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Coincidence of Small-scale Spatial Discontinuities in Leaf Morphology and Nuclear Microsatellite Variation of *Quercus petraea* and *Q. robur* in a Mixed Forest

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• *Background and Aims* The taxon complex comprising *Quercus petraea* and *Q. robur* shows distinct morphologies and ecological preferences, but mostly low differentiation in various types of molecular markers at a broad spatial range. Local, spatially explicit analyses may reveal patterns induced by microevolutionary processes operating mainly over short distances. However, no attempts have been made to date to explore the potential of spatial analyses combining morphological and genetic data of these oaks.
• *Methods* A mixed oak stand was studied to elucidate the small-scale population genetic structure. All adult individuals were classified and putative hybrids were identified using multivariate discrimination analysis of leaf morphological characters. Likewise, all trees were genotyped with five nuclear microsatellites, and a Bayesian assignment method was applied based on maximum likelihood of multilocus genotypes for taxon and putative hybrid classification.
• *Key Results* Multivariate analyses of leaf morphological data recognized two groups with few individuals as putative hybrids. These groups were significantly differentiated at the five microsatellites, and genetic taxon assignment coincided well with morphological classification. Furthermore, most putative hybrids were assigned to the taxon found in their spatial neighbourhood. When grouping trees into clusters according to their spatial positions, these clusters were clearly dominated by one taxon. Discontinuities in morphological and genetic distance matrices among these clusters showed high congruence.
• *Conclusions* The spatial–genetic analyses and the available literature led to the assumption that reproductive barriers, assortative mating, limited seed dispersal and microsite-induced selection in favour of the locally adapted taxon at the juvenile stage may reinforce taxon-specific spatial aggregation that fosters species separation. Thus, the results tend to support the hypothesis that *Q. petraea* and *Q. robur* are distinct taxa which share a recent common ancestry. Occasional hybrids are rarely found in adults owing to selection during establishment of juveniles.

Key words: Genetic boundaries, leaf morphology, local genetic differentiation, nuclear microsatellites, *Quercus* spp., spatial analysis.

INTRODUCTION

For several decades, the genus *Quercus* (Fagaceae) has served as a model system for studying hybridization in plants, using a great variety of morphological, ecological, physiological and genetic approaches (e.g. Stebbins *et al.*, 1947; Whittemore and Schaal, 1991; Rushton, 1993; Feuillat *et al.*, 1997; Howard *et al.*, 1997; Craft *et al.*, 2002; Ponton *et al.*, 2002; Petit *et al.*, 2003; Tovar-Sánchez and Oyama, 2004; Muir and Schlötterer, 2005). The two European white oaks studied here, *Q. petraea* (Matt.) Liebl. (sessile oak) and *Q. robur* L. (pedunculate oak), form a well-studied complex throughout their largely overlapping natural ranges. Their leaf morphologies are distinctive in a multivariate sense (Kremer *et al.*, 2002; Ponton *et al.*, 2004), both taxa are predominantly outcrossing, and they are known to be interfertile, with siring success biased towards *Q. petraea* in experimental pollination trials (Steinhoff, 1993; Bacilieri *et al.*, 1996). However, the natural hybridization rate appears to be low, as indicated by paternity and parentage assignment of acorns and seedlings using nuclear microsatellites (nSSRs; Streiff *et al.*, 1999; A. Kremer and the Oakflow consortium, unpubl. data). Streiff *et al.* (1999) identified 23 cases of interspecific fertilizations in 310 unequivocally assigned paternities (7·4 %), but the hybridization rate could have been as low as 2·3 % when offspring with unassigned paternity were included in the calculations and if all these external paternities were considered as intraspecific. Moreover, 16 of the 23 hybridization events reported were generated by a single maternal tree, and only six of the adult trees showed intermediate leaf morphology, i.e. could be considered putative hybrids. By contrast, maternally inherited chloroplast (cp) DNA markers are shared between these taxa on both local and regional scales (Dumolin-Lapègue *et al.*, 1999; Mátyás and Sperisen, 2001; Petit *et al.*, 2002*b*; Finkeldey and Mátyás, 2003). To explain this pattern of cpDNA distribution across Europe, survival in shared refugial areas during at least the last glacial period and re-colonization along similar routes, but also genetic introgression when in sympatry, have been proposed (Petit *et al.*, 2002*a*, *b*).

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Petit *et al.* (2003) described the genetic interaction between the two *Quercus* species as pollen swamping or nuclear capture. They argued that *Q. robur* is a better colonizer, owing to its specific ecological requirements and longer seed dispersal distances, whereas *Q. petraea* should later introgress the *Q. robur* gene pool via pollen gene flow. These authors expect that introgression is directional owing to asymmetric fertilization probability (Steinhoff, 1993; Bacilieri *et al.*, 1996) and reduced pollen viability in hybrids, i.e. low probability of backcrossing (Rushton, 1993). Such nuclear capture, allowing the resurrection of the late-successional species, would result in a pattern of cpDNA haplotypes that does not differentiate between the two taxa, as was found in a European-wide population screening (Petit *et al.*, 2002*b*). Moreover, hybrids should be frequent, and most nuclear loci should indicate low taxon differentiation owing to high levels of gene flow, whereas disruptive selection at a few loci maintains species distinction (Petit *et al.*, 2003).

As an alternative hypothesis to nuclear capture, Muir and Schlötterer (2005) suggested that the genetic relationship between these two white oaks was a consequence of recent shared ancestry. They found that genetic differentiation in 20 nSSRs was stronger between geographically proximate populations of different taxa than among populations within taxa on a range-wide scale (Muir *et al.*, 2000). Sequence analyses of nSSR alleles further suggested that shared genetic variation is better explained by common ancestry than by high rates of interspecific gene flow (Muir and Schlötterer, 2005). This model suggests that nuclear genetic taxon separation should be maintained also on a local scale. However, both of the above hypotheses are difficult to substantiate (Lexer *et al.*, 2006; Muir and Schlötterer, 2006) and are mainly based on patterns of range-wide genetic variation, while microevolutionary processes act locally. In fact, local gene flow between the taxa when in sympatry should leave a detectable imprint in the genetic structure of a mixed stand. Hence, under the nuclear capture hypothesis, frequent hybridization would obscure taxon differentiation owing to introgression. Unless there is strong disruptive selection, the two taxa should be difficult to separate based on phenotypic or molecular markers, with frequent hybrids, and trees of the taxa are likely to be spatially intermingled. By contrast, the model of recent shared ancestry with no or low gene flow would result in small-scale differentiation, few hybrid individuals and clear spatial taxon separation. Therefore, we consider that exhaustive and spatially explicit analyses on a small scale, complementary to range-wide sampling, may provide new evidence towards the on-going debate on white oak genetics (Lexer *et al.*, 2006).

Multivariate analyses have great power in taxon delimitation and hybrid identification when species-specific or diagnostic characters are absent (Nason *et al.*, 1992; Tovar-Sánchez and Oyama, 2004; Valbuena-Carabaña *et al.*, 2005). In *Q. petraea* and *Q. robur*, leaf characters have been routinely applied for morphological classifications (Kremer *et al.*, 2002). Although the ranges of single variables widely overlap between taxa, multivariate analyses result in two clusters with little overlap, suggesting small numbers of hybrids. At the molecular level, differences in allelic frequencies lead to significant interspecific differentiation only when a large number of nuclear markers is analysed, with a small number of loci strongly contributing to overall taxon differentiation. This has been shown for isozymes (Gömöry *et al.*, 2001), amplified fragment length polymorphisms (Bakker *et al.*, 2001; Coart *et al.*, 2002; Mariette *et al.*, 2002; Kelleher *et al.*, 2005), sequence-characterized amplified regions (Bodénès *et al.*, 1997) and nSSRs (Muir *et al.*, 2000; Muir and Schlötterer, 2005). Again, most of these studies considered large geographical areas and mostly pure stands so that allele frequencies are more likely to have differed between taxa than on a small scale. Hence, if taxon differentiation is maintained also at the local level, i.e. where taxa are mixed within a single stand, this could be taken as evidence for a low hybridization rate.

Locally, microsite variation may induce non-random spatial patterns of intraspecific genetic variation, which in turn may be enforced or blurred by directional or stochastic processes such as inbreeding, dispersal or genetic drift (Streiff *et al.*, 1998). Even stronger spatial signals may be expected when investigating interspecific spatial separation so that species-specific environmental requirements come into play. Several studies have addressed the fine-scale genetic structure in European white oaks, using various molecular marker types (Bacilieri *et al.*, 1994; Streiff *et al.*, 1998; Mariette *et al.*, 2002; Cottrell *et al.*, 2003; Jensen *et al.*, 2003; Muir *et al.*, 2004). However, mainly intraspecific and spatially implicit genetic patterns were studied. Consequently, there is little indication of how well *Q. petraea* and *Q. robur* are separated within single stands, which in turn may shed light on microevolutionary processes. Assuming that selection acts at the microsite level, i.e. seedlings are eliminated according to their taxon-specific environmental requirements, we expect to find small-scale spatial aggregation of conspecifics. Furthermore, limited seed dispersal may enforce spatial autocorrelation. Hybrid seedlings may have lower survival than pure seedlings in their respective maternal environment (Anderson, 1948; Galloway, 2005). As suggested above, in relation to selectively neutral processes, selection probably imposes a spatial imprint on the neutral genetic structure if interspecific gene flow is low. Should frequent hybridization lead to nuclear capture, we would expect to find morphological and genetic patterns that are not congruent, owing to a gradual genomic transition from *Q. robur* to *Q. petraea*. Alternatively, the model of recent shared ancestry and low gene flow between taxa should invoke relatively sharp and coinciding morphological and genetic boundaries.

The goal of the present study was to determine how well the two taxa can be separated locally in a spatial–genetic context, and whether leaf morphological discontinuities coincide with genetic discontinuities based on nuclear microsatellites. This approach required limiting our detailed, spatially explicit investigation to a single study population. Specific questions were as follows. (1) Are *Q. robur* and *Q. petraea*, as identified from their leaf morphology, genetically differentiated? (2) Do leaf morphology and nuclear molecular markers indicate hybrid individuals?

(3) Does posterior assignment of the multilocus genotypes coincide with morphological classification? (4) Are the two taxa spatially structured, and does the genetic structure reflect historical interspecific gene flow? Using various multivariate and spatial–genetic approaches, the two taxa are shown to remain separated morphologically and genetically in a spatial context even at a local scale, possibly due to low levels of effective interspecific gene flow and small-scale environmental selection. From this it is concluded that our findings better support the hypothesis of recent shared ancestry than that of nuclear capture.

MATERIALS AND METHODS

Study site and sampling

Given finite resources, sampling efforts for a population genetic study can be allocated to either the number of sites or the number of individuals. The two extreme ends of such a sampling gradient can be termed, in allegory to conservation strategies, as 'single large or several small' (SLOSS). To date, most studies on European white oaks have addressed the large scale, i.e. small samples were taken from several populations ('several small'), whereas we chose to study a single site extensively ('single large'), disentangling local patterns of species mixture and putative hybridization.

Our sampling site close to Büren, western central Switzerland (47°07′28″N, 7°22′52″E), was located in a mixed forest consisting of about 20 % oaks along with other abundant tree species such as beech (*Fagus sylvatica*), ash (*Fraxinus excelsior*), silver fir (*Abies alba*), Norway spruce (*Picea abies*) and maple (*Acer* spp.). Based on information from the local forest service, the oaks represent natural regeneration. Using three cpDNA microsatellites (DT1, DT3, DT4; Deguilloux *et al.*, 2003), we determined three haplotypes (F. Gugerli *et al.*, unpubl. data) indicative of different postglacial origins (Deguilloux *et al.*, 2004). This haplotypic composition let us assume that the study stand was not at an equilibrium regarding possible nuclear swamping so that hybrids, if at all, should be present. On an area of about 9 ha, all oak trees were mapped that reached the canopy ($n = 414$; Fig. 1). Leaves were collected from the sun-exposed crown area, using a shotgun, for both morphological and molecular analysis. In a few cases, sampling for DNA extraction had to be repeated owing to technical difficulties in the laboratory. Among these resampled trees, 18 individuals had been felled after the first sampling or their leaves were not readily accessible from the ground. In both situations, wood cores from the base of the trunk were used and the cambial region was taken for DNA extractions.

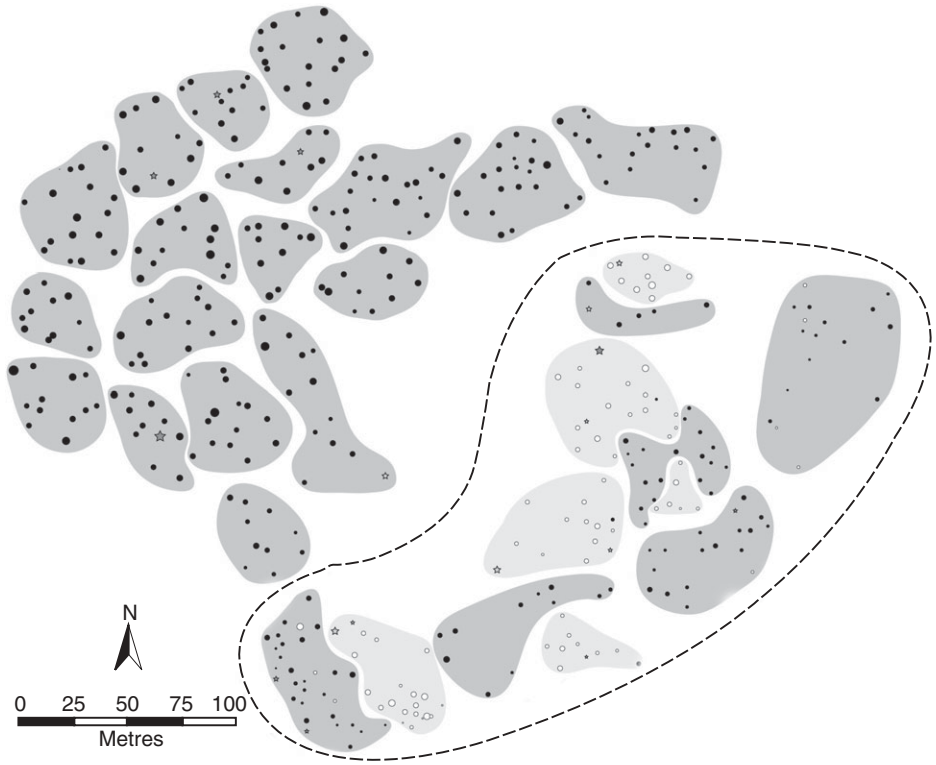

FIG. 1. Position of oak trees (open circles: *Quercus petraea*, closed circles: *Q. robur*, shaded stars: 'unclassified') within a mixed forest of *Quercus* spp. in Switzerland. Taxon classification was based on leaf morphology (Fig. 2); light and dark grey refer to the multilocus genotype assignment (cf. Figs. 2 and 3). The symbol sizes are proportional to the diameter at breast height. Light grey areas indicate 'petraea' clusters, dark grey areas indicate 'robur' clusters. The dashed line delineates the subsample of trees and clusters used to evaluate a possible sampling bias (for details see text).

Leaf morphology

Leaf morphology was used to assign individuals taxonomically and to identify putative hybrids. The strategy was to (1) classify individuals *a priori* using a well-established method (Kremer *et al.*, 2002), (2) run a canonical discriminant analysis (CDA) and (3) test for concordance with the *a priori* classification. For this purpose, 14 leaf morphological characters for 3–5 leaves per tree were recorded as described in Kremer *et al.* (2002).

First, the species score (*ID*) was calculated for each individual based on petiole length and number of veins, as given by the discriminant function from Kremer *et al.* (2002). Trees with positive *ID* values were assigned to *Q. robur*, whereas trees with negative values were assigned to *Q. petraea*. This *a priori* grouping was used for the subsequent CDA (SyStat Vers. 10·0; SPSS, 2000). The new discriminant function obtained excluded those four variables that were directly dependent on the two variables included in calculating *ID* (petiole length, number of veins, petiole ratio, percentage venation) to avoid overdetermination. Finally, the classification analysis assigned individuals to the two *a priori* groups ('petraea', 'robur'). Trees that exhibited a leaf morphology atypical for the respective taxon group were considered as putative hybrids ('unclassified').

DNA isolation and microsatellite analysis

Total DNA was extracted from approximately 50 mg fresh leaf material or from approx. 15 mm^3 of sapwood/cambium, using the Qiagen Plant Tissue kit (Qiagen, Hilden, Germany). DNA extracts were fluorometrically quantified (DyNA Quant 200, Hoefer Pharmacia Biotec, Dübendorf, Switzerland) and adjusted to 2 ng μL^{-1}, or approximately diluted according to visual quantification on agarose gels.

Five nuclear dinucleotide microsatellite loci were analysed, namely QpZAG9, QpZAG104 (Steinkellner *et al.*, 1997), QrZAG30, QrZAG96 (Kampfer *et al.*, 1998) and MSQ13 (Dow *et al.*, 1995). These loci were previously mapped onto different linkage groups (Barreneche *et al.*, 2004). The polymerase chain reaction (PCR) with fluorescently labelled primers was based on the protocols given by the respective authors with minor adjustments (e.g. $MgCl_2$ concentration, DNA amount) and multiplexing two or three loci in some cases. For fragment length analysis, an automated sequencer (ABI377 or ABI3100-Avant; Applied Biosystems, Rotkreuz, Switzerland) was used and in most cases 2–3 loci from single-plex PCRs were multiplexed. Size determination, relative to the internal size standard ROX400HD (Applied Biosystems), and allele assignment were carried out using GeneScan and Genotyper software (Applied Biosystems). Fragment lengths detected on the two different automatic sequencers, ABI377 (gel-based) and ABI3100-Avant (capillaries), were not identical but could be easily cross-referenced using standard samples. Genotypes obtained from DNA of wood cores were further confirmed based on comparisons with those previously or simultaneously obtained using DNA extracted from leaves.

Population genetic analyses

In 16 cases (0·8 %), three PCR-amplified fragments were observed at particular loci. These loci were subsequently coded as having missing values.

Different classification criteria for the partitioning of genetic variation among groups were compared (Excoffier *et al.*, 1992), presuming that the highest differentiation value indicates the biologically most meaningful classification. Analyses of molecular variance (AMOVA, based on *F*-statistics; default values used for permutation tests) were calculated with ARLEQUIN version 2000 (Schneider *et al.*, 2000). Individuals were classified according to either their leaf morphological status ('petraea', 'robur', 'unclassified') or the genetic assignment to two taxa (see below). The robustness of the genetic differentiation observed was tested by jackknifing over the five loci using Fstat version 2.9.3.2 (cf. Goudet, 1995).

To account for the unbalanced representation of *Q. petraea* and *Q. robur* in the stand, all the AMOVAs were run for a subset of data, i.e. only considering the southeastern part of the study area (81 'petraea' and 'unclassified', 89 'robur'; Fig. 1). However, this subsampling had only a minor effect on the results (F. Gugerli, unpubl. data).

We checked for the coincidence of taxon assignment based on leaf morphology vs. multilocus genotype with a simple method for individual genotype assignment that was applied to the multilocus nSSR data (ARLEQUIN, upgrade 2001). This approach computes the log-likelihood for each individual to belong to one of two groups which are characterized by their respective allele frequency estimates (Paetkau *et al.*, 1997; Waser and Strobeck, 1998). The data can then be displayed in a $x-y$ plot. Because this assignment method relies on two *a priori* groups and 'petraea' trees were already under-represented in the sample, 'unclassified' trees were grouped with 'petraea' for group-wise allele frequency computation. However, including the 'unclassified' trees in 'robur' did not alter the outcome (data not shown). Additionally, the nSSR data were analysed using a Bayesian approach implemented in the software STRUCTURE (Pritchard *et al.*, 2000). This software uses an algorithm which constructs a specified number of groups (K) to optimize consistency of allele frequencies assuming Hardy–Weinberg equilibrium within these groups. The posterior probabilities to belong to each of the K groups are then given for each individual. We ran 500 000 Markov Chain Monte-Carlo simulations with the length of burnin period set to 10 000, and allowed admixture for $K = 2$ (two taxa) and $K = 3$ (two taxa plus putative hybrids). Means over 30 iterations were used to assign individuals to either of the gene pools identified. Because the taxa were little differentiated, posterior probabilities of $<0{\cdot}2$ for an assignment to the morphology-based expectation were taken as indicative of misclassification in 'petraea' and 'robur'. 'Unclassified' trees were assigned to either of the former two taxa when the respective probability was $>0{\cdot}8$.

Spatial analyses

Assuming spatial segregation of taxa, it was predicted that 'unclassified' individuals represented morphological

outliers rather than putative hybrids, and that these could be assigned to the taxon by which they were surrounded in the stand. Therefore, the taxonomic neighbourhood of the 'unclassified' individuals was determined as the percentage of 'petraea' and 'robur' trees occurring within 20 m of each 'unclassified' tree. This distance was considered to be a reasonable minimum neighbourhood size based on the pollen dispersal in these oaks (Streiff *et al.*, 1999). A Pearson's correlation coefficient was then computed between the respective taxon percentages and the distances (D) from the diagonal in the assignment scores (ARLEQUIN) obtained for an 'unclassified' tree ($D = y - x$; see above).

Based on a purely geographical distance matrix, all individuals were grouped into spatial clusters (UPGMA, SyStat version 10.0; SPSS, 2000). The resulting cluster borders were minimally adjusted to obtain clusters of similar sample size ($n \geq 10$ except for one cluster with $n = 6$), giving six 'petraea' and 24 'robur' clusters (Fig. 1). Using these tree clusters, distance matrices were calculated based on either morphological or genetic data. For morphological data, we ran a principal component analysis (PCA) using all 14 leaf morphological variables, took the means of the first two canonical factor scores from the PCA over all individuals per cluster, and calculated Euclidean distances for all pairs of clusters (D01 in R PACKAGE version 4.0; http://www.bio.umontreal.ca/Casgrain/en/labo/R/v4/index). For the genetic distance matrix, nSSR allele frequencies of each cluster were used to calculate a matrix of pairwise Φ_{ST} values (ARLEQUIN; Schneider *et al.*, 2000). The few negative Φ_{ST} values were converted to zero values. The respective matrix correlation coefficient (Mantel, 1967) was calculated in R PACKAGE, estimating its significance based on 999 permutations.

In a next step, the central coordinates for each cluster were taken to delineate Voronoï polygons (Manni *et al.*, 2004). Using Monmonier's algorithm (Monmonier, 1973) implemented in the software BARRIER version 2.2 (Manni *et al.*, 2004), boundaries were determined based on both of the two above distance matrices. Five boundaries were calculated including external virtual points for triangulation as provided by the program BARRIER. Bootstrap support for the genetic distance matrix was calculated by importing 126 permuted distance matrices obtained from MICROSAT version 2 (distance measure F_{ST}; http://hpgl.stanford.edu/projects/microsat/). To find discontinuities in the spatial genetic pattern of nSSR genotypes, we relied on an IAM-based differentiation statistic (F_{ST} or its analogue Φ_{ST}), which has been shown to be more reliable than the SMM-based R_{ST} for detecting fine-scale genetic patterns (Streiff *et al.*, 1999).

RESULTS

Leaf morphology

The CDA with ten morphological characters effectively separated the two taxa, misclassifying only 17 (=4·1 %) of the 414 individuals [*Q. petraea:* 75 trees, five trees (6·7 %) misclassified; *Q. robur:* 322/12 (3·7 %); Fig. 2].

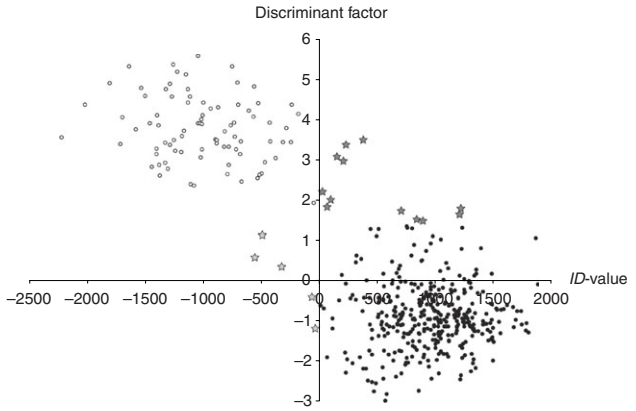

FIG. 2. Taxon assignment of *Quercus* spp. trees from a mixed stand in Switzerland, based on *ID* values (*x*-axis; following Kremer *et al.*, 2002) and the score for the discriminant function using ten leaf morphological characters (*y*-axis). Small open ('petraea') and small closed ('robur') circles represent individuals assigned to their *a priori* group. Large stars indicate individuals, *a priori* classified as 'petraea' (light grey) or 'robur' (dark grey), but misclassified in the canonical discriminant analysis ('unclassified').

These misclassified trees were grouped as 'unclassified'. Note that two characters with the highest discrimination power in Kremer *et al.* (2002), i.e. petiole length and number of veins, were not included in our CDA because they were used in *a priori* group identification (see Material and methods). When running the same analyses with all 14 variables, only seven individuals were misclassified (data not shown).

Population genetic statistics and multilocus genotype assignment

For the five loci analysed, allele numbers per locus varied between 15 (MSQ13) and 53 (QrZAG30). The exceptionally high allele number identified in QrZAG30 resulted from insertions/deletions in the flanking region of the microsatellite motif (F. Gugerli and S. Brodbeck, unpublished data). Taxon-specific alleles were mainly detected at very low frequencies except for one of two irregularly sized alleles in QpZAG104 (194 bp), which was exclusive to 'petraea' trees at a relative frequency of 10 %.

The three morphology-based taxonomic groups contributed a significant partition to the total genetic variation ($\Phi_{ST} = 0{\cdot}090$; $P < 0{\cdot}001$; Table 1). When grouping 'unclassified' trees according to their respective genetic multilocus taxon assignment (see below), the among-species component accounted for a slightly higher partition of the total genetic variation ($\Phi_{ST} = 0{\cdot}103$; $P < 0{\cdot}001$; Table 1). The five nSSR loci contributed unevenly to this morphology-based pattern of differentiation. In locus-by-locus AMOVAs, this component increased in the order QpZAG9 < QpZAG104 < QrZAG30 < MSQ13 < QrZAG96. However, jackknifing over loci confirmed that the genetic differentiation between two groups was significant (for assigned 'unclassified' individuals: $\theta_{mean} = 1{\cdot}02$, s.e. = 0·04, 95 % confidence interval = 0·017–0·187).

TABLE 1. *Results of the analyses of molecular variance (AMOVA) using nuclear microsatellite data of trees from a mixed oak stand (*Quercus *spp.) in Switzerland*

Grouping	Source of variation	d.f.	SS	Percentage variation
Morphological taxa	Among taxa (Φ_{ST})	2	63·2	9·0***
	Within taxa	825	1651·6	91·0
Species	Among species (Φ_{ST})	1	63·3	10·3***
	Within species	826	1651·5	89·7

Groupings refer to three morphological taxa ('petraea', 'robur', 'unclassified' trees) or two species (*Quercus petraea*, *Q. robur*).
*** $P < 0{\cdot}001$; d.f., degrees of freedom; SS, sum of squares.

At the multilocus level, the assignment tests (ARLEQUIN, STRUCTURE with $K = 2$) were highly congruent and supported the *a priori* taxon grouping. The two taxa were well separated, with only two (2·7 %; both methods) of the 'petraea' and six (1·9 %; ARLEQUIN) or five (1·6 %; STRUCTURE) of the 'robur' individuals misplaced (data from ARLEQUIN results presented in Fig. 3). 'Unclassified' trees were placed in both taxon groups (Fig. 3). The STRUCTURE-based results were interpreted with posterior probability limits set to 0·2 and 0·8 (see Material and methods). When applying more stringent limits of 0·05 and 0·95, only five (1·2 % of all trees) of the 17 putative hybrids showed admixture. With $K = 3$, the 'robur' group displayed an admixture of two gene pools in most trees, whereas this additional gene pool barely contributed to the posterior probabilities of 'petraea' trees.

Spatial structure

In the assignment tests, 'unclassified' trees were in most cases allocated to the taxon group that corresponded to their *in vivo* neighbouring trees. In other words, an 'unclassified' individual genetically assigned to the 'petraea' population was mainly surrounded by 'petraea' individuals in the stand (Fig. 1). Deviations from this pattern were mostly found in those trees that stood next to a forest clearing in the centre of the stand (data not shown), for which the taxonomic composition remained unknown. A significant Pearson's correlation coefficient was obtained between the percentage of 'petraea' or 'robur' individuals within 20 m distance and the distance from the diagonal in the graph of the assignment diagram (Fig. 3; $|r| = 0{\cdot}586$, $P = 0{\cdot}013$).

The tree clusters (Fig. 1) had only seven 'petraea' trees in 'robur' clusters and three 'robur' trees in 'petraea' clusters. 'Unclassified' trees occurred in both cluster types (but see preceding paragraph). These spatially delimited tree clusters were clearly supported by the morphological and genetic boundaries detected (Fig. 4). The first four boundaries determined with the allelic data were identical to those found with the morphological data, indicating high congruence between morphological and genetic separation of tree clusters in space. As expected, the matrices of morphological and genetic distances among tree clusters were highly correlated ($r_m = 0{\cdot}906$, $P < 0{\cdot}001$). Bootstrap support for the genetic boundaries was ≥ 70 % in all cases where 'petraea' and 'robur' clusters abutted (Fig. 4).

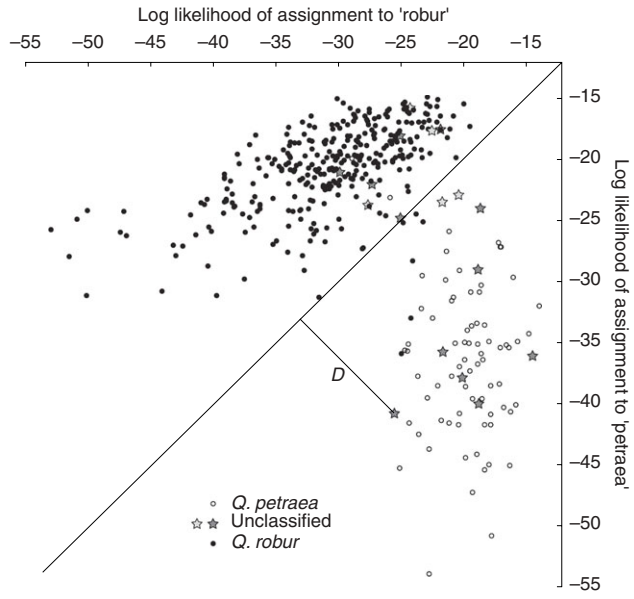

FIG. 3. Scattergram of log-likelihoods of assignment to the respective taxon 'petraea' or 'robur' for individual multilocus genotypes of oak trees (*Quercus* spp.) in a mixed stand in Switzerland. 'Unclassified' trees were *a priori* grouped with 'petraea'. The distance (*D*) from the diagonal is an indication for putative hybrid individuals. Symbols are as in Fig. 2.

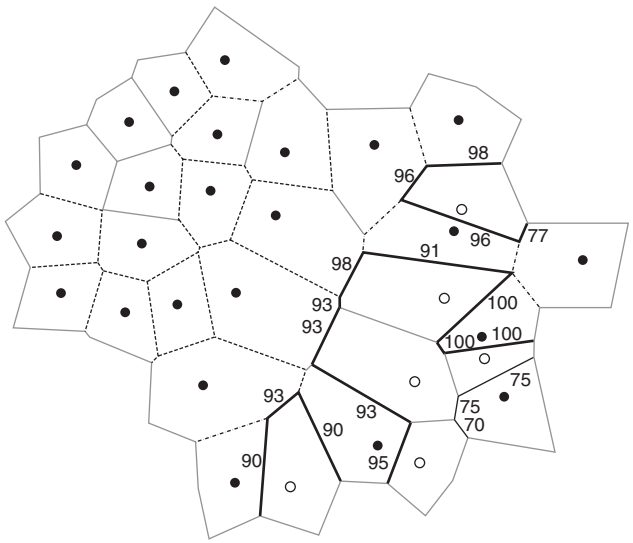

FIG. 4. Delimitation of 'petraea' (open circles) and 'robur' tree clusters (closed circles; Fig. 1) in a mixed stand of *Quercus* spp. in Switzerland. Thin grey lines delimit the Voronoï polygons obtained by the Delaunay triangulation (Manni *et al.*, 2004). Boundaries were obtained with distance matrices of both morphological and genetic traits. Bootstrap support, based on permuted matrices of genetic distances, are indicated for the respective boundaries if ≥ 70 % (thick black lines) or < 30 % (dashed black lines).

DISCUSSION

Our spatially explicit, multivariate analyses of morphological and nuclear microsatellite data, conducted on a mixed oak stand (*Quercus petraea*, *Q. robur*) in Switzerland, provide evidence that this taxon complex remains distinct even on a small scale. Little overlap between the two taxa in morphology and multilocus genotypes was found, underpinned by high congruence between morphological and genetic spatial structure in the study population. The multilocus genotypes of a few morphologically misclassified individuals, considered as putative hybrids, could be reasonably assigned to either of the two taxa. These findings are taken as clear indication that gene flow between taxa has little effect on the genetic composition in the adult stage. In the light of published evidence and our own results, we assume that a relatively small number of genomic regions under selection, in concert with strong reproductive barriers and limited seed dispersal, minimize interspecific gene flow and maintain taxon separation. It is recognized that we cannot specifically prove this assumption with our single-stand study. But, as will be outlined in the following paragraphs, our data provide a further piece of evidence to the still disputed puzzle of white oak genetics (Lexer *et al.*, 2006; Muir and Schlötterer, 2006). The low, but distinct, genetic differentiation observed better reflects the hypothesis of shared polymorphism by common ancestry than that of nuclear capture through frequent interspecific hybridization.

Spatial structure of interspecific genetic variation

The two taxonomic groups (*Q. petraea* and *Q. robur*) in our study plot displayed a non-random spatial distribution, with only a few individual trees growing in a spatial cluster of the other taxon (Fig. 1). Morphological discontinuities were consequently detected between pairs of clusters of *Q. petraea* and *Q. robur* (Fig. 4). An almost complete agreement was also found between morphological and genetic boundaries between tree clusters (Fig. 4), consistent with our expectations under the hypothesis of recent shared ancestry. These results were supported by a highly significant Mantel correlation between morphological and genetic distances among clusters.

Spatial analyses of the genetic structure within taxa have been conducted in many plant species, among them the two oaks under study (Cottrell *et al.*, 2003; Jensen *et al.*, 2003). Here, we highlight the value of spatially explicit data to elucidate genetic structure in relation to interspecific gene flow within a mixed oak community. The spatial aggregation found may be explained by several factors acting towards a non-random distribution of the trees of each taxon. Among these are (1) assortative mating among conspecifics, (2) limited seed dispersal and (3) microsite-induced selection. Assortative mating, often coupled with reproductive barriers such as shifts in flowering phenology (Fox, 2003), may contribute to taxon separation in space and time (Jiggins and Mallet, 2000). Pollen dispersal curves further suggest that siring success is related to spatial proximity even in wind-pollinated taxa like *Quercus* (Streiff *et al.*, 1999). Acorns are mostly dispersed by gravity, although secondary seed dispersal does occur, occasionally including long-distance transfers (Davies *et al.*, 2004). Nevertheless, the majority of the acorns are deposited beneath or close to their mother tree. Subsequent germination and establishment success depends on each species' ecological requirements. *Quercus robur*, susceptible to severe drought, prefers considerable soil moisture, whereas *Q. petraea* is often found in drier microsites (Ponton *et al.*, 2001). Soil moisture and associated vegetation clearly reflected environmental differences among microsites in our study plot (F. Gugerli, pers. observ.). Accordingly, we suspect a positive feedback of maternal site conditions on seedling survival and establishment. Taken together, all these processes favour the spatial aggregation of taxa, as detected in our study stand.

Interspecific differentiation and hybridization

In our search for criteria to separate taxa and to identify putative hybrids, we relied on multivariate analyses of morphological and molecular data. Under the assumptions that leaf morphology is representative of taxon status and that hybrids should show intermediate trait expression, the data revealed only a few putative hybrid trees in the study stand. These morphologically 'unclassified' trees further tended to be clearly assigned to either of the two taxa 'petraea' or 'robur' based on their multilocus genotypes (Fig. 3). Furthermore, these putative hybrids were mainly surrounded by trees of the taxon to which they had been genetically assigned (Fig. 1; see above). When grouping these individuals with either 'petraea' or 'robur' as suggested by the assignment tests, the highest among-group component of genetic variation was obtained (Table 1), which we take as evidence for a biologically meaningful assignment. We therefore suggest that the trees misclassified in the CDA represent morphological outliers rather than hybrids. Taxonomic status (assessed by leaf morphology or multilocus genotype assignment) also better explained the genetic structure than did cpDNA lineages (data not shown). This confirms earlier findings that cpDNA lineages, which are indicative of glacial survival areas, do not coincide with nuclear differentiation in the two oak taxa (Finkeldey and Mátyás, 2003). In their study of *Q. lobata* and *Q. douglasii*, Craft *et al.* (2002) also obtained close agreement between morphological and genetic taxonomic assignment using a Bayesian approach. They interpreted their result as indicating low hybrid occurrence. Similarly, our results support the view of relatively clear species separation (Figs 2 and 3) that tends to be in conflict with the nuclear capture hypothesis.

Conversely, only a few trees were found that were assigned to 'petraea' or 'robur' according to their leaf morphology, but assigned to the other gene pool (Fig. 3). Although these individuals might be considered hybrids from a genetic point of view, they did not show intermediate leaf morphologies (data not shown). However, we realize that this result is equivocal, given the limited power for hybrid detection when performing assignment tests with five microsatellite loci (Vähä and Primmer,

2006). In one case, a tree was detected that was morphologically classified as 'robur' and grew among 'robur' trees, but was assigned to 'petraea' and showed cpDNA lineage C. This lineage was otherwise only found in 'petraea' trees (F. Gugerli, unpubl. data). Accordingly, one might assume that this individual represents a hybrid with *Q. petraea* as mother. However, such a hybrid would not conform to the direction of nuclear capture suggested by Petit *et al.* (2003).

A suite of pre- and post-zygotic selection factors may maintain or foster species separation and act against interspecific hybridization in sympatric or parapatric populations (Lowe *et al.*, 2004). Pollen incompatibilities at genotype or species level (Bacilieri *et al.*, 1996; Buiteveld *et al.*, 2001), juvenile growth (Ponton *et al.*, 2002) or other ecological constraints such as predominantly short-distance seed dispersal (Petit *et al.*, 2003) have been proposed as effective mechanisms enforcing the distinctiveness of *Q. petraea* and *Q. robur* in mixed stands and other pairs of oak species (e.g. Stebbins *et al.*, 1947; Howard *et al.*, 1997; Williams *et al.*, 2001; Ishida *et al.*, 2003; González-Rodríguez *et al.*, 2004). Indeed, natural gene exchange between *Q. petraea* and *Q. robur* has been documented in acorns and seedlings from mixed oak stands across western Europe (Streiff *et al.*, 1999; A. Kremer and the Oakflow consortium, unpubl. data). Although hybrid seedlings occur, we believe they are eliminated by selective forces owing to maladaptation to the differential microsite requirements of either of their two parental taxa (Ponton *et al.*, 2001, 2002). There is supporting evidence of selective removal of inbred or hybrid oak seedlings during growth from a Danish oak stand where juvenile and adult oak cohorts were genetically differentiated (Jensen *et al.*, 2003).

Only a small number of selective loci or genomic regions might affect pre- and post-zygotic reproductive isolation and ecophysiological separation. These are hard to detect, particularly when analysing neutral genetic markers, which need to be linked to genomic regions under selection in order to reflect this differentiation. We believe that the locus with the highest differentiation value (QrZAG96) among those studied, shown to be linked to highly differentiating leaf morphological traits (Saintagne *et al.*, 2004), may represent such a case. The few genomic regions differentiating the two taxa, however, appear to occur at multiple sites across the genome (Saintagne *et al.*, 2004; Scotti-Saintagne *et al.*, 2004). Similarly, Howard *et al.* (1997: 754) concluded that in the *Q. gambelii* – *Q. grisea* complex '... selection acts to maintain coadapted complexes of alleles in the two species', and suggested that co-adapted alleles are quite localized in the genome.

Among other arguments put forward by Petit *et al.* (2003) in favour of their hypothesis of nuclear capture are the higher pollination success of *Q. petraea* in interspecific crosses (e.g. Bacilieri *et al.*, 1996) and the reduced pollen viability observed in *Quercus* hybrids (Rushton, 1993). These factors should hamper backcrossing, whereas asymmetric introgression should be possible through successive pollination of F_1 or later generation hybrids with pollen from *Q. petraea*. On the other hand, *Quercus robur* more often sired *Q. petraea* mother trees than *vice versa* according to paternity analyses of naturally pollinated acorns in our study plot (F. Gugerli *et al.*, unpubl. data), which disagrees with results from artificial pollinations (Steinhoff, 1993; Bacilieri *et al.*, 1996). Further work, for example on the genomic organization related to hybridization and on *in situ* hybrid recruitment, is thus needed to elucidate the status of this taxon complex conclusively.

In conclusion, our spatially explicit analyses have demonstrated that *Q. petraea* and *Q. robur* can be clearly separated on a local scale, and hybridization most probably plays a minimal role at least at the adult stage. According to Jiggins and Mallet (2000), this pattern may be interpreted as a bimodal hybrid zone with only few hybrids. For our study stand, we consider that low gene flow between taxa and subsequent directional selection on seeds and seedlings has led to the spatial, morphological and genetic taxon separation observed in the adults. Although hybridization cannot be completely ruled out with our data, the model of nuclear capture is not supported by our findings. The pattern detected conforms to the combination of the three scenarios – low gene flow among taxa, microsite selection and shared ancestry – suggested by Muir and Schlötterer (2005). As a next step, the spatially explicit approaches presented here should be tested in other mixed stands across the natural range of these European white oaks to check for the generality of our findings or for regional deviations.

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