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Natural hybridisation between *Populus nigra* L. and *P. x canadensis* Moench. Hybrid offspring competes for niches along the Rhine river in the Netherlands

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Abstract Black poplar (*Populus nigra* L.) is a major species for European riparian forests but its abundance has decreased over the decades due to human influences. For restoration of floodplain woodlands, the remaining black poplar stands may act as source population. A potential problem is that *P. nigra* and *Populus deltoides* have contributed to many interspecific hybrids, which have been planted in large numbers. As these *Populus x canadensis* clones have the possibility to intercross with wild *P. nigra* trees, their offspring could establish themselves along European rivers. In this study, we have sampled 44 poplar seedlings and young trees that occurred spontaneously along the Rhine river and its tributaries in the Netherlands. Along these rivers, only a few native *P. nigra* L. populations exist in combination with many planted cultivated *P. x canadensis* trees. By comparison to reference material from *P. nigra*, *P. deltoides* and *P. x canadensis*, species-specific AFLP bands and microsatellite alleles indicated that nearly half of the sampled trees were not pure *P. nigra* but progeny of natural hybridisation that had colonised the Rhine river banks. The posterior probability method as implemented in *NewHybrids* using microsatellite data was the superior method in establishing the most likely parentage. The results of this study indicate that offspring of hybrid cultivated poplars compete for the same ecological niche as native black poplars.

Keywords Natural regeneration · Microsatellite · AFLP · NewHybrids · Posterior probability

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Introduction

Black poplar (*Populus nigra* L.) is a characteristic species for European riparian forests. The forests have been reduced strongly in the past by human developments along the rivers and by management aiming at narrowing the path of the river (Lefèvre et al. 1998). During the last 10 years, views on river management have changed and now the aim is to give rivers more space and to restore floodplain woodlands (Hughes et al. 2001). Remaining black poplar stands may act as source populations for recolonisation in the newly created riparian ecosystems. These new populations should contain sufficient genetic diversity to allow them to adapt to changing environmental conditions and for long-term survival of the populations (Booy et al. 2000).

P. nigra also plays a central role in poplar breeding programmes and has contributed to many successful interspecific hybrids (Frison et al. 1995). The introduction of large numbers of some *P. x canadensis* clones (offspring

of *Populus deltoides × P. nigra* crosses) and *P. nigra* varieties, which have the possibility to intercross with wild *P. nigra* trees, has raised concern about the genetic diversity and integrity of *P. nigra* in Europe (Frison et al. 1995; Heinze 1997; Lefèvre et al. 1998, 2002; Arens et al. 1998; Vanden Broeck et al. 2006). *Populus x canadensis* trees produce seeds sired by either *P. x canadensis* or *P. nigra* (Heinze and Lickl 2002). *P. nigra* produces seeds sired by *P. x canadensis* if no *P. nigra* pollen is available (Vanden Broeck et al. 2004). A few studies have tried to quantify to what extent such hybrid offspring is actually able to establish itself in nature. They found variable percentages: no establishment in natural populations (Fossati et al. 2003), only a few hybrid trees, estimated at 0–5% (Heinze and Lickl 2002), 9.7% (Benetka et al. 1999), 12% (Pospíšková and Šálková 2006) and 19.3% (Ziegenhagen et al. 2008). Heinze and Lickl (2002) doubted whether the genomes of *P. deltoides* and *P. nigra* are sufficiently congruent to allow F2 and BC in all directions, but Vanden Broeck et al. (2004) found evidence for gene flow between cultivated hybrid poplars and native black poplar. Indeed, several of the 27 hybrid offspring trees identified by Ziegenhagen et al. (2008) had combinations of the *P. deltoides* DT chloroplast haplotype and/or the *P. x canadensis* WIN3 genotype that were consistent with F2 or backcrosses.

A major problem in identifying hybrid offspring is the sensitivity of the methods used, especially if further generation hybrids such as F2 or backcrosses have to be identified. Although the morphological plant descriptor for *P. nigra* (Van Slycken 1996) was suitable to distinguish mature *P. nigra* from hybrids in genebank collections (Storme et al. 2002, 2004), its resolution is limited for (repeated) backcrosses between the hybrid and either parent. Identification of young seedlings is even more difficult, as features such as catkin and fruit morphology, bark structure and canopy shape are not present yet, and some leaf characters are of no use for identifying young trees. A cuneate leaf base is typical for *P. nigra*, but small leaves of seedlings and young trees of *P. x canadensis* also have a more cuneate base than leaves of vigorous longshoots on older trees. Glands near the top of the petiole are characteristic for *P. deltoides* and *P. x canadensis*, but these glands are mostly absent on leaves of young trees and seedlings.

Since the late 1980s, isozyme and random amplified polymorphic DNA (RAPD) markers have been successfully used for identification of clones and determination of the interrelationships among poplar species (Rajora 1989; Castiglione et al. 1993; Janssen 1997). However, isozyme markers are limited by their low level of polymorphism, and RAPD markers have problems in reproducibility (Jones et al.1997). Chloroplast markers can identify the female contribution by *P. deltoides* (e.g. Heinze 1998; Holderegger

et al. 2005) but cannot detect hybridisation events of *P. nigra* females with male hybrid clones (Ziegenhagen et al. 2008).

AFLP markers are considered appropriate for hybrid detection because of the large number of loci sampled across the whole genome (Arens et al. 1998), and this method can establish species and hybrid detection using a single primer pair, producing some diagnostic (species-specific) bands. Hybrids often have an intermediate position between the two parents (van Raamsdonk et al. 2000), but the position may become more complicated once F2 and backcrosses occur. Microsatellites are excellent markers for clone and cultivar identification in poplars (Storme et al. 2004), and some loci contain species-specific alleles (Fossati et al. 2003). However, species-specific bands can only trace backcrosses until they are lost, so a sufficiently large number of loci with species-specific alleles must be available, otherwise the classification error rate can be high (Epifanio and Phillipp 1997).

A more sophisticated way to distinguish between hybrid × hybrid and various *P. nigra* × hybrid backcrosses is to calculate posterior probabilities of ancestry using all available allele or band frequencies. In this way, all information contained in allele frequency differences between the parental species can be used. For this, computer programmes are now available, such as *NewHybrids* (Anderson and Thompson 2002) and *HINDEX* (Buerkle 2005), but their use in poplar hybrid analysis has just started (Lexer et al. 2007; Van Loo et al. 2008).

The aim of this study was to use species-specific AFLP bands and microsatellite DNA markers to identify progeny of natural hybridisation established along the Rhine river and to determine their most likely ancestry. We used samples of seedlings and young trees recruited spontaneously along the Rhine river and its tributaries in the Netherlands, where only a few native *P. nigra* populations remain (Arens et al. 1998) in combination with many planted *P. x canadensis* trees. In addition, we recorded leaf characters (colour and cilia on the leaf margins) to determine whether they can be of use for hybrid identification of young trees out in the field. For reference material, we included a series of *P. x canadensis* and *P. deltoides* trees, as possible introgression from *P. x canadensis* is probably of larger magnitude than that from *Populus x generosa* or from *P. nigra* var. Italica.

Materials and methods

Plant material

In June 1999, 44 young poplar trees were sampled along the river Rhine and its tributaries in the Netherlands (Fig. 1)
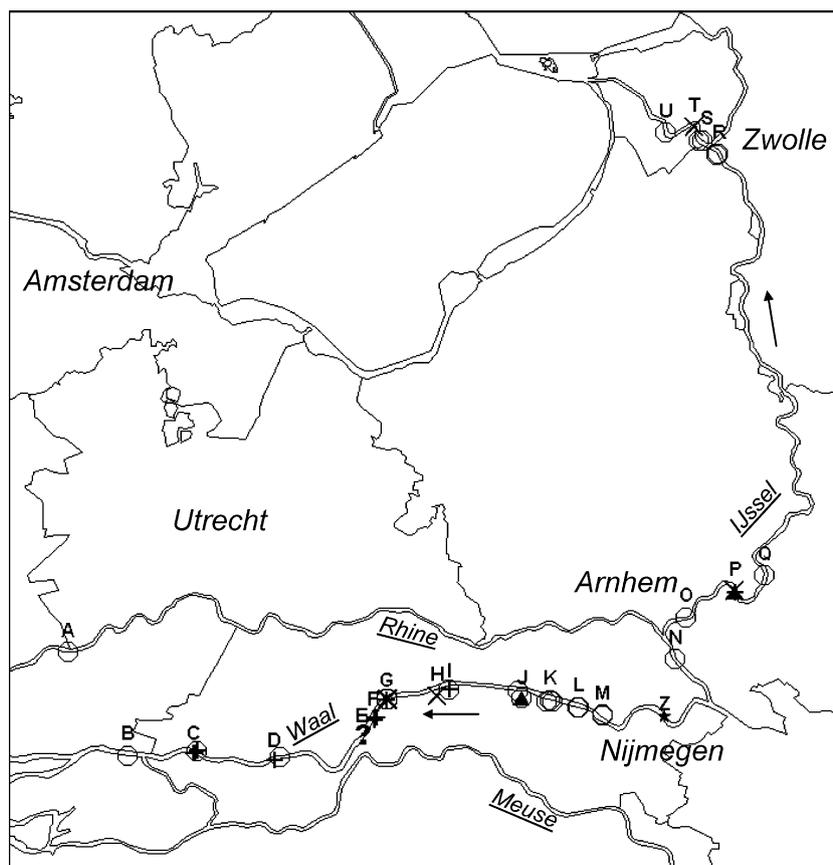
Fig. 1 Location of the sampled trees along the Rhine and its tributaries in The Netherlands. *Letters* refer to locations where groups of trees were sampled. The trees samples are listed in Table 2. The names of the rivers are *underlined*. Note that beyond the point where the IJssel side river branches off, the Rhine water level is regulated. The water level of the IJssel and the Waal (*B–Z*) is not regulated and the sides contain many sandy spots along the river or along pools that have an open connection to the main river, although the IJssel river sides between Q and R are mostly fortified with stones. *Z* is the location of the *P. nigra* population near Gendt (Smulders et al. 2008). The Meuse was not sampled. The direction of water flow is indicated by *arrows*

during a botanical survey of river floodplains in the frame of a biological monitoring programme by the Institute for Inland Water Management and Waste Water Treatment (Odé and Beringen 2000). The trees, denoted RB, were sampled at sites with apparent natural regeneration along riverbanks, banks of clay or sandpits or between the stones of embankments. They were sampled randomly, whenever encountered during the survey and likely to have originated from seedlings. Some trees (RB23 and RB27) could have grown from twigs or branches forming roots after being washed ashore. Two somewhat older trees (RB12 and RB15) may have been planted and could have been parents of nearby sampled younger trees.

Samples were taken by cutting the ends of branches of young trees. As the sampled trees were too young for features such as catkins, fruit morphology, bark structure and canopy shape to be assessed, we recorded the two leaf characters that can be assessed: the color of the leaves and the absence–presence of minute curved hairs (cilia) on the leaf margins (Beringen 1998). Young leaves of *P. nigra* are green. Young leaves of *P. deltoides* and most *P. x canadensis* are conspicuously red–brown or bronze coloured, although those of some *P. x canadensis* clones (e.g. *P. x canadensis* "Marilandica") are rather green (Houtzagers 1937). Hairs on the leaf margins are absent in *P. nigra*. Leaf margins of *P. deltoides* are permanently minutely pubescent, leaf margins of *P. x canadensis* only when they are young (Boom 1982; Stace 1997). The latter character is not always reliable. The hairs are tiny (a magnifying glass is needed) and some *P. x canadensis* form few short-living cilia. Trees with colored young leaves with cilia are traditionally classified as undoubtedly *P. x canadensis* or *P. deltoides*.

For reference samples of pure *P. nigra*, young leaves were sampled from *P. nigra* trees from the European Forest Genetic Resources Programme (EUFORGEN) Core Collection (Turok et al. 1998), from *P. nigra* plants from Dutch populations along the Rhine, sampled in 1997 (Arens et al. 1998) and 1999 (Smulders et al. 2002, 2008), from 30 *P. deltoides* Bartr. trees that have been used in hybrid poplar breeding programmes and that were available at Research Institute for Nature and Forest (INBO), Belgium and from *P. x canadensis* Moench. (syn: *Populus x euramericana*

Dode (Guinier)) clones (Table S1, supplementary material Online). These *P. x canadensis* clones represent commercial clones grown in The Netherlands. Their genotype is consistent with an F1 hybrid status at microsatellite locus PTR6 (Rajora and Rahman 2003), but genetically they are very likely of mixed origin: they may be F1, F2, BC1 or BC2. When selected from open pollinations, the parentage is not really known, or it was sometimes inferred from trees that were locally present. The dataset did not include *P. x generosa* clones. It did include one copy of *P. nigra* var. Italica, the Lombardy poplar, from which also introgression may occur (but not with alleles typical for another species). This is the subject of a separate study (Boerjan et al., manuscript in preparation).

Molecular analyses

The young leaves were stored at −80°C, lyophilised and ground with five to seven glass pearls in a Retch shaking mill directly prior to DNA extraction with the DNeasy kit (Qiagen, Helden, Germany).

AFLP analysis was performed essentially as described by Vos et al. (1995) with modifications (Arens et al. 1998; Smulders et al. 2002) using primer combination E32-M36 (5′-GACTGCGTACCAATTCAAC-3′ and 5′-GAT GAGTCCTGAGTAAACC-3′). This primer combination is sufficient to distinguish genotypes and identify clones (Storme et al. 2004; Smulders et al. 2008). Presence of a band was scored as '1', absence of a band by '0'. Reproducibility was confirmed by analysing reference samples in duplicate (Arens et al. 1998)

Microsatellite analysis was performed on the reference samples and on a part of the RB trees (listed in Tables 1 and 2) using seven loci: PMGC14, WPMS09, WPMS12, WPMS14, WPMS16, WPMS18, and WPMS20. PMGC14 (5′-TTCAGAATGTGCATGATGG-3′ and 5′-GTGAT GATCTCACCGTTTG-3′) was developed by the Poplar Molecular Genetics Co-operative. The other loci have been described in Van der Schoot et al. (2000) and Smulders et al. (2001). Samples were analysed using a primer labelled with a fluorescent group as described in Cervera et al. (2001) on an ABI3700. Raw data were analysed using Genescan (Applied Biosystems). The assignment of single-letter codes to the alleles follows Fossati et al. (2003) and Storme et al. (2004).

Data analysis

Genetic diversity parametres were calculated with Fstat 2.9.3.2 (Goudet 1995). Microsatellite allele frequencies

Table 1 Microsatellite genotypes. Alleles in italics at loci PMGC14, WPMS09 and WPMS18 are specific for *P. deltoides*

Tree	Locus						
	PMGC14	WPMS09	WPMS12	WPMS14	WPMS16	WPMS18	WPMS20
RB01	KM	..	JL	..	CE	GI	CG
RB04	EL	XZ	LL	DM	GH	CJ	CF
RB05	KM	..	LL	MM	CC	CJ	CF
RB07	..	Bb	LL	MU	CI	DG	DG
RB22	IM	QX	LL	MP	..	GI	CG
RB23	KK	..	HH	DO	DE	H*L*	HH
RB27	KL	TT	LL	LO	EI	GI	CH
RB28	K*O*	*Gg*	LM	OO	HH	G*L*	HH
RB32	KK	*Gg*	KK	MO	CE	J*L*	FH
RB34	JK	UZ	HK	PP	EE	GI	DH
RB35	EK	*Gg*	LL	KO	HJ	GI	CH
RB36	KK	UW	LL	MR	EG	GH	EE
RB37	IK	QW	LL	MO	EG	HI	EG
RB38	*PP*	*Gg*	BI	FI	HH	*LL*	GH
RB39	K*P*	*Gg*	LL	MO	HJ	I*L*	FH
RB40	K*P*	Q*g*	LL	MO	EJ	I*L*	FH
RB41	IK	..	LL	MQ	CC	GI	CF
RB42	IK	QX	LL	OP	EH	DG	EH
RB43	K*P*	QQ	LL	OO	HH	I*L*	FF
RB44	KM	XX	DK	MQ	CG	DG	CD
RB45	KK	*Gg*	LL	OO	EE	I*L*	FH
RB46	EP	..	LL	LO	HJ	I*L*	FH

Dots signify missing value

Table 2 Most likely genotype class for the RB trees sampled. Map locations are indicated in Fig. 1

Location on the map	Tree	Most likely genotype class	Probability in NewHybrids (based on either AFLP (A) or microsatellite (M) data)
A	RB01	*P. nigra*	0.99 (M)
B	RB44	*P. nigra*	0.99 (M)
C	RB40	BC to *P. nigra*	0.70 (M)
C	RB42	*P. nigra*	0.99 (M)
C	RB43	BC to *P. nigra*	0.84 (M)
C	RB45	F2	0.99 (M)
C	RB46	F2	0.57 (M)
D	RB39	F2	0.99 (M)
D	RB41	*P. nigra*	0.99 (M)
E	RB21	?	unclear (A)
F	RB28	F2	0.97 (M)
F	RB30	BC to *P. nigra*	0.83 (A)
F	RB31	identical to *P. x canadensis* 'Blanc de Poitou' (M)	
F	RB32	F2	0.99 (M)
G	RB23	BC to *P. nigra*	0.70 (M)
G	RB24	Hybrid	0.79 (A)[a]
G	RB25	Hybrid	0.66 (A)[a]
G	RB26	*P. nigra*	0.97 (M)
G	RB27	*P. nigra*	0.99 (M)
H	RB29	Hybrid	0.93 (A)[a]
I	RB20	BC to *P. Nigra*	0.80 (A)
I	RB22	*P. nigra*	1.00 (M)
J	RB06	*P. nigra*	0.95 (A)
J	RB09	*P. nigra*	0.95 (A)
J	RB11	*P. deltoides*	0.95 (A)
K	RB02	*P. nigra*	0.95 (A)
K	RB03	*P. nigra*	0.82 (M)
K	RB07	*P. nigra*	1.00 (M)
L	RB04	*P. nigra*	0.99 (M)
L	RB17	*P. nigra*	0.95 (A)
M	RB05	*P. nigra*	0.99 (M)
M	RB08	*P. nigra*	0.95 (A)
N	RB36	*P. nigra*	0.99 (M)
O	RB34	*P. nigra*	0.99 (M)
P	RB33	Hybrid	0.99 (A)[a]
P	RB35	F2	1.00 (M)
P	RB38	*P. deltoides*	1.00 (M)
Q	RB37	*P. nigra*	1.00 (M)
R	RB15	*P. nigra*	0.95 (A)
R	RB16	*P. nigra*	0.93 (A)
S	RB12	*P. nigra*	0.95 (A)
S	RB18	*P. nigra*	0.95 (A)
T	RB14	Hybrid	0.79 (A)[a]
U	RB19	*P. nigra*	0.95 (A)

[a] Analysed for dominant AFLP data only, as no microsatellite data were available. Result based on AFLP data was F1, but as for all plants for which also microsatellite data were available this was invariably changed into F2 or BC to *P. nigra*, the exact hybrid classification is in fact unknown (see text)

were calculated with Popgene version 1.31 (http://www.ualberta.ca/~fyeh/info.htm), which was also used to calculate the genetic distances between individual samples. The Unweighted Pair Group Method for Arithmetic Averages (UPGMA) dendrogramme of AFLP data was made in NTSYS (http://www.exetersoftware.com/) using a genetic distance matrix based on the Nei 1972 genetic distance.

Hybrid and backcross genotype posterior probabilities were estimated using NewHybrids 1.0 (Anderson and Thompson 2002) for microsatellite as well as AFLP data. NewHybrids calculates the posterior probability that sampled individuals fall into each of a set of hybrid categories (Parent 1, Parent 2, F1, F2, BC to Parent 1, BC to Parent 2, thus covering parents and two generations of offspring).

The probabilities add up to 1; therefore, if a given genotype has probability 0.8 for one category, the probability of the other five classes together is only 0.2. AFLP data can be used as input in Newhybrids 1.0, but the programme assumes all data to be homozygous (1 becomes 11, 0 becomes 00). This probably does not influence the probabilities for F1 hybrids, but it might affect those of various backcrosses.

As Anderson and Thompson (2002) indicate, the difference between NewHybrids and other programmes using Bayesian methods is that NewHybrids assumes that the sample consists of pure individuals and recent hybrids of two species and uses an inheritance model defined in terms of genotype frequencies for the various hybrid categories. This sets the number of a priori categories; it may improve the calculations of probabilities, and it also means that the groups produced are clearly labelled in terms of genetic contributions.

Results

AFLP analysis of *P. nigra* and hybrids

First, we determined the power of the selected AFLP primer combination to distinguish between *P. nigra*, *P. deltoides* and their hybrids. For this, 73 *P. nigra* individuals from the Dutch population (Arens et al. 1998) and 30 plants of *P. deltoides* that have been used in a commercial hybrid breeding programme at INBO were analysed together with ten commercial *P. x canadensis* hybrid varieties. Amongst the 51 AFLP bands scored, 48 (94%) were polymorphic in the dataset. Only one band was fully diagnostic for *P. nigra* (i.e. present in 100% of the *P. nigra* plants and completely absent in *P. deltoides*), but most bands did have frequency differences. Several bands were specific for *P. deltoides*.

The genetic differentiation between the two parental species *P. nigra* and *P. deltoides* was considerable (G_{st}= 0.371, Ht=0.198 and Hs=0.125). The differentiation between *P. deltoides* and *P. x canadensis* was G_{st}=0.311, which was very similar to the G_{st}=0.298 between *P. nigra* and *P. x canadensis*. This genetic differentiation is relatively large compared to that between the two parental species. This may be due to the dominant nature of AFLP markers.

To determine in which group the trees sampled along the Rhine (denoted RB) would fit best, a UPGMA clustering was performed on the basis of the genetic distance amongst all pairs of plants (Fig. 2). In the clustering, RB31 turned out to be identical to the commercial *P. x canadensis* clone Blanc du Poitou. As it was found at a site along the shore with many poplar and willow seedlings, where obviously no planting had taken place, this tree is most probably the

result of vegetative distribution through branches that manage to root and sprout.

In the cluster analysis of AFLP data, the two parental species formed separate groups, with the *P. x canadensis* group at an intermediate branch. Ten of the sampled RB trees clustered together with the *P. x canadensis* trees; the remaining 34 clustered together with the *P. nigra* trees from the Gendt population. This *P. nigra* group, however, seemed to consist of three clusters grouped together loosely, with different numbers of RB trees: the largest cluster (labelled 'N') included 48 *P. nigra* plus four RB trees, a second cluster (labelled 'M') consisted of 20 *P. nigra* plus 19 RB trees and a third cluster (denoted 'R') contained three *P. nigra* trees plus 11 RB trees. All 11 RB trees in the latter cluster showed the one discriminating AFLP marker for *P. nigra*, as well as two AFLP bands that are very frequent in *P. nigra* and very rare in *P. deltoides*.

Application of diagnostic and informative microsatellite alleles

Allele frequencies in *P. nigra* and *P. deltoides* were determined for seven microsatellite loci on a subset of the samples (Table S2, Supplementary material Online). The average number of alleles per locus was lower in *P. deltoides* than in *P. nigra*. This was not unexpected because six of the seven markers were developed in *P. nigra*, and often polymorphic microsatellite markers are less polymorphic in related species (Smulders et al. 1997). Consistent with less repeat expansion, the average allele size in *P. deltoides* was smaller.

Two loci were monomorphic in *P. deltoides*: WPMS09 and WPMS18. For both loci, the *P. deltoides* allele ('g' at WPMS09 (234 bp), 'L' at WPMS18 (220 bp)) was not found in *P. nigra*, so these alleles are diagnostic markers for *P. deltoides*. This corroborates the results of Fossati et al. (2003). For locus PMGC14, the most frequent *P. deltoides* allele "O" (270 bp) is very rare in *P. nigra*; the second to one most frequent *P. deltoides* allele "P" (268 bp) is unique to *P. deltoides*. We classify these alleles as informative, as they contain information on the likelihood of the sample belonging to a certain species or hybrid. Alleles specific for *P. deltoides* were also present at loci WPMS12, WPMS16 and WPMS20, but the frequencies of these informative alleles were too low to be useful. Locus WPMS14 has many common alleles in both species.

Using the diagnostic and informative alleles, we made inferences on the genetic background of the trees in the study. The alleles found in four out of six commercial *P. x canadensis* clones (Table S2, Supplementary Material Online, Pxc column) were consistent with an F1 hybrid because the alleles from both parental species were present. The other two had a different genotype. *P. x canadensis*
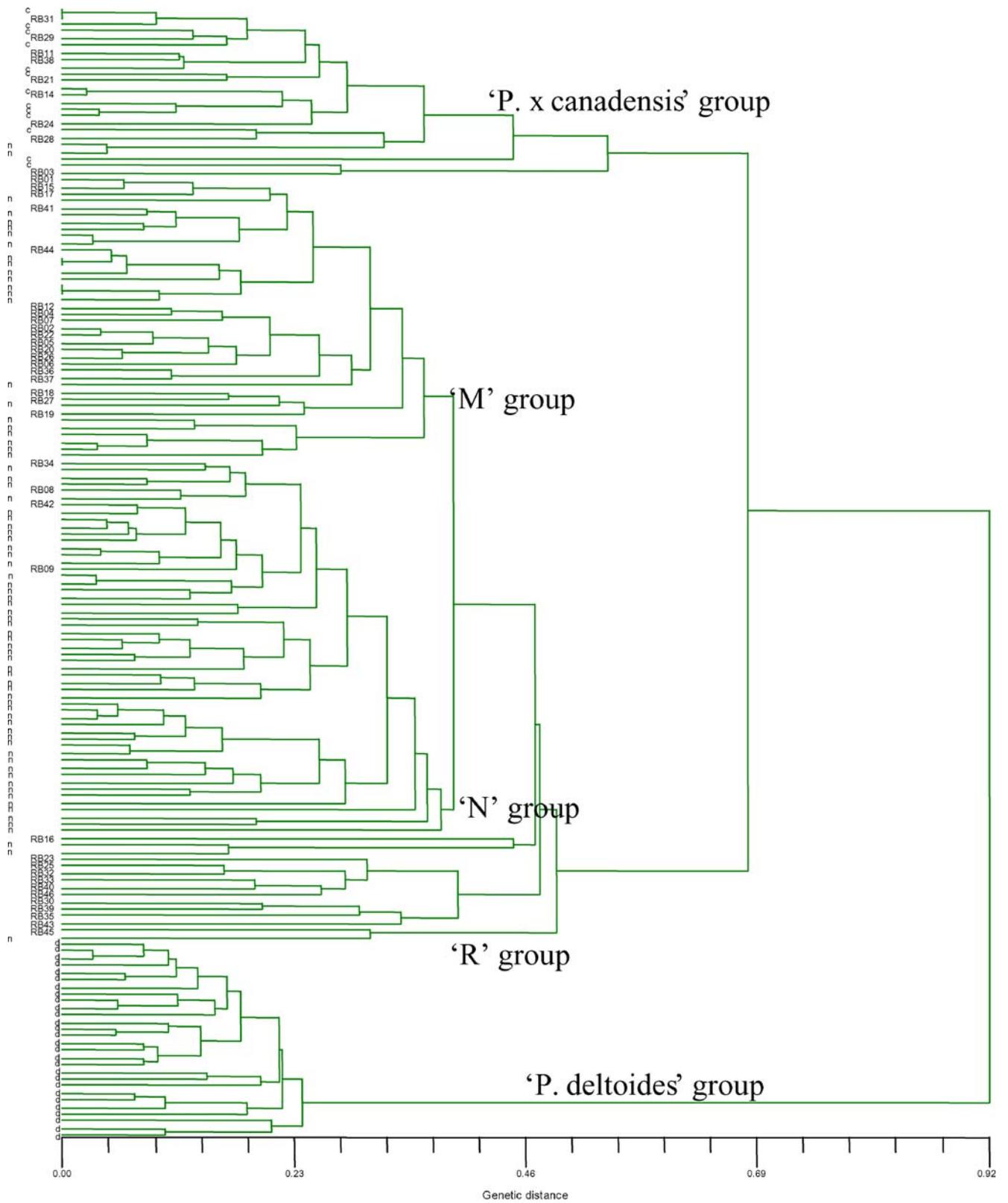
Fig. 2 UPGMA dendrogramme based on AFLP data. Next to the RB trees sampled for this study, the analysis included *P. nigra* (*n*) trees are from the Gendt population (Z in Fig. 1), *P. deltoides* (*d*) trees are from the reference collection of INBO, and a number of *P. x canadensis* cultivars (*c*)

cultivar I-214, for instance, was homozygous for the *P. deltoides* allele 'g' at WPMS09.

The genotype of 24 of the RB trees was consistent with *P. nigra* (Table 2). Some others (such as RB40 and RB46) have one *P. deltoides*-specific allele at loci WPMS09 and WPMS18, which is consistent with a hybrid category (F1, F2 or various backcrosses). RB28, RB35 and RB38 contained *P.-deltoides*-specific alleles at loci WPMS09 or WPMS18 in homozygous state (RB38 even at both loci). They may be F2 hybrids or backcrosses to *P. deltoides*.

Overall, the trees originally placed in the *P. nigra* 'N' cluster based on AFLP data were confirmed to be *P. nigra*, and the trees in the *P. x canadensis* cluster indeed contained *P. deltoides* alleles. However, many RB trees from the other two *P. nigra* clusters contained some *P. deltoides* alleles as well. In these cases, neither AFLP clustering nor inspection of the microsatellite genotype at these seven loci could differentiate between F2 and various backcrosses.

Interestingly, the hybrid trees were not distributed randomly across the sampled sites but located mainly in locations C, F, G and P (Table 2). Possibly more hybrid clones have been planted near those locations, which have functioned as parents.

Hybrid analysis using Bayesian posterior probabilities with NewHybrids

The above analyses do not use all available genotype information. Clustering of AFLP data forces a decision on nodes in the tree based on most common patterns and may override intermediate or alternative positions. In addition, the existence of groups in a dendrogramme does not immediately indicate what common ancestry the members may have. When analysing microsatellite loci, the ancestry is based on the presence of a few diagnostic or informative alleles at only some of the loci, while information from allele frequency differences on other loci is ignored. For instance, the presence of diagnostic alleles at WPMS09 and WPMS18 in heterozygous state is consistent with an F1 but also with many other classes of hybrids (F2 and both types of backcrosses). Anderson and Thompson's (2002) programme *NewHybrids* enables calculating the posterior probability that a sample belongs to each of the parental and various hybrid classes (F1s, F2s and backcrosses with each parental species), based upon a genetic model, using all information in frequency differences between alleles, and incorporating the uncertainty due to sampling error in the allele frequencies.

NewHybrids analysis of the natural population near Gendt produced the highest probabilities (>>0.9) to be *P. nigra* for all trees in the natural population near Gendt

except two trees. These two trees were already under suspicion (Arens et al. 1998). The inferred probabilities for the commercial *P. x canadensis* varieties were interesting, as two of them were classified as F2, not F1, with high probability ($P=0.65$ for 'Gelrica', 0.91 for I-214). 'Heidemij' may be an F2 ($P=0.36$) or a BC to *P. nigra* ($P=0.49$). These three clones have originally been selected from natural hybridisation events (Houtzagers 1937; http://www.populus.it/ipcipccloni.php). 'Blanc de Poitou' is most likely ($P=0.71$) a BC to *P. nigra*. This indicates that they represent the progeny of more advanced crosses rather than being F1 hybrids. When we ran *NewHybrids* with the hybrid cultivars and only ten samples from each of the other groups (not shown), the exact probabilities changed somewhat but always concerned F2 and/or BC to *P. nigra* classes, indicating the stability of the assignments. All EUFORGEN Core Collection plants were clearly *P. nigra* (Fig. 3), except for KAE-N.90.013 from Turkey, which was BC to *P. nigra* ($P=0.54$) or F2 ($P=0.32$).

When we included the RB trees sampled along the Rhine together with the *P. nigra* and *P. deltoides* trees, *NewHybrids* almost always assigned the RB trees clearly to one genotype class, often with probability >0.8. Using AFLP data, 24 of the 44 RB samples (which is only slightly more than 50%) were pure *P. nigra*, 13 were F1 hybrids, five were backcrosses to *P. nigra*, while two classified best in the category pure *P. deltoides*. Nearly all non-*P. nigra* RB trees are located in the '*P. x canadensis*' and 'R' groups in the AFLP-based dendrogramme, while both 'M' and 'N' groups are nearly completely pure *P. nigra* (Fig. 2).

For those RB plants for which also microsatellite data were available (listed in Table 2), the *NewHybrids* analysis of seven loci produced high posterior probabilities to classes *P. nigra*, F2 and BC to *P. nigra* (an example of the probability output is given in Fig. 3; all results included in Table 2). The high probability using microsatellite data was based on the allele frequencies at all seven loci because the two or three diagnostic loci were heterozygous in nearly all of these plants, on which basis one cannot distinguish between F1, F2 or BCs. In addition, the plants that were homozygous at locus WPMS09 for the *P. deltoides* allele 'g' were given high probability for being F2 not BC to *P. deltoides*. This is consistent with the presence of many more *P. x canadensis* clones compared to pure *P. deltoides* trees.

Conspicuously absent was the class F1 because the plants that were labelled F1 based on the AFLP data were given high probabilities for F2 or BC to *P. nigra* classes based on the microsatellite data. This is not unexpected, as the *P. x canadensis* trees themselves are already more advanced than F1. In this case, the AFLP result may have been affected by the fact that the *NewHybrids* programme
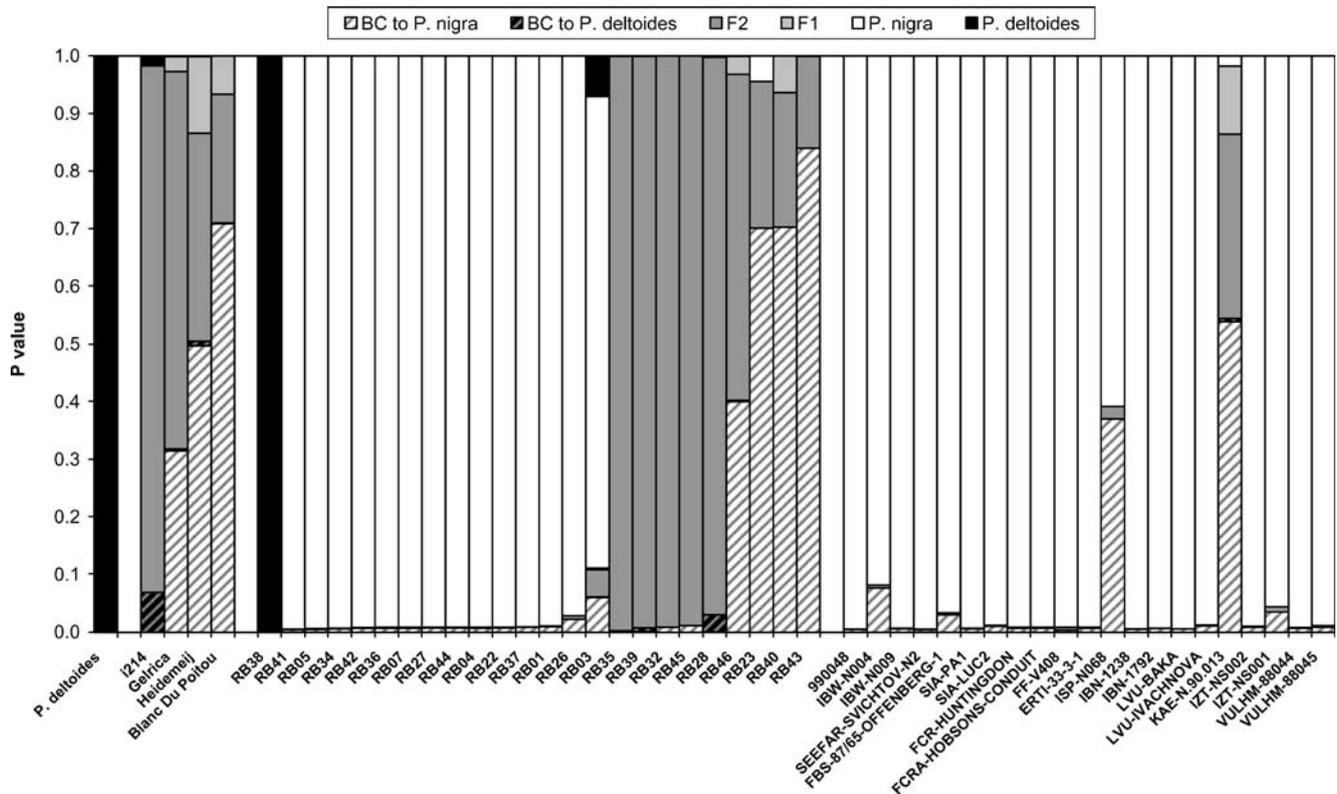
Fig. 3 Posterior probabilities of genotype frequency classes for microsatellite data of a subset of the samples

assumes that the (dominant) bands are homozygous. As could be expected, the class BC to *P. deltoides* was absent with both marker systems.

Correlation with morphology

For 21 RB trees, we could compare detailed morphological data of very young leaves with the most likely genotype as classified by NewHybrids (Table 3). Six out of eight trees classified by NewHybrids as F1, F2 or BC to *P. nigra* did contain some *P. x canadensis* morphological characteristics (leaf colour or pubescense of leaf margins). However, of 12 trees that were classified as *P. nigra* by NewHybrids only six had been identified as such based on morphology.

Discussion

Hybrid detection

AFLP and microsatellite markers were both able to distinguish *P. deltoides* from *P. nigra* and to detect the presence of hybrid trees. Of 48 AFLP polymorphic bands produced with one primer pair, only one was diagnostic between *P. nigra* and *P. deltoides*, but most bands frequencies were different, and as such they were informative to various degrees. Similarly, next to three diagnostic microsatellite loci (PMGC14, WPMS09 and WPMS18, as in Fossati et al. 2003), we could use the allele frequency differences at the other loci in the Bayesian probability

Table 3 Comparison of morphology and NewHybrids inferred genotype class

Groups based on morphological characteristics		NewHybrids inferred genotype				
Colour of young leaf hairs on leaf margin		*P. nigra*	F2 or BC	F1	*P. deltoides*	Total
Green	absent	6	2	0	0	8
(Red)brown	absent	5	3	1	0	9
Green	pubescent	0	1	0	0	1
(Red)brown	pubescent	1	1	0	1	3
Total		12	7	1	1	21

calculations using *NewHybrids*. For the *P. x canadensis* hybrid cultivars, this clearly showed that they were a mixed group of variable parentage, with various probabilities for F2, BC to *P. nigra* and BC to *P. deltoides* classes. Heinze (1998) observed that all *P. x canadensis* clones sampled in Austria had isozyme patterns that were consistent with F1 hybrids because they were heterozygous at the four to five loci tested. However, using that information, F2 and BC's are possible as well. It is known that several cultivated poplar clones are selected from half-sib open pollinated progeny resulting from natural hybridisation. The parental origin for these clones is not known. This is for example the case for the clones "Gelrica", "Heidemij" and "I-214". Our results show that hybrid cultivars from these programmes could represent, in genetic terms, second backcrosses (BC2) or crosses of F2 trees. This type of analysis can help to identify the genetic origin of cultivated clones.

Hybrids in natural populations

Amongst the young trees sampled, we found *P. nigra* trees, but nearly half of them classified as F2 between *P. nigra* and *P. deltoides* or BC to *P. nigra*. The latter trees could be the result of outcrossing of *P. nigra* as a mother with *P. x canadensis* pollen donors, or vice versa. Siring by *P. x canadensis* pollen is a real possibility for solitary *P. nigra* trees but not for those in large populations, as Vanden Broeck et al. (2003) found that the percentage of outcrossing of *P. nigra* with non-*P. nigra* trees was almost zero as long as *P. nigra* males were nearby. Therefore, it is also possible that young hybrid poplars are offspring of female *P. x canadensis* trees, with *P. x canadensis* or *P. nigra* cv Italica as a father. These two groups are by far the most common trees in the sampled area.

P. x canadensis trees often do not produce seeds, but if they do these seeds can be germinated easily (R. Beringen, personal observation). Consistent with this notion is the observation that many poplar seedlings of hybrid morphology occur along the Meuse in regions in which there are no *P. nigra* populations, but many *P. x canadensis* trees do occur in these region (Vanden Broeck et al. 2004; R. Beringen, personal observation).

Because we focused our sampling on plants that had established themselves spontaneously, most probably from seed, the identification of many F2 hybrids or backcrosses to *P. nigra* indicates that it is possible for seeds with hybrid origin to establish under more or less natural conditions. In addition, we detected one tree that was identical to a commercial hybrid clone, Blanc du Poitou. This is in itself not surprising because cuttings of poplars readily root and establish. Alternatively, the tree may have been planted. Two other trees classified best as 'pure' *P. deltoides*. *P. deltoides* cultivars are rare in The Netherlands compared to hybrid

clones, but 'deltoides' alleles can also be derived from *P. x generosa* hybrids, which are *Populus trichocarpa* × *P. deltoides* (=TxD). The female clones (commercial cultivars) of *P. x generosa* 'Beaupre', 'Boelare','Donk' and 'Raspalje' have been frequently planted in the Netherlands.

Former studies already indicated that cultivated poplars can produce viable seeds and seedlings in the field. Vanden Broeck et al. (2004) studied the genetic origin of young poplar seedlings that voluntary colonised the gravel banks of the river Meuse on the Dutch–Belgium border. Although they observed that cultivated poplars were reproductive along the Meuse river and produced seedlings that could survive the water dynamics over several years, they did not find pure black poplar seedlings along a stretch of the Meuse where only relict individuals of black poplar were present. In this study, we showed that seedlings of black poplar and hybrid poplars can be found on the same river banks and therefore may compete for the same habitat.

Fossati et al. (2003), using the same diagnostic microsatellite alleles, did identify some old trees in their Italian population as hybrids, but they concluded it was not introgression but human-mediated planting of cuttings of hybrid poplars. This was partly based on the fact that the same genotypes were present as more than one tree and that no trace of any of the *P. deltoides* alleles could be found in younger trees. In the UK, young natural populations of hybrid poplars were found on damp waste grounds. At one site, the parentage was inferred to be most likely *P. x canadensis* "Marilandica" × *P. x canadensis* "Serotina", as these were the only mature trees in the surrounding area (Stace 1997). Similarly, at another site, the parentage was determined to be probably *Populus candicans* Ait. (Balsam poplar) × *P. nigra* (Gilbert 1993). *P. x canadensis* "Marilandica" is also believed to be the seed parent of *P. x canadensis* "Gelrica", a cultivar originated in the Netherlands around c. 1865 (Houtzagers 1937).

Use of morphological characters

Because offspring of crosses between *P. nigra* and *P. x canadensis* trees can be, in genetic terms, quite diverse types of backcross or crosses between hybrid plants beyond F1, one may expect that the morphological characteristics of such plants could be quite variable, as many morphological characteristics in plants are thought to be unlinked and based on few genes, as was observed in, e.g. wild apple hybrids (Larsen et al. 2006). The observations made on the RB trees (Table 3) are consistent with this hypothesis. In general, there was a correlation between morphological traits and the genetic status, as more than 80% of the genetically not pure *P. nigra* plants (F1, F2 or BC) were morphologically also assigned as being non-*P. nigra*. However, also about 50% of the trees that were genetically

identified as *P. nigra* with high probability had been under suspicion on account of morphology (e.g. RB3, which had red–brown young leaves). These trees might be F3 or further backcrosses. Young trees with a morphology such as RB3 are observed frequently along the Rhine river. The two-way misclassification may indicate that the morphological criteria are not very useful, as they do correctly identify *P. x canadensis* and *P. deltoides* based on cilia (present) and young leaf colour (red–brown), but they cannot identify *P. nigra* trees as their morphology is variable. It may, however, also indicate that many intermediate genotypes exist alongside'pure' *P. nigra* trees. Progressive backcrosses of hybrids with *P. nigra* would produce trees with predominantly *P. nigra* traits but with variable presence of some hybrid-like traits.

Consequences for nature management

The results from this paper corroborate what botanists already suspected: that poplar trees found in natural and semi-natural habitats are mostly pure *P. nigra*, but some are offspring of hybrid clones (here category F2) or offspring of *P. nigra* sired with *P. x canadensis* pollen (category BC to *P. nigra*). Currently, we do not know the exact parentage of such hybrids. When mother and seed genotypes are determined with microsatellite makers, the partial genotype of the father can be inferred. Tabbener and Cottrell (2003) performed such an analysis on trees of various *Populus* species in the Royal Botanical Garden in Edinburgh and Vanden Broeck et al. (2004) on offspring of a single *P. nigra* tree. In general, these studies showed that, given sufficient pollen of the own species, the black poplar females do not produce hybrid seeds. Only when the *P. nigra* mother plant is solitary and surrounded by many plants of hybrid origin or of other species does hybridisation takes place. Such situations do occur. The offspring of such crosses cannot be identified by cpDNA, as they contain *P. nigra* chloroplasts.

In view of the occurrence of hybridisation and establishment as observed in our study, in which hybrid trees are nearly as numerous as *P. nigra* trees, an efficient conservation method may be to enhance the number of pure *P. nigra* trees and populations by planting (Vanden Broeck et al. 2005). The number of *P. nigra* trees does not automatically increase in nature development projects, as the results of this study indicate that hybrid cultivated poplars may compete for, and subsequently occupy, the same ecological niche as native poplars.

An alternative view is to acknowledge that the establishment of hybrid and mixed origin offspring on natural locations that used to be exclusively for *P. nigra* is something that started with human activity (planting of hybrid poplars by man) but that now occurs spontaneously. The establishment of hybrid poplars may initially be non-adaptive or even maladaptive but

may also lead to adaptive evolution through the production of hybrid genotypes that are more fit than their parents (Arnold 1997). This may also be important in the light of a changing climate. Progressive hybridisation, germination and selection will lead to a new type of poplar. In Germany, hybrid poplar is already being called a 'anökophyt'—a species that develops under human influence (Keil and Loos 2004). A second possibility is that the hybrids actually have an increased fitness, whose heterosis may be maintained by clonal propagation. Increased clonal propagation was found in *Populus x canescens*, the natural hybrid of *Populus alba* and *Populus tremula*, in the Austrian Danube valley (Van Loo et al. 2008). Our dataset concerned seedlings, so we do not yet know how extensive they will propagate clonally. The large natural population of mature *P. nigra* trees near Gendt did show extensive clonal propagation, but these trees could clearly be classified as pure *P. nigra*.

In both scenarios, further research is needed to determine whether poplar hybrid seedlings have a competitive advantage over pure black poplar seedlings or vice versa, especially if river flow dynamics change as a result of increased weather variability due to climate change. Factors such as fertility, seed dispersal distance, seed and seedling establishment and vegetative reproduction have a strong effect on gene flow and merit further considerations. With regard to the occurrence of advanced backcrosses of *P. x canadensis* (and *P. x generosa*) clones to *P. nigra* an in-depth study is warranted. Such a study would have to start with a detailed analysis of the commercial clones planted across northwestern Europe during the last century, using a large number of markers spread evenly across the genome that have frequency differences between the parental species.

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