Philipps Universität Marburg

Population genetic processes of a *Populus nigra*/*P. x canadensis* hybrid complex

spatially explicit studies on gene flow as a basis for conservation measures

Dissertation
Zur Erlangung des Doktorgrades
der Naturwissenschaften
(Dr. rer. nat.)

dem Fachbereich Biologie
der Philipps-Universität Marburg
vorgelegt von

Georg Rathmacher
aus Neuss am Rhein

Marburg/Lahn, 2008

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The research pertaining to this thesis was carried out at the Department of Conservation Biology at the Philipps-University of Marburg from August 2005 to November 2008 under the supervision of Prof. Dr. Birgit Ziegenhagen and Dr. Ronald Bialozyt. It was funded by the Federal Ministry of Education and Research (BMBF – Bundesministerium für Bildung und Forschung) within the framework of the project 'Modelling (trans)gene flow of poplar in a real landscape' which is part of a joint project on 'Biosafety of transgenic woody plants'.

Vom Fachbereich Biologie
der Philipps-Universität Marburg als Dissertation am …….. angenommen

Erstgutachter	Prof. Dr. B. Ziegenhagen
Zweitgutachter	Prof. Dr. G. Kost

Tag der mündlichen Prüfung am:

In Liebe für meine Familie

Nature is ancient but surprises us all.

Björk

This thesis is based on the following publications and manuscripts: They will be referred to in the text by the term ‘paper’ and their roman numerals.

I. Allelic ladders and reference genotypes for a rigorous standardisation of poplar microsatellite data

G. Rathmacher, M. Niggemann, H. Wypukol, K. Gebhardt, B. Ziegenhagen and R. Bialozyt

Trees - Structure and Function (in press), DOI: 10.1007/s00468-008-0302-z

II. Short-distance gene flow in *Populus nigra* L. accounts for small scale spatial genetic structures – implications for in situ conservation measures

G. Rathmacher, M. Niggemann, M. Köhnen, B. Ziegenhagen and R. Bialozyt

Manuscript, submitted to *Conservation Genetics*

III. Subtle invasion of *Populus deltoides* genes into endangered *P. nigra* L. populations – challenges for quantitative diagnostic methods

G. Rathmacher, M. Niggemann, B. Ziegenhagen and R. Bialozyt

Manuscript

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German summary – Zusammenfassung

Eingeschränkter Genfluss in Populationen führt zur räumlichen Klumpung von verwandten Individuen. Dies hat Inzucht und einen Verlust der genetischen Diversität zur Folge. Die theoretischen Folgen dieser Prozesse sind grundsätzlich nachvollziehbar. Die konkreten Folgen für reale Organismen in einer realen, vom Menschen veränderten Landschaft sind jedoch nicht sofort offenkundig. Zusätzlich ergibt sich aus dem anthropogenen Einbringen von exotischen Arten oder gezüchteten Kulturpflanzen in die Landschaft die Möglichkeit von Introgression durch Genfluss zu ihren einheimischen nahen Verwandten.

Die beiden Taxa *Populus nigra* (Europäische Schwarzpappel) und *P. x canadensis* (‚Kanadapappel', Hybrid aus *P. deltoides* und *P. nigra*) wurden als Modellsystem benutzt, um Kenntnis über quantitative Ausbreitungsmuster innerhalb des Mosaiks von verbliebenen natürlichen Vorkommen von *P. nigra* und Plantagen von *P. x canadensis* zu gewinnen. Hierfür wurden genetische Untersuchungen mit Hilfe von nSSR-Makern (nuclear simple sequence repeats, Mikrosatelliten-Orte des Zellkerns) durchgeführt. Dabei konnten Eigenschaften wie ein hoher Polymorphiegrad, kodominante Vererbung und das Vorhandensein von artspezifischen Allelen dazu verwendet werden, eine einfach zu handhabende Methode zur Gewinnung von genetischen Fingerabdrücken der Individuen zu erstellen. Es wurden öffentlich erhältliche allelische Leitern für jeden Locus hergestellt. Auf diese Weise kann das Markersystem dazu genutzt werden, verlässliche und eindeutige Ergebnisse für genetische Studien an Pappel zu erzeugen, die zwischen unterschiedlichen Genotypisierungssystemen übertragbar sind. Auf diese Weise ist es möglich, Klonvarietäten zu zertifizieren und molekulare Datenbanken zu vergrößern. Dies ist insbesondere für die Forstwirtschaft von großem Nutzen.

In dieser Studie wurde das Markersystem außerdem dazu verwendet, die genetische Diversität und die räumlich-genetische Struktur einer natürlichen Schwarzpappelopulation zu bestimmen. Im Zusammenhang mit Plantagen in der Umgebung wurde die Hybridisierung zwischen *P. nigra* und *P. x canadensis* untersucht. Elternschaftsanalysen von Sämlingen sowie von Jungwuchs aus räumlich begrenzter Naturverjüngung wurden dazu genutzt, den pollen- und samenvermittelten Genfluss zu quantifizieren. Die räumlich-genetische Struktur der Schwarzpappel-Altbaumpopulation deutet darauf hin, dass Genfluss über kurze Entfernungen vorherrscht, dessen Hauptanteil von 70 % innerhalb von 1 km Umkreis um die Quelle stattfindet. Anhand dieser Ergebnisse lässt sich die verminderte genetische Diversität des untersuchten Jungwuchses erklären. Genfluss zwischen *P. nigra* und *P. x canadensis*

wurde in beide Richtungen gefunden. Die jeweiligen Raten schwankten allerdings stark, was auf stochastische Umweltbedingungen sowie vor allem auf die räumliche Verteilung der Altbäume zurückgeführt wurde. Grundsätzlich begünstigt war die Hybridisierung von weiblichen *P. x canadensis*- und männlichen *P. nigra*- Bäumen. Zusätzlich konnte die Etablierung von Nachkommen mit Hybridisierungshintergrund nachgewiesen werden. Bezüglich dieser Ergebnisse werden praktische Konsequenzen sowohl für zukünftige Studien als auch für effektive Schutzmaßnahmen der Europäischen Schwarzpappel vorgeschlagen. Die Ergebnisse werden außerdem im Bezug auf Hybridisierungsprozesse und im Hinblick auf die Risikoeinschätzung für die Ausbreitung von Transgenen durch Genfluss von gentechnisch veränderten Kultursorten diskutiert. Die Ergebnisse dieser Studie können außerdem auf das Management von Pflanzenarten übertragen werden, die der Pappel ähnliche Ausbreitungs-mechanismen aufweisen.

English summary

Limitations of gene flow in populations lead to spatial aggregation of related individuals, which implicates inbreeding and a loss of genetic diversity. The theoretical consequences of these processes are generally understood. However, consequences for real populations, distributed across man made landscapes are not immediately apparent. The anthropogenic introduction of exotic species or domesticated cultivars into landscapes has increased the possibility of introgressive gene flow to their wild relatives. The two taxa *Populus nigra* and *P. x canadensis* were used as a model system to obtain knowledge on the quantitative patterns of dispersal within and between the mosaic of remnant native individuals of *P. nigra* and plantations of *P. x canadensis*. For this purpose, key features of nSSR markers (nuclear simple sequence repeats), namely high polymorphism, codominant inheritance and availability of species-specific alleles were utilised to create an easy-to-handle tool for obtaining multilocus genetic fingerprints. As publicly available allelic ladders of each locus have been created, the presented marker system enables the generation of transferable genetic data on poplar in different laboratory settings. By this means, the marker assay can help to enlarge present clonal molecular data bases. It is also essential for certification purposes in commercial forestry.

Using the marker assay, a natural population of *P. nigra* was analysed for genetic diversity and spatial genetic structure. Parentage analyses of seedlings as well as juveniles from a restricted area of natural regeneration enabled the quantification of pollen and seed-mediated gene flow, respectively. Consequences for genetic diversity could be concluded and consequences for the management of natural recruitment could be deduced. Spatial genetic patterns of the *P. nigra* adult tree population suggest prevailing short-distance gene flow, the major part of which (i. e.70 %) takes place within distances of less than 1 km. This helps to explain the reduced diversity in investigated juveniles.

In the context of surrounding plantations, introgressive gene flow between *P. nigra* and the bred taxon *P. x canadensis* was studied. For this purpose, progeny of both taxa was analysed. Introgressive gene flow was found in both directions. Particular rates varied greatly and were probably due to stochastic environmental conditions and the spatial distribution of trees. However, preferential hybridisation was found between female *P. x canadensis* and male *P. nigra.* Moreover, introgressed individuals could be found in natural recruitment. Practical consequences for both upcoming studies and the conservation of natural *P. nigra* populations

are implicated. Results of this research are discussed with respect to hybridisation processes and concerning the risk assessment of transgene flow from genetically modified taxa. Findings may also be transferred to management plans of plant species exhibiting similar dispersal mechanisms as poplar.

1. Introduction

1.1 Gene flow in contemporary real landscapes: sustaining genetic diversity and 'risking' hybridisation and introgression at the same time

Gene flow among populations of a species is an important force that influences their genetic structure. When gene flow is restricted and populations become fragmented and genetically isolated, they risk to lose their genetic diversity. Nevertheless, it is critical to the long-term survival of populations - especially under changing environmental conditions (Primack 2000; Frankham et al. 2002). Limitations of gene flow are leading to spatial aggregation of related individuals that is called 'isolation by distance' (Hardy & Vekemans 1999; Born et al. 2008). Theoretically, consequences of such changes are understood. However, consequences for real organisms that are distributed across real landscapes are not immediately apparent. Knowledge about the genetic diversity and the gene flow inside the remaining populations of endangered species provides key information for managing their population dynamics (Lowe et al. 2005). Hence, there is an increasing interest in gene dispersal in landscapes that have been modified by human activities concerning the impact that habitat loss, disturbance and fragmentation may have on plant populations (e.g. Sork & Smouse 2006; Brunner et al. 2007; Meagher 2007). Additionally, the introduction of exotic species or bred taxa into landscapes for agricultural, ornamental and forestry purposes has increased the possibility of hybridisation and introgressive gene flow between species that are normally isolated (Olden et al. 2004). For the sake of clarity, definitions for certain terms are given below. In this thesis, natural species that are non-native in their present habitat are referred to as 'exotic species'. The term 'bred taxa' represents bred cultivars of pure species (native and exotic) as well as hybrids. 'Hybridisation' is the mating of genetically distinguishable groups or taxa leading to the production of progeny. These groups or taxa may include individuals of two different species, varieties of the same species or individuals that are already hybrids (Hails & Morley 2005; Mallet 2005). The transfer of genetic material between hybridising taxa in many generations of backcrosses is called 'introgression'. It is the invasion of foreign genetic material into a genome. The method of introduction is usually sexual contact. The result of introgression is the transfer of traits found in one species to another (Hails & Morley 2005; Mallet 2005). Planting of exotic species or bred taxa within the natural range of a related native species therefore leads to hybridisation and introgressive gene flow if the two taxa are able to produce viable offspring (Ellstrand et al. 1999; Hails & Morley 2005). Consequently,

evolutionary effects on native populations may arise as allele frequencies are shifted or new alleles are introduced (Ellstrand et al. 1999). Much attention has been given to introgressive gene flow as a potential way for the escape of transgenes into natural populations (e.g. Colwell et al. 1985; Hails & Morley 2005; Kuparinen & Schurr 2008). Hybridisation with bred taxa has also been implicated in the extinction of certain wild relatives (Ellstrand 1992b; Rhymer & Simberloff 1996; Levin et al. 1996).

In order to assess gene flow patterns as well as the risks of introgressive gene flow, knowledge is required on the patterns of dispersal within and between the mosaic of remnant populations and plantations. For the study of gene flow, highly polymorphic genetic markers such as nuclear simple sequence repeats (nSSRs, syn. nuclear microsatellite markers) are required. They are reproducible, codominant and highly polymorphic. If an adequate number of loci is combined, they allow the unambiguous identification of each individual (Jones et al. 1997; Dayanandan et al. 1998; Jones & Ardren 2003). Their allelic information can be used for the statistical procedures of parentage analyses, spatial autocorrelation and Bayesian assignment analyses (Sampson & Byrne 2008). Parentage analysis may deliver concrete distances of effective pollen and seed dispersal (Dow & Ashley 1998). In order to get significant results, sample sizes have to be large enough for the detection of gene flow at biologically significant levels (>1 %) (Ellstrand et al. 1999). Autocorrelation analyses of spatial genetic structure (SGS) may reveal patterns of isolation by distance. In Bayesian assignment methods, the calculation of posterior probabilities of ancestry makes it possible to distinguish between pure *P. nigra*, F2 and various backcrosses of *P. nigra* and *P. x canadensis* (Anderson & Thompson 2002; Buerkle 2005).

It has been reported that particularly tree species are likely to be resilient to landscape changes because they already contain high genetic diversity. Especially wind-pollinated trees have successful mechanisms for extensive gene flow (Hamrick 2004). However, extensive gene flow coincides with the enhanced likelihood of introgressive gene flow if related bred taxa are present (Kuparinen & Schurr 2008). This is because many tree species reproduce for a long time before they are harvested and compatible native relatives can frequently be found in close proximity to plantations of bred taxa. Consequently, hybridisation can occur easily (James et al. 1998; Williams & Davis 2005). The question is whether genetically isolated, sometimes small remnant populations risk to lose their genetic diversity due to restricted gene flow and to what extent they are affected by introgressive gene flow. As a model system in gene flow studies, members of the genus *Populus* are suitable for a number of reasons. These include the possibility to hybridise different species (Stettler et al. 1996) as well as

commercial use and extensive plantation of bred taxa (Zsuffa et al. 1996). Additionally, in the European context, the European black poplar (*P. nigra* L.) is an important native keystone species of habitats that have been modified extensively due to anthropogenic needs (Guilloy-Froget et al. 2002).

1.2 The genus *Populus*: a model forest tree in commercial exploitation

Populus L. (family Salicaceae) is a genus comprising about 30 species, which are distributed throughout the temperate and subtropical zones of the Northern Hemisphere (Eckenwalder 1996; Farmer 1996). *Populus* species are predominantly dioecious and obligatory outcrossers. Poplar pollen is wind-dispersed and its seeds are dispersed by wind and water in light hydrophobic cotton (Legionnet et al. 1997). Old trees can supply over 50 million seeds in a single season (OECD 2000). Therefore, gene flow that occurs through the effective dispersal of pollen and seeds, migration rates, and genetic diversity of poplar populations are assumed to be high (Legionnet & Levèvre 1996). The genus *Populus* is considered a model forest tree in plant biology (Bradshaw et al. 2000; Taylor 2002). The main reasons are that poplars grow fast, they are easy to propagate clonally through cuttings and they have a relatively small genome size (x = 19, 2n = 2x = 38, 450-550 mega base pairs) (Bradshaw et al. 2000). In 2003, the poplar genome was the first tree genome that was fully sequenced (Taylor 2002). Within ongoing research, it is possible to identify specific gene sequences and to clarify their corresponding phenotypic traits, such as stress response. This is one advantage of poplar over any other tree species, especially with regard to breeding purposes (Cervera et al. 2001; Taylor 2002; Sims et al. 2006; Gaudet et al. 2008). Using controlled hybridisation, breeders are able to combine the favorable traits of different parental species in one cultivar (Bradshaw et al. 2000). The heterosis in growth characteristics of these so-called ‘F1-hybrids’ makes them attractive for commercial use (Zsuffa et al. 1996). Individual cultivars of these bred taxa are typically represented by a single clone (Rajora & Rahman 2003). Poplar plantations usually represent one single cultivar or clone in order to minimise the variability in growth and wood quality within the plantation. They are thus easily processed in industry (Zsuffa 1974; Fossati et al. 2005). These qualities have led to a widespread production of cultivated poplar plantations in Europe. The utility of bred poplar taxa ranges from their use as windbreaks to the production of wood, such as plywood, packages, structural timber, matches, chopsticks and paper (Castiglione et al. 1993; Miko 1993). Additionally, plantations of hybrid

poplar have already been appreciated for a long time as an alternative resource for wood production due to increasing demands for renewable energy (Zsuffa 1975; Bekkaoui et al. 2003; Rajora & Rahman 2003; Sims et al. 2006). Recently, the conversion of abandoned agricultural areas to woodland has been promoted by the European Community (De-Lucas et al. 2008). Poplars can also be used for monitoring the level of pollution of trace elements (Madejón et al. 2004). Aside from conventional breeding, new highly productive poplar cultivars have also been created by transgenic engineering. Benefits of genetically modified (GM) trees arise from the transfer of traits that are not readily available either in the breeding population or the genetic resource. By this means, beneficial traits such as stress tolerance or resistance to pests and herbivores can be transferred. Therefore, GM taxa gain importance in commercial forestry (Hoenicka & Fladung 2006; Brunner et al. 2007).

Poplar species that are frequently used in breeding programmes in order to produce hybrid cultivars are *P. nigra*, the North American cottonwoods *P. deltoides* Marsh. (both belonging to section Aigeiros Duby) and three species of the sect. Tacamahaca, namely *P. trichocarpa* Torr. Ex Gray, *P. balsamifera* L. and *P. maximowiczii* Henry, also classified as *P. suaveolens* s.l. (Eckenwalder 1996). More than 90 percent of all cultivated poplars are assumed to belong to the interspecific hybrid taxon *P. x canadensis* Moench (syn. *P. x euramericana* (Dode) Guinier) and their parental species *P. deltoides* Marsh. and *P. nigra* L. (FAO 1979). *P. nigra* has many desirable characteristics that lead to its inclusion as a parent in breeding programmes: wide adaptability to many environments and different kinds of soil, excellent rooting ability of cuttings and fair resistance to common diseases. Today, 63 % of the poplar cultivars descend from *P. nigra*, either as a pure species or from interspecific hybrids (Cagelli & Lefèvre 1997).

Due to their immense commercial relevance, poplar breeders, growers and industry call for proper identification of poplar cultivars in order to avoid commercial frauds (Fossati et al. 2005; De-Lucas et al. 2008). Therefore, European regulations such as the German Law on Forest Reproductive Material (Forstvermehrungsgutgesetz) demand certificates of origin and clonal identity (FoVG 2006). The traditional method for this is based on morphological, phenological and floral characteristics (UPOV 1981). However, it turned out that phenotypic methods of identification are not always satisfying. They were described as difficult, ambiguous, time-consuming and subjective (Rajora & Rahman 2003). This is caused by the instability of morphological characters due to environmental and management factors as well as by the age of the tree and its state of health (Fossati et al. 2005; De-Lucas et al. 2008). Currently, molecular markers are used for differentiation and identification purposes (e.g.

Rajora & Dancik 1992; Castiglione et al. 1993; Fossati et al. 2005). Thereby, highly polymorphic nSSR markers turned out to supply the largest amount of reliable information (Rajora & Rahman 2003; Fossati et al. 2005; De-Lucas et al. 2008). SSR markers were isolated and characterised, and they have already been used for clone identification and the assessment of genetic relationships in poplar (e.g. Dayanandan et al. 1998; De-Lucas et al. 2008). However, due to the common problem of reproducibility of absolute DNA fragment lengths in different electrophoretic platforms and settings (de Valk et al. 2007), these studies have not produced any unambiguously transferable results so far.

1.3 The European black poplar: a riparian pioneer in manipulated habitats

The European black poplar is a keystone species of riparian ecosystems in Europe, Northern Africa and Central and West Asia (Fig. 1). In Europe, it is the only native member of the sect. Aigeiros. Together with other members of the family Salicaceae, it dominates the early successional stage of floodplain woodlands in many temperate areas (Fig. 2). Natural occurrences of *P. nigra* mainly exist along rivers and streams, their distribution is sometimes very scattered and extended natural populations are rare (Guilloy-Froget et al. 2002).

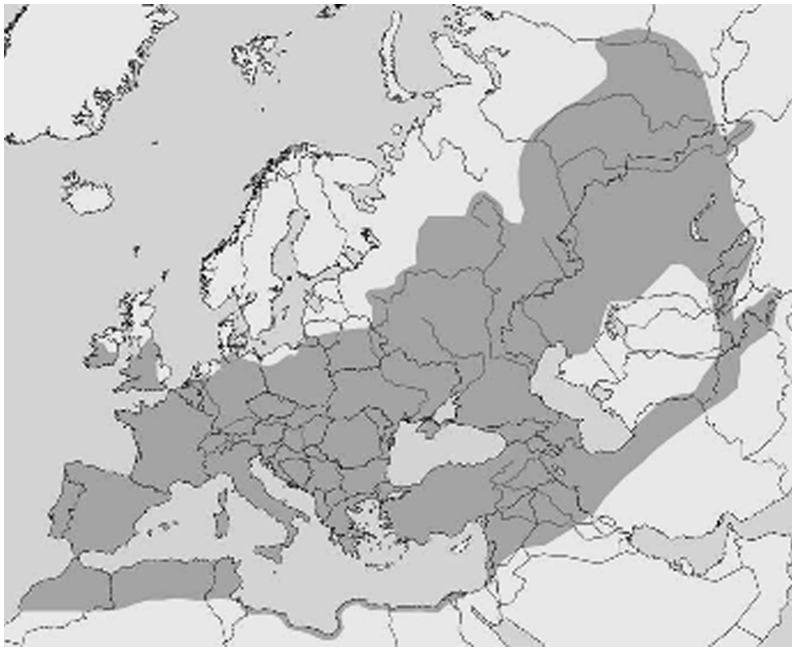

Figure 1 Geographic distribution range of *P. nigra* (Vanden Broeck et al. 2003)

As a pioneer species, it colonises bare, moist soil on riverbanks by both, sexual reproduction through seeds and asexual reproduction of cuttings or root suckers (Legionnet et al. 1997). Prior to leaf shooting in early spring (March-April) male and female trees produce flowers clustered in pendulous catkins (Vanden Broeck et al. 2003).

The wind- and water-dispersed seeds have a short viability period. They need very specific water/soil conditions for germination (Braatne et al. 1996; Guilloy-Froget et al. 2002). Successful regeneration only occurs when the moisture of sediment remains high enough for seedling roots to establish (Legionnet et al. 1999). Regeneration is generally poor within old established stands as the riparian forest naturally evolves towards hardwood formations (Lefèvre et al. 1998). By means of asexual vegetative reproduction, genotypes can persist for long periods of time beyond the longevity of single trees (Jenik 1994). For instance, mechanical damage stimulates sprouting of dormant primordial tissue in roots and shoots (Barsoum 2001), or branches that are sheared off are deposited downstream and subsequently take root to become viable clonal saplings (Rood et al. 2003).

Figure 2 Floodplain woodland, the typical habitat of *P. nigra*

Because of human activities such as wood cutting and habitat loss due to drainage of rivers or management of riverbanks, natural populations of black poplar in Europe have declined or completely disappeared. The artificial regulation of floods has limited the regeneration capacities of the species and favoured the succession of native poplar stands by hardwood forests (Lefèvre et al. 1998). Furthermore, many suitable habitats have been transformed into plantations mainly of productive *P. x canadensis* cultivars. Accordingly, habitat loss and poor natural regeneration have caused that today the European black poplar is one of Europe's most endangered native trees (Tabbener & Cottrell 2003; Pospíškova & Šalková 2006). In Germany, it is categorised as 'vulnerable' in the National Red List (BfN 2008). The goals of the conservation of genetic resources are both to maintain a large gene pool for evolution that will ensure the potential for adaptation and to provide base material for further breeding programmes (Cagelli & Lefèvre 1997). Much theoretical work on conservation of *P. nigra* has been carried out in Europe on a national and international scale. A combined conservation strategy was developed by the EUFORGEN (European Forest Genetic Resources Programme) *Populus nigra* Network (Cagelli & Lefèvre 1997; Arbez & Lefèvre 1997).

Genetic analyses of *P. nigra* populations have been performed with the use of different molecular markers, such as isozymes and AFLPs (e.g. Legionnet & Levèvre 1996; Cervera et al. 1996). Recent studies mainly focussed on the use of nSSRs (e.g. Imbert & Levèvre 2003; Fossati et al. 2003; Pospíškova & Šalková 2006; Smulders et al. 2008b). Gene flow studies on *P. nigra* revealed a SGS that was caused by isolation by distance (Legionnet & Levèvre 1996; Imbert & Levèvre 2003; Pospíškova & Šalková 2006). Furthermore, due to their immense polymorphism and the resulting ability to distinguish between single individuals, nSSR markers have been used successfully in paternity analyses in *Populus* (e.g. Tabbener & Cottrell 2003; Pospíškova & Šalková 2006; Vanden Broeck et al. 2006). However, in all cases, estimates of effective pollen and seed dispersal limitations could only be specified by single findings or indirectly by statistical analysis of estimated SGS (Imbert & Levèvre 2003; Pospíškova & Šalková 2006). Therefore, quantitative measures of effective pollen and seed dispersal distances which cause spatial genetic patterns in poplar populations are still missing.

Apart from intraspecific loss of genetic diversity, the gene pool integrity of *P. nigra* is threatened by interspecific introgressive gene flow from bred poplars of the F1 hybrid generation (e.g. Heinze 1997; Heinze 1998b; Levèvre et al. 2001; Vanden Broeck et al. 2004; Ziegenhagen et al. 2008). Due to the fecundity of F1 cultivars, mating among themselves or mating with pure *P. nigra* generates either F2 or backcross progenies respectively (Bradshaw et al. 2000). Thus, under certain circumstances, F1 hybrids of *P. x canadensis* may swamp the

gene pool of native black poplar, thereby reducing its genetic diversity and causing introgressive gene flow of *P. deltoides* genes into the *P. nigra* gene pool (Cagelli & Lefèvre 1997; Vanden Broeck et al. 2004).

Introgressive gene flow can be analysed using different types of genetic markers. Chloroplast (cp) markers can identify the female contribution by *P. deltoides* (e.g. Heinze 1998b; Holderegger et al. 2005) but cannot detect hybridisation events of *P. nigra* females and male hybrid clones (Ziegenhagen et al. 2008). Some nSSR loci can be used as diagnostic markers to identify hybridisation and introgressive gene flow since they contain species-specific alleles (Bekkaoui et al. 2003; Fossati et al. 2003; Khasa et al. 2005). Due to their codominant inheritance, hybridisation can be detected in either direction. Field studies on introgressive gene flow in openly pollinated *P. nigra* and *P. x canadensis* stands revealed varying results. Several studies showed no evidence for introgressive gene flow (e.g. Fossati et al. 2003; Imbert & Levèvre 2003; Tabbener & Cottrell 2003). In contrast, Vanden Broeck et al. (2003) reported on the production of viable seeds under specific field conditions. Furthermore, recent studies revealed the establishment of both F2 and backcrossed poplars in the field (Pospíšková & Šalková 2006; Heinze 2008; Smulders et al. 2008a; Ziegenhagen et al. 2008). A first estimate of the degree of introgression was obtained by Ziegenhagen et al. (2008). They demonstrated the benefit of combined diagnostic cp and SSR markers for the identification of juveniles with *P. x canadensis* parental background. To date, all studies that reported on mating of *P. nigra* and *P. x canadensis* qualitatively revealed the existence of spontaneous hybridisation or the survival of progeny in principle. However, quantitative studies are still missing. They would help to assess the risk of introgressive gene flow. This would contribute to an effective management of conservation activities for black poplar.

1.4 Objectives of this thesis

The aim of this study was to systematically analyse pollen and seed mediated gene flow in the model system *P. nigra*/*P. x canadensis*. Key features of SSR markers, namely high polymorphism, codominant inheritance and availability of species-specific alleles were used to create a tool for obtaining high-definition multi-locus genotypes. These so-called genetic fingerprints allow the unambiguous identification of individuals and the assessment of species affiliation concerning *P. nigra* and *P. deltoides*. Therefore, an easy-to-handle assay of seven SSR markers was established which offered all of these requested features (paper I). This

marker system was applied in several genetic analyses. Seedlings, juveniles from natural recruitment and an adult tree population were studied. This allowed a detailed insight into gene flow patterns and their consequences for spatial genetic structure, diversity and introgression of the *P. nigra* population. I focussed on the following aims:

i) I used the high information content offered by the seven-loci SSR genetic fingerprints to establish a fast but reliable and unambiguous identification of individuals belonging to the hybrid complex of *P. nigra* and *P. x canadensis*. Allelic ladders and reference genotypes allow an identification that is transferable to different laboratory settings. Therefore, I established a first standardised hybrid clone register consisting of 65 different clones of commercial interest (paper I). The marker assay was also the basis for subsequent studies as laboratory routines were established that allowed large sample throughput.

ii) I systematically analysed quantitative measures of effective pollen- and seed-mediated gene flow inside a natural population of *P. nigra*. I compared these results to spatial genetic patterns of the adult trees. Consequences for genetic diversity could be concluded and consequences for the management of natural recruitment could be deduced. For this, I used a stepwise approach in examining different ontogenetic stages (seedlings, juveniles and adult trees): In a first step, I characterised and compared genetic diversity of both an adult *P. nigra* population and the juveniles, its offspring population. Hereby, the results are discussed in relation to other European studies on black poplar. Secondly, I used two different approaches for measuring gene flow: Indirect measurement on the basis of SGS in the adult tree population as well as direct monitoring of the effective pollen and seed dispersal as obtained by parentage analysis of seedlings. For the first time, both effective pollen and seed dispersal inside a *P. nigra* population could be quantified in the context of the surrounding area. I used this to explain and classify SGS and to give implications for in-situ conservation measures (paper II).

iii) Finally, the choice of several markers exhibiting species-specific alleles markers allowed to study introgressive gene flow between *P. nigra* and *P. x*

canadensis and to assess the hybrid status of individuals. I explored and quantified introgressive gene flow systematically as both species grow in direct vicinity to each other at the study site. Analyses focussed on both, produced viable seeds and juveniles. I analysed large sample sizes of progeny originating from seeds of known mothers of both poplar taxa and juveniles at four diagnostic nSSR marker loci. Maternal species affiliation of juveniles from natural recruitment was assessed by a cp-marker (DT (Demesure et al. 1995)). By this means, rates of progeny production with *P. x canadensis* ancestry could be estimated in total and the two different maternal origins could be analysed separately (paper III).

All papers are discussed regarding natural conservation of *P. nigra* against the background of gene flow and hybridisation. A transfer of results may contribute to the general understanding of gene flow and hybridisation processes. This includes possible consequences for managing populations of wind-pollinated trees and pioneer species on a landscape level. Results are also discussed concerning GM trees and the risk assessment of transgene flow.

2. Publications and manuscripts

2.1 Paper I:

Allelic ladders and reference genotypes for a rigorous standardisation of poplar microsatellite data

G. Rathmacher, M. Niggemann, H. Wypukol, K. Gebhardt, B. Ziegenhagen and R. Bialozyt

Trees – Structure and Function (in press), DOI: 10.1007/s00468-008-0302-z
The original publication is available at www.springerlink.com

Allelic ladders and reference genotypes for a rigorous standardisation of poplar microsatellite data

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Abstract

A correct identification of members of the poplar hybrid complex *Populus x canadensis* is essential in breeding programmes and studies in introgressive gene flow. Molecular marker protocols have been developed for this purpose. However, due to missing standards, these techniques have so far not been suited to the transfer of results between different laboratories. We present here a powerful system of nuclear microsatellite DNA (nSSR) fingerprints, standardised by allelic ladders and reference genotypes. Seven nSSR loci provided fingerprints of 65 commercial poplar clones. Their alleles were used to construct allelic ladders. Thus, a first standardised register of poplar clones is now available. All procedures were optimised according to simplified DNA extraction protocols, multiplexed PCR and electrophoresis procedures. Corresponding data originating from two different electrophoretic platforms in different laboratories were congruent when the allelic ladder was used. Unambiguous differentiation of the clones was based on a very low probability of identity (*PI*) of 1.95×10^{-8}. Our results revealed discrepancies between clone denotations and genetic fingerprints. This suggests that, potentially, members of the clone collection could have been

mixed up, thus confirming the demand for rigorous standards. The protocol presented can be exploited in a manifold way, e.g. to enlarge the present clonal molecular data base, or to use it for purposes of certification and control. Furthermore, the allelic ladders are recommended for use in poplar population genetic studies across different laboratories. The allelic ladders and single sample reference genotypes can be obtained on demand.

Introduction

Members of the genus Populus (Salicaceae) are major suppliers of industrial wood worldwide. They are fast-growing, and many poplar species are suitable for clonal forestry. For breeding purposes, one advantage of poplar over any other tree species is that both physical and molecular genetic maps are available from which links between phenotypic traits and genes can be deduced (Cervera et al. 2001; Taylor 2002; Sims et al. 2006; Gaudet et al. 2008).

Through controlled hybridisation (Taylor 2002), breeders are able to combine the favorable traits of different parental species in one cultivar. Simple and cost-effective vegetative propagation of cultivars can be exploited for clonal distribution. Clonal plantations exhibit fast growth as well as homogeneity of size and wood quality. They are thus easily processed in industry (Zsuffa 1975; Fossati et al. 2005). Plantations of hybrid poplar have since long been appreciated as an alternative resource for wood production due to increasing demands for renewable energy (Zsuffa 1975; Bekkaoui et al. 2003; Rajora & Rahman 2003; Sims et al. 2006). Recently, the conversion of abandoned agricultural areas to woodland has begun to be promoted by the European Community (De-Lucas et al. 2008).

More than 90 percent of all cultivated poplars are assumed to belong to the interspecific hybrid *P. x canadensis* Moench (syn. *Populus x euramericana* (Dode) Guinier) and their parental species *P. deltoides* Marsh. and *P. nigra* L (FAO 1979). Consequently, many poplar breeding programmes have focused on *P. deltoides* x *P. nigra* controlled crosses which has led to a large number of cultivars and clones.

The proper identification of the highly productive cultivars ensures the correct assignment between the declared and the true identity of a clone. Such interest, shown by poplar breeders, growers and industry, is legitimate and needs to be supported (Fossati et al. 2005; De-Lucas et al. 2008). In addition, European regulations such as the German Law on Forest Reproductive

Material (Forstvermehrungsgutgesetz), demand certificates of origin and clonal identity (FoVG 2006).

The traditional method adopted by the International Poplar Commission for identification, registration and certification of poplar clones is based on a total of 64 morphological, phenological and floral characteristics (UPOV 1981).

Phenotypic methods of identification are not always satisfactory because of the instability of morphological characters caused by environmental and management factors as well as by the age of the tree and its state of health (De-Lucas et al. 2008). Individual descriptions are of limited use if two or more similar clones cannot be observed at the same time and in the same environment (Fossati et al. 2005). On the whole, this method for clonal identification is difficult, ambiguous, time-consuming and subjective (Rajora & Rahman 2003). Currently, molecular markers are used for differentiation and identification purposes. These are allozyme polymorphism (Rajora & Dancik 1992) or DNA markers such as RAPDs (Castiglione et al. 1993; Rajora & Rahman 2003), AFLPs (Cervera et al. 1996; Cervera et al. 2005; Fossati et al. 2005; Zhou et al. 2005) or nuclear simple sequence repeats (nSSR, syn. nuclear microsatellite markers) (Rajora & Rahman 2003; Fossati et al. 2005; De-Lucas et al. 2008). The latter are the markers of choice. They are reproducible, codominant, highly polymorphic, and if an adequate number of loci is combined, they allow the unambiguous identification of each individual (Jones et al. 1997; Smulders et al. 1997; Dayanandan et al. 1998; Gerber et al. 2000; Jones & Ardren 2003). SSR markers were isolated, characterised and have already been used for clone identification and the assessment of their genetic relationship in poplars (Dayanandan et al. 1998; Bekkaoui et al. 2003; Rajora & Rahman 2003; Fossati et al. 2005; De-Lucas et al. 2008). Due to the common problem of reproducibility of absolute DNA fragment lengths in different electrophoretic platforms and settings (de Valk et al. 2007), these studies did not present any unambiguously transferable results so far.

The aim of our research was to enable a fast, but reliable and transferable identification of commercial poplar clones belonging to the hybrid complex of *P. x canadensis*. For that purpose, seven-loci SSR genetic fingerprints were used for composing allelic ladders and establishing a first standardised clone register consisting of 65 different clones.

Material and methods

DNA extraction

Leaves of 91 trees representing commercially relevant poplar clones of different species (*P. x canadensis*, *P. deltoides*, *P. nigra*, *P. trichocarpa* Torr. & Grey and *P. nigra* x *P. maximoviczii* Henry, see Table 1) were kindly provided by the holders of four German clone collections. The plant material was dried for 24 hours at 36 °C. Approximately 0.5 cm² of leaf material was homogenised using a Retsch shaking mill (Retsch, Hilden, Germany), according to the protocol described by Ziegenhagen et al. (1993).

The DNA was extracted in two different ways:
The first subset of samples was extracted according to the ATMAB procedure (Dumolin-Lapegue et al. 1997). The DNA concentration was measured using the BioPhotometer (Eppendorf, Hamburg, Germany). The quality of the extracted DNA was estimated by calculating the 260:280 OD ratios and by checking the suitability of the DNA as a template in the PCR procedures.
The second subset of samples was extracted using a more time- and cost-effective method of DNA extraction following Jump et al. (2003). Deviant from that protocol, homogenised leaf tissue was mixed with 300 µl of 0.5 M NaOH + 2 % Tween 20.

Optimisation of PCR and genotyping procedures

Populus DNA was analysed at seven highly polymorphic nSSR loci: WPMS05 and WPMS09 were described by van der Schoot et al. (2000), WPMS14, WPMS18, WPMS20 were taken from Smulders et al. (2001). Loci PMGC14 and PMGC2163 were selected from the IPGC (International Populus Genome Consortium) SSR Resource (http://www.ornl.gov/sci/ipgc/ssr_resouce.htm). All markers are completely unlinked (Cervera et al. 2001; Gaudet et al. 2008).
For optimisation purposes, the seven loci were subjected to multiplex PCR reactions comparable to studies in *Quercus* and *Pinus* (Dzialuk & Burczyk 2004; Dzialuk et al. 2005). Three groups of marker loci were formed according to three different panels of annealing

temperatures. The marker loci PMGC14 and WPMS05, further referred to as the Temp50 panel, were amplified at annealing temperature of 50 °C. The marker loci PMGC2163, WPMS09 and WPMS18, further referred to as the Temp55 panel, were amplified at annealing temperature of 55 °C. The marker loci WPMS14 and WPMS20, further referred to as the Temp60 panel, were amplified at annealing temperature of 60 °C. Primers were labelled with different fluorescent colors (FAM, HEX and TAMR) in order to distinguish amplification products in the following automated multiplex electrophoresis.

PCR was performed in a volume of 13 µl containing 10 ng of genomic DNA (extraction following Dumolin-Lapegue et al. (1997)) or preferably 1 µl of DNA extract (extraction following Jump et al. (2003)), 2.4 µM of all amplification primers (2.9 µM (WPMS05) and 2.15 µM (PMGC14) for the Temp50 panel), 0.2 mM of each dNTP, 1.75 mM $MgCl_2$ (2.5 mM for the Temp55 panel), and 0.2 U of Promega GoTaq DNA polymerase (Promega, Madison, WI, USA) in 1 × Green GoTaq Flexi Buffer (Promega, Madison, WI, USA). Thermocycling was performed in a T1 thermocycler (Biometra, Göttingen, Germany) following the protocol described in Dayanadan et al. (1998): After an initial denaturation of 1 min at 94 °C, five cycles were performed for a duration of 1 minute each at 94 °C (denaturation), annealing temperature depending on the marker panel and 72 °C (extension), followed by 30 cycles for a duration of 30 seconds each. To check the success of amplification, several PCR products were controlled. 6 µl each were run on 1 % agarose gel and then stained with GelRed (Biotum, Hayward, CA, USA).

Automated multiplex capillary electrophoresis

For automated multiplex capillary electrophoresis, 2 µl Temp55 panel PCR were treated separately while each 1 µl of Temp50 and Temp60 panels were merged. This was possible, as the DNA fragments of locus WPMS05 were expected to be far longer than the fragments expected from the other three loci present in the PCR cocktail. Hence, both primers of the panel Temp50 (WPMS05 and PMGC14) were labelled with the same fluorescent color. By this means, four loci could be genotyped in only one step of electrophoresis. Each cocktail of PCR products was mixed with 7.75 µl of distilled water and 0.25 µl of ET-ROX 400 marker (GE Healthcare, Diegem, Belgium) each. Following denaturation of the samples for 1 min at 95°C and rapid cooling to 4 °C they were injected onto a MegaBACE 500 equipped with a 96 capillary array. Injection and running parameters were performed according to the instructions

of the manufacturer (GE Healthcare). The fragment size of each PCR product was estimated using Genetic Profiler 2.2 software (GE Healthcare).

Validation of the system

In order to rate the discriminatory power of the multilocus allelic information of the commercial clones, several parameters were calculated; the number of alleles as well as the observed heterozygosity (H_o) and the Probability of Identity (*PI*) for each locus and combined loci. All parameters were calculated using the software package GenAlEx 6 (Peakall & Smouse 2006).

Composing allelic ladders

Allelic ladders were composed by merging the PCR products of samples displaying one out of all the alleles occurring in the clone collection. If one allele did not occur in a homozygous status in the clone collection, a homozygous sample of *P. nigra* (own unpublished data) was taken whenever possible. This procedure was used for each locus. The volume of PCR product taken for the ladder depended on the intensity of amplification as seen in the peaks of the MegaBACE fluorogram. Identification and scoring of the alleles was standardised by a one-letter code (allele A, B, C, etc.). Due to significant stutter peaks of WPMS05 PCR products, the construction of an allelic ladder failed. Instead heterozygous single-sample reference genotypes were selected with main peak distances of at least six base pairs (bp). In electrophoresis, each of them had to be analysed in a single lane.
The allelic ladders were run on the MegaBACE 500 and in a second laboratory on a LI-COR 4300 DNA Analysis System together with samples of known DNA fragment length. Both PCR products of single samples and the allelic ladders were reamplified using 1 µl of PCR product directly as a DNA template in the standard PCR protocol. For the transfer to the LI-COR-system, all markers were labelled with the LI-COR infra-red dye 700. As this system only allows the detection of one fluorescent color, amplified fragments of multiplex PCR reactions can not be distinguished by different label colors but only by substantial differences in bp length. Therefore, multiple PCR reactions could only be performed for the Temp50 panel, as expected fragments of WPMS05 and PMGC14 differed in length of at least 50 bp. All other PCR reactions had to be conducted separately for each locus and consequently

amplified fragments had to be run separately in electrophoresis as well. Preparation of samples and running parameters were performed according to manufacturer's recommendations (LI-COR). The fragment size of each PCR product was estimated using the Saga^{GT} 3.3 genotyping software (LI-COR).

Table 1 Species affiliation, clonal groups (=samples with congruent multilocus genotypes), clone collection of origin (Ori 1-4), licensure of clone variety in Germany (cat. D) and allelic letter code for 91 poplar clones.

Species/ clonal groups	Official name of variety FAO-register	Reference Accession name/No.	Ori	cat. D	WPMS05		WPMS09		WPMS14		WPMS18		WPMS20		PMGC14		PMGC2163	
N/1	Blom	Blom10	4	×	G	L	J	J	C	S	C	F	E	F	H	M	J	S
	Not registered	Neunburg	2		G	L	J	J	C	S	C	F	E	F	H	M	J	S
D × N/2	Brabantica	Brabantica	1		M	R	A	K	I	K	A	D	D	F	B	F	A	R
	Brabantica	Brabantica	3		M	R	A	K	I	K	A	D	D	F	B	F	A	R
D × N/3	Baden	Baden (30/58)	1		G	N	A	O	J	K	A	I	D	F	A	H	A	W
	Drömling	Drömling	1	×	G	N	A	O	J	K	A	I	D	F	A	H	A	W
D × N/4	Dorskamp	Dorschkamp	4		G	N	A	J	P	S	A	I	D	E	A	M	A	J
	Grandis	Grandis9	4	×	G	N	A	J	P	S	A	I	D	E	A	M	A	J
D × N/5	Flachslanden	Flachslanden	3	×	N	X	A	J	K	S	A	I	D	E	B	H	A	J
	Flachslanden	Flachslanden	1	×	N	X	A	J	K	S	A	I	D	E	B	H	A	J
	Forndorf	Forndorf	1		N	X	A	J	K	S	A	I	D	E	B	H	A	J
D × N/6	Grandis	Grandis	1	×	L	M	A	Z_5	I	K	A	E	D	D	A	F	A	R
	Grandis	Grandis	3	×	L	M	A	Z_5	I	K	A	E	D	D	A	F	A	R
D × N/7	Gelrica	Gelrica	2	×	N	N	A	M	F	K	A	F	D	I	B	F	A	S
	Löns	Löns3	4	×	N	N	A	M	F	K	A	F	D	I	B	F	A	S
D × N/8	Löns	Löns	1	×	N	N	A	Z_5	K	Q	A	I	D	D	B	E	A	R
	Löns	Löns	3	×	N	N	A	Z_5	K	Q	A	I	D	D	B	E	A	R
D × N/9	Marilandica	Marilandica	1	×	N	R	A	N	I	K	A	F	D	D	A	F	A	R
	Marilandica	Marilandica	2	×	N	R	A	N	I	K	A	F	D	D	A	F	A	R
D × N/10	Robusta	Robusta	1	×	G	N	A	J	P	S	A	I	D	E	A	M	A	J
	Robusta	Robusta	3	×	G	N	A	J	P	S	A	I	D	E	A	M	A	J
	Robusta	Robusta	2	×	G	N	A	J	P	S	A	I	D	E	A	M	A	J
	Zeeland	Robusta Zeeland (231/54)	1		G	N	A	J	P	S	A	I	D	E	A	M	A	J
D × N/11	Selys	Selys	1		M	M	A	N	I	K	A	F	D	F	B	E	A	H
	Serotina	Serotina	1		M	M	A	N	I	K	A	F	D	F	B	E	A	H
D	Not registered	4/45 (1)	2		N	N	A	A	K	L	A	A	D	D	A	B	A	A
D	Marquette	Marquette	1	×	N	N	A	A	K	N	A	A	D	D	B	B	A	A
D	Alcinde	Alcinde	2		M	N	A	A	K	O	A	A	D	D	B	B	A	A
N	Not registered	**Harvard**	**2**		**R**	**T**	**H**	**J**	**I**	**K**	**F**	**F**	**E**	**F**	**D**	**E**	**H**	**R**
D	Harvard	**Harvard**	**1**		**N**	**T**	**A**	**A**	**K**	**N**	**A**	**A**	**A**	**D**	**B**	**B**	**A**	**A**
D × N	Not registered	**Harvard19**	**4**		**M**	**R**	**A**	**K**	**I**	**K**	**A**	**D**	**D**	**F**	**B**	**F**	**A**	**R**
N	Not registered	Plantierensis (101/49)	1		G	X	J	N	J	S	I	I	F	F	H	M	J	J
N	Italica	Italica	2		G	X	J	N	J	S	F	I	E	F	H	M	J	W
N	Not registered	Pyramidalis (134/49)	1		N	N	J	N	D	I	D	F	G	G	H	H	H	W
N	Blanquillo de Bucos	Blanquillo de Bucos	1		N	X	K	N	J	J	D	F	D	F	H	M	J	S
N	Not registered	Erlbach	2		M	P	J	J	F	T	D	J	F	G	E	H	K	S
N	Not registered	Erlbach17	4		L	R	J	N	K	K	D	F	D	G	D	H	L	S
N	Vereecken	Vereecken	2		L	X	J	Z_5	K	S	C	I	F	F	F	M	U	W
T[a]	Fritzi Pauley	Fritzi Pauley	2	×	L	L	Z	Z	F	M	C	C	F	F	E	E	B	B
N × M[a]	Rochester	Rochester	2		M	M	E	S	E	K	B	B	D	D	C	M	Q	Q
D × N	Not registered	Karolina (79/54)	1		L	M	A	N	K	T	A	J	D	H	B	G	A	V
D × N	Allenstein	Allenstein	1	×	N	X	A	O	P	S	A	I	D	F	A	M	A	W
D × N	Bietigheim	Bietigheim	1		G	N	A	O	Q	S	A	I	D	F	B	H	A	J
D × N	Blanc du Poitou	Blanc du Poitou	2	×	N	N	A	J	I	Q	A	D	D	F	B	F	A	T
D × N	Büchig	Büchig	1	×	G	N	A	J	P	S	A	F	D	F	A	H	A	J
D × N	Carpaccio	Carpaccio	1		N	X	A	J	J	M	A	F	D	F	B	H	A	J
D × N	Dolomiten	Dolomiten	1	×	G	P	A	J	J	Q	A	I	D	F	A	M	A	J
D × N	Dorskamp	Dorskamp	2		G	G	A	Z	K	P	A	F	D	G	A	E	A	H
D × N	Drömling	Drömling14	4	×	P	X	A	N	Q	S	A	F	D	E	A	M	A	J
D × N	Drömling	Drömling	3	×	G	N	A	N	P	S	A	F	D	E	B	H	A	J
D × N	Eckhof	Eckhof	1		G	N	A	N	J	K	A	I	D	F	A	H	A	W

Table 1 continued

Species/ clonal groups	Official name of variety FAO-register	Reference Accession name/No.	Ori	cat. D	WPMS05		WPMS09		WPMS14		WPMS18		WPMS20		PMGC14		PMGC2163	
D × N	Florence	Florence	1		D	J	A	I	D	I	A	F	D	E	B	H	A	N
D × N	Not registered	Floßgrün	1		N	Y	A	H	I	I	A	F	D	D	B	H	A	W
D × N	Gelrica	Gelrica B12	1	×	L	M	A	Z_5	I	K	A	J	D	F	B	H	A	S
D × N	Gelrica	Gelrica2	4	×	N	N	A	Z_5	K	Q	A	I	D	D	B	E	A	R
D × N	Not registered	Goldgrund	1		G	N	A	J	J	K	A	I	D	F	A	M	A	W
D × N	Guardi	Guardi	1		N	N	A	J	M	S	A	D	D	D	B	H	A	R
D × N	Harff	Harff	1	×	G	N	A	N	Q	S	A	F	D	E	A	H	A	J
D × N	Harff	Harff	3	×	G	N	A	N	Q	S	A	F	D	E	B	H	A	J
D × N	Heidemij	Heidemij	3	×	Q	X	A	N	I	P	A	F	F	F	B	F	A	J
D × N	Heidemij	Heidemij	2	×	Q	X	A	O	J	P	A	F	F	F	B	M	A	J
D × N	I-154	I-154 Casale	1		G	P	A	J	K	S	A	I	D	E	A	M	A	J
D × N	I-214	I-214	2	×	P	R	A	H	D	I	A	D	D	E	A	G	A	S
D × N	I-214	I-214 Casale	1	×	R	R	A	H	D	I	A	D	D	E	B	H	A	S
D × N	I-262	I-262 Casale	1		P	X	A	J	Q	S	A	I	D	F	A	M	A	W
D × N	I-45/51	I-45/51 Casale	1		N	N	A	H	I	Q	A	K	D	F	A	H	A	S
D × N	I-455	I-455 Casale	1		P	X	A	J	Q	S	A	I	D	F	B	H	A	W
D × N	I-476	I-476 Casale	1		M	M	A	H	I	Q	A	K	D	F	B	H	A	S
D × N	I-488	I-488 Casale	1		G	M	A	J	J	Q	A	I	D	F	A	E	A	J
D × N	Not registered	I-92/40 Casale	1		G	P	A	Z_1	D	Q	A	H	D	F	B	G	A	H
D × N	Jacometti-78-b	Jacometti-78-b	1	×	G	M	A	H	I	I	A	H	D	E	B	H	A	W
D × N	Not registered	Kastenwörth	1		M	R	A	J	K	N	A	D	D	G	B	D	A	R
D × N	Lampertheim	Lampertheim	1	×	Q	X	A	J	P	S	A	I	F	F	A	H	A	J
D × N	Not registered	Lampertheim Findl. (56/55)	1		G	N	A	N	P	S	A	I	E	E	A	M	A	J
D × N	Leipzig	Leipzig	1		M	R	A	Q	F	K	A	E	D	F	B	G	A	R
D × N	Lingenfeld	Lingenfeld	1	×	Q	X	A	J	P	S	A	F	E	E	A	H	A	J
D × N	Marilandica	Marilandica	3	×	N	N	A	Z_5	K	Q	A	I	D	D	B	F	A	R
D × N	Neupotz	Neupotz	1	×	G	M	A	N	K	S	A	I	D	E	B	H	A	W
D × N	Neupotz	Neupotz	3	×	G	M	A	N	K	S	A	I	D	E	A	H	A	W
D × N	Ostia	Ostia	1	×	M	X	A	J	I	Q	A	D	D	D	A	F	A	W
D × N	Régénéré	Regenerata Kew (73/56)	1		M	N	A	J	I	Q	A	E	D	D	B	H	A	T
D × N	Rintheim	Rintheim	1	×	L	M	A	K	I	Q	A	D	D	D	A	D	A	I
D × N	Serotina	Serotina	2		L	L	A	J	I	K	A	F	D	F	B	E	A	H
D × N	Not registered	Speyer 02 (49/59)	1		G	M	A	N	K	S	A	F	D	F	A	M	A	J
D × N	Not registered	Sprengen (55/58)	1		N	X	A	J	Q	S	A	F	D	E	B	M	A	W
D × N	Tannenhoeft	Tannenhöft	1	×	G	N	A	J	J	P	A	F	D	F	A	M	A	J
D × N	Tardif de Champagne	Tardif de Champagne	1	×	N	N	A	Q	G	K	A	F	D	F	B	H	A	R
D × N	Tardif de Champagne	Tardif de Champagne	2	×	N	N	A	Q	G	K	A	F	D	F	A	G	A	R
D × N	Virginie de Frignicourt	Virginie de Frignicourt	1		M	M	A	J	I	Q	A	E	D	D	B	F	A	T
D × N	Virginie de Nancy	Virginie de Nancy	1		G	N	A	N	P	S	A	I	F	F	A	M	A	J
D × N	Zürich	Zürich	1		M	X	A	J	J	Q	A	D	D	D	B	H	A	W

Clones of the name “Harvard” are highlighted by bold typing. According to literature, “Harvard” is part of *P. deltoides* and “Blom” is part of *P. trichocarpa* (Fossati et al. 2005).
D = *P. deltoides*, N = *P. nigra*, T = *P. trichocarpa*, DxN = *P x canadensis*, NxM = *P. nigra x P. maximowiczii*
[a] species affiliation according to Fossati et al. (2005).

Results

DNA extraction

DNA solutions of both extraction methods produced congruent PCR products. When applied on the same sample, the results were reproducible between experiments. The DNA extraction of dried and powdered wood was also successful (own unpublished data).

PCR procedure and sizing of amplification products

The optimised amplification protocol of PCR reactions and MegaBACE 500 runs constantly yielded clearly identifiable peaks of different colors and sizes. A significant portion of stutter peaks only occurred in the amplification products of WPMS05 but did not hinder the identification of the prominent peak in each sample. With the help of species-specific allele information (Bekkaoui et al. 2003; Fossati et al. 2003; Khasa et al. 2005) species affiliation according to *P. nigra*, *P. deltoides* and *P. x canadensis* could be assigned.

Diversity estimates

All SSR markers showed high allelic diversity and observed heterozygosity in the studied clones. Among the 91 samples, 77 separate multilocus genotypes could be obtained. The number of alleles ranged from seven alleles of WPMS20 up to 17 alleles of WPMS14, with an average of 12.14 alleles per locus (Table 2). The observed heterozygosity ranged from 0.67 to 0.96 (average: 0.87) with WPMS20 displaying the lowest and WPMS14 the highest allelic diversity values.

Probability of identity (PI)

The probability of two unrelated individuals displaying the same multilocus genotypes by chance is represented by *PI* (Taberlet & Luikart 1999; Waits et al. 2001). Single locus *PI* ranged from 0.03 (PMGC2163) to 0.19 (WPMS20). With all seven loci considered, the combined probability of identity was calculated as 1.95×10^{-8} (Table 2).

However, multilocus genotypes of samples originating from different collections but being declared the same clone identity often differed (Table 1). Most strikingly, the three samples of the clone "Harvard" turned out to belong to *P. nigra* and *P. deltoides* as well as to *P. x canadensis*. These samples originate from three different clone collections. Additionally, several clone samples with different names surprised by showing identical genetic fingerprints, such as with "Gelrica" and "Löns3". Both labels represent nationally licensed clones in Germany. Clone "Blom" (in fact *P. trichocarpa* (Fossati et al. 2005)) showed typical patterns of *P. nigra* which were identical to clone "Neunburg". Congruent genetic data of all samples was shown by three clone identities ("Brabantica", "Flachslanden" (two trees each) and "Robusta" (four trees).

Table 2 Genetic parameters of nSSR loci combined in three marker panels for the batch of 91 trees of commercial relevant poplar clones. N_a: Number of alleles, H_o: observed heterozygosity; *PI*: Probability of identity.

Marker panel	**Locus**	N_a	**Allelic size range in bp (MegaBACE data)**	H_o	***PI***
Temp50	**WPMS05**	13	266-308	0.76	0.05
	PMGC14	9	193-229	0.95	0.06
Temp55	**WPMS09**	14	237-297	0.91	0.10
	WPMS18	10	220-253	0.92	0.11
	PMGC2163	15	190-252	0.93	0.09
Temp60	**WPMS14**	17	232-283	0.96	0.03
	WPMS20	7	206-254	0.67	0.19
combined *PI* for all loci		-	-	-	1.95×10^{-8}
	mean	12.14	-	0.87	0.09

Reference genotypes and Allelic ladders

Reference genotypes with allele sizes and letter codes of WPMS05 are displayed in Table 3. One reference genotype (ref2) displays three main peaks.

The combined fluorograms and letter codes of the allelic ladders for loci WPMS09, 14, 18, 20 and PMGC14 and 2163 are displayed in Figure 1. Not every allele occurring in the clone collection was added to the ladder, e.g. allele "E" in the ladder for PMGC14 (Fig. 1e).

Table 3 Featured alleles of reference genotypes (ref 1-5) for locus WPMS05.

Size (MegaBACE data)	Letter	Reference Genotype
252	A	
260	B	
262	C	
264	D	ref 1
266	E	
268	F	
272	G	ref 2
274	H	
276	I	ref 3
278	J	ref 1
280	K	
282	L	ref3/ref5
284	M	ref 2
286	N	ref 4
288	O	
290	P	ref 2
292	Q	
294	R	ref 4
296	S	
298	T	
300	U	
302	V	
304	W	
306	X	ref 5
308	Y	
310	Z	

Due to differences in DNA fragment concentration, the peaks show varying intensity. The allelic ladders enabled standardisation of genotyping. Allele lengths of the examined poplar clones could be transferred to the allelic letter code (Table 1).

By reamplifying the allelic ladders, the signals of single fragments declined with the increase in fragment size. Due to this obstacle, the DNA of long fragments from single sample PCR products in particular needs to be added at regular intervals to the allelic ladder. Reamplification of single sample PCR products proved to be entirely unproblematic. Due to the increasing intensity of stutter peaks during reamplification of WPMS05 single sample PCR products, original DNA extracts had to be used when refilling reference samples.

Without the usage of allelic ladders, corresponding allele sizes of the same samples run on LI-COR and on MegaBACE respectively, showed differences of up to 4 bp. The corresponding data was congruent when the letter code of the allelic ladders was applied. Control samples could clearly be assigned. These results were compared to published data from De-Lucas et al. (2008) (Table 4).

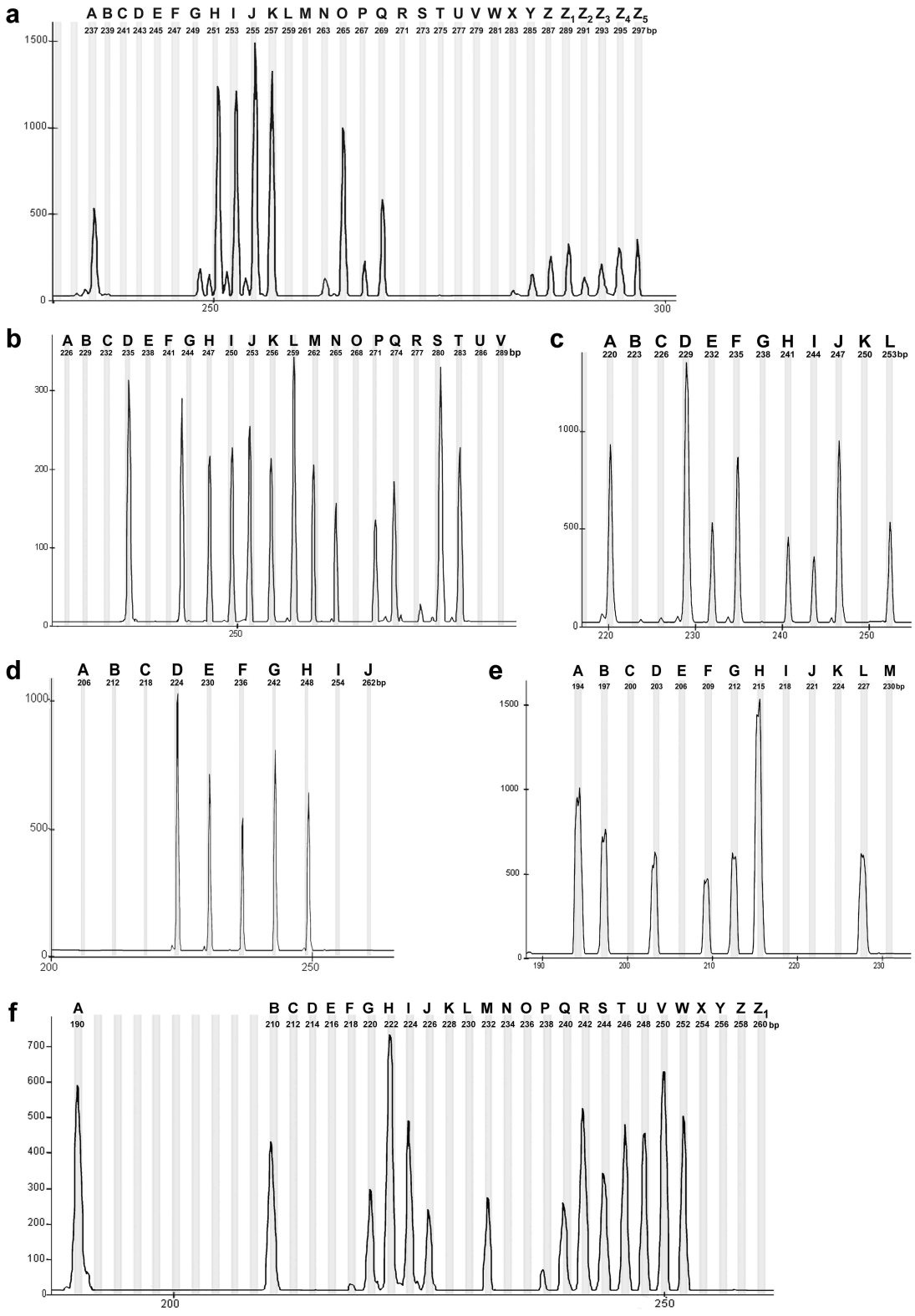

Fig. 1 Allelic ladders of SSR loci. *x*-axis: fragment length in base pairs (bp) (MegaBACE data); *y*-axis: relative intensity of the fluorescent signal. *Letters* are assigned to allele length in ascending order. **a** WPMS09, **b** WPMS 14, **c** WPMS18, **d** WPMS20, **e** PMGC14, **f** PMGC2163. Species specific alleles for *P. deltoides* are alleles A (WPMS09, WPMS18, PMGC2163 (Fossati et al. 2003; Khasa et al. 2005)) and alleles A and B (PMGC14 (Fossati et al. 2003)), respectively.

Table 4 Allele sizes of *P.deltoides* specific alleles (Fossati et al. 2003; Khasa et al. 2005; Bekkaoui et al. 2003) of the three loci WPMS09, 18 and PMGC14 as obtained in different laboratory settings and their differences in base pairs (bp).

	WPMS09	**WPMS18**	**PMGC14**	
LI-COR	233	218	195	198
MegaBACE	237	220	194	197
ABI- PRISM 310	232	215	190	193
difference LI-Cor/ MegaBACE	4	2	1	1
difference LI-Cor/ ABI- PRISM 310	1	3	5	5
difference MegaBACE/ ABI- PRISM 310	5	5	4	4

Data of ABI-PRISM 310 originating from De-Lucas et al. (2008)

Discussion

Our aim was to present a poplar genotyping procedure, which is fast and effective but at the same time reliable, meeting the requirements of rigorous standardisation across different laboratories. We report here on the construction of the first allelic ladders available for a forest tree.

Our DNA extraction according to Jump et al. (2003), using dried tissue instead of frozen samples, enables easy sample taking. Whenever possible, multiplexing PCR reactions minimise the effort with regards to time and financial means. Subsequent multiplexing of different PCR products during electrophoresis additionally speeds up genetic fingerprinting. It offers a fast and cheap genotyping protocol with reliable results and the opportunity of large sample throughput (de Valk et al. 2007). In our study, seven loci genotypes could be obtained by just three PCR reactions and two steps of electrophoresis by using the MegaBACE system.

The analysis of genetic parameters shows that estimates of diversity are comparable to other studies dealing with the identification of commercial polar clones (Fossati et al. 2005; De-Lucas et al. 2008). In these studies, locus WPMS20 was also found to display the smallest number of six alleles while locus WPMS 14 displayed the largest number of alleles, 18 (De-Lucas et al. 2008) or 15 alleles respectively (Fossati et al. 2005). Observed heterozygosity was high in the present study (0.67 to 0.96) indicating that a huge amount of information is displayed in samples which are heterozygous at multiple loci. This could be due to the fact that analysed clones displayed hybrid effects of an elevated level of heterozygosity, typical in crop plant breeding (Stark et al. 2006). Other studies revealed H_o of a clone collection ranging from 0.53 to 0.89 (De-Lucas et al. 2008).

Low rates of probability of identity are required for an unambiguous individual identification. The time and cost limitations of studies require a trade-off between best necessary power of discrimination and a minimum number of loci. In the study of De-Lucas et al. (2008), the combined *PI* of twelve loci revealed a value of 1.18×10^{-9}. The usage of seven loci in our study results in a *PI* differing in only one decimal power. Regarding three loci of the same study (De-Lucas et al. 2008), *PI* resulted in 2.4×10^{-4}. This level was considered sufficient for significant identification of the 28 poplar clones in their study. Therefore, the marker combination of the present study represents a useful trade-off between high information content and simple application.

The question arises whether the markers as such are stable enough with regard to somatic mutations. Despite the significance and stability of SSR markers, somatic mutations may occur especially in old clonal lineages (Thomas 2002). This is important when several poplar clones have been propagated for some 250 years (Rajora & Rahman 2003). Mutation rates for microsatellite loci in plants have been estimated to fall within the range of 10^{-2}-10^{-3} for nuclear-encoded loci with tri- and dinucleotide repeats (Kovalchuk et al. 2000; Udupa & Baum 2001). However, mutations are spontaneous and non-directional. Clones mismatching in their SSR genotypes due to somatic mutations may also have changed their morphological traits (Franks et al. 2002). A formerly productive clone may (through somatic mutation) actually show weak features, although clonal propagation was performed properly and mislabelling can be excluded. Samples carrying the same label but differing in only one allele length may be results of somatic mutations. A molecular genetic fingerprint can therefore even be useful for detecting the stability of an established clone.

The most substantial evidence for the need of standardised molecular clone identification techniques are the diverging genotypes of the samples labelled “Harvard”. In this case, even declared species affiliation was incorrect for at least two of the three samples. Accurate multilocus genotypes representing one distinct clone identity were impossible to achieve because mislabelling could not be retraced.

Furthermore, it was not retraceable, whether identical genetic fingerprints of different samples in our results represented true clonal duplicates or just mislabelled trees of one clone in only one specific collection. Clonal group 1 (“Blom”/”Neunburg”) clearly demonstrates discrepancies between clone denotations and genetic fingerprints: According to the literature, clone “Blom” belongs to species *P. trichocarpa* (Fossati et al. 2005). However, allelic patterns of locus WPMS18 can be used to attest both samples belonging to *P. nigra*, as this locus amplifies for species *P. nigra* and *P. deltoides* but not for *P. trichocarpa* (Smulders et

al. 2001). Species affiliation to *P. deltoides* can be excluded as species-specific alleles did not occur (Fossati et al. 2003). Our results suggest that many trees in clone collections could have been mislabelled and are currently merchandised under the wrong labels. Evidence is given for poplars in Spain (De-Lucas et al. 2008) as well as for apple varieties in Germany (Mosch et al. 2008).

The only reliable way to prevent mislabelling is the use of allelic ladders or reference genotypes. In our study, this method ensured matching results from the determination of correct allele sizes across two laboratories. Without molecular standards, variations in sizing were unpredictable. Consequently, data could not be adjusted by a fixed correction scheme. According to De-Lucas et al. (2008), variation of allele sizes in different laboratory settings is negligible. Our comparison of the lengths of *P. deltoides* specific alleles of WPMS09, 18 and PMGC14 run in different settings illustrates the opposite: Our findings show that differences can range between 2 and 4 bp. A comparison to De-Lucas et al. (2008) even yielded differences up to 5 bp.

Conclusions and perspective

Concerning nationally-licensed clone varieties, congruent samples with different names are not desired: Either varieties are synonymous or certification errors have occurred. In both cases, commercial damage is predetermined. Our molecular certification protocol can be used in solving these problems. Identities can now be clearly recognised by a significant seven-loci SSR marker assisted genetic fingerprint. Now, duplications among previously registered poplar clones, labelling errors and evolved genetic deviation from the originally established clone variety can be recorded. Technically, all procedures were optimised to the effect that the volume of information obtained per sample was maximised while the effort in terms of costs and time was minimised. With the help of allelic ladders and reference genotypes, clone identities of the hybrid complex *P. x canadensis* can be assigned independently of the research facility. The standardisation protocol presented may, therefore, contribute to the establishment of new certification systems in the European Community. In this way, legitimate interests of poplar breeders, growers and industries can be protected efficiently.

Future investigations need to clearly identify specific commercial clone identities. These outcomes will then need to be backed up by reference samples and should be compared to the present allelic table. In the case of congruent information, it will be possible to confirm allelic

code and variety name for use as adjustment data in the future. Subsequently, trees of unknown identity can be assigned to the "true" specific clone identity. Depending on national interests, different clones or hybrid crossings may be commonly used in other countries. However, additional alleles of new varieties or different poplar species yet missing in the presented list of clones can easily be included in order to enlarge the allelic ladders or to offer additional reference genotypes, respectively.

In addition to the registration of elite clones, the protocol presented can also be used for other applications such as the identification of putative parents, the study of biodiversity, geneflow studies and the verification of crosses. Since species-specific alleles are available for four out of seven loci, species affiliation and hybrid status concerning *P. nigra* and *P. deltoides* can be easily diagnosed (Bekkaoui et al. 2003; Fossati et al. 2003; Khasa et al. 2005).

The allelic ladders and single sample reference genotypes can be obtained on demand at www.picme.at. Postal address: Repository centre/PICME; Austrian Research Centers GmbH –ARC; 2444 Seibersdorf, Austria and Silvia.fluch@arcs.ac.at.

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2.2 Paper II:

Short-distance gene flow in *Populus nigra* L. accounts for small-scale spatial genetic structures – implications for in-situ conservation measures

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Manuscript, submitted to *Conservation Genetics*

Short-distance gene flow in *Populus nigra* L. accounts for small-scale spatial genetic structures – implications for in-situ conservation measures

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Running title:

Gene flow and spatial genetic structure patterns of *P. nigra*

Keywords:

parentage analysis, SSR, microsatellite, natural regeneration, pollen dispersal, seed dispersal

Abstract

The European black poplar (*Populus nigra* L.) is a major species of riparian softwood forests. Due to human influences, it is one of the most threatened tree species in Europe. For restoration purposes, remaining stands may act as source populations. We analysed a natural population of *P. nigra* for genetic diversity and spatial genetic structure using seven microsatellite markers. For the first time, paternity analysis of seedlings as well as juveniles from a restricted area of natural regeneration was used to quantify pollen- and seed-mediated gene flow, respectively. Spatial genetic patterns of the adult tree population suggest small-scale isolation by distance due to short-distance gene flow, the major part of which (i.e. 70 %) takes place within distances of less than 1 km. This helps to explain the reduced diversity in the juveniles. It has implications for the spatial management of natural regeneration areas within in-situ conservation measures.

Introduction

The European black poplar (*Populus nigra* L.) is dioecious and wind-pollinated. It is a keystone species for softwood floodplain forest ecosystems. As a pioneer species, it regenerates through colonisation of bare soil along the riverbank created by heavy flooding events. The wind- and water-dispersed seeds have a short viability period. They need highly specific water/soil conditions for germination (Braatne et al. 1996; Guilloy-Froget et al. 2002). Successful regeneration therefore only occurs when the moisture of sediment remains high enough for seedling roots to establish (Legionnet et al. 1999). Due to inappropriate conditions in many years, successful regeneration is absent. Accordingly, the history of flooding is reflected in a strong age structure that frequently exists in naturally occurring stands (Heinze 1998). The natural regeneration of *P. nigra*, which is already patchy and sporadic, is additionally restricted by human activities. The drainage of rivers or management of riverbanks prevents natural flooding dynamics, which causes a lack of suitable areas for seedling establishment. Furthermore, today, many native populations of *P. nigra* have been replaced or fragmented by the widespread cultivation of commercially exploited hybrid poplars. From an economic point of view the most important hybrid combination is *P. x canadensis* (Dode) Guinier produced by crossings of *P. nigra* and the North American species *P. deltoides* Bartr. (FAO 1979). Forest fragmentation leads to a breakup of pollen- and seed-mediated gene flow (Jump & Peñuelas 2006). Limitations of pollen and seed dispersal result in spatial aggregation of related individuals that is called “isolation by distance” (Hardy & Vekemans 1999; Born et al. 2008). However, genetic diversity is important to allow a population to survive and reproduce under changing environmental conditions (Primack 2000).

Due to the reasons outlined above, the European black poplar is one of Europe’s rarest native trees (Tabbener & Cottrell 2003; Pospíškova & Šalková 2006) although it has a wide distribution, ranging from Central and Southern Europe to Central Asia and North Africa (Zsuffa 1974). In Germany, it is categorised as “vulnerable” in the National Red List (BfN 2008).

The conservation of in situ genetic resources of *P. nigra* is limited to some restricted areas. Knowledge about the genetic variation and the gene flow inside these remaining populations provides key information for managing their population dynamics (Lowe et al. 2005). For that purpose, genetic analyses of *P. nigra* populations are performed with the use of different molecular markers (e.g. Legionnet & Levèvre 1996; Cervera et al. 1996; Fossati et al. 2003).

Gene flow studies on *P. nigra* revealed a spatial genetic structure (SGS) that was traced back to isolation by distance through limited gene flow (e.g. Imbert & Levèvre 2003; Pospíškova & Šalková 2006). However, in all cases, estimates of effective pollen and seed dispersal distances and their consequences could only be specified by single findings or indirectly by the statistical analysis of present SGS. To date, the knowledge about gene flow patterns in *P. nigra* is therefore only fragmentary.

Hence, the aim of this study was to deliver explicit quantitative and spatially explicit measures of effective pollen- and seed-mediated gene flow inside a natural population of *P. nigra*. In this context, influences on spatial genetic patterns and genetic diversity of regenerating *P. nigra* populations can be discussed and consequences for the management of natural recruitment can be deduced. We followed a stepwise approach to investigate these aspects:

In a first step, we characterised and compared the genetic diversity of both a natural adult *P. nigra* population and an offspring population of juveniles.
Secondly, we used two different approaches to measure gene flow: Indirect measurement on the basis of SGS as well as direct monitoring of pollen and seed dispersal through parentage analysis. For the first time, both effective pollen and seed dispersal inside a *P. nigra* population and in the surrounding area were quantified. This was used to explain and classify patterns of SGS. All results are discussed with respect to implications for in-situ conservation measures.

Material and methods

Study site and plant material

We investigated a black poplar population located in Hesse, central Germany, at the Eder River east of the city of Fritzlar, within a nature protection area. River regulation activities have severely restricted the natural regeneration of black poplars for a long time (Lübcke 1993). Juveniles of black poplar could only be found in a gravel pit area (Fig. 1) with a size of approximately 100 x 100 m. The black poplar population is interspersed with and surrounded by numerous hybrid poplars (*P. x canadensis*).

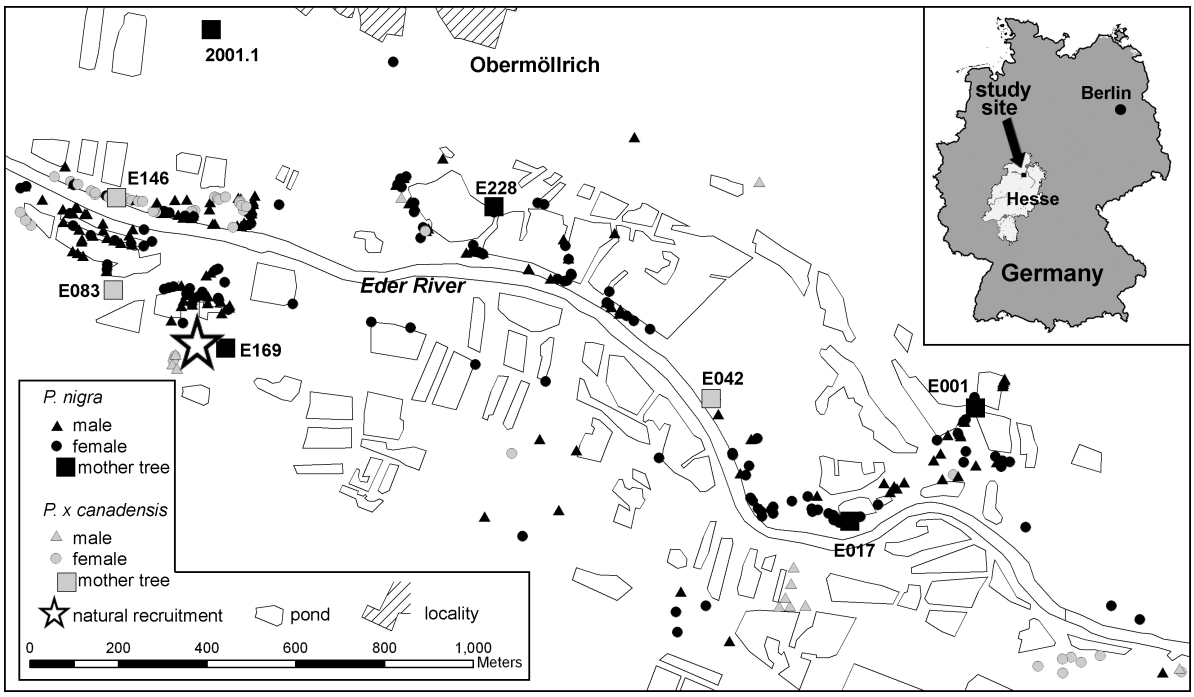

Fig. 1 Distribution of the adult trees and the location of the natural recruitment area containing the juveniles at the study site along the Eder River indicated by the star. Sample names of the mother trees are indicated.

In this study, we sampled adult black poplar and hybrid poplar trees inside the protected area as well as in a radius of 15 km around it. Sex ratio of the natural *P. nigra* adult tree population and the sampled hybrid clones was determined through examination of their flowering phenology. In two consecutive years, seeds were collected from mature catkins of a total of five mother trees of *P. nigra* and three mother trees of *P. x canadensis* (Fig. 1). These seeds were sown on filter paper and placed on waterlogged vermiculite. A few days after germination, seedlings were harvested. Additionally, juveniles of different ages were sampled from the gravel pit area. In total, leaves of 566 adult trees, 2839 seedlings and 380 juvenile plants were sampled and dried for 24 hours at 36 °C.

DNA extraction and genotyping

Approximately 0.5 cm² of dried plant material were homogenised using a Retsch shaking mill (Retsch, Hilden, Germany), following the protocol described by Ziegenhagen et al. (1993). Total DNA was extracted according to Jump et al. (2003). Deviations from that protocol were performed as described by Rathmacher et al. (in press).

Poplar DNA was analysed at seven highly polymorphic nuclear simple sequence repeats (nSSR, syn. nuclear microsatellite markers): WPMS05 and WPMS09 were described by van der Schoot et al. (2000) while WPMS14, WPMS18 and WPMS20 were taken from Smulders et al. (2001). Loci PMGC14 and PMGC2163 were selected from the IPGC (International Populus Genome Consortium) SSR Resource (http://www.ornl.gov/sci/ipgc/ssr_resouce.htm). SSR markers are reproducible, codominant and highly polymorphic. If an adequate number of loci is combined, they allow the unambiguous identification of each individual (Smulders et al. 1997; Jones & Ardren 2003). Loci WPMS09, WPMS18, PMGC14 and PMGC2163 can be used to confirm species affiliation as they contain diagnostic alleles for *P. deltoides* (Bekkaoui et al. 2003; Fossati et al. 2003; Khasa et al. 2005). Hence, they make it possible to distinguish between *P. nigra* and *P. x canadensis*. All markers are completely unlinked (Cervera et al. 2001; Gaudet et al. 2008). PCR and automated multiplex capillary electrophoresis were performed as described in Rathmacher et al. (in press).

Data analysis

Explanatory power of the marker system and diversity parameters

With the information of the diagnostic markers, it was possible to assess species affiliation of the adult poplar trees. To quantify the discriminatory power of the seven-locus allelic information, the Probability of Identity (*PI*) for each locus and of all loci combined were calculated. *PI* represents the probability of two unrelated individuals displaying the same multilocus genotypes by chance (Taberlet & Luikart 1999; Waits et al. 2001). Identical multilocus genotypes (clones) were identified and for calculations, only one ramet of each genet was left inside the data set, as clones are not results of sexual reproduction. Subsequently, the standard parameters of genetic variability according to both the *P. nigra* adult population and the juvenile population were calculated: mean number of alleles per locus (N_a), mean effective number of alleles per locus (N_e), allele frequencies, observed (H_o) and expected heterozygosity (H_e). H_e is considered to represent a measure of gene diversity (ElMousadik & Petit 1996; Comps et al. 2001; Widmer & Lexer 2001). Hybrid poplar clones were excluded from these calculations as they do not belong to a natural population. All parameters were obtained using the software package GenAlEx 6 (Peakall & Smouse 2006). For each locus, deviation from Hardy-Weinberg equilibrium (HWE) was examined by

calculating Weir and Cockerham´s inbreeding coefficient, F_{IS} (Weir & Cockerham 1984), which is equivalent to Wright´s fixation index, F. The significance of F_{IS} was tested using the programme GENEPOP version 4.0 (Raymond & Rousset 1995).

Spatial genetic structure (SGS) and SP *statistic*

SGS is characterised by the relation of kinship or relatedness estimates between pairs of individuals and the physical distances between them (Loiselle et al. 1995; Rousset 2000; Hardy 2003). Its strength can be measured as the rate of decrease of genetic similarity with distance, that is the slope of a kinship-distance curve.

The intrapopulation genetic structure of the black poplar adult tree population was determined using a spatial autocorrelation analysis (Hardy & Vekemans 1999; Vekemans & Hardy 2004). Isolation by distance at the individual level was tested using the software SPAGeDi 1.2 (Hardy & Vekemans 2002). To test for SGS, pairwise kinship coefficients (F_{ij}) were computed between adult individuals for each locus and a multilocus-weighted average using the statistic described in Loiselle et al. (1995). Pairwise kinship coefficients were plotted against the logarithm of spatial distance (d_{ij}) (d is the distance between individual i and individual j) to estimate the logarithmic regression slope b_{log}. The significance of b_{log} was tested by permuting the spatial positions of individuals 10,000 times. Thus the frequency distribution of b could be obtained under the null hypothesis that F_{ij} and d_{ij} were uncorrelated (cf. Mantel test), since a linear decrease is expected in a two-dimensional habitat (Rousset 2000). All seven SSR markers were used for SGS analysis. The average kinship coefficient for pairs of adult individuals was computed for the following distance classes (in m): 0-20; 20-40; 40-80; 80-160; 160-320; 320-640; 640-1280; 1280-2560. Margins of distance classes result from balanced numbers of pairwise distances in each class. Average kinship coefficients between pairs of individuals for each distance interval were plotted against distance classes in a diagram. The extent of SGS was estimated using the *SP* statistic following Vekemans & Hardy (2004). *SP* can be quantified by the regression slope (b_{log}) of F_{ij} on $\ln d_{ij}$ or by the ratio $SP = -b_{log}/(1-F_{(1)})$, where $F_{(1)}$ is the mean F_{ij} between individuals belonging to a distance interval of direct neighbours (0-10 m).

Parentage analysis

Two different parentage analysis calculations were carried out – one with seedlings of harvested seeds from known mothers and a second one with the juvenile population where both parents were unknown. The analysis was performed following a likelihood-based approach, using the software Cervus 3.0 (Kalinowski et al. 2007). This method involves calculating a logarithm of the likelihood ratio (*LOD* score) by determining the likelihood of a pair of individuals being the parents of a given offspring divided by the likelihood of these individuals being unrelated. Offspring is assigned to the parental pair with the highest *LOD* score. To find the confidence level of paternity analysis, simulations with 10,000 repeats were conducted, with 0.01 as the proportion of loci mistyped and 0.8 as the proportion of candidate parents sampled. Simulations also required an estimation of the average number of candidate parents per offspring. For seedlings with known mother, 250 individuals were set as probable candidate fathers. For the juvenile population where both parents were unknown, 250 individuals each were set as probable candidate fathers and mothers. The allele frequencies that were used in the simulation step were calculated based on the *P. nigra* adult tree population data set. Alleles private to seedlings or the juvenile population were added to the frequency data file with $p = 0.0001$. We used 95 % as strict and 80 % as relaxed confidence level as suggested by Marshall et al. (1998).

Identical SSR genotypes of potential parents were treated as one potential parent in parental analysis. Adult trees of undetermined sex were treated as both potential fathers and mothers. We assumed that pollen and seed flight characteristics of both species are the same. Therefore, parental assignments of both species and both years were merged in order to integrate the individual effect of the mother tree and the effect of annual differences. From the paternity analysis of the seedlings, effective pollen dispersal distances were calculated based on the position of the mother tree and the putative pollen parent within the study site. The parentage analysis of the juvenile population was used to calculate effective seed dispersal distances based on the positions of the putative mother tree and recruitment plant.

Kolmogorov-Smirnov test

In order to find out whether the success of male mating was a function of distance between all males and mother trees, we compared the frequency distribution of the distances of assigned fathers to the mother trees with the frequency distribution of the distances among all sampled males and the eight mother trees. This process was carried out using the Kolmogorov-Smirnov test (Crawley 2002) and the programme package "R" (R Development Core Team 2005). Here, the maximum vertical deviation between two frequency distribution curves (*D*) is tested on its statistical significance. The same method was used to assess patterns of effective seed dispersal distances. In this case, we compared the frequency distribution of the distances between assigned mothers and the juvenile population with the frequency distribution of the distances among all sampled female trees and the juvenile population.

Results

Explanatory power of the marker system and diversity parameters

From the 566 adult trees analysed, the presence of diagnostic alleles revealed 320 trees as being *P. nigra*, while 246 turned out to be *P. x canadensis* clones. The sex ratio of the *P. nigra* population exhibited a female excess of 1:0.83, with 149 female and 124 male trees. Due to the absence of flowers, the sex of 47 trees remained undetermined. The hybrid poplar trees had a male proportion of 143 trees, while 95 trees were females and 8 remained undefined (see Fig. 1).

All nSSR markers were highly polymorphic. In both the *P. nigra* adult tree population and its juveniles, WPMS18 showed the smallest number of alleles (8 and 7, respectively). The largest number of alleles was shown at locus WPMS09 for the *P. nigra* adult tree population (17 alleles). In case of the juveniles, it was PMGC2163 (14 alleles). On average, there were 11.6 (*P. nigra* adults) and 10.1 (juveniles) alleles per locus (Table 1, Table 2). Considering all seven loci, the combined probability of identity of the *P. nigra* adult population alone was 1.98×10^{-7}.

Table 1 The characteristics of the genetic diversity of the black poplar population (standard deviation in parentheses). *n*: sample size; N_a: number of alleles per locus; N_e: number of effective alleles per locus; H_o: observed heterozygosity; H_e: expected heterozygosity; F_{IS}: inbreeding coefficient with exact test of departure from Hardy-Weinberg genotypic proportions: *: P ≤ 0.05; **: P ≤ 0.01; ***: P ≤ 0.001; all other values were not significant; S.E.: standard error.

Locus	***n***	N_a	N_e	H_o	H_e	F_{IS}	**P-value**	**S.E.**
WPMS05	285	10	4.19	0.73	0.76	0.0478	0.0610	0.0099
WPMS09	289	17	3.86	0.67	0.74	0.0959***	0	0
WPMS14	290	12	3.19	0.69	0.69	-0.0032***	0.0005	0.0005
WPMS18	290	8	2.4	0.62	0.58	-0.0709*	0.0231	0.0075
WPMS20	290	10	5.13	0.68	0.8	0.1577***	0	0
PMGC14	287	9	4.22	0.78	0.76	-0.0163	0.1211	0.0189
PMGC2163	287	15	3.8	0.72	0.74	0.0183	0.3966	0.056
Mean	288.29 (0.75)	11.57 (1.25)	3.83 (0.33)	0.7 (0.02)	0.73 (0.03)	0.03*** (0.08)		

Table 2 The characteristics of the genetic diversity of the juvenile population (standard deviation in parentheses). *n*: sample size; N_a: number of alleles per locus; N_e: number of effective alleles per locus; H_o: observed heterozygosity; H_e: expected heterozygosity; F_{IS}: inbreeding coefficient with exact test of departure from Hardy-Weinberg genotypic proportions: *: P ≤ 0.05; **: P ≤ 0.01; ***: P ≤ 0.001; all other values were not significant; S.E.: standard error.

Locus	***n***	N_a	N_e	H_o	H_e	F_{IS}	**P-value**	**S.E.**
WPMS05	374	8	3.7	0.69	0.71	0.0211	0.2457	0.0191
WPMS09	377	12	2.8	0.62	0.64	0.0367***	0	0
WPMS14	380	11	2.71	0.61	0.63	0.0378*	0.0348	0.0129
WPMS18	379	7	2.16	0.56	0.54	-0.0399	0.3322	0.0265
WPMS20	376	8	4.75	0.75	0.79	0.0546***	0	0
PMGC14	380	11	3.39	0.67	0.71	0.049	0.0845	0.0184
PMGC2163	380	14	4.05	0.74	0.75	0.0192**	0.0053	0.0032
Mean	378 (2.38)	10.14 (2.54)	3.32 (0.87)	0.66 (0.07)	0.68 (0.08)	0.028*** (0.032)		

Various clonal groups of *P. x canadensis* could be detected, including different numbers of ramets ranging from 2 to 36 each. In the *P. nigra* population, 15 clonal groups were identified; ten groups occurring as two ramets, four groups as three ramets and one group as four ramets. In most cases, clones were located in close vicinity (several meters) except for two cases, where there were distances of more than 1 km between them.

The observed and expected heterozygosities for single loci according to the *P. nigra* adult tree population ranged from 0.62 to 0.78 and from 0.58 to 0.8, respectively (Table 1). In the case of the juvenile population, values were slightly lower and ranged from 0.56 to 0.75 and from 0.54 to 0.79, respectively (Table 2). The positive mean values of the inbreeding coefficient F_{IS} in both populations (0.03 for the adult trees and 0.028 for the juveniles) indicate a weak overall excess of homozygotes. F_{IS} values for single loci in the adult population reflected an

excess of homozygotes for four loci (WPMS05, WPMS09, WPMS20 and PMGC2163), but only two of these loci (WPMS09 and WPMS20) showed significant deviations from zero. By contrast, there was a significant excess of heterozygotes for loci WPMS14 and WPMS18. In case of the juvenile population, all loci except for WPMS18 showed an excess of homozygotes. Four of them were significantly deviating from zero (WPMS09, WPMS14, WPMS20 and PMGC2163).

Spatial genetic structure

The spatial distribution of *P. nigra* trees is shown in Figure 1. Spatial genetic structure was tested using genotype data from all seven microsatellite loci. In agreement with isolation-by-distance models (Hardy & Vekemans 1999), a significant linear decrease of pairwise kinship coefficients with the logarithm of geographical distance was detected. The average kinship coefficients F_{ij} between pairs of individuals for each distance interval were plotted against the logarithm of mean distance classes (Fig. 2). The value of the *SP* statistic was 0.0146 and the value of the regression slope was negative (-0.0136), both indicating positive autocorrelation in the kinship coefficient. Therefore, it turns out that spatially more closely situated individuals were also genetically more related than more distant trees. The first six F_{ij} values were significantly different from a random assignment enclosed by the permutation envelope ($P \leq 0.05$), despite the fact that confidence intervals for the first three distance classes were large because of smaller numbers of pairs in these classes.

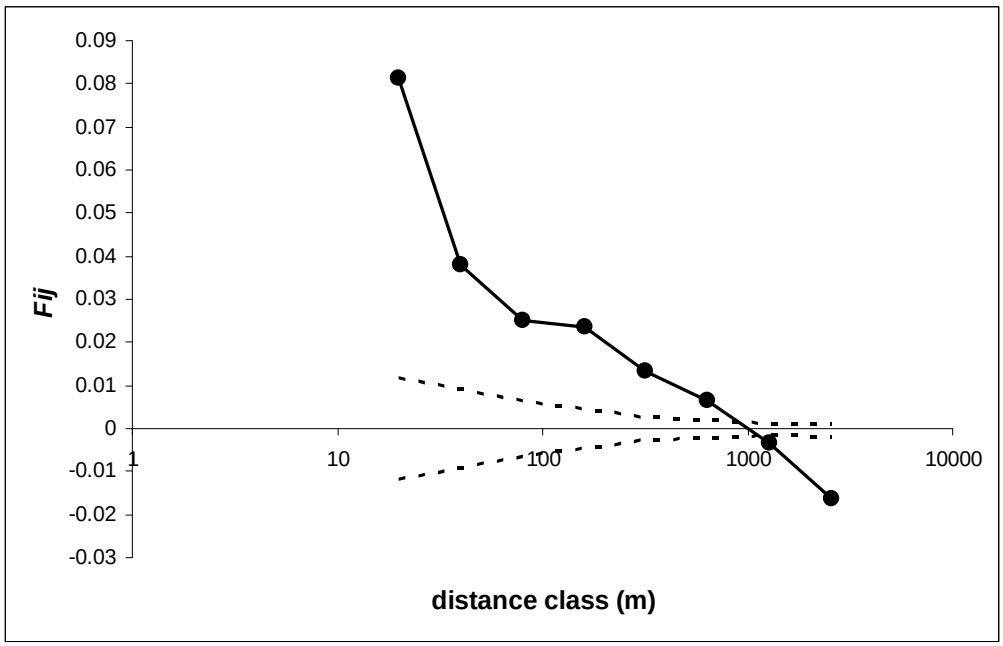

Fig. 2 Analysis of spatial genetic structure of the *P. nigra* adult tree population by means of spatial autocorrelation analysis using F_{ij} kinship coefficients. The correlogram shows mean kinship coefficients between individuals for eight different distance classes. Broken lines delimit 95 % confidence intervals defined through 10,000 permutations around the null hypothesis of random distribution in space.

Parentage analysis

The overall germination rate of collected seeds was high (average 87.5 %), with no significant impact of tree individual or species affiliation. With the help of paternity analysis, 2060 seedlings of the total number of 2839 could be assigned (73 %). Of these, 971 were assigned at 95 % and a further of 1089 assigned at an 80 % confidence level (Table 3). The overall distance of effective pollen movement, inferred from the distance between the mother trees and the most probable pollen fathers, ranged from about 5 to 8200 m with a median of 582 m (Table 3). Both the range and median of effective pollen dispersal occasionally varied greatly between years and mother trees. Altogether, about 50 % of the assigned effective pollen traveled less than 500 m and about 75 % less than 1000 m. Approximately 4 % of the effective pollen was dispersed further than 2 km (Fig. 3).

Table 3 Profiles of effective pollen flow for each mother tree. *n*: sample size; *LOD**: number of seeds assigned to pollen fathers with 95 % confidence level; *LOD+*: number of seeds assigned to pollen fathers with 80 % confidence level; range of effective pollination distance (median).

year	tree ID	*n*	*LOD**	*LOD+*	distance in meters
2006	E001	95	50	26	39-5174 (131)
	E017	105	32	38	95-1908 (823)
	E042	89	18	32	39-6198 (1124)
	E083	96	43	27	46-5763 (217)
	E146	53	23	17	44-2077 (219)
	E169	136	39	73	79-3552 (337)
	E228	51	14	16	387-1901 (586)
2007	E001	391	110	147	39-5607 (1172)
	E017	451	151	186	87-8198 (840)
	E083	447	177	139	46-7568 (270)
	E146	354	107	161	136-6198 (1126)
	E169	191	74	79	79-3552 (392)
	E228	190	51	73	5-1288 (702)
	2001.1	190	82	75	382-4299 (620)
	Total	2839	971	1089	5-8198 (582)

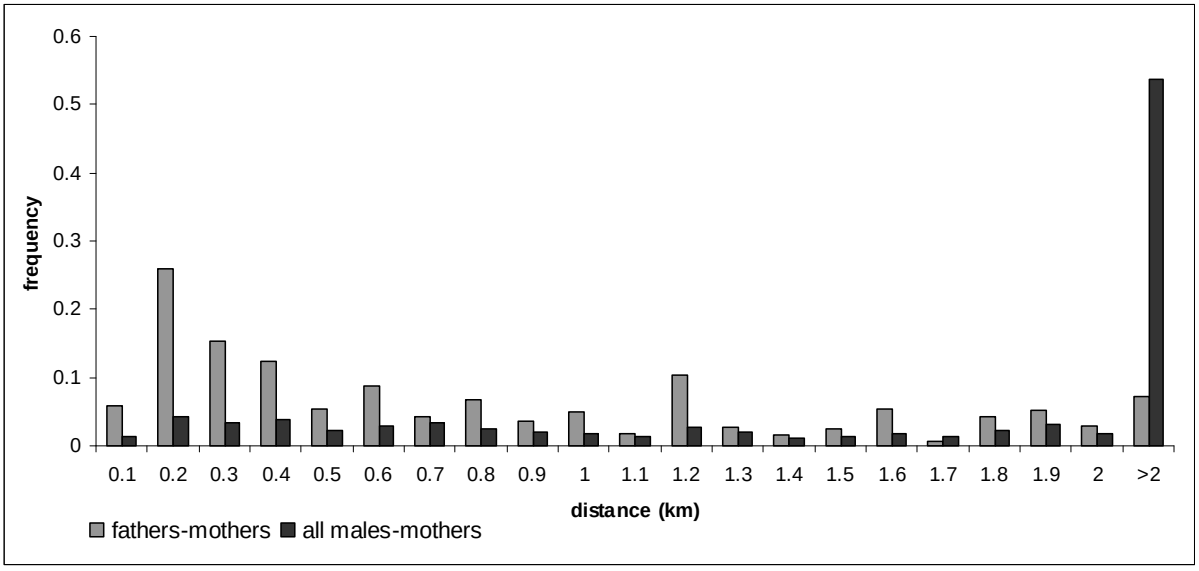

Fig. 3 Frequency distribution of effective pollen dispersal distances based on paternity analysis of the seedlings (grey bars) and the distances between all males and the mother trees (black bars).

For 380 plants sampled from the juveniles, parent pairs could be assigned for 169 plants (44.5 %). From these, four were assigned based on a 95 % confidence level along with 165 parent pairs at an 80 % confidence level. The distance of effective seed dispersal, which was

inferred from the distance between the determined mother trees and the natural recruitment area, ranged from 23 to 6923 m. About 70 % of the assigned effective seeds travelled less than 500 m and about 86 % travelled less than 1000 m. Approximately 1 % of the effective seeds were dispersed further than 2 km (Fig. 4).

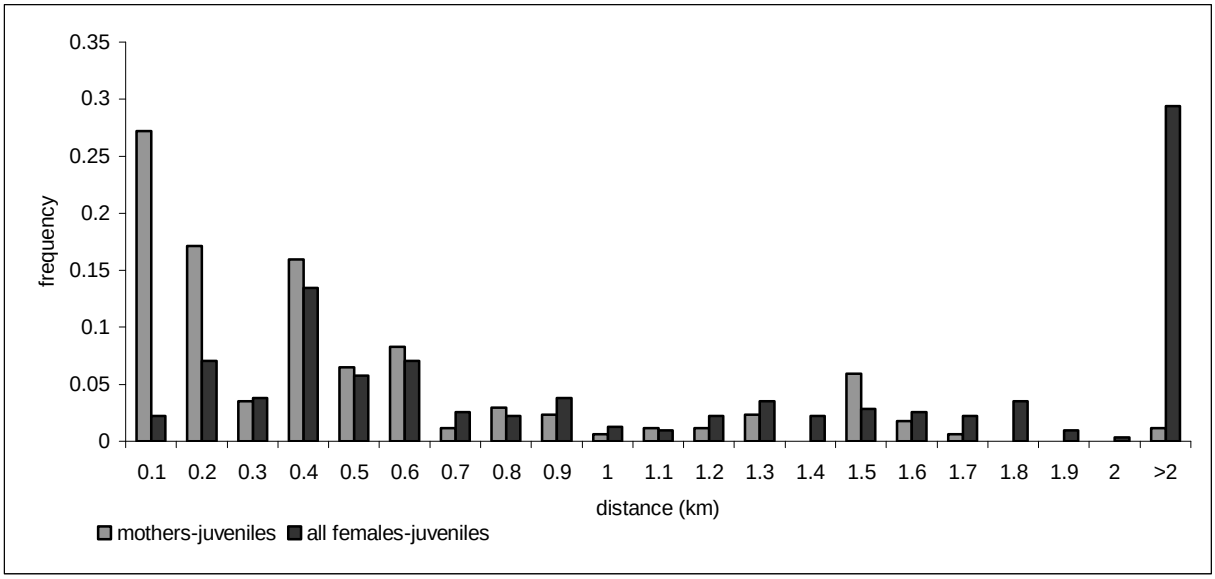

Fig. 4 Frequency distribution of effective seed dispersal distances based on parental analysis of the juveniles (grey bars) and the distances between all female trees and the juveniles (black bars).

Cervus 3.0 was also used to estimate null allele frequencies of the *P. nigra* population. The adult tree population exhibited substantial values of null alleles for loci WPMS09 and WPMS20 (p = 0.054 and p = 0.069, respectively). In the juvenile population, substantial frequencies of null alleles were estimated for the four loci WPMS09, WPMS14, WPMS20 and PMGC14 (p = 0.014, p = 0.017, p = 0.027 and p = 0.025, respectively).

Kolmogorov-Smirnov test

Using the Kolmogorov-Smirnov test, the difference between the frequency of distances of assigned fathers and all males in relation to the mother trees (Fig. 3) was found to be significant (D = 0.512, $P \leq 0.001$). Furthermore, the difference between the frequency distance of assigned mothers and all females in relation to the juveniles (Fig. 4) was significant as well (D = 0.404, $P \leq 0.001$). These results indicate that the observed genetic patterns of effective pollen dispersal cannot be explained by the spatial distribution of male

trees and mother trees alone. Additionally, the genetic patterns of effective seed dispersal cannot be explained by the spatial distribution of female trees alone. This could be an indication that isolation by distance plays an important role in both pollen and seed dispersal.

Discussion

Our results contribute to a better understanding of gene flow in poplar. They can be related directly to genetic diversity in both the adult and juvenile populations of *P. nigra*. The question arises whether *P. nigra* may still use these life history traits to rejuvenate successfully under man-made disturbance regimes. The knowledge gained from this genetic study has implications for in-situ conservation measures.

Spatial genetic structure of the adult tree population

In this study, SGS was estimated using the *SP* statistic (Vekemans & Hardy 2004; Kalinowski et al. 2007), an approach which has been shown to be appropriate in many studies (Vekemans & Hardy 2004; Jones & Hubbell 2006; Chung 2008). Although SGS can result from several factors, gene flow through pollen and seed dispersal are key determinants of its establishment (Streiff et al. 1998; Vekemans & Hardy 2004).

Weak spatial genetic structures have been reported for some forest trees, such as species of the genera *Fagus* and *Quercus* (Streiff et al. 1998; Streiff et al. 1999; Vornam et al. 2004). With respect to poplar, one of the smallest *SP* values ever reported was 0.00414 for *P. alba* L., which was ascribed to wind pollination of the species (van Loo et al. 2008). In *P. nigra* populations, isolation by distance has been reported in several European studies (Legionnet & Levèvre 1996; Imbert & Levèvre 2003). Significant SGS of a *P. nigra* population was also reported by Pospíškova and Šalková (2006). *SP* in their study was 0.0166 and the value of the regression slopes was negative (-0.0158). The results of the present study show that SGS plays a role as well, but to a lesser extent (*SP* = 0.0146, regression slope = -0.0136). The logarithmic relation between the kinship coefficients and the distance between trees (Fig. 2) clearly proves that isolation-by-distance patterns show a continuous variation of allele frequencies (Born et al. 2008).

Reduced genetic diversity in the juvenile population

The results presented in this paper show that the adult trees of the studied black poplar population are characterised by a high level of genetic diversity at the microsatellite loci. Comparing adults and juveniles, the adults exhibit higher values of both observed and expected heterozygosity. This is probably due to the small area for natural regeneration at the study site. According to spatial limitations in seed dispersal, only few mother trees in direct vicinity contribute large amounts of seeds. Consequently, only a small part of the genetic diversity of the adult population is represented in the offspring. Apparently, the loss of genetic diversity is promoted by restricted natural regeneration. The measure of 0.73 for H_e (gene diversity) in the adult population reported in our study is comparable to genetic diversity of *P. nigra* in other European studies (Fossati et al. 2003; Imbert & Lefèvre 2003; Pospíškova & Šalková 2006; Smulders et al. 2008b).

Some authors have emphasised the importance of allelic richness rather than heterozygosity for measuring diversity, especially in the context of genetic conservation (Schoen & Brown 1991; ElMousadik & Petit 1996). Allelic richness is particularly sensitive to a decrease in population size, for example via a bottleneck or founder effect (Petit et al. 1998), in contrast to those measures of diversity that are determined primarily by the more frequent alleles, such as expected heterozygosity. According to the number of alleles per locus, Pospíškova and Šalková (2006) and Fossati et al. (2003) found averages of 11 and 7.25, respectively. The results of these two studies are comparable to our findings, as in each case a single isolated population was the subject of their study. In our case, the black poplar population is completely isolated as there were no other notable black poplar populations inside the studied radius of 15 km that could enrich the local gene pool. Studies of multiple local populations found higher average values of 12.1 (Imbert & Levèvre 2003) and 15.7 (Smulders et al. 2008b). These results are due to the fact that the amount of allelic richness is positively correlated with both population size and sample size (Gregorius 1983; Gapare et al. 2008).

The observed positive value of the mean fixation coefficient indicates a slight excess of homozygotes in both our adult population and the juvenile population. The excess of homozygotes might result from biparental inbreeding, Wahlund effect or null alleles (Dakin & Avise 2004; Makrem et al. 2006; Vakkari et al. 2006; Alves et al. 2007; Chapuis & Estoup 2007). Positive F_{IS} values in most populations suggest inbreeding, but only when all studied loci show equally high values (Pospíškova & Šalková 2006). In the case of the studied adult

tree population, the average F_{IS} of 0.03 was caused by the presence of null alleles in the WPMS09 and WPMS20 loci. The positive, high values for these two loci strongly influenced the calculation of the average F_{IS} value across all loci. When they are excluded from F_{IS} calculation, the overall inbreeding coefficient is no longer significant at -0.086. A similar interpretation was suggested by Pospíškova and Šalková (2006). In the case of the juvenile population, F_{IS} calculation without loci WPMS09 and WPMS20 still reveals a significant positive value of 0.017. Overall tendencies towards positive F_{IS} over loci are more distinct here, as only locus WPMS18 shows a negative value of -0.04. Thus, the significant positive F_{IS} of the juveniles might actually derive from weak inbreeding tendencies, which also indicates a loss of genetic diversity. This yields heterozygote deficits, which reflect deviations from Hardy-Weinberg equilibrium. A misinterpretation of a heterozygote deficiency can result in the detection of null alleles as the relationship between heterozygote deficit and null allele presence in a local population is used for the calculation of null allele frequencies in Cervus (Dakin & Avise 2004; Kalinowski et al. 2007). The increased number of loci displaying significant null allele frequencies in the juveniles is therefore likely to derive from a heterozygote deficit.

Quantifying pollen- and seed-mediated gene flow

Pollen from wind-pollinated trees, as well as poplar's tiny cottony seeds, has usually been assumed to travel very long distances (Legionnet et al. 1997; Guilloy-Froget et al. 2002). Our observations also revealed long-distance dispersal. However, both reproductively effective pollen and seed were predominantly dispersed over short distances (Fig. 3, Fig. 4). The overall shorter dispersal distances of effective seed are probably due to its larger size and weight in comparison with pollen. Comparable studies quantifying gene flow of poplar populations have not yet been published. However, parentage analysis of poplar offspring relying on singular findings revealed pollen dispersal distances of 230-350 m (Tabbener & Cottrell 2003; Pospíškova & Šalková 2006) and seed dispersal distances of 370 m (Pospíškova & Šalková 2006). Imbert & Lefèvre (2003) reported on gene flow among black poplar populations occurring essentially through pollen. In their study, they found single events of seed flow being limited for distances beyond 1-3 km. Additional evidence for non-random mating in black poplar is supplied by a Belgian study (Vanden Broeck et al. 2006), which states that female trees are pollinated by a restricted number of males.

Deviations from random mating and more frequent near-neighbour mating were also observed in other wind-pollinated tree species (Kaufman et al. 1998; Mantovani et al. 2006). Studies on *Araucaria angustifolia* (Bertol.) Kuntze and *Pinus echinata* Mill., respectively, attributed pollen gene pool heterogeneity to high forest density, producing physical barriers to pollen transport (Dyer & Sork 2001; Bittencourt & Sebbenn 2008). As the size of *P. nigra* pollen (diameter of 26 µm (von Blohn et al. 2005)) lies at the lower end of the general range of pollen sizes of wind-pollinated trees (17-58 µm (Sousa & Hattemer 2003)), poplar pollen dispersal may also be restricted by forest density. In open-pollinated trees, a limited number of proximate male trees can therefore be considered to contribute the main portion of effective pollen. The resulting embryos are mixtures of full and half-sibs sired by a very limited number of fathers. Short distance pollen and seed dispersal can produce spatial genetic subdivision. As a result, genetic variability may be lost and a small effective pollination neighbourhood area can create the opportunity for genetic drift. The reduced genetic diversity of the studied juvenile population represents the result of such a small effective pollination neighbourhood. But even in the worst case of genetic inbreeding, recent studies found no clear signal of fecundity or progeny fitness being consistently affected (Kramer et al. 2008). Short-distance dispersal of limited pollen might be common and might play an important role in causing the highly variable seed production in some wind-pollinated tree species (Sork et al. 2002; Koenig & Ashley 2003). Therefore, local pollen and seed dispersal may also be a natural feature of the life-history of *P. nigra*. By this, single local patches of natural regeneration may of course exhibit decreased genetic diversity. However, on the population level, genetic diversity on the whole may be promoted by river dynamics which allow time-displaced reproductive success of all adult trees: As flooding events locally create patchy areas of bare soil, which are colonised by seeds of local parents, integrating patches of different years and different locations may finally exhibit the entire genetic diversity of the original adult tree population.

Conclusions

In the studied *P. nigra* population, we found a genetic diversity which is in the range of other European populations. This is the first important condition for successful conservation. Conservational attention should focus on the maintenance of such large and diverse populations, which can be considered as a source population for any restoration project.

Therefore, local diversity can be preserved and a capacity for natural regeneration can be offered. Sufficient natural regeneration is urgently needed to compensate for the loss of genetic diversity caused by population obsolescence. Therefore, conditions for natural recruitment should be provided in situ. This includes more variation in hydrological conditions, proper water flow and active erosion-deposition processes (Guilloy-Froget et al. 2002; Hughes et al. 2005). Thus, the restoration challenge is to find ways of enhancing this natural potential for regeneration. Apparently, our findings of reduced genetic diversity and inbreeding in the investigated juvenile population illustrate that singular, small-sized areas for natural regeneration may not be sufficient for the effective conservation of present genetic diversity. Our results show that only a minor part of gene flow takes place at distances beyond 1 km. Due to the limitation of both pollen and seed dispersal, patterns of SGS are present in the surrounding adult tree population. By limited local regeneration, only few local genotypes are able to contribute to maintain the genetic diversity. Concerning natural regeneration of *P. nigra*, which is considered to be patchy, it is necessary to provide multiple appropriate habitats for seedling establishment at considerable distances. If small relict populations are to be conserved, it is important to know how far apart populations can grow and still remain in contact via pollen and seed dispersal. Furthermore, knowledge of effective pollen and seed dispersal distances for poplar is also of major importance in terms of introgressive gene flow, which is likely to occur in the vicinity of *P. nigra* and hybrid poplar cultivars (Vanden Broeck et al. 2004; Smulders et al. 2008a). Interspecific introgressive gene flow poses a potential threat to natural *P. nigra* populations (Cagelli & Lefèvre 1997). This is the subject of ongoing studies. Combining several approaches for estimating contemporary gene dispersal in landscapes provides a solid basis for decisions about *P. nigra* population management for conservation purposes.

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2.3 Paper III:

Subtle invasion of *Populus deltoides* genes into endangered *P. nigra* L. populations – challenges for quantitative diagnostic methods

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Manuscript

Subtle invasion of *Populus deltoides* genes into endangered *P. nigra* L. populations – challenges for quantitative diagnostic methods

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Abstract

The European black poplar (*Populus nigra* L.) is among the most threatened tree species in Europe. Introgressive gene flow from cultivated poplars represents a potential threat to the persistence of *P. nigra*. In this study we investigated hybridisation between *P. nigra* and the hybrid cultivar *P. x canadensis* by means of combined diagnostic molecular markers from the chloroplast and nuclear genome. Open-pollinated progeny of both species as well as juveniles from natural regeneration were analysed. The results indicate introgressive gene flow in both directions with preferential hybridisation between female *P. x canadensis* and male *P. nigra.* Moreover, introgressed progeny has become established at the study site. Mating events in subsequent generations will complicate the monitoring of introgressive gene flow. The processes of swamping and the formation of hybrid swarms are discussed with respect to the management of *P. nigra* populations. Conservation strategies need to prevent introgressive gene flow in order to save the species integrity of *P. nigra*.

Introduction

Hybridisation and introgressive gene flow are considered as evolutionary drivers. Some authors consider introgressive hybridisation between rarer and more numerous species as a potential genetic enrichment of the endangered form (e.g. Arnold 1997; Mallet 2005; Reusch & Wood 2007). According to their theory, introgressive hybridisation could increase the fitness and the genetic variability of the rare form and could facilitate its habitat expansion. However, habitat reduction followed by hybridisation can lead to the extinction of rare plant species (Ellstrand 1992).

For the European black poplar (*Populus nigra* L.) such approaches become more relevant, as it is believed to be the most threatened forest tree species of old, natural floodplain forests in the temperate zones (Levèvre et al. 2001; Tabbener & Cottrell 2003; Pospíškova & Šalková 2006). Due to economic reasons, the hybrid combination *P. x canadensis* (Dode) Guinier was largely cultivated in the neighbourhood to natural *P. nigra* populations. These F1 hybrids were produced by crossing the North American *P. deltoides* Bartr. with male *P. nigra* (FAO 1979). Postzygotic barriers and hybrid mortality only allow for unidirectional controlled crossing of *P. deltoides* females and *P. nigra* males (Melchior & Seitz 1968; Zsuffa et al. 1999). Like many other poplar cultivars, *P. x canadensis* is propagated and distributed through vegetative clones. They are fertile and able to generate F2 and backcross progenies by either mating among themselves or mating with pure *P. nigra*, respectively (Bradshaw et al. 2000). Thus, under certain circumstances, F1 hybrids may swamp the gene pool of native black poplar (Cagelli & Lefèvre 1997; Vanden Broeck et al. 2004). Indeed, some field studies have provided evidence for introgressive gene flow in openly pollinated *P. nigra* and *P. x canadensis*. Vanden Broeck et al. (2003) were able to show that crosses between *P. nigra* females and *P. x canadensis* males are compatible and can produce viable seeds under specific field conditions. Furthermore, recent studies revealed the establishment of both F2 and backcrossed poplars in natural sites (Pospíškova & Šalková 2006; Smulders et al. 2008; Ziegenhagen et al. 2008). A first estimate of the degree of introgression in a population was obtained by Ziegenhagen et al. (2008). They used a combined approach of diagnostic nSSR markers (nuclear simple sequence repeats, syn. nuclear microsatellite markers) and a chloroplast (cp) marker. Chloroplast markers can expose the female contribution by *P. deltoides* (Heinze 1998; Holderegger et al. 2005) as they are maternally inherited in poplar (Mejnartowicz 1991). The approach of Ziegenhagen et al. (2008) turned out to be promising for the detection of backcross and F2 generations. However, species-specific alleles can only

trace backcrosses as long as they are passed on. Therefore, it is difficult to distinguish between F2, backcrosses and later generation hybrids. A sufficiently large number of diagnostic loci is required. Otherwise the error rate for classification/identification can be high (Epifanio & Philipp 1997; Boecklen & Howard 1997).

Another strategy to reveal introgressive gene flow is the use of maximum-likelihood approaches provided by computer programmes such as *NewHybrids* (Anderson & Thompson 2002) or *HINDEX* (Buerkle 2005). A couple of studies have already used this technique in the analysis of poplars (Lexer et al. 2007; van Loo et al. 2008; Smulders et al. 2008).

So far, all studies that reported on mating of *P. nigra* and *P. x canadensis* qualitatively revealed the existence of spontaneous hybridisation in principle. However, there are still no quantitative studies. The aim of this study was therefore to explore and quantify gene flow between *P. nigra* and *P. x canadensis* systematically. For that purpose, we focussed on large sample sizes of both produced viable seeds and established juveniles. The combination of four diagnostic nSSR marker loci and a cp marker (DT (Demesure et al. 1995)) was used to obtain a detailed insight into mating scenarios. By this means, rates of progeny with *P. x canadensis* ancestry could be estimated in total and the two maternal origins of *P. nigra* and *P. x canadensis* could be analysed separately. Results of this quantitative study allow a suitable risk assessment with regard to introgressive gene flow. They could therefore enhance the effective management of conservation activities for black poplar.

Material and methods

Study site and plant material

The study site is located in Hesse, Central Germany, at the Eder River around the east of the city Fritzlar (Fig. 1). It represents the nature protection area “Ederauen bei Obermöllrich und Cappel”, which includes a natural *P. nigra* population. This population is interspersed with and surrounded by numerous trees of *P. x canadensis*. Other non-indigenous poplar taxa are present only occasionally. In two consecutive years, seeds were collected from mature catkins of six mother trees from both taxa (Fig. 1, Tables 1 & 2).

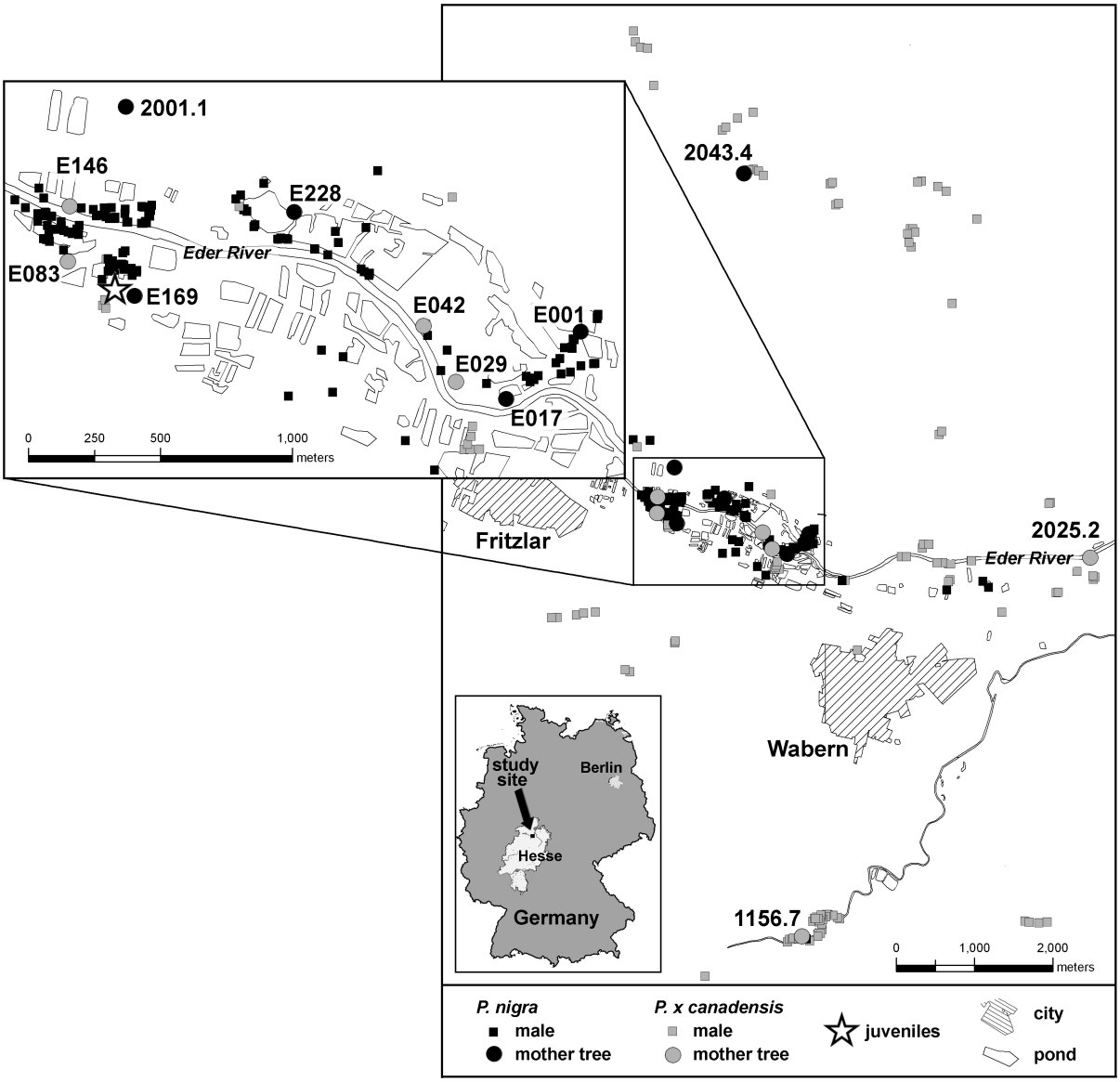

Fig. 1: Distribution of the mother and male poplar trees and the location of the juveniles at the Eder River study site. Sample names of the mother trees are indicated. Species affiliation corresponds to Rathmacher et al. (submitted).

Species affiliation was analysed and reported in another study (Rathmacher et al., submitted). The harvested seeds were sown on filter paper that was placed on waterlogged vermiculite. A few days after germination, seedlings were collected. In total, 2606 seedlings were sampled and dried at 36°C for 24 hours. Natural regeneration within the study site could only be found at a gravel-pit area (Figs. 1 & 2) with a size of approximately 100 x 100 m. Here, leaves of 380 juvenile plants of different ages were sampled (Table 3). These samples were dried at 36°C for 24 hours as well. Approximately 0.5 cm² of dried plant material were homogenised using a Retsch shaking mill (Retsch, Hilden, Germany), following the protocol described by Ziegenhagen et al. (1993). Total DNA was extracted according to Jump et al. (2003) with slight modifications (Rathmacher et al., in press).

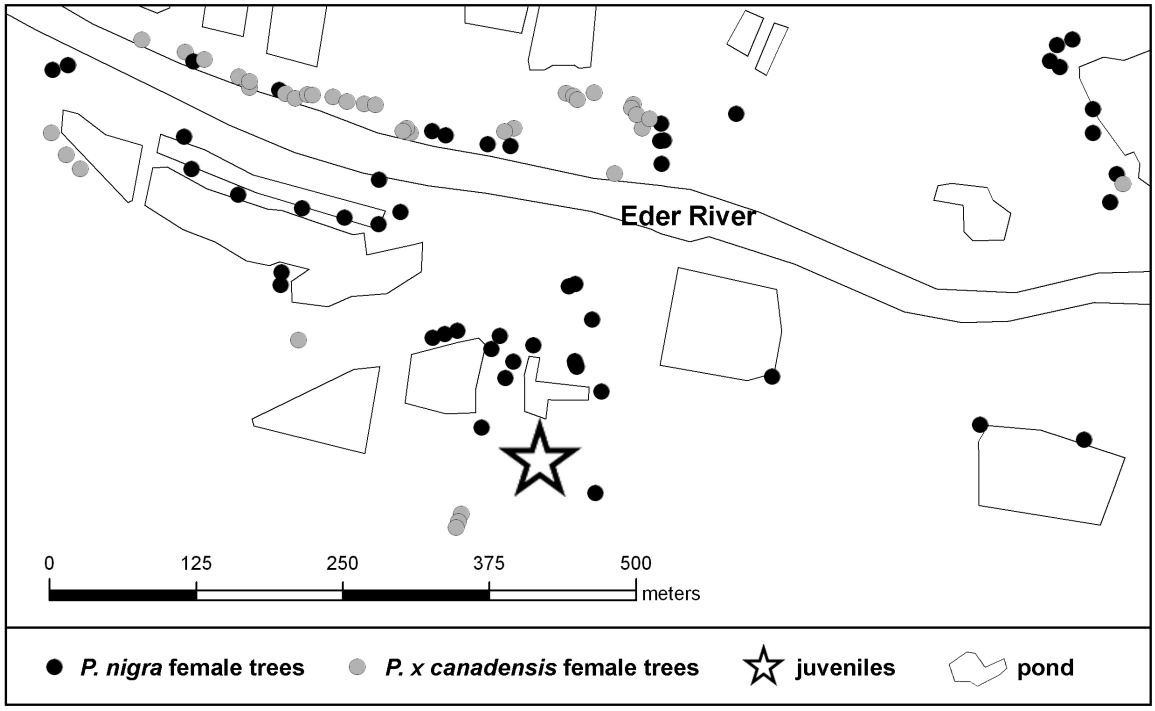

Fig. 2: Distribution of the poplar trees near the location of the juveniles at the Eder River study site. For clarity purposes, only the surrounding females are indicated. Species affiliation corresponds to Rathmacher et al. (submitted).

Table 1: Diagnostic marker patterns of seedlings from *P. nigra* mothers. total can: individuals with a *P. x canadensis* paternity background showing at least one allele specific for *P. deltoides* in the SSR markers; BC = backcross.

tree ID	**2001.1**		**2043.4**		**E001**		**E001**		**E017**		**E017**		**E169**		**E169**		**E228**		**E228**	
year	2007		2007		2006		2007		2006		2007		2006		2007		2006		2007	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%
	190	100	95	100	95	100	136	100	105	100	191	100	136	100	191	100	51	100	190	100
number of diagnostic alleles for *P. deltoides* in the SSR loci																				
0	190	100	21	22.11	89	93.68	132	97.06	104	99.05	191	100	136	100	191	100	51	100	188	98.95
1	0	0	25	26.32	6	6.32	4	2.94	1	0.95	0	0	0	0	0	0	0	0	2	1.05
2	0	0	20	21.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	24	25.26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	5	5.26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
total can	0	0	74	77.89	6	6.32	4	2.94	1	0.95	0	0	0	0	0	0	0	0	2	1.05
BC to P. nigra[a]	**0**	**0**	**79**	**83.16**	**6**	**6.32**	**4**	**2.94**	**1**	**0.95**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**1.05**

[a] we actually recorded 93.75 % of the effective hybrid poplar pollination. This corrected value represents 100 %.

Table 2: Diagnostic marker patterns of seedlings from *P. x canadensis* mothers F2: *P. x canadensis* x *P. x canadensis*.

tree ID	**1156.7**		**2025.2**		**E029**		**E029**		**E042**		**E083**		**E083**		**E146**		**E146**	
year	2007		2007		2006		2007		2006		2006		2007		2006		2007	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%
	190	100	190	100	173	100	146	100	89	100	96	100	194	100	53	100	95	100
F2[a]	**2**	**1.05**	**12**	**6.32**	**4**	**2.31**	**0**	**0**	**1**	**1.12**	**2**	**2.08**	**2**	**1.03**	**0**	**0**	**1**	**1.05**
corr[b]	**3**	**1.58**	**18**	**9.47**	**6**	**3.47**	**0**	**0**	**1**	**1.12**	**3**	**3.13**	**3**	**1.55**	**0**	**0**	**1**	**1.05**
number of diagnostic alleles for *P. deltoides* in the SSR loci																		
0	8	4.21	14	7.37	21	12.14	8	5.48	5	5.62	6	6.25	9	4.64	4	7.55	6	6.32
1	45	23.68	48	25.26	40	23.12	31	21.23	19	21.35	16	16.67	49	25.26	21	39.62	22	23.16
2	69	36.32	67	35.26	65	37.57	53	36.30	25	28.09	47	48.96	62	31.96	18	33.96	43	45.26
3	57	30	49	25.79	39	22.54	42	28.77	12	13.48	19	19.79	54	27.84	10	18.87	20	21.05
4	10	5.26	6	3.16	5	2.89	11	7.53	8	8.99	4	4.17	20	10.31	0	0	4	4.21
5	0	0	4	2.11	1	0.58	0	0	0	0	1	1.04	0	0	0	0	0	0
6	2	1.05	1	0.53	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	1	0.53	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Samples that are homozygous for a *P. deltoides* specific allele in at least one diagnostic marker certainly have a *P. x canadensis* father and therefore represent F2.
[b] We actually recorded 68.36 % true F2 proportion. This corrected value represents 100 %.

Molecular analysis

SSR markers

Poplar DNA was analysed at seven highly polymorphic nSSR loci: WPMS05 and WPMS09 (van der Schoot et al. 2000), WPMS14, WPMS18 and WPMS20 (Smulders et al. 2001) as well as PMGC14 and PMGC2163, which were selected from the IPGC (International Populus Genome Consortium) SSR Resource (http://www.ornl.gov/sci/ipgc/ssr_resouce.htm). All markers are completely unlinked (Cervera et al. 2001; Gaudet et al. 2008). PCR and automated multiplex capillary electrophoresis were performed as described by Rathmacher et al. (in press). Loci WPMS09, WPMS18, PMGC14 and PMGC2163 contain diagnostic alleles for *P. deltoides* (Fossati et al. 2003; Bekkaoui et al. 2003; Khasa et al. 2005). Combining the data of the diagnostic nuclear loci allows the detection of backcrosses or F2 hybrids to a certain degree.

Chloroplast DNA marker

The chloroplast (cp) DNA marker DT (Demesure et al. 1995) was used to identify the maternal origin of the juveniles. The marker variation is characterised by a fragment length polymorphism in the intergenic spacer region between the trnD and trnT genes. At the DT region, *P. nigra* and *P. deltoides* are characterised by diagnostic alleles (Heinze 1998). All *P. x canadensis* F1 hybrids bred for commercial purposes are supposed to carry the *P. deltoides* allele since *P. deltoides* was always used as the mother tree. Female hybrid clones therefore transmit the marker allele to all of their offspring.

For verification purposes, reference samples of pure *P. nigra* and *P. deltoides* as well as *P. x canadensis* were included into genotyping. These samples were taken from Rathmacher et al. (submitted). The total PCR volume of 16 µl contained 1 µl of DNA extract, 1 x PCR reaction buffer, 2 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP and 0.25 U of *Taq*-polymerase (Bioline USA Inc.). The PCR cycle started with an initial denaturation at 94°C for 4 min and was followed by 25 cycles of denaturation at 94°C, annealing at 55°C and elongation at 72°C for 45 sec each. Finally, products were cooled down to 4°C.

A volume of 5 µl PCR product was subjected to 2 % (0.5 x TBE (TRIS-borate-EDTA)) agarose gel electrophoresis at 70 V for 30 min and at 120 V for 90 min. For UV visualization of the DNA fragments, the gels were stained with ethidium bromide.

Data analysis

Due to Mendelian segregation of alleles at the nSSR loci, a certain proportion of mating with *P. x canadensis* will remain undetected. Offspring may solely display alleles that are diagnostic for *P. nigra* at all four diagnostic nSSR markers, even in cases where both parents were *P. x canadensis* (Fig. 3). As all marker loci are transmitted independently (Cervera et al. 2001; Gaudet et al. 2008), each parental allele is passed on to the offspring with a probability of 0.5. Based on the observed proportions of species specific alleles for *P. deltoides* in the individual offspring, the theory of probability could be used to estimate the "de facto fraction" of *P. x canadensis* paternity. This was done for both the seedlings and the juveniles. In case of the juveniles, the species affiliation of the mother was already known. In case of the juveniles, the species affiliation of the mother was identified using the DT marker (Figs. 3a & b). The theory of probability allowed an iterative scheme for the following two cases:

Backcrosses P. nigra x P. x canadensis

After mating of a *P. x canadensis* male tree with a *P. nigra* female, a heterozygous occurrence of a specific allele for *P. deltoides* in the offspring indicates *P. x canadensis* paternity. Half of the offspring exhibits specific *P. deltoides* alleles for the first diagnostic marker locus and can therefore be assigned to *P. x canadensis* backcrosses (Fig. 3a). By the use of a second diagnostic marker, again 50 % of the remaining unassigned offspring can be identified as backcrosses, which is a proportion of 25 % of the de facto fraction of backcrosses. Considering a third and fourth diagnostic marker, additional proportions of 12.5 % and 6.25 % respectively can be assigned. Using four diagnostic markers, about 93.75 % of the offspring that actually has a hybrid poplar father can be identified for sure while a proportion of 6.25 % remains undetected. In order to obtain the de facto fraction of *P. x canadensis* paternity in *P. nigra*, the yet missing portion of 6.25 % was estimated using cross-multiplication in the rule of three:

$a/b=c/d$, where a = number of assigned backcross progeny using four diagnostic markers, b = 93.75 %, c = total number of de facto fraction individuals (unknown), d = 100 %.

By solving this equation, it was possible to estimate the total number of individuals of the de facto fraction of backcrosses. The remaining seedlings were assigned to pure *P. nigra* (Fig. 3a).

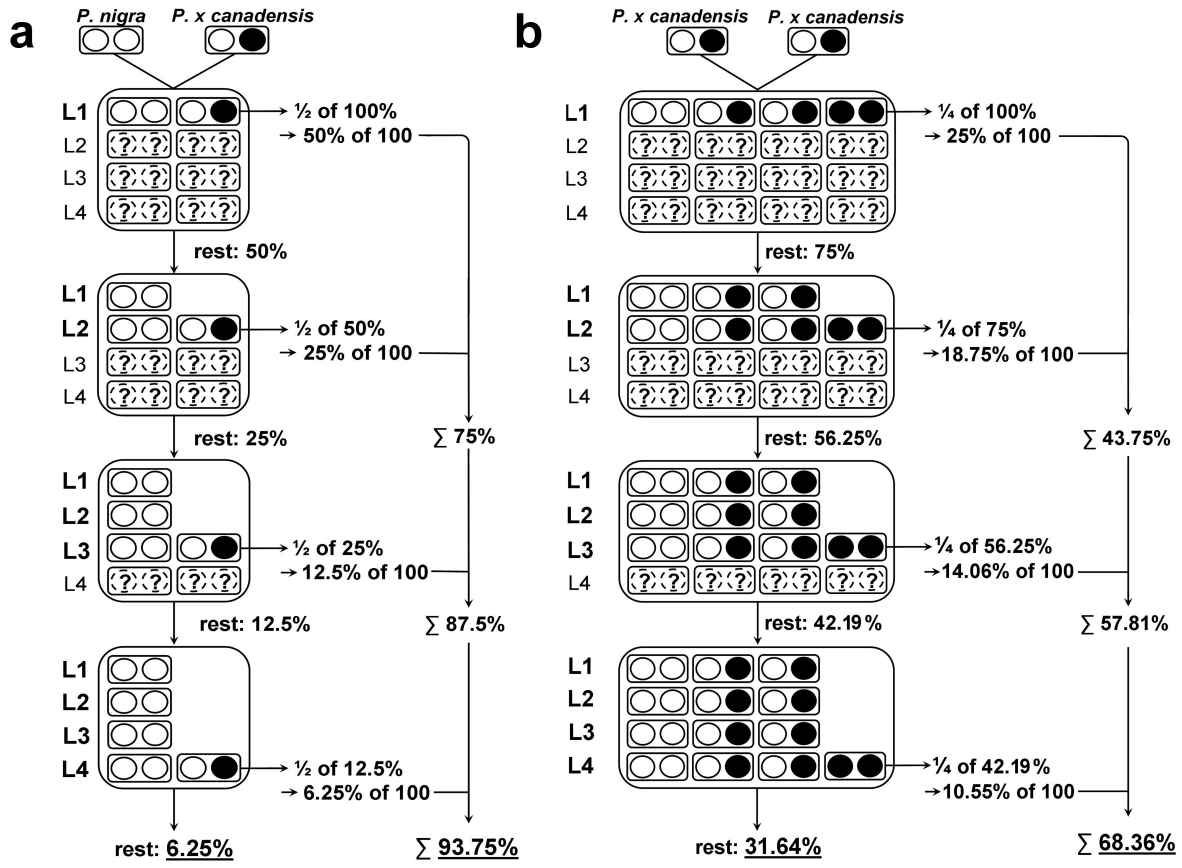

Fig. 3: Iterative scheme for identifying fractions of **a)** backcross and **b)** F2 progeny of *P. nigra* and *P. x canadensis* parents. This figure is used as the basis for the calculation of the de facto rates. Four nuclear diagnostic marker loci containing species specific alleles for *P. deltoides* are used cumulatively. Each box represents the allele combination of one marker locus. Species specific alleles of *P. deltoides* are marked by black dots. A heterozygous allele specific for *P. deltoides* indicates *P. x canadensis* ancestry. The parents are the individuals at the top. The fractions of possible genotypes concerning their offspring are illustrated underneath. L1-L4: diagnostic marker loci, those under consideration are represented in bold letters.

F2 generation crossing of P. x canadensis x P. x canadensis

Considering only one diagnostic marker locus, an F2 crossing of two hybrid poplars reveals that 25 % of offspring are homozygous for the allele that is species specific for *P. deltoides* (Fig. 3b). A proportion of 75 % of the de facto F2 fraction remains undetected, as its allele combinations could possibly be produced also by crossings of *P. nigra* and *P. x canadensis* (Fig. 3a). If a second diagnostic marker is used, again one quarter of the remaining 75 % can be assigned, namely 18.75 % of the original F2 fraction. Considering a third and fourth diagnostic marker, additional fractions of 14.06 % and 10.55 % respectively can be assigned. Using four diagnostic markers, about 68.36 % of the offspring that actually has a hybrid

poplar father can be assigned for sure while 31.64 % still remain undetected (Fig. 3b). Against this background, seedlings that were homozygous for at least one specific *P. deltoides* allele were counted. In order to obtain the de facto fraction of F2, the yet missing proportion of 31.64 % was estimated using cross-multiplication in the rule of three (see above), where a = number of assigned F2 progeny using four diagnostic markers, b = 68.36 %, c = total number of de facto fraction individuals (unknown) and d = 100 %. By solving this equation, it was possible to estimate the total number of individuals of the de facto fraction of F2 progeny.

The rate of backcrosses with pure *P. nigra* can only be estimated indirectly through the rate of F2, since the only possible mating in the study area takes place either between *P. nigra* and *P. x canadensis* or *P. x canadensis* x *P. x canadensis*. Consequently, the fraction of samples that are not F2 has to be backcrossed with pure *P. nigra*. Regardless of the parental gender, all mating events of *P. nigra* and *P. x canadensis* produce offspring that features rates of 0.75 *P. nigra* genes and 0.25 *P. deltoides* genes. The only difference is that the progeny of female *P. x canadensis* exhibits *P. deltoides* chloroplasts (see above).

Projection of scenarios for further generations

Referring to the calculations of detectable fractions of backcrosses and F2 that are part of the current generation (see above), scenarios could be projected for further generations (Figs. 4 & 5). They help to estimate the additional number of diagnostic loci to detect repeated backcrosses in further generations (Fig. 5).

The projection of the present proportions of detectable crossing events for further generations revealed constant proportions for F2 crossings. In case of crossings solely between F2 generation individuals, the fraction of *P. deltoides* genes sticks to the original 50 % over several generations. The resulting portion of the F2 generation that is detectable with the help of four diagnostic loci remains at 68.36 % (Figs. 3b & 4). In case of repeated backcrosses, the proportion of *P. deltoides* genes halves from generation to generation, and so does the portion of detectable backcrosses. Generation one still exhibits 25 % of *P. deltoides* genes. By using four diagnostic markers, 93.75 % of the de facto backcrossed individuals can de detected (Figs. 3a & 4). Over several generations, proportions decline until in generation seven, the de facto fraction of *P. deltoides* genes is 0.39 % of which 1.46 % can be detected with four diagnostic marker loci (Fig. 4).

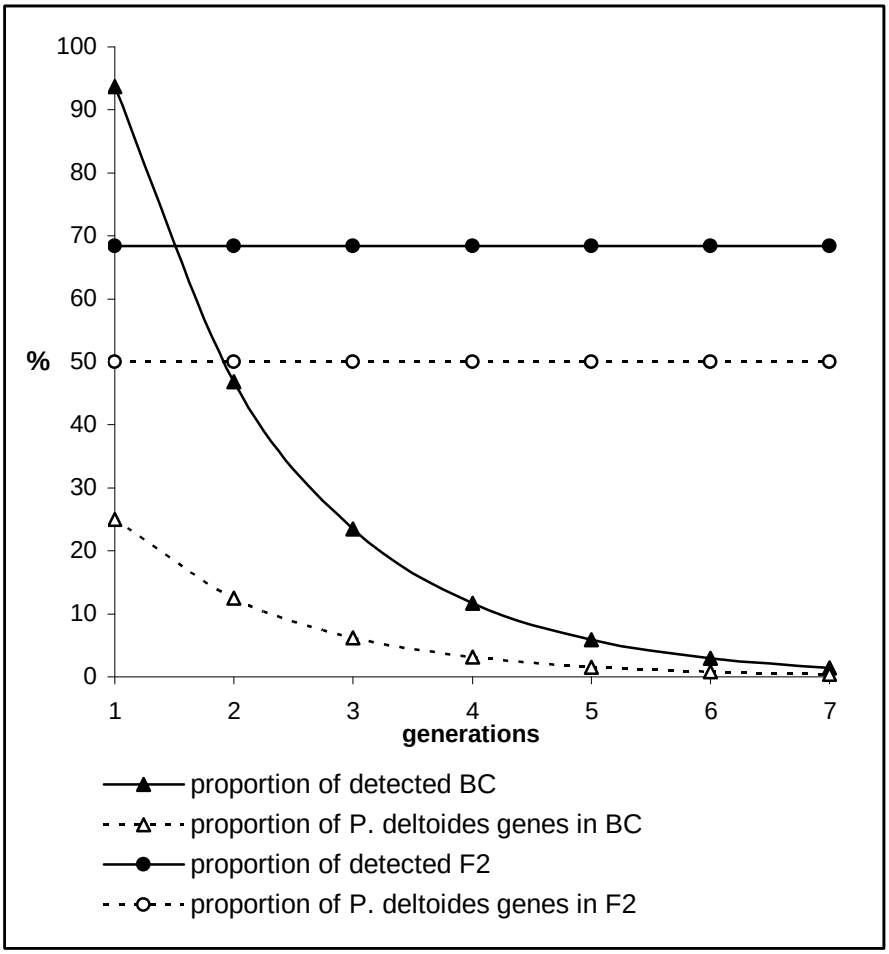

Fig. 4: Proportions of detectable backcrosses and F2 crossings with their corresponding proportions of *P. deltoides* genes over the course of seven generations. Four diagnostic marker loci are used. It is assumed that backcrosses (BC) took place between *P. nigra* and *P. x canadensis* to produce generation 1. The subsequent generations are the result of repeated backcrosses with mating of previous generation crossings and pure *P. nigra*. In case of F2 crossings, the first generation was produced by *P. x canadensis* x *P. x canadensis*. The following generations result from mating of trees from the particular previous F2 generation.

In case of proceeding backcrosses, more and more diagnostic markers are needed for the identification of introgressed individuals. While in backcross generation 1, four diagnostic markers are needed for the identification of 93.75 % of the de facto backcross fraction (Figs. 4 & 5), nine markers are needed to achieve a detection rate of 92.5 % in the second backcross generation (Fig. 5). In the third backcross generation, the detection of more than 90 % is only possible with the use of more than 17 diagnostic markers. In the fifth backcross generation even 17 diagnostic markers enable the detection of 42 % of the de facto fraction of introgressed individuals.

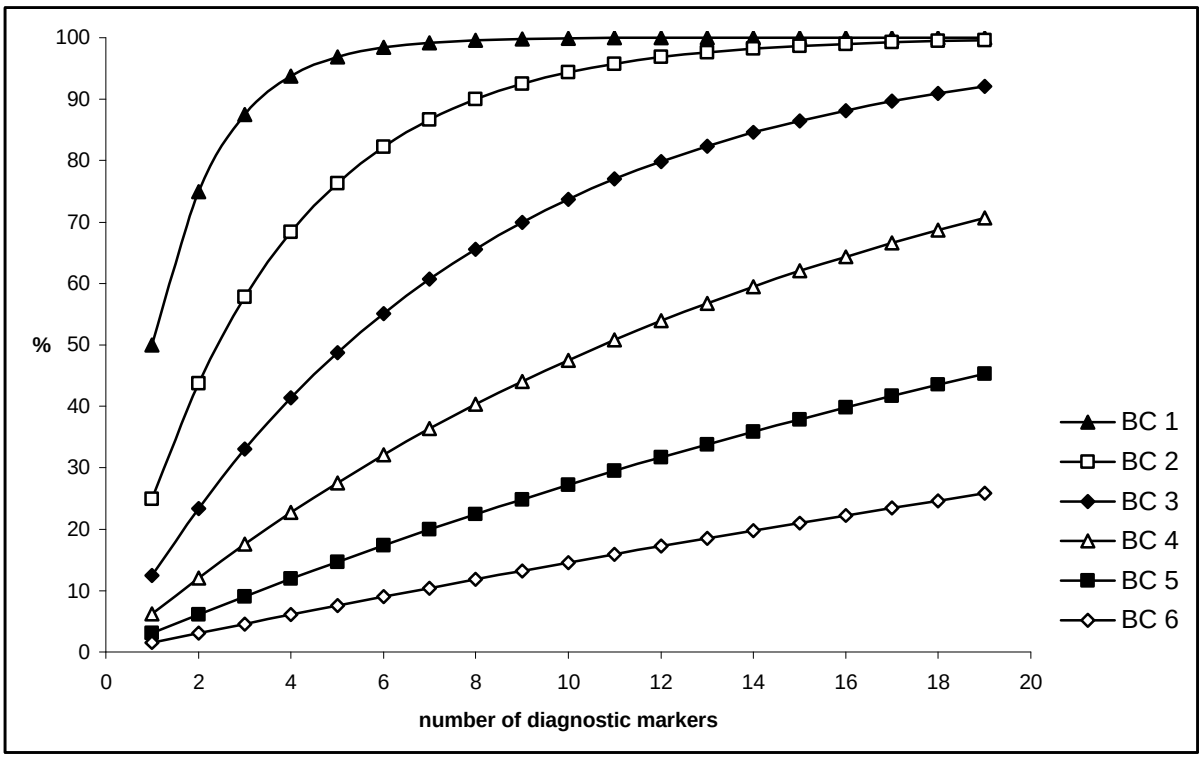

Fig. 5: Portions of individuals with *P. x canadensis* ancestry in proceeding backcross generations that can be detected by the cumulative use of diagnostic markers. It is assumed that backcrosses (BC) took place between *P. nigra* and *P. x canadensis* to produce generation 1. The subsequent generations are the result of repeated backcrosses with mating of previous generation crossings and pure *P. nigra*. BC1-BC6: backcross generations 1-6.

Results

For the assessment of the de facto introgression rates, two subsets of poplar progeny were used. According to the analysed seedling genotypes, species affiliation of only the father tree had to be determined by using the four nSSR diagnostic marker loci, as the mother was already known. In case of the juveniles, species affiliation of both parents was unknown.

Juveniles

Considering all diagnostic markers, a total of 15 juvenile poplars (3.95 %) exhibited *P. x canadensis* parental background (Table 3). No F2 crossings could be identified, as species specific alleles for *P. deltoides* were always heterozygous (Table 4).

According to the DT marker, eight of 380 juvenile trees (2.11 %) could be assigned to *P. x canadensis* maternal origin (Tables 3 & 4). One of these did not have any nSSR specific *P.*

deltoides allele, while the other samples with *P. x canadensis* maternal background exhibited one to four alleles specific to *P. deltoides*.

In the remaining fraction of 372 juvenile plants with *P. nigra* mothers, *P. x canadensis* paternity was detected for seven samples (1.88 %). Thus, the de facto fraction of *P. x canadensis* paternity revealed eight individuals (2.15 %, Table 4). With regard to all 380 sampled juveniles, the de facto fraction of individuals exhibiting *P. x canadensis* parentage (15 individuals, 3.95 %) could be corrected to 16 individuals (4.21 %), using the iterative scheme of Fig. 3a. Each half of the 16 individuals is represented by *P. x canadensis* maternity and *P. nigra* maternity (making up 8 individuals each).

Table 3: Diagnostic marker patterns for the juveniles. DTn: DT marker specific allelic pattern of *P. nigra*; DTd: DT marker specific allelic pattern of *P. deltoides*; can: individuals with a *P. x canadensis* parental background showing at least one allele specific for *P. deltoides*.

	#	%
	380	100
DTn	372	97.89
DTd	8	2.11
can	15	3.95

Table 4: Diagnostic marker patterns for juveniles with either *P. nigra* or *P. x canadensis* maternal background. F2: *P. x canadensis* x *P. x canadensis*; total can: individuals with a *P. x canadensis* paternity background showing at least one allele specific for *P. deltoides* in the SSR markers; BC: backcross.

		mother: *P. nigra* (=DT n)		mother: *P. x canadensis* (=DT d)	
		#	%	#	%
		372	100	8	100
number of	0	365	96.32	1	12.5
diagnostic	1	7	1.88	4	50
alleles for	2	0	0	1	12.5
P. deltoides	3	0	0	2	25
in the SSR					
loci	4	0	0	0	0
F2[a]		**-**	**-**	**0**	**0**
total can		7	1.88	-	-
BC to *P. nigra*[b]		**8**	**2.15**	**-**	**-**

[a] Samples that are homozygous for a *P. deltoides* specific allele in at least one diagnostic marker certainly have a *P. x canadensis* father and therefore represent F2.

[b] We actually recorded 93.75 % of the effective hybrid poplar pollination. This corrected value represents 100 %.

Seedlings of P. nigra mothers

The overall fractions of *P. nigra* mother trees that were pollinated by *P. x canadensis* ranged from 0 % to 77.89 %. In detail, *P. x canadensis* paternity was assigned to seedlings of mother trees E001 (6.32 % in 2006, 2.94 % in 2007), E017 (0.95 % in 2006), E228 (1.05 % in 2007) and 2043.4 (77.89 % in 2007) (Table 1). The calculation of the de facto fraction of *P. x canadensis* paternity (Fig. 3a) did not change these outcomes substantially. After both the addition of the undiscovered 6.25 % and rounding, the numbers of individuals remained the same except for the seedlings of tree 2043.4 (77.9 % vs. 83.16 %, Table 1).

Seedlings of P. x canadensis mothers

According to the diagnostic nSSR markers, rates of F2 crossings in the seedlings of *P. x canadensis* mother trees ranged from 0 % to 6.32 %. F2 samples were found for seedlings of all mother trees except for E029 in 2007 and E146 in 2006. The portions of F2 crossing ranged from 1.03 % (E083, 2007) to 6.32 % (2025.2, 2007). Larger de facto fractions (Fig. 3b) were calculated for the seedlings from mother trees E029 (2.31 % vs. 3.47 %) and E083 (2.08 % vs. 3.13 %) in 2006 and for seedlings of mother trees 1156.7 (1.05 % vs. 1.58 %), 2025.2 (6.32 % vs. 9.47 %) and E083 (1.03 % vs. 1.55 %) in 2007 (Table 2).

Discussion

Our main concern was to contribute to establish a diagnostic marker system for introgressive gene flow into natural *P. nigra* populations. The diagnostic markers we used gave a detailed insight into different mating scenarios. The cp marker revealed the maternal origin of progeny with unknown maternity. In combination with four diagnostic nuclear markers, hybridisation events beyond the F1 generation could be retraced. Our results have crucial implications for in situ conservation measures concerning effects of swamping and the formation of hybrid swarms.

Low introgression rates in P. nigra offspring

The results of diagnostic nSSR markers reveal *P. x canadensis* paternity in seeds of multiple open pollinated *P. nigra* mothers, although its rates are small. All *P. nigra* mother trees are located directly inside a large *P. nigra* population, except for mother tree 2043.4, which is relatively isolated in terms of direct male *P. nigra* neighborhood (Fig. 1). Thus, tree 2043.4 was pollinated by *P. x canadensis* to a large extent (83.16 %). Phenology observations at the study site support these findings, as in both years, the flowering periods did not constitute a barrier to hybridisation between *P. nigra* and *P. x canadensis* (data not shown). For the determination of the introgression rate, the small-scale availability of hybrid pollen produced by *P. x canadensis* males that are cultivated nearby is therefore most important. The hypothesis of pollen competition (Rajora 1989), where in a mixed pollen cloud, pollen from *P. nigra* may be more successful in pollinating female black poplar than that from *P. x canadensis*, is therefore still valid. However, our findings demonstrate that the simultaneous presence of *P. nigra* pollen will not completely prevent mating between *P. x canadensis* males and *P. nigra* females. These findings are contrary to those of Vanden Broeck et al. (2004). According to their study spontaneous hybridisation between female *P. nigra* and male *P. x canadensis* only occurred when the presence of *P. nigra* was minimal in the pollen cloud. They found evidence for the massive introduction of *P. deltoides* genes (91 %) in progeny of an isolated *P. nigra* female (Vanden Broeck et al. 2004). Because of postzygotic barriers (Melchior & Seitz 1968) direct gene flow from *P. deltoides* males to *P. nigra* females is not possible. However, via fertile male F1 hybrids, there is no reproductive barrier and genes of *P. deltoides* are able to invade progeny of female *P. nigra.* Beyond the F1 generation, introgression of *P. deltoides* genes into the gene pool of *P. nigra* is therefore possible in any direction. Contrary to our findings, numerous studies on seedlings of *P. nigra* did not find any evidence of introgression from *P. deltoides* (Imbert & Levèvre 2003; Fossati et al. 2003; Tabbener & Cottrell 2003; Vanden Broeck et al. 2006).

P. deltoides genes in established juveniles

The frequency of juvenile poplars that exhibited *P. deltoides* specific alleles was relatively low at 3.95 %. Each half of this rate appeared to originate from *P. nigra* and *P. x canadensis* maternal origin respectively. The lack of F2 individuals may be due to the overall low rates of

both *P. x canadensis* progeny in the juveniles and F2 in the seeds of female *P. x canadensis*. It seems that the paternity of *P. x canadensis* does not have a detrimental impact on the establishment of seeds at least from *P. nigra* mothers. With the exception of the differing introgression rate of the seeds from mother tree 2043.4, the average introgression rate of all seeds from *P. nigra* mothers equaled the rate of introgressed juveniles with *P. nigra* maternal background (≈2 %). *P. x canadensis* females only contributed a proportion of 2.11 % to the juveniles although potential mother trees often grow in direct proximity to the area of natural recruitment. Therefore, progeny from *P. nigra* females seems to establish favourably. However, it must be mentioned that the area of natural seed recruitment is relatively small. Hence, it can not be representative. Other studies on juvenile poplars reported on larger rates of introgression. For example, 5.7 % hybrid genotypes of young trees along the Rhine river in the Netherlands were found to exhibit *P. deltoides* ancestry (Arens et al. 1998). More recent studies on juvenile poplars also revealed high rates of individuals with genetic background of *P. deltoides* (Ziegenhagen et al. 2008, 24 %; Smulders et al. 2008, 45 %). These introgression rates may be quite variable, but they all show that in spatial proximity, *P. nigra* and *P. x canadensis* mating does happen and seeds will establish eventually.

In subsequent generations, *P. deltoides* genes may spread in the genome of *P. nigra*. They may establish permanently, thereby altering the genetic structure of *P. nigra* populations and affecting the fitness of individuals (Ellstrand et al. 1999). As poplar plantations represent only a limited number of genotypes, frequent mating events are considered to reduce the genetic diversity of *P. nigra* populations, especially as its rarity contrasts with the widely planted hybrid cultivar plantations (e.g. Cagelli & Lefèvre 1997; Arens et al. 1998; Levèvre et al. 2001). Once hybridisation has begun, it is difficult to stop, especially if hybrids are fertile and mate both among themselves and with parental individuals. By this means, poplar populations are at risk to become hybrid swarms, as they consist of a mixture of hybrids, backcrosses, and later-generation recombinant types (Grant 1981). The monitoring of introgressive gene flow in a hybrid swarm is an effort that hardly any study will provide, even if enough diagnostic markers were available. Mating of backcrosses with established F2 generation individuals may increase the proportion of *P. deltoides* genes in the local gene pool. By this means, F2 crossings act as a reservoir of *P. deltoides* genes even if their cultivated parents have disappeared.

P. nigra swamps into P. x canadensis offspring

Seedlings of *P. x canadensis* females have not yet been the subject of any studies on introgressive gene flow. Basically all seedlings of *P. x canadensis* mothers exhibit portions of both *P. nigra* and *P. deltoides* genes and are therefore "introgressed" in principle. Accordingly, determining the de facto fraction of paternity of both *P. nigra* and *P. x canadensis* in this progeny is a difficult task. More diagnostic markers are needed to clearly detect substantial rates of the de facto F2 fraction (Fig. 3b). The extremely low de facto fractions of F2 mating events and high *P. nigra* mating fractions are surprising. Even hybrid mother tree 2025.2, which is located quite far from the black poplar population, was heavily pollinated by *P. nigra*. The amount of *P. nigra* paternity is probably due to the pollen cloud composition produced by the male neighbourhood but overall, it exceeded *P. x canadensis* x *P. x canadensis* mating by far. These findings suggest that the hypothesis of pollen competition (Rajora 1989) can be applied also to the pollination of *P. x canadensis* females, probably even on a larger scale. Through such 'pollen swamping' (Petit et al. 2004) of *P. nigra* into offspring of *P. x canadensis*, local gene pools of both taxa are merged and offspring evolves. Although the larger part of its nuclear genes (i.e. 75 %) originates from *P. nigra*, 25 % still represent *P. deltoides* genes. By this means, alleles of *P. deltoides* may invade the *P. nigra* gene pool in subsequent generations. This way of introgressive gene flow seems to be especially important, as our findings reveal that the paternity of *P. nigra* in *P. x canadensis* offspring is rather the rule than the exception.

Although paternity analysis has been used successfully in other studies on introgressive gene flow in black poplar (Tabbener & Cottrell 2003; Wheeler et al. 2006; Pospíškova & Šalková 2006; Vanden Broeck et al. 2006), this technique was not appropriate for our data set, as identical genotypes of potential fathers are widely spread in terms of the clonal hybrid poplar cultivars. According to Rathmacher et al. (in press), about 76 % of all male *P. x canadensis* cultivars at the study site could be identified to be ramets of a single male genet referred to as clone "Robusta" (own unpublished data). Additionally, due to their frequent presence at the study site, hybrid poplars could only be sampled randomly. Distinct *P. x canadensis* fathers were therefore impossible to find.

Hybrid identification using a Bayesian assignment method

By way of comparison, hybrid and backcross genotype probabilities of all poplar progeny were estimated using the software "*NewHybrids*" (Anderson & Thompson 2002). For this purpose, data of the four diagnostic markers and three additional nSSR loci was used (Rathmacher et al., submitted). In both subsets, i.e. the seedlings of *P. nigra* mothers and the juveniles, the portion of *P. x canadensis* ancestry was underestimated if not overlooked at all (data not shown). This phenomenon was even more pronounced in seedlings of *P. x canadensis*, where large portions were assigned to pure *P. nigra*. It seemed that not even the presence of species specific alleles of *P. deltoides* inevitably led to the assignment of *P. x canadensis* ancestry. Moreover, seedlings were assigned to categories which are unlikely to exist in the study area, such as "pure *P. deltoides*" and "backcross to pure *P. deltoides*". Hence, our results show that such findings have to be interpreted with caution at least in this case. Generally, assignment depends strongly on the coincidental combination of the number of reference samples and their specific allelic settings. Additionally, seven microsatellite loci including four diagnostic markers may not be sufficient for an unbiased assignment (Anderson, personal communication).

Conclusions

Introgressive gene flow takes place in two directions as *P. nigra* females are pollinated by *P. x canadensis* males and vice versa. Because of stochastic events and settings, the particular rates may vary in space and over time. The major part of introgressive gene flow is represented by *P. nigra* males pollinating *P. x canadensis* females. These findings support the hypothesis of preferential hybridisation between female *P. x canadensis* and male *P. nigra* (Pospíškova & Šalková 2006). Therefore, our results demonstrate that this marker combination allows to identify parental taxa of progeny from the hybrid complex of *P. nigra*/*P. x candensis*. It proved to be efficient even for advanced hybrid generations, such as the F2 and first backcross generation.

The establishment of *P. x canadensis* progeny poses a fundamental risk in terms of the species integrity of *P. nigra* even though it rarely occurs. In any case, *P. deltoides* alleles are included. With respect to the massive presence of *P. x canadensis* in the landscape, hybridisation with pure *P. nigra* is therefore most likely. It represents an important genetic

risk for the ‘species *P. nigra*’ as such, especially for small populations with huge *P. x canadensis* neighbourhood. In the long run, such populations are at risk to turn into hybrid swarms.

For a conclusive evaluation of the longevity of these introgression processes, further issues need to be considered, such as the viability and fertility of produced offspring. Further research on the genetic background of seedling establishment and fitness is needed to evaluate the consequences for natural regeneration of *P. nigra* populations. Introgressive gene flow is only retraceable during initial generations. Therefore, species affiliation of juvenile populations of natural recruitment that are believed to consist of pure *P. nigra* progeny needs to be reassessed. Altogether, detailed mating scenarios under field conditions are hard to predict. However, introgressive gene flow seems to occur frequently. As suitable data on distances of effective pollen dispersal in poplar are available (Rathmacher et al., submitted), our results could be regarded as a call for a spatially oriented management of conservation programmes. By this means, it is possible to avoid introgressive gene flow and natural stands of *P. nigra* can be prevented from becoming hybrid swarms. This research may also provide valuable information for evaluating the risk of introgression from plantations of genetically modified poplar cultivars.

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3. Discussion

There is an increasing interest in gene dispersal in landscapes that have been modified by human activities. This is due to concerns about the impact that habitat loss, disturbance and fragmentation may have on plant populations. The introduction of exotic species, which is associated with potential hybridisation and introgressive gene flow, may additionally have an impact on local native plant populations. The established high-resolution marker system (paper I) turned out not only to enable a reliable identification of poplar varieties but also to allow a detailed analysis of gene flow inside a *P. nigra* population (paper II) and introgression of *P. deltoides* genes into the local gene pool of *P. nigra* (paper III). This robust system offers a fast and cheap genotyping protocol with reliable results and the opportunity for large sample throughput (papers I, II & III). These large sample sizes were essential for the detection of gene flow at biologically significant levels (>1 %) (Ellstrand et al. 1999). The unambiguous individual identification turned out to be the crucial prerequisite for subsequent analyses of gene flow.

3.1 Intraspecific gene flow in *P. nigra*

Gene flow can be a potent evolutionary force. A small amount of gene flow is able to counteract the other evolutionary factors of mutation, drift and selection (Slatkin 1987). Its impact depends on its magnitude. The magnitude of gene flow among natural plant populations is described to be widely diverse, varying among species, populations, individuals, and even between years (Ellstrand 1992a). Anthropogenic landscape change and habitat fragmentation have become so ubiquitous that they may threaten the genetic connectivity of many plant species (Sork & Smouse 2006). As populations become genetically isolated they are consequently at the risk of losing genetic diversity (Frankham et al. 2002). However, genetic diversity is required for the response to environmental changes that influence evolutionary processes (Sork & Smouse 2006). Many plant populations that were once continuous are now highly fragmented, exhibiting a collection of local populations with different degrees of connectivity (Harrison & Hastings 1996). This obviously holds true also for *P. nigra* as it naturally occurs in floodplain habitats, which have already been altered heavily by river management activities (Cagelli & Lefèvre 1997; Guilloy-Froget et al. 2002). Natural floodplain forests, with *P. nigra* as keystone species, represent the type of potential

natural vegetation along rivers in Western Europe (BfN 2008). They are of major interest in the ecological network of the river ecosystem and may serve as habitat for many organisms (Guilloy-Froget et al. 2002).

In the studied *P. nigra* population of paper II, estimates of genetic diversity were comparable to findings of other studies on black poplar. This is the first important condition for successful conservation measures focussing on natural regeneration without improvement by additional afforestation. Conservational attention should focus on the maintenance of such large diverse and locally adapted populations. They can be considered as a starting point for restoration projects.

Since *P. nigra* was and still is used in breeding programmes (Levèvre et al. 2001), it also represents a very valuable species for poplar breeders. Hence, a special effort must be made to maintain a large gene pool even from a commercial point of view. Considering the wide natural range of this species, particular attention should be devoted to preventing the loss of *P. nigra* resources in the whole distribution area as selection may have favoured particular variants and local adaptation has emerged (Cagelli & Lefèvre 1997). By using markers with links to candidate genes that are involved in the expression of physiological traits, the complete range of *P. nigra* ecotypes could be captured. Subsequently, special ecotypes exhibiting desired traits can be selected. They represent valuable resources for breeding programmes in the future.

Patterns of SGS in the adult tree population could be attributed to the spatial limitations of effective pollen and seed dispersal (paper II). Paternity analyses for retracing mating events and the estimation of pairwise relationships for assessing gene dispersal are methods of direct genealogical inference in population biology (Meagher 2007). Results of paper II demonstrate the effectiveness of such direct methods for analysing and interpreting genetic patterns of natural populations. The majority of dispersal distances of both effective seed and pollen was spatially limited as most mating occurred within local populations in a comparatively small neighbourhood (i.e. ≈1 km). These results were unexpected, as both poplar pollen and seeds are wind-dispersed. Therefore, generally large distances of distribution are assumed for pollen and seed of black poplar (Pospíškova & Šalková 2006). Dispersal distances of paper II differed as larger portions of seed were found in spatial vicinity of the mother tree than it was the case for effective pollen in proximity to the father tree. According to Meagher (2007) a difference in magnitude between seed and pollen dispersal has a strong impact on the spatial genetic neighbourhood composition of local populations. As effective seed dispersal is more

restricted than pollen-mediated gene flow it may be more relevant to population structure (Crawford 1984). Consequently, if seed dispersal is limited relative to the average spacing between maternal trees, spatial clustering of family members in the offspring generation might be expected (Adams 1992). Apparently, the findings of reduced genetic diversity and inbreeding tendencies according to the investigated juvenile population in paper II reflect these consequences. However, sufficient natural regeneration is urgently needed to avoid the loss of genetic diversity due to population senescence. In natural systems, this can be achieved by the colonisation of patchy areas of bare soil that were created by river dynamics. As such events occur stochastically, it follows that in many years, successful regeneration does not occur. Consequently, there is often a strong age structure in natural stands, reflecting the history of flooding (Heinze 1998a). However, combining different regeneration patches over time may eventually reflect the amount of genetic diversity of the original parental population. This kind of regeneration strategy of *P. nigra* can be interpreted as an adaptation to the extremely dynamic system of natural floodplains. Disturbance is not only a destructive factor. In case of *P. nigra* it is rather urgently needed for successful longevity of populations.

The positive effects of disturbance on genetic diversity has also been reported for other forest trees (e.g. Hubbell et al. 1999; Kelly & Bowler 2002). The long life spans of tree species relative to the scale of temporal variability of disturbances and seedling establishment provides a buffer or storage effect (Beckage et al. 2005). By this means, tree species can persist through periods of low recruitment, when years of high fecundity or survivorship do not coincide with suitable areas for seedlings to establish. On the long run, population diversity is promoted by large annual fluctuations of patchy recruitment processes.

Apparently, the findings of reduced genetic diversity and inbreeding tendencies according to the investigated juvenile population in paper II also illustrate that singular, small-sized areas for natural regeneration do not seem to be sufficient for the effective conservation of present genetic diversity. These results should therefore be considered in the concept of conservation measures. According to species whose regeneration strategy is adapted to such dynamic habitats, the restoration challenge turns out to find ways to enhance the natural potential for regeneration. In populations where natural recruitment is to be promoted, conditions for the establishment of juveniles in multiple regeneration patches need to be optimised in situ. This can be achieved by permitting more variation in hydrological conditions, proper water flow and active erosion/deposition processes (Guilloy-Froget et al. 2002; Hughes et al. 2005).

In case of predominating short-distance gene flow, as reported in paper II, the potential for gene flow between nearby individuals and populations is great (Adams 1992). For the

conservation of small relict populations, it is important to know how far apart target populations of conservation measures can grow and yet remain in contact by effective pollen and seed dispersal. If the amount of pollen produced by near neighbours is small in relation to more distant pollen sources, as it is the case for small relict populations or solitary trees, the advantage of proximity may be eliminated or compromised (Adams 1992; Ellstrand et al. 1999). In such cases, accumulated long-distance dispersal of small amounts of pollen from many trees can result in considerable pollen distribution over long distances when whole stands are considered as pollen sources (Ellstrand & Elam 1993).

3.2 Interspecific gene flow: hybridisation and introgression

As plantations of cultivars containing numerous plants frequently grow in direct vicinity to their wild relatives (James et al. 1998; Williams & Davis 2005), knowledge of effective pollen and seed dispersal distances is of major importance in terms of introgressive gene flow, which is likely to occur in the vicinity of *P. nigra* and hybrid poplar cultivars (Vanden Broeck et al. 2004; Smulders et al. 2008a). As gene flow generally exhibits a tendency to homogenise population structure (Slatkin 1987) hybridisation accompanied by introgression can be discussed controversially with regard to its evolutionary consequences. Even low rates of hybridisation can have important evolutionary consequences if species that hybridize are common (Mallet 2005). Therefore, interpreting the evolutionary significance of hybridisation and determining the role of hybrid populations in developing conservation plans is a very difficult issue (Allendorf et al. 2001).

Genetic data shows that hybridisation and introgression between species are ongoing and regular, if not always common processes in nature (Arnold 1997). At least 25 % of plant species and 10 % of animal species are involved in these processes (Mallet 2005). Some authors consider introgressive hybridisation between rarer and more numerous species as a potential genetic enrichment of the endangered form (e.g. Arnold 1997; Mallet 2005; Reusch & Wood 2007). According to their theory, introgressive hybridisation could increase the fitness and the genetic variability of the rare form and could facilitate its habitat expansion. Given the fact that our environment will continue to change, the process of natural hybridisation may therefore actually be an ‘ark’ for the genetic variability contained in some species.

Natural hybridisation is a frequent and important component of plant evolution and speciation (Rieseberg & Ellstrand 1993; Mallet 2005). A large number of plant species may be descended from hybrids (Arnold 1997). Whitham et al. (1999) illustrate that plant hybrid zones are dynamic centres of ecological and evolutionary processes for plants and their associated communities. The genetic differences between the parental species will result in the greatest genetic variation in the hybrid zone, which will in turn have a positive effect on biodiversity (Whitham et al. 1999). Variation introduced via introgression is said to contribute regularly to adaptation and diversification. It may occasionally allow adaptive combinations to evolve at a higher rate than in the absence of an input of variation from hybridisation (Mallet 2005). Natural zones of hybridisation are considered to be unique and worthy of special efforts to promote their conservation and protection (Whitham et al. 1999; Martinsen et al. 2001). Taxa resulting from natural hybridisation events should therefore be protected, just like any other species (Allendorf et al. 2001). Accordingly, natural interspecific hybridisation is not rare.

On the other hand, hybridisation has also led to the extinction of many populations and species (Ellstrand 1992b). Rare species may be threatened by hybridisation in two ways: Outbreeding depression from detrimental gene flow will reduce the fitness of a locally rare species that is mating with a local common one. An alternate route to extinction is by swamping, which occurrs when a locally rare species loses its genetic integrity and becomes assimilated into a locally common taxon as a result of repeated events of hybridisation and connected introgression. By this means, poplar populations are at risk to become hybrid swarms, as they consist of a mixture of hybrids, backcrosses, and later-generation recombinant types (Grant 1981). Genetic assimilation involves the loss of the genotypes/phenotypes of the rare form through asymmetric gene flow from the more numerous taxon (Ellstrand & Elam 1993). The individuals that are increasing in frequency would actually be hybrids and thus their increase might be seen as the loss of the rare form (Arnold 1997). Both phenomena can lead to extinction rapidly in only a few generations (Ellstrand et al. 1999). How the data on hybridisation and introgression are interpreted is therefore as important as the data themselves. It is important to differentiate between natural hybridisation among native species in the absence of human activities and hybridisation that occurs after the anthropogenic introduction of a domesticated related species.

In *Populus,* natural hybridisation among native species is common (Eckenwalder 1996; Zsuffa et al. 1996). However, in Europe, there are no native mating partners for natural hybridisation with *P. nigra* (Stettler et al. 1996). In natural conservation, hybridisation that is

enabled by anthropogenic transfer of species and bred taxa is generally considered to be a threat for native populations (Vanden Broeck et al. 2005; Kothera et al. 2007; Carlson & Meinke 2008). Cases of gene flow from bred taxa to their native relatives provide examples of contemporary microevolution that have important practical implications (Ellstrand et al. 1999). Hence, detailed knowledge about introgressive gene flow into natural populations is of great value for establishing conservation measures. If any hybridisation is detected within distances that are typical for those occurring between the bred taxon and the native species, it provides evidence for successful mating under field conditions in principle.

Initially, introgressive gene flow depends on pollen exchange between the cultivar plantation and the natural population. Hence, it takes place mostly at the border between these populations (Kuparinen & Schurr 2008). In paper III, these effects are demonstrated impressively, as a solitary native tree was heavily pollinated by pollen of hybrid cultivars. Subsequently, introgressive gene flow is either caused by pollen dispersal between the F2 generation hybrids or by pollen exchange between these hybrids and native plants (backcrosses) (Kuparinen & Schurr 2008). Thereby, the original plantation of F1 hybrids itself may act as 'depot' for exotic genotypes (paper III).

So far, no reports on hybrid swarms in adult populations of *P. nigra* and *P. x canadensis* are available. This may be due to the fact that anthropogenic hybridisation and introgression might have begun only recently so that to date, advanced backcross generations among *P. nigra* and *P. x canadensis* are unlikely. However, as in Europe plantations of bred poplars already exist for some 250 years (Rajora & Rahman 2003) there has already been a long period of time for extensive gene flow between these cultivars and natural *P. nigra* populations. The existence of poplar hybrid swarms in European landscapes therefore needs further investigation. Admittedly, several studies reported on introgressed individuals in juvenile populations (Ziegenhagen et al. 2008; Smulders et al. 2008a, paper III). The enlarged set of nuclear markers (paper I) combined with organelle markers has proven to provide detailed information of parent species contributions at least in the F2 and first backcross generations (paper III).

Against the background that introgressive gene flow emerges spontaneously, the next step is to determine the rate at which it occurs (Hails & Morley 2005). If bred taxon-specific alleles occur at biologically important frequencies (>1 %) in adjacent individuals of the native species, these alleles are likely to be permanently incorporated into natural populations via hybridisation and introgression (Ellstrand et al. 1999). In cases where natural populations are large compared to cultivated poplar plantations anthropogenic introgression seems to be

restricted (Imbert & Levèvre 2003). This could be explained by the presence of many conspecific mating partners combined with the effects of conspecific pollen advantage in *P. nigra* (Rajora 1989).

By contrast, the assessed hybridisation rates between *P. nigra* and *P. x canadensis* in a large black poplar population reported in paper III exceeded 1 % in both seedlings and juveniles. The rate of incorporation of foreign alleles under such levels of hybridisation is likely to be several orders of magnitude higher than typical mutation rates (Kovalchuk et al. 2000; Udupa & Baum 2001). Therefore, gene flow in such systems can be expected to be much more important than mutation (Ellstrand et al. 1999). Hence, the studied *P. nigra* population is at risk to be permanently contaminated with alleles of *P. deltoides*.

The major part of introgressive gene flow in the studied population (paper III) is represented by *P. nigra* males pollinating *P. x canadensis* females. The amount is probably due to the pollen cloud composition produced by the male neighbourhood. However, on average it exceeded *P. x canadensis* only mating by far. Although these results of quantitative gene flow between *P. nigra* and *P. x canadensis* (paper III) are congruent to the hypothesis of preferential hybridisation between female *P. x canadensis* and male *P. nigra* (Pospíškova & Šalková 2006), mating in both directions was found. Levels of gene flow from bred taxa to their native relatives may be highly variable and may depend on a variety of spatiotemporal factors. Differences in the relative sizes of the source and sink populations result in different rates of hybridisation and gene flow (Ellstrand & Elam 1993). With respect to the massive presence of *P. x canadensis* in the landscape, patterns of conspecific pollen advantage in *P. nigra* (Rajora 1989) can be concluded. Hybridisation of pure *P. nigra* and *P. x canadensis* is a logical consequence. This introgressive gene flow is only retraceable during the first couple of generations (see above). However, the assessment of quantitative rates turned out to be more laborious for the progeny of *P. x canadensis* mothers (paper III). This was due to the fact that half of the genome of the mother (as an F1 hybrid) already consists of *P. nigra* genes. These F1 hybrids can be identified reliably with molecular markers. Due to Mendelian segregation during meiosis, there is a certain probability that offspring exhibits only *P. nigra*-specific alleles in all considered diagnostic markers. It is therefore more difficult to distinguish between F2s, backcrosses and later generation hybrids (Epifanio & Philipp 1997; Boecklen & Howard 1997, paper III). In progressing backcross generations, advanced mating events can only be monitored with growing efforts in terms of costs and time (paper III). Although offspring may be checked regarding its hybrid status, introgressed offspring may remain

undiscovered as a limited number of diagnostic markers was used (paper III). Hence, the general presence of maternal *P. x canadensis* offspring shall be rated as a risk for natural *P. nigra* populations. It may exhibit undetected genes of *P. deltoides* although it was classified to be pure *P. nigra*. In any case, *P. deltoides* alleles are included. This poses an important genetic risk for the 'species *P. nigra*' as such, especially for small populations with a huge *P. x canadensis* neighbourhood. Altogether, detailed scenarios of mating under field conditions are hard to predict but occur frequently (paper III).

For a conclusive evaluation of the longevity of these introgression processes, further issues need to be considered, such as the viability and fertility of produced offspring. Common post-mating reproductive barriers of hybridisation include hybrid sterility, hybrid weakness or inviability and hybrid breakdown. First generation (F1) hybrids are vigorous (hybrid superiority due to heterosis), robust and fertile, but later generation hybrids are weak or inviable (Rieseberg & Carney 1998). Interestingly, studies on several species report both outbreeding depression and enhanced fecundity of hybrids (reviewed in Ellstrand et al. 1999; Hails & Morley 2005). In the majority of such studies, the fitness of the hybrid admittedly tends to be the same as or even higher than that of the wild parent. Poplar hybrids are generally characterised by reduced fertility with respect to parental species; pollen and seed viability is significantly lower in F1 hybrids (Stettler et al. 1996). However, due to the immense presence of cultivated F1 hybrid poplars, even extremely low fertility or viability of subsequent hybrid generations (e.g. F2, first backcross generation) would not necessarily prevent extensive gene flow (Arnold et al. 2001).

Hybridisation of *P. nigra* and *P. deltoides* only results in viable *P. x canadensis* offspring if *P. deltoides* is the mother tree. Due to postzygotic barriers (Melchior & Seitz 1968) direct gene flow from *P. deltoides* males into offspring of *P. nigra* is impossible. As both, male and female individuals of *P. x canadensis* are able to produce viable offspring with *P. nigra*, mating barriers seem to be irrelevant beyond the F1 generation. By this means, even male mating partners are able to introduce *P. deltoides* genes into the *P. nigra* gene pool (paper III). A comparative study on performance parameters of progeny from open-pollinated *P. nigra* and *P. x canadensis* mothers revealed competition to be advantageous for seedlings of *P. x canadensis* mothers (Köhnen 2008). Further research on the genetic background of seedling establishment and fitness is needed for the evaluation of consequences for natural regeneration of *P. nigra* populations. However, considering that habitat reduction followed by hybridisation can lead to the extinction of a rare plant species (Ellstrand 1992b), interspecific introgressive gene flow poses a potential threat to natural populations of *P. nigra* (Cagelli &

Lefèvre 1997). Ultimately, changes in diversity of natural *P. nigra* populations will depend on levels of allelic richness and genetic diversity of the source population (in our case, the poplar cultivars) in relation to that of the sink population. Cultivar plantations typically contain substantially less genetic variation than populations of their wild relatives (Ladizinsky 1985). Consequently, levels of neutral variation in a natural population will often decrease under gene flow from a related bred taxon (Ellstrand et al. 1999). The presence of many hybrid poplar plantations representing a limited number of genotypes is therefore frequently considered to pose a severe threat to the native populations of *P. nigra*, especially as its rarity contrasts the widely planted hybrid cultivar plantations (e.g. Cagelli & Lefèvre 1997; Arens et al. 1998; Levèvre et al. 2001). Once hybridisation processes have begun they are difficult to stop, especially if hybrids are fertile and mate both among themselves and with parental individuals. There is also the fact that a successful monitoring of these advanced hybridisation events is almost impossible (paper III).

3.3 The risk of transgene flow

Monitoring and risk assessment of introgressive gene flow additionally gain importance as hybrid poplar plantations may soon include GM trees. Benefits of GM trees can arise from the transfer of traits that are not readily available either in the breeding population or the genetic resource (Hoenicka & Fladung 2006). A major concern is that genetic resources of wild relatives will be altered through transfer of the transgene by hybridisation (DiFazio 2002; Ellstrand 2003). Therefore, particularly GM trees are confronted wordwide with increased attention regarding biosafety issues (Hoenicka & Fladung 2006).

In case of introgressive alleles the conditions for homogenisation through gene flow (Slatkin 1987) will vary depending on whether these alleles are neutral, detrimental or beneficial in the ecological and genomic environment of the population that receives them (Ellstrand et al. 1999). Therefore, the risk of transgene spread from GM to natural populations depends on the genotypic and phenotypic effects of the transgene: If transgenic taxa have higher fitness, e.g. due to decreased mortality, their establishment in natural populations is favoured (James et al. 1998; Hails & Morley 2005; Chapman & Burke 2006). For instance, genes for resistance against abiotic stress, native herbivores or pathogens might provide great ecological benefits (Whitham et al. 2006). However, even neutral or deleterious genes are considered to persist

and become fixed in wild populations in situations where GM cultivars numerically swamp native genes (Haygood et al. 2003).

Cross-pollination of transgenic and exotic taxa with their native local relatives is therefore a major concern in crops (Ellstrand et al. 1999) and is becoming a concern for conservationists and resource managers (Allendorf et al. 2001; Anderson & Thompson 2002). Plantations have multiple ecological connections with other managed and natural ecosystems. Dispersal of pollen and seeds, or vegetative spread from intensively bred, exotic, or recombinant DNA from modified forest plantations may cause either detrimental or beneficial ecological impacts on wild or managed ecosystems. Because of their size and the large number of organisms that use trees as food and habitat (even in plantations), non-target effects from the release of pollen, leaves and other parts of GM trees are of greater ecological concern than for annual crops (Strauss et al. 2001).

The scale of gene flow affects how efficiently transgene flow can be prevented by limiting the presence of conventional genotypes in the proximity of a GM population (Chapman & Burke 2006). Additionally, insertion of genes designed to prevent or substantially reduce dispersal could reduce the risk and extent of undesired impacts (Brunner et al. 2007). One approach, mitigation (e.g. Kuparinen & Schurr 2008), is a directed form of plant domestication which causes that the fitness benefits of transgenes are effectively cancelled by tight linkage to a gene that is beneficial within farms or plantations, but deleterious elsewhere. It has the advantage of being applicable to vegetative and sexual dispersal, which is useful for species like poplars that can spread vegetatively (Hoenicka & Fladung 2006). For example, genes that reduce the rate of height growth in forest trees, especially for shade-intolerant species like poplars (Guilloy-Froget et al. 2002), are expected to provide a very powerful competitive disadvantage in competition with wild trees (Strauss et al. 2004). However, by crossing-over events, mitigation gene and transgene might become separated (Brunner et al. 2007). By this means, the transgene could subsequently be inherited without the mitigation gene. In this case the control mechanism of transgene spread is interrupted. Mitigation genes can also be combined with sterility genes to provide a second layer of containment (Brunner et al. 2007). Sterility technology may contribute to minimising transgene flow from plantations and increasing wood production. The loss of flowers and seeds in polar agriculture would appear to be without consequence in terms of biodiversity (Strauss et al. 2001). Producing GM poplars without flowers or with infertile flowers is highly desirable because it reduces the ecological complications of introgressive gene flow (Strauss et al. 1995). Gene flow in poplar plantations and its implications for transgenic risk assessment have been studied by DiFazio

(2002). In his study, he combined large-scale field studies, genetic analyses and simulation modelling. Resultant levels of gene flow from hybrid poplar plantations in the field studies were on average lower than reported in paper III. He concluded that a reduction of pollen and seeds in non-native species/bred taxa can minimise or even prevent gene flow (DiFazio 2002). Even if the sterility mechanism is imperfect and some sterility genes are released into wild populations, impacts are local and short-lived. This is due to the fact that fertility is a major component of fitness, and trees with sterility genes would probably have a significant disadvantage (Strauss et al. 2001).

However, neither the most effective containment approaches nor their reliability can be defined on the basis of current genomic knowledge and technological tools (Brunner et al. 2007). In the time frame relevant for commercial decision making, it is not possible to empirically assess the risks and factors related to transgene spread and to estimate the long-term effects of gene flow on the ecosystem (Williams & Davis 2005). So far, safety mechanisms work reliably and genes of GM poplars are reported to be relatively stable (Hoenicka & Fladung 2006; Brunner et al. 2007).

In safety studies, various threats facing forests and natural tree populations, such as biological invasions, horizontal and vertical gene flow, still need to be considered in a case-by-case evaluation. The impact on associated organisms such as herbivores, mycorrhiza and pathogenes should not be excluded from the experiments as they are common features of the field environment (Hoenicka & Fladung 2006). In the case of perennial species with occurring long-distance pollen dispersal, such as many wind-pollinated trees including poplar (James et al. 1998; Williams & Davis 2005), the risk management measures would need to be applied over large areas and for long periods of time, which may give rise to several practical difficulties and considerable costs (Snow et al. 2005).

4. Conclusions and perspective

This thesis provides valuable insights into gene flow and into the problem of introgression in *P. nigra*. The approach of using a well-optimised combination of SSR markers has a widespread application, including genetic management in tree plantations (Burczyk et al. 2002; De-Lucas et al. 2008, paper I), conservation biology (Pospíškova & Šalková 2006; Bittencourt & Sebbenn 2008, papers II & III) and the analysis of introgressive gene flow

(paper III). The results of paper III also contribute to a risk assessment of potential gene flow from GM crops to adjacent natural plant populations (DiFazio 2002; Brunner et al. 2007).

Knowledge of factors that influence gene flow in real landscapes is important for evaluating impacts of forest management on the genetics of populations. Understanding relationships between effective pollen and seed dispersal and tree spacing is necessary for assessing the genetic implications of alternative natural regeneration systems and for designing areas for seedling establishment. In that way, mating among genotypes can be maximised and contamination from undesired sources, such as GM taxa can be limited (Adams 1992).

Genetic diversity of *P. nigra* in Europe has been the subject of multiple studies of single populations as well as comparative studies of multiple populations (e.g. Smulders et al. 2008b). However, knowledge about gene flow patterns and spatial genetic structure of *P. nigra* populations to date is only rudimental. Results of paper II can be regarded as a first systematic approach. Future research should therefore focus on comparative studies of multiple natural populations of *P. nigra* in order to verify gene flow patterns and resulting SGS. Results shall be used as a basis for the conceptual design of natural regeneration projects on international scales.

Habitat degradation and introgression pose a serious threat to natural *P. nigra* populations. Therefore, conservation measures should focus on several issues:

As genetic diversity of local juveniles is reduced by river regulations that inhibit natural processes of patchy natural regeneration, processes that sustain biodiversity need to be conserved or restored. Accordingly, fluvial geomorphologic processes need to be promoted in order to create sites that are suitable for patchy regeneration, which are probably not only needed by *P. nigra* alone but also by several floodplain species. This is concordant with the definition of an efficient strategy for ‘dynamic conservation’ (recombination and selection) of pioneer species, that naturally grow in disturbed ecosystems (Cagelli & Lefèvre 1997). Therefore, efforts should focus on maintaining and expanding the remaining pure native populations.

As several studies assume that tree populations are able to maintain diversity in the face of environmental change (Hamrick 2004; Sork & Smouse 2006), it may also hold true for *P. nigra*. However, the extent of resilience to fragmentation is determined by both the amount of gene flow and the diversity of the pollen and seed pool from which immigration is drawn (Sork & Smouse 2006). Therefore, population structure, connectivity and area for possible natural regeneration should be considered carefully for the elaboration of conservation programmes for *P. nigra*. Remaining native populations need to be in spatial proximity to

each other in order to obtain extensive gene flow. By this means, the main cause of the decrease in black poplar populations, namely the destruction and fragmentation of its natural habitat, can be attenuated.

By habitat restoration combined with afforestation programmes in small populations, the risk of introgressive gene flow with plantations of cultivated poplars can be limited as well. All of the practices of poplar cultivation, as well as those of conventional and organic agriculture, cause dramatic changes in ecological processes when compared to natural ecosystems. For tree plantations, examples include short-rotation cultivation, controlling competing vegetation and planting evenly-spaced trees at high density (Zsuffa et al. 1996). The use of highly productive bred taxa constitutes a major divergency from the locally adapted populations that might have existed on the site historically. However, eliminating poplar cultivars is not an option because of the economic importance of hybrid poplar plantations.

Nevertheless, the results of this thesis can be regarded as a call to the management of conservation programmes. They should consider the risk of introgressive gene flow regarding distances of effective pollen and seed dispersal in managing natural populations of *P. nigra* and potential seed establishment areas. In doing so, introgressive gene flow in any direction can be limited and the establishment of juveniles with a genetic *P. x canadensis* background can be prohibited. Long-distance introgressive gene flow may be prevented by preferential conspecific mating of *P. nigra*. If no conservation actions are taken, the results of paper III indicate that natural poplar populations are at risk of becoming hybrid swarms. This applies especially to black poplar stands that become very small compared to the widespread hybrid poplar plantations. Such populations may go extinct through genetic assimilation.

As the total removal of fertile hybrid poplars is not possible, further monitoring of hybridisation processes in natural black poplar populations is of crucial importance. Thereby, knowledge on long-term consequences for the conservation of black poplar can be obtained. As F2 and backcross hybrids establish in natural regeneration (paper III) these advanced hybrid generations may compete with progeny of *P. nigra* for the same ecological niche (Smulders et al. 2008a). Further studies on progeny of *P. x canadensis* mothers need to be performed in order to assess its role in establishing *P. deltoides* genes in hybrid swarms, as this progeny may mate extensively with pure *P. nigra*. Additionally, the establishment and survival as well as the vegetative establishment of progeny with any kind of parental background should be compared under field conditions. Advanced hybrid generations may lead to adaptive evolution by producing hybrid genotypes that are fitter than their parents in the parental or novel habitats (Arnold 1997). As competitive ability and initial frequency are

factors that have a strong effect on the risk of extinction through hybridisation (Wolf et al. 2001), hybridisation may have crucial consequences for the evolution of native black poplar populations. Population size, hybrid status of progeny and associated rates of introgression should therefore be monitored over several generations. By this means, possible advantages of certain hybrid generations can be evaluated. Results can be used to assess the long-term effects of introgressive gene flow in real landscapes.

The findings of introgressive gene flow also provide some insight into the possible consequences of any introductions of GM poplar hybrids into the neighbourhood of native populations of *P. nigra.* If these GM cultivars are still fertile and qualities of pollen and seed dispersal are not modified, results of effective pollen and seed dispersal distances reported in papers II & III can be transferred. This research may therefore help to evaluate the risk of introgression from GM poplar cultivar plantations. Genetic engineering of trees is a relatively new discipline and little has been done regarding their potential for environmental impact (Hoenicka & Fladung 2006). In order to avoid unpredictable consequences for native species and their ecosystem, the escape of transgenes into natural populations and species urgently needs to be prevented.

Natural populations of indigenous poplar species are the ultimate source of genetic variation. They need to be conserved, whenever possible restored and protected from genetic contamination through non-native gene pools (Zsuffa et al. 1996).

Applying the results of papers II & III will contribute to the general understanding of gene flow in wind-pollinated trees. Consequences for spatial configuration and regeneration of genetic diversity, especially in pioneer tree species should be applied in conservation management.

Thereby, distances of effective pollen flow of *P. nigra* (paper II) may also be suitable in conservation measures concerning other wind-pollinated tree species. With respect to the connectivity of subpopulations, they may help in the conceptual design of protection and afforestation areas. Furthermore, they help to estimate the minimum distances between natural populations and plantations of cross-compatible bred taxa or exotic species. The levels of introgressive gene flow presented in paper III can be used as reference points for risk assessment regarding hybridisation and transgene flow in comparable landscapes.

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Declaration

I hereby declare that the dissertation entitled

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is the original and independent work carried out by me. The indicated resources were used exclusively. The dissertation has not been submitted previously to receive any degree, diploma or other similar titles.

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