled crosses. While Rajora (1990a) was unable to detect any linkages in the material available, Müller-Starck's (1992) crosses revealed linkage groups GOT-A with GOT-B, NADH-DH-A with PGM-A, and IDH-B and 6PGDH-D with PGI-B. Among the latter, two loci called GPI(=PGI)-2 and IDH-1 also formed a weakly linked group in the basket willow (*Salix viminalis* L.; Thorsen *et al.* 1997). Linked loci may prove versatile in introgression studies, for instance with var. 'Italica', because in case of a significant contribution of its pollen to *P. nigra* offspring, linkage disequilibrium is expected among linked loci.

In conclusion, isoenzymes are useful for both introgression analyses and the study of genetic diversity in *Populus*. Comparison of results between laboratories remains controversial as far as banding patterns are concerned, while derived parameters of genetic diversity are on a comparable scale (Legionnet and Lefèvre 1996).

## **DNA techniques - basic principles**

### ***DNA-DNA hybridization***

Historically, one of the first techniques to study relationships between related species with DNA was DNA-DNA hybridization. DNA was extracted from both species, heated up to separate the two DNA strands of molecules, then both species' DNAs were mixed and slowly cooled down again. The closer the species were related, the quicker the double strands formed again, which can be monitored by a change in absorbency in a spectrometer. While this method is outdated nowadays, it might be interesting to use with the hybrids and backcrosses in *P. nigra*, because not just single genes but the whole genome would be assessed. One practical difficulty is the need for standardization of the relative amounts of chloroplast and chromosome DNA in the samples (chloroplast DNA is presumably more similar between both species than nuclear DNA).

**Table 1. Some isoenzyme studies in *Populus*.** Enzyme systems applied ('yes'), and whether they were suitable for the purpose ('yes' in bold)

Authors †	<i>Populus</i> species	Tissue	Gel matrix	Main purpose	GOT-								G6PDH					
					LAP	MDH	AAT	EST	PER	PGM	PGI	6PGDH	IDH	ACO	DIA	SKDH	Others	
1	<i>tremula</i>	leaves	starch	clone distinction	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	-	-	-	-	-	-	-	-	-
2	<i>deltoides, nigra, maximowiczii</i>	root tips, leaves	starch	introgression, clone distinction	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	yes	<b>yes</b>	yes	yes	yes	ACP
3	<i>nigra, deltoides</i>	buds	polyacrylamide	introgression	<b>yes (AP)</b>	yes	<b>yes</b>	<b>yes</b>	-	<b>yes</b>	<b>yes</b>	-	yes	<b>yes</b>	<b>yes</b>	-	-	-
4	<i>nigra</i>	buds	starch	introgression	<b>yes</b>	yes	<b>yes</b>	-	-	<b>yes</b>	<b>yes</b>	yes	yes	-	-	-	-	MNR
5	<i>nigra</i> × <i>euramericana</i>	roots	starch	introgression	<b>yes</b>	yes	-	-	yes	-	yes	<b>yes</b>	yes	yes	-	-	-	-
6	<i>trichocarpa</i>	root tips	starch	genetic variation	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	-	-	yes	-	-	<b>ACP, Peptidases, SOD, MNR</b>
7	<i>tremuloides</i>	-	-	genetic variation	<b>yes</b>	-	<b>yes</b>	-	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	-	-	-	<b>ACP, Peptidases, SOD, MNR</b>
8	<i>nigra</i>	buds, leaves	starch	genetic variation	<b>yes</b>	<b>yes</b>	<b>yes</b>	<b>yes</b>	-	<b>yes</b>	-	-	<b>yes</b>	-	<b>yes</b>	<b>yes</b>	-	-
9	<i>Tacamahaca</i> hybrids	buds, leaves	starch	inheritance, linkage	<b>yes</b>	<b>yes</b>	<b>yes</b>	yes	yes	<b>yes</b>	<b>NADH-DH, PEPCA, ACP</b>							

† Instead of including all publications on the subject, only main or early ones are listed. 1=Bergmann 1981; 2=Rajora 1989a, 1989b, 1989c, 1990a, 1990b; Rajora and Dancik 1992a; 3=Malvolti *et al.* 1991; 4=Janßen 1997; 5=Pospíšková *et al.* 1997; 6=Weber and Stettler 1981; 7=Liu and Furnier 1993; 8=Legionnet and Lefèvre 1996; 9=Müller-Starck 1992.

**Table 2. Comparison of isoenzyme analyses of introgression in *Populus nigra* L. and *P. deltoides* Marsh.** (enzymes and loci comparable between the studies are listed)

Enzyme system/locus	Rajora 1989a, b, c, 1990a, b; Rajora and Dancik 1992a	Janßen 1997	Legionnet and Lefèvre 1996	Pospíšková <i>et</i> <i>al.</i> 1997	Malvolti <i>et al.</i> 1991
<b>Summary</b>					
# plants <i>P. nigra</i>	43	37	256	–	12
# plants <i>P. deltoides</i>	140	16	–	–	12
# plants <i>P. × euramericana</i>	42	73	24	–	160 (4 families, 40 each)
plant tissue used	roots, leaves	buds	buds, leaves	root tips	buds
gel matrix	starch	starch	starch	starch	polyacrylamide
<b>Locus AAT-B</b>					
E.C.2.6.1.1	GOT-4	AAT-B	GOT-4	–	GOT-a/b/c
# alleles <i>P. nigra</i>	1	1	–	–	2/3/3
# alleles <i>P. deltoides</i>	2	1	–	–	2/1/1
# alleles <i>P. × euramericana</i>	1	2	–	–	2/2/1
alleles mutually exclusive?	no	yes	–	–	yes/no/no
patterns concordant with	(Janßen)	(Rajora)	–	–	–
<b>Enzyme LAP</b>					
E.C.3.4.11.1	Lap-1	LAP-A	–	LAP(-?)	AP-a/b
# alleles <i>P. nigra</i>	1	1	–	–	1/1
# alleles <i>P. deltoides</i>	1	1	–	–	1/2
# alleles <i>P. × euramericana</i>	2	2	–	–	2/3
alleles mutually exclusive?	yes	yes	–	yes	yes/yes
patterns concordant with	Janßen, (Malvolti)	Rajora, Malvolti	–	–	Janßen, (Rajora)
<b>Enzyme/Locus MDH(-2?)</b>					
E.C.1.1.1.37	MDH-2	MDH	MDH(-?)	–	MDH-a/b/c/d
# alleles <i>P. nigra</i>	1	–	–	–	–
# alleles <i>P. deltoides</i>	–	–	–	–	–
# alleles <i>P. × euramericana</i>	1	–	–	–	–
alleles mutually exclusive?	yes	no	1 species-specific allele ( <i>P. deltoides</i> )	–	no
patterns concordant with	–	–	–	–	–

Enzyme system/locus	Rajora 1989a, b, c, 1990a, b; Rajora and Dancik 1992a	Janßen 1997	Legionnet and Lefèvre 1996	Pospíšková <i>et al.</i> 1997	Malvolti <i>et al.</i> 1991
<b>Locus 6-PGDH</b>					
E.C.1.1.1.44	6-PGD-1/2/3/4/5	6-PGDH-A,B	PGD	6-PGD	—
# alleles <i>P. nigra</i>	1/1/1/5/4	—	—	—	—
# alleles <i>P. deltoides</i>	3/-/3/1/1	—	—	—	—
# alleles <i>P. × euramericana</i>	1/2/1/3/2	—	—	—	—
alleles mutually exclusive?	no/yes/no/yes/ yes	no	1 species-specific allele ( <i>P. deltoides</i> )	yes	—
patterns concordant with	—	—	—	—	—
<b>Locus PGI-B</b>					
E.C.5.3.1.9	Pgi-2	PGI-B	—	—	PGI-b
# alleles <i>P. nigra</i>	1	2	—	—	2
# alleles <i>P. deltoides</i>	2	3	—	—	2
# alleles <i>P. × euramericana</i>	2	2	—	—	2
alleles mutually exclusive?	yes	no	—	—	no
patterns concordant with	(Janßen, Malvolti)	(Rajora), Malvolti	—	—	Janßen, (Rajora)
<b>Locus PGM-A</b>					
E.C.2.7.5.1	PGM-1	PGM-A	—	—	PGM-a
# alleles <i>P. nigra</i>	1	2	—	—	1
# alleles <i>P. deltoides</i>	2	1	—	—	1
# alleles <i>P. × euramericana</i>	2	2	—	—	2
alleles mutually exclusive?	no	yes	—	—	yes
patterns concordant with	(Janßen, Malvolti)	(Rajora, Malvolti)	—	—	(Rajora, Janßen)
<b>Locus ACO-1</b>					
E.C.4.2.1.3	ACO-1	—	—	—	ACO-a
# alleles <i>P. nigra</i>	2	—	—	—	2
# alleles <i>P. deltoides</i>	1	—	—	—	1
# alleles <i>P. × euramericana</i>	1	—	—	—	2
alleles mutually exclusive?	no	no	—	—	no
patterns concordant with	(Malvolti)	—	—	—	(Rajora)

Enzyme system/locus	Rajora 1989a, b, c, 1990a, b; Rajora and Dancik 1992a	Janßen 1997	Legionnet and Lefèvre 1996	Pospíšková <i>et al.</i> 1997	Malvolti <i>et al.</i> 1991
<b>Locus ACO-2</b>					
E.C.4.2.1.3	ACO-2	—	—	—	ACO-b
# alleles <i>P. nigra</i>	1	—	—	—	1
# alleles <i>P. deltoides</i>	2	—	—	—	2
# alleles <i>P. x euramericana</i>	2	—	—	—	2
alleles mutually exclusive?	yes	—	—	—	yes
patterns concordant with	Malvolti	—	—	—	Rajora
<b>Enzyme DIA</b>					
E.C.1.6.4.3	DIA-(1)/2/3/(4/5)	—	—	—	DIA-a/b
# alleles <i>P. nigra</i>	1/2	—	—	—	0/2
# alleles <i>P. deltoides</i>	0/1	—	—	—	1/1
# alleles <i>P. x euramericana</i>	-	—	—	—	1/2
alleles mutually exclusive?	yes/no	—	—	—	yes/no
patterns concordant with	—/Malvolti	—	—	—	—/Rajora
<b>Enzyme EST</b>					
E.C.3.1.1.2	CE-1/2	—	—	—	EST(=1?)
# alleles <i>P. nigra</i>	1/1	—	—	—	2
# alleles <i>P. deltoides</i>	1/1	—	—	—	2
# alleles <i>P. x euramericana</i>	2/2	—	—	—	2
alleles mutually exclusive?	yes/yes	—	—	—	yes
patterns concordant with	(Malvolti)	—	—	—	(Rajora)

A similar modern approach is slot blot hybridization. DNA to be tested is made single stranded and bound to a nylon filter in spots. DNA from pure species is labelled with radioactivity or with a colour-producing marker chemical, then incubated with the filter. Darkening of a photographic film or colour development indicates how much of this labelled DNA has been bound by the DNA on the filter. The relative amounts of signal from the pure species may be compared with the overall contribution of the parent species to a hybrid's genome. While this kind of analysis has been applied to other hybrid problems, it has never been applied in *Populus*. Again, the chloroplast DNA - nuclear DNA ratio would have to be standardized.

#### ***Direct restriction analysis***

Chloroplasts can be separated from nuclei by density gradient centrifugation, and subsequently, pure chloroplast and chromosome DNA fractions can be obtained. Such chloroplast DNA can be cut into defined pieces by restriction enzymes, which cut DNA only at specific base sequences of 4-8 nucleotides. These pieces of DNA can be separated in an electrophoresis gel (agarose) and made visible. Different species will show different patterns. The same type of analysis is not possible with chromosome DNA because after restriction with commonly used enzymes, there are so many different fragments of similar lengths that single restricted DNA fragments cannot normally be separated by current electrophoresis techniques.

#### ***Restriction fragment length polymorphism***

To make such single fragments visible among the whole background of other sequences, or to do chloroplast analysis without chloroplast isolation, techniques called RFLP (restriction fragment length polymorphism) and Southern hybridization are used. The whole load of fragments which appears as a smear after DNA electrophoresis is transferred to a nylon filter and incubated with a defined piece of DNA. This DNA carries a radioactive or colour-producing marker again. The DNA will stick to the smear only where it finds a complementary sequence among the DNA restriction fragments. Dark spots in the case of radioactivity or a coloured band in the other case show how far this matching fragment had moved.

The source of the probing DNA can be single, separated DNA molecules from the species (chloroplast, nuclear, mitochondrial) which are multiplied in bacteria. Most often, further information from these sequences is not available. Sometimes the functions of these sequences are known, e.g. they are known genes that code for certain proteins. One can also try and use sequences with known identity from other, related species. For instance, many probes developed from one *Populus* species will work in many others as well; some probes will work across different genera.

#### ***Polymerase chain reaction***

A recent discovery in molecular biology is that DNA can be multiplied in a way similar to what happens in cell division, without using living cells, but only a single enzyme. This polymerase enzyme uses short pieces of DNA that bind complementary to a longer strand and supplies further nucleotides to elongate the missing strand of DNA from the starter ("primer") onwards. Primers can be synthesized chemically. If primer sites are known within a relatively short distance from each other, on the opposite strands of a DNA double strand, the enzyme will copy both strands. These can be separated from each other again, and primers may bind again. The enzyme will copy again, and this time, there will be (almost) 4 times as many DNA fragments as in the beginning. Further rounds of copying will yield so much of this single sort of DNA that it can be visualized in an electrophoresis gel without the use of probes and radioactivity or dyes. This is called the Polymerase Chain Reaction (PCR). The procedure is simple: all necessary

reaction components are mixed in a reaction tube, and the tube is put into an automated heating block that drives the amplification reaction by shifting between appropriate temperatures for DNA double strand separation, primer binding, and enzyme activity for 25-50 cycles. Anonymous or known genes or DNA fragments can be analyzed depending on which primers are selected: ones that bind specifically only to one or a few spots on the DNA, or ones that bind in a more random fashion and yield more fragments.

### Chloroplast and mitochondrial DNA analysis in *Populus*

Isolation and digestion of pure chloroplast or mitochondrial DNA is hardly being carried out anymore. However, it helped in establishing the approximate size of the *Populus* chloroplast DNA molecule - 150-156 kilobasepairs (Sabsch 1992), and that both cell organelles are inherited from the mother in species hybridizations (Mejnartowicz 1991; Sabsch 1992). Chloroplast and mitochondria isolation requires a lot of work and would present an obstacle if large numbers of plant samples are to be analyzed. This kind of analysis has been replaced by RFLP-Southern hybridization analysis because the latter does not require the isolation of cell organelles. Such chloroplast studies were done by a number of authors in *Populus* (Table 3).

Smith and Sytsma (1990) and Rajora and Dancik (1992b, 1995a, 1995b, 1995c) used cloned chloroplast fragment of *Petunia* as probes. Smith and Sytsma (1990) found no interspecific variation, neither between *P. nigra* var. 'Italica', *P. nigra* var. 'betulifolia' (Pursh) Torr. and two other clones, nor between *P. deltoides* subsp. *monilifera* Eckenw. and *P. deltoides* subsp. *deltoides* (1 clone each). They also calculated that the *P. nigra* chloroplast genome was more closely related to *P. alba*, which challenges currently accepted taxonomic treatments. They invoked hybridization and chloroplast capture as a possible explanation.

Mejnartowicz (1991) tested controlled crosses involving clones 'Muhle Larsen', 'Androscoggin', 'Oxford' and 'Columbia River'. She could establish the maternal inheritance of chloroplast DNA in these crosses. She later extended the investigations and provided data for a phylogenetic comparison (Sabsch 1992). She found intraspecific variation at a low level in *P. trichocarpa*. Her dendrograms show completely differing results in dependence of the enzymes used: *EcoRV* and *PstI* place *P. nigra* and *P. deltoides* next to each other, but when the whole data set is included, a specific artefact of the clustering technique ('chaining', Dunn and Everitt 1982) makes the two mentioned species appear basal to the balsam poplars.

Rajora and Dancik (1992b) confirmed maternal chloroplast inheritance for *P. deltoides* × *P. nigra* and *P. deltoides* × *P. maximowiczii* crosses and extended the work to intraspecific crosses in *P. deltoides*. Again, chloroplast DNA of offspring was identical to the maternal parent. Vornam *et al.* (1994) and Rajora and Dancik (1995a, 1995b, 1995c) compared *P. nigra* and *P. deltoides* more comprehensively. Vornam *et al.* (1994) found a specific marker that distinguishes between the two species' chloroplast types. No intraspecific variation was found. Seven out of 26 mature trees presumed to be *P. nigra* showed *P. deltoides* chloroplast types. Rajora and Dancik (1995a) established clearly that there are indeed intraspecific chloroplast DNA variations for both *P. nigra* and *P. deltoides*, and also for *P. maximowiczii*. They attributed this to 'varieties' of different geographical origin. In *P. deltoides* var. 'deltoides', variation was found between clones of the same variety. In *P. nigra*, var. 'Italica' was different from the other clones. Interestingly, despite the fact that the probes used in Rajora and Dancik (1992b, 1995a, 1995b, 1995c) cover the region of the *psbC-psbD* probe used by Vornam *et al.* (1994), the reported fragment sizes do not match for enzyme *XbaI* which produced the polymorphism in Vornam *et al.* (1994).

Rajora and Dancik (1995b) analyzed 17 *P. × euramericana* clones which had chloroplast types indistinguishable from or similar to *P. deltoides* var. 'deltoides', suggesting that the latter variety served as maternal parent in the 'ancient' hybrid poplar clones in Europe. A

phylogenetic comparison placed *P. deltoides* and *P. maximowiczii* closer to each other, and *P. nigra* further apart. Rajora and Dancik (1995c) report on chloroplast DNA types in *P. × euramericana* clones not present in *P. deltoides*. While the authors invoke recombination of parental chloroplast molecules, this explanation would require the presence, at least shortly after pollination, of parental chloroplasts in the embryo ('paternal leakage'). This phenomenon has been observed in other species, but not in any other *Populus* study. The authors also cite higher mutation rates and sequence instability in hybrids as a further possible mechanism. The novel types appeared only with one particular probe, P10, covering the large single copy DNA region close to the junction with inverted repeat A, which has been found to be a mutation hotspot in other species. However, other possible explanations like probe contamination or the existence of more, as yet undetected, intraspecific chloroplast DNA types in *P. deltoides* cannot be ruled out.

In summary, *Populus* chloroplasts show inter- and intraspecific differences. Intraspecific differences may be correlated with geographic origin of the maternal line of the chloroplast. The chloroplast RFLP method yields data for the whole chloroplast DNA which can be compared fairly well between labs. One caveat is that size determinations of DNA bands are not as accurate as desired. DNA purification is more demanding than for PCR as higher amounts and better quality are necessary. Also, the many steps involved in Southern hybridization analysis mean that handling of large numbers of samples is very work-intensive.

There are fewer investigations on *Populus* mitochondrial DNA. Radetzky (1990) tested 5 heterologous probes in the crosses of the Göttingen laboratory (Müller-Starck 1992). No intraspecific variation and maternal inheritance were the main findings. Paige *et al.* (1991) used three mitochondrial DNA probes to show maternal inheritance of the organelles in a *P. fremontii* - *P. angustifolia* hybrid swarm, with unilateral introgression occurring. Rajora *et al.* (1992) and Barrett *et al.* (1993) tested controlled crosses and clones of *P. deltoides*, *P. nigra*, *P. maximowiczii*, *P. × euramericana*, and *P. tremuloides* for differences in RFLP patterns of two heterologous probes using 16 restriction enzymes. Again, maternal inheritance, and the matching of *P. deltoides* and *P. × euramericana* patterns, was observed. The pattern of one probe only differed for one enzyme in one species (*P. maximowiczii*), while the other probe separated the two *P. deltoides* varieties (var. 'deltoides' and var. 'occidentalis'), and all species from each other. A cluster analysis placed *P. maximowiczii* and *P. deltoides* in a separated group from the one consisting of *P. nigra* and *P. tremuloides*. While this latter result partially reflects the findings of Smith and Sytsma (1990) for chloroplasts (affinity of *P. nigra* with section *Leuce*), several questions remain to be investigated: *P. nigra* and *P. tremuloides* did not cluster closely in Smith and Sytsma (1990); mitochondria show extensive rearrangements in many plant species, making phylogenetic analysis problematic; and an investigation of only two regions may be not representative for the rather large *Populus* mitochondrion. For example, had the authors only analyzed one of their probes, *P. maximowiczii* would stand out as most diverged species.

Table 3. Chloroplast DNA studies in *Populus*

Authors	Species/ hybrids studied; no. of clones <sup>†</sup>	Controlled crosses?	Probe source	No. of probes	No. of enzymes
Smith and Sytsma (1990)	4 N, 2 D, 9 O	no	<i>Petunia</i>	14	23
Mejnartowicz (1991)	4+ O	yes (Müller-Starck 1992)	tobacco: rbcL, psbA+trnH+rpl2+trnI, spinach: psaA	3	5
Sabsch (1992)	2 NM, 1 MN, 15+ O	yes (Müller-Starck 1992)	as above + psbA 0.7	4	11
Rajora and Dancik (1992b)	4+ D, 2 N, 30 DN, 2+ O	yes	<i>Petunia</i>	5	16
Vornam <i>et al.</i> (1994)	30 N, 6+ O	yes (Müller-Starck 1992)	poplar genomic DNA library: 1 chloroplast DNA clone (psbC-psbD region)	1	1
Rajora and Dancik (1995 a, 1995b, 1995c)	14 D, 13 N, 17 DN, 8 O	no	<i>Petunia</i>	6	16

<sup>†</sup> N=*P. nigra*; D=*P. deltoides*; O=other *Populus* species; NM=*P. nigra* × *P. maximowiczii*; DN=*P. deltoides* × *P. nigra*.

### ***Chloroplast PCR with conserved primers***

The high degree of sequence conservation of the chloroplast genes between different plant species has enabled the design of PCR primers that can be used in many species, almost 'universally' (Taberlet *et al.* 1991; Demesure *et al.* 1995; Dumolin-Lapegue *et al.* 1997). Such primers are often placed in such a way that the spacer DNA stretches so that separate genes are amplified. Spacers are not under the same high selection pressure as the genes, so they show a higher mutation rate. DNA sequence variation in these regions can be revealed by observation of length differences in electrophoresis, by mutations at restriction enzyme recognition sites, or by a combination of both (Demesure *et al.* 1996). Many small length differences can be analyzed in simple agarose gels, making the method amenable to analyses of large numbers of samples.

We have analyzed approx. 18 kbp (10 regions) in *P. nigra* (up to 60 clones), *P. deltoides* (up to 10 clones), two artificial crosses between the two species, and commercial hybrids including members of section *Tacamahaca* (up to 110 clones). For some spacers, up to 11 restriction enzymes were tested. Many spacer regions were monomorphic. In the polymorphic spacers (three regions), four restriction site mutations mostly corresponded with length-variable stretches of DNA. One region (ORF62-trnG) was particularly polymorphic, with inter- and intraspecific polymorphisms and a nearby length variation in var. 'Italica' clustering together (Heinze 1998; the var. 'Italica' polymorphisms were also observed by F. Lefèvre, pers. comm.). The analysis of another region (trnT-trnD) is now used routinely as one test for hybrid poplar introgression with *P. nigra*.

To achieve a coverage of the whole chloroplast genome with the PCR method, many single analyses are necessary: for approx. 150 kbp (containing a repeat of approx. 25 kbp) analyzed in pieces of 1.5 kbp at a time, between 80 and 85 reactions. However, it is not necessary to analyze the genes themselves, so this number may actually decrease. Furthermore, if a number of spacers known to be more polymorphic are analyzed, a high degree of resolution may be achieved. Mainly because of its amenity to large sample numbers and automatization, in the long run this method will replace RFLP-Southern hybridization analysis at least for chloroplasts, and maybe also for mitochondria (Demesure *et al.* 1995; Dumolin-Lapegue *et al.* 1997).

## **Genomic DNA markers**

### ***RFLPs of ribosomal RNA genes***

Ribosomes, structures in living cells that are the site of protein synthesis, consist mainly of a number of ribosomal RNAs. These are encoded in special genes (rRNA genes or rDNA) that are highly conserved in structure and sequence. The 18S, 5.8S and 25S RNA genes are arranged in one block. Up to several hundred such blocks are tandemly repeated, separated by an intergenic spacer (IGS). These big gene complexes are found at either one or more than one chromosomal location. In the latter case, loci of different 'size' (repeat number) may be present. Major rDNA loci are associated with the so-called nucleolus-organizing regions. The 5S RNA is also tandemly arranged, but in different blocks. Evolutionary mechanisms that are important for the rDNAs include unequal crossing-over which creates variation, and gene conversion and homogenization of sequences within and between loci which counteracts and slows down evolution. Some of the copies may become nonfunctional pseudo-genes which then have higher mutation rates. The overall number of rRNA genes in a genome can vary over the generations in response to environmental effects. Because of all these features, genetic interpretation of rDNA variation is not straightforward. Rather, rDNA lends itself to taxonomic studies. In other plant genera, variation in the IGS region, sometimes also within the genes, has been utilized to construct a phylogeny of the species.

Smith and Sytsma (1990) reported on rDNA RFLP analysis in *Populus* which allowed them to compare phylogenies for nuclear rDNA and chloroplast DNA. Procedural difficulties reduced the size of their data set: incomplete restriction digestion with Pst I occurred, and length variation in IGS had to be ignored. Length variation (insertions/deletions), however, is often correlated with restriction site mutation, for instance in the poplar chloroplast DNA (Heinze 1998). Their main finding is that *P. nigra* assumes a position between section *Leuce* and a cluster made of *P. deltoides* and two balsam poplars. No restriction map is given in the paper. In the light of later findings of several rDNAs in one species as discussed below, their analysis would need revision.

D'Ovidio *et al.* (1990, 1991; D'Ovidio 1992) studied the structure of rDNA in eight *Populus* species. They found variation between and within species. The ribosomal RNA genes made up approximately 2.4% of the DNA of a cell. Therefore, observation of certain fragments directly on agarose (after restriction with enzyme *SstI*) allowed an assignment of clones to species, or identification of parental species in some hybrids. Major and minor variants of rDNAs were observed within species. Hybrids inherited the variants from both parent species. Faivre-Rampant *et al.* (1992) also constructed restriction maps for several rDNAs of five *Populus* species, using some restriction enzymes identical to those in the former studies. The restriction maps for the 18S-5.8S-25S unit coincide, but new variants are reported for the IGS regions. While the authors constructed a phylogenetic tree for the different gene variants on the basis of these data, this cannot be taken as a clue to poplar taxonomy because of gene conversion and homogenization which increase the number of reversals of character states (i.e. different rDNA loci or genes within a species do not necessarily evolve independently). A simple test – *EcoRI* digestion and probing with a whole rDNA unit – correctly identified *P. nigra*, *P. deltoides* and first generation hybrid clones. Stoehr and Singh (1993) published a restriction map of *P. balsamifera* which largely coincides with the former maps in the coding regions. They also report on length and possible methylation variation in a population sample of *P. balsamifera*. From their Figure 4 (Stoehr and Singh 1993) it appears that more than two alleles may be present in an individual.

An explanation for the complicated patterns of inheritance of rDNA in *Populus* was presented by Prado *et al.* (1996). They hybridized rDNA probes with chromosomes on microscopic slides to detect the number of rDNA loci within several species. One major and one minor locus were found for both *P. nigra* and *P. deltoides*, but their hybrids had one major and two minor loci. This suggests that the minor loci of the two species are not situated at corresponding chromosomal positions. *Populus balsamifera* showed two major and one minor locus, *P. alba* only one locus. Two loci were found for the 5S rDNA in all species.

'Universal' PCR primers have also been described for the rDNA genes (White *et al.* 1990); however, these amplify the spacers within the unit and not the more variable IGS (which for many plant species is too long for routine PCR amplification).

DNA quality and quantity requirements may hamper the broad application of the methods outlined above for quick detection of variation within and between (introgression) species. Further studies are necessary to pinpoint PCR primers to the regions of the rDNAs that account for the variability.

#### **RFLP analysis with nuclear DNA fragments**

The following other sources of DNA probes have been used in *Populus*: anonymous fragments from poplar cloned in bacteria, heterologous probes from other species, and specific poplar genes of known function. Keim *et al.* (1989) used the first kind of probes in a study that could prove unidirectional introgression in a *P. angustifolia* - *P. fremontii* hybrid swarm. Bradshaw *et al.* (1994) constructed a genetic linkage map of a *P. deltoides* - *P. trichocarpa* pedigree with all three probe sources.

Establishing genetic control and allelism for the variants is usually not complicated in this kind of RFLP analysis. Keim *et al.* (1989) could establish species-specific markers in their study. Estimates of genetic diversity can also be derived provided that allelism is clarified, which is the case for most single-locus and also some other probes. In most cases, all the variants will be visible in the analysis (complete codominance). The drawback again is the workload for studies involving the screening of many (50+) individuals.

### **PCR of anonymous sequences**

RAPD (random amplified polymorphic DNA) has become very popular shortly after its introduction in 1990 (Welsh and McClelland 1990; Williams *et al.* 1990). PCR is applied with a single short 10-base primer. At low stringency conditions for primer binding, a number of DNA fragments are amplified from genomic DNA. After electrophoresis, some of these fragments are absent from some individuals on genetic causes. RAPD consequently detects 'presence' and 'absence' alleles for a locus characterized by the size of a DNA band. 'Presence' is dominant over 'absence', hence low-frequency 'absence' alleles remain undetected.

The procedure is quite simple and quick. DNA isolations can be rather crude compared with RFLP analysis (Heinze 1994), but poor DNA quality may lead to artefactual variation. In RAPD analysis, a small quantity of this DNA is subjected to PCR with one or more short RAPD primers, and the resulting fragments are resolved on agarose gels (polyacrylamide gels may be used if higher resolution is desired).

For the interpretation of results (comparisons of banding patterns), classical genetic parameters used in isoenzyme and RFLP analysis are only applicable after imposing some assumptions on the data mainly because of the dominance problem: primers should not be pre-selected (Clark and Lanigan 1993), and large population samples are necessary to correctly estimate underlying allele frequencies (Lynch and Milligan 1994). Seemingly monomorphic fragments may disguise low-frequency 'absence' alleles in the sample, so the number of individuals required to represent a population is quite high (Lynch and Milligan 1994). Alternatively, other ways of estimating diversity from RAPD data can be tried, for instance the (statistical) comparison of the distribution of genetic distances calculated within different populations from RAPD data (Triest *et al.* 1997), but these may not allow a direct comparison with isoenzyme analyses. The AMOVA method (analysis of molecular variance) of Excoffier *et al.* (1992) partitions genetic variation into between- and within-population components. It was developed for haplotype data, e.g. mitochondrial RFLP types, and RAPD data do not correspond to some of the basic assumptions. Huff *et al.* (1993) nevertheless found it useful in RAPD data analysis. An adaptation of the method for RAPD data was introduced by Stewart and Excoffier (1996). They assumed Hardy-Weinberg equilibrium and corrected for a degree of selfing in the population estimated with other data (e.g. isoenzymes). Liu and Furnier's (1993) estimates of RAPD genetic differentiation in aspens (*P. tremuloides* and *P. grandidentata*), assuming Hardy-Weinberg equilibrium, differed markedly from those using isoenzyme or RFLP data. Tuskan *et al.* (1996) based their analysis of *P. tremuloides* populations on a similarity index and clustering. Clustering is a much-used way of analyzing RAPD data. The clustering process, however, reduces the complexity of the data set, which may lead to loss of information and artefacts. For example, clustering is dependent on the choice of similarity index, choice of clones or species studied, selection of bands (monomorphic ones sometimes excluded) and other factors. A common phenomenon is 'chaining' where similarity values at the extremes of a continuous distribution form nuclei of clustering that may lead to erroneous results (Dunn and Everitt 1982). In hybridization and introgression studies, the application of clustering suffers from some theoretical drawbacks: what is the expected outcome of a clustering study involving hybrids and

both of their parent species? First generation hybrids, in theory, should exhibit equal average distances to both parents, which hampers the use of a cluster dendrogram as a 'relationship tree'.

*Populus nigra* in particular has not often been studied by RAPD up to the present. Castiglione *et al.* (1993) included one clone (J. Pourtet) in an exploratory analysis of several important breeding clones. Wang *et al.* (1996) used this method to discover genomic changes in plants derived from tissue culture and after genetic transformation. Studies in other *Populus* species described clone identification (Lin *et al.* 1994), estimation of genetic variation within and between populations (Liu and Furnier 1993; Chong *et al.* 1994; Yeh *et al.* 1995; Tuskan *et al.* 1996), the identification of genotypes in *P. tremula* stands (Bueno *et al.* 1997) and phylogenetic relationships (Su *et al.* 1996). The use of RAPD analysis for phylogenetics is highly controversial (Backeljau *et al.* 1995; van de Zande and Bijlsma 1995). In breeding programmes, Bradshaw *et al.* (1994) and Bradshaw and Stettler (1995) included RAPD markers in a genetic linkage map of *P. trichocarpa* × *P. deltoides* hybrids. Villar *et al.* (1996) identified RAPD markers useful in pinpointing a genomic region involved in rust resistance in the same cross.

Strengths of the RAPD assay include that only minute DNA quantities are necessary, although DNA quality is rather important. No prior information (e.g. DNA sequences) or prior work (e.g. DNA probe isolation) is required, and handling is rather simple. The high number of studied loci usually possible in RAPD often compensates for some of the shortcomings in a sense that more loci give a more accurate representation of the whole genome. The most important application in *Populus* genetic variation studies is clone differentiation. Patterns of multiple bands in a lane make up a kind of 'genetic fingerprint' characteristic for individual clones. One caveat is that such an analysis should be done as a side-by-side comparison. Artefactual variation inherent to the RAPD procedure will make database comparisons of banding patterns problematic.

AFLP (amplified fragment length polymorphism, Vos *et al.* 1995) combines some of the strengths of RFLP and RAPD analyses. Genomic DNA is cut with restriction enzymes. To the end of the fragments, short pieces of DNA are 'glued' (ligated). The sequence of these short adaptors is complementary to PCR primers that then amplify a subset of the restriction fragments. Careful choice of restriction enzymes, primers and detectable labels on the primers reduces the complexity of fragments seen on an electrophoresis gel. Large polyacrylamide gels are necessary to resolve the many bands that appear. AFLP analysis, similar to RAPD, yields a number of polymorphisms from a single analysis run. Sometimes, heterozygotes are identified by bands of half the intensity of homozygotes. Still, it is not yet completely clear whether the method works as well for diverse genetic backgrounds as it does in pedigrees. Cervera *et al.* (1996a) identified three AFLP markers tightly linked to *Melampsora* resistance in a *P. deltoides* × *P. nigra* cross, and described applications in poplar breeding (Cervera *et al.* 1996b).

Storme and Boerjan (pers. comm.) have collected material from a number of trees of var. 'Italica' and compared their AFLP fingerprint patterns among them and in comparison with other *P. nigra* clones. While most of the var. 'Italica' clones group together in cluster analysis by showing identical patterns, some trees show a lower congruence of patterns (approx. 90% only in some cases). This may be due to either somatic mutations accumulated in var. 'Italica', or to procedural causes (a low-level reproducibility error rate). Winfield *et al.* (1998) have addressed this problem directly by including five duplicate samples in their study of AFLP diversity in *P. nigra* in the Upper Severn area (UK). Congruence between patterns in the duplicate tests was between 93 and 100%. A similar level was observed when sampled trees from the same field were compared, so the conclusion is that these trees belong to the same clone (and that overall genetic diversity in the study area is very low).

Some of the RAPD problems like bands originating from unrelated loci with similar electrophoretic mobility, or long-term reproducibility, may be less important in AFLP. The level of expertise required is a little higher than for RAPDs.

#### ***PCR of nuclear genes or gene fragments (STS, sequence tagged sites)***

Bradshaw *et al.* (1994) have determined partial DNA sequences from RFLP probes. PCR primers were designed on that basis. These primers amplified defined DNA fragments in PCR which, after treatment with appropriate restriction enzymes and sometimes even without restriction, showed genetic polymorphisms in their *P. deltoides* – *P. trichocarpa* pedigree. They also demonstrated that several of the primer pairs are useful in amplifying defined DNA fragments from other poplar species. This approach is sometimes called 'sequence tagged sites' or STS.

Legionnet *et al.* (1997) applied nine of these primer pairs in their study of sexual and asexual reproduction in *P. nigra*. Using several restriction enzymes, 44 polymorphic bands were revealed. These allowed the recognition of 50 different genotypes in 118 trees from a single stand.

Faivre-Rampant *et al.* (1995, and pers. comm.) applied these markers to the study of phylogenetic relationships of *P. nigra* and *P. deltoides*. From their data, eight restriction fragments (bands) show highly differentiated distributions in *P. nigra* and *P. deltoides*, respectively: for instance, fragment 757a was absent only in one out of 206 *P. nigra*, while present in none of 11 *P. deltoides* clones analyzed. On the other hand, there were four bands typical of *P. deltoides* present in a single *P. nigra* individual, while absent from the remaining 205. Similarly, I have tested *P. nigra*, *P. deltoides* and a range of hybrids for the banding patterns of *win3*, one of the primer pairs developed by Bradshaw *et al.* (1994). The typical *P. deltoides* allele can easily be distinguished from the one typical for all other poplar species (*P. nigra*, *P. trichocarpa*, *P. maximowiczii*) analyzed to date, on agarose gels (Heinze 1997). The marker behaves in a Mendelian manner in controlled backcrosses to *P. nigra*. Presence of the *P. deltoides* allele in a few *P. nigra* seedlings is taken as an indication of low-level introgression.

Concerning the use of these markers for introgression analysis, the same dilemma as stated for isoenzymes applies: the low-frequency or apparently absent alleles as described above may be products of ongoing introgression, but they may also be present at a very low frequency among 'true' members of the species in the form of polymorphisms.

#### ***PCR analysis of microsatellite DNA sequences***

Wang *et al.* (1996) have used a microsatellite of *P. nigra* to analyze regenerants from tissue culture. The DNA sequence that amplified a product of approx. 140 bp showed extensive length variation. Recently, Rajora (1997) has introduced the analysis of microsatellites in *P. tremuloides*. Three microsatellite sequences could be amplified with primers designed from a total of 20 DNA fragments that hybridized to short tandem repeats. He could also show that these primer sequences are capable of amplifying DNA from *P. nigra* species. Microsatellites would serve best for the purpose of clone identification because the expected variability is higher than in any other marker type. It may be possible to compile databases for lab-to-lab comparisons as the respective allele sizes alone (i.e. numbers) are all that are needed for comparisons.

### **Cytology**

*Populus* species have a nuclear DNA content of approx. 1.1 pg/nucleus (Wang and Hall 1995). Direct observation of chromosomes under the microscope does not normally reveal any polymorphisms; however probing chromosome spreads with appropriate DNA sequences have allowed the identification of the loci coding for the ribosomal RNA genes

(see above; Prado *et al.* 1996). The hybridization patterns are useful in studying poplar hybrids as numbers and locations of these genes differ between species.

### **Discussion and Conclusions – which method to choose?**

There is no single ideal method for the genetic analysis of *P. nigra*. Different questions may demand the application of different methods of analysis. A systematic dilemma, however, concerns the application of markers for introgression studies: how to distinguish between rare genetic marker variants occurring in a species and introgression? In the words of Legionnet and Lefèvre (1996): “indeed the difficult point about distinguishing introgressed trees is that once an allele is rare in a species and common in the other, it is impossible to know if it belongs to the normal polymorphism of the first species or if the genotypes displaying this allele are introgressed”.

We can only observe the status quo – rare or introgressed alleles or markers – and cannot wind back time to find out if this allele has always been there in a species or whether it has been introgressed only recently.

Following is a simplified 'decision tree' that may serve useful for colleagues that intend to start up new studies. First, the aims of a study have to be clear.

#### **What do I want to know? What is the purpose of my study?**

Possible examples explained in more detail below are:

- genetic variation in *P. nigra*
- introgression with other species
- clone differentiation within species (how many genotypes are present)
- clone identification (does a certain plant belong to a certain clone?)

#### **How much detail do I need to know (pilot or in-depth study)?**

- pilot study involving only a few plants (up to 50)
- in-depth study: analysis of large sample numbers (populations; 50+)

#### **What expertise is available?**

- What methods could be tried in one's own laboratory?
- Are there other laboratories around for collaboration on more demanding analyses?

#### **What are the limits in cost and time?**

- Is there a necessity for start-up development or is it possible to start with a 'cook-book'?
- Some methods require more financial input than others, some require more time, and some, both - what is the best in one's own particular situation?

#### **Is the study a one-time effort or is it planned to continuously analyze samples?**

Some methods require a side-by-side comparison which is not practical for long-term studies. In the latter case, methods that allow the compilation of databases (isoenzymes, STS, microsatellites) for comparisons are preferred.

### ***Genetic variation in P. nigra***

#### **Pilot studies:**

For pilot studies, isoenzymes are first choice. Protocols are easily adapted to different laboratory situations, and the body of literature on isoenzyme variation in plants allows a quick comparison of data.

**In-depth study:**

Also for studies involving higher sample numbers, isoenzymes serve well because of relatively low costs and the possibility to streamline and even automate some of the steps in the protocol. A sufficiently high number of loci should be studied (around 10 or more). Microsatellites, once they are available, would have the additional advantage of higher information content, but the start-up development and the higher level of expertise needed are major drawbacks. These are partially avoided in the analysis of known PCR fragments (STS), and in RAPD and AFLP analysis. The latter two methods, however, yield dominant markers, and additional information or assumptions on the biology of the species are necessary to arrive at parameters comparable to other studies. For instance, inbreeding cannot be estimated with dominant markers like RAPDs.

***Introgression with other species*****Pilot studies:**

Analysis of phenolic compounds in leaves and buds is a very quick and simple technique which may answer many of the questions raised by possible hybridization, without involving sophisticated laboratory equipment in the case of thin layer chromatography. Application of a few isoenzyme or PCR markers, chloroplast and nuclear, serves the same purpose. RFLP methods for chloroplast and nuclear DNA, and genomic *in situ* hybridization, are still reasonably effective with lower number of plant samples to analyze.

**In-depth study:**

Detailed studies of introgression need to employ more than just a few markers. In fact, as many as possible should be applied, and nuclear and chloroplast markers should be combined appropriately. Most other techniques may prove too time-consuming for large sample numbers.

***Clone differentiation within species*****Pilot studies:**

For a rough grouping of a limited number of individuals, isoenzymes may be employed, but RAPD is more effective in this kind of studies.

**In-depth study:**

If many plants are to be analyzed, AFLP and microsatellites may be more suitable than either of the two methods above because of the higher level of resolution, which in turn is paid for by increased handling requirements. Practically, once sample numbers beyond 100 are considered, this was where RAPD approached its limits in Legionnet *et al.* (1997) study. The low-level error rate of AFLPs (around 95% reliability) presents a theoretical drawback, but in practice, if a high ratio of the number of AFLP polymorphisms and the number of plants analyzed is maintained, a 95% similarity can safely be considered as identity.

***Clone identification*****Pilot studies:**

If one or a few plants of unknown clonal identity are to be compared with a few known clones for identification, isoenzymes and RAPD will often yield the desired level of resolution. AFLPs will provide sufficient information with substantially less runs necessary.

**In-depth study:**

The more clones and plants involved, the more sophisticated markers have to be utilized. A combination of isoenzymes and PCR analysis of known nuclear DNAs may be useful in establishing a database for future reference. The suitability of AFLP markers for 'databasing' is as yet untested. Also, there is currently no way of distinguishing between somatic mutations and procedural errors in AFLP. Alternatively, microsatellites, once available, will serve the same purpose.

Combinations of different methods may come closer to the ideal situation than just choosing one method that has its strengths in only one of several aspects of the planned study. For example, to resolve the genotypes present in a *P. nigra* stand, Legionnet *et al.* (1997) sequentially applied PCR of known fragments and then RAPD analysis because the first method alone could not give the desired answer due to lower discriminating power, but applying RAPDs to all the samples would have increased the workload substantially. Therefore, RAPD was only employed to differentiate genotypes not resolved by the first type of analysis.

**Poplar Molecular Network**

The University of Washington (Seattle, USA) is maintaining a computer site for *Populus* genetics which includes a lot of information on molecular biology, the Poplar Molecular Network. It can be reached over the Internet at: <http://poplar2.cfr.washington.edu/>. There is also an electronic mailing list where questions regarding poplar breeding and genetics can be sent to a large expert audience (for information, contact Carl G. Riches at the following address: [cgr@poplar1.cfr.washington.edu](mailto:cgr@poplar1.cfr.washington.edu)). A hardcopy newsletter is distributed by Marc Villar of INRA Orléans (E-mail: [villar@orleans.inra.fr](mailto:villar@orleans.inra.fr)).

**Acknowledgements**

I would like to thank all colleagues who have contributed to this review by sending publications or unpublished data, by critically reading draft versions of this manuscript, by sending clones to me for 'calibrating' laboratory tests, and for additional hints received: Veronique Storme and Wout Boerjan, Patricia Faivre-Rampant, Agnes Legionnet and François Lefèvre, Alwin Janßen and Petra Walter, Silvia Fineschi, Stefano Bisoffi, Jos Van Slycken and An Vanden Broeck, Karl Gebhardt, Jozsef Gergác, Sándor Bordács, Georg von Wühlisch, Markéta Pospíšková, Mark Winfield.

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### Laboratories involved or interested in *P. nigra* genetic characterization: an overview

The following questionnaire was given out to Network meeting participants in order to compile a list of laboratories willing to share their expertise and to stimulate mutual collaboration among interested laboratories. The Network encourages the further distribution of the following questions to all laboratories involved or interested. Information should be sent back to Network members for update. The questions included were:

- Which techniques are available?
- Is there any experience with *P. nigra* analyses in the laboratories? In which methods?
- Current or possible sample turnover (how many samples can be analyzed in what time with which techniques?).
- In what ways would an international collaboration be possible/desirable for the laboratories (e.g. standardization, exchange of practical experience, mutual visits, common projects, analysis of samples from other countries, etc.)?
- Contact person(s) and address.
- Recent relevant publications.

The following list includes participants of the newly established EU research project EUROPOP (Genetic diversity in river populations of European black poplar). Contact: Barbara van Dam, Institute for Forestry and Nature Research, IBN-DLO, NL-6700AA Wageningen, the Netherlands; Tel: +31-317-47 78 41, Fax: +31-317-42 49 99).

#### AUSTRIA

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**Available techniques:**  
DNA-based methods  
**Interest in international collaboration:**  
EUROPOP participant

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**Available techniques:**  
RAPD, STS, chloroplast PCR  
**Experience:**  
*P. nigra* introgression, poplar clone identification with mentioned methods  
**Sample turnover:**  
100-200 samples/ day/ marker  
**Interest in international collaboration:**  
standardization of methods, exchange of expertise, mutual visits

#### **Main relevant publications:**

Heinze, B. 1997. A PCR marker for a *Populus deltoides* allele and its use in studying introgression with native European *Populus nigra*. Belg. J. Bot. 129(2):123-130.  
Heinze, B. 1998. PCR-based chloroplast DNA assays for the identification of native *Populus nigra* and introduced poplar hybrids in Europe. For. Genet. 5:33-40.

**BELGIUM**

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Department of Genetics  
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B-9000 Gent  
**Contact person:**  
Wout Boerjan  
E-mail: woboe@gengenp.rug.ac.be

**Available techniques:**  
AFLP, (in development: SAMPLE,  
microsatellites)  
**Experience:**  
AFLP analysis of *P. nigra* and  
particularly var. 'Italica'  
**Sample turnover:**  
100 samples in 3 weeks (including DNA  
preparation)  
**Interest in international collaboration:**  
EUROPOP participant

**Main relevant publications:**

Cervera, M.T., J. Gusmao, M. Steenackers, J. Peleman, V. Storme, A. Vanden Broeck,  
M. van Montagu and W. Boerjan. 1996a. Identification of AFLP molecular  
markers for resistance against *Melampsora larici-populina* in *Populus*. Theor. Appl.  
Genet. 93:733-737.  
Cervera, M.T., J. Gusmao, M. Steenackers, A. van Gysel, M. van Montagu and W.  
Boerjan. 1996b. Application of AFLP(TM)-based molecular markers to breeding  
of *Populus* spp. Plant Growth Regulation 20:47-52.

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Ludwig Triest  
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**Available techniques:**  
isoenzymes, RAPD, AFLP  
**Experience:**  
survey of isoenzyme polymorphisms in  
*P. nigra*  
**Sample turnover:**  
isoenzymes: 20 individuals/20  
stains/day  
RAPD: 150 plant/primer  
combinations/day  
AFLP: 100 plant/primer  
combinations/day  
**Interest in international collaboration:**  
common projects; analysis of samples  
from other countries, scientific  
hypotheses (beyond screening)

Institute for Forestry and Game  
Management  
Gaverstraat 4  
B-9500 Geraardsbergen  
**Contact person:**  
An Vanden Broeck  
Tel: +32 54 43 71 11; Fax: +32 54 41 08 96  
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**Available techniques:**  
AFLP, isoenzymes  
**Sample turnover:**  
(planned) AFLP 120 samples/ 2 weeks  
starting from DNA preparation;  
isoenzymes 30 samples/ day  
**Interest in international collaboration:**  
EUROPOP participant; standardization,  
common projects, mutual visits, analysis  
of samples from other countries

**CZECH REPUBLIC**

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Gardening (VÚOZ)  
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**Contact persons:**

Markéta Pospíšková, Kveta Vacková  
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**Available techniques:**

isoenzymes (starch gels)

**Experience:**

*P. nigra* and introduced hybrids and  
species: isoenzyme analysis

**Sample turnover:**

40 samples per week

**Interest in international collaboration:**

exchange of practical expertise, mutual  
visits

**Main relevant publication:**

Pospíšková, M., V. Benetka and K. Vacková. 1997. Isoenzyme analyses of the genus *Populus*. P. 156 in Abstracts, Molecular Biology for Agriculture, 25-29 August 1997, České Budějovice, Czech Republic. Institute of Plant Molecular Biology, Academy of Science of the Czech Republic, České Budějovice.

**FRANCE**

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faivre@orleans.inra.fr

**Available techniques:**

RAPD, RFLP, AFLP, isoenzymes

**Experience:**

isoenzymes, RAPD, RFLP in *P. nigra*

**Interest in international collaboration:**

mainly exchange of practical experience

**Main relevant publication:**

Legionnet, A., P. Faivre-Rampant, M. Villar and F. Lefèvre. 1997. Sexual and asexual reproduction in natural stands of *Populus nigra*. *Botanica Acta* 110:257-263.

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**Contact person:**

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Fax: +334 90 13 59 59  
E-mail: lefevre@avignon.inra.fr

**Available techniques:**

isoenzyme and DNA analyses

**Experience:**

isoenzymes, RAPD, STS in *P. nigra*

**Interest in international collaboration:**

EUROPOP participant

**Main relevant publications:**

Legionnet, A., P. Faivre-Rampant, M. Villar and F. Lefèvre. 1997. Sexual and asexual reproduction in natural stands of *Populus nigra*. *Botanica Acta* 110:257-263.  
Legionnet, A. and F. Lefèvre. 1996. Genetic variation of the riparian pioneer tree species *Populus nigra* L. 1. Study of population structure based on isozymes. *Heredity* 77:629-637.

**HUNGARY**

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- D'Ovidio, R., M. Sabbatti, E. Iacono and G. Scarascia Mugnozza. 1993. Ribosomal DNA gene and RAPDs analysis in *Populus* species. Proceedings of International Workshop on Ecophysiology and Genetics of Trees and Forests in a Changing Environment, Viterbo, Italy, 23-30 maggio 1993.
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**Interest in international collaboration:**  
 EUROPOP participant; standardization  
 of methods, common projects, exchange  
 of experience, mutual visits

**Main relevant publications:**

- Castiglione, S., G. Wang, G. Damiani, C. Bandi, S. Bisoffi and F. Sala. 1993. RAPD fingerprints for identification and for taxonomic studies of elite poplar (*Populus* spp.) clones. *Theor. Appl. Genet.* 87:54-59.
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**Experience:**

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**Sample turnover:**

60 samples for 12 isoenzyme systems per  
week

**Interest in international collaboration:**

EUROPOP participant; for isoenzymes:  
standardization of methods, exchange of  
practical experience, mutual visits,  
common projects, analysis of samples  
from other countries.

## Morphological variability of the leaves of black poplar (*Populus nigra* L.) in natural stands along the Sava river (Croatia)

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### Introduction

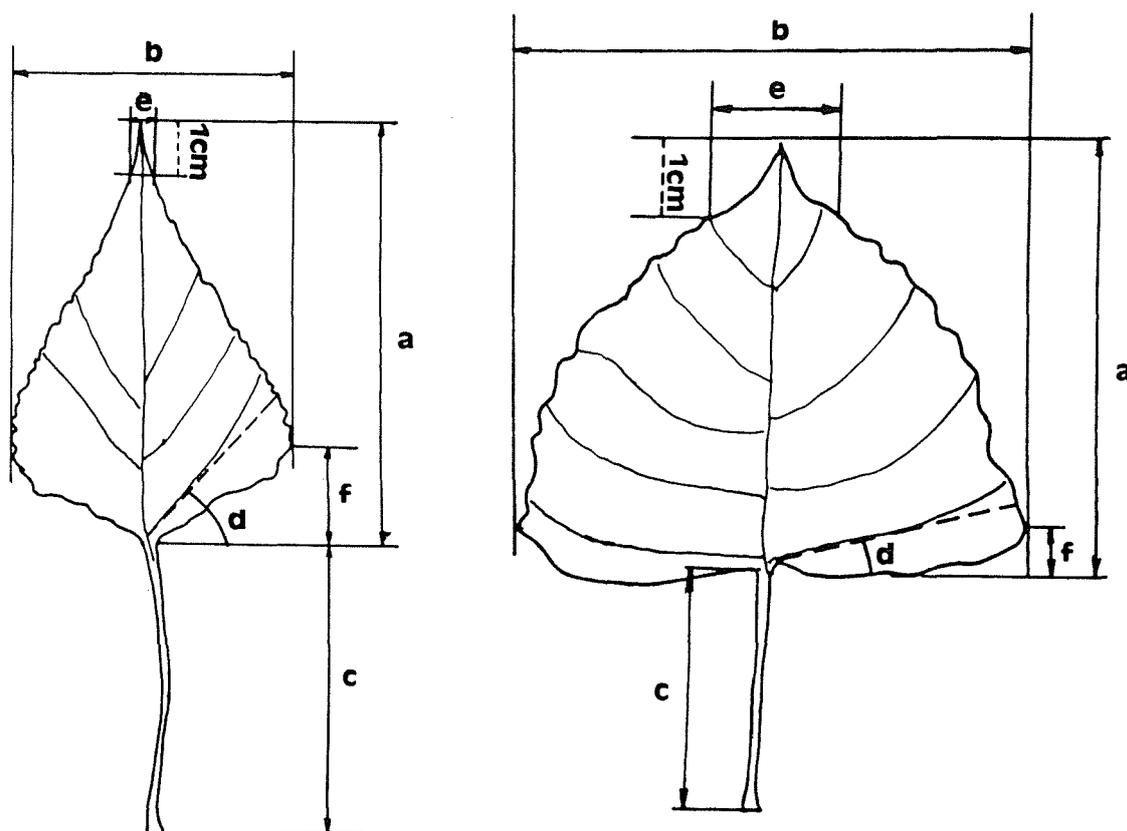
In the past, the black poplar natural populations (*Populus nigra* L.) in Croatia were represented along the Mura, Drava, Sava and Danube rivers. By interventions in the environment, as well as by uncontrolled cutting, the populations of this species have been reduced to fragments, namely to individual trees. Nevertheless, even today along the main streams, the black poplar natural regeneration occurs. To what degree it is a pure European black poplar and to what degree the progeny has been "contaminated" by the genes of *P. deltoides* Bartr. is what this paper aims to discover on the basis of the morphological leaf characteristics in the generative progeny. During the past 80 years, intensive establishing of plantations of the poplar hybrids (*P. × euramericana* (Dode) Guinier) took place. In particular, the hybrid clone 'I-214' was planted on large surface areas. This being a female clone whose flowering is synchronized with the domesticated Lombardy poplar male clone (*P. nigra* var. 'Italica' (Duroi) Mch.), a spontaneous hybridization between these two clones, as well as that of the clone 'I-214' with the autochthonous European black poplar male trees, can be expected. A reciprocal, backcross interspecific hybridization is less likely to occur because the cross between *P. nigra* and *P. deltoides* is very difficult in the case where the European black poplar functions as a female parent but also because of a small number of male hybrids of Euramerican poplars present in the plantations. The progeny could also develop by spontaneous intraspecific hybridization of the autochthonous black poplar. It can be expected that the progeny of the 'I-214' hybrid and the domesticated clone *P. nigra* var. 'Italica', by its morphological characteristics, will be closest to the European black poplar with a phenotypical expression of rare properties, which will characterize *P. deltoides* as well. The variability of this type would suggest a hybrid character of the youngest populations of European black poplar.

An attempt was made to determine the existence of introgression into the European black poplar natural populations on the basis of the modified leaf morphology in its progeny, with the simultaneous use of biochemical analyses (Ronald *et al.* 1973; Eckenwalder 1982, 1984; Hu *et al.* 1985; Rood *et al.* 1986; Rajora and Zsuffa 1990; Greenway *et al.* 1991; Bisoffi and Cagelli 1992). According to the research carried out so far in Europe, the European black poplar was found to be contaminated by the *P. deltoides* genes and therefore, to preserve its genetic resources, the selection and reproduction of autochthonous old trees was started (Bisoffi *et al.* 1987; Krstinić and Kajba 1994a, 1994b, 1996).

The objective of this research was also to determine the efficiency of *in situ* conservation, as well as the possibility of selecting 'plus' individuals with superior performance in the generative progeny. According to the transgression variability, the progeny would present superiority for some economically important properties in relation to the European black poplar, also with better adaptation to site conditions. By cloning superior individuals, positive properties of parental species can be maintained.

### Material and methods

Leaf variability in black poplar was studied within individual adult trees by means of morphometric analysis of leaves from short and long shoots. As within one tree the leaf dimorphism has been determined, for the leaf variability analysis in the generative progeny we took leaves from the short shoots only. The analyzed material was compared with the leaf measurements on the short fertile shoots from one European black poplar tree (*Populus nigra*) of about 200 years of age, one eastern cottonwood (*P. deltoides* Bartr.), a '618' ('Lux') clone, a 'I-214' hybrid clone (*P. × euramericana*), as well as with measurements on the Lombardy poplar clone *P. nigra* var. 'Italica'. Leaf samples were taken from a 3-year old generative progeny of black poplar in three locations close to the Sava river (Jarun, Zaprešić 1 and Zaprešić 2). The analysis included only sound, fully developed leaves, collected in mid-July. From each of the individual adult trees 50 leaves were analyzed, while the generative progeny sample represented 165-260 leaves taken from 33-52 plants per population. The properties measured were maximum leaf blade length, maximum leaf blade width, petiole length, leaf blade width at 1 cm from the leaf tip, distance between the leaf base and the leaf widest part, and  $\alpha$  angle between the first lateral vein and the horizontal (Fig. 1). For the *P. nigra*, *P. nigra* var. 'Italica', *P. deltoides* and the clone *P. × euramericana* 'Neva', observations were made for the number of teeth on a length of 3 cm, from the leaf blade widest part to the leaf tip. In addition to these parameters, some of the typical leaf properties, namely shape of the leaf base, petiole colour and glands at the leaf blade base, were observed.



**Fig. 1.** Overview of the measured leaf parameters: a = leaf blade length; b = leaf blade width; c = petiole length; d = angle between the first lateral vein and the horizontal; e = leaf blade width at 1 cm from the leaf tip; f = distance between the leaf widest part and the leaf base; g = number of teeth on 3 cm of leaf margin length.

## Results and discussion

### *Leaf dimorphism*

The difference in size and shape between the leaves taken from long and short shoots was confirmed by the statistical and graphical analysis for the measured properties of leaf blade length and width, petiole length and angle between the first lateral vein and the horizontal. The leaves from the long shoots have a much longer and wider leaf blade and a longer petiole than those from the short shoots. The mean value of the angle between the first lateral vein and the horizontal in leaves from the long shoots is smaller than that in leaves from the short ones, but significant for this property is also a large variability of leaves from the long shoots compared with those from the short ones. This confirms the data in the literature (Rehder 1940 according to Zsuffa 1974) about a rhombic-oval leaf shape on the long shoots and the rhombic leaf shape on the short ones. For all four measured properties, significant differences between the arithmetical means for leaves from the long and short shoots are present. The existence of the seasonal heterophylly in poplars has also been found. Preformed and neoformed leaves also often differ considerably in texture, shape and tothing. Preformed leaves are generally more diagnostic taxonomically than neoformed leaves and tend to differ more among major sections of poplars than among species within sections (Eckenwalder 1996).

### *Leaf blade length*

For the property of leaf blade length (Table 1), the highest values for coefficient of variation were in the samples from the populations (between 22.3 and 24.9%), while the values in *P. nigra*, *P. deltoides*, the 'I-214' clone and *P. nigra* var. 'Italica' ranged from 13.5 to 19.4%. The higher coefficient of variation (CV) values are linked with the generative progenies, which is understandable because of their being characterized by a much higher variability than individual trees.

According to the statistical indicators, there are no significant differences between the trees *P. nigra*, *P. deltoides* and 'I-214' for the property of leaf blade length, but the differences are significant between the progeny populations and the representatives of assumed parental species as well as the 'I-214' hybrid. Significant differences between natural populations appear between the Jarun population and the Zaprešić populations.

### *Leaf blade width*

From the leaf blade width values (Table 1) it is evident that this property is inherited intermediately. The progeny populations have, similarly to the leaf blade length, significantly lower values (from 33.4 to 37.7 mm). The populations also have the highest CV value (from 23.3 to 28.1%), while lower values were obtained in the trees *P. nigra*, *P. nigra* var. 'Italica', *P. deltoides* and 'I-214' (from 13.9 to 21.0%). The statistical indicators show significant differences in all combinations except between the Zaprešić 1 and Zaprešić 2 populations. The leaves in the progeny populations, according to the properties of leaf blade length and width, are significantly smaller than those in the parental species, which can be due to the hybridization with the male clone *P. nigra* var. 'Italica'.

### *Petiole length*

The average value of petiole length ranged from 62.9 mm in *P. deltoides* to 34.7 mm in *P. nigra* var. 'Italica', so this property also shows an intermediary way of inheritance (Table 1). The progeny populations for this property also show much smaller dimensions (from 21.7 to 25.0 mm). Similarly, as for the preceding two properties, the most variable is the Jarun population (CV=37.3%), followed by Zaprešić 2 (CV = 33.8%) and Zaprešić 1 (CV=33.0%). Much smaller values are those of *P. nigra*, *P. nigra* var. 'Italica', *P. deltoides* and 'I-214', ranging from 18.7 to 27.4%. Again, significant differences are present in all combinations except between two progeny populations from Zaprešić.

Table 1. Morphometric values of leaves of adult poplar trees

	Trait <sup>†</sup>						
	a (mm)	b (mm)	c (mm)	d (°)	e (mm)	f(mm)	g (no)
<b>Clones (age)</b>							
<i>P. nigra</i> (200 yrs)							
Mean	74.1	55.2	46.7	52.3	6.0	21.1	11.2
Min-Max	41-94	25-72	22-75	42-62	4-12	13-28	9-15
SD <sup>‡</sup>	12.7	10.0	11.7	5.0	1.6	3.7	1.6
CV <sup>‡</sup> (%)	17.2	18.1	25.0	9.6	26.3	17.4	14.0
<i>P. nigra</i> var. 'Italica' (70 yrs)							
Mean	58.7	49.7	34.7	51.9	8.4	18.5	10.8
Min-Max	47-87	40-71	24-55	36-61	5-12	13-25	8-14
SD	8.0	6.9	6.5	5.4	2.0	2.9	1.4
CV (%)	13.5	13.9	18.7	10.5	23.8	15.8	13.3
<i>P. deltoides</i> 'Lux 618' (13 yrs)							
Mean	71.3	77.0	62.9	28.8	16.5	11.6	5.8
Min-Max	52-103	52-102	37-95	17-42	6-35	5-19	4-9
SD	12.1	11.9	13.5	5.9	6.8	3.4	0.9
CV (%)	16.9	15.5	21.4	20.5	41.1	29.1	16.4
<i>P. × euramericana</i> 'I-214' (12 yrs)							
Mean	75.0	61.1	56.2	43.4	6.4	15.7	9.2
Min-Max	41-96	25-80	20-84	27-57	4-19	7-26	7-12
SD	14.6	12.8	15.4	7.3	2.4	4.6	1.4
CV (%)	19.4	21.0	27.4	16.8	38.1	29.7	15.1
<b>Populations (age)</b>							
Jarun (3 yrs)							
Mean	43.8	33.4	21.7	56.0	16.0	16.9	–
Min-Max	18-86	15-70	4-47	27-72	4-32	5-30	–
SD	10.9	9.4	8.1	7.8	5.8	4.6	–
CV (%)	24.9	28.1	37.3	13.9	36.0	27.4	–
Zaprešić 1 (3 yrs)							
Mean	49.4	37.7	23.8	57.3	16.6	19.5	–
Min-Max	27-85	27-85	6-47	32-72	5-35	10-33	–
SD	11.0	9.5	7.9	8.2	6.1	5.3	–
CV (%)	22.2	25.1	33.0	14.4	36.5	27.2	–
Zaprešić 2 (3 yrs)							
Mean	48.9	36.4	25.0	57.7	16.5	18.2	–
Min-Max	25-83	21-59	7-54	32-72	5-35	6-37	–
SD	11.1	8.5	8.4	8.4	5.5	5.2	–
CV (%)	22.7	23.3	33.8	14.5	33.6	28.3	–

<sup>†</sup> a = leaf blade length; b = leaf blade width; c = petiole length; d = angle between the first lateral vein and the horizontal; e = leaf blade width at 1 cm from the leaf tip; f = distance between the leaf's widest part and the leaf base; g = number of teeth on 3 cm of leaf margin length.

<sup>‡</sup> SD = standard deviation, CV = coefficient of variation.

Small leaf dimensions of generative progeny in three populations can be explained by the hypothesis that the male parent was the clone Lombardy poplar (*P. nigra* var. *Italica*), which has smaller leaves than the autochthonous black poplar. The extremes of variability range in the populations and in the Lombardy poplar were of 54 and 55 mm, respectively, in relation to *P. nigra* (75 mm), *P. deltoides* (95 mm) and *P. × euramericana* (84 mm).

#### **Angle between the first lateral vein and the horizontal**

*Populus deltoides* has the smallest mean angle value (28.8°), *P. nigra* has an angle of 52.3°, *P. nigra* var. *Italica* 51.8° and the 'I-214' clone 43.4° (Table 1). Mean values for progeny populations are also higher than those for *P. nigra*, the populations Zaprešić 1 and 2 being again very homogeneous (57.3° and 57.7° respectively), while the Jarun population has a slightly lower mean value (56.0°). The lowest variability is shown by *P. nigra* (CV=9.6%) and *P. nigra* var. *Italica* (CV=10.5%). The CVs of progeny populations range from 13.9 to 14.5%.

Significant differences for this property appear in all combinations except between three progenies. As the lowest values, these individuals have the angle of 27-32°, while in the black poplar and the Lombardy poplar the lowest values are at the variability range of 42° and 36°. The progeny populations contain a minor portion of leaves (up to 5%) with a much smaller angle than the average value. This minor leaf sample gravitates towards *P. deltooides*, so this can possibly indicate the existence of introgression.

#### **Leaf blade width at 1 cm from the leaf tip**

This property was chosen for the analysis because of a different leaf tip shape in *P. nigra* and *P. nigra* var. 'Italica', whose leaf tips are distinctly acute, and *P. deltooides*, whose leaf tip is obtuse. The blade width at the distance of 1 cm from the leaf tip was noted to behave differently in relation to the so far described properties. It can thus be seen from Table 1 that, for instance, in *P. nigra* the mean value of this distance is 6.0 mm, in *P. nigra* var. 'Italica' 8.4 mm, and in the 'I-214' clone 6.4 mm. The mean value for *P. deltooides* is 16.5 mm, and for the progeny populations, from 16.0 to 16.6 mm. The variability range in the populations is from 4 to 35 mm, in *P. deltooides* from 6 to 35, in *P. nigra* and *P. nigra* var. 'Italica' from 4 to 12 mm. This property with its mean values and variability ranges also indicates the existence of introgression of *P. deltooides* into the European black poplar generative progeny. CV values ranged from 25.0% in *P. nigra* var. 'Italica' to 41.4% in *P. deltooides*.

#### **Distance between the leaf's widest part and the leaf base**

This is also a typical property since *P. nigra* has a wedge-shaped base, which suggests a considerably larger distance between the leaf base and the leaf blade widest part than in *P. deltooides* whose leaf base is almost straight and very close to the leaf's widest part. The mean values for this property in the clones suggests an intermediary inheritance of this property (Table 1). The progeny populations show various mean values for this property. It is indicative, however, that the mean values in all three populations range between the mean values of potential parental species, but with a shift toward *P. nigra*. This was expected because of the assumption that the progeny was most likely to have been produced by the backcross of the 'I-214' clone with the European black poplar. Thus, the Jarun population has the value of 16.9 mm, the Zaprešić 1 population 19.5 mm and the Zaprešić 2 population 18.2 mm. The lowest CV was observed in *P. nigra* (17.4%) and *P. nigra* var. 'Italica' (15.7%), followed by *P. deltooides* (29.1%) and the 'I-214' clone (29.1%). The variability of progeny shows similar values or even slightly lower values than those in *P. deltooides* and 'I-214', ranging between 27.2 and 28.3%.

According to the statistical indicators, it is seen that there are significant differences in all combinations except between the Jarun population and the 'I-214' clone. This can mean that this clone participated as a female parent in creating the population progeny.

#### **Number of teeth on 3 cm of leaf margin length**

The number of teeth on the length of 3 cm, from the leaf blade's widest part toward the leaf tip, was measured on the leaves of trees *P. nigra*, *P. nigra* var. 'Italica', *P. deltooides*, *P. × euramericana* clone 'Neva' and in the clone *P. × euramericana* 'I-214' which is a backcross hybrid with the black poplar. *P. nigra* and *P. nigra* var. 'Italica' have the largest number of teeth, on average 11.22 and 10.83, respectively, and *P. deltooides* the smallest number of teeth, 5.80 (Table 1). The F<sub>1</sub> generation hybrid, the 'Neva' clone, has on average 7.04 teeth while the hybrid 'I-214' has an average of 9.20 teeth. This property is inherited intermediately. The 'I-214' clone is the closest to the European black poplar with respect to this trait.

### **Shape of the leaf base**

The leaves of parental species have various leaf base shapes. The European black poplar and Lombardy poplar have a wedge-shaped base while the leaf base of *P. deltoides* is straight to slightly cordate. The F<sub>1</sub> generation progeny, the clone *P. × euramericana* 'Neva' has a straight leaf base, as well as the female parent *P. deltoides*, while in the sample of 'I-214' backcross hybrid in addition to the leaves with a more or less wedge-shaped leaf base there are also leaves with a straight base. The progeny population samples show three base shapes, namely: wedge-shaped, straight and an intermediate shape which is formed by starting from the petiole in a wedge-shaped manner to then pass into a straight shape.

### **Petiole colour and glands**

On the trees of *P. nigra* and *P. nigra* var. 'Italica' there are generally leaves with a green petiole, although there are those with a reddish one, too. Leaf petioles on *P. deltoides* are green, although in the literature they are said to be reddish. Most leaves in the 'I-214' clone have a petiole which is green on the lower side and reddish on the upper side. In all three progeny populations the petioles with red upper side and green lower side predominate. Some hybrids of *P. × euramericana* have been noted before to have green petioles in shadow and reddish petioles in the light, which could lead to the conclusion that this property is strongly affected by the environment.

The number of glands found on the leaves of *P. deltoides* and the 'Neva' hybrid is 2-3 and 1-2, respectively. No glands were found on the leaves of the black poplar and the Lombardy poplar, nor on the leaves of the hybrid 'I-214', although the literature reports the existence of 1-2 glands in this clone on the junction of leaf blade with the petiole. No glands on the leaf blade base were found on any plants in the progeny populations.

## **Conclusions**

In the black poplar (*P. nigra*) natural populations in Croatia along the Sava river, with respect to the observed leaf morphological properties, the phenotypes which most resemble black poplar predominate. This is because these progenies could be produced by crossing between the representatives of pure black poplar, as well as by crossing between a female clone, the 'I-214' hybrid, with black poplar, namely with the clone *P. nigra* var. 'Italica'. The existence in the natural progeny of rare individuals with properties of *P. deltoides*, can be explained by recombinations namely by the transgression (F<sub>2</sub> generation). The properties attributable to *P. deltoides*, such as leaf blade width, petiole length, angle between the first lateral vein and the horizontal, suggest the existence of introgression of *P. deltoides* into the black poplar genome. The observed properties are not equally under the genetic control. Thus, for instance, the leaf size property is more susceptible to modifications and this both within the same tree and within the members of the same species of the same age. The properties which are under strong genetic control and, therefore, from this aspect indicative for determining progeny's hybrid character are: angle between the first lateral vein and the horizontal, distance between the leaf base and the leaf widest part, base shape, leaf blade width at the distance of 1 cm from the leaf top, and number and shape of teeth.

By selection of best-performing variants in natural progenies and by their cloning, the genotypes containing positive properties can be produced. The activities on the preservation of the black poplar genetic resources should be carried out with focus on the very old trees because they are not contaminated by the *P. deltoides* genes.

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## Agenda

1. Introduction (F. Lefèvre, J. Van Slycken, J. Turok)
2. Brief country updates (all participants) and a synthesis (F. Lefèvre)
3. Network discussion: Dynamic processes in the riparian ecosystem and its implications for *P. nigra* gene conservation strategies (P. Tabbush; participants)
4. Joint research (S. de Vries)
5. Presentation of the ongoing work at IBW in Geraardsbergen/ Excursion (J. Van Slycken)
6. EUFORGEN core collection of clones (S. Bisoffi)
7. European database (S. Bisoffi)
8. Synthesis of *in situ* gene conservation measures and activities: discussion and update (S. de Vries)
9. Standardized minimum list of descriptors for inventories of *P. nigra* stands (N. Alba)
10. Characterization of *P. nigra* with molecular methods (B. Heinze)
11. Public awareness activities of the Network (S. de Vries)
12. Other tasks of the Network
13. Miscellaneous
14. Conclusions

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