Differential patterns of morphological and molecular hybridization between *Fraxinus excelsior* L. and *Fraxinus angustifolia* Vahl (Oleaceae) in eastern and western France

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Abstract

We examined large-scale patterns of morphology, genetic structure and ecological correlates of *Fraxinus excelsior* and the closely related species *Fraxinus angustifolia* in France, in order to determine the degree of hybridization between them. We sampled 24 populations in two putative hybrid zones (Loire and Saône), and five control populations of each species. We measured foliar characteristics of adult trees and used five nuclear microsatellites as molecular markers. Canonical discriminant analysis indicated that the two species differ in morphology, but that intermediate types are common in the Loire region but less frequent in the Saône region. Bayesian population assignment identified one *F. angustifolia* and two *F. excelsior* gene pools. Most Loire individuals clustered genetically with the *F. angustifolia* gene pool. In contrast, the Saône region presented individuals belonging mostly to *F. excelsior* pools, although the *F. angustifolia* type was frequent in certain populations. The lowest $F_{ST}$ values were found between the Loire and *F. angustifolia* controls that also exhibited no significant isolation by distance. The proportion of the *F. angustifolia* gene pool in each locality was negatively correlated with winter temperatures, suggesting that a cold climate may be limiting. Hybridization is probably favoured by the intermediate climatic conditions in the Loire region that allow both species to occur, but is somewhat hampered by the harsher winters in the Saône area where morphological introgression has apparently not yet occurred.

Keywords: France, *Fraxinus*, hybridization, microsatellites, morphology, Oleaceae

Received 27 November 2005; revision accepted 17 March 2006

Introduction

The origin of large-scale hybridization patterns in closely related species are difficult to understand as multiple processes may have given rise to the present-day distribution of species and their potential hybrids. In the Northern Hemisphere for example, historical species distribution was influenced by multiple isolation and recolonization events during the glacial events of the Quaternary Period (e.g. Williams et al. 2004) that largely shaped the genetic structure of parental and hybrid populations (Hewitt 2004). In particular, population divergence may have occurred during cold periods when populations survived in refugia where the combined effects of drift and local selection may have favoured within-species divergence (Soranzo et al. 2000; Davis & Shaw 2001; Brewer et al. 2002; Petit et al. 2003). However, during expansion periods, populations may have continued to evolve as individuals began to exploit new niches, taking advantage of the ecological opportunities present in unsaturated floras with low levels of competition (Monasterio & Sarmiento 1991; Levin 2003a, b). Thus, the present patterns of hybrid populations reflect the likelihood of colonization of such expanding lineages, largely determined by the spatial distribution of favourable habitats and the diversity surviving in refugia, but it is still difficult to determine the unambiguous origin of differences among the lineages.

Given enough time, the distribution of neutral markers in the present range of a species may reflect the levels of gene flow among hybridizing groups and the probable environmental associations that have promoted (or limited) the possibility of gene exchange among groups. For example,
in *Quercus* spp. in North America, the association between levels of admixture and climatic variables has provided indirect support for environmental selection, because specific molecular markers were more frequent in regions that corresponded well with the habitat preferences of each species and their potential hybrid populations (Dodd & Afzal-Rafii 2004). Morphological introgression on the other hand, may follow distributions which differ from those of neutral markers, because the morphological traits may be controlled by other, independent genes and be subject to selection. Thus, large-scale hybridization patterns may not always display concordance between neutral markers that in principle have no barriers to introgression among groups, and morphological types that may be heavily influenced by local conditions.

*Fraxinus excelsior* L. (common ash) and *Fraxinus angustifolia* Vahl. (narrow-leaved ash) (Oleaceae) are two widespread trees in western Europe whose present distribution suggests an important influence of isolation and recolonization processes shaped by different ecological preferences. Common ash abounds in central and northern Europe towards the Atlantic seaboard, being gradually replaced towards the Mediterranean basin by the narrow-leaved ash. Common ash grows in well-drained soils on low and middle elevation hills, while narrow-leaved ash prefers areas along rivers where flooding may occur (Marigo et al. 2000). In France, *F. excelsior* normally flowers during the first 2 weeks of March and disperses its samaras in the fall of the same year, while *F. angustifolia* begins flowering in late December and disperses the seeds late in the fall. Seed dormancy appears to be frequent in the common ash for up to 6 years, but seems to be almost nonexistent in the narrow-leaved ash (Wardle 1961). The present-day distribution of these two species indicates a long contact zone where hybridization may occur, running from Turkey to northern Spain and Portugal in the Mediterranean basin.

Evidence of hybridization between these species has been obtained from frequent observations of intermediate morphologies (Rameau et al. 1989), the genotyping of natural populations with random amplified polymorphic DNA (RAPD) markers (Jeandroz et al. 1996) and experimental crosses that were successful in both directions (Morand-Prieur et al. 2002; Raquin et al. 2002). It is worth pointing out that artificial hybridization requires the storage of pollen from *F. angustifolia* because it usually flowers several months before the common ash.

Pollen records show that an undifferentiated pollen type that cannot be assigned exclusively to either species was mainly concentrated in northern Italy, south of the Alps, about 15 000 years before present (BP) (Huntley & Birks 1983). Later, during retreat of the glaciers at about 6000 BP, three groups of *Fraxinus* were present: one north of the Iberian Peninsula on the Mediterranean side, one southeast of the Alps in northern Italy, and one in the Balkans. These groups putatively gave rise to the present-day *F. excelsior*, *F. angustifolia*, and *Fraxinus oxycarpa*, respectively. At least with respect to the common ash, chloroplast haplotype distribution and nuclear microsatellites have confirmed a postglacial expansion from southern refuges (Heuertz et al. 2004a, b), but the historical phylogeography of *F. angustifolia* remains unknown. Both species share at least three chloroplast haplotypes, suggesting a close evolutionary history of the common ash and *F. angustifolia* (Fernández-Manjarres & Gérard, unpublished; Vendramin, personal communication). Indeed, both species cluster closely in phylogenetic analyses (e.g. Jeandroz et al. 1997; Wallander & Albert 2000).

Using morphological and nuclear microsatellite data, we examined the extent and magnitude of hybridization between *F. angustifolia* and *F. excelsior* populations in continental France. The specific questions we addressed were: (i) What is the morphological and genetic structure of *F. excelsior*, *F. angustifolia* and putative hybrid populations? (ii) Is morphological structure related to genetic groupings? (iii) If so, what are the ecological variables that may determine the distributions of any groups found?

**Materials and methods**

**Population samples**

Samples were collected in central France in 2002–2004 at 34 sites, focusing on the Loire valley and the Saône region (Table 1). The Loire valley runs east–west from north-central France to the Atlantic, and is an extensive plain without any significant mountain systems. Samples from the Loire zone were mainly collected along riverbanks, except for those from Seiches (population 18, Fig. 1). The Saône region of eastern France is more heterogeneous as it includes a valley that runs southwards, surrounded by mountains of 600 m or more which give the area a more continental climate. The Saône River drains into the Rhône River that runs into the Mediterranean sea. Various preliminary morphological observations had suggested the existence of *Fraxinus angustifolia* and/or putative hybrid individuals in these areas. At each site, we selected an adult ash tree at random as the starting point of the sampling. We then sampled the closest tree within 30 m of the starting point. The procedure was repeated in a straight line, if possible, until around 30 trees had been identified (Table 1). Trees were sampled regardless of their resemblance to either species.

**Control populations**

As a control for *Fraxinus excelsior*, we used the Dourdan forest in north-central France, which has been thoroughly studied (Morand-Prieur et al. in preparation). This population is situated on a low-altitude plateau, and geographically pertains to the Parisian basin that drains to the Seine River.
In addition, four populations were sampled in eastern (Morschwiller) and central northern (St Gatien, La Romagne, and St Martin d’Abois) regions of France, all of them considered as good *F. excelsior* provenances (Bilger et al. 1995). The easternmost population of Morschwiller (population 14, Table 1, Fig. 1) was included in the Saône group because of its geographical proximity to mountain populations in this area.

As controls for *F. angustifolia*, we sampled one population in the Tech valley close to the Pyrenees and four populations to the west of the Rhône River (Table 1, Fig. 1). The southernmost population of the Pyrenees coincides approximately with the satellite population that appeared 6000 BP, and which may have given rise to the *F. angustifolia* group (*sensu* Huntley & Birks 1983).

**Table 1** Sampling zone, population name, number of individuals sampled per population, geographical position (Lambert coordinates with origin 46.8°N, 2.3°E in hundreds of m.a.s.l.), for 34 populations of *Fraxinus* spp. collected between 2002 and 2004 in continental France

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*control populations for each species.

Morphological data

During the mid-summers of 2002 and 2003, fully expanded leaves were collected. The minimum and maximum numbers of leaflets on each tree were estimated by counting all leaflets on 3–6 terminal branches. Next on one chosen leaf, we measured the length and width of one of the second leaflets next to the terminal one. Tree diameter at breast height (d.b.h.) was also recorded. Generally, adult trees with 9–13 rather elliptical leaflets are a fairly good indicator of common ash (*F. excelsior*), while individuals with 5–7 lanceolate leaflets belong to the narrow-leaved ash, *F. angustifolia* (*sensu* J. Dufour, personal observation). Floral morphology is identical in both species, rendering it useless for group discrimination, although *F. excelsior* may present female or male flowers.
through a reduction of stamens or stigmas, respectively, while *F. angustifolia* bears both. Phenological traits, which constitute a possible means of discriminating both species and hybrids, have been intensively recorded during a parallel, 2-year study (Gérard et al. submitted) of the hybrid population in St Dye (population 21, Fig. 1).

**Molecular methods**

Total DNA was extracted from 25 to 50 mg dried leaf disks of 12.5 mm diameter and immediately soaked in 2 mL of a 1:1 solution of pure ethanol and acetone in Eppendorf tubes. The samples were left for 24–48 h and then the solution was poured out and the tubes left open to dry. DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) following the manufacturer’s instructions. We used five primer pairs of nuclear microsatellites (FEMSATL 4, FEMSATL 11, FEMSATL 16, FEMSATL 19 and M2–30; Brachet et al. 1999; Lefort et al. 1999) that had previously been used successfully to characterize common ash populations (Heuertz et al. 2001, 2004a; Morand et al. 2002). Molecular biology methods follow Morand et al. (2002).

**Statistical analyses**

Zones (Loire, Saône, common ash controls and narrow-leaved ash controls) and populations were compared with respect to morphological characters with ANCOVA, using the d.b.h. as the covariable to account for tree size. We analysed the minimum and maximum numbers of leaflets per tree, the average number of leaflets per tree, leaflet length, leaflet width, and the arc-sine transformed leaflet length/width ratio. We also used the derived variables of the leaflet area (estimated by multiplying the length and width of the leaflet observed) and the total leaf area (estimated by multiplying the measured topmost leaflet area by the average number of leaflets per tree). We acknowledge that derived variables are correlated with the original variables, but we considered that an estimate of the total leaf area was crucial to our analyses.

In order to characterize species and putative hybrids, these morphological characters were also employed in a canonical discriminant analysis (CDA) using PROC DISCRIM (SAS V8) with the population as the classification variable. We also recorded the INTO variable provided by the output of the discriminant analysis that classifies individuals as a function of the posterior probabilities of canonical scores in one of the predefined populations.

**Genetic analyses**

The number of alleles \( A \), effective number of alleles \( A_e \), observed heterozygosity \( H_O \), expected heterozygosity \( H_E \), population inbreeding coefficient \( f_{is} \) and population differentiation \( F_{ST} \) were all estimated using GENEPOP version 3.4 (Raymond & Rousset 1995). Standard deviations of the estimated parameters were obtained by bootstrapping the original data 1000 times with replacement. We also
estimated population differentiation ($F_{ST}$) and tested for isolation by distance (IBD) by comparing the pairwise $F_{ST}/(1 - F_{ST})$ matrix to the log of the pairwise values of geographical distances, using the Mantel test with 1000 permutations. Finally, we determined population genetic distances using the parameter $(\Delta \mu)^2 = (\mu - \mu)^2$, defined as the squared difference in allele size of any two populations (Goldstein et al. 1995).

We performed population assignment using the Bayesian methods implemented in the software STRUCTURE version 2.0 (Pritchard et al. 2000; Falush et al. 2003). This is a clustering method that uses multilocus data from a sample of individuals to infer population structure. Individuals are assigned probabilistically to one of the predefined $K$ populations (gene pools), being the prior hypothesis $1/K$ for each individual. The program was run with 10 000 iterations as the burn-in period, and 100 000 iterations for Markov chain convergence. Population origin was taken into account in the analysis and allele frequencies were set as being uncorrelated. Previous runs without considering population origin and correlated allele frequencies had yielded results which were similar to those obtained with the chosen settings.

Ecological correlations of genetic groups

In order to detect any ecological determinants of the genetic groups found by the STRUCTURE software, we performed a CCA at the population level. Although climatic variables (i.e. altitude and mean temperature) and genetic groupings may be correlated among themselves, canonical correlation allowed us to detect spurious results because the model is only valid if a between-groups correlation (genetic and ecological) is stronger than any within-group correlation. Thus, the average per population percentages of genetic clusters from the STRUCTURE output were correlated with the altitude of the sampled population, the mean annual temperature, mean January and July temperatures (strength of winter and summer respectively), precipitation and number of frost days. Analyses were conducted using PROC CANCORR (SAS V8, SAS Institute). The same ecological variables were also used for visual inspection of the climatic characteristics of each population by its principal components using PROC PRINCOMP from the same software package.

Results

Morphological data

All morphological variables exhibited significant effects with respect to populations the most significant being the minimum leaflet number ($F_{34,863} = 20.55, P < 0.0001, R^2 = 0.46$), and leaflet area ($F_{34,863} = 17.63, P < 0.0001, R^2 = 0.42$). Results were similar for zones (Loire, Saône, and control populations) indicating significant differences among these large regions (see Table S2, Supplementary material). The minimum number of leaflets appeared to be about five in *Fraxinus angustifolia* control populations, and nine or more in *Fraxinus excelsior* control populations. Saône valley populations had up to 11 leaflets, while populations in the Loire valley tended to have about seven leaflets, except for those in St Pryvé and Seiches which had nine (see Table S1, Supplementary material). Leaflet size was smaller in the narrow-leaved ash (~70 × 20 mm) when compared to common ash control populations or some populations in the Saône valley with much larger leaves (e.g. Vernois sur Mance ~90 × 32 mm). Again, Loire individuals displayed intermediate sizes. The leaflet length/width ratio tended to increase in northern and eastern populations, suggesting more rounded leaves for ashes growing in more temperate areas. All morphological characters appeared to be unaffected by tree size, as the d.b.h. was statistically nonsignificant regarding all the variables examined (results not shown).

The CDA yielded four out of seven significant canonical correlations that explained 95.9% of the variation in the model, the first canonical correlation contributing 71.3% (Table 2A). This first canonical variable had a correlation of 90.6% with the minimum leaflet number and a correlation of 82.4% with the average number of leaflets per population. The second canonical variable had a correlation of 68.0% with the total leaflet area, and 61.2% with leaflet width, suggesting that discrimination in this axis was closely related to total leaf size. Finally, the third canonical correlation had the highest input from the leaflet length/width ratio (56.1%), suggesting that leaf shape (ranging from oval to lanceolate) also determined group characteristics (Table 2B).

The control populations of *F. excelsior* and *F. angustifolia* exhibited the most marked morphological differentiation, and formed two separate groups that nevertheless overlapped at the extreme of their distribution (Fig. 2A, B). Individuals from the Saône valley lay within the ‘*excelsior*’ side of the distribution (Fig. 2C) indicating that there were no intermediate morphological individuals in that region. In fact, many Saône individuals displayed morphologies that differed from both control populations, primarily because of the presence of numerous and large leaflets (see Table S1, Supplementary material). Most of the Loire valley individuals exhibited clear hybrid morphologies with intermediate values between the chosen control populations (Fig. 2D). Individual examination of the histograms per population showed that two populations from the Loire valley displayed mostly an ‘*excelsior*’-like morphology: in Seiches, which is situated at distance from the Loire, and St Pryvé, the northernmost and easternmost sampled population. The distribution of the other canonical variables was less clear-cut, with broad variations regarding the second axis in Saône populations that did not exist in other groups, while individuals largely overlapped in the third canonical axis (results not shown).
Multivariate classification of individuals according to their canonical scores (INTO variable output from the CDA) placed 80% of the trees sampled in *F. angustifolia* control populations within the same control populations, 18% of individuals in the Loire zone and 2% elsewhere. Among common ash control populations, only 43% were classified within control populations, 32% within the Saône region, 24% within the Loire, and only 1% within *F. angustifolia* populations. Individuals from the Saône populations were also mainly classified within their own zone (69%), followed by the Loire (17%), common ash control populations (13%), and only 1% within *F. angustifolia* controls. Loire individuals were also mostly classified in their own zone (63%), followed by the Saône (17%) and narrow-leaved ash controls (13%), and only 6% in the common ash controls. This classification reinforces the notions that morphology barely overlapped between the chosen control populations (about 1%), that more *F. angustifolia*-like individuals were found in the Loire than in the Saône regions (13% vs. 1%), and that morphology was highly specific to zones.

### Genetic diversity and structure

Nuclear microsatellite variability in all sampled zones was comparable to that found in other European populations (Table 3), but a reduced number of alleles (both effective and total) was found in control populations of *F. angustifolia* ($A = 24.2$). Intermediate values of allelic richness were found in Loire populations ($A = 33.6$) and in common ash control populations ($A = 37.4$), and the highest level being in the Saône zone ($A = 56.2$). This pattern also applied to standardized minimum sample sizes, where the upper limit of the standard deviation overlapped more or less with the lower limit of the distribution of bootstraps (results not shown), thus confirming the trend observed in raw values. Heterozygote deficiency values were lower for *F. angustifolia* (0.087), intermediate in the Loire valley (0.132) and *F. excelsior* populations (0.154), and much higher for the Saône valley (0.192). High $f_{st}$ values in common ash can be explained by skewed sexual type ratios (Miyamoto *et al.* in preparation) that are unknown in *F. angustifolia*.

Values for the overall genetic structure in all 34 populations were moderate ($F_{ST} = 0.0428$). Pairwise values among species and zones (Table 4) were smallest between *F. angustifolia* control populations and the Loire ($F_{ST} = 0.028$), and biggest between Saône and Loire zones ($F_{ST} = 0.045$). Similarly, IBD was always significant, except when comparing *F. angustifolia* and the Loire valley, suggesting extensive gene flow between southern Mediterranean France and the Loire. Interestingly, northern populations of *F. excelsior* exhibited significant IBD with Saône valley populations, indicating that within common ash, IBD can also be present. Our $F_{ST}$ values were similar to those reported for the common ash in France ($F_{ST} = 0.043$, Morand *et al.* 2002), but lower than those from Bulgaria ($F_{ST} = 0.087$, Heuertz *et al.* 2001) and in a comparison between the Balkans and the British Isles ($F_{ST} = 0.078$, Heuertz *et al.* 2004a). The mean divergence of populations between control *F. angustifolia* and common ash populations was estimated at ($\Delta q^2 = 93.3$). In relative terms, Loire populations and the narrow-leaved ash appeared...
to display equal divergence, and Saône populations and the common ash appeared to be four to five times more divergent (results not shown).

**Population assignment**

Population assignment suggested that three was the most likely number (K) of gene pools. The results of iterations showed a rapid increase from panmixis, attaining a maximum at K = 3 [ln(probability of data) = −23825, SD = 20.9]. For values of K > 3, the variance of the likelihood estimates became much larger than with K = 2 or 3 (results not shown). For K = 2, gene pools were clearly divided into homogeneous *F. excelsior* and *F. angustifolia* control populations, showing a widespread presence of the latter in the Loire valley and in some Saône valley populations.

With K = 3 possible gene pools, the clustering of ‘excelsior’-like individuals was broken down into two groups, one containing numerous individuals from the highest elevation sampling sites in the Saône, and one containing common ash control populations (see Fig. 3 for the average gene pool composition of populations). For most of the analyses and in the discussion, we will be focusing on the ‘angustifolia’ component in order to evaluate the degree of molecular introgression of this species into the common ash, bearing in mind the presence of two ‘excelsior’ components in the total gene pool. On average, individuals from the common ash control populations contained 22% (SD = 8%) of the *F. angustifolia* gene pool, those from the narrow-leaved ash contained 75% (SD = 3%), Saône region trees contained 21% (SD = 15%) and those from the Loire 59% (SD = 16%).

From a marker perspective, it was observed that grouping was achieved by the presence of small alleles around 130 bp in *F. angustifolia* for FEMSATL 19, and less than 160 bp for M2-30, some of them private to *F. angustifolia*.

### Table 3 Overall genetic variation at five nuclear microsatellite loci in the two transects examined in central France for *Fraxinus excelsior* populations, potential hybrid populations, and *Fraxinus angustifolia* control populations. SD represents the standard deviations of the average for five loci

<table>
<thead>
<tr>
<th>Zone</th>
<th>$A_e$</th>
<th>$A_o$</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$f_is$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saône</td>
<td>56.2</td>
<td>17.3</td>
<td>0.73</td>
<td>0.89</td>
<td>0.19</td>
</tr>
<tr>
<td>SD</td>
<td>10.9</td>
<td>10.6</td>
<td>0.19</td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>Loire</td>
<td>33.6</td>
<td>12.6</td>
<td>0.77</td>
<td>0.87</td>
<td>0.13</td>
</tr>
<tr>
<td>SD</td>
<td>13.7</td>
<td>8.8</td>
<td>0.18</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td><em>F. excelsior</em></td>
<td>37.0</td>
<td>18.3</td>
<td>0.78</td>
<td>0.92</td>
<td>0.15</td>
</tr>
<tr>
<td>SD</td>
<td>8.9</td>
<td>10.3</td>
<td>0.08</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td><em>F. angustifolia</em></td>
<td>24.0</td>
<td>10.5</td>
<td>0.76</td>
<td>0.83</td>
<td>0.09</td>
</tr>
<tr>
<td>SD</td>
<td>13.0</td>
<td>8.5</td>
<td>0.21</td>
<td>0.17</td>
<td>0.11</td>
</tr>
</tbody>
</table>

### Table 4 Pairwise genetic structure ($F_{ST}$) of the populations analysed, including regions and species, and of control populations. The lowest values for genetic structure were among the Loire and the *Fraxinus angustifolia* populations, and the highest values were among the Loire valley and Saône populations. All $F_{ST}$ values are significantly greater than zero, except between the Loire and *F. angustifolia* from the south.

<table>
<thead>
<tr>
<th></th>
<th>Saône</th>
<th>Loire</th>
<th><em>F. angustifolia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. excelsior</em></td>
<td>0.038</td>
<td>0.045</td>
<td>0.037/0.3288***</td>
</tr>
<tr>
<td><em>F. angustifolia</em></td>
<td></td>
<td></td>
<td>0.043/0.6957***</td>
</tr>
<tr>
<td>Saône</td>
<td>0.3288</td>
<td>0.3896</td>
<td>0.052/0.1755n.s.</td>
</tr>
<tr>
<td>Loire</td>
<td>0.2141</td>
<td>0.4697</td>
<td>0.042/0.0986n.s.</td>
</tr>
</tbody>
</table>

†The second number in each entry represents the correlation of the pairwise regression analysis of genetic and geographical distances (IBD). The asterisks represent the level of significance of the correlation after 1000 permutations of the distance and genetic matrices of a Mantel test (**P < 0.01, *P < 0.05, n.s.**).
This tendency for small alleles in the narrow-leaved ash has been confirmed in other samples (Gérard et al. submitted) and appears to be a good indicator for the species, at least in French populations.

Joint consideration of morphological structure and the amount of the ‘angustifolia’ gene pool indicated that the majority of individuals from the control populations in each species clustered in opposite places, but that overlapping of extreme genotypes and/or morphologies was possible (Fig. 4A). Most of the individuals from the Saône valley displayed morphologies and genetic types close to F. excelsior control populations (Fig. 4B), while Loire valley individuals more frequently exhibited a significant proportion of the F. angustifolia gene pool (Fig. 4C). We also found that our chosen F. excelsior populations possessed about 22% of the narrow-leaved ash gene pool, indicating the possibility of gene flow between species in northern-central France.

**Ecological correlations**

Overall CCA between the population-level proportion of gene pools and ecological variables was highly significant (Wilks’ Lambda = 0.096, $F_{24,673} = 3.49$, $P < 0.001$). Of the three possible correlations, only the first was significant (3.49, $P < 0.001$) explaining 78% of the variation (Table 5A). This canonical correlation (0.887) was higher than any of the correlations within the two sets of variables (results not shown), thus validating use of the model. Standardized canonical coefficients indicated that the first canonical variable was a weighted difference of loadings in the ‘angustifolia’ group, followed closely by the ‘excelsior 2’ group and then by the ‘excelsior 1’ group (12.78, 12.53 and 11.30, respectively). The correlation between the ‘angustifolia’ group and the first canonical variable was negative ($-0.887$), but it was positive for the two ‘excelsior’ groups, suggesting opposite responses of the gene pools to ecological conditions (Table 5B). The first transformed ecological variable was mainly made up of the average temperature, number of frost days and mean January temperature (loadings of $-1.35$, $-0.97$, $-0.60$, respectively). Altitude, latitude, longitude and July temperature contributed less, while rain was the least informative of all. The first canonical correlation revealed that the January temperature was the most strongly correlated ecological variable ($-0.83$), followed closely by altitude ($0.80$), mean temperature ($-0.76$), and frost days ($0.73$). Latitude, longitude, precipitation, and July temperature were moderately correlated and with the same magnitude ($0.50 – 0.60$), except for the July temperature, where the correlation was negative (Table 5B). Thus, the multivariate correlation suggested that F. angustifolia may be limited by severity of the winter, altitude and by mean temperatures during the year, while opposite trends applied for the common ash groups.
Ecological characteristics of sampling sites

Principal Component Analysis (PCA) of the data from meteorological stations sited nearest to the sampled populations revealed distinct climatic conditions at the sampled sites, with Loire valley stations intermediate between the northeastern and the southeastern populations, and South and Saône zones representing extreme climatic conditions (Fig. 5). Because the first principal component for climatic data was correlated mostly with the temperature in January (0.458), the number of frost days (−0.442) and the mean temperature (0.440), this axis represented a generally cold to warm weather gradient. The second axis was positively correlated to July temperature (0.617) and the amount of precipitation (0.613), suggesting a gradient towards warmer and wetter summers. Thus, extreme populations in the south and Saône regions shared the same summer heat and rainfall, but with colder winters in the mountains, as expected. *F. excelsior* control sites and Loire sites have intermediate temperatures between the Saône and *F. angustifolia* control sites, but they probably have dryer summers than both.

Discussion

Hybridization evidence

We present here strong evidence that hybridization has occurred among *Fraxinus excelsior* and *Fraxinus angustifolia* in Atlantic and Continental/Mediterranean France, but that introgression patterns are not the same for the hybrid zones of the Loire and Saône. Furthermore, we found that isolation between species appeared to be weak, and the gene pools detected for different sampling zones may be influenced by ecological conditions.

The first line of evidence for hybridization comes from the morphological differences between ash species (Fig. 2A, B) and the intermediate state of individuals in the Loire valley (Fig. 2D). Morphological separation regarding canonical variables was bimodal in both species, while individuals from the Loire could be considered as having a hybrid phenotype. Our results differ from those obtained on *Quercus robur* and *Quercus petraea* (Kremer et al. 2002) where overlapping bimodality of synthetic characters was found within populations that included putative hybrids. At a local level (Saint-Dyé in the Loire valley), we found evidence that intermediate morphological characters are significantly correlated with phenological patterns which may highlight hybrid individuals (Gérard et al. submitted).

For example, trees with a series of *F. angustifolia* characters tended to flower early in winter, while individuals with a more *F. excelsior*-like morphology flowered later in spring, and intermediate ones flowered profusely in February. Whether this pattern holds for other hybrid populations.

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*Fig. 4* Dispersion graph of morphological characteristics of each individual as represented by its first canonical variable (Can1), and the probability of pertaining to the *Fraxinus angustifolia* gene pool (ang). (A) Control populations represented with red triangles for *F. angustifolia* and blue circles for *Fraxinus excelsior*; (B) Putative hybrid individuals from the Saône region (squares); and (C) Putative hybrid individuals from the Loire (diamonds). Control populations were left in light shading to facilitate comparison with putative hybrid zones.
needs to be verified, as hybrid segregation tends to produce mosaics of traits that may change from one hybrid zone to another (Rieseberg & Ellstrand 1993). The morphological diversity found here was mainly due to a reduction in leaflet numbers and size in *F. angustifolia*, and consequently in total leaf surface area. This reduction in leaf area may be related to water use efficiency (e.g. Casper *et al.* 2001; Knight & Ackerly 2003), coping with leaf overheating (Bragg & Westoby 2002), and resistance to wind (e.g. Niklas 1996), that may be particularly strong in the Mediterranean basin. Smaller leaves may be part of a suite of characters that also includes physiological responses, e.g. *F. angustifolia* depends more on mannitol and *F. excelsior* more on malate for stomata closure (Oddo *et al.* 2002). A genetic basis for smaller leaves is plausible, as green ash (*Fraxinus pennsylvanica*) seedlings in a common garden from a more xeric environment had smaller leaves, were more xerophytic and displayed higher net photosynthetic rates than seedlings from more mesic habitats (Abrams *et al.* 1990). We do not know, however, if *F. angustifolia* compensates for smaller leaflets with a larger number of leaves. Similarly, the abundance of large and numerous leaflets in many of the Saône individuals, may have originated from the need to equilibrate physiological processes in colder environments. In fact, common ashes growing in mountainous areas and on plains have been long differentiated by foresters and even receive different common names in the German language (see Marigo *et al.* 2000). Clearly, phenotypic plasticity plays a role in the physiological and morphological responses referred to here, and the genetic basis of these changes is still to be explored.

The abundance of intermediate phenotypes in the Loire region (Fig. 2D), where the weather is clearly intermediate (Fig. 5), suggests that both species may overcome their

### Table 5: Ecological correlates of the genetic groups found for common ash, narrow-leaved ash and putative hybrid populations in France, calculated using CCA

#### A. Canonical correlation analysis

<table>
<thead>
<tr>
<th>Canonical correlation</th>
<th>Standard error</th>
<th>Eigen value</th>
<th>Proportion</th>
<th>Cumulative</th>
<th>Likelihood ratio</th>
<th>F value</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.887664</td>
<td>0.036914</td>
<td>3.7158</td>
<td>0.7884</td>
<td>0.0960</td>
<td>3.49</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>2</td>
<td>0.639461</td>
<td>0.102895</td>
<td>0.6918</td>
<td>0.1468</td>
<td>0.4527</td>
<td>1.67</td>
<td>0.0952</td>
</tr>
<tr>
<td>3</td>
<td>0.483796</td>
<td>0.133333</td>
<td>0.3056</td>
<td>0.0648</td>
<td>0.7659</td>
<td>1.27</td>
<td>0.3050</td>
</tr>
</tbody>
</table>

#### B. Correlation of canonical variables

![Fig. 5: Principal components of climatic data from the 34 sampled populations. Climatic data originated from the closest available weather centre, and more than one field station could be assigned to the same weather station. Dots (·) represent the Saône valley stations, stars represent the *Fraxinus excelsior* control populations, circles represent Loire valley stations, and triangles represent southeastern *Fraxinus angustifolia* stations. It is evident that the Loire valley climate is intermediate between the northeastern planes of France and the south, although closer to the former.](image)
physiological limits, and interspecific gene exchange would be likely in the region as both ashes can co-exist. We observed the same morphological variation elsewhere, in a small 70 km transect, to the East of the Pyrenees in the Tech Valley, where F. angustifolia individuals thrive on riparian vegetation from sea level up to an altitude of 85 m. A narrow hybrid zone between this altitude and the beginning of the ecotonal piedmont at an altitude of about 110 m is rapidly replaced by F. excelsior, which increases its frequency up to an altitude of 1100 m (Dufour et al., personal observation). Thus, large-scale latitudinal patterns of hybridization in these two ashes can be repeated at smaller scales when mountain ranges provide more rapid transitions among vegetation types.

The second line of evidence for hybridization arises from the presence of F. angustifolia gene pool elements in many of the Loire valley and in the Saône individuals. Many individuals from the Loire exhibited an F. angustifolia-like morphology (13%), with components of the narrow-leaved ash gene pool (59% on average). In the case of Saône individuals, the narrow-leaved ash gene pool was more concentrated in certain populations, where it constituted up to 50% of the local gene pool (e.g., Conflandey), while only a few individuals resembled F. angustifolia (1%). However, the different morphological and marker introgression patterns may have had different origins, as discussed below.

**Differential hybridization patterns, markers or history?**

A first hypothesis to explain the different introgression patterns observed in the two hybrid zones may simply be marker resolution. If the microsatellites that we used during this study segregate independently of the genes controlling morphology (as seems to be the case), there is no reason for markers to follow the same geographical distribution as morphological traits. Allele-rich markers are efficient in detecting groups, especially if private alleles are present, as was the case in this study with the locus FEMSATL 19. However, genome-wide screening is still required to detect gene interactions that may control morphological characters.

From an historical point of view, differential hybridization patterns may also indicate that the interspecific gene flow is more ancient in the Loire than in the Saône region. The Loire conditions are intermediate (Fig. 5) and may even be milder since the riverbank rarely freezes, although the nearby plains experience frost (J. Dufour, personal observation). In addition, the Loire delta has always been protected from freezing, even during glacial periods (Maupin 1993), which suggests that no climatic barrier has existed to southern colonizing communities. Since F. angustifolia is a riparian specialist (Marigo et al. 2000), it is possible that this southern group may have migrated freely, and several times, from the western Pyrenees up to the Loire. So there might have been enough time for both neutral alleles and morphological traits to introgress in the Loire, whereas in the Saône region, a hybridization process may only be in its initial stages. In fact, divergence estimates indicate that control populations and Saône populations are between four- and fivefold more isolated than Loire and southern F. angustifolia populations. In view of the fact that permafrost completely covered the Saône region about 18 000 yr (Krebs et al. 2004) and that the Saône valley is sometimes only 20 km wide and surrounded by mountains displaying a continental climate, it is not unrealistic to suppose that migration might be difficult for F. angustifolia, a species adapted to warmer environments.

Geographical patterns of introgression in the Saône region appear to be more complex than those in the Loire. While the F. angustifolia gene pool is quite widespread in the Loire region, in the Saône valley, this gene pool is concentrated in populations from the north of our transect (Verœois) and the south (Broye and Tavaux), resembling a mosaic of gene pools (Fig. 3). This patchy distribution of gene pools can be assigned to the more complex geography of the region, which may entail not only different migration patterns at a small scale, but also the possibility of a second source of F. angustifolia. The first source for the migration of F. angustifolia into the Saône region is very probably the Rhône River, which receives waters from the Saône and then drains directly into the Mediterranean sea (Fig. 1). Thus, contact between the F. excelsior arriving from the southern Alps refuge, and F. angustifolia originating from Pyrenean populations is a likely scenario, as was proposed by Huntley & Birks (1983). A second, northern source of F. angustifolia could be explained by the fact that the Loire and the Seine rivers flow at the west of the Saône region, where altitudes are low. As a consequence, the F. angustifolia gene pool that was already established in the Loire valley may take advantage of some mild protected lowlands, and arrive in northern areas of the Saône, but the actual routes are difficult to identify. In fact, many species appear to have migrated from the west to the east, as the limits to postglacial colonization between Iberian groups and those from the east were the border between France, Germany and the Alps (Dumolin-Lapègue et al. 1998). For these reasons, independent origins for the narrow-leaved ash and hybrids in the Saône valley are possible.

**Ecological correlations of gene pools**

Because the detected gene pools were correlated with the ecological conditions prevailing at the sampling sites (and notably winter temperatures), the levels of interspecific gene flow may also have been influenced by the environment. Two years of field observations in a highly introgressed population showed that F. angustifolia-like individuals that flower in early winter (Gérard et al. submitted), may lose their flowers to frost and produce no seed at all. However, a small fraction of trees flower again, releasing pollen while...
the later-flowering *F. excelsior* is in bloom, and thus generating the possibility of interspecific gene flow. In principle, *F. excelsior* early-flowering trees can sire late-blooming *F. angustifolia* at the southern part of the common ash distribution where winters are milder and followed by an early spring, but we do not have enough data at the time to favour one hybridization direction or the other. In Spain, *F. angustifolia* is known to require a minimum of chill to trigger flowering (Jato et al. 2004), so its flowering time and successful seed production may vary from year to year depending on winter conditions. Thus, winter temperatures may not only constitute a physiological limit for *F. angustifolia*, but also impose demographic constraints on this species (i.e. risk of no seed production), and control the degree of interspecific hybridization.

In the Saône region, we found that seeds from large, typical *F. excelsior* trees from Tavaux presented early germination without seed dormancy, like those in the hybrid population at St Dye (Gérard et al. in press), which was a clear sign of introgression. Seed dormancy with variable germination is usually interpreted as a bet-hedging evolutionary strategy to deal with a variable environment (Forbis et al. 2002). For this reason, if dormancy release is one of the first physiological characters to introgress, *F. angustifolia* and its hybrids will have a demographic advantage in establishing populations if the climatic conditions become favourable and seed dormancy may no longer be advantageous. In this sense, hybridization will promote ecological invasion in the same way as the Californian species of Carpobrotus (Weber & D’Antonio 1999) in which hybrids appear to gain resistance against soil salinity and can survive in the native niche conditions.

In summary, we have shown that intermediate morphologies and gene pool components that can be attributed to *F. angustifolia* have been found in areas that are usually considered as providing natural conditions for distribution of the common ash. Our results call for a redefinition of the distribution of the narrow-leaved ash towards northwestern France, where the limits with *F. excelsior* appear to be weak. Indeed, our chosen common ash populations exhibited a non-negligible amount of the *F. angustifolia* gene pool and an overlapping morphology which implied that ‘pure’ common ash may only be found further to the north. Furthermore, we have shown that hybridization processes do not follow the same patterns in the two studied regions, probably because of historic climatic conditions that have produced different isolation levels in eastern and western France. In the case of the Saône valley, where hybridization was only detected using nuclear markers, it is not known whether morphological introgression will also occur, if sufficient time is allowed. Future studies of large-scale hybridization patterns of common and narrow-leaved ash populations should examine whether phenological isolation also varies across climatic gradients, whether a lack of dormancy is widespread in introgressed populations, and whether those characters have genetic-based responses.

### Acknowledgements

The authors would like to thank the comments by M.E. Morand, A.L. Caicedo, J.A. Shykoff and three anonymous reviewers that greatly improved the quality of the manuscript. J.F.F-M. was supported by the European research contract RAP-QLK5-2001–00631 as part of a post-doctoral position at the Laboratoire Ecologie, Systematique et Evolution, Paris XI. French Ministry of Agriculture (DGFAR) also provided funds for this study. We would also like to thank I Bilger (CEMAGREF) for her aid in population sampling, and P. Bertolino and L. Nowak for their very useful aid in the laboratory and field collections.

### Supplementary material

The supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2975/MEC2975sm.htm

**Table S1.** Summary of morphological variation for the analyzed populations in central France.

**Table S2.** Morphological analyses on *Fraxinus* spp. populations. *F* corresponds to Fisher’s *F* test for the complete model, *p* corresponds to the overall significance, *R*\(^2\) indicates the proportion of explained, *p* zones the significance value of the type I squares error for groups, and the last two columns represent the *p* value of the type III squares error for populations and diameter at breast height (d.b.h.) respectively.

### References


Juan F. Fernández-Manjarrés research interests include the study of the genetic structure of tropical and temperate tree species at the local and population level, focusing in mating systems. At the moment, he collaborates with the Forest genetics group at Orsay examining the genetic basis of phenological responses in European ashes. Pierre Gérard is a doctoral student at the same laboratory whose interests lie in the study of fine-scale mechanisms of reproductive isolation. Jean Dufour participates actively in the collection and evaluation of French and European timber species provenances. Christian Raquin interests are the physiology of seed germination and micro propagation processes in Ash and related species. Nathalie Frascaria-Lacoste coordinates the research group on Ashes at Orsay whose overall objectives are to determine the genetic gene flow and adaptive variation in *Fraxinus* species.