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Chloroplast DNA variation of oaks in France and the influence of forest fragmentation on genetic diversity

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Abstract

Chloroplast DNA variation was studied in a total of 878 French oak populations from four different species. Three main cpDNA lineages were found, which have well-demarcated distributions in the country. The study of the distribution of haplotypes in each species supports the view that the four species were restricted to different refugia during the last ice-age. This is evident despite the fact that extensive cpDNA introgression occurred during and after postglacial recolonisation. Nevertheless, the individual species have different ecological requirements and also differ in their ability to hybridise, resulting in heterogeneous levels of partitioning of cpDNA diversity and incomplete cpDNA introgression. The first analysis of the effect of the landscape structure on genetic diversity in these oak species is presented here. The only discernible effect of landscape structure on cpDNA diversity was found in *Quercus robur*, and is very weak and rather counterintuitive. The biology and abundance of these oak species may make them particularly resistant to fragmentation; in addition, artificial seed flow may complicate the picture, and will require more direct investigations.

Keywords: Genetic differentiation; Interspecific gene flow; Landscape structure; Postglacial recolonisation routes; *Quercus* sp.; Seed dispersal

1. Introduction

France lies to the north of two peninsulas (Iberia and Italy) that have played an important role as ice-age refugia for the terrestrial biota of western Europe (Huntley and Birks, 1983). Many organisms moved from there northward into France once the climate warmed, together with others that came from further east and notably from the Balkan peninsula (Hewitt, 1999). For many terrestrial taxa, the immigration into France brought together differentiated gene pools that had been evolving separately during the colder periods, resulting in a potentially complex spatial genetic structure, that may have been further modified by human activities. In the case of forest trees, the latter include fragmentation and seed transfers, but also within-stand selection practices, which may have affected adaptative traits such as growth or architecture (Kremer, 1994). By far the more conspicuous of these activities was deforestation; indeed, during historical times, the deforestation that took place in many temperate and Mediterranean regions was comparable in magnitude if not in rapidity to that currently occurring in the tropics. Although what typically comes to the mind while thinking of *global changes* is the global warming induced by the greenhouse effect, deforestation at this scale constitutes an equally large and profound change, although of completely different nature. In France, as in the rest of Europe, it has brought about the change from the

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largely continuous forest cover in prehistoric times to the fragmented forest landscape of today. It is not known, however, whether it may have already influenced the distribution of genetic variation in species inhabiting these forest fragments, beyond the obvious reduction in effective population sizes. An accurate evaluation of the long-term ecological and evolutionary consequences of fragmentation is important in view of the current climatic changes caused by human activities. Most of the literature has focused on the ecological consequences of fragmentation, such as species composition and richness or population dynamics, whereas the study of the population genetic consequences has remained largely theoretical (Templeton et al., 1990; Young et al., 1996). The predicted effects of fragmentation are an increase in drift and inbreeding, a reduction in interpopulation gene flow and finally an increased probability of extinction within the metapopulation. The first empirical evidence, however, based largely on isozymes, indicates that the genetic consequences of fragmentation are more varied than predicted from simple theoretical models.

Oaks provide appropriate species in which to test such theoretical predictions as they comprise key tree species which form a significant part of the forest landscapes in Europe. According to Holsinger (1993), the plant species most likely to suffer from additional habitat fragmentation are those that are abundant and distributed continuously across a broad geographic range rather than those that have a naturally patchy distribution. In the case of the long-lived oaks, due to the limited seed flow into established forests, the footprints of postglacial recolonisation are still discernible using maternally inherited markers such as chloroplast DNA (cpDNA) polymorphisms (Petit et al., 1993, 1997). However, large scale deforestation and secondary, human induced mixing of populations, may have partly blurred this pattern.

Furthermore, in view of the economic importance of several of the European oak species, some knowledge of their genetic variation is important to optimise the management of their genetic resources. Up to 4,000,000 ha in France are covered by white oak forests, and even in rather open, agricultural areas, oak is often a conspicuous element of the landscape, found in hedges or as isolated trees. Few regions in France are completely devoid of white oaks, as almost every region can supply the ecological requirements of at least one of the four species that can grow in this country: the pedunculate oak (*Quercus robur* L.), the sessile oak (*Q. petraea* (Matt.) Liebl.), the pubescent oak (*Q. pubescens* Willd.), and the Pyrenean oak (*Q. pyrenaica* Willd.). For instance, in the Mediterranean region, where the two most widespread species (*Q. robur* and *Q. petraea*) are absent or very rare, *Q. pubescens* constitutes large stands of great ecological importance, even if of little economic value. Each year, French foresters plant about 20 million oak seedlings, representing half the regeneration of oak forests in the country, the remaining half is regenerated naturally, by successive thinning of older stands in order to favour the production of a seed crop and the rapid growth of the resulting seedlings.

To study the processes responsible for the generation of geographic patterns of genetic variation, the first step is to obtain detailed information on the spatial genetic patterns themselves. In the case of the oaks, comparisons with the overall patterns of cpDNA variation observed in Europe (Petit et al., 2002a,b) and with palaeobotanical information (Brewer et al., 2002) should help to interpret the initial phase of oak establishment into France during the Lateglacial interstadial and the Holocene period. To evaluate the indirect influence of man on this genetic structure, the impact of forest fragmentation on genetic diversity can be investigated; indeed, measures of landscape structure could conveniently summarise the cumulative effects of human impacts through time, which would be difficult to measure directly.

In this paper, we present the results of an extensive survey of cpDNA variation in four white oak species in France. We first describe the spatial patterns of genetic variation and attempt to interpret postglacial movements of colonisation on this basis. Levels of cpDNA diversity and its partitioning within and among populations are compared among the different oak species studied; moreover, genetic similarities between species are investigated in order to measure to what extent these species are evolving independently. This information forms the basis for the next step of our study, as interspecific and intraspecific gene flow need to be considered while trying to account for the effect of the structure of the landscape on cpDNA diversity.

Part of the data used here has been published previously. About 100 populations from France were included in the European study of Dumolin-Lapègue et al. (1997). Another 188 populations were used to study the regional pattern of spatial variation for cpDNA in western France (Petit et al., 1997), whereas 378 populations distributed in the southern part of France were sampled to study the association between the maternally inherited chloroplast and mitochondrial lineages in these oaks (Dumolin-Lapègue et al., 1998) and to measure the extent of introgression between the various oak species sampled (Dumolin-Lapègue et al., 1999). Additional sampling was, however, necessary in order to achieve a homogeneous sampling throughout the country, a prerequisite for spatial genetic studies.

2. Material and methods

2.1. Plant material

A total of 878 populations (3605 individual trees) belonging to four oak species (*Q. robur*, *Q. petraea*, *Q. pubescens* and *Q. pyrenaica*) have now been studied in continental France, including 221 new populations from the northern part of France. The data from Corsica are discussed by Fineschi et al. (2002). Some material was sampled in provenance tests (see Kremer et al., 2002), but the majority of the populations were sampled directly in the forests; sampling strategy was as described in Petit et al. (2002a): five to six rees growing between 50 and 500 m apart were sampled in the additional populations. Trees of obvious artificial origins (young plantations, trees in parks or on roadsides, etc.) were not sampled. However, no thorough study based on historical documents was made to attempt to differentiate material of exotic provenance. During the sampling, trees presenting several intermediate morphological characters, which could not be identified readily, were avoided. We attempted to find populations at least every 50 km (often every 20–30 km) with the help of vegetation maps. Longitude and latitude (transformed in decimal degrees Greenwich) were taken with a GPS or from topographical maps (IGN 1/100,000). This material was collected over a period of several years (mostly, 1995–1997).

2.2. PCR-RFLP procedure

Procedures are as indicated in Dumolin-Lapègue et al. (1998). PCR amplification of four cpDNA fragments (AS, CD, DT, TF, see Petit et al., 2002a) were used throughout, followed by digestion with the endonucleases *Hin*fI, *Taq*I, *Taq*I and *Hin*fI, respectively. For part of the material this was done in a stepwise manner, as a limited number of combinations had been found in previous screenings in this part of the range (Dumolin-Lapègue et al., 1997). The first analysis always started with the combination DT-*Taq*I. This allowed the unambiguous identification of some variants characterised by unique mutations (autapomorphies): 7, 12 and 26 (see Petit et al., 2002a). When the fragment DT-*Taq*I-3 had length variant 3, the combination TF-*Hin*fI was used to distinguish between haplotypes 10 and 11. Similarly, when the fragment DT-*Taq*I-1 had the point mutation (9), the combination CD-*Taq*I was used to differentiate between haplotypes 1 and 2. If there were other patterns than the most common ones observed with these combinations, complete characterisation of the haplotypes were made using all four fragments.

2.3. Genetic diversity analysis

Because the chloroplast genome is strictly maternally inherited in oaks (Dumolin-Lapègue et al., 1998), it is equivalent to a single haploid locus, so all analyses are based on the haplotype rather than the single restriction-site level. The methods of analysis were as described in Dumolin-Lapègue et al. (1999). Haplotype frequencies were used to estimate the mean within-population diversity (h_S), the total diversity (h_T), and the ratio $G_{ST} = (h_T - h_S)/h_T$ (which is a measure of the partitioning of diversity among populations), and their standard deviations following Pons and Petit (1995). These parameters were computed for the pooled samples regardless of species, for each of the three more common species (*Q. robur*, *Q. petraea* and *Q. pubescens*), as well as for both pure or mixed species populations. Furthermore, the French range was divided latitudinally to compare diversity in four regions, using as limits the parallels 45, 47 and 49°N, and comparisons were made across these regions. Due to the low sample numbers, the two

southernmost regions were pooled in the case of *Q. petraea*, and the three northernmost were pooled for *Q. pubescens*. In all cases, only populations that provided at least three sampled individuals were taken into account, to permit variances for these parameters to be computed. The coefficient G_{ST} was compared between categories by computing the difference between two G_{ST} values and comparing it with zero using a *t*-test.

2.4. Test of species, population and admixture effects

The tests of more specific hypotheses followed the methods used in Dumolin-Lapègue et al. (1999), which are briefly summarised here. These tests are based on the estimation of genetic identities (or genetic similarities) J ($J = \sum_i p_i^2 = 1 - h$, where i indexes the haplotypes and h is the classical measure of diversity). The test of the *population effect* is designed to evaluate whether oak trees belonging to different species are genetically more similar than expected by chance when present in the same population. Pairwise interspecific genetic similarities are compared within (J_1) and between (J_2) populations. However, the between population comparisons are restricted to nearby populations (located less than 50 km apart), given the strong cpDNA geographic structure. These two types of measures are then compared with each other to test this population effect. The test of the *species effect* is designed to evaluate whether individuals from the same population but belonging to different species are less similar genetically than individuals belonging to the same species; the intraspecific similarity within population (J_3) is compared with J_1, the equivalent measure for interspecific comparisons. For each pair of species, the two intrapopulation intraspecific similarity estimates (J_3) were computed, on the basis of those populations used to derive J_1, so that results can be compared (provided that there were at least two individuals per species). Finally, the *admixture effect* was tested by comparing, for each species, the intraspecific intrapopulation similarity (J_4) in mixed and in pure populations. The means of J_1, J_2, J_3 and J_4 (called M_1, M_2, M_3 and M_4, respectively) are computed and the whole distribution of J values are compared using a nonparametric Mann–Whitney–Wilcoxon test.

2.5. Test of the effect of landscape structure on cpDNA diversity

To test the effect of the fragmentation of the landscape on cpDNA diversity within each oak species, three variables have been measured at each sampling location: (1) the area (in km^2) of the sampled forest or wood (called *local forest area*), (2) the cumulative forest area within 5 km of the sampling point, in percentage of the total (*total forest area*), and (3) the percentage of 1 km^2 squares occupied by forests in the same area (divided into 76 squares) (*occupancy*). That is, only the landscape structure was taken into consideration, and no attempt was made to distinguish between oak forests and forests composed of other species. Additional factors were also considered: the *latitude*, the *longitude*, the presence of related oak species (*mixture*), as well as the following ratios: *local forest area/occupancy*, *total forest area/occupancy* and *local forest area/total forest area*. In a first approach, simple correlation analyses were made for each of the three species separately. There were 538 populations that could be used in total (i.e., comprising two or more individuals and sampled in a comparable way), 322 for *Q. robur*, 135 for *Q. petraea* and 180 for *Q. pubescens*. To examine if several factors could explain part of the level of intrapopulation cpDNA diversity, stepwise multiple regressions were carried out using the Splus statistical package. Various stepwise procedures were used (*Forward*, *Backward*, *Exhaustive* and *Efroymson*).

3. Results

3.1. Species distribution

Oaks are so common throughout France that it was in most cases possible to find a population every 50 km, and often every 30 km. There are, however, a few exceptional areas where oaks are rare and these include some mountainous areas, the lower Rhone region, and some very open landscapes in northern France. However, this largely uniform distribution (at this scale) includes four different species, which have very different distribution ranges, as can be seen from the species composition of the samples obtained for this study from the whole of France (Fig. 1). *Q. robur* covers 2,400,000 ha in France and is the most

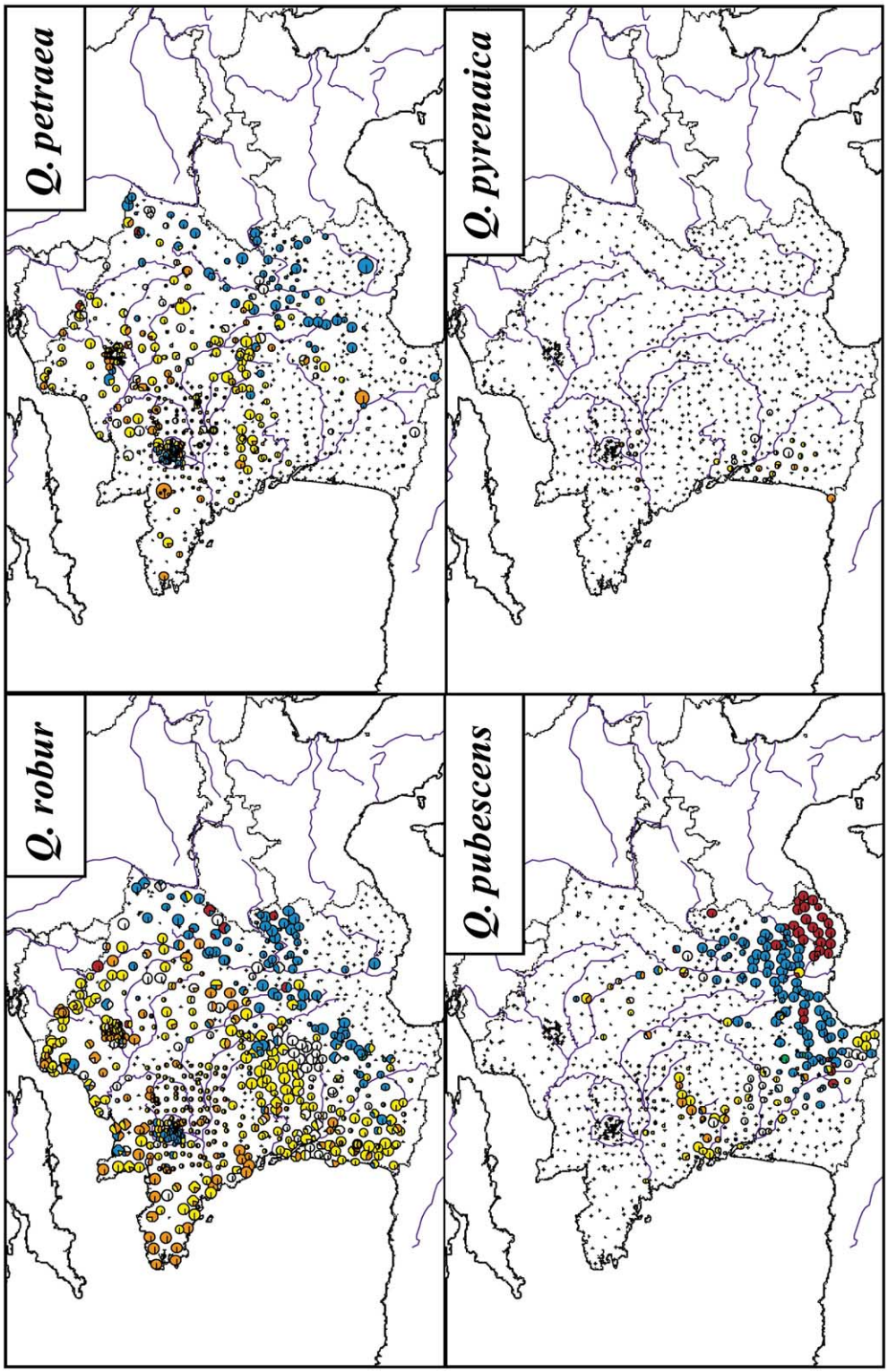

Fig. 1. cpDNA variation by species. The different haplotypes are represented by the same colours as given in Figs. 2 and 3.

Table 1
Number of populations where each haplotype has been detected

	Haplotype (lineage)											
	1 (C)	4 (A)	5 (A)	7 (A)	10 (B)	11 (B)	12 (B)	21 (D)	24 (B)	25 (B)	26 (A)	All (4)
Q. robur	10	1	1	157	276	110	158	2	1	1	1	584
Q. petraea	7			120	185	62	101		1			426
Q. pubescens	36			106	58	34	29	3				232
Q. pyrenaica					13	13	7					31
All	50	1	1	290	385	177	226	4	2	1	1	878

abundant of these oak species. *Q. robur* is rare only in the south-eastern part of the country where it is represented by scattered populations located along the Rhone valley. *Q. petraea* is the second most important species in France (1,750,000 ha); like *Q. robur*, it is more abundant in the north. Elsewhere the distribution of the two species is different: *Q. petraea* is more diffuse in the south, and can be found in the Massif Central, the Pyrenees, and somewhat further south in the Alps. In contrast, it is much less abundant than *Q. robur* along the Atlantic coast. *Q. pubescens* (850,000 ha), is more abundant in the south (especially the south-east), and along the Garonne river, but is rare further north: populations can be found in the vicinity of the Loire and of the Seine river, and at other sites with suitable micro-climate and edaphic conditions, such as in the south of Alsace or in Picardie (not represented in the sample). Finally, *Q. pyrenaica* is a typically Atlantic species, and is much less abundant than the three other species. It is found in regions characterised by poor, acidic soils (such as the Landes in south-western France, or part of Anjou, north of the Loire river).

3.2. cpDNA variation

A total of 11 cpDNA haplotypes were detected during this survey (11 in *Q. robur*, six in *Q. petraea* and in *Q. pubescens*, and three in *Q. pyrenaica*, see Table 1). Compared to the previous surveys in southern France (Dumolin-Lapègue et al., 1998), two new variants were found in northern France (haplotypes 4 and 5), each in a single population. They had been detected previously further east in Europe (Dumolin-Lapègue et al., 1997, see also Petit et al., 2002a,b). The restriction profiles of all haplotypes are provided in Annex 1 and 2 of Petit et al. (2002a). The 11 haplotypes belong to four lineages, as defined in Figs. 1 and 2 of Petit et al. (2002a): lineage A (haplotypes 4, 5, 7 and 26), B (haplotypes 10, 11, 12, 24 and 25), C (haplotype 1) and D (haplotype 21), as indicated in Table 1. The distribution of these haplotypes according to species is presented in Fig. 1, and their cumulative distribution (i.e., independent of species) is illustrated in Fig. 2.

3.3. Geographic distribution of haplotypes

Based on cpDNA variation, three main regions can be distinguished in France (Fig. 2). These consist of: (1) the south-east corner (Var and maritime Alps), where haplotype 1 is almost exclusively present, (2) the region situated east of a line running approximately from Toulouse to Strasbourg, where haplotype 7 dominates, except for the south-east corner, and (3) the remaining part of France (about two-third of the range), where three closely related haplotypes (10, 11 and 12) dominate, but are interspersed with each other. One of the four oak species (*Q. pyrenaica*) is situated completely within the third zone, due to its western distribution. In contrast, the distribution of *Q. pubescens* encompasses all three areas, whereas *Q. robur* and *Q. petraea* have distribution ranges that overlap the two main areas. Maps of distribution of individual haplotypes (Fig. 3) allow some finer patterns of distribution to be distinguished. For instance, a few populations fixed for haplotype 1 are found further west outside the first zone. These include populations near the Pyrenees, as well as others near Switzerland, and along the border between France and Belgium or Germany. Points more inland correspond to populations that are not fixed for this haplotype.

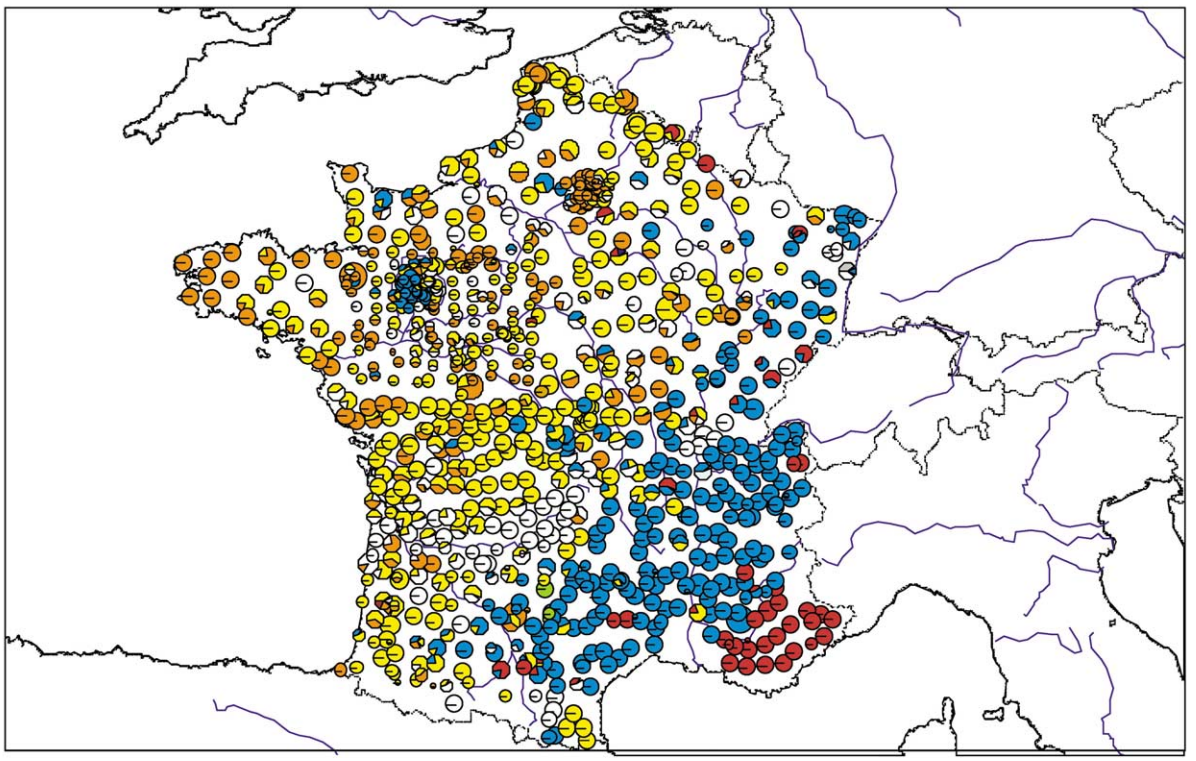

Fig. 2. Overall cpDNA variation in France (regardless of species). The different haplotypes are represented by the same colours as given in Fig. 3.

Haplotype 7 also appears some considerable distance outside of its zone as defined previously, as in the case of the patch found in the Sarthe region, in north-western France; contrary to the situation with haplotype 1, however, these outlying populations are fixed for haplotype 7. Haplotype 10 is the most abundant of the three main haplotypes belonging to lineage B. Haplotype 11, which is less frequent, appears to be more patchily distributed; it is especially abundant along the Dordogne river in western France. Both haplotypes 10 and 11 are found in the eastern Pyrenees, near the Mediterranean sea, in a somewhat isolated location. Compared to the two previous haplotypes, haplotype 12 has generally a more westerly distribution. Despite these small differences, the overall similarity in the distribution of the three haplotypes remains striking. Most of the other haplotypes occur in only one or two locations, so that it is difficult to appreciate if there is any geographic structure; haplotype 21 appears, however, to be somewhat clustered, as it is found in three neighbouring populations, near Toulouse, but also in the Alps in one locality where it is fixed.

3.4. cpDNA sharing among species

Among the 878 populations analysed, 367 (42%) consist of two or more species (mixed populations). These populations are especially useful for characterising the level of cytoplasmic exchange. For instance, to quantify the propensity of the species to share the same haplotype when in sympatry, interspecific similarities can be contrasted between and within population (Table 2). In each of the three interspecific comparisons considered (excluding *Q. pyrenaica*), the mean intrapopulation similarity was significantly higher than the corresponding interpopulation similarity (by 20–30%). Hence, when two trees from different oak species are growing in the same population, they will have a strong tendency to share the same haplotype (the probability varies from 0.67 to 0.85). The interspecific similarities were greatest in

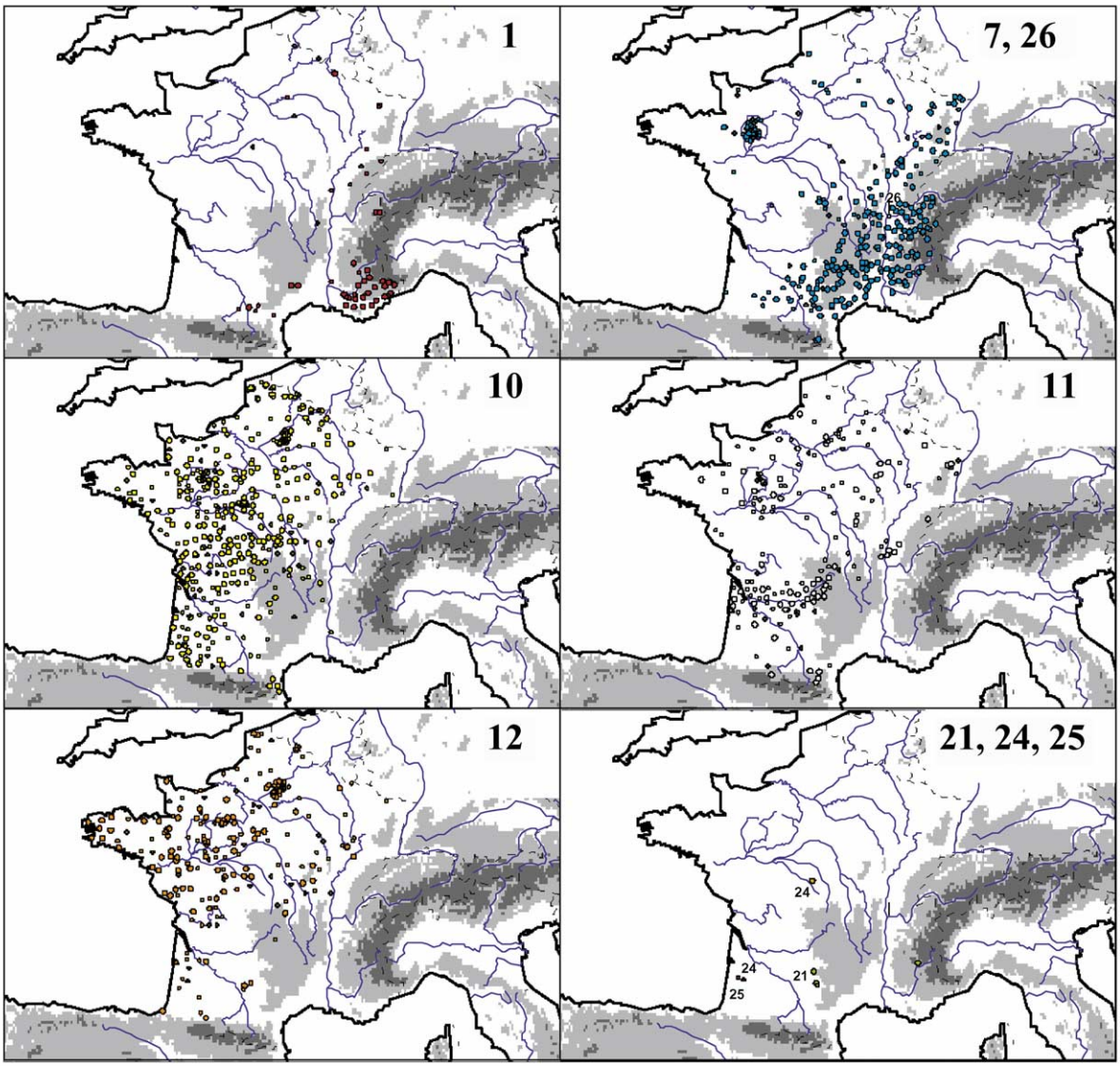

Fig. 3. Distribution of the haplotypes detected during the survey. Regions above 500 and 1000 m are represented by different shades of greys. Symbols sizes are roughly proportional to the likeliness that the population is autochthonous: populations fixed for a given haplotype are represented by the larger circles, those of dubious status by the smaller ones, and those populations where more than one haplotype have been identified are represented by intermediate symbols.

the pair *Q. petraea*/*Q. pubescens* and lower in the pair *Q. robur*/*Q. pubescens*. These results are very similar to those obtained in the previous, more limited study in southern France (Dumolin-Lapègue et al., 1999), except that the interspecific similarities between *Q. robur* and *Q. petraea* are somewhat lower in the present study (note however that few new populations of *Q. pubescens* have been added, as this species is rather rare in northern France).

To check if there is a species effect, a comparison of the intrapopulation similarities within and between species was made. There are actually six possible comparisons (two intraspecific similarities by species pair) (Table 2). In four of six cases, the intraspecific

Table 2
Test of population and species effects

Pair of species	Number of populations	Mean of the genetic parameters[a]			Local sharing, Z_{12}[b]	Species effect, Z_{23}[b]
		M_1	M_2	M_3		
Q. robur/*Q. petraea*	274[c]	0.56	0.81		-8.98^{***}	
Q. robur	155[d]			0.80		$+0.29^{n.s}$
Q. petraea	146[d]			0.88		-2.82^{**}
Q. robur/*Q. pubescens*	92[c]	0.51	0.67		$+3.26^{**}$	
Q. robur	49[d]			0.84		-5.16^{***}
Q. pubescens	53[d]			0.74		-3.69^{***}
Q. petraea/*Q. pubescens*	40[c]	0.58	0.85		-3.36^{***}	
Q. petraea	17[d]			1.00		$-1.89^{n.s}$
Q. pubescens	20[d]			0.80		$+0.17^{n.s}$

[a] Mean of the genetic parametres: M_1, mean of the interspecific gene identities between nearby (<50 km) populations J_1; M_2, mean of the interspecific gene identities within population J_2; M_3, mean of the intraspecific gene identities within population J_3 (based on those populations used to compute J_2, provided that sample sizes were sufficient).

[b] Mann–Whitney–Wilcoxon statistics for the distributions of the genetic parameters: comparison of J_1 and J_2 (Z_{12}) and J_2 and J_3 (Z_{23}), respectively, testing the local sharing and the species effect. n.s: not significant, ** $0.001 < P < 0.01$, *** $P < 0.001$.

[c] Number of populations where both species are present.

[d] Number of populations where each species is represented by more than one individual when both species are present.

similarity was higher than the corresponding interspecific similarity, with three of these tests being highly significant: in particular, the similarity between *Q. petraea* trees belonging to the same population is higher than the corresponding interspecific similarity with *Q. robur*, and both *Q. robur* and *Q. pubescens* individuals, when found in mixtures, are more similar to their conspecifics than to individuals of the other species. Furthermore, in a mixed population of *Q. petraea* and *Q. pubescens*, *Q. petraea* trees are also more likely to share the same haplotype with other conspecifics than with *Q. pubescens* individuals, although the test does not reach the 5% significance level, but samples sizes are lower for this species pair. Surprisingly, despite the relatively high sample sizes, *Q. robur* trees found in mixture with *Q. petraea* are not more similar to each other than to *Q. petraea* trees; similarly, although sample sizes are not as large, *Q. pubescens* trees are not more related to each other (as judged by their cpDNA haplotype) than they are to *Q. petraea* trees growing with them.

The data also allow the existence of an ''admixture effect'' to be tested, whereby the presence of other related oak species could affect the level of *intraspecific* diversity. Comparisons between mean genetic similarities for mixed or pure populations of each of the three species studied are given in Table 3. As in Dumolin-Lapègue et al. (1999), *Q. pubescens* is found to be more variable in mixed populations. More surprisingly, and despite the increased sample sizes, no effect is apparent for the two other species; if any, the indication is rather that pure populations are more variable than mixed ones.

3.5. Distribution of cpDNA diversity within and among populations

cpDNA diversity and its partitioning among populations have been studied and compared for the total population and then separately for the three main

Table 3
Within-population intraspecific gene identities

	Number of populations	M_4[a]	Z[b]
Q. robur mixed	211	0.812	$+1.23^{n.s}$
Q. robur alone	232	0.798	
Q. petraea mixed	153	0.892	$+1.44^{n.s}$
Q. petraea alone	132	0.857	
Q. pubescens mixed	59	0.763	-3.76^{***}
Q. pubescens alone	124	0.939	

[a] Mean of the within-population intraspecific gene identity J_4.

[b] Mann–Whitney–Wilcoxon statistics for the comparison of the distributions of J_4 for each species (mixed versus single-species populations). n.s: not significant, *** $P = 0.001$.

Table 4
Genetic diversity and differentiation, by region and by species

	Number of populations	Mean sample size	Number of haplotypes	h_S	h_T	G_{ST}
Pure populations	435	4.33	10	0.132 (0.012)	0.756 (0.008)	0.826 (0.016)
Mixed populations	295	4.29	7	0.198 (0.017)	0.720 (0.010)	0.725 (0.022)
Q. robur	286	3.98	9	0.177 (0.016)	0.733 (0.011)	0.758 (0.022)
$x < 45°N$	56	3.66	6	0.167 (0.037)	0.669 (0.036)	0.750 (0.055)
$45°N < x < 47°N$	104	4.12	6	0.162 (0.027)	0.719 (0.019)	0.774 (0.037)
$47°N < x < 49°N$	68	4.14	6	0.151 (0.031)	0.742 (0.019)	0.797 (0.041)
$x > 49°N$	77	3.81	5	0.230 (0.035)	0.639 (0.033)	0.640 (0.054)
Q. petraea	170	3.73	6	0.118 (0.019)	0.712 (0.017)	0.835 (0.026)
$x < 47°N$	71	3.96	5	0.094 (0.027)	0.647 (0.028)	0.854 (0.040)
$47°N < x < 49°N$	54	3.71	5	0.122 (0.034)	0.741 (0.028)	0.836 (0.045)
$x > 49°N$	45	3.43	5	0.150 (0.041)	0.691 (0.039)	0.783 (0.059)
Q. pubescens	152	4.32	6	0.089 (0.018)	0.687 (0.028)	0.870 (0.025)
$x < 45°N$	107	4.43	6	0.053 (0.016)	0.602 (0.038)	0.913 (0.027)
$x > 45°N$	45	4.08	5	0.176 (0.045)	0.757 (0.022)	0.767 (0.059)
All populations	733	4.31	11	0.159 (0.010)	0.743 (0.006)	0.786 (0.013)

species (Table 4). Altogether, it appears that mixed populations are more variable within population and hence less differentiated among populations, compared to pure ones; this was an expected result, since it was shown previously that cpDNA types were not completely homogenised across species. In addition, the three species partition cpDNA diversity differently. The highest level of subdivision is found in *Q. pubescens*, followed by *Q. petraea* and then by *Q. robur*; the differences between *Q. robur* and the two other species are significant. In the previous study (Dumolin-Lapègue et al., 1999), the ranking of the three species was the same although the difference was only significant between the two extremes (*Q. robur* and *Q. pubescens*). Furthermore, for all three species, there is a tendency for G_{ST} to be higher in the south. In particular, for *Q. robur*, the populations situated above the 49°N parallel have a significantly lower G_{ST} than those situated further south, whereas for *Q. pubescens*, populations situated above the 45°N parallel had a lower G_{ST} than those situated below. The trend was similar but not significant in *Q. petraea*.

3.6. Landscape analysis

The mean size of the 532 forests for which measures of landscape were obtained was 1050 ha, and the forest cover around each sampled point was 23% (Table 5). Woods occurred in 73% of the 76 neighbouring 1 km^2 cells. These three measures were correlated with each other, especially the size of the sampled forest and the forest cover ($r = 0.82$), whereas the correlation between the presence of woods and forest cover or size were lower ($r = 0.46$ in both cases). All these measures varied greatly, as indicated by the large standard errors associated with these values (Table 5). The forests sampled were larger in the south (1370 ha) than in the south-central part of France (670 ha). However, forest size increased again towards the north, but this was not accompanied by an increase in the overall forest area around each sampled point, as the percentage of forest occurrence gradually decreased from south to north. That is, larger but more isolated forests were sampled more frequently in the north than in the south of France. Single species *Q. robur* forests that were sampled are, on average, smaller (780 ha) than those where pure *Q. petraea* or *Q. pubescens* populations were sampled (1090 and 1050 ha, respectively). In addition, they were found in more open landscapes (18% of forest cover, versus 23% for *Q. petraea* and 26% for *Q. pubescens* forests). Mixed species forests were relatively small when *Q. pubescens* was present (550–710 ha), but larger in cases of mixtures of *Q. robur* and *Q. petraea* (1020 ha). These measures illustrate the variety of the landscapes where oaks are growing.

Table 5
Measures of landscape characteristics according to latitude or to species

Category	Population	Mixture (%)	Total forest area, in % (S.E.)	Local forest area, in 10^3 ha (S.E.)	Occupancy, in % (S.E.)	Ratio local forest area/total forest area (S.E.)
By geographic zone						
$x < 45°N$	198	0.26	0.29 (0.30)	13.7 (22.6)	0.79 (0.23)	8.3 (10.1)
$45°N < x < 47°N$	200	0.49	0.18 (0.19)	6.7 (11.5)	0.75 (0.24)	8.3 (7.3)
$47°N < x < 49°N$	72	0.49	0.20 (0.17)	9.8 (13.0)	0.70 (0.25)	7.4 (8.0)
$x > 49°N$	75	0.40	0.21 (0.18)	12.9 (13.7)	0.55 (0.21)	4.6 (4.8)
By species						
Q. robur	167	–	0.18 (0.20)	7.8 (14.6)	0.71 (0.27)	9.2 (9.4)
Q. petraea	36	–	0.23 (0.20)	10.9 (14.5)	0.75 (0.22)	22.6 (16.6)
Q. pubescens	122	–	0.26 (0.23)	10.5 (15.1)	0.76 (0.24)	24.4 (18.4)
Q. robur + *Q. petraea*	131	–	0.19 (0.18)	10.2 (13.6)	0.68 (0.24)	6.5 (6.3)
Q. robur + *Q. pubescens*	74	–	0.19 (0.20)	5.5 (10.6)	0.76 (0.21)	9.8 (9.6)
Q. pubescens + *Q. petraea*	33	–	0.18 (0.15)	7.1 (11.0)	0.78 (0.23)	8.4 (8.1)
All	332	0.39	0.23 (0.23)	10.5 (17.0)	0.73 (0.26)	7.7 (8.3)

Table 6
Correlation between intrapopulation cpDNA diversity and other parameters

Species	Population	Longitude	Latitude	Mixture	Total forest area	Local forest area	Occupancy	Ratio 1[a]	Ratio 2[b]	Ratio 3[c]
Q. robur	322	−0.02	0.06	−0.01	+0.00	+0.05	−0.05	+0.01	+0.03	+0.14**
Q. petraea	135	−0.07	0.13	−0.10	−0.04	−0.06	+0.03	−0.04	−0.06	−0.03
Q. pubescens	180	−0.22**	0.25**	+0.30**	−0.14*	−0.10	−0.10	−0.16*	−0.10	−0.07
Total	538	−0.16**	0.12**	+0.11**	−0.03	+0.02	−0.09*	−0.03	+0.02	+0.10*

[a] Total forest area/occupancy.
[b] Local forest area/occupancy.
[c] Local forest area/total forest area.
* $0.01 < P < 0.05$, ** $P < 0.01$.

Significant positive correlation with cpDNA diversity was found for the factors latitude, mixture and the ratio local forest area/total forest area, whereas negative correlation was observed with the variables occupancy and longitude (Table 6). For *Q. robur*, only the ratio local forest area/total forest area was significantly positively correlated with cpDNA diversity, whereas for *Q. pubescens* the variables mixture and latitude were positively correlated with cpDNA diversity, and the variable longitude was negatively correlated. Finally, no variable was found to be correlated with cpDNA diversity in *Q. petraea* (Table 6). Stepwise multiple regression procedures were used for all three species. In *Q. robur*, the combination of two variables (total forest area and occupancy) was shown to explain part of cpDNA diversity ($P = 0.04$), but has a low predictive value ($R^2 = 0.025$). For *Q. petraea*, no combination of factors was identified (results not shown), whereas for *Q. pubescens*, the variables mixture and latitude accounted for 32% of the variation and were highly significant, whereas the variables describing the landscape had no detectable influence on cpDNA diversity.

4. Discussion

The geographic distribution of *Q. robur* in France seems to radiate from the south-western part of the

country and closely resembles that of haplotypes of lineage B (except that haplotype 7 seems to have been incorporated along its eastern edge of distribution in France, from Toulouse to Alsace). The abundance of this species along the Atlantic coast in Spain and Portugal suggests its continued presence in the Iberian peninsula during the last ice-age (Petit et al., 2002b), from where it would have migrated into France. The western side of the Pyrenees, in the Basque country, did not constitute a major barrier, as the three most frequent haplotypes belonging to lineage B, and some rarer ones (such as haplotype 24) could immigrate easily into France. The distribution of the thermophilous *Q. pyrenaica* suggests that its colonisation route followed the same path. In contrast, the scattered distribution of *Q. petraea* in the south of France but also in Spain does not suggest an early migration from an Iberian refugium, similar to that of *Q. robur*, but rather a movement from east to west, for instance in association with haplotype 7 (Petit et al., 2002b). Currently, the distribution of haplotype 1 in south-eastern France seems to obstruct the passage along the Mediterranean coast at the border with Italy, but redistribution of haplotypes during the cold transition between the Lateglacial and the Holocene (the Younger Dryas period, see Brewer et al., 2002) may have obscured the path taken by haplotype 7 from Italy into France.

Assuming that the thermophilous *Q. pubescens* reached France from Italy, and was initially associated with haplotype 1 (Petit et al., 2002b), all the populations of this species characterised by haplotype 7 or haplotypes of lineage B (10, 11, 12) would then represent a secondary expansion of this species, by pollen swamping, at the expense of *Q. petraea* in the east or of *Q. robur* in the west. Moreover, given its ecology, the species must have tracked regions where limestone is abundant, and avoided others such as the Landes in south-western France or the Massif Central, except in its southern part (the Causse region). The Rhone and then the Seine river allowed this species to penetrate into northern France, taking advantage of calcareous bedrocks with favourable exposition.

The analyses based on cpDNA similarities also indicate that it is important to distinguish between the various oak species, although these analyses probably reflect more the recent dynamics, and not so much the postglacial one. Despite extensive local interspecific exchanges of cytoplasm, differences between species still exist, as shown by the test of the ''species effect''; the barriers to gene flow seem to depend on the species pair considered. These results confirm those obtained previously (Dumolin-Lapègue et al., 1999); in particular, three of the six tests measuring the species effect were significant, instead of only one in the previous study. In view of the increased sample sizes, however, it is surprising that within a given forest *Q. robur* trees are more likely to share their cpDNA haplotype with *Q. petraea* trees than with other conspecifics. Along the same lines, the finding that both *Q. petraea* and *Q. robur* found in mixed populations do not have increased levels of intrapopulation cpDNA diversity (the tendency seems in fact to be the opposite, although not significantly so) is also surprising. A possible explanation is that, in this part of the range, the presence of several oak species in one place points to a more ancient forest. Indeed, recently created woods will often be composed of a single species (generally *Q. robur*) whereas the presence of the more successional *Q. petraea* could indicate a more mature forest, characterised mostly by trees with haplotypes of local origin. The fact that *Q. petraea* trees are more similar to each other than to *Q. robur* trees from the same forest can also be explained with similar arguments. *Q. robur*, being more pioneer, is found more often in forest edges or in more open areas where seed flow is more likely to occur. Secondary human-mediated acorn movement (directly through plantations or indirectly through the opening of the landscape), involving mostly *Q. robur* trees, would decrease the similarity within *Q. robur* and between *Q. robur* and *Q. petraea*, but less so within *Q. petraea*.

In the case of *Q. pubescens*, the results are different: there is still a population effect, i.e., *Q. robur* and *Q. pubescens* trees growing in the same place are more similar than expected by chance, but only slightly so (the similarity is 0.67, versus 0.51 when they are growing in nearby populations). This contrasts with the interspecific similarities measured between *Q. pubescens* and *Q. petraea* (0.85) or between *Q. robur* and *Q. petraea* (0.81). As a consequence, there is a strong species effect between *Q. pubescens* and *Q. robur*, but not between *Q. pubescens* and *Q. petraea*. The fact that both *Q. robur* and *Q. pubescens* are relatively pioneer species, so that both may settle independently at a given place, with seeds originating

from different places and hence having potentially different cpDNA haplotypes, could explain this pattern. Furthermore, studies based on morphological characters of the leaves have shown that *Q. petraea* and *Q. pubescens* are closely related and represent a morphological continuum, whereas *Q. robur* and *Q. pubescens* form more clear-cut entities (Dupouey and Badeau, 1993), which fits well with the pattern observed here with cpDNA.

In the case of *Q. pubescens*, the stepwise multiple regression analyses identified two factors accounting for a significant part of cpDNA diversity: the presence of related oak species and the latitude. Fig. 1 illustrates this point well: in the area where only pure *Q. pubescens* populations exist, in south-eastern France, mixtures of cpDNA haplotypes are rarely observed, whereas in the zone of sympatry with the other species, mixed haplotype populations become more frequent. This could correspond to a secondary expansion of *Q. pubescens* into areas already colonised by other oak species, as discussed above (see Petit et al., 2002b).

Although there are numerous studies that address the ecological consequences of forest fragmentation, few are based on quantitative measures (Groom and Schumaker, 1993); when this is done, the size of the forests often constitutes the sole measure of fragmentation. Although many sophisticated measures can be proposed, such as those based on assessments of edge effects, we have chosen to summarise the level of fragmentation of the forest landscape by the size of the forest sampled (in hectares), and by two other ''local'' measures of forest distribution around the sampling point: the forest area and the distribution of forest fragments, both measured within 5 km of the sampling point. There was some degree of arbitrariness in the size of the area considered, but it allowed us to measure unique features of each landscape, as there was generally no overlap between neighbouring sampling points (most sampled forests are located more than 10 km apart). Moreover, movements of jays, the main long-distance vectors of acorns, are of this magnitude (up to 8 km, Schuster, 1950). It should be pointed out also that our sampling strategy was biased towards larger forests, and can provide therefore only relative information. Nevertheless, by combining measures of forest area with the measure of forest presence, some idea of the fragmentation can be obtained. Actually, in the only case where there were some indication of an effect of the landscape characteristics on cpDNA diversity within population (i.e., in the case of *Q. robur*), these two measures have been identified by the stepwise regression procedure. Another bias could exist, as landscape parameters can vary geographically. However, the inclusion of the latitude and the longitude in the model did not modify the conclusions in the case of *Q. robur*. The larger and more isolated forests displayed increased cpDNA diversity as compared to small forests in less opened landscapes.

It may seem surprising to find that isolated forests display (slightly) larger levels of cpDNA diversity than those located in more densely forested landscapes: drift and reduced gene flow should indeed reduce diversity when isolation increases. However, such forests are precisely those expected to have been more intensively managed by man, because they would constitute the sole resource of wood in the region. Moreover, the more open the landscape, the more likely it is that the nearest forest will harbour oaks characterised by a different cpDNA haplotype. Indeed, cpDNA variation was shown to present a characteristic pattern with patches of 30–50 km fixed for a single haplotype, a consequence of the stratified dispersal that characterised postglacial oak migrations (Petit et al., 1997). If plantations are established in such isolated forests, the seeds or seedlings used will likely differ genetically from the local trees. As a consequence, fragmentation of the landscape could result in increased intrapopulation diversity in these oaks. Although this result is the opposite to that expected by simple theoretical models, it is not unique. For example, in the case of a mistletoe in Australia, simulations have shown that fragmentation was promoting invasion by this parasitic plant (Lavorel et al., 1999). Empirical studies of *Acer saccharum* in Ohio (USA), using allozyme loci (Foré et al., 1992; Young et al., 1993), have also concluded that fragmentation resulted in an increase rather than a decrease in interpopulation gene flow and within-fragment genetic diversity. The ensuing redistribution of genetic variation may have negative consequences, such as outbreeding depression (Young et al., 1996).

Actually, Ellstrand and Elam (1993) argue that ''in most cases, recognition and consideration of gene flow as a potential hazard by plant conservation

decision-makers will prevent future problems''. The counterintuitive result that fragmentation could increase genetic diversity deserves to be investigated further, but should not be considered a priori as a positive by-product of human activities. Furthermore, this result may hold for cpDNA in oaks but not for other organisms living in oak ecosystems. For instance, in a population genetics study of the winter moth (*Operophtera brumata*) in oak woodlands in Belgium, Van Dongen et al. (1998) report increased differentiation and decreased diversity with increasing isolation of forest fragments (but no effect of the size of forest fragments).

It should, however, be kept in mind that landscape parameters explained only a small part of cpDNA diversity in one of three oak species studied, and had no significant effect in the two other species. As discussed elsewhere (Petit et al., 1997; Le Corre et al., 1997), the distribution of cpDNA variation in oaks was established during postglacial recolonisation and has remained largely unchanged since then. Only minor modifications are to be expected through fragmentation, except for cases of direct plantations with nonlocal material, which will be only weakly dependent on the landscape shape, resulting in highly idiosyncratic patterns of cpDNA variation where oak plants have been introduced. These should now been directly investigated by comparing information on past forest management for each forest compartment in a given forest with results of cpDNA variation. Simultaneously, the genetic consequences of forest fragmentation should be examined in organisms other than oaks, as they will depend on the life history of the species investigated.

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