LOW GENETIC DIFFERENTIATION AMONG ITALIAN POPULATIONS OF *POPULUS TREMULA* L. (SALICACEAE) ESTIMATED USING CHLOROPLAST PCR-RFLP AND MICROSATELLITE MARKERS

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ABSTRACT

Chloroplast (cp) DNA polymorphism was analysed in ten Italian populations of *Populus tremula* L. by PCR-RFLP and microsatellites (SSR) chloroplast markers. Amplified fragments were digested by restriction endonucleases and the resulting restriction fragments were separated by gel electrophoresis. Three cpDNA amplification products showed polymorphism after digestion. By analysing this polymorphism we identified six haplotypes. Five universal pairs of primers were used for the amplification of specific cpDNA microsatellite regions, which are supposed to contain mononucleotide stretches (A/T). Two microsatellite loci showed polymorphism, resulting in a total of six haplotypes. Both PCR-RFLP and SSR chloroplast markers revealed very low genetic differentiation among populations, with $G_{ST} = 0.22$ and $N_{ST} = 0.24$ (PCR-RFLP), and $G_{ST} = 0.07$ and $N_{ST} = 0.07$ (SSR), resulting about 4–10 times lower than the mean G_{ST} value estimated in broad-leaved forest species for maternally inherited markers. This could be mainly explained by the high efficiency of *P. tremula* seed wind dispersal over long distances, determining little geographic structure of the genetic diversity.

Key words: PCR-RFLP, SSR, *Populus tremula* L., cytoplasmic polymorphism, genetic differentiation.

INTRODUCTION

The genus *Populus*, Salicaceae, consists of about 40 species widely distributed throughout the northern hemisphere; in Europe it is represented by three autochthonous species: *P. alba* L., *P. tremula* L., and *P. nigra L.* and the hybrid *P.* × *canescens* (Aiton) Smith (GELLINI & GROSSONI 1997; HARLOW *et al.* 1996).

P. tremula, the European trembling aspen, is the most widespread among European *Populus* species and the least demanding in terms of soil moisture, it is a typical species of moist sites within woodlands. In Mediterranean Europe it is mostly diffused in mountain areas, where it is particularly efficient in colonising marginal, abandoned, and poor soils. The combination of tolerance to waterlogging and flooding, and suckering habit, enables *P. tremula* to stabilise the often unstable media on which it grows (GELLINI & GROSSONI 1997; HUNTLEY & BIRKS 1983).

Like other *Populus* species, the European trembling aspen produces anemophilous, imperfect flowers (plants are doiecious) in hanging catkins that bloom in the spring before the leaves unfold. The fruit is a capsule containing tufted seeds, bearing long, white hairs, which give them buoyancy in air dispersal (HARLOW *et al.* 1996).

During the last decade several studies reported results on chloroplast DNA (cpDNA) variation in a wide range of plants, including trees (for a review see SOLTIS *et al.* 1992, PETIT 1999). CpDNA polymorphism and its geographic distribution has been described in some European angiosperm species: oaks, *Quercus robur* L. and *Q. petraea* (Matt.) Liebl., KREMER & PETIT 1993, PETIT *et al.* 1993, FERRIS *et al.* 1993 1995 1998, DUMOLIN-LAPÈGUE *et al.* 1997); beech, *Fagus sylvatica* L., (DEMESURE *et al.* 1996); common alder, *Alnus glutinosa* (L.) Gaertn. (KING and FERRIS 1998); sweet chestnut, *Castanea sativa* Mill. (FINESCHI *et al.*, 2000); and one species from the austral hemisphere, the southern beech, *Nothofagus nervosa* (Phil.) Dim. et Mil. (MARCHELLI *et al.* 1998).

Both cytoplasmic genomes (chloroplasts and mitochondrial), are maternally inherited in most angiosperms. Maternal inheritance of chloroplast DNA in *Populus* was confirmed by MEJNARTOWICZ (1991) and RAJORA & DANCIK (1992). Studies on organelle DNA

markers demonstrated that when seed flow is less efficient than pollen flow, as it is the case in many angiosperms, organelle polymorphisms are highly structured in comparison with the nuclear ones (PETIT *et al.* 1993). This is particularly true for angiosperm trees which produce heavy seeds, usually dispersed by weight; *Populus* species have a more efficient seed dispersal mechanism, which can result in a very efficient seed flow.

In this paper, chloroplast markers, both restriction fragments (PCR-RFLP) and microsatellites (SSR), were used in order (i) to analyse the haplotypic diversity in Italian populations of European trembling aspen; (ii) to evaluate the role of species life history traits in shaping the distribution of genetic diversity.

MATERIALS AND METHODS

Dormant buds and leaves were collected from 10 natural stands, located in northern, central and southern Italy (Table 1). Ten individuals per population were analysed by means of both PCR-RFLP and SSR chloroplastic markers.

Trees were sampled at a minimum distance of 100 m apart. Total genomic DNA was extracted according to the procedure described in DUMOLIN *et al.* (1995).

PCR-RFLP

Five non-coding cpDNA regions were amplified *via* PCR using specific pairs of universal chloroplastic primers: CD, CS, DT, HK, K_1K_2 (DEMESURE *et al.* 1995) (Table 2).

PCR reactions were carried out on a template of genomic DNA (20 ng for each reaction). The reaction mixture was composed by 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.8 mM $MgCl_2$ (Gibco BRL, Life Technologies), 2 $mg \cdot ml^{-1}$ BSA, 100 mM of each dNTP, 0.28 mM of each primer of the specific pair, 2 units of *Taq* polymerase (Gibco BRL, Life Technologies), for a final volume of 25 ml.

Amplification cycles, as described in DEMESURE *et al.* (1995), consisted of an initial denaturation (94 °C for 4 min), followed by 30 cycles of denaturation (93 °C for 1 min), annealing (1 min at temperature specific for each pair of primers), extension (72 °C for 2 min) followed by a final extension (72 °C for 10 min). Specific annealing temperatures are reported in Table 2. The reactions were performed in a DNA thermal cycler *Genius* Techne (Cambridge, UK).

PCR products were digested by 3 to 5 restriction endonucleases (*Alu* I, *Cfo* I, *Hinf* I, *Rsa* I, *Taq* I) (Gibco BRL, Life Technologies). 5ml of the amplification product were digested by 5 units of each restriction enzyme. The combinations of PCR products with the enzymes assayed are specified in Table 2. The resulting restriction fragments were separated by means of polyacrylamide gel electrophoresis (Acrylamide Bis Solution 30 % w/v, Gibco BRL, Life Technologies, final concentration 8 % w/v) carried out in Tris Borate EDTA buffer (1×), at constant voltage (300 V) for 90 to 120 min, depending on the size of the fragments.

After electrophoresis, gels were stained in ethidium bromide and photographed under UV fluorescence (MP4 Polaroid Land Camera, Polaroid 667 film).

Different combinations of fragment length variants defined corresponding haplotypes.

SSR

Five chloroplastic microsatellite regions were amplified using universal primer pairs (ccmp2, ccmp3, ccmp5, ccmp7, ccmp10) (WEISING & GARDNER 1999).

PCR amplifications were performed on a template of 25 ng of genomic DNA. PCR reaction solution contained 10× PCR buffer (Pharmacia), 2.5 mM $MgCl_2$, 200 mM of each dNTP, 0.2 mM 5'-fluoresceine labelled primers, 1 unit of *Taq* polymerase (Pharmacia), for a final volume of 25 ml.

Amplification cycles, as described in VENDRAMIN & ZIEGENHAGEN (1997), consisted of an initial denaturation (95 °C for 5 min), enzyme addition (80 °C for 5 min), followed by 25 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min), extension (72 °C for 2 min) terminated by a final extension (72 °C for 8 min). The reactions were performed in a Perkin Elmer thermal cycler, model 9600.

PCR products were loaded on a 20 cm long denaturing polyacrylamide gel (Pharmacia, final concentration 6% w/v) and run at constant power (35 Watt) for approximately 80 min using an ALF automatic sequencer. Two and three microsatellites were simultaneously loaded with internal size standards (50, 100, 200 and 250 bp). Fragment sizes were obtained using Fragment Manager version 1.2 conversion software (Pharmacia).

Analysis of genetic diversity

Haplotype frequencies were used to calculate the level of population subdivision for cytoplasmic genomes for unordered alleles (G_{ST}) and for ordered alleles (N_{ST}), using the softwares HAPLODIV (PONS & PETIT 1995) and HAPLONST (PONS & PETIT 1996), respectively.

As a measure of phylogeographic structure of the populations (PONS & PETIT 1996) a comparison was made between G_{ST} and N_{ST}, using a permutation test to confirm the difference: 500 random permutations of

Table 1. Details on sample size, location, area code (N=north, C=centre, S=South), and number of individuals per haplotype detected in the analysed *P.tremula* populations.

Area	Population name	Pop. number	Long. E	Lat. N	Haplotypes PCR-RFLP						Sample size
					A	B	C	D	E	F	
N	Sovazza	1	8.47	45.82	5	5	0	0	0	0	10
N	Albino	2	9.80	45.76	0	5	5	0	0	0	10
N	Ala	3	11.01	45.75	0	6	4	0	0	0	10
N	Demonte	4	7.30	44.31	0	6	4	0	0	0	10
C	Monghidoro	5	11.32	44.22	2	3	2	0	3	0	10
C	Borgo Pace	6	12.30	43.66	0	4	6	0	0	0	10
C	Montegallo	7	13.48	43.53	0	0	10	0	0	0	10
S	Moliterno	8	15.87	40.24	0	4	3	0	3	0	10
S	Camigliatello	9	16.44	39.34	0	2	0	2	2	2	8
S	Politrea	10	16.71	39.25	0	8	0	0	2	0	10
	Total sample size				7	43	34	2	10	2	98
	Frequency				0.071	0.439	0.347	0.020	0.102	0.020	1

Area	Population name	Pop. number	Long. E	Lat. N	Haplotypes SSR						Sample size
					R	S	T	U	V	Z	
N	Sovazza	1	8.47	45.82	2	8	0	0	0	0	10
N	Albino	2	9.80	45.76	0	10	0	0	0	0	10
N	Ala	3	11.01	45.75	0	10	0	0	0	0	10
N	Demonte	4	7.30	44.31	0	9	1	0	0	0	10
C	Monghidoro	5	11.32	44.22	0	8	0	0	0	2	10
C	Borgo Pace	6	12.30	43.66	0	9	0	1	0	0	10
C	Montegallo	7	13.48	43.53	0	10	0	0	0	0	10
S	Moliterno	8	15.87	40.24	0	7	0	0	1	2	10
S	Camigliatello	9	16.44	39.34	0	4	2	0	1	1	8
S	Politrea	10	16.71	39.25	0	6	1	2	0	1	10
	Total sample size				2	81	4	3	2	6	98
	Frequency				0.02	0.827	0.041	0.031	0.02	0.061	1

haplotype identities were made, maintaining the haplotypes frequencies and the matrix of pairwise haplotype differences as in the original study (BURBAN *et al.* 1999).

RESULTS

PCR-RFLP

The digestion of the cpDNA amplified regions with 3 to 5 restriction endonucleases revealed polymorphism in three loci out of five, as revealed by 5 primer-enzyme combinations out of 17 (Table 2). In particular (Table 3), the fragment CD alone, with its three polymorphic restriction fragments (with length variations and restriction-site point mutations), accounted for the major part of the variability detected. The other two amplified fragments which resulted polymorphic (DT and CS) showed a length variation.

Restriction analysis identified 13 variants (Table 3), which combined into 6 cpDNA haplotypes (Table 4). The distribution of the observed haplotypes in each population is indicated in Table 1 and Fig. 1a.

Two haplotypes were peculiar of one population (Camigliatello), whereas all the others appeared in at least two populations. Only one population (Montegallo) was monomorphic. Haplotype frequencies are reported in Table 1. The most frequent one, haplotype B (0.44), appeared in all populations except for the monomorphic one, in which only the second most frequent haplotype (C = 0.35) occurred. This last haplotype was present in seven populations, mostly located in the centre-north of Italy. Haplotype E, with a frequency of 0.10, was scored in all southern populations (Moliterno, Camigliatello and Politrea) and only in one central population (Monghidoro). Haplotype A

Table 2. Details on primers, restriction enzymes, amplification conditions, and indication of polymorphic sites.

Gene	Primer pairs and sequence	Code	Ref.	Ann.temp.	Ext/time	Restriction enzymes	Polymorphism
trn C trn D	CCA GTT CAA ATC TGG GTG TC GGG ATT GTA GTT CAA TTG GT	CD	(1)	58 °C	4'	**Taq I, Alu I, Hinf I** Cfo I, Rsa I	yes (*)
psb C trn S	GGT CGT GAC CAA GAA ACC AC GGT TCG AAT CCC TCT CTC TC	**CS**	(1)	57 °C	2'	**Alu I** Cfo I, Rsa I	**yes** no
trn D trn T	ACC AAT TGA ACT ACA ATC CC CTA CCA CTG AGT TAA AAG GG	**DT**	(1)	54.5 °C	2'	**Taq I** Alu I, Hinf I	**yes** no
trn H trn K1r	ACG GGA ATT GAA CCC GCG CA CCG ACT AGT TCC GGG TTC GA	HK	(1)	62 °C	2'	Alu I, Hinf I, Rsa I	no
trn K1 trn K2r	GGG TTG CCC GGG ACT CGA AC CAA CGG TAG AGT ACT CGG CTT TTA	K1K2	(1)	53.5 °C	2'	Alu I, Hinf I, Rsa I	no

(1) DEMESURE *et al.* 1995

(*) The enzymatic digestion of the CD amplification product with Hinf I produces two polymorphic fragments, indicated as electrophoretic bands CD Hinf I (I) and CD Hinf I (VIII). The polymorphism shown by CD Hinf I (I) is also present in the digestion of the same amplification product with Cfo I and Rsa I, therefore indicating the same mutation.

Table 3. Size of the amplified fragments, number and type of variants detected. Primer codes correspond to those indicated in Table 2.

Primer code	Approximate size of PCR product (bp)	Restriction enzymes	Total RFLP bands (1)	Polymorphic bands (2)	Total number of variants (3)	Type of variants
CD	3169	*Taq I*	6	I	2	Point mutation
CD	3169	*Alu I*	2	I	3	Point mutation and indel
CD	3169	*Hinf I*	8	I	2	Indel
		Hinf I		VIII	2	Indel
DT	1213	*Taq I*	5	II	2	Indel
CS	1611	*Alu I*	6	I	2	Indel

(1) Indicates the total number of bands detected by electrophoresis after digestion of each amplified fragment.
(2) Indicates the position of the polymorphic band on the gel according to decreasing molecular weight.
(3) Indicates the number of electrophoretic variants detected for each single polymorphic band.

(0.07) was present in two populations localised in the centre-north of the country (Sovazza and Monghidoro). The two unique haplotypes, D and F, shared the same frequency (0.02) and appearred in the same southern population (Camigliatello), which, together with Monghidoro, was the most polymorphic population (four haplotypes detected).

SSR

Two microsatellite regions out of five showed polymorphism, with two and four length variants respectively (Table 5). In particular, the amplification product of ccmp2 revealed the presence of four different variants (203, 211, 212 and 233 bp), and the product of ccmp5 showed two variants (104 and 105 bp), which combined into a total of 6 haplotypes (Fig. 1b). One haplotype (R) was specific of one northern population (Sovazza). Three populations (Albino, Ala, and Montegallo) were found monomorphic (haplotype S), while the remaining populations showed two to four haplotypes.

Haplotype frequencies are reported in Table 1. The

DISCUSSION

The PCR-RFLP haplotypes distribution showed that: (i) the most common haplotype (B) is present in all but one population ii) only one population, Montegallo, is monomorphic (fixed for haplotype C). These observations revealed that the genetic diversity of *P. tremula* in Italy is not distributed according to a strong geographic structure. However, it is worth to analyse the haplotypes distribution more in details. Populations from the central and northern part share haplotypes B and C whereas populations from the south share haplotypes B and E. This last haplotype is also present in the central population Monghidoro. If haplotype E originated in the south, it seems to have migrated northwards, because of its presence in one Apennine population; on the other hand, the two unique haplotypes (D and F) apparently were not able to migrate from the Calabrian population Camigliatello. At this stage, lack of information on cpDNA variation from the other part of the European distribution of the species does not allow to consider this population as a possible refugial area yet.

Fragment CD, as previously mentioned, appears in two variants, differing for a very large insertion / deletion mutation.

The distribution of microsatellite haplotypes partially confirms that of restriction fragments: (i) there is one haplotype (S) present in all populations, and (ii) population Montegallo is monomorphic. Furthermore, the three southern populations share two haplotypes, S and Z, the latter one also being present in the central population Monghidoro. Higher diversity was detected in the populations from South Italy than in the other parts of the peninsula: the high haplotypes richness in the southern populations could suggest the hypothesis of a refugial area in this region.

Both PCR-RFLP and cpSSR markers exhibited a low level of genetic differentiation among populations, much lower than expected for maternally inherited markers. In a comparative study on cytoplasmic diversity (PETIT 1999), the mean value of population subdivision measured for 97 plant species was $G_{ST} = 0.70$; in particular the G_{ST} value was = 0.73 for angiosperm tree species. The low values of genetic differentiation estimated in this species can be due to the very efficient mechanism of seed dispersal of *Populus tremula*. In fact, the genetic differentiation among populations reported in the present study represents, to our knowledge, the lowest values estimated for maternally inherited markers in tree species studied so far. Species belonging to the family of Fagaceae and producing heavy seeds, like *Fagus sylvatica, Quercus robur* and *Q. petraea*, are characterised by higher values of G_{ST}, ranging between 83 % and 90 % (DEMESURE *et al.* 1996; DUMOLIN-LAPÈGUE *et al.* 1997). Even for *Alnus glutinosa*, a species characterised by wind dispersal of tiny winged seeds, a high value of genetic differentiation estimated using chloroplast markers was reported (G_{ST} = 87%, KING & FERRIS 1998). Interestingly, also *Sorbus aucuparia*, a species whose seeds are efficiently dispersed by birds, showed higher values of genetic differentiation than *Populus tremula* (G_{ST} = 29%, RASPÉ *et al.*, 2000). A larger survey of haplotypic diversity in trembling aspen at European scale showed similar low values of genetic differentiation (C. BITTKAU, pers. com.). The high number of haplotypes detected within populations seems also to exclude a predominant role of the agamic propagation, typical of poplars, in shaping the distribution of diversity in this species.

The distribution of chloroplast haplotypic diversity in *Populus tremula* seems therefore to be strongly affected by its life history traits, in particular by the efficient seed dispersal mechanism. Frequent extinction and re-colonisation events characteristics of pioneer species could have disrupted the spatial structure of cpDNA which might have originated during the recolonization processes in the post-glacial period (LE CORRE *et al.* 1997). On the other hand, in European trembling aspen the role of the human activities in shaping the distribution of the diversity through admixture of genetic material of non autochthonous origin could be considered marginal, because of its limited economical importance.

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