Theor Appl Genet (2004) 109:1–9
DOI 10.1007/s00122-004-1609-9

ORIGINAL PAPER

C. Vettori · G. G. Vendramin · M. Anzidei · R. Pastorelli · D. Paffetti · R. Giannini

Geographic distribution of chloroplast variation in Italian populations of beech (*Fagus sylvatica* L.)

Received: 4 August 2003 / Accepted: 16 January 2004 / Published online: 10 March 2004

Abstract The distribution of chloroplast DNA (cpDNA) variation in Italian beech (*Fagus sylvatica* L.) populations was studied using PCR-RFLP and microsatellite markers. In total, 67 populations were analysed, and 14 haplotypes were identified by combining the two marker types. A remarkable subdivision of cpDNA diversity in Italian beech was found, as indicated by a high level of genetic differentiation (G_{st}=0.855). The highest level of total haplotype diversity (h_t=0.822) was estimated for southern Italian populations. The highest number of haplotypes was found in the central-southern region of the peninsula. The nested clade analysis provided evidence for past fragmentation events that may have been occurred during the Quaternary glaciations and had a major role in defining the genetic structure of the central-southern Italian beech populations. Only one haplotype apparently spread towards the north of Italy along the Apennine chain and reached the Italian slope of the western part of the Alps (Maritime Alps, Liguria). All haplotypes found along the Apennines remained trapped in the Italian peninsula. Southern and central Italy represent hotspots of haplotype diversity for Italian beech.

Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s00122-004-1609-9

Communicated by O. Savolainen

C. Vettori · G. G. Vendramin · M. Anzidei · R. Pastorelli · R. Giannini (✉)
Plant Genetics Institute, Division of Florence,
National Research Council, Via Madonna del Piano,
50019 Sesto Fiorentino-Florence, Italy
e-mail: raffaello.giannini@igv.cnr.it
Tel.: +39-55-5225704
Fax: +39-55-5225729

D. Paffetti · R. Giannini
Department of Forest Environmental Science and Technologies,
University of Florence,
Via San Bonaventura 13, 50145 Florence, Italy

Introduction

Pollen and genetic data suggest that the Italian peninsula was one of the main European refugia during the last glaciation for many forest tree species (Petit et al. 2003). For this reason, Italian forests display high population differentiation and, therefore, can be considered to be extremely important with respect to the conservation of genetic resources. Due to these characteristics and as a consequence of the migration processes in the post-glacial period, the Italian populations of many forest tree species are typically different from their counterparts found in other parts of Europe (Demesure et al. 1996, for beech; Palmé and Vendramin 2002, for hazelnut; Fineschi et al., 2002, for oak).

For European beech, *Fagus sylvatica* L., both pollen (Huntley and Birks 1983; Magri 1998; Brewer 2001) and genetic (Demesure et al., 1996; Comps et al. 2001) data indicate the presence of two main refugia during the last glaciation—one located in the south of the Italian peninsula and another one in the Balkan peninsula—from where the recolonisation of the Italian peninsula and of the rest of Europe, respectively, presumably started. In southern and central Italy, beech has been continuously found since the late glacial period (Magri 1998). Watson (1996) reported that *Fagus* appeared earlier in the eastern than in the western part of the region during the mid-Holocene and become dominant in the northern Apennine forests about 4,000–3,000 years Before Present (BP). In the Alps, where immigration from the east was observed, *Fagus* pollen has been dated to about 6,000–7,000 years BP and has been found in much older strata extending towards Slovenia than in that extending towards France. Therefore, on the basis of the palynological data, a different origin of the Apennine and Alpine Italian populations was hypothesised (Magri 1998).

The analysis of molecular markers, in particular those localised in the organelle genomes, has significantly contributed to our understanding of the migration history of many forest tree species (Ferris et al. 1999; Petit et al. 2003). The geographic distribution of organelle markers

gives a clearer picture of past migration history than do nuclear markers because of the uniparental mode of inheritance of chloroplast (cp) DNA and because the effective population size is reduced in haploid markers. Moreover, in angiosperms, the cpDNA is generally maternally inherited, and their analysis subsequently provides a stronger signal of seed migration (Demesure et al. 1996; Petit et al. 2003).

In this paper, the geographic distribution of haplotype diversity of Italian populations of *F. sylvatica* was analysed. Our main objectives were (1) to examine the extent of genetic variation and the genetic structure of Italian populations of *F. sylvatica*; and (2) to detect any possible trend of variation of haplotype diversity related to the recolonisation process of the Italian peninsula. For these specific objectives, both PCR/RFLP (restriction fragment length polymorphism) and microsatellite chloroplast markers were used.

Materials and methods

Plant material

Sixty-seven Italian beech populations (five individuals each) were sampled along their natural range following a north-south and east-west gradient, for a total of 335 individuals (Fig. 1; see also Appendix 1 in ESM). Dormant buds were taken from each individual and stored at −20°C.

DNA extraction

DNA was isolated from dormant buds (100 mg as starting material) using the NucleoSpin Plant kit (Macherey Nagel, Germany) and following the manufacturer's specifications.

PCR/RFLP analyses of cpDNA

Amplifications of the cpDNA regions were performed in 20-μl volumes as reported by Paffeti et al. (2001) using the primer pairs for the intergenic spacers between the *trn*D-*trn*T (DT) region (Demesure et al. 1995) and for the *orf*184-*pet*A (OA) region (Grivet et al. 2001) (Table 1). The DT and OA amplification products (1 μg DNA) were digested with 30 U of the restriction enzymes, *Hae*III and *Hinf*I (Takara, Japan), respectively, following the manufacturer's specifications. The mixtures were analysed by gel electrophoresis on 8% (w/v) sequencing non-denaturing polyacrylamide gels (Sambrook et al. 1989). The gels were then stained with ethidium bromide, photographed and analysed with an UVP gel scanner (Photo-Capt, Vilbert Loormat, France). The digestion profiles of the two fragments were compared simultaneously with the digestion fragments of control DNA of known haplotypes (kindly provided by Remy Petit, INRA, Pierroton, France, and by Thomas Geburek, FBVA, Vienna, Austria).

Microsatellite analysis of cpDNA

Four primers pairs were used for the amplification of chloroplast microsatellite markers: ccmp4, ccmp6, ccmp7, and ccmp10 (Weising and Gardner 1999). These markers were selected because they display variation and/or because they can be multiplexed by size. PCR amplifications were carried out using a Perkin Elmer model 9700 thermal cycler (Perkin Elmer, Foster City, Calif.) following the protocols reported by Palmé and Vendramin (2002).

Sizing of the PCR products was carried out using a Pharmacia ALF Express automatic sequencer and the Pharmacia conversion software FRAGMENT MANAGER ver. 1.2 (Amersham Pharmacia Biotech, Piscataway, N.J.). Sizing was repeated at least twice and performed according to Vendramin et al. (2000).

DNA cloning and sequencing

The amplification products were gel purified using the QIAquick Gel Extraction kit (Qiagen, Germany) following the manufacturer's specifications. The DT and OA fragments and the four-microsatellite regions were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, Calif.) using the TOPO TA Cloning kit (Invitrogen). Sequencing of the cloned fragments was carried out in both directions from independent amplification reactions using the dideoxy-chain termination method (Sanger et al. 1977), the Sequenase kit (USB), the M13 universal primers and DT and OA internal primers (Table 1). The four DT, four OA, and four microsatellite regions sequenced have the following accession numbers, respectively: AF525755, AF525756, AF525757, AF525758, AF525759, AF525760, AF525761, AF52762, AY563558, AY563559, AY563560, AY563561.

The computer software BLAST (http://www4.ncbi.nlm.nih.gov/BLAST/) was used to determine the genes and the coding domain region (CDS) by comparison with the sequence of *Nicotiana tabacum* (Z00044) in order to evaluate the similarity among the DT and OA fragments of *F. sylvatica*, *N. tabacum* and other species, to translate the CDS regions and to identify, by similarity, the identity of the hypothetical CDS product (results are reported in Appendix 2 as ESM). DNA sequences were multiply aligned using the CLUSTAL X programme (Thompson et al. 1997) among themselves and with the sequence of *N. tabacum*. The multiple alignments of the DT and OA regions of *F. sylvatica* L. indicated that their size varied

Table 1 Primers used for the amplification and sequencing of the DT and OA cpDNA regions

Name	Primer F (5′-3′)	Sequences	Primer R (5′-3′)	Sequences	Position in *Fagus sylvatica*[a]	Size (in basepairs)	References
DT	*trn*D	accaattgaactacaatccc	*trn*T	ctaccactgagttaaaaggg	1–1,661 (31,988–33,200)	1,661–1,664	Demesure et al. 1995
DT_1-DT_2	DT_1	GTAGACATATTGCC-AACG	DT_2	CCCGAAAGATCCTAT-ACC	518–1,032 (32,119–32,667)	515	This study
OA	*orf*184	tggcgatcagaacayatatggatag	*pet*A	ccctckgaaacaagaagtt	1–2,889 (62,644–65,092)	2,889–2,893	Grivet et al. 2001
OA_1-OA_2	OA_1	AACTGAAAAGATTG-AATGC	OA_2	ATATATATCATGAGC-GATGC	562–2,208 (63,202–64,411)	1,647–1,651	This study
OA_3-OA_4	OA_3	TATTCGTTAGTATCG-CATGG	OA_4	GATACAATCCTCATT-GTGC	1147–1523 (63360–63726)	377	This study

[a] The position referred to is the sequence with accession no. AF525755 for the DT, and with accession no. AF525759 for the OA. The position in the *Nicotania tabacum* (Z00044) sequence is reported in parenthesis

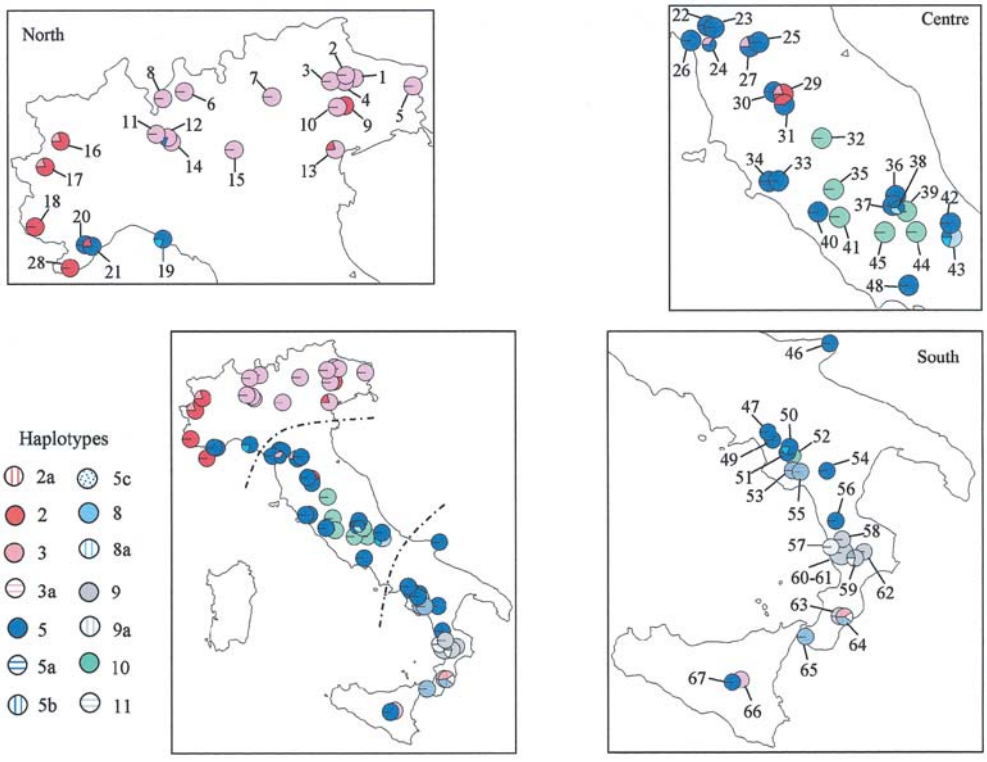

Fig. 1 Geographical distribution of chloroplast haplotypes in Italian populations of *Fagus sylvatica*. The *number* indicates the populations; see Appendix 1 in the ESM for names of populations and their locations

from 1,661 bp to 1,664 bp and from 2,889 bp to 2,893 bp, respectively. Sequence analyses revealed the presence of two microsatellites [DT-CDR1: mononucleotide A repeat (9/12); DT-CDR2: mononucleotide A repeat (7)] in the DT_2 polymorphic fragments (from 496 bp to 499 bp) and three microsatellites [OA-CDR1: mononucleotide T repeat (9/10); OA-CDR2: mononucleotide T repeat (8/9); OA-CDR3: mononucleotide T repeat (9/12)] in the OA_2 polymorphic fragment (from 462 bp to 466 bp). The alignment of the entire DT and OA regions sequenced with the same regions of *N. tabacum* showed an identity of 90% and 88%, respectively.

The sequencing of all four fragments amplified using chloroplast microsatellite primer pairs confirmed the presence of the mononucleotide stretches. The microsatellite was represented by a A/T (CCMP4, CCMP6, CCMP10) or G/C (CCMP7) repeat. Length variation among the different cpSSR (simple sequence repeat) haplotypes detected in the Italian populations was due to differences in the number of repeats within the microsatellite stretches. No mutations were observed in the flanking regions.

Sequencing of identical haplotypes sampled in different populations revealed the same composition of the microsatellite regions, which seems to indicate the absence of size homoplasy.

Genetic diversity

Polymorphisms were scored as point mutations or insertion/deletions characters, and, together with the sizes of the chloroplast microsatellites, were used to define chloroplast haplotypes. Parameters of genetic diversity (h_s and h_t) and differentiation (G_{st} and N_{st}) in the chloroplast genome were estimated following the methods described by Pons and Petit (1995, 1996). These two parameters were compared using the U test (Pons and Petit 1996). The contribution of a given population to total diversity (C_t) was calculated as reported by Petit et al. (1997).

A hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed to estimate the amount of variation due to differences within and among geographic regions (northern, central and southern Italy). For this analysis the populations (pops) were assigned a priori on the basis of their geographic locations (north: pop 28, and from pop 1 to pop 21; central: pop 48, from pop 22 to pop 27 and from pop 29 to pop 45; South: pops 46 and 47, and from pop 49 to pop 67) (Fig. 1).

Nested clade analysis

The parsimony criterion was used to infer a haplotype network with the method described in Templeton et al. (1992) and implemented in TCS ALPHA, ver. 1.01 (Clement et al. 2000). A nested clade analysis of the spatial distribution of genetic variation was performed as such an analysis permits discrimination between gene flow and historical events (past fragmentation, range expansion, colonisation) that have produced the observed patterns at the population and species levels (Templeton et al. 1995; Templeton 1998). The haplotype network was converted manually into a nested design using the rules defined by Templeton et al. (1987) and Templeton and Sing (1993). Once the cladogram had been converted into the nested design, a categorical test for geographical association was performed. An exact permutational contingency analysis was performed for each clade, and a Chi-square statistic was calculated. An analysis that utilises information on geographical distance was used following Templeton et al. (1995). The statistical analyses applied to the constructed nested design were done with the programme GEODIS 2.0 (Posada et al., 2000). The interpretation of statistically significant patterns was made using the inference key reported in Templeton et al. (1995) and Templeton (1998). The TCS and GEODIS softwares are available at http://bioag.byu.edu/zoology/crandall_lab/programs.htm.

Results

Italian beech populations were studied by combining the PCR/RFLP and microsatellite cpDNA data, and a total of 14 different haplotypes were identified (Table 2). The ccmp6 and ccmp10 microsatellites were monomorphic.

Table 2 Description of the 14 haplotypes identified by combining PCR/RFLP and microsatellite polymorphic fragments

Haplotypes[a]	Number of polymorphic fragments			
	DT_2	OA_2	ccmp4	ccmp7
2	1	3	1	1
2a	1	3	1	2
3	1	4	1	1
3a	1	4	1	2
5	2	5	1	2
5a	2	5	2	2
5b	2	5	1	1
5c	2	5	1	3
8	2	4	1	2
8a	2	4	1	1
9	4	5	1	2
9a	4	5	1	1
10	2	7	1	2
11	5	5	1	2

[a] Letters indicate the contribution given by chloroplast microsatellite polymorphism in differentiating the haplotypes

The geographic distribution of the cpDNA haplotypes of the populations studied is reported in Fig. 1. The most frequent haplotypes are Hap 3 in northern Italy (20.5%), Hap 5 in central and southern Italy (21.7% and 11.8%, respectively), Hap 10 in central Italy (9.1%) and Hap 9 in southern Italy (8.5%). In particular, the frequency of Hap 5 is high along the Apennine chain and decreases along a line from the centre-south to the north (from 21.7–11.8% to 4.2%).

Most of the cpDNA differentiation was distributed among populations (G_{st}=0.855). The hierarchical analysis of molecular variance (AMOVA) indicated that the amount of variation was higher among and within geographic regions (southern, central and northern Italy) than within populations (Table 3). The total diversity for the southern Italian region was higher than in the other regions (h_t=0.82±0.050, 0.57±0.079, 0.57±0.090 for southern, central, and northern regions of Italy, respectively). The southern and central populations having a C_t greater than 0.005 contributed significantly to the total diversity, mostly as the result of their own divergence from the other populations. In some populations, intrapopulation polymorphism was also found (pops 12, 13, 16, 17, 19, 21, 24, 27, 29, 31, 38, 43, 50, 59 and 64), and the majority of the northern and central polymorphic populations were located in the north-west zone (pops 16, 17, 19, 21, 24, 27, 29 and 31). N_{st} (0.867) was similar to G_{st} (0.855), indicating that the haplotypes are phylogenetically equivalent [U test (N_{st}/G_{st})=1.23, not significant] and that only a geographical structure is present (Fig. 1).

The probability of parsimony (as defined in Templeton et al. 1992) for pairwise differences until the probability exceeds 0.95% was calculated automatically by the TCS programme. This analysis suggested that a most parsimonious relationship of four steps has a probability of greater than 95% of being true.

The haplotype network obtained from the TCS programme reported in Fig. 2 showed some ambiguities for the presence of more than one parsimonious connection of a haplotype to the rest of the network. These ambiguities were resolved following the criteria suggested by Crandal and Templeton (1993): (1) rare haplotypes are more likely to be found at the tip, and common haplotypes at interior nodes, of a cladogram; (2) a singleton (i.e., a haplotype represented by a single individual) is more likely to be connected to haplotypes from the same population than to haplotypes from different populations. The nested design was constructed manually following the rules defined in Templeton et al. (1987) and Templeton and Sing (1993) on the resolved haplotype network (Fig. 2). The nested contingency analysis indicated significant geographical associations within five clades and for the total cladogram (Fig. 2). Therefore, only these were interpreted using the inference key of Templeton et al. (1995) and Templeton (1998) (Table 4). Contiguous range expansion was inferred for the haplotypes nested in clade 1-2 and for the one-step level nested in clade 2-1 that is restricted to northern Italy. Past fragmentation events were hypothesised for the haplotypes nested in clades 1-4 and 1-5 and for the one-step level nested in clade 2-2 located throughout central-southern Italy and for the total cladogram.

Table 3 Analysis of molecular variance (AMOVA). Each geographic region (southern, central and northern Italy) corresponds to a group

Source of variation	*df*	Sum of squares	Percentage of variation
Among groups	2	234.260	49.21***
Among populations within groups	64	285.229	41.49***
Within populations	264	51.067	9.30***
Total	330	570.556	

***$P \leq 0.001$

Table 4 Interpretation of the results presented in Fig. 2 using the key of inference of Templeton et al. (1995) and Templeton (1998)

Clade	Chain of inference	Demographic event inferred
Haplotypes nested in 1-2	1-2-11-12-NO	Contiguous range expansion
Haplotypes nested in 1-4	1-2-3-5-15-NO	Past fragmentation
Haplotypes nested in 1-5	1-2-3-4-9-NO	Past fragmentation
One-step clades nested in 2-1	1-2-11-12-NO	Contiguous range expansion
One-step clades nested in 2-2	1-2-3-4-9-NO	Past fragmentation
Total cladogram	1-2-11-17-4-9-NO	Past fragmentation
	1-2-3–5-15-NO	Past fragmentation

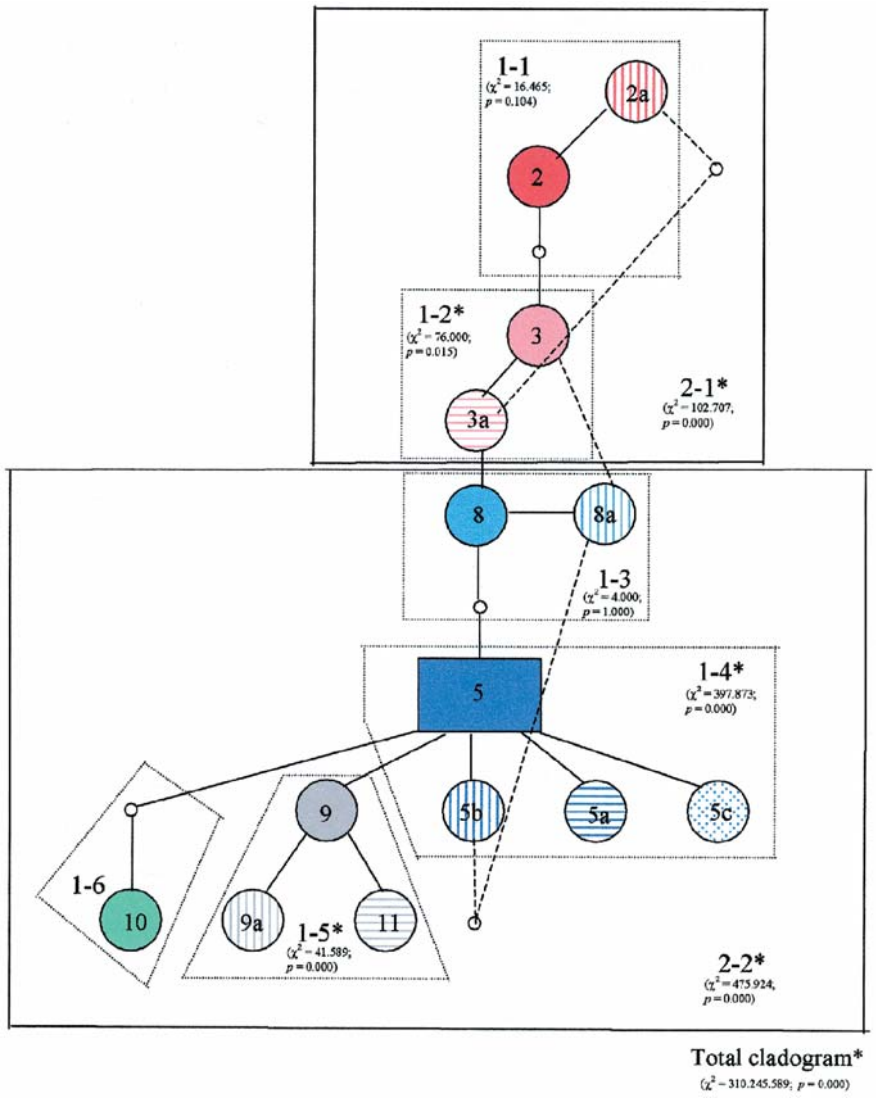

Fig. 2 The preferred resolution of the ambiguities (haplotypes with more than one parsimonious connection are represented by *dashed lines*) for the haplotype network, with the associated nested design used for the statistical analyses and inferred using the rules defined by Templeton et al. (1987) and Templeton and Sing (1993). A *circle* represents a haplotype, and a *number* identifies each haplotype. *Small empty circles* indicate intermediate haplotypes that are not present in the sample but are necessary to link all observed haplotypes to the network. Each *solid line* represents a single mutation change that interconnects two haplotype states that have a probability greater than 95%. *Thin-dashed-lined boxes* and *polygons* enclose one-step clades, which are designed by "*1-x*", where x is a number assigned to identify the clade; *thick-lined boxes* enclose two-step clades ("*2-x*"). The chi-square statistic (χ^2) is given *in parenthesis*, as is the probability (*P*) for each clade of the nested contingency analysis of geographical associations. *Value is significant at the 0.05 level

Discussion

Variation in cpDNA is geographically structured in Italian populations of beech (*F. sylvatica*), with a higher diversity estimated in the central-southern populations. The distribution of haplotype diversity in Italy appears to be associated with the migration history of this species. A different origin of the Apennine and Alpine populations is evident from the genetic data obtained in this study. Our results are in agreement with Magri (1998), who hypothesised, based on pollen analysis, that the populations of the northern Apennines originated from the southern-central Apennine region, while Slovenia (the Balkans) might have been the starting point of the recolonisation of the Alps. A previous study conducted on a European scale using cpDNA markers (Demesure et al. 1996) detected the presence of two main groups of haplotypes—one originated from the Balkans and spread all over Europe (Alps included) and the second was restricted to the Italian peninsula (Apennines), where it remained trapped. This study confirmed the presence of the typical divergent Italian haplotypes in the screened populations located along the Apennine chain.

As already pointed out by Grivet and Petit (2003), *F. sylvatica* and *Carpinus betulus* have interesting similarities, considering that both species show (1) a very high level of genetic differentiation among populations for chloroplast markers (a G_{st} greater than 0.85) and a similar distribution of chloroplast haplotypes across Europe; (2) haplotypes that remained trapped in the Italian peninsula, probably because of the earlier arrival of populations spreading from the other refugia.

The level of subdivision of cpDNA diversity in *Fagus* is remarkable. This high level of genetic differentiation is still evident at the level of the Italian populations (a G_{st} greater than 0.85). In general, the Italian populations are characterised by a high degree of fixation of a few haplotypes. In the central-southern part of the Italian peninsula, different haplotypes are found. The reduced cpDNA diversity within Italian beech populations may

also linked to the life history traits of the species. Beech is characterised by heavy seeds whose dispersal mechanisms are based mainly on gravity and partly mediated by animals, potentially resulting in a strong genetic differentiation among populations. The important role of the pollen and seed dispersal mechanisms in shaping the diversity has been clearly demonstrated (Petit 1999; Petit et al. 2003).

A drastic reduction in the population size of European beech has been documented to have occurred at the onset of post-glacial colonisation (Demesure et al. 1996). This event also affected allelic richness, as measured by isozyme markers, even if heterozygosity has remained at high levels in the more recently colonised populations (Comps et al. 2001). There are several recent studies showing that, in the temperate zone, post-glacial recolonisation processes have not necessarily been associated with large losses in genetic diversity (Comps et al. 2001; Widmer and Lexer 2001), in contrast to what has often been reported (Hewitt 1996). In our study, some evidence of a decrease in diversity from the south to the north was observed. In fact, the central-southern Italian beech populations showed higher intra-population polymorphism than the northern ones, and 8 out of 14 haplotypes were found exclusively in the central-southern populations.

Nested clade analysis indicated that past fragmentation events (Table 4) had the most important role in shaping the genetic structure of the Central-southern Italian beech populations. During the glacial periods of the Quaternary, the beech populations in Italy were mainly distributed at altitudes lower than where they are found today and they were restricted to a few and/or small areas (especially in the centre-south) (Magri 1998). Therefore, it can be supposed that with the retreat of the glaciers the populations spread to higher altitudes and to northern latitudes, thereby coming into contact and mixing haplotypes. Both past historical events and the orthography of the territory may have determined the actual beech distribution in Italy and the presence of unique haplotypes in the central-southern populations. The mixing of haplotypes present in the suture zones (north-west of the Italian distribution range) can be ascribed to the confluence between the routes coming from southern Italy (Hap 5) with that coming from the east through the Alps (probably from the Balkans; principally Hap 2 and Hap 3). This is supported by another nesting series where contiguous range expansion is the inferred explanation for the observed distribution pattern of haplotypes of clades 1-2 and 2-1. This result could be related to a scenario of population expansion following European Pleistocene glaciations. Magri (1998) suggested that beech populations might have survived during the last glacial period at different locations in the peninsula, so that no clear large-scale migration trends can be recognised in southern and central Italy. On the contrary, the data from northern Italy suggest that migrations took place from the eastern (older interior clade nested in 2-1) part of the Alps towards the western (younger tip clade) part. The results of our study seem to confirm these hypotheses. The presence of populations displaying high divergence in central-southern Italy may be associated to the fact that beech populations have persisted in these regions since the middle Pleistocene, as also suggested by Follieri et al. (1988). During the Saint Germain I period (about 110,000–95,000, BP), beech represented a major component of the vegetation in central Italy. Pollen data have revealed that the Italian peninsula might have been an important area for the survival of beech not only during the last glaciation but also during previous interglacial periods (Magri 1998).

Some haplotypes detected in the Balkan peninsula (principally Hap 2a and Hap 3) were also found in some populations of southern Italy. This seems to be the result of the presence of a connection, during the Pliocene, between the Italian and Balkan peninsulas, thereby confirming previous isoenzyme analysis (Gömöry et al. 1999), which favoured gene flow and allowed genetic admixture between the gene pool originated from two different refugia areas. Similar evidence has been observed for some other tree species. For example, western Greek populations of *Pinus halepensis* share haplotypes with Apulia (Italy) populations but not with all the other populations across the circum-Mediterranean natural range (Morgante et al. 1998).

The results obtained in this study demonstrate the importance of the Italian refugia in shaping the actual distribution of diversity as well as the relevance of this area for the conservation of the genetic resources of this species. This study has revealed that beech populations from central and southern Italy deserve high conservation priority, considering that these populations have unique haplotypes not observed in other European populations. Of course, adaptive traits need to be analysed for a more precise definition of the conservation value of these populations. A strong genetic differentiation at nuclear microsatellites markers between Italian and European populations was also observed (data not shown).

Acknowledgements The study was carried out with financial support from the Commission of the European Union (EU project FAIR3 CT96 1464). We thank R. Petit (INRA, Pierroton, France) and T. Geburek (FBVA, Vienna, Austria) for providing control DNA samples and useful suggestions in the laboratory work organization, and G. Stotzky (New York University, USA), L. Paule (University of Zvolen, Slovakia), G. Mastromei and C. Indorato (University of Florence, Italy) for helpful comments on the manuscript. We express our gratitude to A. M. Proietti, P. Giaimi, R. Mercurio, F. Mancini, and the Italian Forest Services for assistance during the collection of materials.

Appendix 1

Italian *Fagus sylvatica* populations analysed using PCR/RFLP and SSRs cpDNA markers The two last capital letters of the population names indicate the provinces (Table 5).

Table 5

	Population name	Latitude	Longitude	Altitude (m.a.s.l.)
1	Giau Merlot, Valvisdende (Santo Stefano di Cadore, BL)	46°37′	12°41′	1,350
2	Passo Zovo (Auronzo, BL)	46°33′	12°26′	1,350–1,150
3	Val da Rin (Auronzo, BL)	46°32′	12°24′	1,300
4	Foresta Sommadida (Auronzo, BL)	46°32′	12°18′	1,150
5	Laghi di Fusine (Tarvisio, UD)	46°29′	13°43′	930
6	Bagni di Masino (Traona, SO)	46°10′	9°39′	1,000
7	Molveno (TN)	46°10′	11°00′	860–1,040
8	Monte Lugone (Carlazzo, CO)	46°04′	8°13′	1,000
9	Cansiglio, Pian Pannocchia (Fregona, TV)	46°03′	12°2′	1100
10	Passo San Boldo (Cison di Valmareno, TV)	46°01′	12°13′	1,000
11	Monte Generoso (Orimento, CO)	45°56′	9°06′	1,700
12	Ballabio (Piani Resinelli, LC)	45°53′	9°26′	697
13	Montello (Nervesa della Battaglia, TV)	45°49′	12°12′	370
14	Valle Imagna (Roncola, BG)	45°48′	9°33′	950
15	Monte Magno (Gargnano, BS)	45°43′	10°40′	1,040
16	Monte Arvic (Issogne, AO)	45°38′	7°45′	1,100
17	S. Ambrogio, Colle Braida—Laghi Avigliana (Giaveno, TO)	45°05′	7°23′	1,010
18	Val Maira—Rio Droneretto (San Damiano Macra, CN)	44°31′	7°16′	1,100–1,300
19	Monte Penna (Rezzoaglio, GE)	44°29′	9°30′	1,350–1,500
20	Alta Val Tanaro (Bognasco, CN)	44°15′	8°02′	1,400
21	Passo Melogno (Calizzano, SV)	44°14′	8°13′	900–1,030
22	Val di Luce (Fiumalbo, MO)	44°10′	10°38′	1,420–1,780
23	Monte Gomito (Abetone, PT)	44°08′	10°38′	1,700
24	Pian di Novello (Cutigliano, PT)	44°07′	10°42′	1,200
25	Passo della Futa (Scarperia, FI)	44°05′	11°17′	900
26	Monte Altissimo (Castelnuovo Garfagnana, LU)	44°03′	10°14′	1,000
27	Aquerino (Pistoia, PT)	43°59′	11°04′	850
28	Monte Ceppo (Molini di Triora, IM)	43°57′	7°49′	1,400
29	Passo dei Mandrioli (Bagno di Romagna, FO)	43°50′	11°57′	1,100
30	Falterona (Dicomano, FI)	43°52′	11°43′	1,250
31	Montete Castello (Caprese Michelangelo, AR)	43°39′	11°59′	1,300
32	Monte Subasio (Assisi, PG)	43°04′	12°17′	1,140
33	Monte Cetona (Sarteano, SI)	42°56′	11°55′	1,050
34	Monte Amiata (Abbadia San Salvatore, SI)	42°54′	11°41′	1,680
35	Monte Martano (Giano dell'Umbria, PG)	42°49′	12°37′	1,030
36	Frazione Valloni (Valle Castellana, TE)	42°44′	13°29′	450
37	Lago di Campotosto (Campotosto, AQ)	42°33′	13°25′	1,300
38	Prati di Tivo (Pietracamela, TE)	42°31′	13°32′	1,450
39	Colle Mesola (Arsita, TE)	42°27′	13°45′	1,100
40	Monte Cimino (Soriano, VT)	42°24′	12°15′	1,030
41	Monte Tancia (Monte San Giovanni in Sabina, RI)	42°19′	12°47′	1,250
42	Passo Lanciano (Pretorio, CH)	42°11′	14°10′	1,630
43	Valle Favorana (Guardiagrele, CH)	42°10′	14°12′	960
44	Monte Morrone (Sulmona, AQ)	42°05′	13°60′	1,200
45	Marsia (Tagliacozzo, AQ)	42°04′	13°14′	1,500
46	Foresta Umbra (Vieste, FG)	41°53′	16°10′	700
47	Monte Pecoraro (Capracotta, CB)	41°48′	14°18′	1,400
48	Val Fondillo (Opi, AQ)	41°47′	13°50′	1,200
49	La Montagnola (Civitanova del Sannio, CB)	41°38′	14°26′	1,050
50	Laghi di Monticchio (Rionero Vulture, PZ)	40°56′	15°39′	650
51	Passo Crocelle (San Fele, PZ)	40°48′	15°35′	1,100
52	Monte Caruso (Avigliano, PZ)	40°44′	15°44′	1,220
53	M.te Volturino (Marsico Nuovo, PZ)	40°25′	15°44′	1,400
54	Bosco Monte Pasquino (Laurenzana PZ)	40°24′	16°02′	1,000
55	Alta Val d'Agri (Viggiano, PZ)	40°23′	15°57′	1,200
56	Cugno Curnone (Terranova Pollino, PZ)	39°59′	16°16′	1,300–2,000
57	Acqualonga (Fuscaldo, CS)	39°25′	16°07′	1,020
58	Varco S. Mauro (Rose, CS)	39°25′	16°26′	1,200
59	Fago del Soldato (Camigliatello Silano, CS)	39°20′	16°27′	1,500
60	Macchialonga (Spezzano Piccolo, CS)	39°17′	16°24′	1,300
61	Sila di Tasso (Spezzano Sila, CS)	39°17′	16°24′	1,400
62	Foresta Gariglione (Mesoraca, KR)	39°08′	16°42′	1,400
63	M. te Pecoraro (Serra S. Bruno, CZ)	38°35′	16°20′	1,200
64	Bosco di Stilo (San Nicola di Ferdinandea, CZ)	38°34′	16°28′	1,150
65	Monte Basilicò (Reggio Calabria, RC)	38°09′	15°53′	1,400
66	Annunziata (Randazzo, CT)	37°53′	14°57′	1,650
67	M. te Soro, Nebrodi (Cesarò, ME)	37°50′	14°43′	1,600

Appendix 2

Blast results. The BLAST of each DT and OA sequence enabled us to determine: (1) the partial *trn*D (tRNA-Asp) gene, the *trn*Y (tRNA-Tyr) gene, the *trn*E (tRNA-Glu) gene, and the partial *trn*T (tRNA-Thr) gene in the DT region; (2) the partial *orf*184, the *orf*229, and the partial *pet*A gene in the OA region. The genes and open reading frames (ORFs) determined in the DT and OA regions had a very high homology with those sequenced in *Nicotiana tabacum* L. (Z00044) and other species. In fact, BLAST analyses gave identity values ranging from 100% for tRNA to 84% for ORF229. The DT-CDR1 and DT-CDR2 microsatellites are contained in the *trn*D-*trn*Y intergenic spacer, which is longer than that found in *N. tabacum* (476 bp vs. 108 bp). The OA-CDR1, OA-CDR2, and OA-CDR3 microsatellites are located in the ORF184-ORF229 intergenic region, which is longer than that in *N. tabacum* (667 bp vs. 221 bp).

References

Brewer S (2001) Recolonisation postglaciaaire de quelques taxons tempérés en Europe: approche spatiale et temporelle. PhD thesis, Université d'Aix-Marseille, France

Clement M, Posada D, Krandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1659

Comps B, Gömöry D, Letouzey J, Thiébaut B, Petit RJ (2001) Diverging trends between heterozygosity and allelic richness during postglacial colonization in the European beech. Genetics 157:389–397

Crandall KA, Templeton AR (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. Genetics 134:959–969

Demesure BN, Sodzi BN, Petit RJ (1995) A set of universal primers for amplification of polymorphic noncoding regions of mitochondrial and chloroplast DNA in plants. Mol Ecol 4:129–131

Demesure B, Comps B, Petit RJ (1996) Chloroplast DNA phylogeography of the common beech (*Fagus sylvatica* L.) in Europe. Evolution 50:2515–2520

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Ferris C, King RA, Hewitt GM (1999) Isolation species and history of glacial refugia. In: Hollingsworth PM, Bateman RM, Gornall RJ (eds) Molecular systematics and plant evolution. Taylor and Francis, London, pp 20–34

Fineschi S, Taurchini D, Grossoni P, Petit RJ, Vendramin GG (2002) Chloroplast DNA variation of white oaks in Italy. For Ecol Manage 156:103–114

Follieri M, Magri D, Sadori L (1988) 250,000-year pollen record from Valle di Castiglione (Roma). Pollen Spores 30:329–356

Gömöry D, Paule L, Brus R, Zhelev P, Tomović Z, Gračan J (1999) Genetic differentiation and phylogeny of beech on the Balkan peninsula. J Evol Biol 12:746–754

Grivet D, Petit RJ (2003) Chloroplast DNA phylogeography of the hornbeam in Europe: evidence for a bottleneck at the outset of postglacial colonization. Conserv Genet 4:47–56

Grivet G, Heinze B, Vendramin GG, Petit RJ (2001) Genome walking with consensus primers: application to the large single copy region of chloroplast DNA. Mol Ecol Notes 1:345–349

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. Biol J Linn Soc London 58:247–276

Huntley B, Birks HJ (1983) An atlas of past and present pollen maps for Europe, 0–13,000 years ago. Cambridge University Press, Cambridge

Magri D (1998) Quaternary history of *Fagus* in the Italian peninsula. Ann Bot 61:147–154

Morgante M, Felice N, Vendramin GG (1998) The analysis of hypervariable chloroplast microsatellites in *Pinus halepensis* reveals a dramatic genetic bottleneck. In: Karp A, Isaac PG, Ingram DS (eds) Molecular tools for screening biodiversity: plants and animals. Chapman and Hall, London, pp 407–412

Paffetti D, Vettori C, Giannini R (2001) Relict populations of *Quercus calliprinos* Webb on Sardinia Island identified by chloroplast DNA sequences. For Genet 8:1–11

Palmé AE, Vendramin GG (2002) Chloroplast DNA variation, post glacial recolonization and hybridization in hazel, *Corylus avellana*. Mol Ecol 11:1769–1779

Petit RJ (1999) Diversité génétique et histoire des populations d'arbres forestiers. Dossier d'habilitation a diriger des recherches, Université de Paris-Sud, U.F.R. Scientifique d'Orsay

Petit RJ, El Mousadik A, Pons O (1997) Identifying populations for conservation on the basis of genetic markers. Conserv Biol 12:844–855

Petit RJ, Aguinagalde I, de Beaulieu J-L, Bittkau C, Brewer S, Cheddadi R, Ennos R, Fineschi S, Grivet D, Lascoux M, Mohanty A, Müller-Stark G, Demesure-Musch B, Palmé A, Martín JP, Rendell S, Vendramin GG (2003) Glacial refugia: hotspots but not melting pots of genetic diversity. Science 300:1563–1565

Pons O, Petit RJ (1995) Estimation, variance and optional sampling of gene diversity. I. Haploid locus. Theor Appl Genet 90:462–470

Pons O, Petit RJ (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. Genetics 144:1237–1245

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. Mol Ecol 9:487–488

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. Mol Ecol 7:381–397

Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. Genetics 134:659–669

Templeton AR, Boerwinkle E, Sing CF (1987) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and an analysis of alcohol dehydrogenase activity in *Drosophila*. Genetics 132:659–669

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132:619–633

Templeton AR, Routman E, Philips CA (1995) Separating structure from population history: a clade analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrium*. Genetics 140:767–782

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Vendramin GG, Anzidei M, Madaghiele A, Sperisen C, Bucci G (2000) Chloroplast microsatellite analysis reveals the presence of population subdivision in Norway spruce (*Picea abies* K.). Genome 43:68–78

Watson CS (1996) The vegetation history of the northern Apennines, Italy: information from three new sequences and a review of the regional vegetational change. J Biogr 23:805–841

Weising K, Gardner RC (1999) A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome 42:9–19

Widmer A, Lexer C (2001) Glacial refugia: sanctuaries for allelic richness, but not for gene diversity. Trends Ecol Evol 16:267–269