Genetic variation and differentiation of populations within the *Quercus affinis* – *Quercus laurina* (Fagaceae) complex analyzed with RAPD markers

Antonio González-Rodríguez, Dulce M. Arias, and Ken Oyama

Abstract: The population genetics of two hybridizing Mexican red oaks, *Quercus affinis* Schweid. and *Quercus laurina* Humb. & Bonpl., was investigated with 54 randomly amplified polymorphic DNA (RAPD) markers scored in 415 individuals from 16 populations representing the distribution area of the two species and a probable secondary hybrid zone. Genetic relationships among populations, depicted in a unweighted pair group method with arithmetic averaging (UPGMA) dendrogram, were largely incongruent with the morphological classification of populations as *Q. affinis*-like or *Q. laurina*-like that was obtained in previous studies. In contrast, the two main population clusters in the UPGMA dendrogram corresponded to the location of populations in two distinct geographical areas: southwestern and northeastern. A Mantel test confirmed a significant association between geographic and genetic distances among populations. Analyses of molecular variance (AMOVA) indicated that most genetic variation is contained within populations (84%), while 10.5% (*P* < 0.0001) is among populations, and 5.1% (*P* = 0.007) is between the two morphological groups. Differentiation between the southwestern and northeastern geographical groups (as recognized by the UPGMA), was 7.8% (*P* < 0.0001). The incongruence between genetic and phenotypic patterns suggests that introgression of neutral markers has been considerable between the two species in the hybrid zone, while morphological differentiation has remained comparatively stable.

Key words: hybridization, population genetics, *Quercus*, RAPD markers.

Résumé : Les auteurs ont étudié la génétique des populations chez deux chênes rouges capables d’hybridation, le *Quercus affinis* Schweid. et le *Quercus laurina* Humb. & Bonpl. À cette fin, ils ont utilisé 54 marqueurs ADN polymorphiques aléatoires repérés chez 415 individus, appartenant à 16 populations représentatives de l’aire de distribution des deux espèces, ainsi qu’à une zone probable d’hybridation secondaire. Les relations génétiques entre les populations, décrites par une méthode de groupes de paires non pondérées avec dendrogramme basé sur la moyenne mathématique (UPGMA), ne concordent pas avec la classification morphologique des populations, telle qu’obtenue pour les types *Q. affinis* et *Q. laurina* des études précédentes. Au contraire, les regroupements des deux populations principales du dendrogramme UPGMA correspondent à la localisation des populations dans deux zones géographiquement distinctes : sud-ouest et nord-est. Un test de Mantel confirme une association significative des distances géographiques et génétiques, entre les populations. Les analyses de variance moléculaire (AMOVA) indiquent que la majeure partie de la variation génétique se retrouve dans la population (84 %), alors qu’elle est de 10,5 % (*P* < 0.0001) entre les populations, et de 5,1 % (*P* < 0.007) entre les deux groupes morphologiques. La différenciation entre les groupes géographiques, sud-ouest et nord-est (tel qu’indiquée par l’UPGMA), est de 7,8 % (*P* < 0.0001). Le manque de correspondance entre les patrons génétiques et géographiques suggère que l’introgression de marqueurs neutres a été considérable entre les deux espèces dans la zone d’hybridation, alors que la différenciation morphologique est demeurée relativement stable.

Mots clés : hybridation, génétique des populations, *Quercus*, marqueurs RAPD.

[Traduit par la Rédaction]

Introduction

Population genetics studies of species in genus *Quercus* L. (the oaks) using neutral markers of nuclear origin have shown that, as with other highly outcrossing, wind-pollinated, and long-lived forest tree species, oaks generally have high levels of within-population genetic variation and low differentiation among populations (Hokanson et al. 2005).
in the genetic composition of populations from one species to the other along the macrogeographic gradient was observed, with genetically intermediate populations situated in the area of overlap (González-Rodríguez et al. 2004a). Foliar variation was also continuous between the two species, but only a comparatively small fraction of the individuals was intermediate, and a particular morphology predominated in most populations (i.e., *Q. affinis*-like or *Q. laurina*-like individuals). The observed patterns were interpreted as consistent with the originally proposed hypothesis (Valencia 1994) of an origin for the area of intergradation through secondary contact between the two oak species and subsequent hybridization and introgression.

The previously described results have provided insight into the probable origin and structure of the hybrid zone between *Q. affinis* and *Q. laurina*. In this study, we used a larger, random sample of molecular markers constituted by 54 RAPD bands (loci) to estimate genetic diversity in these oaks and to analyze genetic differentiation among populations, and between morphologically defined groups of populations representing the two species (i.e., *Q. affinis*-like and *Q. laurina*-like populations). We also examined the pattern of genetic relationships among populations and determined how this pattern is related to the morphological classification of populations and (or) to the geographic distances separating them.

### Materials and methods

#### Plant material

Sixteen populations of the *Q. affinis*–*Q. laurina* complex were sampled throughout the geographic distribution of both species. Figure 1 illustrates the location of the sampled populations and the three main mountain ranges where oaks of this complex are distributed. Morphologically representative populations of *Q. affinis* are distributed along the Sierra Madre Oriental, while morphologically representative populations of *Q. laurina* occur in the Sierra Madre del Sur and the western region of the Trans-Mexican Volcanic Belt (Valencia 1994). The area of overlap is situated in the eastern region of the volcanic belt. At each site, young, intact leaves were collected from randomly chosen adult trees separated from each other by at least 100 m to avoid sampling related individuals. The leaves were immediately frozen in liquid nitrogen and then transferred to a −80 °C freezer until further analyses were performed. Sample size per location varied between 20 and 30 trees (Table 1). Each population was classified as "*Q. affinis*-like" or "*Q. laurina*-like" according to the predominant morphological type in the locality, determined by morphological analyses (González-Rodríguez et al. 2004a; González-Rodríguez and Oyama 2005).

#### DNA extraction and PCR conditions

Approximately 100 mg of frozen leaf tissue were used for DNA extraction following the protocol of Lefort and Douglas (1999) with only minor modifications. The concentration of DNA in solution was estimated using a DNA fluorometer (Hoefer Pharmacia Biotech, San Francisco, California).

All amplification reactions contained 10 ng of template DNA, 1× PCR buffer, 2 mmol/L MgCl₂, 0.1 mmol/L each
dNTP (Fermentas, Hanover, Maryland), 0.2 µmol/L of a single 10-mer oligonucleotide (Operon Technologies, Alameda, California.), 5 µg bovine serum albumin, and 1 U Taq DNA polymerase (Invitrogen, San Diego, California) in a total volume of 25 µL. The thermal cycling program was run on a MJ Research (Waterton, Massachusetts) thermal cycler. The program was as follows: 1 cycle of 2 min at 94 °C; followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C. A final extension step at 72 °C for 7 min was included.

Polymerase chain reaction (PCR) products were electrophoresed at 200 V for 2 h in 1.5% agarose gels with 0.5× Tris–borate–EDTA buffer and photographed under UV light after staining with ethidium bromide. A 123–bp ladder

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**Table 1.** Name, geographic coordinates, morphological group, sample size (N), percentage of polymorphic loci (%P), and Shannon diversity index (S$_S$) for 16 populations within the *Quercus affinis* – *Quercus laurina* complex.

<table>
<thead>
<tr>
<th>Population number and name</th>
<th>Latitude, longitude</th>
<th>Morphological group</th>
<th>N</th>
<th>%P</th>
<th>S$_S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tequila</td>
<td>20°50’N, 103°48’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>23</td>
<td>48.15</td>
<td>0.218</td>
</tr>
<tr>
<td>2. Mil Cumbres</td>
<td>19°40’N, 100°55’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>25</td>
<td>51.85</td>
<td>0.239</td>
</tr>
<tr>
<td>3. Cuernavaca</td>
<td>19°05’N, 99°15’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>24</td>
<td>50</td>
<td>0.195</td>
</tr>
<tr>
<td>4. Santa Inés</td>
<td>17°03’N, 96°55’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>24</td>
<td>50</td>
<td>0.223</td>
</tr>
<tr>
<td>5. Amealco</td>
<td>20°10’N, 100°20’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>27</td>
<td>53.7</td>
<td>0.244</td>
</tr>
<tr>
<td>6. Ozumba</td>
<td>19°05’N, 98°42’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>29</td>
<td>57.41</td>
<td>0.252</td>
</tr>
<tr>
<td>7. Llano de Flores</td>
<td>17°30’N, 96°30’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>20</td>
<td>42.59</td>
<td>0.219</td>
</tr>
<tr>
<td>8. Pinal de Amoles</td>
<td>21°01’N, 99°40’W</td>
<td><em>Quercus affinis</em>-like</td>
<td>22</td>
<td>46.3</td>
<td>0.204</td>
</tr>
<tr>
<td>9. Puerto Aire</td>
<td>18°45’N, 97°30’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>30</td>
<td>64.81</td>
<td>0.284</td>
</tr>
<tr>
<td>10. Jacala</td>
<td>20°50’N, 99°05’W</td>
<td><em>Quercus affinis</em>-like</td>
<td>27</td>
<td>59.26</td>
<td>0.255</td>
</tr>
<tr>
<td>11. Real del Monte</td>
<td>20°05’N, 98°40’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>28</td>
<td>57.41</td>
<td>0.229</td>
</tr>
<tr>
<td>12. Cerro Navajas</td>
<td>20°12’N, 98°30’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>30</td>
<td>64.81</td>
<td>0.285</td>
</tr>
<tr>
<td>13. Zembo</td>
<td>19°15’N, 98°32’W</td>
<td><em>Quercus laurina</em>-like</td>
<td>29</td>
<td>62.96</td>
<td>0.277</td>
</tr>
<tr>
<td>14. Jalacingo</td>
<td>19°30’N, 97°15’W</td>
<td><em>Quercus affinis</em>-like</td>
<td>28</td>
<td>61.11</td>
<td>0.293</td>
</tr>
<tr>
<td>15. Zacapoaxtla</td>
<td>19°50’N, 97°40’W</td>
<td><em>Quercus affinis</em>-like</td>
<td>20</td>
<td>46.3</td>
<td>0.221</td>
</tr>
<tr>
<td>16. Zacualtipán</td>
<td>20°39’N, 98°40’W</td>
<td><em>Quercus affinis</em>-like</td>
<td>29</td>
<td>62.96</td>
<td>0.273</td>
</tr>
</tbody>
</table>
(Invitrogen, San Diego, California) was included in all gels as a reference to estimate the size of the amplified fragments.

To assess the reproducibility of PCR products, the DNA from 38 individuals was first amplified independently three times with each primer. A total of 54 bands (loci) appeared consistently in the three assays and were later scored in all individuals. These 54 fragments included the nine semi-diagnostic markers (produced by seven primers: OP-A05, OP-A07, OP-A08, OP-A10, OPB-17, OP-C09, and OP-I07) of the two species used in the previous study (González-Rodríguez et al. 2004a) plus another 45 bands amplified simultaneously by the same seven primers, and two additional primers (OP-D10, OP-G19). Only a few of the fragments showed an association with chloroplast DNA variation assessed in the same individuals (González-Rodríguez et al. 2004b), and the association was in all cases very weak. Thus, all the fragments were assumed to be of nuclear origin.

Data analysis

Amplified fragments were recorded as absent (0) or present (1) in all individuals. For each fragment, these two possible states were considered as the molecular phenotypes resulting from the expression of two alleles at a single locus, one dominant and one recessive, the dominant being the one that determines the presence of the band. Although the frequency of the two alleles at each locus can be inferred from the frequency of presence and absence of the band (e.g., Lynch and Milligan 1994), analyses do not rely on knowing these frequencies were preferred in this study to avoid the uncertain assumption of Hardy–Weinberg equilibrium. Molecular diversity within each population was assessed by calculating the percentage of polymorphic fragments (%P) and the Shannon diversity index (Sh) using POPGENE version 1.31 (Yeh et al. 1999). The Shannon index was also calculated at the level of the total population (Sh), and from this value and the average of Sh over all populations, the proportion of the total genetic variation found among populations was calculated as 1 – (average Sh / Sh) (Martin and Hernández-Bermejo 2000). To identify possible differences in population levels of genetic diversity between morphological groups and geographic areas, values of %P and Sh were compared using a Wilcoxon test (Sokal and Rohlf 1995).

To determine genetic relationships among populations, a matrix of pairwise Manhattan distances (this distance is based on the frequency of absence or presence of bands and does not assume allelic frequencies; Swofford et al. 1996) was generated with the RAPD program (Black 1995), and a dendrogram based on this matrix was constructed with the NEIGHBOR procedure in the PHYLIP 3.5C package (Felsenstein 1993), using the unweighted pair group method with arithmetic averaging (UPGMA).

The influence of spatial separation on the degree of differentiation among populations was investigated with a Mantel permutation test (Mantel 1967). Because in our case a spurious correlation between geographic and genetic distances could potentially arise from the fact that populations from the same morphological group are expected to be genetically more similar and also tend to occur in similar geographical areas, the partial version of the Mantel test was used (Smouse et al. 1986). In this version, the goal is to test the correlation between two matrices while controlling the effect of a third matrix, to remove possibly confounding effects (Smouse et al. 1986). This test was performed between the matrices of pairwise Manhattan distances and the corresponding geographical distances, controlling the effect of a third matrix indicating the morphological classification of the populations. The elements of this third matrix were 1 for pairs of populations belonging to the same morphological group, or 2 for pairs of populations belonging to interspecific groups.

Analysis of molecular variance (AMOVA) was used to investigate the partitioning of genetic variation among groups of populations, among populations within groups, and within populations. The significances of the different variance components were estimated from distributions generated from 10 000 random permutations. These analyses were carried out using ARLEQUIN version 2000 (Schneider et al. 2000).

Results

Fifty-one (94.44%) out of 54 amplified fragments were polymorphic within or between populations (Table 1). In single populations, the percentage of polymorphic fragments (%P) ranged from 42.59% to 64.81% with an average of 54.98%. The Shannon diversity index within populations (Sh) varied between 0.195 and 0.293, with an average (SD) of 0.244 (0.031). The overall value of the Shannon index (Sh), when considering the whole sample as a single population, was 0.299 (Table 1). From these values, the proportion of the total genetic variation residing among populations (1 – (average Sh / Sh)) was 0.184.

The UPGMA dendrogram generated from the matrix of pairwise Manhattan distances is shown in Fig. 2. Two main groups of populations were defined in this dendrogram, which clearly corresponded to two distinct geographical areas; one group was formed by populations situated in the Sierra Madre del Sur and the western region of the volcanic belt (southern group), while the other clustered populations from the eastern region of the volcanic belt and the Sierra Madre Oriental (northeastern group). Within these two main groups there was also some tendency for geographically proximate populations to cluster together (Figs. 1 and 2). This pattern was confirmed by the partial Mantel test, which detected a significant correlation between the matrix of pairwise genetic distances and the matrix of corresponding geographical distances, even when controlling for the taxonomic grouping of populations (r = 0.341, P = 0.01). In contrast, the genetic relationships among populations, as depicted in the UPGMA dendrogram, were largely non-congruent with their morphological classification. For example, the southwestern group contained seven Q. laurina-like populations and one Q. affinis-like population, and the northeastern cluster contained four populations from each morphological group (Fig. 2, Table 1). Nevertheless, it might be significant that three of the four Q. laurina-like populations included in the northeastern group (Real del Monte,
Cerro Navajas, and Puerto Aire) clustered together and separated from the other five populations of this group (Fig. 2).

The AMOVA performed over all 16 populations for partitioning of RAPD variation between the two main morphological groups (\textit{Q. affinis}-like and \textit{Q. laurina}-like groups), among populations within these two groups, and among individuals within populations revealed that most of the variation (84.4\%) is found within populations. Differences between the two population groups (5.07\%, \( P = 0.007 \)) and among populations within groups (10.49\%, \( P < 0.0001 \)) accounted for comparatively smaller amounts of the total variance, although both effects were significant. The overall differentiation among populations (\( \Phi_{ST} \)) was 0.16. A second AMOVA analysis, conducted for populations arranged according to the two main groups defined in the UPGMA dendrogram that are concordant with geographic location (i.e., southwestern and northeastern population groups), indicated that 7.85\% of the variation was distributed between the two groups (\( P < 0.0001 \)), 8.85\% of the variation was among populations within groups (\( P < 0.0001 \)), and 83.3\% was among individuals within populations. Within the northeastern group, differentiation between the cluster formed by the three populations Real del Monte, Puerto Aire, and Cerro navajas and the other five populations was 2.88\% (\( P = 0.017 \)).

A one-tailed Wilcoxon two-sample test was used to compare levels of within-population genetic diversity between the \textit{Q. affinis}-like and \textit{Q. laurina}-like groups of populations, as well as between the southwestern and northeastern groups. These comparisons revealed significantly higher levels of genetic diversity in populations of the northwestern group for both the proportion of polymorphic loci (\( Z = -2.635, P = 0.008 \)) and the Shannon index (\( Z = -2.626, P = 0.009 \)) (Table 1). In the case of the geographic groups, the populations of the northeastern group were the ones characterized by higher genetic diversity (Table 1). In contrast, genetic diversity levels were equivalent between the morphological groups (\( Z = -0.114, P = 0.913 \) for the proportion of polymorphic loci; \( Z = -0.397, P = 0.743 \) for the Shannon index).

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Discussion

The distribution patterns of putatively neutral nuclear genetic variation within and between species in the *Quercus affinis* – *Q. laurina* complex are similar to those described for other oak species. A large proportion of the total genetic variation is found within populations of these two Mexican red oaks. Although most of the previous studies reporting this result in oak species, as well as in other trees with similar life history traits (Hamrick et al. 1992), have been conducted using allozymes, the few analyses using RAPD markers have found that both methods usually provide comparable values of population subdivision (Le Corre et al. 1997; Nybom and Bartish 2000). The two estimates of overall genetic differentiation among populations of the *Q. affinis* – *Q. laurina* complex obtained in this study with different analytical methods were \((1 - (\text{average } S_{ST} / S_{T})) = 0.18\) (using the Shannon diversity index) and \(F_{ST} = 0.16\) (from AMOVA). The AMOVA further revealed that this \(F_{ST}\) can be significantly partitioned into a component of differentiation between *Q. affinis*-like and *Q. laurina*-like populations (0.05) and a component of average differentiation among populations within these two groups (0.105). Although it is necessary to be careful when comparing results across studies because differences in sampling strategies, geographic scale considered, markers employed, and analytical procedures are customary, the values of interspecific differentiation and population differentiation within *Q. affinis* and *Q. laurina* obtained in this study seem to fit within the general literature on oak population genetics. For example, the overall interspecific differentiation among the four California species of red oak estimated with AMOVA from AFLP data (with dominant expression as RAPD markers) was 0.087 (Dodd and Kashani 2003). Coart et al. (2002) estimated allelic frequencies at AFLP loci and obtained a \(F_{ST}\) value of 0.073 for the differentiation between *Quercus robur* and *Quercus petraea*. In another study on these two species, which used codominant allozyme markers and considered a very different geographic area, an interspecific \(F_{ST}\) of 0.021 was estimated (Gömöry et al. 2001). Intraspecific population differentiation in oak species has been estimated at 0.11 (Hamrick et al. 1992, based on data from 28 studies) and 0.07 (Kremer and Petit 1993, based on data from 25 studies), although it should be noticed that these values come almost exclusively from allozyme markers. These means vary a great deal among studies, ranging from population differentiation values as low as 0.018 in *Quercus chrysolepis* (Montalvo et al. 1997) and 0.02–0.03 in *Q. petraea* and *Q. robur* (Zanetto and Kremer 1995; Le Corre et al. 1997; Coart et al. 2002) to the remarkably high values recently reported for three of the four species of California red oak, *Quercus wislizeni* (0.18), *Quercus kelloggii* (0.34), and *Quercus agrifolia* (0.24), although the authors of this last study cautioned about the reliability of the estimates for the last two species because of limited sampling (Dodd and Kashani 2003).

Our results also suggest that the subdivision of genetic variation in the *Q. affinis* – *Q. laurina* complex may be more strongly related to geography than to the morphologically defined classification of populations. In an UPGMA dendrogram based on Manhattan distances (Fig. 2), the genetic relationships among our sampled populations appeared to be more closely associated with geographic location than with taxonomic grouping. This correspondence between geography and the distribution of genetic variation was further indicated by the partial Mantel test, which detected a significant correlation between geographic and genetic distances, independently of the morphological grouping of populations. According to a second AMOVA, the degree of differentiation between the two main population groups defined in the dendrogram (i.e., southwestern and northeastern population groups) was 0.078. This value is higher than the amount of genetic variation that differentiated the morphologically defined *Q. affinis*-like and *Q. laurina*-like population groups (0.05), although this comparison should be taken with caution because the two values are not completely independent since there is some degree of overlap between the geographic and the morphological groups analyzed in the two AMOVAs. Another observation supporting that the southwestern and northeastern groups represent an actual genetic subdivision between populations was the detection of significant differences between the two groups in within population diversity levels, as measured by the proportion of polymorphic loci (%P) and the Shannon diversity index (\(S_{E}\)). In contrast, no differences in genetic variation were found between the *Q. affinis*-like and *Q. laurina*-like groups.

On the other hand, there is also some evidence of the taxonomic effect in the clustering pattern in the UPGMA dendrogram. This is particularly illustrated by the three *Q. laurina*-like populations (Real del Monte, Cerro Navajas, and Puerto Aire) that belong to the northeastern group but formed a clearly separated subcluster, indicating genetic affinity among them and a degree of differentiation from the other five populations within this group.

In conclusion, although the two factors are difficult to separate in some analyses, it appears that the distribution of nuclear genetic variation among populations in the *Q. affinis* – *Q. laurina* complex is firstly a function of geography and secondly, but also significantly, a reflection of the morphologically based taxonomic subdivision of populations. This result implies that gene flow and isolation by distance are the predominant forces shaping the population structure of neutral, nuclear genetic variation within this complex. According to previous work (Valencia 1994; Gonzalez-Rodriguez et al. 2004a), a hybrid zone between previously diverged *Q. affinis* and *Q. laurina* putatively formed after secondary contact in the eastern region of the volcanic belt. As was originally proposed by Valencia (1994), *Q. laurina* populations probably migrated along the volcanic belt in an eastward direction and established within the area already occupied by populations of *Q. affinis*. After this happened, the weak reproductive barriers between the two oak species permitted interspecific gene flow that resulted in the bidirectional genetic introgression of both species in this area. The increased levels of genetic diversity in populations of the northeastern group are consistent with this scenario. During the formation of the hybrid zone, geographical distances, according to a pattern of isolation-by-distance, largely influenced genetic relationships among populations. However, the existence of the weaker taxonomic effect suggests that the amount of interspecific genetic exchange may be constrained to some extent in com-
parison to intraspecific gene flow or that the time since secondary contact has been insufficient to erode genetic differentiation completely and homogenize populations.

The incongruence between genetic and morphological relationships among populations suggests that interspecific genetic exchange has affected morphological differentiation between *Q. affinis* and *Q. laurina* to a lesser extent. In contrast to what was observed with genetic distances, morphological similarities among populations did not show any association with the geographical distances separating them (González-Rodríguez and Oyama 2005). Although there are several possible explanations for this incongruence between molecular and phenotypic patterns (Rieseberg and Ellstrand 1993), it seems likely that if foliar morphology has experienced restricted introgression despite interspecific gene flow and exchange of neutral markers, it is probably because selective factors are operating against the recombination of genomic regions controlling adaptively relevant traits, indicating a semipermeable species barrier (Arnold 1992).

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