PCR-BASED CHLOROPLAST DNA ASSAYS FOR THE IDENTIFICATION OF NATIVE *POPULUS NIGRA* AND INTRODUCED POPLAR HYBRIDS IN EUROPE

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ABSTRACT

Populus nigra L., native to Europe, is under threat from being replaced by, and genetically introgressed with, cultivated hybrid poplars. Chloroplast DNA variation between *P. nigra* and the most common hybrid poplars (*P.* × *euramericana* (Dode) Guinier and balsam poplars from section *Tacamahaca*) was studied with assays based on the polymerase chain reaction (PCR). After PCR amplification of intergenic spacers using conserved primers, followed by restriction digestion, inter– and intraspecific variation was detected. In total, 18,000 basepairs were analysed. Many spacer regions analysed were monomorphic. In the polymorphic spacers, small length mutations indicative of insertion or deletion events were more common than restriction site, i.e., point, mutations. Three length-variable spacer regions, two of which were coupled with restriction site mutations, and one more intra-specific restriction site change were detected. One particular spot in the large single copy region exhibited a concentration of several small insertions/ deletions and one restriction site change. This region lies between the genes for trnG and ORF 62, and shows inter- and intraspecific polymorphisms. A small insertion/ deletion close by distiguishes the widely cultivated Lombardy poplar, *P. nigra* var. *italica*, from all other trees analysed. Another group of clones bred in Japan from a *P. nigra* mother tree of unknown origin carries a mutation in a restriction site close to the junction of inverted repeat B and the large single copy region.

The abundance of small insertions / deletions relative to point mutations in the poplar chloroplast genome could explain taxonomic discrepancies reported in previous studies based on RFLP data.

Key words: *Populus nigra*, chloroplast DNA, PCR, hybrid poplars, conservation, *P. deltoides*

INTRODUCTION

Populus section *Aigeiros* is represented by a single member in Central Europe, *P. nigra* L., and no member of the closely related section *Tacamahaca* is native to this area (ECKENWALDER 1996). However, poplar clones which are easily propagated by cuttings have been introduced to Europe since many centuries. For instance, the Lombardy poplar, *P. nigra* var. *italica*, is thought to originate in Western or Central Asia and comprises a group of clones with a narrow genetic basis. Hybrid poplars spread widely across the continent after the discovery of spontaneous hybrids in the offspring of female *P. deltoides* Marsh. transferred to France around 1600 (ZSUFFA *et al.* 1996). While the taxonomic grouping of such hybrids was for a long time unclear, it emerged that in Europe they comprise a relatively small group of clones (MÜLLER & SAUER 1958), mainly from first generation *P. deltoides* × *P. nigra* crosses (syn. *P.* × *euramericana* (Dode) Guinier). Around the turn of the century, systematic crossing experiments started and resulted in the production of a larger set of clones with additional parental species. Such clones are now widely planted in plantations at alluvial sites across Europe, where they have often replaced the native stands of *P. nigra* and willow species. There is concern for the conservation of *P. nigra* (CAGELLI & LEFÈVRE 1995) owing to no apparent fertilization barriers between hybrids and pure *P. nigra*. Species identification or analysis of hybrid status is necessary for conservation projects because spontaneous crossings between hybrids and pure *P. nigra* result in plants with a range of morphological characteristics, but generally difficult to assign to the hybrids or *P. nigra*. The investigations reported here are part of a project to assess the impact of hybrid poplar cultivation on Austrian *P. nigra*.

The *Populus* chloroplast genome has been studied by RFLP analysis. Maternal inheritance (MEJNARTOWICZ 1991, RAJORA & DANCIK 1992, SABSCH 1992) and inter- and intraspecific variation (SMITH & SYTSMA 1990, SABSCH 1992, VORNAM *et al.* 1994, RAJORA &

DANCIK 1995a,b,c) were reported. We work on the substitution of RFLP assays by PCR-restriction analysis. Conserved PCR primers that direct the amplification of chloroplast genes or intergenic spacers have been described that allow the analysis of a great range of taxa (TABERLET *et al.* 1991, DEMESURE *et al.* 1995, DUMOLIN-LAPEGUE *et al.* 1997). While screening for polymorphisms between native black poplars and hybrid poplars, a particularily polymorphic region was detected, while other regions provided little or no polymorphism at all. This paper discusses the significance of such a finding for the analysis of introgression in European poplars, and for phylogeny in general.

MATERIAL AND METHODS

Poplar plants and clones

Clones were obtained from the Tulln nursery stoolbeds of the Federal Forest Research Centre in the form of dormant winter cuttings. This collection of around 130 clones comprises many entries from the European Catalogue (PINON & VALADON 1997) with pure and hybrid specimen from *Populus* sections *Aigeiros* and *Tacamahaca* (mainly *P. nigra*, *P. deltoides*, *P. maximowiczii*, and *P. trichocarpa*). Further material, including controlled crosses "Sv–1" and"Sv–2" and their respective *P. nigra* and *P. deltoides* parents (GERGÁCZ 1988), was obtained from the ERTI Experimental Station in Sárvár, Hungary (Dr. Józef Gergácz). Buds were also collected from poplar stands in the Vienna area (described in HEINZE 1997).

DNA was prepared from dormant buds or newly emerged leaves essentially as described (HEINZE & GEBUREK 1995).

PCR primers and programmes

The "universal" set of chloroplast primers is given in TABERLET *et al.* (1991), DEMESURE *et al.* (1995), and DUMOLIN-LAPEGUE *et al.* (1997). Primer pairs HK, DT, CS, SfM, ST (DUMOLIN-LAPEGUE *et al.* 1997), ucp a/d and ucp c/d (TABERLET *et al.* 1991) were used in this study. Further primers were designed based on alignments of angiosperm DNA sequences from GenBank/EMBL DNA databanks in the region between trnfM and trnS (SUGIURA 1992) with the help of the PCR-PLAN program of the PC/GENE package version 6.85 (Oxford Molecular Group); their respective 5'->3' sequences are: ORF62-P, CTT GCT TTC CAA TTG GCT GT; ORF62-M, TTG GTT TTG GGT CGT CGA C; trnG-P, GCC AAG GAG AAG ATG CGG G; trnG–M, AAC CCG CAT CTT CTC CTT GG. Primer trnG–P was also used in combination with the appropriate psaA-targeted primer of DEMESURE *et al.* (1995). The region around the junction of inverted repeat B and the large single copy region (SUIGURA 1992) was amplified with primers psbA3' (5'–CTA GCA CTG AAA ACC GTC TT–3') and rpl23p (5'–TAA GAC AGA AAT AAA GCA TTG CGT CGA AC–3'), and an overlapping neighbouring region with psbA5' (5'–TAC GTT CGT GCA TAA CTT CC–3') and K2 (DEMESURE *et al.* 1995), respectively.

Standard PCR reaction conditions employed 10–50 ng DNA in a 10–20 µL reaction containing 200 nM of each primer, 2.0 mM $MgCl_2$, 200 µM of each dNTP, 10–50 ng/µL RNAase A (HEINZE 1994), and 0.5 U of DNA polymerase enzyme (DynaZyme, FinnZymes Oy) in 1x reaction buffer as supplied along with the enzyme.

The thermal profile in an MJR Programmable Thermal Cycler (Model PTC 100-60 or PTC 100-96v) was either 94 °C – 50 sec, 55 °C – 50 sec, 70 °C – 2 min (35 cycles), then 4°C until recovery, or 94 °C – 50 sec, 70 °C – 1 min (10 cycles), followed by 94 °C – 30 sec, 55 °C – 50 sec, 70 °C – 2 min (35 cycles), and 4 °C until recovery.

Restriction analysis

Table 1 lists PCR fragments and which restriction enzymes (purchased from Pharmacia Biotech and Life Technologies) were tested on them. For each PCR fragment – restriction enzyme combination, at least 5 clones of pure *P. nigra* , 2 of *P. deltoides*, 3 of *P.* × *euramericana*, 2 of *P. trichocarpa*, and 1 of *P. maximowiczii* were tested along with F_1 hybrids of known pedigree and clones from the Tulln collection of uncertain breeding records. The ORF62-P – trnG-M / Eco RI, Dra I combination was additionally tested with 90 *P. nigra* clones collected in the Vienna area. The trnD – trnT length polymorphism was tested on the 90 *P. nigra* clones mentioned above, and on 40 commercial hybrid clones (mainly *P.* × *euramericana*) from the Tulln nursery. Restriction reactions were carried out by adjusting the buffer to >10 mM Mg_2+ after PCR, adding of 0.1 U restriction enzyme per 10 µL PCR reaction, and incubating at 37 °C for at least 4 hours. As an exception, digests with Xba I needed to be done on ethanol-precipitated PCR-products or DNA and with the buffer recommended by the enzyme suppliers.

The restriction map of the polymorphic region between trnfM and trnS is based on partial and double digests with the enzymes indicated.

Table 1. PCR primer pairs for amplification of chloroplast DNA fragments, size of the resulting fragments, and restriction enzymes employed in the analyses. Total length of non-overlapping fragments analysed: approx. 18,150 / 18,350 bp. Restriction site mutations (gains or losses) are shown in bold, length-variable fragments are underlined.

Primer pair	Approx. size (bp)	Restriction enzymes
ucp a+d	1050	Hae III, Rsa I, Msp I, Hha I
ucp c+d	650	Bam HI, Bgl I, Eco RI, Hae III, Hha I, Hind III, Msp I, Pst I, Xho I
psbA 5' + trnK 2	6000	Bam HI, Bgl I, Eco RI, Hind III, Pst I, Xho I
psbA 5' + trnK1	1350	Hha I, Msp I, Rsa I
psbA 5' + psbA 3'	900	Hinf I
psbA 3' + rpl23p	3000	Hae III, **Hha I**, Hinf I, Msp I
psbA 3' + trnH	1300	Rsa I
trnH + trnK	1800	Msp I, Rsa I
trnS + trnfM	1600	Bam HI, Bgl I, Dra I, Eco RI, Hae III, Hha I, Hinf I, Msp I, Pst I, **Rsa I**, Xho I
ORF62–P + trnfM	1040	Bam HI, Eco RI, Hae III, Hha I, Hinf I, **Rsa I**, Xho I
ORF62–P + trnG–M	800 250	Dra I, Eco RI, **Rsa I** Bam HI, Bgl I, Eco RI, Hae III, Hha I, Hinf I, Msp I, Xba I, Xho I
trnG–P + trnfM	3500	Hae III, Hha I, Hinf I
trnG–P + psaA	850/1050	**Hinf I**, Msp I, Rsa I, **Xba I**
trnD + trnT	1600	Hinf I, Xba I
psbC + trnS trnS + trnT	1700	Bam HI, Bgl I, Eco RI, Hae III, Hha I, Hind III, Hinf I, Msp I, Pst I, Rsa I, Xho I

Electrophoresis

Reaction products were analysed in 1.5–2.5 % agarose gels (NuSieve GTG 3:1, FMC) using 0.5 × TBE buffer and compared to a 100 basepair ladder (Life Technologies) or to a lambda DNA Hind III digest for size estimation. Gel images were captured with a video device (GelPrint 2000i, MWG Biotech) and stored electronically. Band sizes were calculated using RFLP-Scan software (Scanalytics). Average sizes determined from several electrophoresis runs are presented.

The highly variable and one monomorphic region were subjected to single-strand conformation polymorphism analysis (SSCP) by electrophoresis through a polyacrylamide derivate (0.5x MDE gel matrix, FMC, Rockland) in a BioRad Protean II 20 cm cell using 0.5 × TBE running buffer and tap water cooling as described (HEINZE 1997). This gel matrix is capable of resolving bands differing in their internal DNA sequence. Bands were visualized by silver staining according to HEINZE (1997).

Inverse PCR

This technique was tested to quickly identify mutations in restriction sites based on reported RFLP polymorphisms between *P. nigra* and *P. deltoides* (SABSCH 1992, VORNAM *et al.* 1994, RAJORA & DANCIK 1995b). Genomic DNA of both species and of several hybrids was digested with Xba I, diluted, ligated to facilitate the formation of intramolecular circles, and subjected to PCR using primer pairs that point away from each other following the experimental conditions suggested by OFFRINGA and VAN DER LEE (1995). Primers employed in this experiment were: psbC (from pair TC) + trnT (from pair DT), psbC + trnD (DT), trnT + trnS (primers from both pairs ST and SfM), ucp e + ucp b, ucp c + trnS (ST), trnS (ST) + psaA, and trnfM + psaA (DEMESURE ET AL. 1995, DUMOLIN-LAPEGUE *et al.* 1997, TABERLET *et al.* 1991).

RESULTS

PCR amplification, gel resolution

The primer combinations listed in table 1 produced amplification products in a robust manner. Only a few PCR reactions failed, and in most cases poor DNA quality was the reason. Furthermore, while the PCR programmes given may not be the optimal ones for each primer pair, they still work well enough for routine analyses. Time-consuming optimization of reaction conditions for each single primer combination could thus be avoided. In total, more than 18,000 non-overlapping basepairs (bp) were amplified.

Agarose gels as employed in this study resolve band size differences down to approx. +/– 5%. A similar variation was observed when band sizes were compared between runs. This level of precision is sufficient for the assays presented here, as size differences become readily visible when samples are analysed side by side.

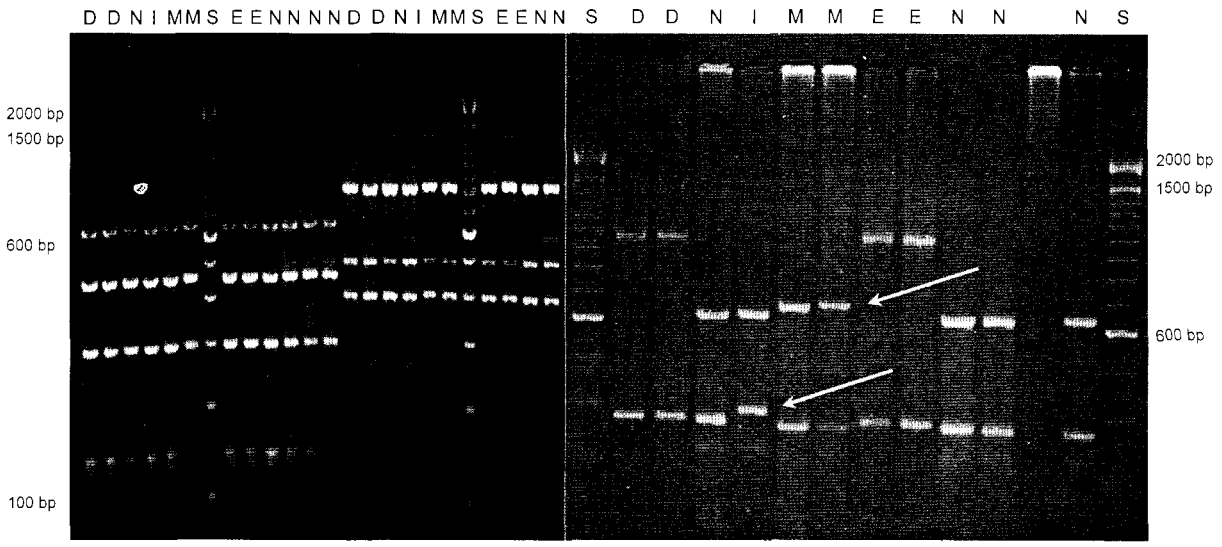

Figure 1. Monomorphic and polymorphic restriction patterns from two chloroplast DNA regions in *Populus*. Left panel: DNA fragments amplified with primers trnS + trnT (ST, DEMESURE *et al.* 1995) and restriction digested with enzymes Hinf I (left) and Hha I (right). Right panel: DNA fragments amplified with primers trnS and trnfM (SfM, DEMESURE *et al.* 1995) and restriction digested with enzyme Rsa I. D, *P. deltoides*; N, *P. nigra*; I, *P. nigra* var. *italica*; M, *P. maximowiczii*; E, *P.* × *euramericana*; S, DNA size standard (100 bp ladder). Note variable fragments in the SfM / Rsa I analysis (arrows).

Polymorphisms: lenght and restriction pattern

Two types of polymorphisms were detected between clones or species: simple length differences, some only detectable after restriction digests, and apparent restriction site mutations (gain or loss of recognition sites). Hybrids shared the chloroplast genome of their respective female parents.

A cluster of such polymorphisms was detected in the region between trnS and trnfM, including several size differences between species, a restriction site mutation (Rsa I), and two intra-specific length polymorphisms (Figure 1). A restriction map of this region is presented below.

Other regions analysed yielded less polymorhism: a restriction site mutation in the region of the junction between inverted repeat B and the large single copy region (SUGIURA 1992, genes rpl23 – psbA) differentiated a group of *P. nigra* × *P. maximowiczii* clones from the rest of the samples analysed. These clones, known as "NM101 – 106" or "Max 1–6" (WEISGERBER 1983, KARNER 1984), and "Kamabuchi I" were presumably derived from the same *P. nigra* mother of unknown provenance, at Oji Paper Company, Kuriyama, Japan (H. Kohda, personal communication). They share another small length polymorphism in the trnS – trnfM region with *P. nigra* clone "Vert de Garonne" from France, but not with any other *P. nigra* clone analysed.

An inverse PCR protocol as described by OFFRINGA and VAN DER LEE (1995) was employed with a view to quickly find additional polymorhisms previously described in RFLP assays (VORNAM *et al.* 1994, RAJORA & DANCIK 1995b). These authors cite Xba I as an enzyme recognizing several polymorphisms in the large single copy region of the poplar chloroplast genome, where the primers used in this study are situated. One such polymorphism was readily detected after cutting poplar genomic DNA with Xba I, ligating in diluted solution, and amplifying with primers pointing away from each other: primers trnS and trnT showed differences between *P. nigra* and *P. deltoides* clones after a second round of PCR amplification as suggested by OFFRINGA and VAN DER LEE (1995). Primers psbC and trnT gave an amplification product only from *P. deltoides* in the range of 2500–3500 bp which is also a significant size limit under the PCR reaction conditions used in this study. The trnT primer, together with trnD of the appropriate primer pair (DT, DEMESURE *et al.* 1995), gave a length polymorphism between *P. nigra* and *P. deltoides* / *P.* × *euramericana* with the *P. nigra* variant being approx. 200 bp shorter, and a smaller size difference between *P. deltoides* and members of the *Tacamahaca* section. This length polymorphism was associated with the loss of an Xba I site in *P. nigra*. A Hinf I digest of this region showed up to six restriction fragments, some of them polymorphic, others conserved in length between species.

All other combinations of primers and restriction enzymes listed in table 1 did not reveal any further polymorphisms in the clones tested (Fig. 1). In order to quickly check PCR fragments for polymorphisms, it turned out that treating the fragments with four-base

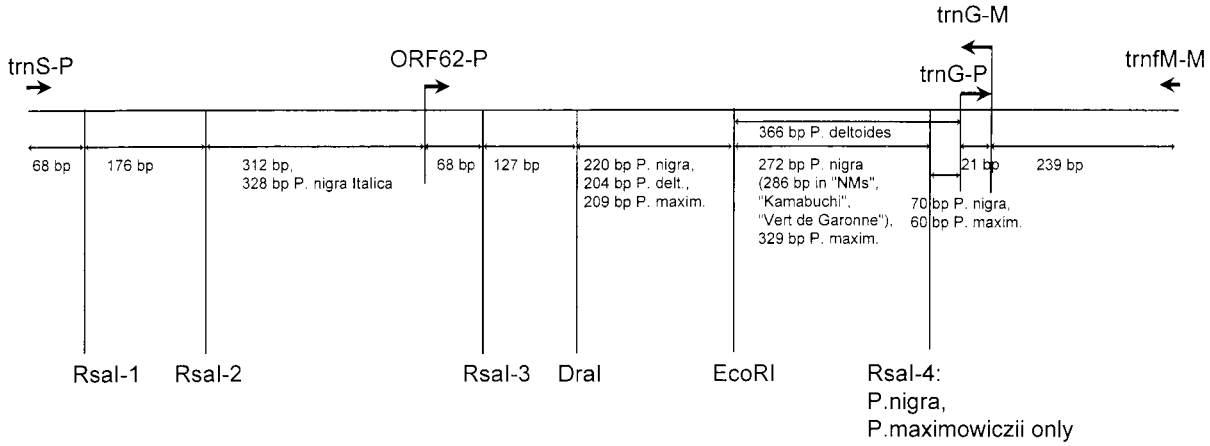

Figure 2. Restriction map of the chloroplast DNA region between trnS and trnfM in *Populus*. Overall length: approx. 1550 – 1600 bp. Arrows indicate position and orientation of primers, which were based on conserved gene sequences as indicated. Restriction enzyme sites and polymorhisms are given. Lengths are average values from several agarose electrophoresis runs. The polymorphic region lies between ORF 62 and trnG. A further polymorphism destinguishes *P. nigra* var. *italica* form all other poplars analysed. The order of the 68 and 176 bp fragments between trnS and Rsa I 1 and 2 could not be established with certainty.

cutting restriction enzymes, and in particular Hinf I, resulted in a range of smaller sized fragments that were readily screened for size differences in electrophoresis gels. Electrophoretic separation of single strands of DNA, SSCP analysis, was tested as an alternative mutation screening method for the polymorphic trnS–trnfM region, and for the region between ucp a and ucp b. While the latter fragment was monomorphic, SSCP pattern diversity in the OFR 62 – trnG region coincided with restriction patterns in the samples analysed.

In summary, three length-variable spacer regions, two of which were coupled with restriction site mutations, and one more intra-specific restriction site change were detected in a survey of more than 18,000 bp of the *Populus* chloroplast genome in this study.

Restriction mapping of the region between trnS and trnfM

The gene sequence in and around this region in tobacco (*Nicotiana tabaccum* L.) is: psbC – trnS – ORF105 – ORF62 – trnG – trnfM – rps 14 – psaB – psaA (SUGIURA 1992). As all primer combinations that were tested in poplar behaved in the same way as predicted from the tobacco data, and as observed fragment sizes were also similar in range to those predicted from the tobacco sequence, there is no doubt that the genes run colinear between *Nicotiana* and *Populus* in this region of the chloroplast genome. A detailed restriction map of the poplar polymorphic region is given in figure 2. It accounts for the small insertion / deletion polymorphisms between the species, the intra-specific polymorphisms in *P. nigra*, and the restriction site mutation (Rsa I 4). While it was not possible to design "universal" primers for the proposed open reading frame (ORF) 105 due to not obtaining meaningful alignments of DNA sequence from several species in this region, the approximate locations of the mutations relative to the other genes can be deduced (Fig. 2).

DISCUSSION

Introgression analysis

Genetic markers offer a tool to assess introgression in plant populations independent of environmental impact that often confunds morphological analyses. Polymorphisms can be identified and the variants attributed to the appropriate pure species. Previously, such markers have been described for *P. nigra* based on isozymes (RAJORA 1990, RAJORA & ZSUFFA 1990), rDNA (D'OVIDIO *et al.* 1991, FAIVRE-RAMPANT *et al.* 1992), mitochondrial (RAJORA *et al.* 1992, BARRETT *et al.* 1993) and chloroplast RFLPs (SMITH & SYTSMA 1990, SABSCH 1992, RAJORA & DANCIK, 1992, VORNAM *et al.* 1994, RAJORA & DANCIK 1995a,b,c), and more recently, a PCR assay for a nuclear gene (HEINZE 1997). Nuclear genes recombine beyond the first hybrid generation and markers based upon them need to be applied in greater numbers or in combination with extrachromosomal markers. Here, simple PCR-restriction assays are described for chloroplast DNA markers to help in the identification of variants originating from other poplar species in *P. nigra* seedlings collected in the field. Due to maternal inheritance, and the female

contribution of *P. deltoides* to the poplar clones most prominently planted in Central Europe over the past decades, presence of these markers in poplar offspring is indicative of a hybrid poplar in the maternal lineage of the pedigree of the respective plant. On the other hand, the contribution of pollen from male hybrid clones cannot be estimated with this marker. A combination of nuclear and chloroplast markers, however, will result in increased detection of introgression. Occasionally, we do find plants in the field, but also in clone collections, that appear to assume an intermediate position between first generation hybrids and pure *P. nigra* as assessed with this chloroplast and a nuclear (HEINZE 1997) marker. This would indicate backcrosses of hybrids to *P. nigra*. An enlarged set of nuclear markers is desirable to estimate parent species' contributions to single clones and plants more accurately.

Types and distributions of chloroplast polymorphism

The results presented above indicate that small insertions and deletions are the most common type of DNA polymorphisms in the poplar chloroplast genome. Similar results were obtained by DEMESURE *et al.* (1996). In contrast, lower levels were detected for restriction site mutations in this study. VORNAM *et. al.* (1994), RAJORA & DANCIK (1995b), and SMITH & SYTSMA (1990) list a number of such mutations, however. They also report on length variations, but with the RFLP employed by them, these cannot be determined accurately. Neither can length variations be distinguished from restriction site mutations that create fragments too short to be detected with this method. It can be anticipated that a detailed analysis of the poplar chloroplast genome will reveal much more small deletions/ insertions than those reported here.

A restriction site mutation (Rsa I) in the polymorphic region co-maps with insertions / deletions. A second restriction site mutation, between trnT and trnD, is coupled with the loss of an Xba I site in *P. nigra*. Based on these findings, it seems plausible that many of the restriction site mutations previously reported are the result of such small insertions/ deletions.

A cluster of polymorphisms is reported here in an intergenic region on the chloroplast DNA circle, between genes trnG and ORF 62. Of particular interest is that var. *italica*, a *P. nigra* clone, carries a mutation very close to this cluster, apparently between trnS and ORF 62 (ORF 105 which is situated in this region could not be placed on the restriction map). In contrast, mutations where rare in the other regions analysed. With the limited survey presented here, it is not yet justified to talk of a mutation hotspot, but uneven distributions of chloroplast DNA polymorphisms have been reported in previous studies (*e.g.*, MORTON & CLEGG 1993, HIPKINS *et al.* 1995, JOHNSON & HATTORI 1996).

Implications for phylogeny

The data presented here should mark a rethinking of current chloroplast-based poplar phylogenetic trees (SMITH & SYTSMA 1990, RAJORA and DANCIK 1995b). In contrast to SMITH & SYTSMA (1990), this study confirms that there is intraspecific DNA variation in the poplar chloroplast (RAJORA & DANCIK 1995a). Keeping in mind the preponderance of small insertions/ deletions, and the clustering of several of these in a single region, it seems possible that a more detailed analysis of the poplar chloroplast genome will resolve the apparent discrepancies reported by SMITH & SYTSMA (1990) and RAJORA & DANCIK (1995b). SMITH and SYTSMA (1990) proposed a close phylogenetic relationship between the chloroplast genomes of *P. nigra* and *P. alba* L. RAJORA & DANCIK (1995b) concluded from their data that *P. deltoides* and *P. maxiowiczii* Henry are closer relatives than *P. nigra* and *P. deltoides*. In the light of results presented here, their calculations of genetic distances between species may need re-assessment. Small insertions/ deletions as reported here cannot be recognized in RFLP analyses. They are single mutational events occurring with equal probabilities relative to each other. The most commonly used formula for genetic distance estimation from restriction site data (NEI 1987), however, gives different weights to longer and shorter restriction enzyme recognition site mutations. This would only be justified if the mutations that created or deleted the sites were true point mutations, but not if insertions/ deletions were the mechanism of mutation. There is, however, no consensus as how to treat insertions / deletions in phylogenetic calculations.

It also appears that reassessed *Populus* chloroplast phylogeny data would contribute to a better understanding of the taxonomic placement of *P. nigra* (FAIVRE-RAMPANT *et al.* 1995, ECKENWALDER 1996, RAJORA & DANCIK 1995B, SMITH & SYTSMA 1990). While *P. nigra* is established as a member of section *Aigeiros*, together with *P. deltoides* and others, it shows some affinity with section *Tacamahaca*, and a peculiar relationship in artificial crossing with *P. deltoides*. Only the fertilization of *P. deltoides* by *P.nigra* pollen is possible in the first generation (= F_1 cross), but not vice versa. ECKENWALDER's (1996) phylogenetic analysis based on morphological features places *P. nigra* in section *Aigeiros*, but basal to it, with the *Tacamahaca* clade joining next. This arrangement was

questioned on the basis of chloroplast RFLP data by RAJORA and DANCIK (1995b) and SMITH and SYTSMA (1990). Ultimate resoluton, *i.e.*, DNA sequencing, will be required to resolve this disagreement. The ORF 62 – trnG region will provide a good starting point for such an investigation.

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