Molecular Ecology (2002) **11**, 377–385

A generalized heterozygote deficiency assessed with microsatellites in French common ash populations

M.-E. MORAND,* S. BRACHET,* P. ROSSIGNOL,* J. DUFOUR† and N. FRASCARIA-LACOSTE*
ENGREF, Laboratoire d'Ecologie, Systématique et Evolution (ESE), UPRESA CNRS 8079, Bâtiment 362, Université Paris-Sud, 91 405 Orsay Cedex, France, †INRA — Centre de Recherches d'Orléans, Unité Amélioration, Génétique et Physiologie forestières, BP 20619, 45 166 Olivet Cedex, France

Abstract

Common ash is a temperate forest tree with a colonizing behaviour, a discontinuous spatial distribution and a peculiar and poorly known mating system. Microsatellite markers were used to study the genetic structure in natural populations of common ash. Twelve populations located in northeastern France were analysed at five loci. Levels of genetic variability within and among stands were estimated for the seedling and adult stages. As expected for a forest tree, our results reveal high levels of intrapopulation diversity and a low genetic differentiation between stands. However, a general and significant heterozygote deficiency was found, with a mean F_{IS} of 0.163 for the seedlings and of 0.292 for the adult trees. The different explanations for such an excess homozygosity are discussed: a nonMendelian inheritance of alleles, the presence of null alleles, a Wahlund effect and assortative mating.

Keywords: common ash, *Fraxinus excelsior*, genetic variation, heterozygote deficiency, microsatellites, temperate trees

Received 22 June 2001; revision received 15 November 2001; accepted 29 November 2001

Introduction

Recently, microsatellite markers have been used to examine spatial patterns of genetic variability in temperate forest tree species. For example, focusing on one population, White & Powell (1997) for *Swietenia humilis*, Streiff *et al*. (1998) for *Quercus petraea* and *Q. robur* and Ueno *et al*. (2000) for *Camellia japonica* found a high level of within-population diversity. Focusing on several populations, Bodénès *et al*. (1997) found in the European oaks (*Q. petraea* and *Q. robur*) a high level of within population genetic diversity at the European scale. Moreover, they revealed a low genetic differentiation between populations. The same results were also shown for populations of *Melaleuca alternifolia* (Rossetto *et al*. 1999), *Pithecellobium elegans* (Chase *et al*. 1996) and, in conifers, for *Pinus sylvestris* (Karhu *et al*. 1996). As suggested by these studies, high intrapopulation variation and low genetic differentiation seem to be the general pattern for tree species.

Common ash (*Fraxinus excelsior* L., Oleaceae) is a forest tree that is widespread in France, except in the Mediterranean region and in Corsica (Franc & Ruchaud 1996). It is a colonizing species and is often found with a spatial discontinuous distribution in mixtures with other tree species, contrary to tree species that form continuous pure stands (Franc & Ruchaud 1996). Nevertheless it can also be encountered in pure stands over small areas when ecological conditions become very favourable. Common ash pollen and fruits, which are samaras, are wind-dispersed. Sexual types vary across a continuum from pure male individuals to pure female individuals, with all kinds of hermaphrodites in between (Wardle 1961; Picard 1982; Binggeli & Power 1999; Morand *et al*., personal observation).

All the biological features of common ash mentioned above (colonizing species, spatial distribution, mating system) make it a model of great interest for population genetics as it differs from the other tree species widely studied up until now. Moreover, although common ash is widespread in France, genetic variability in this species has not yet been investigated.

In this study, we used five polymorphic microsatellite loci (Brachet *et al*. 1999; Lefort *et al*. 1999) to assess the structure of genetic variability of common ash on a large geographical scale. The 12 studied stands revealed a high level of intrapopulation diversity and a low genetic differentiation

Correspondence: Marie-Elise Morand. Fax: 33 (0)1 69 15 73 53; E-mail: marie-elise.morand@ese.u-psud.fr

between populations, as is often shown in forest tree species. Nevertheless, a significant excess homozygosity was found for both seed and adult stages and explanations for this are discussed.

Material and methods

Population samples

Twelve natural populations of *Fraxinus excelsior* were sampled by the French National Institute of Agronomic Research (I.N.R.A.) with the aim of starting an improvement programme in this species. These populations are located in northeastern France (Fig. 1). In these mixed stands, common ash is the dominant species and represents on average 50% of the total number of individual trees, with important variations between stands according to the ecological conditions. They ranged from nearly pure stands (Ath, Sma, Sps and Vsp), constituted by 50–80% ash, to stands where ash was scattered (Hut, Mar and Sgo) with mixed stands in between (Fig. 1). In three out of the 12 stands (Hut, Lar and Mwr), both adult trees and seeds, were genotyped (Fig. 1). The remaining stands were investigated by analysis of seeds. In each of the 12 stands, 20 fruiting trees were chosen at a minimal distance of 20 m from one another. The sampling for the analysis of seeds comprised gathering samaras from each mother tree. Then, for each maternal tree, two randomly chosen seeds were

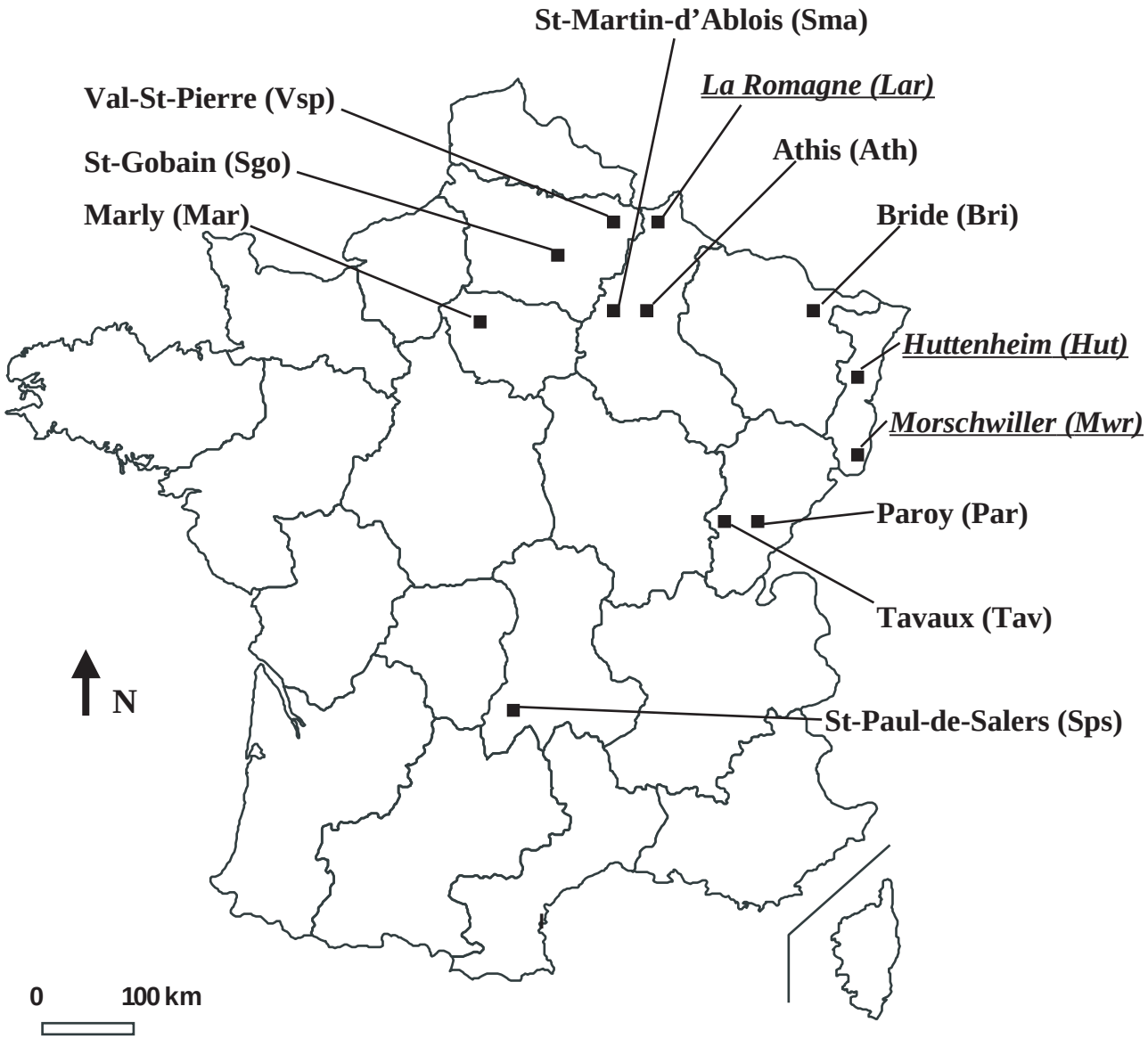

Fig. 1 Location, name and abbreviation of the 12 populations of common ash studied in France. The three populations in italic and underlined are those in which both seeds and adult trees were analysed.

subjected to a method of *in vitro* germination developed in our laboratory (Raquin *et al*., 2002), the first seed being destined for genetic analysis and the second one in case the first one died. The obtained seedlings were grown and used for genetic analysis. For the analysis of adult trees, in each of the three considered stands (Hut, Lar and Mwr), we sampled buds from the 20 maternal trees previously sampled for seeds. These three stands were chosen for their F_{IS} values in the seed samples (see Results). Buds and leaves for the seedlings were frozen at −20 °C before DNA extraction.

Molecular analysis

Total DNA was extracted from 100 mg of bud or leaf material using the DNeasy Plant Mini Kit (Qiagen). Among the 16 polymorphic microsatellites isolated by Brachet *et al*. (1999) and Lefort *et al*. (1999), five loci were chosen for their high polymorphism and quality of banding pattern (Brachet & Frascaria-Lacoste, personal communication): M2–30, FEMSATL4, FEMSATL5, FEMSATL11 and FEMSATL19. Amplification reactions were carried out as described in Brachet *et al*. (1999) and Lefort *et al*. (1999). Polymerase chain reaction (PCR) products were separated by electrophoresis in 6% sequencing polyacrylamide gels and visualized by silver staining according to Streiff & Lefort (1997). In order to determine accurately the allelic sizes, we used as a reference on each acrylamide gel the sequence of the plasmid vector pGEM®*-3Zf(+) (Promega, Silver Sequence™ DNA Sequencing System) (Fig. 2). In order to compare our method of allele scoring with another one, we analysed a subset of the DNA samples of this study (n = 33) with the fluorescent technique for microsatellite detection used by Heuertz *et al*. (2001).

Analysis of gene frequencies and linkage disequilibria

We calculated the observed number of alleles (A_O) and allele frequencies. Both expected and observed heterozygosities were estimated according to Nei (1987) and using the correction of Levene (1949), with the software GENEPOP (version 3.2a, Raymond & Rousset 1995). GENEPOP was also used to test for genotypic linkage disequilibrium among loci.

Hardy–Weinberg equilibrium and structure of genetic variability

We tested for the deviation of observed genotypes from Hardy–Weinberg equilibrium using GENEPOP. The structure of genetic variability in common ash was estimated using Wright's *F*-statistics (Wright 1921; Wright 1969). *F*-statistics were calculated according to Weir and Cockerham's method (Weir & Cockerham 1984) and their significance was tested with the software FSTAT (version 2.9.1, Goudet 1995).

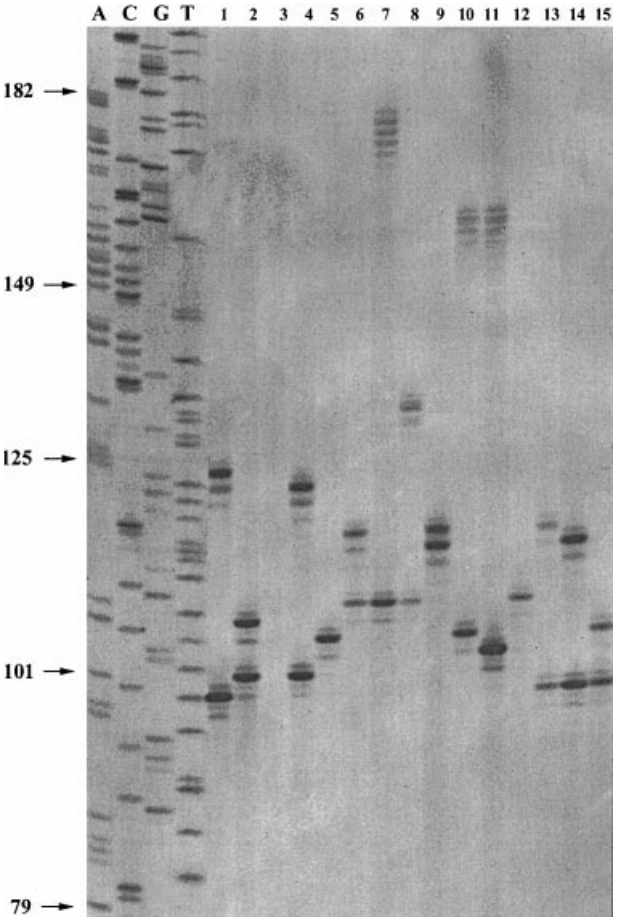

Fig. 2 An example of allelic polymorphism observed at locus FEMSATL4, after silver staining. On the left is the sequence of the plasmid vector pGEM®*-3Zf(+) (Promega) which was used to determine accurately the allelic sizes (given in base pairs). On the right, each lane (numbered from 1 to 15) shows the genotype at locus FEMSATL4 of one analysed individual. For example, the individual in lane 9 was scored as a heterozygote with its two alleles being 116 and 118 bp, respectively. The heterozygote individuals in lanes 7, 10 and 11 show a 'long-sized' allele of weaker intensity than the 'short-sized' alleles.

Correlation between genetic distance and geographical distance

The relationship between genetic distance and geographical distance was examined with GENEPOP by estimating the Spearman Rank correlation coefficient between $F_{ST}/(1 - F_{ST})$ and the distance in km. Using this program, the significance of the correlation coefficient was tested with a Mantel test (50 000 permutations were performed).

Results

The comparison of the subset of DNA samples analysed both with fluorescent detection and silver staining allows us to conclude that both methods are equivalent in terms of

Table 1 Levels of genetic diversity and deviation of observed genotypes from Hardy–Weinberg equilibrium (HWE) assessed for the seeds with five microsatellite loci

	Population												
Locus	Hut	Mar	Sgo	Ath	Sps	Sma	Mwr	Lar	Par	Tav	Vsp	Bri	All pop.
M2–30													
N	14	14	15	18	19	14	14	20	18	19	20	19	
A_O	17	16	16	21	20	18	18	17	16	21	23	25	56
H_E	0.942	0.955	0.952	0.959	0.959	0.963	0.966	0.946	0.932	0.932	0.968	0.969	0.953
H_O	0.929	0.857	0.600	0.889	0.947	0.786	0.786	0.700	0.778	0.737	0.900	0.789	0.800***
4													
N	19	16	17	13	18	14	17	19	16	19	19	19	
A_O	11	11	13	13	13	12	12	17	12	12	17	16	37
H_E	0.853	0.847	0.868	0.920	0.894	0.884	0.841	0.916	0.907	0.905	0.930	0.917	0.891
H_O	0.789	0.750	0.647	0.769	0.889	0.643	0.765	0.947	0.688	0.737	0.789	0.842	0.800***
5													
N	18	17	14	18	20	15	14	16	14	15	17	18	
A_O	14	10	5	15	13	13	14	19	17	13	21	13	52
H_E	0.719	0.708	0.561	0.916	0.763	0.749	0.926	0.931	0.966	0.874	0.966	0.856	0.827
H_O	0.778	0.882	0.429	0.444	0.600	0.733	0.929	0.500	0.500	0.533	0.647	0.389	0.600***
11													
N	18	17	19	20	15	18	17	19	17	20	20	19	
A_O	11	10	21	15	13	13	13	15	13	18	14	12	40
H_E	0.873	0.865	0.962	0.897	0.901	0.908	0.911	0.899	0.939	0.938	0.904	0.883	0.907
H_O	0.889	0.882	0.842	0.850	0.933	0.833	0.706	0.737	0.882	0.750	0.650	0.632	0.800***
19													
N	17	17	11	13	18	11	16	20	15	13	19	17	
A_O	17	15	10	14	16	15	17	12	12	11	15	18	36
H_E	0.952	0.904	0.848	0.932	0.916	0.952	0.948	0.909	0.892	0.898	0.935	0.936	0.920
H_O	0.941	0.765	0.636	1.000	0.611	0.636	0.750	0.750	0.933	0.692	0.842	0.882	0.800***
All loci													
H_E	0.863	0.852	0.849	0.924	0.884	0.888	0.916	0.920	0.927	0.912	0.940	0.912	
H_O	0.860	0.827	0.645	0.780	0.789	0.736	0.782	0.734	0.763	0.698	0.768	0.707	
HWE	ns	ns	***	***	***	***	**	***	***	***	***	***	

For each locus in each population the number of samples successfully genotyped (*N*), the observed number of alleles (A_O), and the observed (H_O) and expected (H_E) heterozygosities are reported. For each locus H_O and H_E and departure from Hardy–Weinberg equilibrium (HWE) tested with the program GENEPOP (Raymond & Rousset 1995) are shown. For each population H_O and H_E, A_O are shown and the departure from Hardy–Weinberg equilibrium is indicated by asterisks.
ns, nonsignificant; $^{**}P < 0.01$; $^{***}P < 0.001$.

allele scoring. The two techniques seem to have a similar power for allele detection.

Inheritance of microsatellite alleles within test crosses

The segregation of alleles was investigated for the five loci used in this study (M2–30, FEMSATL4, FEMSATL5, FEMSATL11 and FEMSATL19) by analysis of control-pollinated full-sibs ($n = 68$). None of the four following loci (M2–30, FEMSATL4, FEMSATL11 and FEMSATL19) deviated significantly from expected Mendelian segregation proportions ($P > 0.05$). As to FEMSATL5, for all pairs of crossed parents, the father was homozygote and some progenies (nearly 50%) appeared to be homozygous for one allele of the mother, then lacking the allele of the homozygous father. This may result either from the presence of a null allele at this locus or from punctual and random amplification flaws. If we take into account the existence of this null allele, the locus FEMSATL5 also shows a Mendelian inheritance of alleles ($P > 0.05$).

Genetic diversity within the seed stage

The distributions of allele frequencies revealed that the five loci are highly polymorphic. Loci have between 36 (FEMSATL19) and 56 (M2–30) alleles over the 12 populations (Table 1). Across all five loci, the linkage disequilibrium test revealed the statistical independence of our loci.

Table 2 Heterozygote deficiency [$F_{IS}(f)$] in *Fraxinus excelsior* as revealed by five microsatellite loci

	Seeds ($N = 235$)	Adult trees ($N = 60$)
Locus		
M2–30	0.156***	0.450***
4	0.131***	0.077ns
5	0.266***	0.415***
11	0.127***	0.307***
19	0.144***	0.196***
Population		
Hut	0.003ns	0.266**
Mar	0.034ns	—
Sgo	0.254***	—
Ath	0.149***	—
Sps	0.104***	—
Sma	0.191***	—
Mwr	0.147***	0.463**
Lar	0.215***	0.171**
Par	0.190***	—
Tav	0.247***	—
Vsp	0.190***	—
Bri	0.230***	—
Overall	0.163 ± 0.024***	0.292 ± 0.069***

F_{IS} was calculated according to Weir & Cockerham (1984) using the software FSTAT (Goudet 1995). For both seeds and adult trees, F_{IS} is reported per locus, per population and across all loci and populations with standard deviation. The significance of F_{IS} is estimated using FSTAT.
ns, nonsignificant; **$P < 0.01$; ***$P < 0.001$.

Hardy–Weinberg equilibrium was rejected for 10 populations ($P < 0.01$), all showed a significant deficiency of heterozygotes (Table 1). Only for two populations (Hut and Mar), the hypothesis of Hardy–Weinberg equilibrium could not be rejected ($P = 0.74$ and $P = 0.12$, respectively). Hardy–Weinberg equilibrium was also rejected for all loci ($P < 0.001$).

This heterozygote deficiency can also be pointed out with the values of F_{IS}. The global F_{IS} was estimated as 0.163 and differed significantly from zero ($P < 0.001$) (Table 2). Across populations, F_{IS} showed high, positive and significant values. It ranged from 0.104 (Sps, $P = 0.0008$) to 0.254 (Sgo, $P = 0.0033$) (Table 2). The Hut and Mar populations showed a low and nonsignificant value of F_{IS} ($F_{IS} = 0.003$, $P = 0.49$ and $F_{IS} = 0.034$, $P = 0.18$, respectively). Across loci, the estimator of F_{IS} varied from 0.127 (FEMSATL11) to 0.266 (FEMSATL5) and is highly significant for all six loci ($P < 0.001$) (Table 2).

Across populations, expected and observed heterozygosities were high, indicating high variability within populations. The values ranged from 0.849 (Sgo) to 0.940 (Vsp), and from 0.645 (Sgo) to 0.860 (Hut), respectively (Table 1). The value of θ, the estimator of F_{ST} (Weir & Cockerham 1984), across all samples was low, showing low interpopulation differentiation. It was estimated as 0.043 ± 0.017 and differs significantly from zero ($P < 0.001$). This greater variation within populations than among populations is consistent with the lack of correlation between the geographical distance and genetic differentiation (Spearman Rank correlation coefficient = 0.045; $P = 0.24$).

Genetic diversity within the adult stage

The three adult stands we analysed were chosen for their F_{IS} values in the seed samples: Hut with a F_{IS} close to zero (0.003, $P > 0.05$), Mwr with an average F_{IS} value (0.147, $P < 0.001$) and Lar with a high F_{IS} value (0.215, $P < 0.001$).

Across all five loci, the linkage disequilibrium test here also revealed the statistical independence of our loci. Hardy–Weinberg equilibrium was rejected for the three populations ($P < 0.001$), which showed an excess homozygosity. It was also rejected for all loci ($P < 0.05$), except for those from FEMSATL4 ($P = 0.46$).

The global F_{IS} was estimated as 0.292 and differed significantly from zero ($P < 0.001$) (Table 2). F_{IS} across populations also showed high, positive and significant values ($P < 0.01$). F_{IS} across loci varied from 0.077 (FEMSATL4) to 0.450 (M2–30) and is highly significant for all five loci ($P < 0.001$) except for FEMSATL4 ($P = 0.10$) (Table 2).

The adult stands showed a level of intrapopulation variability of the same order as the one found for the seed stands. The values for the observed and expected heterozygosities ranged from 0.500 (Mwr) and 0.741 (Lar) and from 0.896 (Mwr) and 0.900 (Hut) for H_O and H_E, respectively (Table 3).

Discussion

Our study revealed high levels of intrapopulation diversity and low genetic differentiation between common ash populations for both seed and adult stages. These results are very close to those reported by Heuertz *et al*. (2001) on 10 Bulgarian common ash populations analysed with five microsatellites. Indeed, they found a high level of total diversity, with an H_T value of 0.793 (± 0.300) and a low overall differentiation among populations ($F_{ST} = 0.087$, $P < 0.001$). Our results are also consistent with the peculiar distribution of genetic variability for nuclear genes in forest trees. Some usual explanations for this pattern refer to the biological features of forest trees: large population sizes, mating systems usually close to strict allogamy and pollen or seed dispersion over great distances (Hamrick *et al*. 1992; Le Corre & Kremer 1998). More recently, Austerlitz *et al*. (2000) have demonstrated that high levels of pollen flow associated with life cycle characteristics of trees (longevity and length of the juvenile phase) allow us to explain this observed structure of genetic diversity.

Table 3 Levels of genetic diversity and deviation of observed genotypes from Hardy–Weinberg equilibrium (HWE) assessed for the adults with five microsatellite loci

Population		Locus M2–30	4	5	11	19	All loci
Hut	N	13	19	14	16	19	
	A_O	12	13	12	9	16	
	H_E	0.938	0.888	0.905	0.833	0.937	0.900
	H_O	0.385	0.895	0.571	0.750	0.737	0.691
	HW						***
Mwr	N	13	17	15	19	8	
	A_O	12	12	12	11	9	
	H_E	0.938	0.825	0.936	0.892	0.917	0.896
	H_O	0.462	0.647	0.333	0.526	0.500	0.500
	HW						***
Lar	N	18	16	12	18	17	
	A_O	16	12	13	12	12	
	H_E	0.932	0.905	0.859	0.862	0.918	0.897
	H_O	0.667	0.875	0.750	0.556	0.882	0.741
	HW						***
All populations	A_O	29	18	26	20	22	
	H_E	0.936	0.873	0.903	0.864	0.926	
	H_O	0.523	0.808	0.537	0.604	0.750	
	HW	***	ns	***	***	***	

For each locus in each population, the number of samples successfully genotyped (N), observed number of alleles (A_O), observed (H_O) and expected (H_E) heterozygosities are reported. For each locus, H_O and H_E and departure from Hardy–Weinberg equilibrium (HWE) tested with the program GENEPOP (Raymond & Rousset 1995) are shown. For each population, H_O and H_E, A_O and departure from HWE are given.
ns, nonsignificant; ***$P < 0.001$.

This study also showed excess homozygosity within our common ash populations at the level of both seeds and adults. Heuertz *et al*. (2001) also detected a significant heterozygote deficiency (mean F_{IS} value of 0.014 ± 0.045, $P < 0.001$) in the Bulgarian populations, although the level was lower than that detected in the French stands. It would be interesting to confirm these results with studies in common ash populations from other regions of Europe. Except in species where selfing is known to happen, many forest trees show no significant departure from Hardy–Weinberg equilibrium (Hamrick *et al*. 1992; with allozymic markers). For the European oaks (*Quercus petraea* and *Q. robur*), Streiff *et al*. (1998) found for microsatellite markers fixation indices close to zero, with a global value of F_{IS} being 0.07. For the same species and studying two populations, Degen *et al*. (1999) obtained the similar results, with mean F_{IS} values around −0.02. Oaks differ from ash by their continuous spatial distribution and by their mating system (monoecious species, seed dispersal by animals). Further information on other forest tree species will help understand if different strategies in reproductive biology come to various genetic structures in tree species.

To know if this heterozygote deficiency remains constant over the life stages of the ash, we analysed the mother trees in three of our stands. In fact, many authors have shown for various forest trees the presence of a positive F_{IS} at the seed stage that becomes null or negative at the adult stage (Farris & Mitton 1984; Yazdani *et al*. 1985; Plessas & Strauss 1986). They put forward the existence of inbreeding depression that would eliminate between the two stages the inbred individuals, which have the highest homozygosity rate. We also found for the adults a significant excess homozygosity, which does not seem to be consistent with a process of inbreeding depression acting between the juvenile and mature phases.

It remains to us to explain the origin of this excess homozygosity. Many explanations can be put forward.

The heterozygote deficiency may be linked with the nature of the markers used. First, it cannot be explained by biased transmission of alleles as our controlled crosses showed that our microsatellite loci present a Mendelian inheritance of alleles. Secondly, the presence of null alleles (alleles that are never amplified because of nonmatching primer sequences) can be suspected for microsatellite loci, especially when using heterologous primers (Pemberton *et al*. 1995). Yet, the primers used in this study were specifically defined in common ash (Brachet *et al*. 1999; Lefort *et al*. 1999). Controlled crosses are one experimental way

to find evidence for null alleles when at least one of the parents is a homozygote at the focusing locus. If some progenies fail to yield an amplification product for the allele of the homozygote parent, showing the noninheritance of one parental allele, it means that the homozygote parent is in fact heterozygote and holds a null allele. In this way, our controlled crosses, which test for only a sampling of alleles present at each locus, suggest that four out of the five loci do not show null alleles. The locus FEMSATL5 remains ambiguous and needs further investigation to confirm the presence of a null allele assumed from the results of the controlled crosses.

Nevertheless, as this experimental test is not sufficient for conclusions to be drawn from it, a short calculation can be made in order to quantify the presence of null alleles in our 12 seed stands. At a given locus, the frequency p^2 of the individuals that failed to amplify any fragment also represents the frequency of the homozygotes for the supposed null allele. Assuming then Hardy–Weinberg equilibrium, the frequency of the null allele, if it exists, should be p. According to the number of nonamplifying individuals per locus in the analysis of the seed stands, the frequency of a null allele was estimated for each locus. It ranges from 0.25 (FEMSATL11) to 0.38 (FEMSATL19). Given these high null alleles frequencies compared to the frequencies we observed for the other alleles at each locus, it seems unlikely that the entire heterozygote deficiency in common ash French populations is due to null alleles.

Another, more putative, source of nondetection of microsatellite alleles is the short allele dominance, where, in heterozygote individuals that present a short and a long allele, only the short allele is detected and scored (Wattier *et al*. 1998). In the common ash microsatellites, looking at Fig. 2, it appears that for FEMSATL4 some heterozygote individuals (lanes 7, 10 and 11) show a 'long-sized' allele of weaker band intensity than is the 'short-sized' allele. These types of microsatellite profiles can also be found for some of the other loci used in this study. We can then assume that for some individuals these long alleles may be totally suppressed during PCR and thus not scored. To see if this phenomenon might happen for our microsatellite loci, we achieved a little test on a few samples. At a given locus (M2–30, FEMSATL4, FEMSATL5, FEMSATL11 and FEMSATL19 were tested), two types of individuals were chosen: some heterozygotes for two short alleles and some heterozygotes for two long alleles. Each combination 'short allele heterozygote — long allele heterozygote' was amplified in the same PCR reaction tube and analysed on a 6% polyacrylamide gel. In the sample tested, we just observed a small decrease in band intensity for the alleles of the 'long' heterozygotes compared with the alleles of the 'short' heterozygotes. However, these long alleles were still scorable and were most often identified as a 'real' allele. Sometimes, they were identified as a 'suspected' allele. In this later case, the sample was reamplified and regenotyped in order to check the presence of the long allele. So, even if this test is only a preliminary work, it suggests that this short allele dominance phenomenon does not happen clearly in our case.

Finally, the presence of null alleles cannot be completely rejected and may act as part of a combination of factors. A definitive conclusion would require numerous experiments testing all combinations of alleles in controlled crosses. Another way to conclude on the presence of null alleles would be to design sets of new primers, located upstream or downstream from the original ones, to see if the individuals previously scored as homozygotes remain as such when using the new primers, as Gibbs *et al*. (1997) did. We carried out some preliminary experiments of this kind by designing new primers for the M2–30, FEMSATL4, FEMSATL5, FEMSATL11 loci. For these four loci, we did not detect any problem related to the presence of null alleles. The results of these tests do not then question our genotyping with the initial primers.

Secondly, we can assume the existence of a Wahlund effect, due to the presence of breeding subunits inside the studied population. This Wahlund effect may be either spatial or temporal. In our study, each population represented a large area with mother-trees being at least 20 m from one another. Thus, each population may consist of several spatial breeding subpopulations, generating a spatial Wahlund effect. A temporal Wahlund effect could instead be produced either by a difference in within-year flowering phenology, which we observed in common ash, or by variations in flowering expression of individuals between years. Each population may then represent two or more subunits of reproductive individuals flowering at different times the same year or over the years. Moreover, the F_{IS} can be considered as an F_{ST} value, which measures the differentiation between the population subunits that are at Hardy–Weinberg equilibrium. The low level of interpopulation differentiation that we observed (global F_{ST} of 0.043 for seeds) is far less than the mean estimated F_{IS} (F_{IS} = 0.163 for seeds). As a consequence, it seems difficult to assume that, inside the stands, there are subunits that would create an F_{ST} value about four times greater than the value found between the stands. Thus, the existence of a Walhund effect, either spatial or temporal, seems unlikely here (see also García de León *et al*. 1997). Nevertheless, further information, especially with regard to the distribution of sexual phenotypes and the flowering phenology in common ash populations, will help to provide a definitive conclusion.

Finally, excess homozygosity within populations could be explained by mating among relatives, or partial selfing. To our knowledge, there is no information about inbreeding in common ash in the literature. We tested for selfing possibilities on the few trees located in our campus.

Branches were bagged before the emergence of stigmas and the bags were removed after the receptive stage of the female flowers. No fruits were obtained. This suggests that selfing does not occur in common ash under controlled conditions. However, it does not exclude the occurrence of a few selfing events in natural populations, as was found for the European oaks (Bacilieri *et al*. 1995; Streiff *et al*. 1999).

As a conclusion, the present study revealed an original result for a temperate tree, which remains to be definitively explained: the existence of excess homozygosity in the studied common ash populations. No single hypothesis could explain this. Instead, two explanations out of the three discussed here may act jointly to produce the observed heterozygote deficiency: the presence of null alleles for some loci (in particular FEMSATL5) on the one part, and assortative mating on the other. Our current study on a large native population will allow us to test for spatial or temporal structuring. More generally, we are estimating gene flow by pollen and seeds and clarifying the mating system of this species. Such information will help us understand how common ash populations function.

Acknowledgements

We would like to thank B. Jung-Muller, E. Lacey, P. Taberlet and two anonymous reviewers for critical reading and helpful comments for improving previous versions of the manuscript. We also thank C. Raquin and I. Normand for constructive discussion of the results, M. Heuertz and J. F. Hausman for their welcome in their laboratory to compare the two microsatellite detection techniques, O. Jonot, P. Bertolino and O. Cudelou for laboratory work, F. Vedel and R. Boyer for the time they spent on the photographs and L. Nowak for his help in the field. The first author acknowledges an FCPR grant from the French Ministry of Agriculture (FMA). The DERF and the ENGREF institutions from the FMA also provided financial support.

References

Austerlitz F, Mariette S, Machon N, Gouyon PH, Godelle B (2000) Effects of colonization processes on genetic diversity: differences between annual plants and tree species. *Genetics*, **154**, 1309–1321.

Bacilieri R, Ducousso A, Kremer A (1995) Genetic, morphological, ecological and phenological differentiation between *Quercus petraea* (Matt.) Liebl. & *Quercus robur* L. in a mixed stand Northwest of France. *Silvae Genetica*, **44**, 1–10.

Binggeli P, Power J (1999*) Gender variation in ash (Fraxinus excelsior* L.). http://members.tripod.co.uk/WoodyPlantEcology/species/ash.htm.

Bodénès C, Labbé T, Pradère S, Kremer A (1997) General vs. local differentiation between two closely related white oak species. *Molecular Ecology*, **6**, 713–724.

Brachet S, Jubier MF, Richard M, Jung-Muller B, Frascaria-Lacoste N (1999) Rapid identification of microsatellite loci using 5′ anchored PCR in the common ash *Fraxinus excelsior*. *Molecular Ecology*, **8**, 160–163.

Chase M, Kesseli R, Bawa K (1996) Microsatellite markers for population and conservation genetics of tropical trees. *American Journal of Botany*, **83**, 51–57.

Degen B, Streiff R, Ziegenhagen B (1999) Comparative study of genetic variation and differentiation of two pedunculate oak (*Quercus robur*) stands using microsatellite and allozyme loci. *Heredity*, **83**, 597–603.

Farris MA, Mitton JB (1984) Population density, outcrossing rate, and heterozygote superiority in ponderosa pine. *Evolution*, **38**, 1151–1154.

Franc A, Ruchaud F (1996) Le Frêne commun. In: *Autécologie Des Feuillus Précieux: Frêne Commun, Merisier, Erable Sycomore, Erable Plane* (ed. CEMAGREF), pp. 15–68. Imprimerie Louis Jean, Gap.

García de León FJ, Chikhi L, Bonhomme F (1997) Microsatellite polymorphism and population subdivision in natural populations of European sea bass *Dicentrarchus labrax* (Linnaeus, 1758). *Molecular Ecology*, **6**, 51–62.

Gibbs HL, Prior KA, Weatherhead PJ, Johnson G (1997) Genetic structure of populations of the threatened eastern massasauga rattlesnake, *Sistrurus c. catenatus*: evidence from microsatellite DNA markers. *Molecular Ecology*, **6**, 1123–1132.

Goudet J (1995) FSTAT (Version 1.2): a computer programme to calculate F-statistics. *Journal of Heredity*, **86**, 485–486.

Hamrick JL, Godt MJW, Sherman-Broyles SL (1992) Factors influencing levels of genetic diversity in woody plant species. *New Forests*, **6**, 95–124.

Heuertz M, Hausman JF, Tsvetkov I, Frascaria-Lacoste N, Vekemans X (2001) Assessment of genetic structure within and among Bulgarian populations of the common ash (*Fraxinus excelsior* L.). *Molecular Ecology*, **10**, 1615–1623.

Karhu A, Hurme P, Karjalainen M *et al.* (1996) Do molecular markers reflect patterns of differentiation in adaptive traits of conifers? *Theoretical and Applied Genetics*, **93**, 215–221.

Le Corre V, Kremer A (1998) Cumulative effects of founding events during colonisation on genetic diversity and differentiation in an island and stepping-stone model. *Journal of Evolutionary Biology*, **11**, 495–512.

Lefort F, Brachet S, Frascaria-Lacoste N, Edwards KJ, Douglas GC (1999) Identification and characterization of microsatellite loci in ash (*Fraxinus excelsior* L.) and their conservation in the Olives family. *Molecular Ecology*, **8**, 1088–1090.

Levene H (1949) On a matching problem arising in genetics. *Annals of Mathematics and Statistics*, **20**, 91–94.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Pemberton JM, Slate J, Bancroft DR, Barrett JA (1995) Non-amplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology*, **4**, 249–252.

Picard JF (1982) Contribution à l'étude de la biologie florale et de la fructification du Frêne commun (*Fraxinus excelsior* L.). *Revue Forestière Française*, **XXXIV**, 97–107.

Plessas ME, Strauss SH (1986) Allozyme differentiation among populations, stands, and cohorts in Monterey pine. *Canadian Journal of Forest Research*, **16**, 1155–1164.

Raquin C, Jung-Muller B, Dufour J, Frascaria-Lacoste N (2002) Rapid seedlings obtaining from European ash species *Fraxinus excelsior* L. & *Fraxinus angustifolia* Vahl. *Annales Des Sciences Forestières*, **59**, in press.

Raymond M, Rousset F (1995) GENEPOP (Version 1.2): population genetics software for exact tests and eucumenism. *Journal of Heredity*, **86**, 248–249.

Rossetto M, Slade RW, Baverstock PR, Henry RJ, Lee LS (1999) Microsatellite variation and assessment of genetic structure in

tea tree (*Melaleuca alternifolia* — Myrtaceae). *Molecular Ecology*, **8**, 633–643.

Streiff R, Lefort F (1997) A protocol for higher contrasted DNA silver staining. *Castaway Times (Stratagene)*, **6**, 2.

Streiff R *et al.* (1998) Within-population genetic structure in *Quercus robur* L. & *Quercus petraea* (Matt.) Liebl. assessed with isozymes and microsatellites. *Molecular Ecology*, **7**, 317–328.

Streiff R *et al.* (1999) Pollen dispersal inferred from paternity analysis in a mixed oak stand of *Quercus robur* L. & *Q. petraea* (Matt.) Liebl. *Molecular Ecology*, **8**, 831–841.

Ueno S, Tomaru N, Yoshimaru H, Manabe T, Yamamoto S (2000) Genetic structure of *Camellia japonica* L. in an old-growth evergreen forest, Tsushima, Japan. *Molecular Ecology*, **9**, 647–656.

Wardle P (1961) Biological flora of the British Isles. *Fraxinus excelsior* L. *Journal of Ecology*, **49**, 739–751.

Wattier R, Engel CR, Saumitou-Laprade P, Valero M (1998) Short allele dominance as a source of heterozygote deficiency at microsatellite loci: experimental evidence at the dinucleotide locus Gv1CT in *Gracilaria gracilis* (Rhodophyta). *Molecular Ecology*, **7**, 1569–1573.

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

White G, Powell W (1997) Isolation and characterization of microsatellite loci in *Swietenia humilis* (Meliaceae): an endangered tropical hardwood species. *Molecular Ecology*, **6**, 851–860.

Wright S (1921) Systems of mating. *Genetics*, **6**, 111–178.

Wright S (1969) *Evolution and the Genetics of Populations, The Theory of Gene Frequencies*, Vol. 2. University of Chicago Press, Chicago.

Yazdani R, Muona O, Rudin D, Szmidt AE (1985) Genetic structure of a *Pinus sylvestris* L. seed-tree stand and naturally regenerated understory. *Forest Sciences*, **31**, 430–436.

This work is part of a broader project on the evolution of genetic diversity of trees in Nathalie Frascaria-Lacoste's team. One part of the project aims at understanding genetic structure, gene flow and mating system of *Fraxinus excelsior*. Marie-Elise Morand is a PhD student with an ENGREF graduate, Stéphanie Brachet is an ENGREF doctor-engineer at the laboratory, Pascale Rossignol is a student at the Paris XI University and Jean Dufour is a researcher-engineer at the INRA institution (Orléans). Nathalie Frascaria-Lacoste is a lecturer in forest genetics at the ENGREF institution (Paris) and her laboratory is situated in Orsay.
