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Interspecific gene flow in a multispecies oak hybrid zone in the Sierra Tarahumara of Mexico

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• *Background and Aims* Interspecific gene flow can occur in many combinations among species within the genus *Quercus*, but simultaneous hybridization among more than two species has been rarely analysed. The present study addresses the genetic structure and morphological variation in a triple hybrid zone formed by *Q. hypoleucoides*, *Q. scytophylla* and *Q. sideroxyla* in north-western Mexico.
• *Methods* A total of 247 trees from ten reference and 13 presumed intermediate populations were characterized using leaf shape variation and geometric morphometrics, and seven nuclear microsatellites as genetic markers. Discriminant function analysis was performed for leaf shape variation, and estimates of genetic diversity and structure, and individual Bayesian genetic assignments were obtained.
• *Key Results* Reference populations formed three completely distinct groups according to discriminant function analysis based on the morphological data, and showed low, but significant, genetic differentiation. Populations from the zone of contact contained individuals morphologically intermediate between pairs of species in different combinations, or even among the three species. The Bayesian admixture analysis found that three main genetic clusters best fitted the data, with good correspondence of reference populations of each species to one of the genetic clusters, but various degrees of admixture evidenced in populations from the contact area.
• *Conclusions* The three oak species have formed a complex hybrid zone that is geographically structured as a mosaic, and comprising a wide range of genotypes, including hybrids between different species pairs, back-crosses and probable triple hybrids.

Key words: Altitudinal cline, hybridization, introgression, leaf shape variation, Mexico, nuclear microsatellites, *Quercus scytophylla*, *Quercus sideroxyla*, *Quercus hypoleucoides*, red oak.

INTRODUCTION

Hybrid zones are considered an important source of genetic recombination and diversity in plant evolution (Arnold, 1997; Rieseberg, 1997). These zones are most often the product of secondary contact between populations or species that have differentiated previously in allopatry, or may also arise *in situ* in response to spatially varying selection (Durrett *et al.*, 2000). Nearly all of the hybrid zones that have been so far studied involve two parental taxa and their hybrids. Nevertheless, more complex instances of simultaneous hybridization among three or more parental taxa also occur in nature, but these have been rarely analysed (Arnold, 1993; Kaplan and Fehrer, 2007). Consequently, the genetic structure and the dynamics of gene flow in multispecies hybrid zones are poorly known (Dodd and Afzal-Rafii, 2004; Curtu *et al.*, 2007; Kaplan and Fehrer, 2007; Lepais *et al.*, 2009). These situations require the production of some fertile hybrid genotypes (F_1 and/or backcrosses) between at least one species pair. Subsequently, these individuals may disperse throughout the hybrid zone and crosses can occur between

hybrids and a third species, or between hybrids from different species combinations (Kaplan and Fehrer, 2007).

According to theoretical models, the evolution of hybrid populations will depend on the relative fitness of the various hybrid and parental genotypes (Barton and Gale, 1993; Arnold, 1997). Nevertheless, the nature and intensity of selective factors are not necessarily homogeneous across space. Both exogenous and endogenous selection have been invoked to explain the structure of hybrid zones (Barton and Hewitt, 1985; Moore and Price, 1993). Exogenous selection implies adaptation to local environments, whereas endogenous selection occurs when hybrids have low fitness due to incompatibilities between the parental genomes (Bronson *et al.*, 2003). Therefore, the selective pressures acting on hybrids may result from the combined effects of environmental factors such as climate, soil and interactions with other organisms (Fitzpatrick and Schaffer, 2004; James and Abbott, 2005; Raudnitschka *et al.*, 2007), and factors independent of environmental variation such as the disruption of co-adapted gene complexes and the action of genes associated with sterility (Barton

and Gale, 1993; Rieseberg and Wendell, 1993). If the main selection pressures are exogenous, the likely result is a clinal or mosaic hybrid zone, in which certain genotypes could be spatially segregated according to the distribution of habitats (Endler, 1977). In contrast, if the selection pressures are endogenous, the generation of tension zones is expected, wherein clines are maintained by the equilibrium between the movement of parental individuals and selection against the hybrid genotypes (Barton and Gale, 1993). However, it is not clear which of these models best describe multispecies hybrid zones (Dodd and Afzal-Rafii, 2004).

Although a high frequency of interspecific gene flow in many combinations has been inferred within the genus *Quercus* from morphological variation, relatively few studies have used genetic markers considering several species simultaneously (Whittemore and Schaal, 1991; Dumolin-Lapègue *et al.*, 1999; Dodd and Afzal-Rafii, 2004; Curtu *et al.*, 2007; Lepais *et al.*, 2009). Of particular interest has been the detection of widespread cytoplasmic introgression even among distantly related species at local scales (Whittemore and Schaal, 1991; Dumolin-Lapègue *et al.*, 1999). Nevertheless, few studies have been conducted using nuclear microsatellites, which allow the identification of different genealogical classes and detailed characterization of the genetic structure of hybrid zones (i.e. Gugerli *et al.*, 2008; Lepais *et al.*, 2009).

Mexico is considered a centre of species diversification for the genus *Quercus*, with 161 species (32–40 % of the worldwide diversity). In particular, 76 species of red oaks have been reported in Mexico, including 61 endemic species (Valencia, 2004). In recent years, several studies conducted on Mexican red oaks have focused on hybridization between two species (Valencia and Delgado, 2003; González-Rodríguez *et al.*, 2004, 2005; Tovar-Sánchez and Oyama, 2004; Tovar-Sánchez *et al.*, 2008). However, several oak species complexes comprising three or more species that probably hybridize simultaneously have been described in Mexico (Dodd and Kashani, 2003; McCauley *et al.*, 2007). In this context, understanding of the evolutionary dynamics and consequences of hybridization requires the consideration of such multispecies interactions as this is the way the phenomenon occurs in nature. The present study investigated a complex of three red oak species formed by *Q. hypoleucoides*, *Q. scytophylla* and *Q. sideroxyla*. These species are well delimited by diagnostic morphological characters, although some individuals exhibit morphological intermediacy in different combinations in several localities across the Sierra Tarahumara in north-west Mexico where the species coexist. In this region, the species overlap partially in distribution along an altitudinal gradient (1800–2500 m) and also in the timing of their flowering periods (from March to August) (Valencia, 2004).

The purpose of this study was to analyse this multispecies oak hybrid zone by combining morphometric analysis of leaf shape and nuclear microsatellite molecular markers. The particular questions addressed here were: (1) Do hybridization and introgression occur between *Q. scytophylla*, *Q. sideroxyla* and *Q. hypoleucoides*?; (2) What is the geographical structure of morphological and genetic variation among the three species in the contact area; and (3) are there associations between molecular and phenotypic variation with the altitudinal gradient encompassed by the hybrid zone?

MATERIALS AND METHODS

Study species

The three species belong to the red oak section *Lobatae* (Nixon, 1993). *Quercus scytophylla* Liebm. is a tree about 20 m in height included in the series *Scytophyllae* (Trelease, 1924). The leaves of this species are elliptical or obovate, 5–17 cm in length and 2·5–8 cm in width, with 1–6 teeth with aristae. The petioles are 9–35 mm in length. Acorns are produced singly or in groups and have a peduncle 3–10 mm in length, with maturation periods of 2 years. It is the most widely distributed of the three species in the complex, present in the Sierra Madre Occidental, the Eje Neovolcánico Transversal and the Sierra Madre del Sur, at altitudes between 1400 and 2500 m (Fig. 1). *Quercus sideroxyla* Humb & Bonpl is a tree about 10 m in height that belongs to series *Sideroxylae* (Trelease, 1924), with populations occurring in the Sierra Madre Occidental and the Eje Neovolcánico Transversal, with an altitudinal distribution from 1800 to 2700 m (Fig. 1). It has obovate or oblanceolate leaves 3–6 cm in length and 2·5–3·5 cm in width, with 1–5 distal teeth with aristae, and flattened petioles 3–9 mm long. Fruits are biennial, almost sessile, and solitary or produced in pairs. Finally, *Quercus hypoleucoides* Camus is a tree 10–20 m in height included in series *Hypoleucae* (Trelease, 1924), and has a narrower geographical distribution than the other two species, with populations in the north of the Sierra Madre Occidental and in Arizona, New Mexico and Texas, at altitudes ranging from 2000 to 2500 m (Fig. 1). The species has narrow leaves that are lanceolate, elliptical or obovate, 5–11 cm long and 1·5–3 cm wide, with entire margins. Acorns are produced annually or biennially, are sessile or almost sessile and solitary or produced in pairs.

Sampling procedure

Leaf samples were collected in 13 populations located in the zone of contact (Fig. 2). As reference, two morphologically representative populations situated within the zone of contact were also sampled, and two representative isolated populations situated out of the zone of contact were sampled per species (Fig. 2), with the exception of *Q. hypoleucoides*, because its geographical distribution within Mexico is restricted to the study zone. Representative populations were chosen based on individuals with typical diagnostic characters of each species. In each locality, 8–12 individuals sampled haphazardly were chosen but at least 50 m apart from each other. Three branches from each individual were collected: one for taxonomic identification, one for morphometric analysis and the third to obtain fresh intact leaves for molecular analysis, which were placed on ice and then at −80 °C until genetic analyses were conducted.

Leaf shape morphometric analysis

Photographs were taken of the abaxial side of five intact mature light-exposed leaves from each of eight to ten individuals per population. Coordinates '*x, y*' of 21 unambiguous and repeatable marks (i.e. landmarks and semi-landmarks) were registered along the border of each leaf image using the
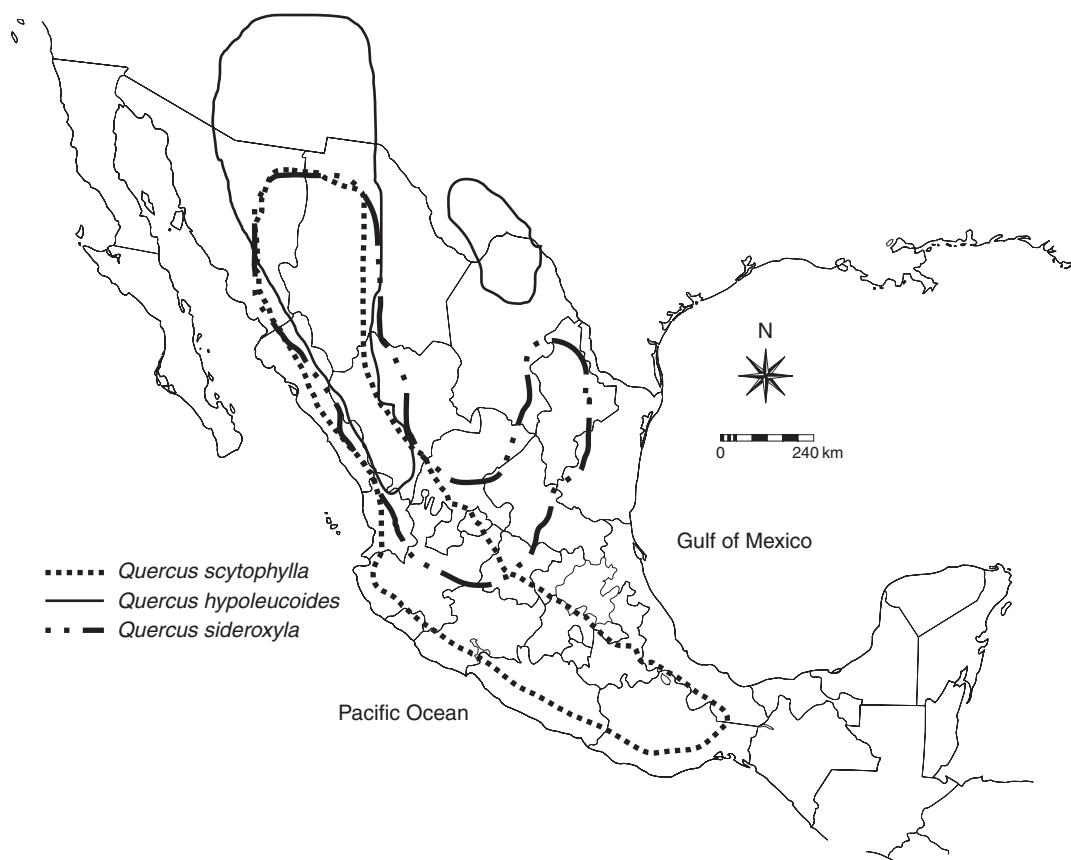
FIG. 1. Geographical distribution of the three red-oak species studied, *Quercus scytophylla*, *Q. hypoleucoides* and *Q. sideroxyla*.

program TpsDig (Rohlf, 2004). Three marks (apex, lamina base and petiole extreme) corresponded to homologous loci ('landmarks' *sensu* Bookstein, 1991), and the other 18 were semi-landmarks, i.e. morphological points that incorporate information about leaf contour in morphometric analysis, in areas that lack landmarks for all the individuals (Zelditch *et al.*, 2004). Landmarks '1' (lamina base) and '12' (apex) were used to construct a 'fan' (radial guidelines with equal angular spacing on images) with 80 radial guidelines covering the whole leaf contour, which was used to digitize the 18 semi-landmarks. The program MakeFan6 within the IMP software package (Integrated Morphometrics Package; http://www.canisius.edu/~sheets/morphsoft.html) was used for this procedure. A Procrustes superimposition analysis was developed with the CoordGen program in IMP. This analysis allows the calculation of leaf shape variation without the effect of the size. The resulting shape variables (Procrustes distances) for all individuals were subjected to a canonical discriminant analysis to determine the variation in leaf shape among individuals of each species and intermediate populations using SPSS 11·0 (Ferran, 1997). Individuals from reference populations of the three species were first analysed to obtain the canonical discriminant functions, and then the discriminant scores calculated with these functions were obtained for individuals from all populations. In this way, five morphological groups were identified, three of which corresponded to pure species individuals and two corresponded to intermediate individuals (see Results).

Microsatellite amplification

Genomic DNA was extracted from 100 mg of leaf material using the method proposed by Lefort and Douglas (1999). Seven nuclear microsatellite loci (OC11, OA01, 1C08, 2M04, OE09, 1H14, 1F07) previously designed for *Quercus rubra* (Aldrich *et al.*, 2002) were selected based on the quality of preliminary amplification trials. Polymerase chain reactions were carried out in a volume of 25 µL containing 20 ng of template DNA, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 9), 0·1 mM of each dNTP, 0·5 mg mL^{-1} bovine serum albumin, 2 µM of each primer and 0·3 units of TaqDNA polymerase (Gibco, Invitrogen, San Diego, CA, USA). The thermal cycling conditions consisted of 40 cycles, each at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min. A final extension at 72 °C for 10 min was included. Amplified fragments were separated in polyacrylamide gels (6 %) in a semi-automatic sequencer LI-COR 4300 (LI-COR Biosciences, Lincoln, NB, USA). Fragment sizes were calculated using the program ImageJ by comparisons with internal and external standards. All individuals sampled from the 23 populations were genotyped.

Genetic analysis

The mean number of alleles per locus (N_a), mean effective number of alleles (N_e), mean observed heterozygosity (H_O), mean expected heterozygosity (H_E), mean fixation index (F)
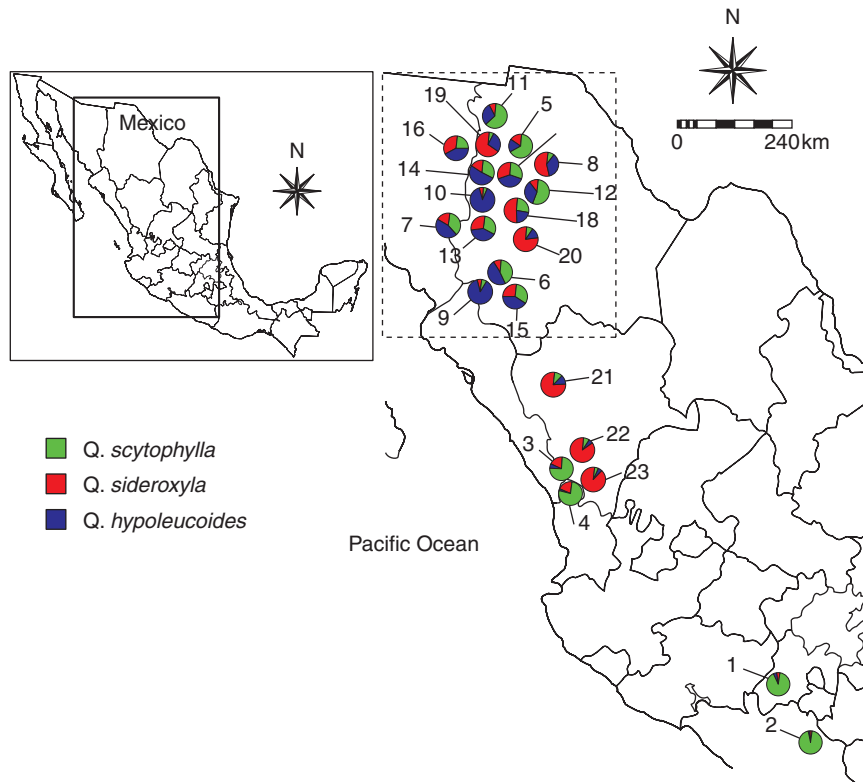
FIG. 2. Map showing sampling localities representing parental and intermediate populations. Each pie chart represents the proportions in each population of the three genetic groups as assigned by the program STRUCTURE. Green, red and blue represent the genetic groups corresponding to *Quercus scytophylla*, *Q. sideroxyla* and *Q. hypoleucoides*, respectively. Numbers next to each symbol correspond to the population numbers given in Table 1. The area of species contact is delineated with a dashed rectangle.

and their respective standard errors were calculated for each population using FSTAT (Goudet, 1995). Average values were also obtained for each of the five morphological groups defined on the basis of canonical discriminant function analysis. Pairwise genetic differentiation among the five morphological groups was calculated using the method of Weir and Cockerham (1984) with the GENETIX 4 program (Belkhir *et al.*, 2004) with 10 000 permutations for statistical significance. Within each of the morphological groups genetic differentiation among populations was estimated with analyses of molecular variance (AMOVA). The significances of the different variance components were estimated from distributions generated from 10 000 random permutations. These analyses were carried out using ARLEQUIN 3·0 (Excoffier *et al.*, 2005).

Bayesian admixture analysis

The genetic ancestry of each individual was inferred with the program STRUCTURE 2·3·1 (Pritchard *et al.*, 2000; Falush *et al.*, 2003; Hubisz *et al.*, 2009). This program is based on a Bayesian model clustering procedure to determine the proportions of ancestry of individuals derived from multiple populations. In this analysis, different probable values of K (number of genetic groups) were assayed, increasing the probable number from $K = 1$ to 13 (ten times for each value). The program was run using the admixture model with correlated allelic frequencies without prior population information. For all runs, a length of the burn-in period of 50^3 iterations was used, followed by 10^6 Markov chain Monte Carlo iterations. Additionally, the most probable value of K was determined using the maximum value of ΔK, following the rules of Evanno *et al.* (2005), and by the estimated ln probability of data, ln$P(D)$.

Genotype simulation and assignment

To evaluate the ability of the assignment procedure to recognize accurately the different genotypic classes potentially present in our samples (i.e. pure parental, hybrids between the different pairs of species and triple hybrids), the procedure developed by Vähä and Primmer (2006) was followed. Individuals that in the assignment analysis described above had an assignment coefficient (Q) higher than 0·90 (indicating a high probability of belonging to a single genetic cluster) were used to estimate allelic frequencies of the three species. Thirty-nine such individuals were identified for *Q. scytophylla*, 21 for *Q. hypoleucoides* and 44 for *Q. sideroxyla*. Pure species and hybrid genotypes were then simulated with HYBRIDLAB 1·0 (Nielsen *et al.*, 2006) using the calculated allelic frequencies. Four hundred genotypes were simulated for each species, 37 for the different possible F_1 hybrids, 113 for backcrosses and 14 for triple hybrids (triple hybrids were considered the product of crossing an F_1 hybrid from a pair of species with a pure individual of the third species). The number of simulated hybrid genotypes

is similar to the number observed in the real data. The simulated data were then analysed with the program STRUCTURE, with the same number of genetic groups previously inferred ($K = 3$), and the same settings. Thereafter, the performance of STRUCTURE in hybrid and pure-bred individual identification was evaluated via the parameters of efficiency (number of individuals correctly assigned), accuracy (proportion of an identified group that truly belongs to that category) and performance (efficiency multiplied by accuracy), following Vähä and Primmer (2006). Finally, the optimal threshold values of Q to assign individuals to the different genotypic categories were determined.

Geographical patterns of variation

To determine if variation patterns in genetic composition and morphology of populations could be explained by geographical variables, we conducted stepwise regression analyses of the population average values of canonical discriminant scores and proportions of genetic ancestry in each of the genetic groups inferred against the latitude, longitude and altitude of the populations.

RESULTS

Leaf shape morphometric analysis

The first two discriminant functions (F1 and F2), derived from the Procrustes analysis of leaf shape, explained all the observed variation (55·1 and 44·9 %, respectively), indicating that discrimination among reference populations of the three species was absolute and highly significant (for F1, Wilks' $\lambda = 0.027$, d.f. $= 82$, $P < 0.001$; for F2, Wilks' $\lambda = 0.180$, d.f. $= 40$, $P < 0.001$), supporting the taxonomic delimitation of the three taxa. This is clearly observed in Fig. 3, with the three species forming completely separated clusters. F1 contributed most to the discrimination between *Q. hypoleucoides* and the other two species, while F2 discriminated between *Q. scytophylla* and

FIG. 3. Scatterplot of individual scores from discriminant function analysis of populations in the oak complex based on foliar geometric morphometric data. Closed circles, squares and triangles symbolize representative populations of *Quercus scytophylla*, *Q. hypoleucoides* and *Q. sideroxyla*, respectively, and open circles and crosses are the Mixed 1 and Mixed 2 population groups, respectively. See text for details.

Q. sideroxyla. Individuals from the 13 populations considered to show indications of morphological intermediacy had score values identical to one of the reference clusters or were intermediate either along F1, F2 or both. These populations formed two recognizable groups (Fig. 3). The first group (hereafter called Mixed 1) included individuals from populations Río Chico, Querari and Yécora, most of which were similar to reference individuals of *Q. hypoleucoides*, and individuals from population Madera that were clearly intermediate between *Q. hypoleucoides* and *Q. sideroxyla*. The second group (hereafter Mixed 2) contained individuals similar to *Q. scytophylla* or intermediate between this species and the other two, and belonged to populations Huracán A, Huracán B, Guadalupe, Km 346, Guachochic, Amarilla B, Poleo, Amarilla A and Babícora.

Genetic structure

Seven microsatellite loci revealed high levels of genetic diversity and low but significant genetic differentiation between and within the three species (Tables 1 and 2). The mean number of alleles per locus (N_a) within populations varied from 3·71 to 10·42 and the mean of the effective number of alleles (N_e) was between 2·55 and 7·25 (Table 1). High values of H_O (ranging from 0·633 to 0·905) and H_E (from 0·555 to 0·841) were observed (Table 1). H_O, H_E, N_a and N_e did not differ among the five morphological groups (three reference and two intermediate groups) according to Wilcoxon tests (data not shown). Among 161 inbreeding coefficients (F) calculated (23 populations and seven loci), only four were significant after applying a Bonferroni correction: for locus OA01 in populations Espinazo ($F = 0.744$, $P < 0.00001$) and Tecuán ($F = 0.226$, $P < 0.0001$), and for locus 2M04 in populations Temosachic ($F = -0.337$, $P < 0.0001$) and Tecuán ($F = -0.313$, $P < 0.00001$). The program MICROCHECKER (Van Oosterhout *et al.*, 2004) was used to test whether these deviations could be due to the presence of null alleles, stuttering or large allele dropout at those loci. Only population Espinazo showed signs of the presence of a null allele at high frequency (in four of ten individuals) at locus OA01. For further analysis, this locus was coded as missing data in this population.

According to Weir and Cockerham's θ, genetic differentiation was significant in all pairwise comparisons among the five morphological groups, except between the Mixed 1 and Mixed 2 groups (Table 2). Differentiation among populations was also significant within the three species, but not within the Mixed 1 and Mixed 2 groups (Table 3).

Estimates of admixture

The highest posterior probability was obtained for three genetic clusters according to the values of $\ln P(D)$ (Fig. 4A). This result was also confirmed by the ΔK values (Fig. 4B). Using the admixture model in the program STRUCTURE, the proportion of ancestry (Q) of each individual and population in each of the three genetic groups was also estimated. The allopatric reference populations had a high proportion of ancestry from a single genetic group (Figs 2 and 5). For *Q. scytophylla*, populations Tejupilco and Pozo Largo had genetic ancestries of 0·913 and 0·947 in one genetic cluster
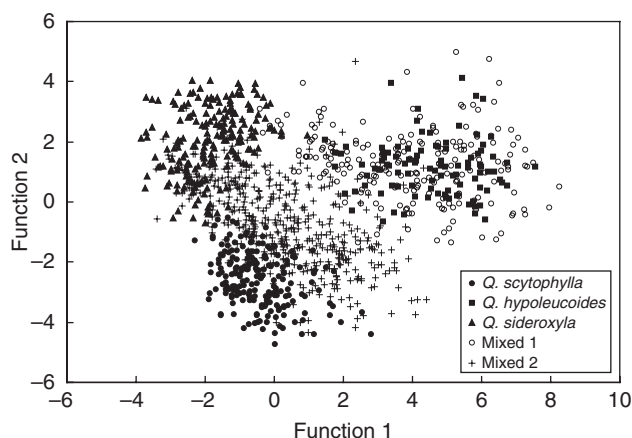
TABLE 1. *Name, geographical coordinates, altitude, mean observed heterozygosity (H$_O$), mean expected heterozygosity (H$_E$), mean number of alleles (N$_a$) and mean number of effective alleles per locus (N$_e$) for 23 populations of* Quercus scytophylla, Q. hypoleucoides *and* Q. sideroxyla

Number and name of locality	State	Coordinates	Sample size	Genetic diversity			
				H_O	H_E	N_a	N_e
Q. scytophylla							
1. Tejupilco	México	19°02′/100°03′	9	0·889 (0·12)	0·772 (0·13)	6·85 (2·96)	5·46 (0·84)
2. Pozo Largo	Guerrero	17°36′/99°05′	10	0·742 (0·19)	0·810 (0·05)	7·14 (2·05)	5·61 (0·55)
3. Espinazo	Sinaloa	23°34′/105°05′	10	0·814 (0·29)	0·805 (0·08)	8·14 (2·26)	5·83 (0·77)
4. Loberas	Durango	23°29′/105°51′	10	0·857 (0·12)	0·832 (0·03)	8·42 (0·61)	6·25 (0·49)
Mixed 1							
5. Río Chico	Chihuahua	29°36/108°10′	12	0·657 (0·35)	0·729 (0·11)	6·71 (3·03)	4·52 (0·94)
6. Querari	Chihuahua	27°11′/107°32′	11	0·831 (0·24)	0·819 (0·08)	9·42 (2·50)	6·41 (0·85)
7. Yécora	Sonora	28°22′/109°02′	12	0·839 (0·13)	0·831 (0·08)	9·85 (3·48)	7·07 (1·03)
8. Madera	Chihuahua	29°32′/108°09′	11	0·889 (0·09)	0·801 (0·05)	7·86 (0·59)	5·35 (0·53)
Q. hypoleucoides							
9. Batopilas	Chihuahua	27°08′/107°34′	10	0·885 (0·16)	0·782 (0·08)	7·00 (2·58)	5·09 (0·58)
10. Temosachic	Chihuahua	28°59′/108°13′	11	0·766 (0·22)	0·728 (0·16)	7·00 (2·76)	4·74 (0·85)
Mixed 2							
11. Huracán A	Chihuahua	29°40′/108°15′	12	0·853 (0·09)	0·824 (0·05)	8·42 (1·51)	6·13 (0·62)
12. Guadalupe	Chihuahua	29°11′/107°57′	10	0·846 (0·17)	0·765 (0·14)	7·14 (2·03)	5·19 (0·68)
13. Km 346	Chihuahua	28°26′/108°32′	11	0·633 (0·47)	0·555 (0·38)	3·71 (2·21)	2·55 (0·67)
14. Amarilla B	Chihuahua	29°12′/108°14′	12	0·657 (0·26)	0·681 (0·14)	6·42 (3·86)	4·09 (0·97)
15. Guachochic	Chihuahua	26°56′/107°08′	8	0·905 (0·12)	0·799 (0·03)	7·57 (1·81)	5·11 (0·32)
16. Poleo	Sonora	29°37′/108°98′	12	0·809 (0·16)	0·829 (0·09)	10·42 (3·40)	7·25 (1·21)
17. Amarilla A	Chihuahua	29°11′/108°14′	10	0·778 (0·09)	0·796 (0·08)	8·14 (2·11)	5·53 (0·68)
18. Babícora	Chihuahua	29°13′/107°49′	10	0·724 (0·22)	0·778 (0·13)	7·57 (2·99)	5·53 (0·85)
19. Huracán B	Chihuahua	29°40′/108°15′	12	0·734 (0·22)	0·764 (0·08)	7·71 (2·21)	4·69 (0·56)
Q. sideroxyla							
20. Maycoba	Chihuahua	28°17′/108°06′	10	0·857 (0·11)	0·783 (0·13)	7·85 (3·02)	5·75 (0·91)
21. Cuevecillas	Durango	25°02′/106°16′	11	0·844 (0·15)	0·841 (0·07)	9·71 (2·75)	7·17 (0·88)
22. Buenos Aires	Durango	23°42′/105°43′	11	0·831 (0·11)	0·841 (0·04)	8·71 (2·13)	6·71 (0·62)
23. Tecuan	Durango	23°55′/105°01′	12	0·821 (0·04)	0·833 (0·06)	10·28 (1·89)	6·64 (0·79)

Standard errors are given in parentheses. The five morphological groups (three parental species and two mixed groups) were defined on the basis of geometric morphometrics.

TABLE 2. *Pairwise values of genetic differentiation (F$_{ST}$) among the five morphological groups estimated with the method of Weir and Cockerham (1984)*

	Q. scytophylla	Q. hypoleucoides	Q. sideroxyla	Mixed 1	Mixed 2
Q. scytophylla	–	**0·022**	**0·071**	**0·031**	**0·039**
Q. hypoleucoides		–	**0·036**	**0·022**	**0·035**
Q. sideroxyla			–	**0·029**	**0·054**
Mixed 1				–	0·014
Mixed 2					–

Numbers in bold indicate statistically significant values ($P < 0.05$) based on 10 000 random permutations.

and, for *Q. sideroxyla*, individuals from populations Buenos Aires and Tecuan also were assigned mostly to a single genetic group (Figs 2 and 5). In contrast, morphologically representative populations of the three species from within the contact zone showed some indications of introgression. Populations of *Q. hypoleucoides* had slight contributions from the other two genetic groups, while populations Espinazo and Loberas, belonging to *Q. scytophylla*, were influenced by the *Q. sideroxyla* genetic group. Finally, some introgression of the *Q. hypoleucoides* genetic cluster was detected in populations Maycoba and Cuevecillas, belonging to

Q. sideroxyla (Figs 2 and 5). In the morphologically mixed populations, there was evidence of admixture among the three genetic clusters in different proportions. The Mixed 1 group was characterized by a transition from a higher proportion of the *Q. scytophylla* genetic cluster at lower altitudes (i.e. populations Río Chico and Huracán A) to more even contributions from the three species at higher altitudes (Km 346 and Yecora). The Mixed 2 group showed a change from even genetic proportions from the three genetic clusters towards a predominance of ancestry from *Q. sideroxyla* at high altitudes (Figs 2 and 5).

TABLE 3. *Genetic structure within the five morphological groups estimated with Φ_{ST} obtained from AMOVA*

Source of variation		d.f.	SS	Variance components	Percentage of variation	Fixation index
F_{ST}						
	Q. scytophylla					
	Among populations	3	18·1	0·157	5·01	$\Phi_{ST} = 0.05$***
	Within populations	74	220·06	2·973	94·99	
	Q. hypoleucoides					
	Among populations	1	8·89	0·292	9·54	$\Phi_{ST} = 0.09$***
	Within populations	40	110·89	2·772	90·46	
	Q. sideroxyla					
	Among populations	3	17·96	0·135	4·26	$\Phi_{ST} = 0.04$***
	Within populations	84	254·39	3·028	95·74	
Mixed 1						
	Among populations	3	7·18	0·008	0·36	$\Phi_{ST} = 0.004$ n.s.
	Within populations	88	194·5	2·21	99·64	
Mixed 2						
	Among populations	7	−3·43	−0·118	−6·44	$\Phi_{ST} = -0.06$ n.s.
	Within populations	158	307·83	1·95	106·44	

Asterisks indicate statistically significant values ($P < 0.05$) and n.s. indicates non-significant values. Tests were based on 10 000 random permutations.
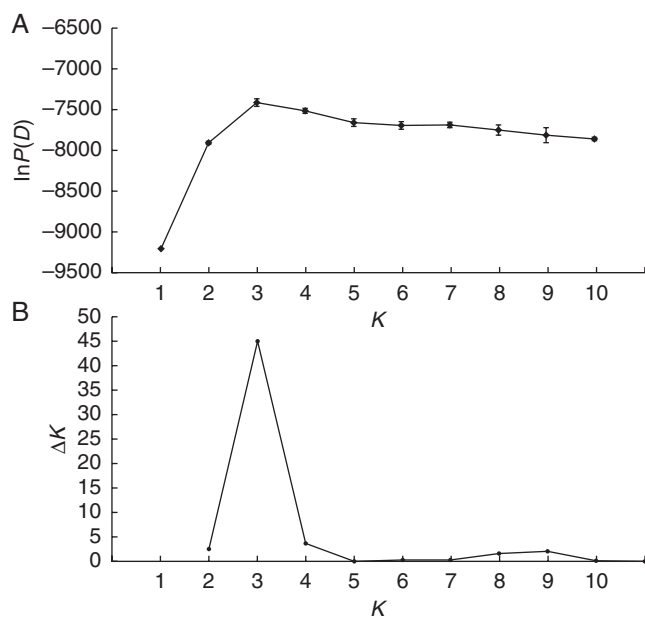

FIG. 4. (A) Mean and standard deviation of $\ln P(D)$ for ten independent runs of STRUCTURE plotted against the number of genetic groups (K) used in the analysis. (B) Values of ΔK plotted against K. In both cases the peak indicates the most probable number of genetic groups given the data.

Performance of STRUCTURE with simulated genotypes

Analysis of the simulated genotypes indicated that a threshold value in the admixture coefficient of $Q \geq 0.90$ allowed adequate separation between pure species and hybrid individuals (Table 4). The genetic assignment of pure individuals from the three species showed high levels of performance, with values of 93 % for *Q. scytophylla*, 96 % for *Q. sideroxyla* and 95 % for *Q. hypoleucoides* (Table 4). For hybrid classes, performance values in most cases were moderately lower in comparison with pure-species assignment. Even for triple hybrids there was a reasonable identification rate.

Given these results, individuals from all populations in the real samples were assigned to a genotypic class as follows: individuals with $Q \geq 0.90$ were considered to be pure bred, trees with $Q < 0.90$ from two genetic groups were considered hybrids between two species, and trees with ancestry from the three genetic groups in more or less equal proportions ($Q = 0.3–0.4$) were considered triple hybrids (Fig. 6). Populations from the contact zone were largely dominated by hybrid individuals (Fig. 6). As previously noted for the admixture coefficient of each population, the proportion of the different genotypes also changed with altitude. At lower altitudes, the predominant genotypes corresponded to crosses between *Q. scytophylla* and *Q. sideroxyla*, which were replaced by crosses between *Q. scytophylla* and *Q. hypoleucoides*, and finally by crosses between *Q. sideroxyla* and *Q. hypoleucoides* with increasing altitude. Triple hybrids were situated in populations at mid altitudes, particularly in populations Km 346 and Amarilla A, where also almost all the other possible genotypes were present.

Associations among leaf shape morphology, genetic ancestry and altitude

The results of the regression analysis indicated that the proportions of genetic ancestry of *Q. scytophylla* and *Q. sideroxyla* in the populations were strongly correlated with altitude ($R^2 = 0.78$, $P < 0.0001$ and $R^2 = 0.72$, $P = 0.0002$, respectively) but not with latitude or longitude. In turn, the proportion of the *Q. hypoleucoides* genetic group was weakly correlated with latitude only ($R^2 = 0.4$, $P = 0.03$). Finally, the canonical scores of morphological variation showed a marginally significant tendency to be correlated with latitude ($R^2 = 0.3$, $P = 0.06$).

Nevertheless, as reference populations of the three species were situated at the extremes of the altitudinal range, it might be that the significant correlations obtained are mostly due to the different altitudinal requirements of the pure species. To verify this explanation, correlations were calculated again without the reference populations and practically the same results were obtained (data not shown).

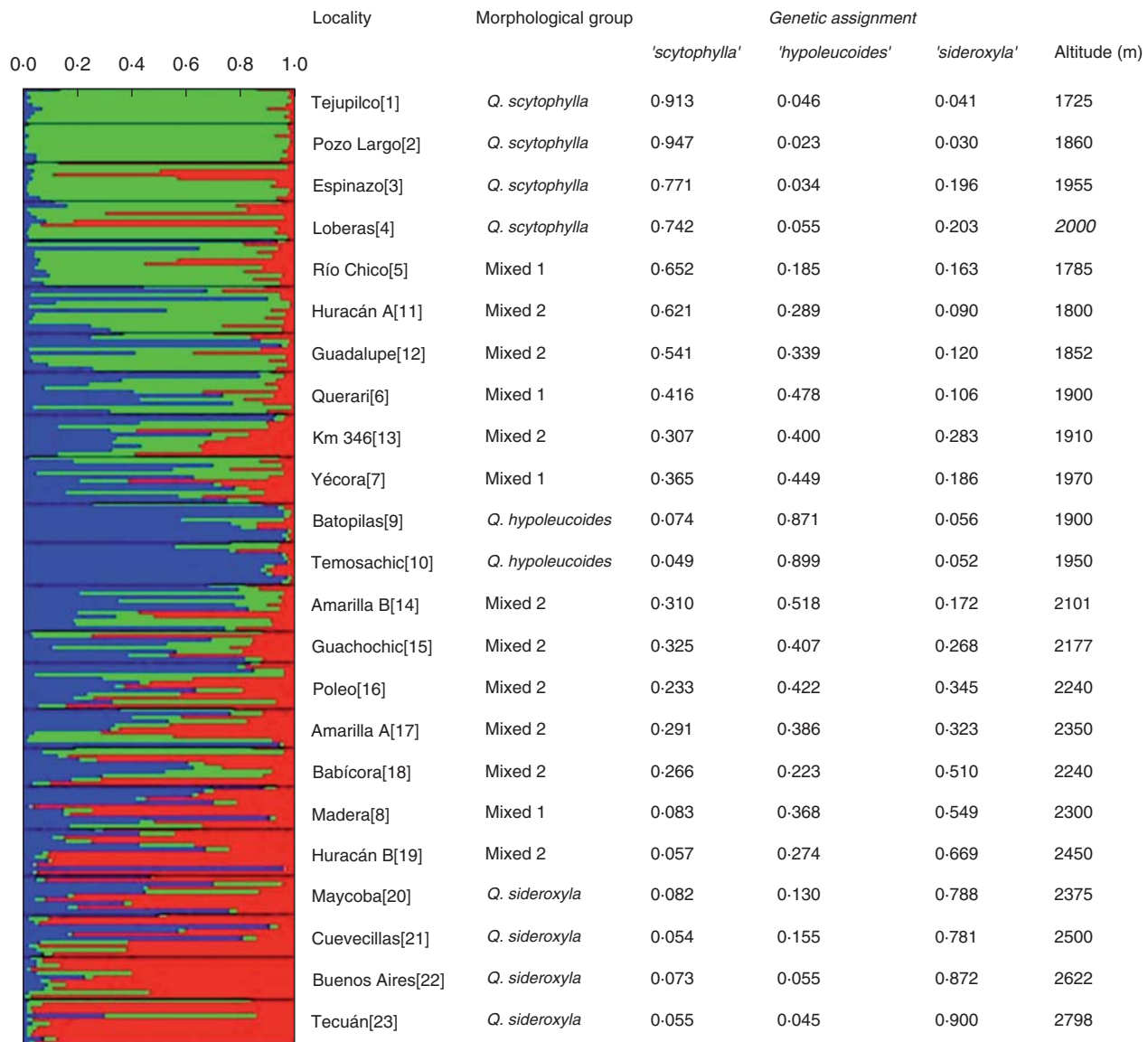| Locality	Morphological group	Genetic assignment 'scytophylla'	'hypoleucoides'	'sideroxyla'	Altitude (m)
Tejupilco[1]	*Q. scytophylla*	0·913	0·046	0·041	1725
Pozo Largo[2]	*Q. scytophylla*	0·947	0·023	0·030	1860
Espinazo[3]	*Q. scytophylla*	0·771	0·034	0·196	1955
Loberas[4]	*Q. scytophylla*	0·742	0·055	0·203	*2000*
Río Chico[5]	Mixed 1	0·652	0·185	0·163	1785
Huracán A[11]	Mixed 2	0·621	0·289	0·090	1800
Guadalupe[12]	Mixed 2	0·541	0·339	0·120	1852
Querari[6]	Mixed 1	0·416	0·478	0·106	1900
Km 346[13]	Mixed 2	0·307	0·400	0·283	1910
Yécora[7]	Mixed 1	0·365	0·449	0·186	1970
Batopilas[9]	*Q. hypoleucoides*	0·074	0·871	0·056	1900
Temosachic[10]	*Q. hypoleucoides*	0·049	0·899	0·052	1950
Amarilla B[14]	Mixed 2	0·310	0·518	0·172	2101
Guachochic[15]	Mixed 2	0·325	0·407	0·268	2177
Poleo[16]	Mixed 2	0·233	0·422	0·345	2240
Amarilla A[17]	Mixed 2	0·291	0·386	0·323	2350
Babícora[18]	Mixed 2	0·266	0·223	0·510	2240
Madera[8]	Mixed 1	0·083	0·368	0·549	2300
Huracán B[19]	Mixed 2	0·057	0·274	0·669	2450
Maycoba[20]	*Q. sideroxyla*	0·082	0·130	0·788	2375
Cuevecillas[21]	*Q. sideroxyla*	0·054	0·155	0·781	2500
Buenos Aires[22]	*Q. sideroxyla*	0·073	0·055	0·872	2622
Tecuán[23]	*Q. sideroxyla*	0·055	0·045	0·900	2798

Fig. 5. Genetic assignment of individuals and populations according to the Bayesian method implemented in the program STRUCTURE. One sample, considered to explain the data best, of ten iterated runs is shown. Each thin horizontal line represents an individual and the proportion of each colour is the proportion of ancestry derived from each of the three main genetic groups ($K = 3$) inferred. Populations are separated by black lines. Values of the proportions of ancestry for each population are given in the table.

DISCUSSION

The combined results of geometric morphometrics of leaf shape and genetic analysis with microsatellites indicate that a high rate of hybridization and introgression have occurred among the three oak species studied, which have resulted in a complex hybrid zone containing a wide array of genotypes. Furthermore, this hybrid zone is geographically structured as a mosaic, with a patchy spatial distribution of pure and mixed populations with different genetic compositions. Altitude was, apparently, the most important geographical variable explaining the distribution of genetic and morphological variation.

The utility of geometric morphometrics to quantify leaf shapes and to assess hybridization in plants is well established (e.g. Jensen *et al.*, 2002). In species of *Quercus*, foliar shape is

particularly informative because other organs (i.e. flowers) show little variation (Kaul, 1985). The morphometric assessment performed in this study, based on Procrustes analysis of landmarks followed by canonical discriminant function analysis, substantiated the complete morphological differentiation of individuals from representative populations of the three oak taxa *Q. hypoleucoides*, *Q. scytophylla* and *Q. sideroxyla*. The morphometric analysis also demonstrated that some individuals were intermediate in various combinations with respect to representative populations, as was initially presumed from field observations. Intermediate individuals were present in a series of populations scattered along a wide area of contact among the three oak species. These populations showed clear differences in their phenotypic composition and could be divided into two groups. The first

TABLE 4. *Number of simulated individuals (rows), which were assigned to one of the three species or into a hybrid category (columns)*

Simulated/ assigned	Qscytop	Qhypole	Qsidero	Hyb QscytoQsidero	Hyb QscytoQhypole	Hyb QsideroQhypole	Triple Hybrid	Total
Qscytop	**391**	–	–	9	–	–	–	400
Qhypole	–	**398**	–	–	2	–	–	400
Qsidero	–	–	**395**	–	–	5	–	400
F_1 Qscyto-Qsidero	2	–	–	**3**	–	2	–	7
BX Qscyto	11	–	–	24	–	2	–	37
F_1 Qscyto-Qhypole	–	–	–	2	**14**	–	–	16
BX Qhypole	–	12	–	–	32	–	–	44
F_1 Qsidero-Qhypole	–	–	–	–	1	**13**	–	14
BX Qsidero	–	–	9	–	1	21	1	32
Triple Hybrid	–	–	–	2	–	3	**9**	14
Total	404	410	404	40	50	46	10	1364
Efficiency	97·75	99·50	98·75	61·3636	76·666	73·913	64·28	
Accuracy	96·782	97·073	97·777	67·50	92·00	73·914	90·00	
Performance	94·60	96·58	96·55	41·42	70·53	54·63	57·85	

Parameters of efficiency, accuracy and overall performance of the assignment method are given in per cent. Note individuals correctly assigned are in bold type. Hyb, hybrids; F_1, first-generation hybrids; BX, backcrosses; Qscyto, *Quercus scytophylla*; Qhypole, *Q. hypoleucoides*; Qsidero, *Q. sideroxyla*.
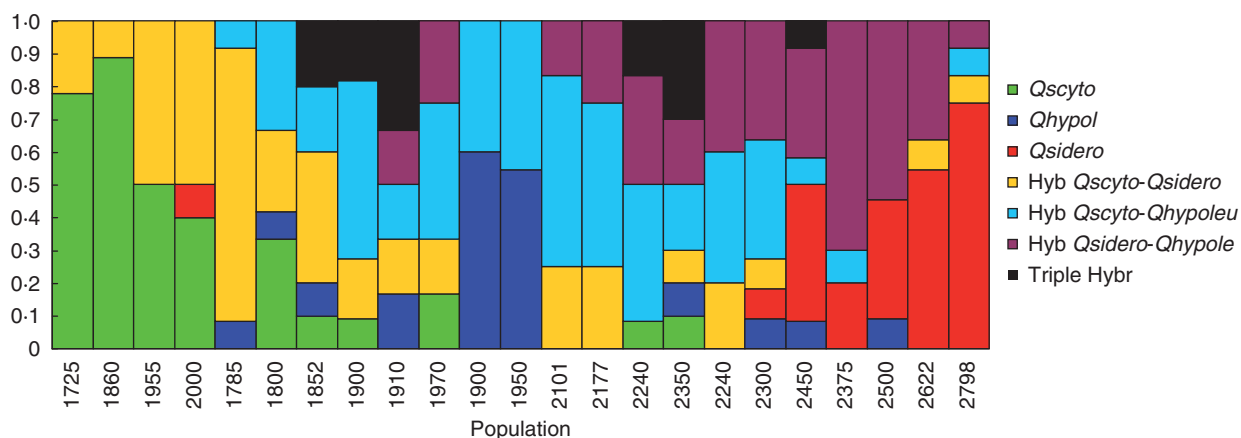

FIG. 6. Frequency of the different genotypic classes observed in each population. Individuals were assigned to each category (*Q. scytophylla*, *Q. hypoleucoides*, *Q. sideroxyla*, hybrids between *Q. scytophylla* and *Q. sideroxyla*, hybrids among *Q. scytophylla* × *Q. hypoleucoides*, hybrids between *Q. sideroxyla* and *Q. hypoleucoides* and triple hybrids, as indicated in the key), depending on their individual coefficient of admixture derived from STRUCTURE. Performance of the assignment procedure was previously assessed by analysing simulated genotypes (see text for details).

group included a series of sites (i.e. Río Chico, Querari, Yécora and Madera) in which the predominant morphology was similar to *Q. hypoleucoides*, *Q. sideroxyla* or intermediate between them. The second group (i.e. Huracán, Guadalupe, Km 346, Guachochic, Amarilla B, Poleo, Amarilla A and Babícora) consisted of populations showing evidence of morphological intergradation between *Q. scytophylla* and the other two species.

The degree of genetic differentiation among the three species was significant but very low (Table 2). However, similar comparisons of oak species belonging to several complexes have repeatedly found low interspecific genetic differentiation based on nuclear markers, ranging from 0·02 to 0·17 (Dodd and Kashani, 2003; González-Rodríguez *et al.*, 2005; Muir and Schlötterer, 2005; Craft and Ashely, 2006; Curtu *et al.*, 2007; Gugerli *et al.*, 2007). Although it has been argued that low genetic differentiation among oak species does not necessarily imply introgression, but can be

accounted for by the sharing of ancestral polymorphisms (e.g. Muir and Schlötterer, 2005), in most instances interspecific gene flow is a more parsimonious explanation for this observation (Lexer *et al.*, 2006).

In the specific case studied here intermediate populations occurred within a defined area with the characteristics of a mosaic hybrid zone (see below). Furthermore, the Bayesian analysis suggested a clustering into three genetic groups, which agrees with the number of taxa involved in this complex, with isolated representative populations assigned to a single genetic cluster. However, the same analysis also suggested the occurrence of various degrees of admixture among the three genetic groups in populations from within the area of contact. The above evidence strongly suggests that introgression has occurred among the three oak taxa and contributed to shape the patterns of variation observed. As shown, both the morphological and the genetic data indicate that some of the populations in the zone of contact are the

result of introgression between a pair of species, but others clearly seem to be an admixture of all three taxa.

At the level of individuals, a high proportion of hybrids resulting from crosses between different species pairs were identified within the contact zone, and 14 trees were assigned as probable triple hybrids. Simulations of genotypes provided reasonable support to the assignment method, providing some confidence on the identification of even these complex hybrids (Vähä and Primmer, 2006). Studies on closely related European white oaks have suggested that five or six microsatellite loci are sufficient to distinguish between pure species and introgressed individuals (Curtu *et al.*, 2007; Gugerli *et al.*, 2007). A recent study involving four oak species suggested that patterns of interspecific crosses may be more complex than those modelled in simulations, possibly leading to the existence of third- or later-generation hybrids as well as to hybridization involving more than two species (Lepais *et al.*, 2009). Triple hybridization first requires the production of fertile hybrid genotypes between at least two species, and then the crossing between hybrids and a third species, or between hybrids from different species combinations (Kaplan and Fehrer, 2007).

Mosaic hybrid zones are described as areas where pure species populations and mixed populations are patchily distributed across a zone of overlap (Harrison and Rand, 1989). The morphological variation in the contact area in the Sierra Tarahumara clearly follows this pattern. Populations with typical parental species morphology and those belonging to the Mixed 1 and Mixed 2 morphological groups are geographically scattered. Genotypic variation was also distributed as a mosaic and showed little correlation with latitude and longitude. Strong associations among specific genotypes and certain habitats are also characteristic of mosaic hybrid zones, and exogenous selection is considered important in such structuring (Harrison and Rand, 1989). From the analyses presented, it was evident that the genetic and morphological composition of the oak populations studied is strongly associated with altitude. However, as can be judged from the lack of similarity among geographically proximate populations, dispersal does not seem to be the factor determining these correlations. Alternatively, the influence of environmental variables on the structure of this hybrid zone can be suggested as an explanation for these results. In their study on four Californian red oak species, Dodd and Afzal-Rafii (2004) obtained results implying that environmental gradients rather than pollen dispersal determine the extent of introgression. In other plant species, hybrid zones associated with altitudinal gradients have been reported and the patterns of variation are often coupled to environmental variables that change with elevation (e.g. James and Abbott, 2005; Kimball, 2008). For oaks, there is some direct evidence indicating that the frequency of hybridization, the direction of introgression and the performance of hybrids depend on habitat conditions (Williams and Ehleringer, 2000; Williams *et al.*, 2001; Himrane *et al.*, 2004), but also on species' relative abundance (Lepais *et al.*, 2009). These factors could be contributing to the structure of the hybrid zone in the Sierra Tarahumara. Nevertheless, ecological studies are required to gain more detailed insight into the dynamics of this hybrid zone. In conclusion, the evidence presented supports the existence of a complex hybrid zone that has formed from pairwise and triple hybridization among *Q. hypoleucoides*, *Q. scytophylla* and *Q. sideroxyla* in the mountains of the Sierra Tarahumara in north-western Mexico, which is structured as a mosaic probably in response to environmental variables associated with altitude.

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