Ann Sci For (1996) 53, 79-91

Original article

Comparison of morphological characters and molecular markers for the analysis of hybridization in sessile and pedunculate oak

R Bacilieri, A Ducousso*, A Kremer

Laboratoire de génétique et d'amélioration des arbres forestiers, INRA, BP 45, 33611 Cestas-Gazinet, France

(Received 20 October 1994; accepted 29 August 1995)

Summary — Interspecific hybridization is common in many plant groups, but the morphology of hybrids has rarely been studied on an experimental basis. The sessile and the pedunculate oak are closely related species which can hybridize in nature. Yet, the morphology of their hybrids is still a matter of conjecture. Here we studied the morphology and the hybridization rate in several open-pollinated progenies collected in a mixed stand of sessile and pedunculate oak. For both species, two types of pollinating environments (intraspecific and interspecific) were compared for their morphological and genetic effects in progenies. The analysis of the molecular markers showed that the contribution of sessile oak to the progenies of pedunculate oak was positive. The genetic effect of the pollinating environment was significant. The morphological characters gave a better image of interspecific gene flow when considered together in multivariate analyses rather than in univariate analyses. This probably occurred because the hybrids were a mosaic of parental and intermediate characters, rather than exactly intermediate forms.

morphology / RAPD / hybridization / *Quercus*

Résumé — Comparaison des caractères morphologiques et des marqueurs moléculaires pour l'analyse de l'hybridation entre les chênes sessile et pédonculé. *L'hybridation interspécifique est un phénomène courant chez de nombreux groupes végétaux, mais la morphologie des hybrides a été rarement étudiée sur des bases expérimentales. Les chêne sessile et pédonculé sont deux espèces étroitement apparentées, qui peuvent naturellement s'hybrider. Toutefois, la morphologie de leurs hybrides reste encore peu connue. Dans ce travail nous étudions la morphologie et le taux d'hybridation chez les descendances issues de pollinisation libre récoltées dans un peuplement naturel de chêne sessile et pédonculé. Les effets de l'environnement pollinique intra ou interspécifique ont été étudiés à l'aide de la morphologie foliaire et de marqueurs moléculaires. Ces derniers ont montré que la contribution du chêne sessile aux descendances de chêne pédonculé est significativement positive mais pas l'inverse. Les effets génétiques des différents environnements polliniques sont significatifs. Les caractères*

* Correspondence and reprints

morphologiques donnent une meilleure image du flux génique interspécifique quand ils sont considérés globablement dans une analyse multivariée que lorsqu'ils sont considérés séparement dans une analyse univariée. Ce résultat laisse penser que les hybrides sont une mosaïque de caractères parentaux et intermédiaires, plutôt que des formes exactement intermédiaires.

***morphologie / RAPD / hybridation /* Quercus**

INTRODUCTION

Natural interspecific hybridization has long been recognized as a common phenomenon in many plant groups (Stebbins, 1950; Lewontin and Birch, 1966; Grant, 1984). It has been traditionally assumed that natural hybrids should have an intermediate morphology between that of the parental species (Anderson, 1953). Nevertheless, this hypothesis has rarely been verified experimentally. Recently, reviewing the effects of interspecific gene flow on plant morphology, Rieseberg and Ellstrand (1993) showed that hybridization does not always produce intermediate forms. In approximately half of the cases studied, the hybrids are more like 1 of the parental species or present phenotypic novelties.

Sessile (*Quercus petraea* (Matt) Liebl) and pedunculate oaks (*Quercus robur* L) are closely related species with a wide sympatric distribution in Europe. They are wind-pollinated and nearly completely outcrossing species (Bacilieri *et al,* 1996). They present slight differences in a series of morphological characters. The differences in the characters of the leaves and seeds are usually used in the literature to discriminate between the two species (Gardiner, 1970; Ietswaart and Feij, 1989). Interspecific gene flow between sessile and pedunculate oak has been inferred in many studies on the basis of the finding, in natural mixed populations, of trees with intermediate morphology (Rushton, 1978, 1979; Minihan and Rushton, 1984; Semerikov *et al,* 1988; Ietswaart and Feij, 1989). Hypothesis of hybridization between these two species is supported by the success of interspecific controlled crosses (Rushton, 1977; Aas, 1991; Steinhoff, 1993). However, the morphology of true hybrids has still not been studied. Among the reasons for this, is the fact that interspecific controlled crosses are difficult to certify free from intraspecific pollution, and no species-specific markers have yet been identified.

The allelic forms of genetic markers such as allozymes or RAPDs present, at best, only small differences in frequencies between sessile and pedunculate oak (Kremer *et al,* 1991; Bacilieri *et al,* 1996; Moreau et al, 1994). If these types of markers cannot be used to directly identify the hybrids, nevertheless the differences in frequencies between species can be exploited to estimate, in mixed forests, the parental genetic contribution to the progenies. This was done in a previous study, in which by studying the allozyme distribution in the seed sets of 1989 and 1992 of a mixed oak stand, we were able to detect asymmetric gene flow between sessile and pedunculate oak in natural conditions (Bacilieri *et al,* 1996). The sessile oak pollinated the pedunculate oak but the reverse did not occur.

Here we studied the morphological characters and the distribution of RAPD markers in several open-pollinated progenies collected in 1989 in the same mixed oak stand mentioned earlier. These progenies were planted in a nursery under homogeneous conditions. To have a greater probability to recognize the morphological effects of hybridization, the families were chosen in order of their provenance in the stand. The families generated by maternal trees encircled by trees of the same species (collected in pure zones of the stand) were compared

with the families collected from maternal trees encircled by trees of the other species (mixed zones; for the definition of mixed and pure zones, see Bacilieri *et al,* 1995). Assuming that the trees mate preferentially with their neighbourhoods, hybridization should be more frequent in the mixed zones, and a difference in morphology should appear among groups. These progenies were analysed for morphological and genetic markers after 3 years of growth. The genetic contribution of the two parental species to these progenies were estimated comparing the RAPD marker frequencies in the progenies and in adults by means of a statistical method presented previously for the study of admixture in human populations (Roberts and Hiorns, 1965; Elston, 1971).

The study of the hybridization and morphology of the hybrid forms in oaks is important to understand the evolution of these taxa and of their genetic resources. Practical consequences concern both the research on the different aspects of the biology of the white oaks, that are based on a preliminary morphological discrimination of the individuals into species, and the management and the sylviculture of the oak stands, that present among their objectives to furnish homogeneous products (seeds, wood, *etc*).

MATERIALS AND METHODS

Sampling

The stand, situated in the Petite Charnie Forêt (Le Mans, France) consists of 426 adult oak trees (about 50% pedunculate oak and 50% sessile oak). A description of the ecology of the stand and the taxonomic discrimination of the adult trees has been presented elsewhere (Bacilieri *et al,* 1995). During autumn 1989, seeds were collected in the crown of several open-pollinated trees of the two species. A map of the positions of the mother trees in the stand is given in figure 1. The families were chosen as a function of the neighbourhood (the ten nearest trees) of their maternal trees (the symbols in parentheses identify each group):

– 11 sessile oaks encircled by trees of the same species *(ses/ses)*; families: 3, 14, 17, 26, 31, 113, 115, 122, 134, 140, 142; the neighourhood was composed, on average, of 85.5% sessile oaks;

– nine pedunculate oaks encircled by trees of the same species *(ped/ped)*; families: 220, 222, 225, 237, 246, 247, 249, 369, 372; the neighbourhood was composed, on average, of 90.0% pedunculate oaks;

– ten sessile oaks encircled by pedunculate oaks *(ses/ped)*; families: 166, 195, 204, 206, 210, 240, 241, 323, 342, 396; the neighbourhood was composed, on average, of 67.8% pedunculate oaks;

– 7 pedunculate oaks encircled by sessile oaks *(ped/ses)*; families: 42, 97, 106, 159, 161, 174, 324; the neighbourhood was composed, on average, of 60.0% sessile oaks.

The seeds were germinated in an incubator, and then transferred to the nursery of Pierroton (Bordeaux). The progenies were randomly distributed in the nursery, in one unitary parcel without repetitions. During the summer 1992, 3 years after germination, a number of leaves was sampled on each of the seedlings of the 37 families for the morphological analysis. The hybridization rate was calculated by means of the comparison of the RAPD marker frequencies of a subsampling of these progenies and of the adult population.

Analysis of the morphological characters

The 41 morphological characters used here are listed in table I. These characters were measured on three leaves per plant. For each family, we studied five randomly chosen seedlings. To determine if the groups presented morphological differences, the means of the morphological characters were compared by means of an *F*-test (Sokal and Rohlf, 1981). The variables 30, 31, 32, 33 were converted by a square root and the frequencies by the angular conversion arcsin $\sqrt{x}$ (Sokal and Rohlf, 1981). The homogeneity of the intragroup variances was analysed with the Bartlett test (Sokal and Rohlf, 1981).

The morphological data were further analysed with different types of discriminant factorial analysis (DFA; Legendre and Legendre, 1984). First,

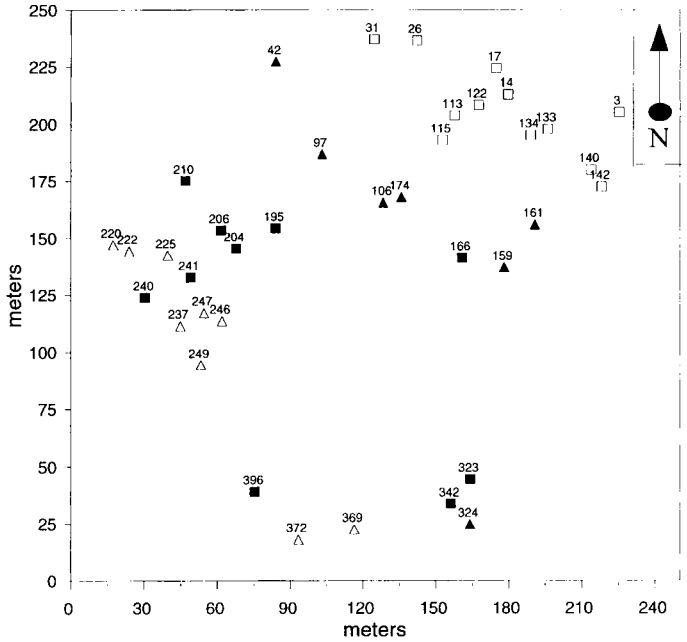

Fig 1. The stand is composed of 426 trees of sessile and pedunculate oak. In this map, only the 37 mother trees from which seeds have been collected are represented. Symbols: △ *ped/ped*; ▲ *ped/ses*; ■ *ses/ped*; □ *ses/ses* (eg, *ped/ses* represents a pedunculate oak surrounded by sessile oaks; see also in the *Materials and Methods* section).

all the individuals and all the characters were included in the analysis (DFAa). Second, the individuals of the groups *ses/ses* and *ped/ped* were considered as principal points, and individuals of the mixed zones *ped/ses* and *ses/ped* as supplementary points (DFAb). In the third DFA, only characters independent of the dimensions of the leaves were considered (character numbers 20, 23, 26, 29, 32, 33, 35, 36, 37, 39, 40, 41; DFAc).

Since we did not dispose of repetitions, it was impossible to estimate the experimental error due to the environmental differences in nursery. Nevertheless, this error was probably small, as the families were randomly distributed in the nursery and the cultural interventions in nursery tended to homogenize growth conditions.

Molecular analysis

The RAPD method (Williams *et al*, 1990; Welsh *et al*, 1991) consists of amplifying part of the DNA of an individual with the PCR technique (polymerase chain reaction), using nucleotidic primers of small size (ten bases). The nucleotide sequences of these primers are chosen at random. With this method, the amplification takes place only if the two primers are, on the chromosomes, at a distance inferior than 2 to 3 Kb. The amplification products are then separated on agarose or acrylamide gels with electrophoretic methods. The polymorphisms revealed by RAPDs correspond generally to the presence or the absence of the amplified fragments. A simple genetic event, such as a mutation or a deletion, at the level of the primer site on the genome is sufficient to impede the amplification. Then, two alleles at each locus are detected: the allele defined by the presence of the amplified fragment (+), and the allele associated with the absence of the fragment, called *null allele (n)*. Since the amplifications are run in saturated conditions, the genotypes +/*n* and +/+ are confounded (in the phenotype *A*+).

With this technique, Moreau et al (1994) found, by analysing several mixed oak populations (among which is the Petite Charnie stand), 12 bands among 419, allowing discrimination between sessile and pedunculate oaks. Among these 12 bands, two (F14a and 174g) were present in small frequencies in pedunculate oak and in high frequencies in sessile oak. The Mendelian heredity of the 2 fragments F14a and 174g was verified in intra- and interspecific controlled

Table I. List of morphological variables.

1) lpet	=	petiole length
2) llimb	=	lamina length
3) ltot	=	lpet + llimb
4) largmax	=	maximal width of the leaf
5) hautmax	=	height of maximal width
6) peri	=	perimeter
7) surf	=	surface
8) tetmoy	=	mean of auricle angles
9) ll1	=	mean length of lobes 1 (left and right)
10) ll2	=	mean length of lobes 2 (left and right)
11) llmoy	=	mean length of the six lobes
12) hl1	=	mean height of lobes 1 (left and right)
13) hl2	=	mean height of lobes 2 (left and right)
14) hlmoy	=	mean height of the six lobes
15) el1	=	mean thickness of lobes 1 (left and right)
16) el2	=	mean thickness of lobes 2 (left and right)
17) elmoy	=	mean thickness of the six lobes
18) an1	=	mean angle of secondary veins 1 (left and right) with the primary vein
19) an2	=	mean angle of secondary veins 2 (left and right) with the primary vein
20) anmoy	=	mean angle of the six secondary veins with the primary vein
21) al1	=	mean angle of lobes 1 (left and right)
22) al2	=	mean angle of lobes 2 (left and right)
23) almoy	=	mean angle of the six lobes
24) as1	=	mean angle of sinus 1 (left and right)
25) as2	=	mean angle of sinus 2 (left and right)
26) asmoy	=	mean angle of the six sinus
27) apexmoy1	=	mean shape of apex of lobes 1 (left and right)
28) apexmoy2	=	mean shape of apex of lobes 2 (left and right)
29) apexmoy123	=	mean shape of apex of the six lobes
30) nblob	=	number of lobes
31) nblub	=	number of lobelets
32) nint	=	number of intercalary veins
33) pillimb	=	density of leaf pilosity
34) lmax:llimb		
35) hlmax:llimb		
36) lpet:llimb		
37) hlmoy:lmax		
38) defic	=	1-4*ll*surf:peri^2
39) llimb:lmax		
40) nblob:peri		
41) nblob:llimb		

crosses (Moreau et al, 1994). Both fragments were dominant over their respective null alleles.

As these fragments have a dominant expression, information on the interspecific gene flow could be obtained only from the progenies born by maternal trees with genotype *n/n*. In these progenies, the amplified fragment (+) found in the phenotype *A+* was brought by the pollen which fertilized the ovule. The seedlings in which the band was present were all heterozygotes; their frequency corresponded to the frequency of the (+) allele in the pollen pool. The necessity to dispose of families with maternal genotype *n/n* restricted the number of the primers to the two cited earlier, F14a and 174g.

The DNA was extracted from dormant buds of adults and seedlings, amplified and then migrated on acrylamide gels following Moreau et

al (1994). In the adult populations, we sampled 41 sessile oaks and 45 pedunculate oaks. The allelic frequencies of the RAPD markers in the adult populations were estimated both considering that these populations were at the Hardy-Weinberg equilibrium, and under the hypothesis that a heterozygote deficit were present in the stand, as described with allozyme markers by Bacilieri *et al* (1994). In the first case, allelic frequencies (p_n and p_+) were estimated on the basis of the frequencies of the genotype *n/n* ($P_{n/n}$).

$$p_n = P_{n/n}{}^{0.5};\ p_+ = 1 - p_n.$$

In the second case of the figure, the heterozygote deficit *(f)* must be considered. The frequency of the phenotype *A+* is the sum of the frequency of the genotype $P_{+/+}$:

$$p_+{}^2 + p_+(1 - p_+)f,$$

and of the genotype $P_{+/n}$:

$$2p_+ (1 - p_+) (1 - f).$$

Knowing the frequency *PA+* of the phenotype *A+* and *f*, the frequency of the allele p_+ could be found solving the equation:

$$p_+{}^2 (f - 1) + p_+ (2 - f) - PA_+ = 0$$

This equation has 2 solutions, but 1 is always greater than unity, if $f > 0$, as was the case here.

As the hybridization was asymmetric (pollen of sessile oak versus ovules of pedunculate oak; Bacilieri *et al,* 1996), with the RAPD markers we studied only the pedunculate oak progenies. Allele frequencies have been estimated in a subsample of 84 individuals from six pedunculate oak progenies (3 *ped/ped* and 3 *ped/ses*) whose parents were homozygotes *n/n* for the two fragments. The relative genetic contribution of the two parental populations (sessile and pedunculate oaks) to the pedunculate oak progenies was estimated using a least-squares procedure developed to describe gene flow among human populations (Roberts and Hiorns, 1965; Elston, 1971). The procedure uses a matrix *X* of the allele frequencies for two parental populations and a row vector *y* of allele frequency differences between the progeny and the parental population. The least-squares estimate of gene admixture, *m*, is a row vector defined as:

$$m = (X'X) - X'y,$$

provided *X'X* is nonsingular. The least-squares estimates of the proportion of genes derived from each parental population are the elements of *m.* The *m* ($0 \leq m \leq 1$) is then an estimate of the hybrid frequency. Standard errors of *m* were used in two-tailed *t*-tests of the null hypothesis H_0: $m = 0$ (no hybridization).

RESULTS

Morphological analysis

All of the 41 morphological characters studied showed an unimodal distribution at the within-group level. Among all of these characters, 31 present significant differences between sessile and pedunculate oak for the *F*-test (table II). Within the sessile oak species, 14 of these 31 variables had a distribution significantly different between groups *ses/ses* and *ses/ped.* In the group *ses/ped*, 12 of these 14 variables showed a shift of the mean in the direction of the other species, the pedunculate oak. In pedunculate oak, 12 variables showed a significant difference between groups; in the group *ped/ses*, all the variables showed a shift in the direction of the sessile oak.

The probability to observe a similar distribution (12/14 and 12/12), in a binomial distribution where the two events ($p >$ mean, $q <$ mean) have the same probability ($p = q = 0.5$) is very small (sign test: $P < 0.001$; Sokal and Rohlf, 1981). We may reject the hypothesis H_0 that the observed differences were exclusively due to chance.

The comparison of the variances of the variables showed a few differences between pollen neighbourhoods. The variances of the variables of the progenies from the mixed neighbourhoods *ses/ped* and *ped/ses* were either greater (two cases in both species) or smaller (three cases in both spcies) than those of the progenies *ses/ses* and *ped/ped*, respectively. The sign test did not show any direc-

Table II. Comparisons of the morphological characters in seedlings.

	Means ses/ses n = *55*	*Means* ses/ped n = *50*	*Prob* F-*test*	*Means* ped/ses n = *35*	*Means* ped/ped n = *45*	*Prob* F-*test*	F-*test comparison between species*
lpet*	9.44	10.00	0.350	4.15	2.93	0.024 (+)	0.000
llimb*	153.49	143.38	0.016 (+)	145.82	133.16	0.015 (+)	0.000
ltot*	162.98	153.41	0.033 (+)	150.06	136.14	0.010 (+)	0.000
largmax*	45.78	40.56	0.000 (+)	41.84	37.01	0.003 (+)	0.000
hautmax*	45.81	43.18	0.089	44.31	41.01	0.046 (+)	0.009
peri*	269.05	234.66	0.000 (+)	239.56	210.07	0.001 (+)	0.000
surf*	2123.46	1765.74	0.001 (+)	1781.43	1443.26	0.007 (+)	0.000
ll1*	36.15	31.54	0.001 (+)	29.91	28.37	0.288	0.000
ll2*	49.33	43.81	0.001 (+)	46.71	42.36	0.017 (+)	0.002
llmoy*	46.32	41.29	0.001 (+)	40.67	36.59	0.008 (+)	0.000
hlmoy*	13.13	11.27	0.007 (+)	14.39	12.20	0.012 (+)	0.050
el2*	29.30	28.28	0.296	37.41	34.89	0.129	0.000
elmoy*	27.45	26.08	0.083	31.77	29.52	0.068	0.000
an2**	54.30	53.28	0.361	50.77	48.54	0.076	0.000
anmoy**	53.89	52.59	0.170	50.84	49.27	0.152	0.000
al1**	71.89	72.74	0.551	65.43	65.29	0.941	0.000
almoy**	72.61	72.36	0.814	69.84	68.87	0.427	0.000
as1**	71.90	70.34	0.378	68.17	65.40	0.182	0.000
as2**	71.79	68.44	0.124	70.41	65.86	0.066	0.020
asmoy**	70.12	68.28	0.295	67.17	62.69	0.030 (+)	0.000
nblob	14.44	13.87	0.136	10.96	10.17	0.074	0.000
nint	1.333	1.029	0.142	1.667	2.200	0.127	0.000
pillimb	3.378	3.228	0.173	1.581	1.267	0.014 (+)	0.000
lmax:llimb	0.300	0.283	0.002 (+)	0.288	0.279	0.173	0.001
hlmax:llimb	0.297	0.300	0.534	0.305	0.309	0.485	0.005
lpet:llimb	0.062	0.070	0.033 (–)	0.028	0.022	0.094	0.000
hlmoy:lmax	0.285	0.276	0.467	0.346	0.328	0.335	0.000
defic	0.627	0.599	0.019 (+)	0.616	0.593	0.126	0.019
llimb:lmax	3.374	3.568	0.002 (+)	3.520	3.642	0.158	0.000
nblob:peri	0.055	0.060	0.006 (–)	0.047	0.050	0.207	0.000
nblob:llimb	0.096	0.098	0.458	0.077	0.079	0.621	0.000

* in millimeters; ** in degrees; *n* = number of seedlings. The sign (+) means that the shift of the mean in the groups *ses/ped* and *ped/ses* of the mixed zones were in the direction of the other species, the sign (–) in the opposite direction. These signs were marked only when the differences between groups within species were significant at the *F*-test. In the last column, the *F*-test was calculated for the differences of mean at the interspecific level (*ses/ses* and *ped/ped*).

tionality in this case (results not shown). The means and the variances of the morphological characters of the maternal plants showed no significant differences between groups within species (results not shown).

The discriminant analysis conducted over all the individuals and all the characters (DFAa) separated the seedlings into two groups along the first axis. This axis explained 33% of the total variance and the second, 6%. The characters normally used

to recognize the species were strongly correlated to the first axis (*lpet, pillimb, nblob, etc*; table III). These characters corresponded to those found in a previous study to best discriminate the adult trees of the two species (Bacilieri *et al,* 1995), except *tetmoy* which, in our sample, did not contribute to the first axis in progenies. We may identify the two groups ordered by DFA as corresponding to the two species, sessile and pedunculate oak. The progenies were classified in the two groups as it was expected knowing the two species of their maternal parent tree, except two seedlings of the family 396 (with sessile oak maternal parent in pedunculate oak zone), one of the family 174 and one of the family 97 (with pedunculate oak as maternal parent in sessile oak zone) which fell in the space of the other species.

The distribution of the seedlings on the first axis of DFAs is shown in figure 2a. The group *ped/ses* had a bimodal distribution, the second peak of which was situated on the side of the species in the majority represented in the neighbourhood, the sessile oak. The comparison of the mean values of the groups on the first axis of DFAa by means of the *F*-test showed a significant difference between the two groups of pedunculate oak progenies ($F = 6.215$, $ddl = 1$ and 81; $P = 0.001$); the mean values on the first axis of the groups *ped/ped* and *ped/ses* were, respectively, 0.017 and 0.014. In contrast, no differences were found in the two sessile oak groups ($F = 0.891$, $ddl = 1$ and 100; $P = 0.650$).

In DFAb, where only the individuals of the pure zones were used as principal points of the analysis, the discrimination between

Table III. Correlation coefficients between morphological characters and the first axis of the discriminant analysis on the progenies (DFAa).

	Correlations		*Correlations*
lpet	−0.776	al2	−0.034
llimb	−0.231	almoy	−0.180
ltot	−0.340	as1	−0.188
largmax	−0.242	as2	−0.096
hautmax	−0.143	asmoy	−0.244
peri	−0.324	apexmoy1	0.044
surf	−0.288	apexmoy2	−0.029
tetmoy	0.008	apexmoy123	−0.094
ll1	−0.242	nblob	−0.731
ll2	−0.099	nblub	−0.039
llmoy	−0.303	nint	0.227
hl1	0.065	pillimb	−0.919
hl2	0.234	lmax:llimb	−0.076
hlmoy	0.103	hlmax:llimb	0.117
el1	0.177	lpet:llimb	−0.765
el2	0.481	hlmoy-lmax	0.307
elmoy	0.374	defic	−0.112
an1	−0.026	llimb:lmax	0.077
an2	−0.247	nblob:peri	−0.425
anmoy	−0.246	nblob:llimb	−0.379
al1	−0.271		

species was improved. The first axis explained, in this case, 39% of the total variance. The two groups of the mixed zones (*ped/ses* and *ses/ped*) presented again a bimodal distribution, the second peak being situated in both cases on the side of the other species (fig 2b). The comparison of the mean values on the first axis showed

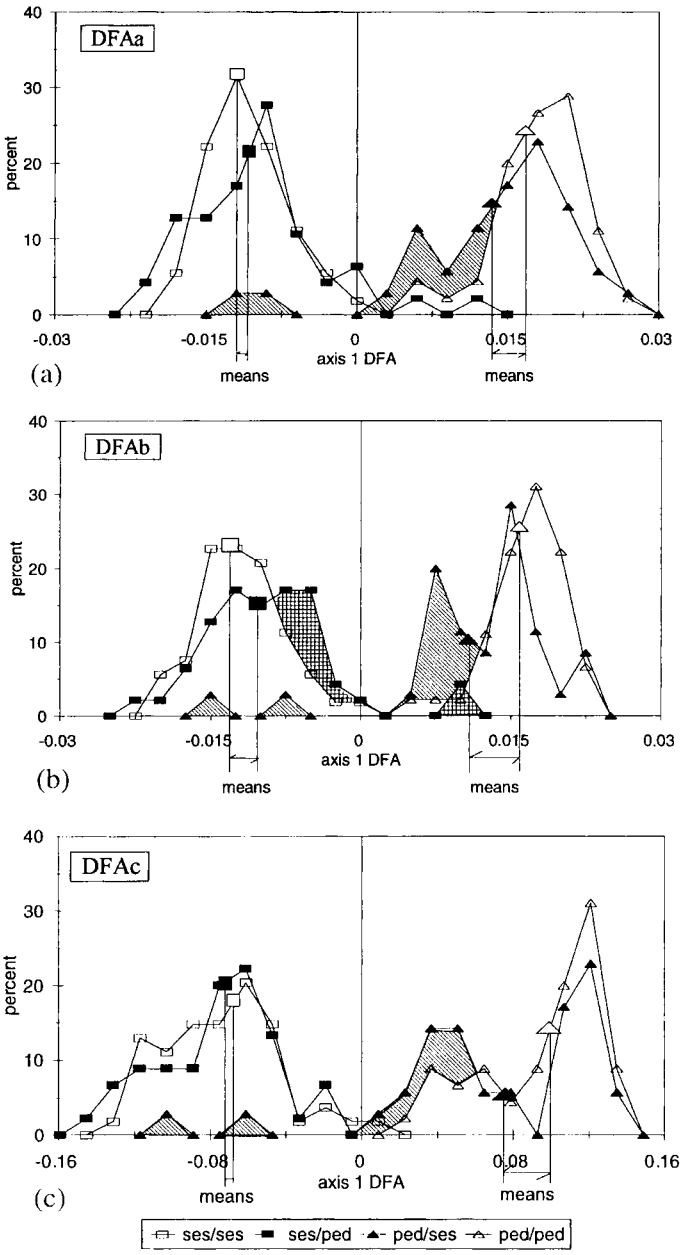

Fig 2. Distributions of the individual values of seedlings on the first axes of the different discriminant analysis. **(a)** In DFAa, all the seedlings and all the characters were considered. **(b)** In DFAb, the seedlings of the mixed zones did not contribute to the construction of the canonical axes. **(c)** In DFAc, only the characters considered independent from the size of the leaf were used.

significant differences in both groups of the 2 species ($P = 0.016$ in sessile oak, $P < 0.001$ in pedunculate oak).

In DFAc, the first axis explained 51.6% of the total variance. The bimodal distribution was shared by the two groups of pedunculate oak. In contrast, the two groups of sessile oak had a very similar unimodal distribution (fig 2c). The difference between the means of the groups was significant in pedunculate oak ($P = 0.005$), and not significant in sessile oak.

In pedunculate oak progenies, the seedlings falling in the sessile oak group were the same over the three analyses. Conversely, in sessile oak only one individual of the family 97 remained classified in the space of the other species over the 3 analyses.

Molecular analyses

The allele frequencies of the RAPD loci in the adult trees of the stand, calculated according to the two hypotheses: i) populations at the Hardy-Weinberg equilibrium, and ii) not at the equilibrium, are shown in table IV, as well as the allele frequencies in the pollen pool. The genetic contribution of the sessile oak to the pedunculate oak was estimated to be positive, independent of the population of reference used (table V). This contribution was greater in the mixed zone *(ped/ses)* than in the pure zone *(ped/ped)*. The comparison of the standard errors of the estimations showed that this difference between zones was significant.

DISCUSSION AND CONCLUSION

The study of the molecular markers showed that the contribution of sessile oak to the pedunculate oak progenies was significantly positive. The hybridization rate obtained with RAPDs had the same magnitude of the rate observed with allozymes in the seeds and natural regeneration of the same stand (Bacilieri *et al*, 1994). This result confirms that hybridization occurs in nature and hybrids survive to the first stage of life.

Both in sessile and pedunculate oak, the seedlings showed significant morphological differences between the intraspecific groups. However, the larger part of the differences between groups was found in char-

Table IV. Frequencies of the allele (+) of the two RAPD markers in adults of both species, and in the pollen which contributed to the seedlings of pedunculate oak (columns 1–5), and significance levels of difference at the chi-square test.

Primers	*Adults (1)* Q petraea *(f = 0.000)* n = *41*	*Adults (2)* Q robur *(f = 0.000)* n = *45*	*Adults (3)* Q petraea *(f = 0.222)* n = *41*	*Adults (4)* Q robur *(f = 0.078)* n = *45*	*Pollen pool (5)* Q robur n = *84*	*Significance level of the χ^2 test* *1–2*	*3–4*	*2–5*	*4–5*
174g	0.319	0.131	0.358	0.136	0.155	0.05	0.05	0.1	ns
F14a	0.688	0.225	0.761	0.234	0.368	0.001	0.001	ns	ns

In adults, the allelic frequencies were calculated under the Hardy-Weinberg hypothesis (columns 1 and 2; $f = 0.000$) and under the hypothesis that a heterozygote deficit was present in both species (columns 3 and 4). In this last case, the f values estimated with allozymes on the whole adult population (Bacilieri et al, 1994) were used. Confidence intervals at the 95% probability level for f were 0.155 and 0.289 in sessile oak and 0.012 and 0.138 in pedunculate oak. n = number of plants analysed.

Table V. Estimations of the genetic contributions of each species to the progenies of pedunculate oak, calculated under the Hardy-Weinberg (HW) hypothesis and under the hypothesis than an heterozygote deficit (HD) was present in both species.

	Contribution (m)		*Variance (m)*		*Probability t-test*	
	Q robur	Q petraea	Q robur	Q petraea	Q robur	Q petraea
HW	0.72	0.28	0.045	0.045	0.0038	0.0241
HD	0.77	0.23	0.043	0.043	0.0030	0.0330

acters linked to the size of the leaf rather than to the discrimination between species. The characters with the stronger power of discrimination between species (this power being measured by the correlations with the first axis of DFA) did not show differentiation between groups, except *pillimb* in pedunculate oak and *lpet:llimb* and *nblod:peri* in sessile oak. These latter two characters showed a shift in their means contrary to that expected under the hybridization hypothesis.

On the other hand, differentiation of the size of the leaf between ecological zones of the parcel had already been observed in adult trees: in both species, individuals from the wetter area have the larger leaves (although these differences were not significant by the statistical tests; Bacilieri et al, 1995). Considered together with the unimodal distribution of the single characters at the within-group level, these results indicate that the morphological differences between groups were probably independent from the interspecific gene flow, but rather related to differences in the ecological environment of maternal trees.

In northern red oak, Sork et al (1993) showed that genetic differentiation for insect resistance occurs over a short distance, in response to environmental heterogeneity in the distribution of insects. However, our experiment did not allow us to attribute the morphological differentiation to an environmental or genetic origin.

In contrast, when the morphological characters were analysed together in a multivariate space by means of a DFA, they gave an image coherent with the hypothesis of hybridization supported by molecular analyses. A common trait joined the 3 analyses used here: their first axes were formed by almost the same combination of characters as the first axis of a DFA on adult trees (Bacileri et al, 1995). However, the effects of hybridization appear to be variable over the analyses. The DFAc, which included only the characters considered independent from the size of the leaf, and in which the individuals of the mixed zones were not considered in the construction of the canonical axes, was the one discriminating at best between sessile and pedunculate oak. This analysis was also the one which best represented the results of interspecific gene flow obtained with allozymes (Bacilieri *et al,* 1994b) and RAPDs. In this analysis, the bimodal distributions of the pedunculate oak was very pronounced. In contrast, the two groups of sessile oak showed a very similar unimodal distribution.

We did not study the progenies of sessile oak with molecular markers; nevertheless, both the studies of controlled crosses (Aas, 1991; Steinhoff, 1993) and of allozymes in progenies (Bacilieri et al, 1996) showed that the gene flow in these species is asymmetric, and that sessile oak can be fertilized by pedunculate oak with difficulty. In this study, the morphological characters were

consistent with the molecular markers, in showing that the pedunculate oak can be hybridized by sessile oak. Furthermore, hybridization seems larger in the zone of the stand where the sessile oak is predominant.

The fact that the morphological characters did not inform on the effect of hybridization when considered individually, but only when considered together in a multivariate analysis, may mean that they have a dominant expression, or that there are maternal effects that make the observation of segregation in the progenies difficult. The hybrids, in this case, are probably a "mosaic of parental and intermediate characters" (Rieseberg and Ellstrand, 1993) and may be detected only when many characters are used conjointly.

The development of dominance in characters linked to the differentiation of interfertile species is frequent (Rieseberg and Ellstrand, 1993). Similarly, its development has been predicted by simulation for those characters submitted to disruptive selection (Dickinson and Antonovics, 1973). Theoretically, dominance may take place if the characters are determined by a small number of genes. On the other hand, the maternal effects on juvenile characters have been shown in a number of species, that is, the size of seedlings is frequently related to the size of the seeds from which they germinate. The maternal effects could also take their origin from the variability of the cytoplasmic organelles (Blada, 1992).

In our experience, the lack of repetitions did not allow more precise conclusions. In spite of this limit, the present study permits us to show that the discrimination of hybrids depends strongly on the type of analysis and characters used. If the DFA was conducted on seedlings of unknown maternal origin, the estimation of hybridization based on morphological characters should have been much lower (approximately 7% or less).

For the white oak species complex, the significance of our findings could be summarized as follows: On the one hand, the asymmetric interspecific gene flow observed previously with allozymes (Bacilieri et al, 1996) is confirmed with another genetic marker. This is important because, in contrast with theoretical predictions, allozymes sometimes appear not to be neutral in relation to selection (Karl and Avise, 1992; Avise, 1994; Pogson and Zouros, 1994), biasing estimations of the population genetic parameters. RAPD markers, relying on a random amplification of DNA independent from the genome functions, are probably less sensitive to selection.

On the other hand, morphology appears not to be a completely reliable criterion of discrimination, because its results depend on the type of analysis and of the characters chosen, and on dominant and maternal effects that probably hide hybrids among the trees of one or the other species. The errors of taxonomic attribution could affect both the studies of biological characters in one given species, and the studies of hybridization. These errors could be reduced in size only by means of the comparison of the distribution of many kinds of characters and of genetic markers in space (over populations) and in time (over generations).

Finally, we have to consider that these European white oak species present, at the same time, an abundant interspecific gene flow and a large level of genetic diversity (Kremer et al, 1991). If the interspecific gene flow is a mechanism contributing to maintain the genetic diversity, as many authors have suggested (Stebbins, 1950; Lewontin and Birch, 1966; Grant, 1984), the networks for the conservation of genetic resources in European white oak must consider the mixed populations among the populations to conserve.

REFERENCES

Aas G (1991) Kreuzungsversuche mit Stiel- und Traubeneichen (*Quercus robur* L und *Q petraea* (Matt) Liebl). *Allgemeine Forst- und Jagdzeitung* 162, 141-145

Anderson E (1953) Introgressive hybridization. *Biol Rev* 28, 280-307

Avise JC (1994) *Molecular markers, natural history and evolution.* Chapman and Hall, New York, NY, USA

Bacilieri R, Labbé T, Kremer A (1994) Intraspecific genetic structure in a mixed population of *Quercus petraea* (Matt) Liebl and *Q robur* L. *Heredity* 73, 130-141

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

Bacilieri R, Ducousso A, Petit R, Kremer A (1996) Mating system and directional gene flow in a mixed stand of sessile and pedunculate oak. *Evolution* (in press)

Blada I (1992) Nuclear and extranuclear genetic effects in *F*1 reciprocal hybrids between *Pinus strobus* and *P peuce. Silvae Genetica* 41, 34-38

Dickinson H, Antonovics J (1973) Theoretical considerations of sympatric divergence. *Am Nat* 107, 256-274

Elston RC (1971) The estimation of admixture in racial hybrids. *Ann Human Genet* 35, 9-17

Gardiner AS (1970) Pedunculate and sessile oak (*Quercus robur* L and *Quercus petraea* (Matt) Liebl). A review of the hybrid controversy. *Forestry* 43, 151-160

Grant WF (1984) *Plant biosystematics.* Academic Press, Toronto, Canada

Ietswaart JH, Feij AE (1989) A multivariate analysis of introgression between *Quercus robur* and *Q petraea* in the Netherlands. *Acta Bot Neerl* 38, 313-325

Karl SA, Avise JC (1992) Balancing selection at allozyme loci in oysters: implications from nuclear RFLPs. *Science* 256, 100-102

Kremer A, Petit R, Zanetto A, Fougère V, Ducousso A, Wagner D, Chauvin C (1991) Nuclear and organelle gene diversity in *Quercus robur* and *Quercus petraea.* In: *Genetic variation of forest tree populations in Europe* (G Müller-Starck, M Ziehe, eds), Sauerländer Verlag, Berlin, Germany, 141-166

Legendre G, Legendre P (1984) *Écologie numérique.* Masson, Paris, France

Lewontin RC, Birch LC (1966) Hybridization as a source of variation for adaptation to new environments. *Evolution* 20, 315-336

Minihan VB, Rushton BS (1984) The taxonomic status of oaks (*Quercus* ssp) in Breen Wood, Co, Antrim, Northern Ireland. *Watsonia* 15, 27-32

Moreau F, Kleinschmit J, Kremer A (1994) Molecular differentiation between *Quercus petraea* and *Q robur* assessed by random amplified DNA fragments. *Forest Genetics* 1, 51-64

Pogson GH, Zouros E (1994) Allozyme and RFLP heterozygosities as correlates of growth rate in the scallop *Placopecten magellanicus*: a test of the associative overdominance hypothesis. *Genetics* 137, 221-231

Rieseberg LH, Ellstrand NC (1993) What can molecular and morphological markers tell us about plant hybridization? *Crit Rev Plant Sci* 12, 213-241

Roberts DF, Hiorns RW (1965) Methods of analysis of the genetic composition of a hybrid population. *Human Biology* 37, 38-43

Rushton BS (1977) Artificial hybridization between *Quercus robur* L and *Quercus petraea* (Matt) Liebl. *Watsonia* 11, 229-236

Rushton BS (1978) *Quercus robur* L and *Quercus petraea* (Matt) Liebl: a multivariate approach to the hybrid problem. 1. Data acquisition, analysis and interpretation. *Watsonia* 12, 81-101

Rushton BS (1979) *Quercus robur* L and *Quercus petraea* (Matt) Liebl: a multivariate approach to the hybrid problem. 2. The geographical distribution of population types. *Watsonia* 13, 209-224

Semerikov LF, Glotov NV, Zhivotovskii LA (1988) Example of effectiveness of analysis of the generalized variance of traits in trees. *Soviet Journal of Ecology* 18, 140-143

Sokal RR, Rohlf FJ (1981) *Biometry,* 2nd edn, Freeman & Co, New York, NY, USA

Sork VL, Stowe KA, Hochwender C (1993) Evidence for local adaptation in closely adjacent subpopulations of northern red oak (*Quercus rubra* L) expressed as resistance to leaf herbivores. *Am Nat* 142, 928-936

Stebbins GL (1950) *Variation and evolution in plants.* Columbia Univ Press, New York, NY, USA

Steinhoff S (1993) Results of species hybridization with *Quercus robur* L and *Quercus petraea* (Matt) Liebl. In: *Genetics of oaks* (A Kremer, PS Savill, KC Steiner, eds), Elsevier, Paris, France

Welsh J, Honeycutt RJ, McClelland M, Sobral BWS (1991) Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR). *Theor Appl Genet* 82, 473-476

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res* 18, 6531-6535