Chloroplast DNA phylogeography of European ashes, *Fraxinus* sp. (Oleaceae): roles of hybridization and life history traits

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Abstract

We investigated range-wide phylogeographic variation in three European ash species (*Fraxinus* sp., Oleaceae). Chloroplast DNA (cpDNA) microsatellites were typed in the thermophilous *Fraxinus angustifolia* and *Fraxinus ornus* and the observed haplotypes and the geographic distribution of diversity were compared to cpDNA data previously obtained in the more cold-tolerant *Fraxinus excelsior*. We found wide-ranging haplotype sharing between the phylogenetically close *F. angustifolia* and *F. excelsior*, suggesting hybridization (i) in common glacial refuges in the Iberian Peninsula, northern Italy, the eastern and/or Dinaric Alps and the Balkan Peninsula, and/or (ii) during postglacial recolonization. The data allowed us to propose additional glacial refuges for *F. angustifolia* in southern Italy and in Turkey, and populations from the latter region were particularly polymorphic. There was evidence for refuge areas in Italy, the Balkan Peninsula and Turkey for *F. ornus*, which did not share any single chloroplast haplotype with the other species. In both *F. angustifolia* and *F. ornus*, cpDNA diversity (*h*<sub>S</sub> = 0.027 and *h*<sub>S</sub> = 0.009, respectively) was lower and fixation levels (*G*<sub>ST</sub> = 0.964 and *G*<sub>ST</sub> = 0.983, respectively) higher than in sympatric *F. excelsior* (*h*<sub>S</sub> = 0.096, *G*<sub>ST</sub> = 0.870). These diversity patterns could be due to temperature tolerance or the demographic history.

Keywords: chloroplast DNA (cpDNA), chloroplast microsatellites, *Fraxinus*, hybridization, life history traits, phylogeography

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Introduction

Hybridization is a common phenomenon in plants (Arnold 1997). The backcrossing of hybrid offspring with a parental species generates introgression, i.e. the integration of foreign genes into a recipient species’ gene pool. In plants, introgression is often more readily observed for maternally inherited genes than for biparentally inherited genes (Arnold 1997; Martinsen *et al*. 2001; Wu & Campbell 2005).

In broadleaved tree genera or species complexes, the sharing of maternally inherited markers like chloroplast DNA was frequently observed, for instance in oaks (*Quercus* spp., Petit *et al*. 1997, 2002; Belahbib *et al*. 2001), birches (*Betula* spp., Palmé *et al*. 2004), poplars (*Populus* spp., Rajora & Dancik 1992; Lexer *et al*. 2005) and willows (*Salix* spp., Hardig *et al*. 2000; Palmé *et al*. 2003) and predominantly explained through hybridization and introgression. Hybridization is an evolutionarily important process because it can contribute to adaptation. This may happen through the transfer of adaptations of one species to another or through the generation of novel adaptive variation (Burke & Arnold 2001; Rieseberg *et al*. 2003). Patterns
of haplotype sharing can inform on the age of hybridization; introgression gradients in hybrid zones most probably reflect hybridization in relatively recent generations (Lexier et al. 2005), whereas on a regional scale, diversity patterns can result from more ancient hybridization events. For instance in oaks a patchy, almost species-independent biogeographic distribution of chloroplast haplotypes was observed and mating patterns and succession behaviour allowed to attribute it to hybridization of Quercus petraea by pollen with already established Quercus robur during postglacial recolonization (e.g. Dumolin-Lapègue et al. 1997; Petit et al. 1997). Mating patterns and natural selection are the main factors that affect the incidence and the direction of hybridization (Loveless & Hamrick 1984; Barton & Hewitt 1985). For gene flow to occur pollen and pollinator abundance are critical; for example, organisms may hybridize more often at species edges, in situations where conspecifics are rare and heterospecifics abundant (Rieseberg 1997; Burke & Arnold 2001; Chan & Lewin 2005). Selection further limits hybridization through pre- and postzygotic barriers (Barton & Hewitt 1985). In marginal habitats, hybrid variants may have a greater probability of survival because selection pressures differ from those in the main species ranges; also, hybrid founder events may play an important role in their establishment (Burke & Arnold 2001). The interaction of these forces results in asymmetric introgression in many plant species (Dumolin-Lapègue et al. 1999; Schweigart & Willis 2003; Palmé et al. 2004; Wu & Campbell 2005).

Three ash species are native to Europe, the common ash, Fraxinus excelsior L., the narrow-leaved ash, Fraxinus angustifolia Vahl, and the flowering ash, Fraxinus ornus L. Fraxinus excelsior and F. angustifolia are both wind-pollinated and wind-dispersed tall tree species. F. excelsior is polygamous (male, female and hermaphrodite individuals, Picard 1982; Wallander 2001) and occurs over most of Europe whereas F. angustifolia is hermaphrodite (M. Palada, M. Verdú & S. Rendell, personal communication, 2005) and thrives in the Mediterranean region (Tutin et al. 1972; Wallander 2001). They are sympatric in the basins of large rivers in Western and Central Europe (Fukarek 1971; Rameau et al. 1989; Morand-Prieur et al. 2002; Volk 2002; French Government 2000, available at www.ecologie.gouv.fr/IMG/natura2000/habitats/pdf/tome1/91F0.pdf), where the flooding-tolerant, warmth-loving F. angustifolia grows in river-bank forests, whereas F. excelsior is an upland species preferring slopes (Jelem 1974). Morphologically intermediate individuals suggest the natural occurrence of interspecific hybrids (Poinso 1972; Jelem 1974; Rameau et al. 1989), and hybridization was experimentally confirmed (Morand-Prieur et al. 2002). Mitochondrial (Morand et al. 2001) and chloroplast DNA markers (Morand-Prieur et al. 2002) failed to discriminate among French samples of the two species, and analysis of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA revealed only few species-specific polymorphisms (Jeandroz et al. 1995; Wallander 2001), confirming their close relatedness. F. ornus is an androdioecious (Dommé et al. 1999; Wallander 2001) sub-Mediterranean species growing up to relatively high altitudes in its essentially eastern Mediterranean distribution (Tutin et al. 1972; Huntley & Birks 1983). It differs from both other European ashes by its life form, as it is an understorey shrub, and by its ambophilius pollination, i.e. combined insect and wind-pollination (Wallander 2001). Hybridization is unlikely to occur between F. ornus and the other ashes because they belong to different sections of the genus (Jeandroz et al. 1997; Wallander 2001). In addition, flowering is most precocious in F. angustifolia (December–February in France) and there is a partial phenology overlap with F. excelsior (March–April in France) but not with F. ornus (June in France, N. Frascaria-Lacoste, personal communication, 2005), which only flowers after leafing, contrarily to the other species (Wallander 2001). In Spain and Greece where F. excelsior is absent, no overlaps were recorded between flowering times of F. angustifolia and F. ornus (M. Verdú, personal communication, 2005).

In the fossil pollen record, F. excelsior and F. angustifolia pollen grains are not distinguished (Huntley & Birks 1983; Gliemeroth 1997; Brewer 2002). Palynological data revealed north and westward expansion of F. excelsior-type ash from 9 14C ka BP, late compared to other tree species, from ice age refuges located in the eastern Alps, in the Balkan Peninsula and possibly in Italy and north of the Black Sea (Huntley & Birks 1983; Gliemeroth 1997; Brewer 2002). Gliemeroth (1997) additionally documented spreading from southern Spain, whereas Brewer (2002) suggested refuges without evidence for postglacial expansion in the Iberian Peninsula and in southern Italy. Chloroplast microsatellite markers were in agreement with postglacial recolonization of F. excelsior from refuges located in the eastern Alps and the three southern European Peninsulas (Heuertz et al. 2004). F. ornus pollen is less abundant in sediments because of the ambophilius pollination. Huntley & Birks (1983) were the only authors to distinguish it and identified postglacial expansion around 9 14C ka BP northward from a single refuge located in western Greece in this species. Palynological information of Fraxinus species in Turkey is scarce (e.g. Eastwood et al. 1999). To date, it is still unclear to what extent glacial refuges and postglacial recolonization routes were shared between F. excelsior and the other two ash species.

In this study, we used chloroplast microsatellites (chloroplast simple sequence repeats, cpSSRs) to investigate patterns of genetic diversity and population genetic structure throughout the distribution ranges of F. angustifolia and F. ornus. The results were compared with cpDNA data previously obtained in F. excelsior (Heuertz et al. 2004) to test for geographic patterns of haplotype sharing reflecting
hybridization and introgression. A joint interpretation of the cpDNA data with fossil pollen data allowed us to outline the roles of life history traits and hybridization during glacial isolation and postglacial recolonization in European ashes.

### Materials and methods

#### Sampling

Leaf or bud samples were collected from an average of 6.11 (± 1.51 SD) *Fraxinus angustifolia* and 6.17 (± 1.26 SD) *Fraxinus ornus* trees in 70 and 59 putatively natural populations covering most of their respective distribution ranges (Fig. 1; Tables S1, S2, Supplementary material). Sampled trees were spaced by at least 80 m to avoid collecting closely related individuals. Leaves were stored dried or frozen at −80 °C whereas buds were frozen at −80 °C.

#### DNA extraction

Total DNA was extracted with the cetyltrimethyl ammonium bromide (CTAB) procedure of the NucleoSpin Plant kit (Macherey-Nagel) from approximately 50 mg of dry leaves, 100 mg of fresh leaves, or from 50 mg fresh weight of buds, ground by hand or in the automatic grinding mill MM200 (Retsch).

#### Chloroplast microsatellite analysis

Mononucleotide (A/T) chloroplast microsatellites (cpSSRs) were amplified with six universal primer pairs for angiosperms (ccmp2, ccmp3, ccmp4, ccmp6, ccmp7, and ccmp10 from Weising & Gardner 1999) as previously described for *Fraxinus excelsior* (Heuertz et al. 2004). Briefly, polymerase chain reaction (PCR) was performed for 25 cycles with GE Healthcare reagents, PCR products were run on an automated ALF Express DNA sequencer (GE Healthcare) and fragment sizes scored in comparison with internal and external size standards using the software fragment manager 1.2 (GE Healthcare). At each cpSSR, all different size variants were cloned into a plasmid vector (TOPO TA Cloning Kit, Invitrogen) and sequenced in both directions on an ALF Express DNA sequencer (GE Healthcare).

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**Fig. 1** Geographic distribution and frequency of chloroplast microsatellite haplotypes in (a) *Fraxinus angustifolia*, (b) *Fraxinus excelsior* (from Heuertz et al. 2004) and (c) *Fraxinus ornus*, and (d) statistical parsimony network representing the minimum number of length differences (bp) between haplotypes (□ *F. ornus*, ▼ *F. excelsior*, △ *F. angustifolia*, ◊ shared between *F. excelsior* and *F. angustifolia*).
Genetic data analysis

The genetic analysis was performed on data from the three European ash species, *F. angustifolia* (*n* = 428), *F. ornus* (*n* = 364), and *F. excelsior* (*n* = 1280 from 201 populations, Heuertz et al. 2004). Sequencing results confirmed that all differences between cpSSR size variants were due to variable numbers of poly(A) or poly(T) repeats. Haplotypes were defined as distinct combinations of size variants at the six cpSSRs, and a statistical parsimony network was built with the software tcs 1.18 (Clement et al. 2000) to visualize the minimum number of length differences (bp) between haplotypes. For the following analyses, the genetic distance between haplotypes *i* and *j* was defined as their squared absolute length difference averaged over the *K* microsatellite regions, $D_{ST}^2(ij) = 1/K\sum_{k=1}^{K}(a_{ik} - a_{jk})^2$, with $a_{ik}$ and $a_{jk}$ being the lengths in bp of variants detected in haplotypes *i* and *j* in region *k*, respectively. $D_{ST}^2(ij)$ is based on Goldstein et al.'s (1995) distance assuming a microsatellite stepwise mutation model and accounts for cpSSRs being linked on the cpDNA molecule (Echt et al. 1998; Vendramin et al. 1998).

In each species, the level of polymorphism within populations was estimated as haplotypic richness $A_S$ (El Mousadik & Petit 1996) using the software estat (Goudet 1995). For a population with sample size *N* and *N*$_i$ occurrences of haplotype *i* among the *N* samples, $A_S = \sum_i [1 - (N_i/N)^N]$/($N_i$) estimates the number of haplotypes expected in a sample of size *n* < *N*. By choosing the same *n* for each $A_S$ estimate, the numbers of haplotypes in samples of different sizes can be compared. We estimated haplotypic diversity within populations according to Pons & Petit (1996), ignoring genetic distances between haplotypes ($h_{ij}$) or taking them into account ($v_{ij}$). Haplotypic diversity statistics were similarly calculated for each total species sample ($h_T$ and $v_T$, respectively). For ease of comparison with *h*-type statistics, all $v$-type statistics were divided by the weighted mean genetic distance between haplotypes, using the respective species' haplotype frequencies as weights (Petit et al. 2002). Differentiation among populations was computed from unordered and from ordered alleles ($G_{ST}$ and $N_{ST}$, respectively) and the contribution of allele phylogeny to total differentiation was assessed with a permutation method (Burban et al. 1999; Hardy et al. 2003). It involves permutation of genetic distances between pairs of haplotypes, and a significant one-tailed test, $N_{ST} \geq N_{ST}$ permuted (i.e. $N_{ST} \geq G_{ST}$), reflects that closely related haplotypes are found together in polymorphic populations more often than expected by chance, which can be interpreted as a phylogeographic signal within populations (Hardy & Vekemans 2002; spagedi online documentation, www.ulb.ac.be/sciences/ecoevol/spagedi.html). All computations were performed with spagedi version 1.2 (Hardy & Vekemans 2002).

In order to compare diversity and differentiation levels between species, results are presented for the southern European region where the three species co-occur. This area excludes populations from Turkey, where no *F. excelsior* samples were available and is hereforth referred to as the ‘sympatric area’.

To investigate whether genetic diversity was geographically structured, haplotype frequency maps were constructed using MapInfo Professional version 4.1 (MapInfo Corporation). We further used spagedi to test for patterns of isolation by distance in each species. Specifically, the differentiation $F_{ST}/(1 – F_{ST})$ between pairs of populations was regressed on the natural logarithm of geographic distance between pairs of populations (Rousset 1997). A significant pattern of isolation by distance occurs if the regression slope $blog$ is larger than 95% of the $blog$ values computed from 10 000 permutations of population locations among populations (one-sided test). This is equivalent to a Mantel test between a matrix of genetic distances and a matrix of geographic distances (Hardy & Vekemans 2002; spagedi online documentation). To test whether the geographic distribution of haplotypes was correlated between species, we performed an analysis of isolation by distance between populations belonging to different species, defining each species as a category using spagedi. This analysis was performed in the sympatric area.

Results

Genetic variation and phylogenetic relationships among haplotypes

The observed cpSSR size variants in the three *Fraxinus* species, their combination into haplotypes and haplotype frequencies are given in Table S3, Supplementary material; phylogenetic relationships between haplotypes are represented in a statistical parsimony network in Fig. 1d. In *Fraxinus angustifolia*, three cpSSRs of the six investigated were polymorphic, with two (ccmp7) or four size variants (ccmp6 and ccmp10), combining into a total of 13 haplotypes. Seven of them were shared with *Fraxinus excelsior* and occurred in 96% of *F. angustifolia* individuals. The private *F. angustifolia* haplotypes (H13–H18 in Table S3) were restricted to Turkey; four of them had variant 105 at ccmp10, which was not encountered in the other ash species. *Fraxinus ornus* displayed three polymorphic cpSSRs (ccmp2, ccmp6 and ccmp7) with two size variants each, giving a total of four haplotypes (H19–H22). Two of them, H19 and H20, were encountered in 95% of *F. ornus* individuals. *F. ornus* haplotypes differ from those in *F. excelsior* and *F. angustifolia* by a 4-bp difference at ccmp2. The statistical parsimony network shows that most haplotypes are separated from 1 to 4 others by just 1 bp and
reticulations reflect wide-ranging homoplasy. For instance, H04, H07, H13 and H16 carry all four size variant combinations at ccmp6 and ccmp10, which requires homoplous origins of at least one of these haplotypes. The distribution of H04 is in agreement with homoplous origins: it was found in Spain in both F. excelsior and F. angustifolia, and in Turkey in F. angustifolia.

Population genetic analysis and geographic distribution of diversity

CpSSR variation in F. angustifolia populations was low, with an average of \( A_s = 1.12 \) haplotypes per population and haplotypic diversity of \( h_s = 0.071 \) and \( v_s = 0.053 \) based on ordered or unordered alleles, respectively (Table 1). Populations from Turkey were much more polymorphic \( (A_s = 1.54, h_s = 0.303, v_s = 0.247) \) than populations from the rest of the range \( (A_s = 1.05, h_s = 0.027, v_s = 0.017; \text{one-tailed } t\text{-tests: } P \leq 0.001, P \leq 0.001, P \leq 0.01) \). In the sympatric area, comprising the three southern European peninsulas and excluding Turkey, F. angustifolia populations were less polymorphic than F. excelsior populations (one-tailed \( t\text{-tests: } A_s, P \leq 0.01; h_s, P \leq 0.01 \), Table 1). In F. ornus, cpSSR diversity was very low overall \( (A_s = 1.029, h_s = 0.018, v_s = 0.027) \), and in the sympatric area, \( A_s \) and \( h_s \) were lower than in F. angustifolia (one-tailed \( t\text{-tests: } P \leq 0.05, P \leq 0.05 \)) and F. excelsior (one-tailed \( t\text{-tests: } P \leq 0.001, P \leq 0.01 \), Table 1). Overall diversity was highest in F. angustifolia and lowest in F. ornus (Table 1). Differentiation among populations was very pronounced in all three species samples, with \( G_{ST} = 0.913 \) in F. angustifolia, \( G_{ST} = 0.888 \) in F. excelsior, and \( G_{ST} = 0.969 \) in F. ornus (Table 1). In F. angustifolia from Turkey, fewer populations were fixed than in the overall sample, resulting in lower among-population differentiation \( (G_{ST} = 0.538, \text{Table 1}) \). The contribution of phylogenetic relationships between haplotypes to among-population differentiation was nonsignificant in all species \( (N_{ST} > N_{ST\text{permuted}} \text{, } P > 0.05, \text{Table 1}) \).

The haplotype distribution maps show strong geographic structuring of haplotypes with distinct regional haplotype groups in all species (Fig. 1). In F. angustifolia, the distribution of haplotypes is very similar to that in F. excelsior (compare Fig. 1a and 1b), with H04 occurring in the Iberian Peninsula, H03 in the north of Italy, H02 in Central Europe and H01 in Eastern Europe. A difference was that H05 restricted to the eastern Alps in F. excelsior was found throughout central and southern Italy, Sicily, Corsica and Sardinia in F. angustifolia. Further, H07 detected in Romania in F. excelsior was widespread from Turkey to Romania in F. angustifolia; and H10 detected in the Czech Republic in F. excelsior was found in Portugal and on Corsica in F. angustifolia. As mentioned above, six additional F. angustifolia haplotypes were detected in Turkey only. In F. ornus, the picture was dominated by H19, which was found all over Italy (Sicily and Sardinia included) and Corsica, and H20, which occurred from Slovenia eastward (Fig. 2). H21 was found in FYR Macedonia and in Turkey and H22 in Turkey only.

Significant patterns of isolation by distance were found in all species for total samples and for the sympatric area (Table 2, Fig. 2); the only exception was for F. angustifolia in Turkey, where differentiation did not increase with geographic distance. In the between-species comparisons, isolation by distance was significant for the F. excelsior and F. angustifolia pair (Table 2), i.e. populations of different species were genetically more similar at short distance than at large distance (Fig. 2). This pattern reflects the geographically coherent extensive sharing of haplotypes between the two species. Conversely, the differentiation between F. ornus and either F. excelsior or F. angustifolia populations was constantly high, whatever the distance separating them.

**Table 1** Chloroplast marker diversity (allelic richness \( A_s \), haplotypic diversities \( h \) and \( v \), number of alleles \( K \)) and differentiation (\( G_{ST} \), \( N_{ST} \)) statistics from ash populations in Europe. \( N \), number of populations; \( \phi \), number of alleles \( \gamma \), total population. Standard deviations over populations are given between brackets. H1: \( N_{ST} > N_{ST\text{permuted}} \text{, permutation test (10 000 permutations)} \) for the presence of phylogeographic structure within populations.

<table>
<thead>
<tr>
<th>Species</th>
<th>Region</th>
<th>N</th>
<th>( A_s )</th>
<th>( h_s )</th>
<th>( v_s )</th>
<th>( K_{T} )</th>
<th>( h_{T} )</th>
<th>( v_{T} )</th>
<th>( G_{ST} )</th>
<th>( N_{ST} ) (perm.)</th>
<th>( N_{ST} &gt; N_{ST\text{permuted}} \text{; } P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. excelsior</td>
<td>Europe (total)</td>
<td>201</td>
<td>1.128 (.280)</td>
<td>0.081 (.188)</td>
<td>0.061 (.221)</td>
<td>12</td>
<td>0.720</td>
<td>0.720</td>
<td>0.888</td>
<td>0.915</td>
<td>0.887</td>
</tr>
<tr>
<td></td>
<td>Sympatric area</td>
<td>134</td>
<td>1.148 (.307)</td>
<td>0.096 (.210)</td>
<td>0.056 (.227)</td>
<td>12</td>
<td>0.736</td>
<td>0.690</td>
<td>0.870</td>
<td>0.919</td>
<td>0.868</td>
</tr>
<tr>
<td>F. angustifolia</td>
<td>Southern Europe with Turkey (total)</td>
<td>70</td>
<td>1.125 (.337)</td>
<td>0.071 (.185)</td>
<td>0.053 (.247)</td>
<td>13</td>
<td>0.812</td>
<td>0.812</td>
<td>0.913</td>
<td>0.934</td>
<td>0.892</td>
</tr>
<tr>
<td></td>
<td>Turkey</td>
<td>11</td>
<td>1.538 (.657)</td>
<td>0.303 (.353)</td>
<td>0.247 (.553)</td>
<td>9</td>
<td>0.657</td>
<td>0.546</td>
<td>0.538</td>
<td>0.547</td>
<td>0.498</td>
</tr>
<tr>
<td></td>
<td>Sympatric area</td>
<td>59</td>
<td>1.048 (.149)</td>
<td>0.027 (.085)</td>
<td>0.017 (.108)</td>
<td>7</td>
<td>0.763</td>
<td>0.605</td>
<td>0.964</td>
<td>0.971</td>
<td>0.953</td>
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<tr>
<td></td>
<td>Southern Europe with Turkey (total)</td>
<td>59</td>
<td>1.029 (.156)</td>
<td>0.018 (.097)</td>
<td>0.061 (.424)</td>
<td>4</td>
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<td>0.552</td>
<td>0.967</td>
<td>0.889</td>
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<tr>
<td>F. ornus</td>
<td>Sympatric area</td>
<td>57</td>
<td>1.015 (.114)</td>
<td>0.009 (.071)</td>
<td>0.057 (.430)</td>
<td>3</td>
<td>0.521</td>
<td>0.439</td>
<td>0.982</td>
<td>0.870</td>
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</tbody>
</table>

Discussion

Genetic diversity and differentiation

Genetic diversity was low in all three ash species analysed with a detected total number of 12, 7 and 3 haplotypes, respectively, in *Fraxinus excelsior, Fraxinus angustifolia* and *Fraxinus ornus* populations from the sympatric area in Europe (excluding Turkey). In comparison, the mean number of haplotypes found in 22 European trees and shrubs in roughly the same geographic area was as high as 16.9 despite a weaker sampling effort (Petit et al. 2003). The differentiation values observed in ash were high with total *G*\textsubscript{ST} = 0.89, 0.91 and 0.97 for *F. excelsior, F. angustifolia* and *F. ornus*, respectively, and even higher values for the two latter species in the sympatric area. In comparison, the mean *G*\textsubscript{ST} for 15 anemochorous woody species was only 0.56 (Aguinagalde et al. 2005).

The low polymorphism and high fixation levels detected in *F. excelsior* cpDNA have earlier been explained by a supposedly low chloroplast mutation rate in the Oleaceae family (Besnard et al. 2002), low *N_e* during glacial times and predominantly short-ranging dispersal (Heuertz et al. 2003; M. E. Morand-Prieur, personal communication, 2003) of the relatively heavy wind-dispersed samaras (Heuertz et al. 2004). In this study, lower polymorphism and higher fixation levels in the thermophilous *F. angustifolia* and *F. ornus* indicate smaller historic population sizes and stronger drift for them than for the more cold-tolerant *F. excelsior* in the sympatric area. Support for this scenario also comes from the divergence of *F. angustifolia* into three geographic subspecies (*F. angustifolia* Vahl ssp. *angustifolia* in Portugal and the western Mediterranean, ssp. *oxycarpa* (M. Bieb. ex Willd.) Franco & Rocha Afonso from northeast Spain to Turkey, and ssp. *syriaca* (Boiss.) Yalt. in Turkey and Asia Minor; Tutin et al. 1972; Huntley & Birks 1983; Wallander 2001). Further, in the sympatric area, *F. excelsior* is the only species that holds haplotypes, which differ by at least two mutations from the common haplotype of the region (H08, H09 and H11). These possibly have ancient origins (Goldstein & Pollock 1997), mirroring larger historical population sizes. Temperature tolerance was highlighted earlier as an important factor of historical population dynamics (e.g. Aguinagalde et al. 2005). For instance, thermophilous pine species (e.g. *Pinus halepensis, Pinus pinea*) harboured lower diversity than those with higher ecological amplitude (*Pinus sylvestris, Pinus pinaster*; Gómez et al. 2005; Robledo-Arnuncio et al. 2005); cold-tolerant species maintained relatively large population sizes during glacial times (Palmé et al. 2003, 2004) and mountain species tolerate harsh and variable climatic conditions by shifting altitude (Davis & Shaw 2001; Robledo-Arnuncio et al. 2005). The three ash species investigated here differ also by their breeding systems. Considering the proportion of individuals with female function in each species and ignoring all other traits, higher diversity would a priori be expected for the hermaphroditic *F. angustifolia* (100% individuals with female function), followed by the polygamous *F. excelsior* (66.7%) and the androdioecious *F. ornus* (50%). Our results do not agree with those a priori.
expectations, suggesting that mating system is not a key determinant of diversity patterns in ash species. 

_Fraxinus angustifolia_ populations from Turkey harboured six private haplotypes and were much more polymorphic and less differentiated than those in the rest of the range. This allows us to propose glacial refuges for _F. angustifolia_ in Turkey which would have remained larger than in the southern European peninsulas, in agreement with warmer temperatures reconstructed for the late glacial maximum (18,000 years BP) in the eastern than in the western Mediterranean (e.g. Hayes et al. 2005). The high diversity in Turkey could partly be due to the co-occurrence of _F. angustifolia_ ssp. _oxycarpa_, which would have had glacial refuges in Turkey, and ssp. _syrriaca_, which may have re-colonized the area from additional refuges in Asia Minor. This hypothesis is consistent with an abundant deposition of temperate tree pollen in the eastern Mediterranean in the early Holocene (Rossignol-Strick 1999).

**Patterns of haplotype sharing and phylogeography of European ashes**

We observed extensive sharing of cpDNA haplotypes between _F. angustifolia_ and _F. excelsior_, but found no common haplotype between these species and _F. ornus_ in agreement with predictions based on taxonomy (Tutin et al. 1972; Jeandroz et al. 1995, 1997; Wallander 2001; Morand-Prieur et al. 2002) and phenology (Wallander 2001; Frascaria-Lacoste, personal communication; M. Verdú, personal communication). In _F. angustifolia_ and _F. excelsior_, different haplotypes are associated with areas of putative glacial refuges, namely H04 with the Iberian Peninsula, H02 with the Eastern or Dinaric Alps, H03 with the north of the Italian Peninsula and H01 with the Balkan Peninsula. This pattern is incompatible with ancestral polymorphism segregating independently in the two species but rather evokes a common glacial history involving expansion of small populations from distinct refugia shared by both species.

Haplotype sharing can have occurred through hybridization in glacial refugia and/or during postglacial recolonization. In the first case, the ecologically favoured _F. excelsior_ could have hybridized through pollen and could have captured the chloroplast of _F. angustifolia_, whose male competitive ability might have been reduced due to environmental stress (environmental emasculation hypothesis, Williams et al. 2001). In the second case, _F. angustifolia_ could have hybridized through pollen and the more cold-tolerant _F. excelsior_ that might have established earlier at higher latitudes (pollen swamping hypothesis, Petit et al. 1997, 2004; Belahbib et al. 2001). The lack of phylogeographic signal and the scarce information about original species association of haplotype in our data preclude privileging one scenario over the other. Nevertheless, there might be weak support for the first scenario, because rare haplotypes remain private to _F. excelsior_ in the sympatric area. Experimental crosses were equally successful in both directions though (Morand-Prieur et al. 2002; N. Frascaria-Lacoste, personal communication). Thorough studies of reproductive biology in both ash species in sympatry and of the genetic architecture of polymorphic hybrid zones would help identifying the preferential direction of hybridization. Additionally, the frequency distributions of nuclear marker polymorphisms in refuge populations might inform further on historical _N_e_ in both species.

Some shared haplotypes had a different distribution in _F. angustifolia_ and _F. excelsior_. This was for instance the case for H05, found in central and southern Italy in _F. angustifolia_ but restricted to the eastern Alpine refuge in _F. excelsior_. The most straightforward explanation would be independent, possibly species-associated, origins of H05 in the two areas. Brewer (2002) suggested two ash refuges in the Italian Peninsula so that H05 in _F. angustifolia_ might originate from the southern Apennine refuge near Naples, and H03 in both species from the northern one close to Florence. The occurrence of H02 and H03 in an isolated _F. excelsior_ population (number 79 in Heuertz et al. 2004) from southern Italy supports different refuges for both species in southern Italy.

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**Table 2** Isolation by distance between ash populations belonging to the same or to different species. _blog_ _obs_, observed slope of the regression of _F_ _ST/(1 – F_ _ST_) on the natural logarithm of geographic distance for pairs of populations (in km); _P_, probability of an isolation by distance pattern (H1: _blog_ _obs_ > _blog_ _exp_ after 10,000 permutations of individuals among populations)

<table>
<thead>
<tr>
<th>Species</th>
<th>Region</th>
<th><em>F</em> <em>ST/(1 – F</em> <em>ST</em>)</th>
<th><em>blog</em> <em>obs</em></th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. excelsior</em></td>
<td>Europe (total)</td>
<td>0.964</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sympatric area</td>
<td>0.667</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td><em>F. angustifolia</em></td>
<td>Southern Europe with Turkey (total)</td>
<td>0.413</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Turkey</td>
<td>0.034</td>
<td>0.257</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sympatric area</td>
<td>1.610</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td><em>F. ornus</em></td>
<td>Southern Europe with Turkey</td>
<td>0.248</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sympatric area</td>
<td>0.850</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td><em>F. excelsior–F. angustifolia</em></td>
<td>Sympatric area</td>
<td>0.886</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td><em>F. excelsior–F. ornus</em></td>
<td>Sympatric area</td>
<td>−0.165</td>
<td>0.908</td>
<td></td>
</tr>
<tr>
<td><em>F. angustifolia–F. ornus</em></td>
<td>Sympatric area</td>
<td>−0.065</td>
<td>0.662</td>
<td></td>
</tr>
</tbody>
</table>
In *F. ornus*, the occurrence of H20 throughout southeastern Europe supports the existence of a glacial refuge in the Balkan Peninsula, as had been suggested from fossil pollen data (Huntley & Birks 1983). The polymorphic population in western Greece would sustain this area as a refuge (Huntley & Birks 1983) in agreement with the retention of higher polymorphism because of temporal persistence of refuge populations (Hewitt 1996; Tzedakis et al. 2002; but see Petit et al. 2003). Alternatively, H21 might have immigrated to Greece from a separate refuge in Turkey supported by our data. We additionally found evidence for an *F. ornus* refuge in Italy.

Common patterns and conclusions

All three species displayed patterns of isolation by distance, resulting from the strong geographic structure of the data. The only exception was for *F. angustifolia* in Turkey, where the widespread H07 prevented among-population differentiation to increase with geographic distance. Isolation by distance was also observed between *F. excelsior* and *F. angustifolia*, reflecting the extensive haplotype sharing between them.

The impact of haplotype phylogeny on the geographic distribution of diversity was weak and no phylogeographic signal was detected in any species. This pattern is probably due to the high fixation levels combined with the low resolution of haplotypes.

Our data suggest the presence of additional, particularly polymorphic glacial refuges for *Fraxinus* species in Turkey, a region for which fossil pollen data was very scarce. This observation confirms the importance of the Eastern Mediterranean area for genetic diversity (Tutin et al. 1972; Fady-Welterlen 2005).

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Supplementary material

The supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2897/MEC2897sm.htm

Table S1 *Fraxinus angustifolia* populations with coordinates, haplotypes and genetic diversity statistics.

Table S2 *Fraxinus ornus* populations with coordinates, haplotypes and genetic diversity statistics.

Table S3 Characteristics of the haplotypes detected with 6 chloroplast microsatellites in three species of *Fraxinus*: *F. excelsior*, *F. angustifolia* and *F. ornus*.
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