Natural hybridization and hybrid zones between *Quercus crassifolia* and *Quercus crassipes* (Fagaceae) in Mexico: morphological and molecular evidence[1]

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Hybrid zones provide interesting systems to study genetic and ecological interaction between different species. The correct identification of hybrids is necessary to understand the evolutionary process involved in hybridization. An oak species complex occurring in Mexico formed by two parental species, *Quercus crassifolia* H. & B. and *Q. crassipes* H. & B., and their putative hybrid species, *Q. dysophylla*, was analyzed with molecular markers (random amplified polymorphic DNA [RAPDs]) and morphological tools in seven hybrid zones (10 trees per taxa in each hybrid zone) and two pure sites for each parental species (20 trees per site). We tested whether geographic proximity of hybrid plants to the allopatric site of a parental species increases its morphological and genetic similarity with its parent. Seventeen morphological traits were measured in 8700 leaves from 290 trees. Total DNA of 250 individuals was analyzed with six diagnostic RAPD primers. *Quercus crassifolia* differed significantly from *Q. crassipes* in all the examined characters. Molecular markers and morphological characters were highly coincident and support the hypothesis of hybridization in this complex, although both species remain distinct in mixed stands. Clusters and a hybrid index (for molecular and morphological data) showed that individuals from the same parental species were more similar among themselves than to putative hybrids, indicating occasional hybridization with segregation in hybrid types or backcrossing to parents. Evidence does not indicate a unidirectional pattern of gene flow.

Key words: Fagaceae; hybrid zones; hybridization; leaf morphology; Mexico; *Quercus*; RAPDs.

Hybridization is a natural phenomenon that occurs frequently in plants and animals (Harrison, 1993). This process produces new genetic combinations by the introduction of semicompatible genes into another genotype, upon which interaction of environment and genetic variation can isolate a novel taxon from parental types. Hybrids may be defined as species, subspecies, variants, or races, depending on the degree of divergence (Futuyma, 1998). Interspecific gene transfer is an important evolutionary force, because the genetic material introduced by introgression exceeds that which is produced directly by mutation (Anderson, 1949).

The genus *Quercus* (Fagaceae) is one of the most diversified groups of temperate trees with more than 500 species distributed worldwide (Nixon, 1993). Hybridization and hybrid zones are common among oaks (Trelease, 1924; Palmer, 1948; Muller, 1952; Tucker, 1961; Cottam et al., 1982; Jensen et al., 1993; Spellenberg, 1995; Howard et al., 1997; Ishida et al., 2003). However, despite the perception that hybrid zones are well documented among oaks, few comparative analyses of oak hybrid zones have used both morphological characters and genetic markers (Howard et al., 1997).

Hybridization in oaks was initially detected based on morphological characters (Stebbins et al., 1947; Barlett, 1951; Tucker, 1961; Benson et al., 1967; Hardin, 1975; Cottam et al., 1982; Rushton, 1993). Leaf morphology in particular has been useful to demonstrate hybridization (Bacon and Spellenberg, 1996). However, in some cases morphological characters alone do not confirm unequivocally the existence of hybridization (Bacilieri et al., 1995; Manos et al., 1999; Mayol and Rosselló, 2001) requiring other methods such as DNA markers (Crawford et al., 1993; Rieseberg and Ellstrand, 1993). Random amplified polymorphic DNA (RAPD) markers have been particularly successful in the detection of interspecific hybridization and introgression in plants (Arnold et al., 1991; Arnold, 1993; Crawford et al., 1993; Cruzan and Arnold, 1993; Marsolais et al., 1993; Fritz et al., 1994; Smith et al., 1996; Samuel, 1999).

Mexico is considered one of the centers of diversification of the genus *Quercus* (oaks) (Muller and McVaugh, 1972; Rzedowski, 1978; González, 1993; Nixon, 1993), with 135–150 species that include 86 endemics (Nixon, 1993). However, hybridization has only recently been reported for some species of Mexican oaks (McVaugh, 1974; Boecklen and Spellenberg, 1990; Spellenberg, 1992, 1995; Spellenberg and Bacon, 1996; González-Rodríguez et al., 2004, in press). The taxonomy and evolutionary relationships of Mexican oaks are currently being studied, and some species complexes formed by potential parental species and putative hybrids have been recently detected by oak specialists. We chose two red oak species (subg. *Erythrobalanus*) for this study, *Q. crassifolia* H. & B. and *Q. crassipes* H. & B. These species have noticeable differences in several morphological characters (Romero, 1993) when they form allopatric pure stands. However, intermediate trees with atypical leaf shapes are observed when both species occur in sympatry, suggesting that hybridization may explain the observed variations. It is important to indicate that other oak species that can be considered as reasonable putative parents
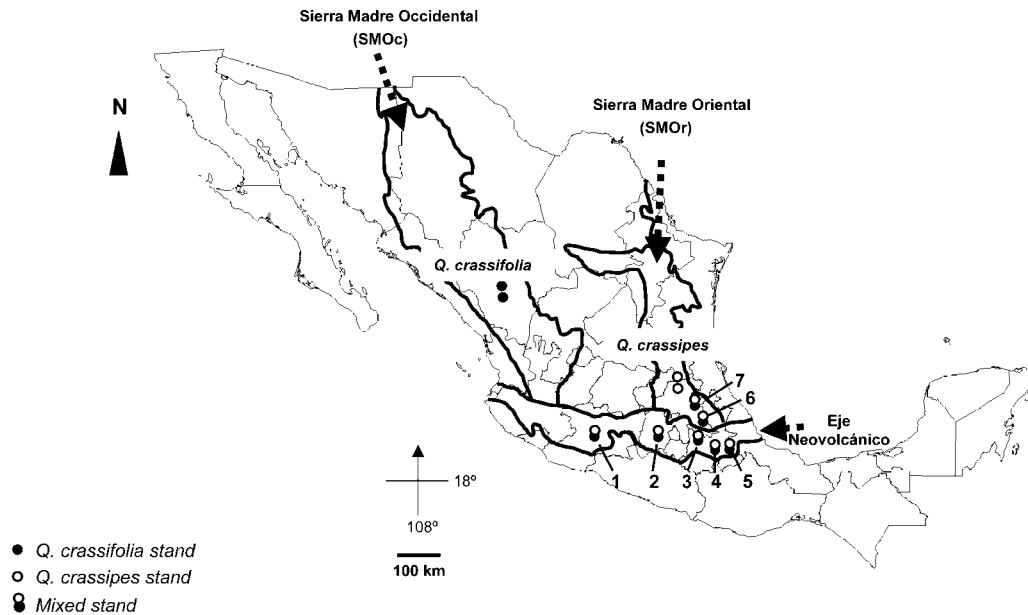
Fig. 1. Map of sampled populations of *Quercus crassifolia* × *Q. crassipes* complex. The mixed stands are represented by numbers, 1 = Cantera, 2 = Canalejas, 3 = Tlaxco, 4 = Acajete, 5 = Esperanza (located at the Eje Neovolcánico), 6 = Agua Blanca, 7 = Palo Bendito (located at the Sierra Madre Oriental).

as judged from the morphological features of *Q. dysophylla* do not occur in the area. The taxonomic status of *Q. dysophylla* is still under discussion; some authors consider this species as a hybrid formed by *Q. crassifolia* × *Q. crassipes* (K. Nixon and S. Valencia, Cornell University and UNAM [Universidad Nacional Autónama de México], respectively, personal communication), while others recognize it as a different species (Romero, 1993; Zavala Chávez, 1995).

In this paper, we describe and compare the patterns of morphological and genetic variation of the *Q. crassifolia* × *Q. crassipes* complex, document the structure of overlapping zones and hybridization in the Eje Neovolcánico and Central Mexico, and assess the taxonomic distinctness of the two species and the putative hybrid.

MATERIALS AND METHODS

Study sites—*Quercus crassifolia* is distributed throughout the Sierra Madre Occidental (SMOc, northwest Mexico), whereas *Q. crassipes* is distributed along the Sierra Madre Oriental (SMOr) on the opposite side of the country. The distribution of these species overlaps along the Eje Neovolcánico, a range of volcanic mountains that traverses central Mexico in an east–west direction, where individuals with intermediate leaf morphology are present (Romero, 1993). Seven sympatric zones were chosen in the Eje Neovolcánico and Sierra Madre Oriental, where *Q. crassifolia* and *Q. crassipes* overlap geographically: Cantera, Canalejas, Tlaxco, Acajete, Esperanza, Agua Blanca, and Palo Bendito. These localities were chosen because they contain the highest numbers of intermediate individuals of this complex. Two localities were also chosen for each parental species, where they are dominant and no hybrids were observed (Fig. 1). In general, the hybrids occur in a very low frequency sporadically interspersed and near to the putative parents. Intermediate trees occur in more disturbed habitats but we cannot assess the preference of hybrids for some type of habitats (e.g., type of soils, forest gaps). It is rare to find intermediate trees outside of the contact zones. No evidence exists on another oak species hybridizing with trees of the species studied. When other oak species were found, they were white oaks that cannot hybridize with red oak species.

Morphological data—Seventeen morphological characters of leaves were measured (Table 1) in 290 trees. A total of 210 trees were sampled in the seven mixed stands, 10 trees for each taxa: *Q. crassifolia*, *Q. crassipes*, and the hybrid. Twenty trees were sampled in each pure site (two sites for each parental species). Thirty mature leaves, without any apparent damage, were randomly sampled in each tree. Seventeen characters were measured in a total of 8700 leaves.

Molecular data—We collected undamaged young leaves from 250 trees of *Q. crassifolia* ($n = 90$), *Q. crassipes* ($n = 90$), and hybrids ($n = 70$), distributed in seven mixed stands and 40 trees in two pure zones for each parental species. Total DNA was extracted using a DNAeasy Plant Mini Kit (Qiagen, Valencia, California, USA). A genetic analysis was performed using RAPD (Welsh and McClelland, 1990; Williams et al., 1990), and the polymerase chain reaction (PCR). Forty-eight 10-base pair (bp) primers of random sequence (Kits A, B, C; Operon Technologies, Alameda, California, USA) were tested to find specific, diagnostic markers for each parental species. Diagnostic markers are those that are present in all individuals of one species and absent in a second species, while species-specific markers are those unique to one species but not necessarily present in all individuals within the species (Howard et al., 1997). Lastly, 250 individuals were tested using six primers (four species-specific markers [two for *Q. crassifolia*, OPA-09 and OPA-13, and two for *Q. crassipes*, OPB-01 and OPC-06], and two diagnostic markers [*Q. crassifolia*, OPA-14, and *Q. crassipes*, OPB-18]). DNA fragments were separated by electrophoresis on 1.5% agarose gel, stained with ethidium bromide, and developed on a UV table. The molecular mass of the DNA fragments was estimated with a 1-kilo base (kb) DNA ladder.

Statistical analysis—Nested variance analyses were conducted (ANOVA) to determine the effects of oak species, locality, and individual (tree) on the morphological leaf variability of each of the 17 studied characters (Table 1). Hybrids were not included in this analysis. Trees were considered as a random factor nested within species, because they were representative of each population. Percentage data were corrected as $X = \arcsin (\%)\frac{1}{2}$, and discontinuous data were transformed as $X = (x)\frac{1}{2} + 0.5$ (Zar, 1999).

To quantify variation in leaf morphology among oak species and hybrids, we randomly selected 30 leaves from vouchered specimens, and all the 17

TABLE 1. List of the leaf morphological characters examined for the *Q. crassifolia* × *Q. crassipes* complex in Mexico.

Abbreviation	Description
Macromorphological characters	
LP	Length of petiole
LL	Length of lamina
TLL	Total leaf length (LL + LP)
MWL	Maximal width of lamina
HMW	Height of maximal width (length of lamina from base to widest part)
PD	Petiole diameter
MD	Midvein diameter
NV	Number of veins
LWB	Leaf width at basal 1/3 of leaf
LWA	Leaf width at apical 1/3 of leaf
NA	Number of aristae
LLW	Length of lamina from base to widest part (LL − HMW)
Combinations of characters	
P%	Length of petiole × 100/total leaf length
HW%	Height of maximal width × 100/total leaf length
DW%	Length of lamina from base to widest part × 100/total leaf length
LL/MWL	Length of lamina/maximal width of lamina
LLW/MWL	Length of lamina from base to widest part/maximal width of lamina

characters were measured. All morphological characters were used for discriminant analysis to assess the most useful character for taxonomic discrimination between *Q. crassifolia* and *Q. crassipes* and to determine how leaf morphological characters separate individuals into groups. Seventeen characters were measured for the character count procedure to determine hybrids with intermediate leaf morphology of the *Q. crassifolia* × *Q. crassipes* complex following the procedure of Wilson (1992). Variation in size and shape due to shape alone was quantified using the ratios of the sums of eigen values from the discriminant analysis (Darroch and Mosimann, 1985).

In the seven mixed stands, the Anderson hybrid index (Anderson, 1949, 1953) was used to identify intermediate individuals and possible backcrosses. A histogram for each mixed stand was obtained. The Anderson hybrid index was calculated using the 17 morphological characters because they demonstrated differences between parental species. The representative characters of *Q. crassipes* received a rank of 2; *Q. crassifolia* characters were assigned a rank of 0; while intermediate characters were assigned a rank of 1 (Wilson, 1992).

A general cluster diagram for all zones was obtained, including pure and mixed stands. STATISTICA 6.0 for Windows was used for all the statistical analyses (Statsoft, 1998).

The maximum likelihood (ML) hybrid index score from RAPD analysis was calculated using Hardig-Hybrid software (Hardig et al., 2000). This index is useful to identify intermediate individuals, showing backcrosses as well as the structures of hybrid swarms. The results were represented in a frequency histogram for each mixed stand and for the pure sites. A Mantel *Z*-test matrix and Tools for Population Genetic Analyses (TFPGA, version 1.3) were used to test isolation by distance, as well as to create a general cluster diagram for the mixed stands and the four pure zones (Miller, 1997).

RESULTS

Morphological analysis for parental and hybrid plants—All of the examined characters in *Quercus crassifolia* differ significantly from those of *Q. crassipes*. Locality and tree also had highly significant effects on each of the measured leaf characters (Table 2). Three characters (NA, LL/MWL, and

TABLE 2. Mean ± standard error and nested ANOVA results (*F* statistics) for all characters of *Quercus crassifolia* and *Q. crassipes* in Mexico. *F* significant at $P < 0.001$ (***); $P < 0.01$ (**); and $P < 0.05$ (*).

Character	Units	*Q. crassifolia*	*Q. crassipes*	Locality *F* (df = 6,4060)	Species *F* (df = 1,4060)	Tree *F* (df = 90,3040)
Macromorphological characters						
LP	cm	1.26 ± 0.019	0.53 ± 0.006	52.32***	1233.57***	5.51***
LL	cm	10.10 ± 0.060	5.54 ± 0.032	30.90***	7579.13***	19.52***
TLL	cm	11.64 ± 0.066	6.07 ± 0.030	23.64***	23792.13***	18.22***
MWL	cm	5.94 ± 0.046	1.63 ± 0.010	14.06***	16810.99***	19.36***
HMW	cm	6.16 ± 0.064	2.68 ± 0.020	49.15***	8089.17**	16.58**
PD	cm	0.23 ± 0.002	0.09 ± 0.001	121.28**	5345.47**	16.86**
MD	cm	0.16 ± 0.002	0.07 ± 0.001	127.87**	2643.26**	40.75**
NV	no.	14.48 ± 0.086	25.25 ± 0.217	121.34**	2156.06**	4.96**
LWB	cm	4.55 ± 0.031	1.44 ± 0.008	13.42***	14684.32***	21.21***
LWA	cm	5.30 ± 0.037	1.41 ± 0.008	22.63***	16552.18***	19.93***
NA	no.	3.15 ± 0.064	1.22 ± 0.000	119.62**	49800.28**	16.14**
LLW	cm	3.94 ± 0.029	2.86 ± 0.018	14.32***	1109.32***	10.98***
Combinations of characters						
P	%	0.11 ± 0.001	0.08 ± 0.001	143.82**	143.07**	9.00**
HW	%	0.54 ± 0.002	0.44 ± 0.002	56.05**	1594.82**	5.99**
DW	%	0.52 ± 0.002	0.27 ± 0.001	37.32***	14809.43***	8.54***
LL/MWL		1.72 ± 0.005	3.47 ± 0.016	242.20**	269715.9**	8.50***
LLW/MWL		0.68 ± 0.004	1.80 ± 0.012	84.50**	255214.3**	4.50**

LLW/MWL) were clearly separated without overlap between the two species in the pure zones as well as in the mixed stands and can be considered as species-specific characters. Hybrids presented intermediate morphological leaf traits between *Q. crassifolia* and *Q. crassipes*. The binomial sign analysis showed that the deviation was highly significant ($P < 0.001$), accepting the hybridization hypothesis. Intermediate characters were found in the 17 examined characters of the samples from Palo Bendito ; 16 of 17 examined characters in the samples from Cantera and Esperanza; and 15 of 17 examined characters in the samples from Canalejas, Tlaxco, Agua Blanca, and Acajete (Table 3).

Discriminant function analysis for size and shape variation showed that in all of the analyzed hybrid zones the hybrids had intermediate characters between their parents. The discriminant axis described a gradient of leaf shapes from elliptic narrow (*Q. crassipes*) to the hybrids and the obovate leaves of *Q. crassifolia* (Fig. 2).

For the *Q. crassifolia* × *Q. crassipes* comparison, one highly significant ($P < 0.001$) discriminant axis accounted from 81.23% (Esperanza) to 94.78% (Acajete) of the variation between taxa. Discriminant function analysis for leaf morphology variation showed that in general the MWL contributed with the highest value to the ordination model for the first axis in Cantera, Canalejas, and Agua Blanca while in Tlaxco and Palo Bendito it was LL, and in Esperanza HW% and in Acajete it was LLW. On the other hand, the character with the highest value for the second axis was LL in Cantera, Canalejas, Agua Blanca, Esperanza, and Acajete, LL/MWL in Palo Bendito, and TLL in Tlaxco (Table 4).

The shape variable produced two significant discriminant axes, and the ratio of the sums of eigenvalues of the Cantera hybrid zone indicated that approximately $4.48/7.16 = 62.57\%$ of shape alone was accountable for the total variation in size and shape. In the other hybrid zones, less than 50% of the total variation in size and shape was attributable to the shape alone: in Agua Blanca, $1.65/6.44 = 25.62\%$; Canalejas, $2.48/8.14 = 30.47\%$; Palo Bendito, $2.88/8.52 = 33.80\%$; Tlaxco, $3.55/9.48 = 37.45\%$; Acajete, $2.28/5.17 = 44.10\%$; and Esperanza, $3.34/7.94 = 42.06\%$.

In a global cluster analysis based on morphological leaf traits for the seven mixed stands and four pure populations (two for *Q. crassifolia* and two for *Q. crassipes*), the populations having the same parental species were more similar to each other. For the five hybrid zones located on the Eje Neovolcánico (mixed stands from 1 to 5), nearness of the hybrid zones to the allopatric putative parent correlated with increasing similarity of the complex to the parental species. Lastly, mixed stands 6 and 7, located on the Sierra Madre Oriental, were more similar to *Q. crassifolia* (Fig. 3, left).

We used 17 morphological characters to estimate the status of *Q. crassifolia* and *Q. crassipes* plants marked previously as "pure" or "hybrid" plants. The results for the Anderson hybrid index support the field identification of 250 plants (Fig. 4). In general, the frequency histogram (Anderson hybrid index) had a pattern similar to the global cluster analysis. In Eje Neovolcánico, the mixed stands (Cantera and Canalejas) nearer to SMOc presented unidirectional introgression towards *Q. crassifolia*. Subsequently, the site closest to central Mexico (Tlaxco) was the only site that showed bidirectional introgression, but the two mixed stands nearest to SMOr (Acajete and Esperanza) registered unidirectional introgression towards *Q. crassipes*. Finally, the two hybrid zones localized north of

Tlaxco (Agua Blanca and Palo Bendito) on SMOr presented unidirectional introgression towards *Q. crassifolia* (Fig. 4).

Individuals A10 (Cantera), A7, A8 (Tlaxco), and A9 (Agua Blanca) were classified as *Q. crassifolia* in the field, but the index showed that they were backcrosses towards *Q. crassifolia*. Individuals B4 and B8 (Tlaxco), B2 (Acajete), and B2 (Esperanza) were originally marked as *Q. crassipes*, but the index analysis confirmed that they were backcrosses toward *Q. crassipes*. Individuals AB1 and AB9 (Agua Blanca), as well as AB2 (Acajete), and AB2 and AB10 (Esperanza), were marked as hybrids, but the index showed that they were individuals of *Q. crassifolia* and *Q. crassipes*, respectively. Also, the individuals AB8, AB9, and AB10 (Cantera); AB1 and AB10 (Canalejas); AB8 and AB10 (Tlaxco); AB6 and AB10 (Agua Blanca); and AB3 and AB5 (Palo Bendito) were classified as hybrid plants, but the index analysis indicated that they were backcrosses toward *Q. crassifolia*. Finally, AB3 (Tlaxco), AB3, AB9, and AB10 (Acajete), and AB3, AB5, and AB9 (Esperanza) were marked as hybrids, but the data showed that they were backcrosses toward *Q. crassipes* (Fig. 4).

Genetic analysis (RAPDs) of parental and hybrid plants—We used six primers to estimate the genetic status of *Q. crassifolia* and *Q. crassipes* plants morphologically identified as "pure" or "hybrid" plants. These primers yielded 49 distinct markers (bands). The RAPD analysis proved to be a powerful tool for characterizing hybrid individuals between *Q. crassifolia* and *Q. crassipes*. The analysis of the ML hybrid index using six RAPD markers supported the field identification of 250 plants (Fig. 5). Individuals A10 (Cantera); A1, A7, and A8 (Tlaxco); and A5 and A6 (Palo Bendito) were classified as *Q. crassifolia* in the field, but the RAPD showed that they were backcrosses toward *Q. crassifolia*. Individuals B4 and B8 (Tlaxco) and B2 (Esperanza) were originally marked as *Q. crassipes*, but RAPDs analysis confirmed that they were backcrosses toward *Q. crassipes*. The individuals AB9 (Agua Blanca) and AB7 (Tlaxco) were marked as hybrids, but RAPDs and subsequent field examinations showed that they were individuals of *Q. crassifolia* and *Q. crassipes*, respectively. Individuals AB10 (Cantera), AB1 (Agua Blanca), and AB3, AB5, and AB7 (Palo Bendito) were classified as hybrid plants, but the molecular analysis indicated that they were backcrosses toward *Q. crassifolia*. Lastly, AB3, AB6, and AB9 (Tlaxco) ; AB4, AB5, AB8, AB9, and AB10 (Acajete); and AB2 and AB10 (Esperanza) were identified as hybrids, but RAPD data showed that they were backcrosses toward *Q. crassipes* (Fig. 5).

Few plants had perfect marker additivity as may be expected in F_1 (12 plants), but 54 individuals were interpreted as F_1, 26 deviated by only one character (0.437–0.562), and 16 plants deviated by only two characters (0.375–0.625). Twelve plants were interpreted as backcrosses toward *Q. crassipes* (0.250–0.312) and 11 as backcrosses toward *Q. crassifolia* (0.687–0.750) (Fig. 5).

The mean ML hybrid index for *Q. crassifolia* individuals was 0.85 (SD 0.07), for *Q. crassipes* was 0.08 (SD 0.06), and for the hybrids, 0.48 (SD 0.09).

The Hardig hybrid index showed the same general pattern as the Anderson hybrid index (see earlier). No introgression was registered only in Canalejas (Fig. 5).

The cluster analysis for genetic (RAPDs) and morphological data showed that these are very similar. The allopatric zones (two zones for *Q. crassifolia* and two for *Q. crassipes*) were

TABLE 3. Character count procedure from the seven hybrid zones of the *Quercus crassifolia* × *Q. crassipes* complex in Mexico. Abbreviations of characters are described in Table 1; + = the hybrid is intermediate between the parental species, − = the hybrid is not intermediate, SD = standard deviation.

Character	Q. crassifolia (mean ± SD)	Hybrid (mean ± SD)	Q. crassipes (mean ± SD)	The hybrid is different from (Tukey, $P < 0.05$)	The hybrid is intermediate?
CANTERA					
LP	1.25 ± 0.62	0.71 ± 0.55	0.41 ± 0.27	both	+
LL	10.89 ± 2.02	9.43 ± 2.08	6.30 ± 1.31	both	+
TLL	12.14 ± 2.19	10.13 ± 2.36	6.71 ± 1.39	both	+
MWL	6.35 ± 1.41	3.70 ± 0.98	1.58 ± 0.40	both	+
HMW	6.83 ± 1.55	4.98 ± 1.32	3.23 ± 0.89	both	+
PD	0.20 ± 0.04	0.14 ± 0.27	0.10 ± 0.02	both	+
MD	0.13 ± 0.03	0.08 ± 0.02	0.07 ± 0.09	Q. crassifolia	+
NV	14.85 ± 4.23	21.16 ± 2.77	28.30 ± 6.15	both	+
LWB	4.72 ± 1.15	3.15 ± 0.83	1.32 ± 0.29	both	+
LWA	5.92 ± 1.42	3.12 ± 0.86	1.33 ± 0.32	both	+
NA	3.36 ± 0.43	1.29 ± 0.25	1.22 ± 0.00	both	+
LLW	4.06 ± 1.13	4.44 ± 1.05	3.07 ± 0.73	both	−
P	18.30 ± 4.49	14.83 ± 2.97	14.43 ± 3.04	Q. crassifolia	+
HW	48.63 ± 4.65	44.44 ± 3.44	43.76 ± 4.42	Q. crassifolia	+
DW	46.38 ± 4.06	37.12 ± 2.74	29.18 ± 3.52	both	+
LL/MWL	1.74 ± 0.25	2.59 ± 0.34	4.07 ± 0.68	both	+
LLW/MWL	0.65 ± 0.16	1.23 ± 0.25	1.99 ± 0.46	both	+
Scores of intermediate characters					16:01
CANALEJAS					
LP	1.62 ± 0.70	0.99 ± 0.51	0.63 ± 0.36	both	+
LL	10.02 ± 2.14	8.40 ± 1.88	5.52 ± 1.33	both	+
TLL	11.64 ± 2.40	9.39 ± 2.08	6.16 ± 1.42	both	+
MWL	6.08 ± 1.47	3.20 ± 0.84	1.58 ± 0.36	both	+
HMW	5.98 ± 1.58	4.02 ± 1.21	2.46 ± 0.76	both	+
PD	0.19 ± 0.05	0.12 ± 0.07	0.08 ± 0.02	both	+
MD	0.13 ± 0.05	0.08 ± 0.01	0.07 ± 0.01	both	+
NV	11.28 ± 2.93	19.71 ± 2.48	26.17 ± 4.36	both	+
LWB	5.06 ± 1.28	2.83 ± 0.67	1.43 ± 0.29	both	+
LWA	5.41 ± 1.41	2.64 ± 0.68	1.39 ± 0.30	both	+
NA	2.92 ± 0.44	1.23 ± 0.05	1.22 ± 0.00	Q. crassifolia	+
LLW	4.04 ± 1.34	4.38 ± 1.14	3.06 ± 0.84	both	−
P	21.67 ± 3.86	18.42 ± 4.72	18.94 ± 3.59	Q. crassifolia	−
HW	45.84 ± 5.40	40.73 ± 4.15	39.04 ± 4.85	both	+
DW	46.43 ± 5.07	35.76 ± 3.93	30.64 ± 3.11	both	+
LL/MWL	1.68 ± 0.25	2.70 ± 0.55	3.53 ± 0.59	both	+
LLW/MWL	0.68 ± 0.22	1.42 ± 0.42	1.96 ± 0.45	both	+
Scores of intermediate characters					15:02
ESPERANZA					
LP	1.49 ± 0.64	0.73 ± 0.44	0.52 ± 0.12	Q. crassifolia	+
LL	10.59 ± 2.35	7.77 ± 2.12	5.14 ± 0.83	both	+
TLL	12.08 ± 2.54	8.50 ± 2.22	5.66 ± 0.85	both	+
MWL	6.10 ± 1.82	3.34 ± 0.92	1.85 ± 0.31	both	+
HMW	6.97 ± 1.80	3.82 ± 1.19	2.33 ± 0.50	both	+
PD	0.20 ± 0.07	0.14 ± 0.03	0.11 ± 0.02	both	+
MD	0.14 ± 0.04	0.09 ± 0.02	0.08 ± 0.02	Q. crassifolia	+
LV	15.43 ± 3.63	20.99 ± 3.28	15.60 ± 2.01	both	+
LWB	4.61 ± 0.90	2.93 ± 0.82	1.70 ± 0.30	both	+
LWA	5.66 ± 1.37	2.71 ± 0.76	1.59 ± 0.24	both	+
NA	2.91 ± 0.36	1.24 ± 0.12	1.22 ± 0.00	Q. crassifolia	+
LLW	3.62 ± 1.22	3.95 ± 1.18	2.80 ± 0.54	both	+
P	20.37 ± 4.13	16.98 ± 3.55	17.65 ± 4.13	Q. crassifolia	−
HW	49.42 ± 4.60	41.90 ± 3.39	39.87 ± 3.18	Q. crassifolia	+
DW	44.93 ± 3.43	39.06 ± 4.27	34.93 ± 1.92	both	+
LL/MWL	1.77 ± 0.23	2.36 ± 0.41	2.78 ± 0.28	both	+
LLW/MWL	0.60 ± 0.17	1.20 ± 0.25	1.52 ± 0.20	both	+
Scores of intermediate characters					16:01
AGUA BLANCA					
LP	0.71 ± 0.44	0.61 ± 0.21	0.41 ± 0.26	both	+
LL	9.01 ± 2.29	8.57 ± 2.09	6.08 ± 1.57	both	+
TLL	8.35 ± 2.03	7.96 ± 2.00	5.67 ± 1.46	both	+
MWL	4.59 ± 1.34	3.46 ± 1.93	1.95 ± 0.43	both	+
HMW	5.13 ± 1.35	4.37 ± 1.28	2.95 ± 1.09	both	+
PD	0.12 ± 0.02	0.22 ± 1.00	0.08 ± 0.01	Q. crassipes	−

TABLE 3.   Continued.

Character	*Q. crassifolia* (mean ± SD)	Hybrid (mean ± SD)	*Q. crassipes* (mean ± SD)	The hybrid is different from (Tukey, $P < 0.05$)	The hybrid is intermediate?
MD	0.09 ± 0.02	0.07 ± 0.02	0.05 ± 0.01	both	+
NV	15.32 ± 2.63	18.95 ± 3.86	21.18 ± 2.94	both	+
LWB	3.54 ± 1.03	2.85 ± 0.64	1.71 ± 0.39	both	+
LWA	4.05 ± 1.29	2.85 ± 0.74	1.65 ± 0.39	both	+
NA	2.64 ± 0.64	0.85 ± 0.39	0.71 ± 0.00	both	+
LLW	3.99 ± 2.07	4.49 ± 2.19	3.72 ± 1.13	*Q. crassipes*	−
P	17.03 ± 7.01	16.15 ± 2.79	15.34 ± 4.30	both	+
HW	51.63 ± 3.74	47.55 ± 3.25	45.73 ± 5.43	both	+
DW	47.86 ± 4.92	40.76 ± 3.80	36.2 ± 2.92	both	+
LL/MWL	1.86 ± 0.30	2.37 ± 0.29	2.91 ± 0.04	both	+
LLW/MWL	0.92 ± 1.24	1.37 ± 0.28	1.91 ± 0.40	both	+
Scores of intermediate characters					15:02
TLAXCO					
LP	1.11 ± 1.23	0.49 ± 0.20	0.56 ± 0.19	*Q. crassifolia*	−
LL	11.26 ± 3.30	7.85 ± 2.17	5.04 ± 1.16	both	+
TLL	12.37 ± 3.76	8.33 ± 2.30	5.61 ± 1.26	both	+
MWL	6.56 ± 2.01	2.72 ± 0.97	1.42 ± 0.34	both	+
HMW	7.07 ± 2.45	3.99 ± 1.37	2.39 ± 0.78	both	+
PD	0.25 ± 0.05	0.11 ± 0.03	0.08 ± 0.02	both	+
MD	0.16 ± 0.04	0.08 ± 0.02	0.06 ± 0.01	both	+
NV	14.01 ± 3.41	21.32 ± 4.07	26.73 ± 5.41	both	+
LWB	4.74 ± 1.72	2.36 ± 0.74	1.29 ± 0.32	both	+
LWA	5.72 ± 2.02	2.37 ± 0.86	1.22 ± 0.31	both	+
NA	3.29 ± 0.45	1.23 ± 0.08	1.22 ± 0.00	*Q. crassifolia*	+
LLW	4.18 ± 1.38	3.86 ± 0.98	2.65 ± 0.79	both	+
P	16.55 ± 4.51	13.85 ± 1.95	18.37 ± 2.86	both	−
HW	49.13 ± 4.96	43.40 ± 3.53	40.67 ± 5.97	both	+
DW	47.12 ± 4.39	34.78 ± 3.32	30.43 ± 3.06	both	+
LL/MWL	1.73 ± 0.21	2.96 ± 0.50	3.59 ± 0.62	both	+
LLW/MWL	0.64 ± 0.15	1.48 ± 0.34	1.90 ± 0.56	both	+
Scores of intermediate characters					15:02
ACAJETE					
LP	0.84 ± 0.68	0.58 ± 0.34	0.56 ± 0.34	*Q. crassifolia*	+
LL	9.66 ± 2.21	6.98 ± 1.76	5.89 ± 1.29	both	+
TLL	10.50 ± 2.16	7.57 ± 1.94	6.44 ± 1.37	both	+
MWL	5.47 ± 1.26	2.62 ± 0.71	1.84 ± 0.42	both	+
HMW	5.52 ± 1.45	3.63 ± 1.17	3.09 ± 0.94	both	+
PD	0.30 ± 0.15	0.12 ± 0.19	0.10 ± 0.05	both	+
MD	0.25 ± 0.19	0.08 ± 0.02	0.06 ± 0.01	*Q. crassipes*	+
NV	16.97 ± 2.39	20.21 ± 3.84	28.33 ± 4.83	both	+
LWB	4.41 ± 1.21	2.25 ± 0.60	1.57 ± 0.34	both	+
LWA	4.84 ± 1.16	2.22 ± 0.62	1.62 ± 0.37	both	+
NA	3.19 ± 0.28	1.25 ± 0.14	1.22 ± 0.00	*Q. crassifolia*	+
LLW	4.14 ± 1.33	3.35 ± 0.95	2.80 ± 0.93	both	+
P	16.65 ± 4.47	15.96 ± 2.66	16.76 ± 3.60	*Q. crassifolia*	−
HW	46.39 ± 4.28	43.59 ± 4.14	43.58 ± 4.94	*Q. crassifolia*	−
DW	46.33 ± 3.87	36.20 ± 3.72	32.45 ± 3.32	both	+
LL/MWL	1.78 ± 0.21	2.71 ± 0.41	3.25 ± 0.53	both	+
LLW/MWL	0.76 ± 0.19	1.31 ± 0.30	1.55 ± 0.47	both	+
Scores of intermediate characters					15:02
PALO BENDITO					
LP	1.20 ± 1.03	0.43 ± 0.60	0.32 ± 0.16	both	+
LL	12.56 ± 2.94	8.59 ± 1.92	6.11 ± 1.43	both	+
TLL	11.36 ± 2.75	8.16 ± 1.77	5.79 ± 1.36	both	+
MWL	6.71 ± 1.89	3.80 ± 2.63	1.88 ± 1.23	both	+
HMW	7.05 ± 2.04	4.40 ± 1.13	2.89 ± 0.92	both	+
PD	0.20 ± 0.08	0.13 ± 0.03	0.07 ± 0.01	both	+
MD	0.17 ± 0.09	0.09 ± 0.02	0.06 ± 0.03	*Q. crassifolia*	+
NV	16.65 ± 2.40	20.21 ± 6.86	24.02 ± 4.35	both	+
LWB	5.13 ± 1.32	3.12 ± 0.93	1.60 ± 0.32	both	+
LWA	6.19 ± 3.31	3.13 ± 1.01	1.61 ± 0.33	both	+
NA	2.93 ± 0.56	1.21 ± 0.25	0.71 ± 0.00	both	+
LLW	4.33 ± 1.26	3.76 ± 0.87	2.89 ± 0.72	both	+
P	18.14 ± 4.53	13.74 ± 2.63	13.49 ± 3.07	*Q. crassifolia*	+
HW	51.52 ± 4.71	47.18 ± 3.27	44.65 ± 4.80	both	+
DW	49.79 ± 5.03	49.47 ± 3.57	34.33 ± 2.87	both	+
LL/MWL	1.73 ± 0.28	2.31 ± 0.35	3.19 ± 0.48	both	+
LLW/MWL	0.67 ± 0.19	1.07 ± 0.22	1.61 ± 0.34	both	+
Scores of intermediate characters					17:00

TABLE 4. Discriminant function analysis for leaf morphology variation from seven hybrid zones of the *Quercus crassifolia* × *Q. crassipes* complex in Mexico. Abbreviations of characters are described in Table 1.

Variable	DF1	DF2
CANTERA		
LL/MWL	−0.48	1.33
LWA	0.76	0.57
LL	0.00	−2.25
PD	0.31	0.44
NV	−0.41	−0.02
HMW	1.24	1.14
MWL	−1.41	1.05
DW%	0.76	0.21
HW%	−0.33	−0.49
LWB	0.09	−0.43
LLW/MWL	0.24	−0.29
LLW	0.00	−0.06
P%	0.06	0.01
Constant	−0.82	−1.83
Eigenvalue	8.97	1.03
% Variation	86.32	13.68
Significance	<0.001	<0.001
CANALEJAS		
MWL	−0.85	1.98
NV	−0.65	0.07
LL	0.66	−2.61
LL/MWL	−0.31	0.90
LWA	0.28	0.55
DW%	0.43	−0.79
PD	0.18	0.14
LWB	0.32	0.15
MD	0.09	0.19
HMW	0.14	0.19
HW%	−0.24	−0.07
LLW/MWL	−0.24	−0.08
P%	0.00	−0.12
Constant	−0.82	−1.83
Eigenvalue	9.63	1.03
% Variation	89.96	10.04
Significance	<0.001	<0.001
TLAXCO		
NA	0.68	−0.38
DW%	1.15	0.29
TLL	1.80	3.75
MWL	−0.91	−1.61
P%	0.00	−1.11
NV	−0.39	−0.30
LL/MWL	−0.03	−0.73
HWM	0.68	−0.98
LL	−1.21	−0.94
LLW/MWL	0.50	0.09
PD	0.05	−0.19
MD	0.12	0.08
HW%	0.24	0.25
Constant	−1.66	10.20
Eigenvalue	17.53	1.93
% Variation	89.06	10.94
Significance	<0.001	<0.001
ACAJETE		
MWL	0.30	0.85
LL/MWL	0.24	1.03
LL	0.81	−2.36
NV	0.28	0.51
LWA	−0.73	0.69
PD	−0.21	0.09
LWB	−0.50	0.41
DW%	−0.47	0.23
P%	−0.31	1.49

TABLE 4. Continued.

| Variable	DF1	DF2
LP	−0.12	−0.99
MD	−0.12	−0.11
HW%	−0.82	1.18
LLW	−0.83	1.04
LLW/MWL	−0.45	0.43
Constant	0.56	0.52
Eigenvalue	6.69	0.52
% Variation	94.78	5.22
Significance	<0.001	<0.001
ESPERANZA		
LLW/MWL	1.20	1.46
NV	−0.29	−0.80
LL	1.83	−2.27
HMW	−1.00	1.28
MWL	−0.86	1.49
DW%	0.39	−1.11
LP	0.87	0.03
HW%	1.92	0.71
LL/MWL	−1.56	−0.93
MD	0.00	0.17
NA	1.83	0.80
P%	−0.72	0.70
LL	0.06	−0.91
PD	1.73	2.75
Constant	−4.98	−1.46
Eigenvalue	7.79	1.54
% Variation	81.23	18.77
Significance	<0.001	<0.001
AGUA BLANCA		
NA	0.81	0.51
LWA	0.37	0.05
LL/MWL	−0.42	1.21
LL	0.83	−4.15
MWL	−0.89	1.67
NV	−0.39	0.26
HW%	0.31	−0.78
MD	0.21	−0.06
HWL	−0.26	2.04
PD	−0.05	−0.21
LWB	0.48	0.29
P%	0.29	−0.19
LP	−0.19	0.21
DW%	0.14	−0.16
Constant	5.13	−2.07
Eigenvalue	9.86	1.32
% Variation	92.57	7.43
Significance	<0.001	<0.001
PALO BENDITO		
NA	−0.81	−0.41
LL/MWL	0.7	3.01
HW%	−0.43	−0.26
LL	−1.47	−1.79
MWL	1.32	−1.81
NV	0.26	−0.04
LP	−0.15	0.08
PD	−0.19	−0.05
LWA	−0.32	0.19
DW%	−0.70	1.04
HWL	0.68	−1.78
P%	−0.07	−0.18
LLW/MWL	−0.39	0.53
LLW	−0.17	−0.55
Constant	5.86	−4.45
Eigenvalue	13.07	0.25
% Variation	88.11	11.89
Significance	<0.001	<0.001
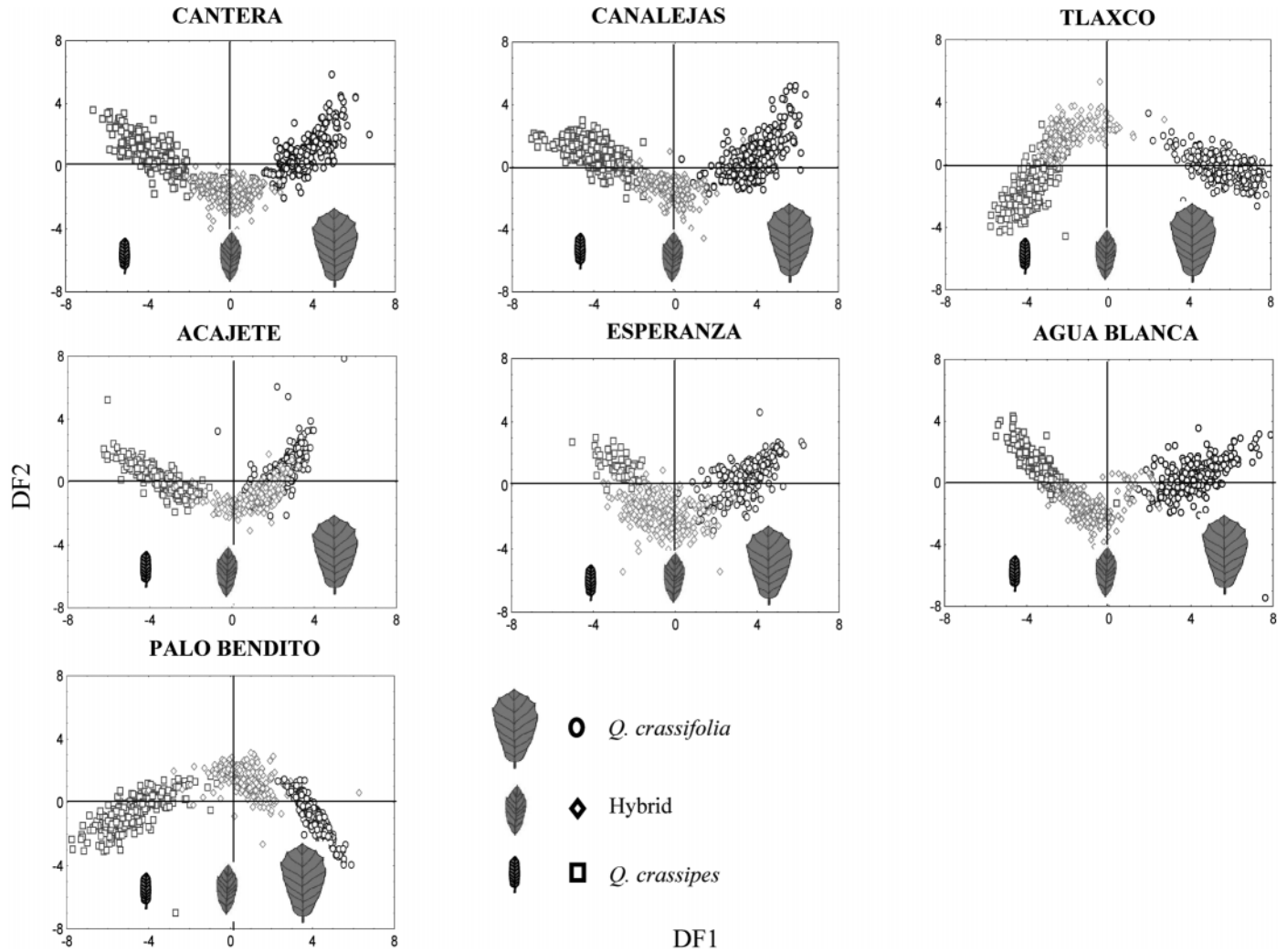
Fig. 2. Discriminant function analysis for leaf morphology variation in *Quercus crassifolia* × *Q. crassipes* complex (17 measured characters) in seven hybrids zones in Mexico. See abbreviations in Table 1.
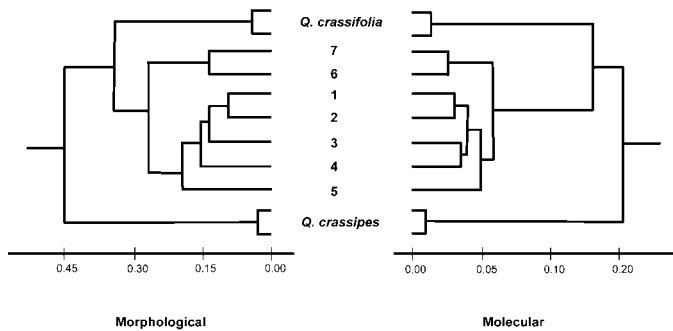

Fig. 3. Phenogram of similarity based on leaf morphology and RAPD data in seven hybrids zones of the *Quercus crassifolia* × *Q. crassipes* complex in Mexico. The mixed stands are represented by numbers, 1 = Cantera, 2 = Canalejas, 3 = Tlaxco, 4 = Acajete, 5 = Esperanza (located at the Eje Neovolcánico), 6 = Agua Blanca, 7 = Palo Bendito (located at the Sierra Madre Oriental).

located at the edges, while the seven hybrid zones were located between them (Fig. 3).

The Mantel *Z*-test matrix showed that no correlation exists between the geographic distances and the genetic distances for any of the species (*Q. crassifolia*, $r = 0.37$, $P > 0.05$; and *Q. crassipes*, $r = 0.14$, $P > 0.05$).

DISCUSSION

Oaks frequently present complex patterns of variation leading to taxonomic problems in differentiating species (Burger, 1975). Interspecific hybridization and shared ancestral polymorphisms are two of the most common explanations for the observed pattern of variation (Jensen et al., 1993; Kleinschmit et al., 1995; Bruschi et al., 2000). *Quercus crassifolia* and *Q. crassipes* form hybrids, but they remain morphologically distinct in their allopatric and sympatric distributions. All the examined morphological leaf characters in these species differed significantly by localities, populations, and individuals. Relatively few diagnostic molecular markers differentiated between *Q. crassifolia* and *Q. crassipes*. However, these markers demonstrated geographic consistency in support of the morpholog-
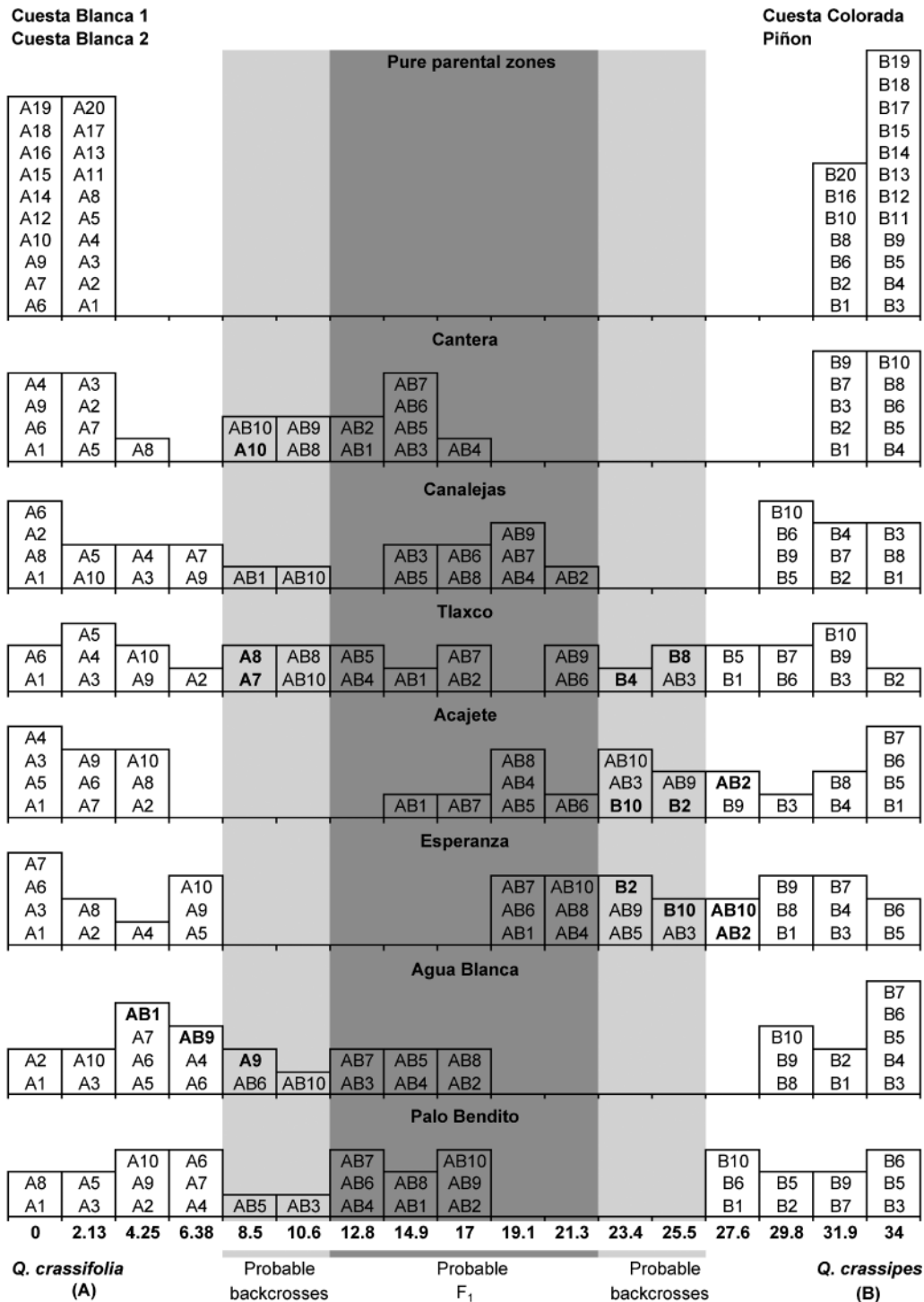
Fig. 4. Frequency distribution of individuals vs. the Anderson hybrid index derived from 17 morphological characters in two pure and seven hybrid zones. The number of each plant evaluated is represented. The figure shows the cluster results of the pure or nearly pure parentals in four zones (index = 0 or 34), a cluster for probable F_1 hybrids (index = 12.75–25.5), probable backcrosses towards *Q. crassifolia* (index = 8.5–10.63), and finally probable backcrosses toward *Q. crassipes* (index = 23.38–25.5). The plants misidentified in the field are indicated in boldface type.

ical evidence, indicating that each species is distinct and that each has some degree of genetic cohesiveness.

Furthermore, the character count procedure has allowed us to confirm statistically that *Q. dysophylla* is the result of hybridization between *Q. crassifolia* and *Q. crassipes* in the seven hybrid zones. The ordination analysis also demonstrated

that hybrids presented intermediate morphology between the parental species and that leaf shape explains a major percentage of variation.

Quercus crassifolia ranges from the Sierra Madre Occidental (SMOc) to the center of Mexico along the Eje Neovolcánico, whereas *Q. crassipes* ranges from the Sierra Madre Ori-
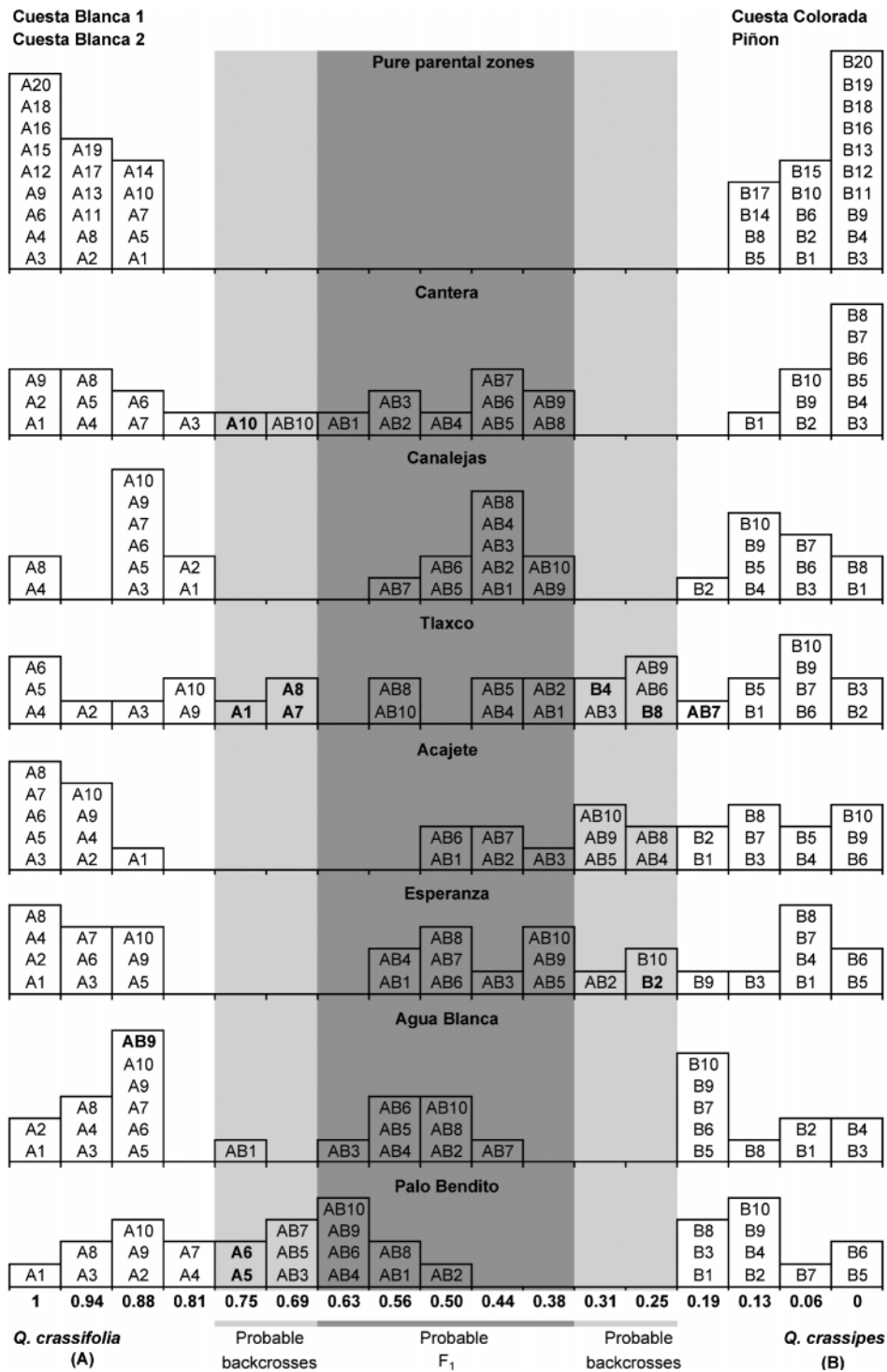
Fig. 5.   Frequency distribution of individuals vs. the Hardig hybrid index derived from the RAPD band data using six primers. The number of each plant evaluated is represented. The figure shows cluster results of the pure or nearly pure parentals in four zones (index = 0 or 1), a cluster of probable F₁ hybrids (index = 0.437–0.562), probable backcrosses toward *Q. crassifolia* (index = 0.687–0.750), and finally probable backcrosses towards *Q. crassipes* (index = 0.250–0.312). The plants misidentified in the field are indicated in boldface type.

ental (SMOr) to the Eje Neovolcánico, where both species overlap producing hybrid zones. The Eje Neovolcánico, an orographic system that traverses the central part of the country in an east–west direction, is considered geologically the youngest mountain range in Mexico and contains valleys higher

than 2000 m in altitude and the tallest mountains in Mexico (Ferrusquia-Villafranca, 1993). Phylogeographic studies are in progress to understand the process of migration and the colonization routes of this oak complex, as has been done for other oak species (e.g., Dumolin-Lapègue et al., 1997).

The genetic results indicate that the introgression process is present in both species, but the direction changes depending on the localization of the hybrid zone. The hybrid zones closest to the SMOc (Cantera and Canalejas) registered unidirectional introgression towards *Q. crassifolia*, while the hybrids from the intermediate locality between the two mountain ranges (Tlaxco) showed bidirectional introgression, and the hybrids from the two closest localities to the SMOr registered unidirectional introgression towards *Q. crassipes*. These findings suggest that the closeness of hybrids to an allopatric site of either parental species is directly related to their similarity and vice versa. Thus, the Eje Neovolcánico acts as a corridor where the proximity to an allopatric site favors the introgression of the hybrid towards the parental species, increasing its variation from the species with which it is maintaining a genetic exchange, diluting the limits with the parental species in the allopatric site. Lastly, the two hybrid zones located north of Tlaxco (SMOr) showed unidirectional introgression towards *Q. crassifolia*. These results confirm that patterns of variation in oaks do not follow simple monotonic clines (e.g., Barton and Hewit, 1985) but form complex mosaic zones characterized by patches of pure populations and mixed populations scattered across a zone of overlap (Howard et al., 1997). A bidirectional hybrid zone was detected for *Q. crassifolia* and *Q. crassipes*.

In the seven hybrid zones studied along the Eje Neovolcánico, where intermediate plants are mixed with their parental species, hybrids are rare and they are in a narrow contact zone between well-differentiated taxa. The presence of hybrid individuals in the hybrid zones was very low (between 10 and 17 trees), requiring an extensive field search. Oak hybrids are produced in an isolated and sporadic manner and they may introgress with parental species (Bacon and Spellenberg, 1996). Hybrid zones with high levels of disturbance (i.e., Canalejas, Acajete, and Esperanza) were the ones with the highest number of hybrid individuals (mostly juveniles). Disturbances produced by human activities such as logging, deforestation, fires, and agriculture, may enhance the establishment of hybrids as they modify reproductive barriers (Arnold et al., 1990; Klier et al., 1991).

Our results suggest that the sympatric zones of *Q. crassifolia* and *Q. crassipes* are mosaic hybrid zones as proposed by Howard (1982, 1986) and Harrison (1986, 1990), because of the patchy distribution pattern of the parental species in sympatric and allopatric sites and the lack of a gradual transition from *Q. crassipes* to *Q. crassifolia*. It is important to mention that hybrid plants were less frequent than putative parents in the mixed stands and that *Q. crassipes* prefers drier habitats and lower sites than *Q. crassifolia*. Ecological divergence rather than genetic incompatibility may maintain hybrid zones (Jiggins and Mallet, 2000) by causing local adaptations to different environmental conditions (e.g., Howard et al., 1997).

In summary, we found that molecular markers (RAPD) and morphological leaf traits are highly coincident and support the phenomenon of hybridization between *Q. crassifolia* and *Q. crassipes* complex (Fig. 3). Inasmuch as hybridization was evident, both species remain distinct in mixed stands. We also observed that the Eje Neovolcánico acts as a corridor where proximity to an allopatric site favors the introgression of the hybrid with its parental species, increasing its divergence from the species with which it maintains a genetic exchange, and thus diluting the limits with parental species in the allopatric

site. Hybrid plants constitute a heterogeneous group in which many individuals were F_1 and others appeared as backcrosses of *Q. crassifolia* or *Q. crassipes*, depending on the locality.

Our data and field observations suggest that the sympatric zones of *Q. crassifolia* and *Q. crassipes* must be considered as mosaic hybrid zones (e.g., Howard, 1982, 1986; Harrison, 1986, 1990), because of the patchy distribution pattern of the parental species in sympatric and allopatric sites, and there is not a gradual transition from *Q. crassipes* to *Q. crassifolia*. Finally, we suggest that *Q. dysophylla* does not deserve the status of species but it must be recognized as an entity of potential evolutionary importance, named as *Quercus* × *dysophylla* Benth. pro sp.

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