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Application of genetic markers to the discrimination of European Black Poplar (*Populus nigra*) from American Black Poplar (*P. deltoides*) and Hybrid Poplars (*P.* x *canadensis*) in Switzerland

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Abstract European Black Poplar (*Populus nigra*) is considered a rare and endangered tree species because of severe reduction of its natural riverine habitat and potential hybridisation with the related non-indigenous taxa *P. deltoides* and *P.* x *canadensis*. As it is difficult to distinguish these taxa solely based on their morphology, we applied a PCR-based assay with an easy-to-use and robust molecular marker set (cpDNA *trn*L-*trn*F/*Rsa*I RFLP, nDNA win3 and nDNA POPX/*Msp*I RFLP) in order to identify pure *P. nigra*. Different plant tissues could be used for fast and standardised DNA extraction. The application of the three marker types was tested on a number of different *Populus* taxa, and they were also used for the verification of pure *P. nigra* in a sample of 304 putative *P. nigra* individuals from Switzerland. Cross-checking of the DNA data with those using a traditional allozyme approach resulted in complete agreement. The availability of molecular identification methods is an important prerequisite for the conservation of European Black Poplar, because pure, non-introgressed plant material can then be used in restoration projects of European floodplains.

Keywords Conservation · Molecular identification · *Populus nigra* · *Populus deltoides* · *Populus* x *canadensis* · Restoration

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Introduction

The European Black Poplar, *Populus nigra* L., is considered a rare and endangered tree species mainly because of two reasons: loss of natural habitats (Rotach 2001) and potential hybridisation along with genetic swamping by non-indigenous related taxa (Heinze and De Vries 1998; Heinze and Lefèvre 2001; Lefèvre et al. 2001; Heinze and Lickl 2002). *Populus nigra* is a typical species of riparian systems, where it dominates annually flooded forests along the banks of larger rivers and their tributaries. During the last two centuries, most European rivers have been braided and their hydroperiods drastically altered. This intensive river management did not only eradicate most natural habitats of *P. nigra* in European floodplains, but strongly disturbed the species' regeneration niche as well (Barsoum 2001). As a result of the strongly altered flooding dynamics of most European rivers, sexual regeneration of *P. nigra* in modern landscapes is low (Barsoum 2001). Habitat change and/or lack of sexual reproduction have caused a severe decline of *P. nigra* both in numbers of individuals as well as entire populations (Van Dam 2002).

Genetic swamping and introgression by related non-indigenous *Populus* taxa is supposed to threaten the species' genetic and evolutionary integrity (Vanden Broeck et al. 2002). In many European countries, American Black Poplar, *P. deltoides* Marsh., and Hybrid Poplar, *P.* x *canadensis* Moench (synonym *P.* x *euramericana* (Dode) Guinier; *P. deltoides* x *P. nigra*), have been widely planted for economic reasons (Heinze and Lefèvre 2001). Quite often, the remaining natural stands of *P. nigra* are small and close to such poplar plantations (Fossati et al. 2003). This is likely to increase gene exchange between *P. nigra* and the other two taxa either leading to first generation F1 or later-generation F2 and BC1 (backcross) hybrids. Thus, hybridisation and potential genetic swamping is seen as an additional threat to the in situ conservation of *P. nigra*. However, the amount of introgression of *P. deltoides* genes into the *P. nigra* gene pool is still debated (Benetka et al. 1999; Vanden Broeck et al. 2002). Recent reports from

different European countries suggest that the probability of genetic swamping of native *P. nigra* is lower than previously thought (Vanden Broeck et al. 2004), and Heinze and Lickl (2002) state that this phenomenon is "rare but steady."

Floodplain restoration projects have now been started all over Europe (Lefèvre et al. 2002). In Switzerland, corresponding long-term and large-scale conservation measures have been or will be undertaken along the river Rhone in the central-alpine Valais and the rivers Aare or Thur in the Swiss Plateau (Dietiker and Jansen 2003; Rohde et al. 2004). These projects aim at restoring natural riparian woodlands including *P. nigra* forests. Hence, there is a demand for regional plant material of known taxonomic status, i.e. pure *P. nigra* seeds or plantlings. Unfortunately, it is not easy to identify pure *P. nigra* individuals from *P. deltoides* or *P.* x *canadensis* using morphological traits only (Van Slycken 1995; Kajba and Romanić 2002). It is thus difficult, first, to determine the actual abundance, state and distribution of pure *P. nigra* in Switzerland and, second, to identify naturally occurring *P. nigra* trees suitable as genetic resources for the above-mentioned restoration projects. Consequently, various molecular markers have been used to identify pure *P. nigra* individuals (Vornam et al. 1994; Janssen 1997; Heinze 1998, 2001; Rajora and Rahman 2003; Storme et al. 2004).

A practicable genetic marker approach should allow for different plant tissues to be used for DNA extraction (sampling in different seasons and from different life stages), use a molecular technique with high throughput and produce results that are easy to interpret (i.e. low number of different banding patterns). Because of the latter point, highly polymorphic markers such as nuclear microsatellites (Van der Schoot et al. 2000) or amplified fragment length polymorphisms, AFLPs (Arens et al. 1998; Winfield et al. 1998), do not seem to be the markers of choice for the purpose of species identification in *P. nigra*, although they are superior to less variable molecular markers in assessing local gene exchange or dispersal patterns (Rajora and Rahman 2001; Imbert and Lefèvre 2003; Tabbener and Cotrell 2003).

Here, we assess the applicability of a set of molecular markers for the discrimination of pure European Black Poplar from American Black Poplar and Hybrid Poplars in Switzerland using three PCR assays on both chloroplast DNA (cpDNA) and nuclear DNA (nDNA). We first show that different plant tissues can be used for fast and standardised DNA extraction, then describe the chosen markers' banding patterns and test their easy applicability on a set of different *Populus* taxa. Finally, we use this marker set for the identification of pure *P. nigra* in a large Swiss sample of morphologically determined, putative *P. nigra* individuals.

Materials and methods

DNA extraction

As a standardised, fast and high throughput DNA extraction procedure usable for different plant tissues of *P. nigra*, we modified the DNeasy 96 plant extraction kit (protocol for fresh plant leaves using a mixer mill; QIAGEN 2002). Fresh plant tissue samples (50 mg) were first lyophilised for 3 days and then crushed in a shaker mill (RETSCH MM 300). DNA extraction followed the instructions of the supplier with the following modifications. Start by adding to the crushed plant material a mixture of 600 µl AP1 buffer, 1 µl RNase and 1 µl Reagent DX preheated to 65°C, vigorously shake for 15 s and centrifuge at 5600 *g* for 15 min; transfer 400 µl of the supernatant to the collection tubes provided and add 130 µl of AP2 buffer, vigorously shake for 15 s, shortly spin down to collect all liquid, incubate for 15 min at −20°C and centrifuge for 10 min at 56,000 *g*; then proceed to step 8 according to QIAGEN (2002). DNA quality and quantity was checked against λ-standards on 1.5% agarose gels stained with ethidium bromide. Extractions were conducted using pealed winter buds, early and late summer leaves and wood cores. In the latter case, care was taken to include the active cambium region of the wood core.

trnL-trnF/*RsaI* RFLP cpDNA marker

The intergenic region *trn*L-*trn*F of cpDNA (Vornam et al. 1994; Demesure et al. 1995) was PCR-amplified and digested with *Rsa*I. The PCR reaction volume of 25 µl contained approximately 10 ng of genomic DNA, 2.5 U Taq DNA polymerase (SIGMA), 1× PCR buffer, 1.7 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of forward and reverse primer (Demesure et al. 1995) and ddH_2O. Amplification was carried out on an MJ RESEARCH PTC-100 PCR machine with initial denaturing at 95°C for 3 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min with a final extension of 72°C for 10 min. Restrictions of 10 µl reaction volume were carried out overnight with 4 µl of amplification product, 10 U of *Rsa*I, 1× restriction buffer and ddH_2O at 37°C. Fragment banding patterns were detected on 2% agarose gels stained with ethidium bromide, and fragment sizes determined against a 100 bp ladder (FERMENTAS). Interpretation of banding patterns followed Vornam et al. (1994).

win3 nDNA marker

The win3 primers and assay for distinguishing *P. deltoides* and *P. nigra* were first described by Bradshaw et al. (1994)

and Heinze (1997). The win3 region of nDNA, activated during wound healing of plants, was PCR-amplified (for primer, see Heinze 1998) with an initial denaturing at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min with a final extension of 72°C for 4 min. PCR mixture, electrophoresis and visualisation were carried out as presented above. Interpretation of banding patterns followed Heinze (1998).

POPX/M*sp*I RFLP nDNA marker

A peroxidase region, POPX, of nDNA was amplified (for primers, see Heinze 1998) and restricted with *Msp*I. PCR amplification used an initial denaturing at 94°C for 4 min, followed by 30 cycles of 94°C for 45 s, 62°C for 45 s and 72°C for 2 min with a final extension of 72°C for 10 min. PCR mixture, restriction mixture and procedure, electrophoresis and visualisation were carried out as presented above, interpretation of banding patterns followed Heinze (1997, 1998).

Cross-checking with allozymes

Allozymes have traditionally been used to identify pure *P. nigra* individuals from cultivated *Populus* taxa (Rajora 1989; Benetka et al. 1999; Storme et al. 2004). In order to cross-check the results obtained with the above three PCR methods, we conducted starch gel electrophoresis of four allozymes, namely, LAP-A (E.C. 3.4.11.1), AAT-B (E.C. 2.6.1.1), PGM-A (E.C. 5.4.2.2) and PGI-B (E.C. 5.3.1.9; Wendel and Weeden 1989) using 12% starch gels. The methods used and subsequent interpretation of banding patterns followed Janssen (1997). We applied allozyme electrophoresis to a sub-sample of the *Populus* individuals studied (see below).

Blind test samples

To "blindly" check whether the combination of the above three PCR assays and the allozyme method enabled the secure identification of *P. nigra* and its delimitation from other *Populus* species or hybrids, an external person sampled winter buds from 79 trees representing diverse *Populus* taxa cultivated in Switzerland. These samples included, amongst others, known *P. deltoides*, *P. nigra*, *P. nigra* var. *Italica* (Cagelli and Lefèvre 1995) and *P.* x *canadensis* individuals. The identity of these samples was unknown to us. We subjected them to genetic analysis and subsequently compared our results with the known taxonomic status of the samples. The aim was to consistently delimit pure *P. nigra* from other *Populus* taxa.

Putative *P. nigra* samples

The three PCR assays were also applied to 304 naturally occurring adult individuals from Switzerland (Cantons of Argovia, Berne, Thurgau and Zurich). Diverse tissues were sampled from these putative *P. nigra* trees identified as *P. nigra* by local foresters on the basis of their morphology during the vegetation period (EUFORGEN s.d.). Our assumption was that most of these individuals were pure *P. nigra*, but that confusion with *P. deltoides* and *P.* x *canadensis* could well have occurred. Therefore, we determined the corresponding mismatch rate between morphological and genetic identification of putative *P. nigra*. For 130 of these samples, for which bud tissue was available, we also conducted allozyme electrophoresis.

Results

DNA extraction

DNA extraction using the QIAGEN 96 plant extraction kit resulted in good quality DNA. Less than 5% of the samples did not properly amplify in PCR and needed additional cleaning (e.g. PROMEGA DNA clean up columns). In samples that supposedly contained high levels of polysaccharides, the addition of 200 µl dichloromethane during the first step of the DNA extraction procedure increased the success rate of amplifications. DNA yield, however, was tissue dependent. Although extractions from buds and both early and late summer leaves typically gave yields of more than 50 ng/µl, those from wood cores typically contained only about 5 ng/µl, which was still sufficient to perform the three PCR assays below. Once collected, the plant material could be stored at room temperature for up to 2 weeks prior to lyophilisation without harming the quality of extracted DNA.

trnL-trnF/*RsaI* RFLP of cpDNA

Here (and below), we only refer to the banding patterns observed in *P. deltoides*, *P. nigra* and *P.* x *canadensis*. The restriction of the *trn*L-*trn*F region of cpDNA with *Rsa*I resulted in two distinct patterns (Vornam et al. 1994). The PCR product (1070 bp in length) of *P. nigra* was not cut (Fig. 1a, pattern A), while that of *P. deltoides* was digested into two bands of about 700 and 370 bp (site mutation; Fig. 1a, pattern B). Thus, the lack of an *Rsa*I restriction site in the *trn*L-*trn*F fragment of the pure *P. nigra* cpDNA robustly delimited this species from *P. deltoides* and from *P.* x *canadensis* stemming from a *P. deltoides* mother plant, since cpDNA is maternally inherited in poplars (Rajora and Dancik 1992).

win3 nDNA

PCR amplification of win3 nDNA resulted in four distinct banding patterns (Fig. 1b). *Populus nigra* either exhibited one band of about 170 bp or two bands of 170 and 200 bp (Heinze 1997, 1998; Fig. 1b, patterns cc and bc), while *P. deltoides* was characterised by one band of about 260 bp

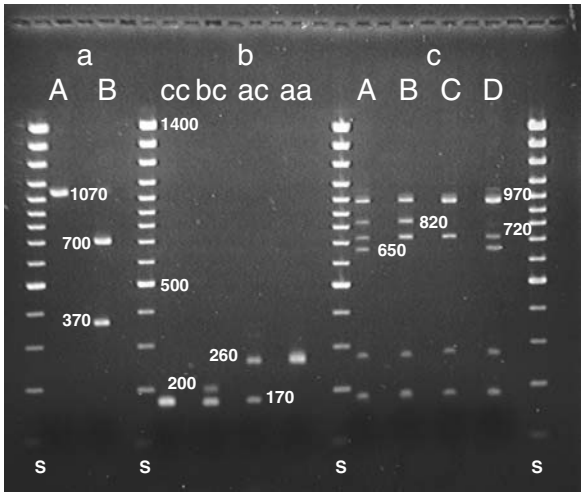

Fig. 1 Banding patterns observed in three PCR assays used for the identification of pure *Populus nigra*. **a** *trn*L-*trn*F/*Rsa*I RFLP of cpDNA; pattern A: *P. nigra* (undigested); pattern B: *P. deltoides* and (typically) *P.* x *canadensis*. **b** win3 nDNA; patterns cc and bc: *P. nigra*; pattern aa: *P. deltoides*; pattern ac: *P.* x *canadensis*. **c** POPX/*Msp*I RFLP of nDNA; patterns B and C: *P. nigra*; patterns A and D: *P. deltoides* and *P.* x *canadensis*. Approximate band sizes are indicated, the standard (S) is a 100 bp ladder (FERMENTAS)

(Fig. 1b, pattern aa). *Populus* x *canadensis* showed a combination of the *P. deltoides* and *P. nigra* patterns with two bands of 170 and 260 bp in length (Fig. 1b, pattern ac). This band combination should be indicative of F1 hybrids. (Note that a 200 bp band was not observed in these putative hybrids). Thus, the presence of a band at 260 bp indicates that an individual is not a pure *P. nigra*.

POPX/*Msp*I RFLP of nDNA

Restriction of the POPX PCR product of nDNA with *Msp*I resulted in four patterns with two to four bands (Heinze 1998). Two bands of about 970 bp (two close, almost co-migrating fragments form a particularly strong band) and 720 bp occurred in all individuals checked (Fig. 1c). *Populus nigra* showed either none or only one additional band of 820 bp (Fig. 1c, patterns B and C). On the other hand, *P. deltoides* and *P.* x *canadensis* had one additional band of 650 bp (Fig. 1c, pattern D) or two additional bands of 650 and 820 bp (Fig. 1c, pattern A). Hence, the presence of a band at 650 bp indicates that an individual is not a pure *P. nigra*.

Cross-checking with allozymes

The allozyme genotypes of the 209 *Populus* trees either from the blind test samples or the putative *P. nigra* samples (see below) were in full agreement with the results of the three PCR assays. We detected a sub-sample of the typical allozyme genotypes indicated by Janssen (1997) in *Populus* taxa from Switzerland. Of particular interest were those for Black Poplars and their hybrids. For *P. nigra,* the genotypes found were LAP-A n1n1, AAT-B n1n1, PGM-A n1n2 and n2n2 and PGI-B n2n2. For *P. deltoides* and/or *P.* x *canadensis*, LAP-A d1n1 and d1d1, AAT-B d1n1 and d1d1, PGM-A d1n2 and d1d1 and PGI-B d3n2 and d3d3 were observed (genotypes are given according to Janssen 1997; banding patterns not shown).

Blind test samples

The blind test resulted in almost complete consistency between the known and the genetically determined taxonomic identity. In particular, this held true for samples of *P. deltoides*, *P. nigra* and *P.* x *canadensis*. Two individuals supposed to represent pure *P. nigra* were determined as *P.* x *canadensis* based on the DNA and allozyme methods applied. All of the other *Populus* taxa within the blind test samples were easily delimited from Black Poplars and their hybrids. *Populus nigra* var. *Italica* showed the typical banding patterns of *P. nigra*.

Putative *P. nigra* samples

Only 8 out of the 304 putative *P. nigra* trees from Switzerland tested with the molecular marker set (and allozymes for about a third of them) were not pure *P. nigra* as indicated by the genetic methods applied. This resulted in a mismatch rate between morphological and genetic identification of pure *P. nigra* of only 2.6%.

Discussion

We reliably distinguished *P. nigra* from *P. deltoides* and the hybrid *P.* x *canadensis* with the three PCR-based marker types used. These previously published markers proved to be easily applied in a large-scale screening, the PCR reactions were robust, and the results were straightforward to interpret (Fig. 1). A benefit was that diverse plant material both in terms of life stage (i.e. seedlings versus adults) and organ type (wood, leaves, buds) could be used for taxon delimitation. Hence, sampling of plant tissue of naturally occurring *Populus* trees is not restricted to a particular season, but can be done year round. This latter point and the distinct banding patterns observed are advantages of the used DNA markers over the traditional allozyme method, which requires buds (Rajora 1989; Janssen 1997). However, it should be noted that for the two nuclear markers win3 and POPX, other banding patterns, additional to those presented in this study (Fig. 1), could be observed in F2 or BC1 *P.* x *canadensis* or in any later-generation hybrids and backcrosses.

The molecular approach that we used, targeting both cpDNA and nDNA, allowed for the identification of *P. deltoides* and, at least, of F1 hybrids of *P.* x *canadensis*. The two nuclear markers should be able to indicate potential F2 or BC1 hybrids with a probability of 75%, but given the combination with a cpDNA marker, the probability to

identify later-generation hybrids is even higher (see below). *Populus* x *canadensis* cultivars in Central Europe appear to be F1 hybrids (Heinze 1998). The relatively recent introduction of Hybrid Poplars is therefore not likely to have resulted in later-generation hybrid individuals so far.

It is well known from cultivar breeding and crossing experiments that *P. nigra* rarely acts as the dam in interspecific crosses of *P. deltoides* and *P. nigra* (Hofmann 2002). High-embryo abortion is induced when *P. nigra* acts as the mother. This seems to hold true for later-generation hybrids as well, although tests under natural conditions are scarce (Vanden Broeck et al. 2002). Vanden Broeck et al. (2003) suggested that the availability of conspecific pollen is the key factor limiting interspecific crosses under natural conditions. As a consequence, the probability that any hybrid *P.* x *canadensis* would exhibit the specific *P. nigra* cpDNA banding pattern A (Fig. 1a) is rather low, which strongly increases the power to detect even later-generation hybrids of *P. nigra* and *P. deltoides*. However, only the application of all three marker types used in this study allows the molecular identification of pure *P. nigra* with high certainty.

The sample set of putative *P. nigra* individuals from Switzerland indicates that specialists, such as local foresters with an adequate botanical training, are able to identify pure *P. nigra* with high accuracy based on a combination of morphological traits alone. It should be noted that our sample set only included putative *P. nigra* trees with rather typical morphological traits (EUFORGEN s.d.) and did not consist of a random sample of *Populus* sp. trees from Switzerland. Our collectors correctly identified pure *P. nigra* in 97.3% of these samples. This value is surprisingly close to the 3% of hybrids detected with allozymes in nine European gene bank collections, which could not be delimited from pure *P. nigra* based on their morphology alone (Storme et al. 2004). However, a direct comparison of individual morphology and genotype, based on the molecular markers presented here, using a large random sample of *P. nigra*, *P. deltoides* and *P.* x *canadensis* would be desirable to thoroughly test the congruity of morphological and genetic identification approaches of pure *P. nigra*.

Not surprisingly, the cultivar *P. nigra* var. *Italica*, forming a clone that originated from a single distinct individual, could not be delimited from natural *P. nigra* using the above DNA marker set. However, this variety is easily identified by its distinct fastigiate growth habit (Cagelli and Lefèvre 1995). Hence, this missing genetic resolution does not pose any practical problems at the adult stage. It also has to be stressed that the markers used do not allow for a test of autochthony of *P. nigra* individuals. For the latter purpose, more variable DNA markers like nuclear microsatellites (Rajora and Rahman 2001) or AFLPs (Winfield et al. 1998) have to be used and to be tested in extensive regional samples of *Populus* sp.

Because the three PCR assays used in this study provide an easy-to-use, robust molecular tool for the identification of pure *P. nigra* from *P. deltoides* and hybrid *P.* x *canadensis*, we advocate their use as a prerequisite for the conservation of European Black Poplar, both in situ and ex situ. Naturally occurring pure *P. nigra* trees can then serve as sources for plant material used in re-establishing riparian woodlands during ongoing restoration projects of floodplains throughout Europe.

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