Micromorphological character expression of the hybrid *Quercus* × *dysophylla* and its parental species (*Q. crassifolia* and *Q. crassipes*)

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Abstract

Background: Hybridization leads to individuals with atypical phenotypes, leading to taxonomic confusion when hybridizing populations are studied. Micromorphological characters may enable taxonomists to discriminate between parental and hybrid categories due to their simple genetic inheritance.
Species study: Three oak taxa distributed in the montane regions of the Mexico were studied: *Quercus crassifolia*, *Q. crassipes* and their hybrid *Q.* × *dysophylla*.
Hypothesis: We describe the leaf micromorphological and macromorphological variation of these taxa. Specifically, we searched for a unique combination of micromorphological characters in hybrids. We hypothesize that spatial micromorphological variation will match the spatial pattern revealed by a previous genetic study.
Study sites: Two allopatric stands for each parental species and seven hybrid zones were studied. Individuals within each stand were identified as belonging to parental or hybrid categories following previous genetic analyses.
Methods: Stomata and trichome types for each taxa were determined through Scanning Electron Microscopy. Eight micromorphological characters for trichomes and stomata and four foliar macromorphological characters were measured.
Results: We found the presence of both multirradiate and simple stellate non-glandular trichomes as a diagnostic feature of *Q.* × *dysophylla*. Overall populations, *Q.* × *dysophylla* showed intermediate phenotypes in 70 % of morphological characters. However, hybrid phenotype exhibited geographical variation. Lastly, spatial hybrid's phenotype variation did not correlate with the spatial genetic pattern previously identified.
Conclusions: The micromorphological features of *Q.* × *dysophylla* may enable taxonomists to accurately discriminate between this taxon and its parental species. Finally, we suggest that micromorphological expression of both parental species and hybrids may be influenced by environmental gradients and microclimates.
Keywords: hybrid phenotype, morphometrics, stomata, trichome.

Resumen

Antecedentes: La hibridación resulta en individuos con morfologías atípica, lo cual lleva a confusiones taxonómica al estudiar poblaciones en las que ocurre hibridación. Los caracteres micromorfológicos pueden ser útiles para diferenciar entre individuos híbridos y parentales.
Especies de estudio: Se estudiaron tres taxones de encinos: *Quercus crassifolia*, *Q. crassipes* y *Q.* × *dysophylla*, el híbrido de estas especies.
Hipótesis: Se describe la expresión de caracteres micromorfológicos y macromorfológicos de estos taxones. En particular, se buscó una combinación única de caracteres micromorfológicos en los híbridos. Asimismo, esperamos que la variación espacial fenotípica sea similar a la encontrada en un estudio genético previo.
Sitio de estudio: Se estudiaron dos poblaciones alopátridas para cada especie parental y siete zonas híbridas. Los individuos fueron considerados como parentales o híbridos de acuerdo al estudio genético previo.
Métodos: Los tipos de estomas y tricomas para cada taxón fueron identificados mediante Micoscopía Electrónica de Barrido. Además, se midieron ocho caracteres micromorfológicos de estomas y tricomas y cuatro caracteres macromorfológicos foliares.
Resultados: Se encontró que la presencia de tricomas multirradiados y estrellados no glandulares son un carácter diagnóstico de *Q.* × *dysophylla*. Asimismo, *Q.* × *dysophylla* mostró un fenotipo intermedio en el 70 % de los caracteres morfológicos. Sin embargo, el fenotipo híbrido mostró variación geográfica. El patrón espacial de la variación morfológica no mostró relación con el patrón genético encontrado previamente.
Conclusiones: Las características micromorfológicas de *Q.* × *dysophylla* pueden ser de utilidad para los taxónomos con el fin de discriminar entre categorías híbridas y parentales. Se sugiere que la expresión micromorfológica puede estar influenciada por gradientes ambientales.
Palabras clave: fenotipo híbrido, morfometría, estoma, tricoma.

Genetic exchange between two differentiated genomes is considered a major source of variation with an important role in bacterial, animal, fungal and plant evolution (Arnold *et al*. 2008). In particular plant hybridization may lead to the origin of new species, an increase of intraspecific genetic diversity, species extinction, among others (Rieseberg & Carney 1998, Arnold 2006). However, besides its importance in evolution, plant hybridization is challenging for taxonomist when it leads to atypical or morphologically intermediate individuals. Atypical individuals are sometimes described as a new species (*e.g*., Romero 1993), and in extreme cases, the presence of morphologically intermediate hybrids causes the merging of two taxa into a single species (López-Caamal *et al*. 2014). However, even when hybridization is known to occur between a species pair, the boundaries between parental species are blurred, creating difficulties when assigning individuals into parental or hybrid categories.

Hybrid identification relies nowadays in neutral molecular markers. The main advantage of molecular markers is that their analysis is straightforward, revealing with great accuracy the ancestry of hybrids and identifying the potential outcomes of hybridization (Hohenlohe *et al*. 2011). However, botanists rely on morphological characters to discriminate between hybrid and parental individuals under field conditions. In this regard, identifying diagnostic morphological characters of hybridizing species would be of great value for taxonomists.

The morphological expression of hybrids is complex (Rieseberg *et al*. 1999). Hybrids may express intermediate characters if under polygenic control, transgressive character expression when complementary gene action occurs, or parental character expression if the character is under the control of one or few genes with complete dominance/recessivity. Given the complexity of hybrid phenotypic expression, morphological markers are rarely used nowadays as the unique tool for hybrid recognition. These markers are used along with molecular or cytological markers to make robust hypothesis of hybridization (López-Caamal & Tovar-Sánchez 2014). However, morphometric studies are important as they reveal diagnostic markers for both parental and hybrid individuals and may give insights of the ecological performance of hybrids (López-Caamal & Tovar-Sánchez 2014).

Natural hybridization between oaks (*Quercus* L., Fagaceae) is frequent. Hybrid zones have been and are continuously being detected in North America (González-Rodríguez *et al*. 2004, Peñaloza-Ramírez *et al*. 2010, Valencia-Cuevas *et al*. 2014), Europe (Muir & Schlötterer 2005) and Asia (Lee *et al*. 2014, Song *et al*. 2015). The involved oak species usually show high levels of gene flow, generating complex patterns of morphological expression within hybrid zones leading to taxonomic confusion. Most oak hybridization studies use a combination of morphological and molecular markers (Tovar-Sánchez & Oyama 2004, González-Rodríguez *et al*. 2004, Song *et al*. 2015). In particular, leaf morphological characters are thoroughly used in these studies, usually supporting the hybridization hypothesis when used along with molecular markers. Leaf micromorphological characters (leaf trichomes, trichome density, stomata type, stomata density) are informative and usually exhibit unique combinations in oak hybrids (Scareli-Santos *et al*. 2007, Scareli-Santos *et al*. 2013, Fortini *et al*. 2015). If these characters are found, they may enable taxonomists to easily differentiate between parental and hybrid types.

Oaks exhibit a high diversity in Mexico. Indeed, the country is considered a center of diversification of the genus with a high proportion of endemic species (Valencia-A. 2004). Mexican oaks have been intensively studied in recent years. Hybrid zones have been found between several *Quercus* species pairs (Tovar-Sánchez & Oyama 2004) and some authors have explored hybridization rates between three or more species of *Quercus* (Peñaloza-Ramírez *et al*. 2010, Valencia-Cuevas *et al*. 2014). Also, phylogeographical analyses have revealed a complex history of hybridization and introgression between oak species (González-Rodríguez *et al*. 2004, Tovar-Sánchez *et al*. 2008). Despite the high number of studies, the genus *Quercus* is still considered a "difficult" group for taxonomists. The recognition of parental species and their hybrids in the wild is usually difficult due to the lack of clearly diagnostic leaf morphological markers. However, leaf micro morphological markers arise as a useful tool to discriminate between hybrid and parental categories due to their usually intermediate pattern of expression in hybrids (Little 2004, Kim *et al*. 2010, Danusevičius *et al*. 2012, Fortini *et al*. 2015), suggesting a simple genetic inheritance (Wei *et al*. 2015).

In this study, we explore the micromorphological patterns of hybridization between two

Author contributions
Alfredo López-Caamal conducted statistical analyses and wrote the manuscript. Luz del Carmen Ruiz-Amaro performed morphological measurements and statistical analyses. Armando Zepeda-Rodríguez characterized the type of stomata and trichomes in leaves through Scanning Electron Micoscopy (SEM), Patricia Mussali-Galante conducted collection of botanical material in field and wrote the manuscript, Efraín Tovar-Sánchez conceived the original idea, performed statistical analyses and wrote the manuscript.

Mexican oaks: *Quercus crassifolia* Bonpl. and *Q. crassipes* Bonpl. (sect. *Lobatae*). *Quercus crassifolia* is distributed mainly in the Sierra Madre Occidental (SMOc), the Faja Volcánica Transmexicana (FVT), and in the southern parts of Sierra Madre Oriental (SMOr) while *Q. crassipes* does it in SMOr and in the FVT (Figure 1). In their sympatric range, atypical individuals with intermediate morphology are found. Initially, these individuals were considered as a separate species (*Q. dysophylla*; Romero 1993, Zavala Chávez 1995). However, Tovar-Sánchez & Oyama (2004) identified through macro morphological and molecular markers that the intermediate forms between *Q. crassipes* and *Q. crassifolia* are hybrid individuals, designating them as *Q.* × *dysophylla* (Tovar-Sánchez & Oyama 2004). Furthermore, these authors found that geographic proximity of hybrid individuals to the allopatric site of a parental species increases their macro morphological and genotypic similitude with the parental species.

The goals of this paper were 1) to describe the micro- and macro-morphological spatial variation of *Quercus* × *dysophylla* and its parental species, 2) to compare the spatial patterns of hybridization revealed by genetic (Tovar-Sánchez & Oyama 2004) macro morphological and micro morphological, and 3) find micro-morphological diagnostic markers of *Q.* × *dysophylla* in order to facilitate its recognition. Due to the simple genetic inheritance found in a number of micro morphological characters, we hypothesize that micro morphological characters will exhibit the same spatial pattern revealed by genetic markers (geographic proximity to a species' allopatric site will increase the similitude individuals to that species).

Materials and Methods

Study species and study sites. *Quercus crassifolia* and *Q. crassipes* belong to the section *Lobatae*. These species show noticeable differences when distributed in allopatry (Valencia-A. 2004). Chloroplast DNA analyses revealed that for both species, colonization routes were in a north – south direction following the formation and retreat of glaciers during the Last Glacial Maximum (Tovar-Sánchez *et al*. 2008). *Quercus crassifolia* colonized initially the northern part of SMOc, the entire FVT and the southern parts of SMOr. Meanwhile, *Q. crassipes* colonized initially the northern parts of SMOr and then the FVT. Atypical individuals with putative intermediate morphology are found where the distributions of these species overlap (FVT and southern parts of SMOr). These individuals were described as *Q. dysophylla*. However, RAPD analysis revealed that these individuals are the result of interspecific gene flow between *Q. crassipes* and *Q. crassifolia*. The name *Q.* × *dysophylla* was proposed for intermediate individuals with additive pattern of RAPD loci (Tovar-Sánchez & Oyama 2004).

For this paper, we chose the same sites and individuals studied by Tovar-Sánchez & Oyama (2004). These include two allopatric sites in SMOc for *Q. crassifolia* and two for *Q. crassipes* in SMOr. Also, five hybrid zones in the FVT and two in the southern part of SMOr were included

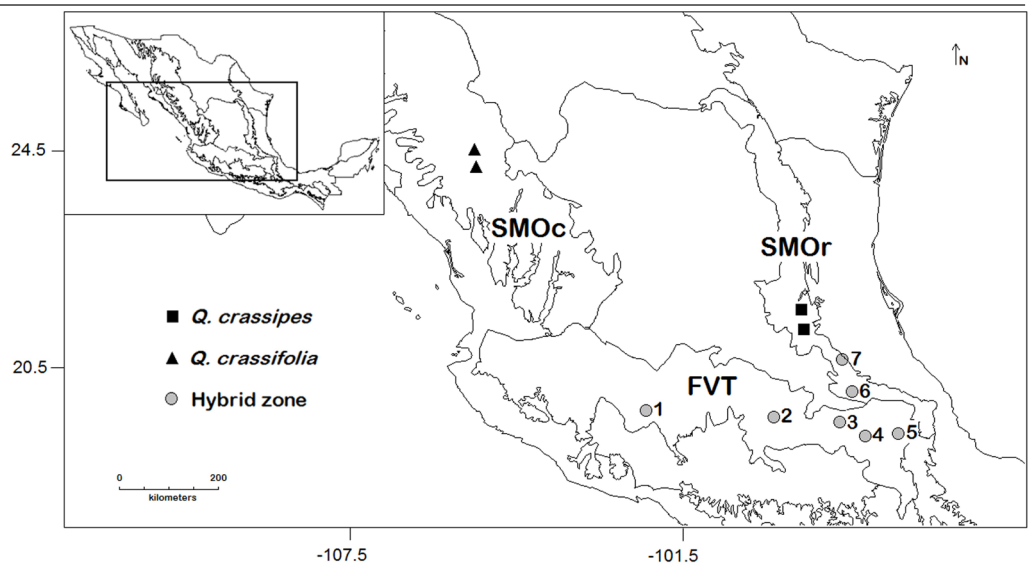

Figure 1. Map of sampled *Quercus crassifolia* and *Q. crassipes* allopatric stands and hybrid zones. 1) Cantera, 2) Canalejas, 3) Tlaxco, 4) Acajete, 5) Esperanza, 6) Agua Blanca, and 7) Palo Bendito. Solid lines represent biogeographical regions.

in the analysis (Figure 1). We labeled individuals as *Q. crassifolia*, *Q. crassipes* or *Q.* × *dysophylla* according to the genetic analysis performed by Tovar-Sánchez & Oyama (2004).

Trichome and stoma characterization. In order to characterize the type of stomata and trichomes in leaves through Scanning Electron Micoscopy (SEM), we randomly chose one individual per taxa in each sympatric site (*Q. crassifolia*, *Q. crassipes* and *Q.* × *dysophylla*) and one individual per taxa in each allopatric site (*Q. crassifolia* or *Q. crassipes*).

For trichome characterization, we explored the abaxial leaf surface. Due to their great density, we manually eliminated a portion of trichomes in order to distinguish them clearly. We followed the nomenclature provided by Jones (1986) for trichome characterization. For stomata characterization, we retired manually all trichomes. SEM Coating System (Polaron) was used to coat the samples with gold and SEM was carried out in a Zeiss DSM 950 microscope.

Leaf micro- and macromorphological measurements. For trichome micro-morphological measurements, we randomly selected three individuals per taxa (*Q. crassifolia*, *Q. crassipes* and *Q.* × *dysophylla*) in each macromorphological allopatric site and hybrid zones (n = 25). For each micromorphological individual, we randomly selected three mature and undamaged leaves. Five trichome slides were prepared for each leaf (n = 1,125 slides). We only measured fasciculate-stipitate trichomes as this was the only type shared across all individuals of *Q. crassipes*, *Q. crassifolia* and *Q.* × *dysophylla* (see Results). Trichome slides were prepared as follows; we carefully retired the leaves' underside trichomes. Next, trichomes were stained with toluidine blue and permanent slides were prepared with glycerinated gelatin. We measured 15 trichomes per leaf, and we included three leaves per individual (n = 135 trichomes). For each trichome, four micromorphological characters were recorded: ray length (RL), stipe length (TSL), ray number (NR) and the relation between stipe length and ray length (TSL%; Table 1). For stomata measurements, we randomly selected five individuals per taxa in each allopatric site and hybrid zone. Three undamaged leaves were selected per individual and three slides were prepared per leaf (n = 1,125). These slides were prepared as impressions of the leaf surface through the replica technique (Wilson 1981). Briefly, the impression was made by placing a drop of cyanoacrylate adhesive in the leaf surface. Once the adhesive dried out (after 1 – 2 hours), the leaf tissue was carefully removed. The Slides were then observed under a microscope at 40X. In total, four characters (stomata length [SL], width [SW], density [SD] and stomata coberture [SC]) were measured in 1875 stomata.

In addition to micromorphological characters, we also measured macromorphological leaf characters (Foliar area [FA], petiole area [PA], secondary vein angle [VA] and the relation between foliar and petiole area [PA%]; Table 1). We selected 10 individuals per taxa in each hybrid

Table 1. List of stomata, trichome and leaf macromorphological characters measured in individuals of *Quercus crassifolia*, *Q. crassipes* and *Q.* × *dysophylla* in Mexico.

Character	Units	Description
Micromorphological		
Stomata		
SL	µm	Stoma length
SW	µm	Stoma width
SD	no./µm^2	Stomata density at 40X
SC	µm^2	[(Stoma length + Stoma width)/4]2 π
Trichomes		
TSL	µm	Stipe length
RL	µm	Ray length
NR	no.	Number of rays
TSL%	-	(Stipe length/Ray length)100
Macromorphological		
FA	cm^2	Foliar area
PA	cm^2	Petiole area
VA	degrees	Secondary vein angle
PA%	-	(Petiole area/Foliar area)100

zone and 20 individuals in each allopatric stand (n = 290 trees). Twenty mature undamaged leaves were randomly selected per individual and four macromorphological characters were recorded (Table 1).

Data analyses. ANOVA was conducted to determine the effect of taxa (*Q. crassifolia*, *Q. crassipes* and *Q.* × *dysophylla*) in each micro- and macromorphological character measured. Percentage data were transformed as [arcsin $(\%)^{1/2}$], and count characters were transformed as $[(x)^{1/2} + 0.5]$ (Zar 2010). Significant mean differences between taxa were determined with a Tukey multiple range test. According to the results of the Tukey multiple range test, we recorded the phenotype of *Q.* × *dysophylla* as intermediate or *Q. crassifolia*-like when significant differences were found with at least one parental species. Otherwise, the character was recorded as Parental-like when no significant differences were found between *Q.* × *dysophylla* and either parental species. All allopatric and hybrid zones were included in the analysis.

Discriminant function analysis (DFA) was carried out using all morphological variables. We performed a separate DFA for trichome, stomata and leaf macromorphological characters including all populations without taking into account their geographic origin. The purpose of this analysis was to determine the most useful characters to discriminate between taxa and to visually assess the separation of individuals into groups. We established taxon (*Q. crassifolia*, *Q. crassipes* and *Q.* × *dysophylla*) as the predictor variable. Separate analyses were performed with trichome, stomata and macromorphological datasets because different sample sizes were obtained with each of these structure measurements, rendering them incomparable. Aditionally, a classification analysis using DFA was performed in order to test the accuracy of each structure's morphology to place each individual in the established pre-defined categories (*Q. crassifolia*, *Q. crassipes* and *Q.* × *dysophylla*).

In order to know the phenotypic expression of *Q.* × *dysophylla* in each hybrid zone, we performed the following analyses. First we conducted an ANOVA with taxa (*Q. crassifolia*, *Q. crassipes* or *Q.* × *dysophylla*) as independent variable and each morphological character as dependent variable. Significant mean differences between taxa were determined with a Tukey multiple range test. If a *Q.* × *dysophylla* character differed significantly between taxa, it was recorded as transgressive (positive or negative) if the value of the putative hybrid exceeded the parental species value. Otherwise, the character was reported as intermediate, *Q. crassifolia*-like or *Q. crassipes*-like when significant differences were found between the mean trait values of *Q.* × *dysophylla* and one or both parental species. Finally, the character was reported as parental-like, when no significant differences were found with either parental species (Schwarzbach *et al*. 2001).

Finally, to test if geographic proximity to an allopatric site of a *Quercus* species increases the morphological similitude of individuals to that species, we performed linear regressions between each macro- and micromorphological character and geographic distance of each hybrid zone in a west-east direction. We expect *Q.* × *dysophylla* morphological characters to increase their similitude to *Q. crassifolia* when there is close proximity to its allopatric stand (west). In a similar fashion, we expect that *Q.* × *dysophylla* morphology will increase its similitude to *Q. crassipes* when it is in close proximity to its allopatric site (east). All statistical analyses were performed with JMP software ver. 12 (SAS Institute, Cary, NC).

Results

Trichome and stoma characterization. We found one type of glandular trichome and five types of non-glandular trichomes (Table 2, Figure 2). In general, all three taxa exhibited simple uniseriate glandular trichomes, however, this type was not present in all individuals of the three taxa. Regarding non-glandular trichomes, *Q. crassifolia* exhibited all types except simple stellate trichomes, while *Q. crassipes* only exhibited fasciculate stipitate and simple stellate trichomes. Lastly, *Q.* × *dysophylla* showed fasciculate stipitate, multirradiate and simple stellate trichomes (Table 2). We found that all individuals of all three taxa exhibited fasciculate stipitate trichomes. Meanwhile, anomocytic stomata were observed in all individuals of *Q. crassipes*, *Q. crassifolia* and *Q.* × *dysophylla*. In some cases, the stomata were covered with dense epicuticular waxes, which made difficult to observe all of their structures.

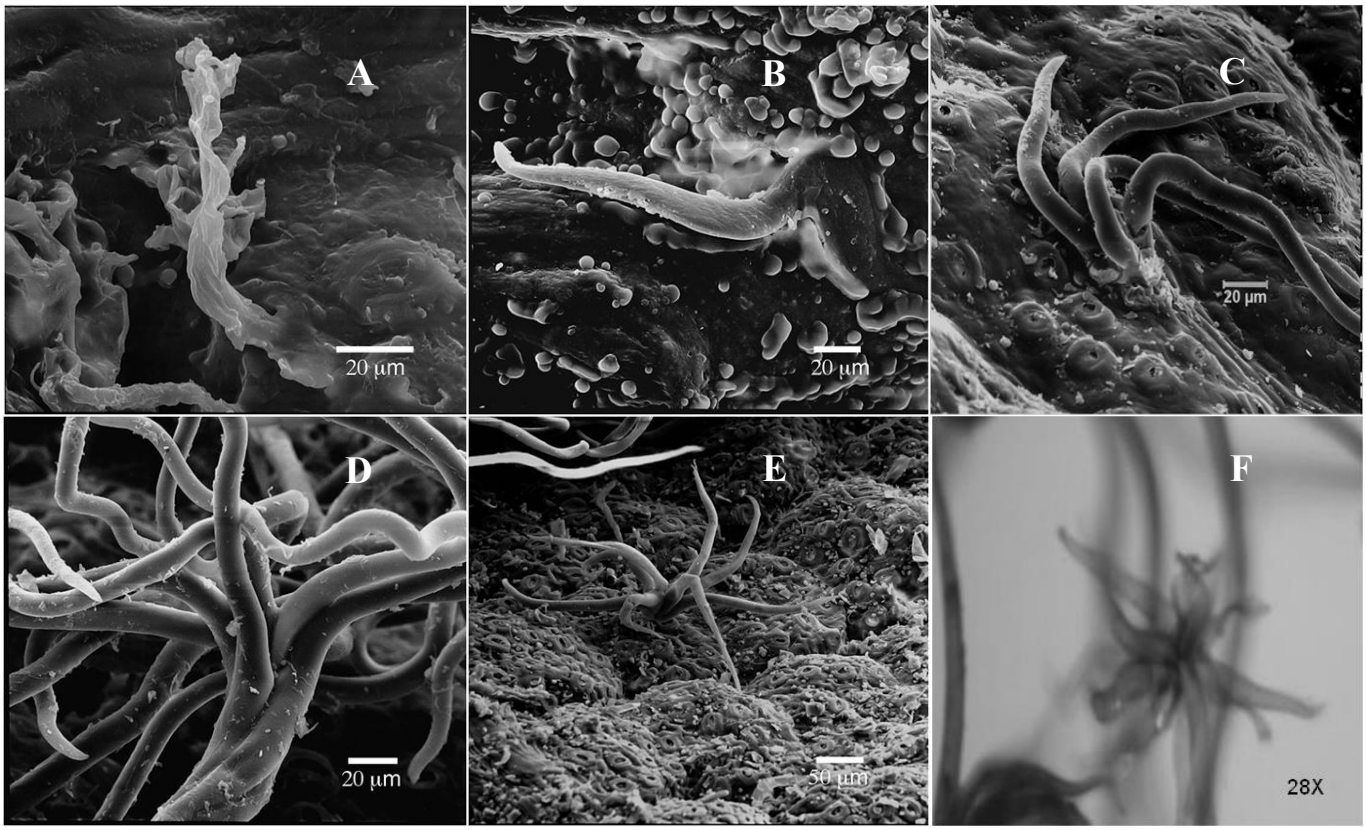

Figure 2. Uniseriate simple glandular trichome (**A**) and non-glandular trichomes (**B-F**) found in *Quercus crassifolia*, *Q. crassipes* and *Q.* × *dysophylla* through SEM. Solitary unicelular non-glandular (**B**), fasciculate sessile (**C**), fasciculate stipitate (**D**), simple stellate (**E**) and multirradiate trichomes (**F**) were found. See text for details.

Quantitative analysis of trichome, stoma and leaf macromorphological characters. ANOVA of trichome, stoma and leaf macromorphological characters revealed that taxa (*Q. crassipes*, *Q. crassifolia* and *Q.* × *dysophylla*) had a significant effect in all morphological characters except for stomatal density (SD; Table 2). Furthermore, after post hoc comparisons, *Q.* × *dysophylla* showed an intermediate phenotype between its parental species in all characters except for stomatal density (parental-like; Table 3) and TSL% (*Q. crassifolia*-like; Table 3).

DFA with taxa as the predictor variable and stomata characters as dependent variables yielded two discriminant functions (DF) that explained 100 % of the variation of the original data set (Figure 3A). The variables with the highest standardized discriminant coefficients in DF1 were Stoma length and Stoma coberture, while Stoma coberture and stoma length had the highest standardized discriminant coefficients in DF2 (Table 4). The plot of DF2 *vs*. DF1 showed that individuals of *Q. crassifolia*, *Q. crassipes* and *Q.* × *dysophylla* show significant overlap in the ordination space (Figure 3A). However, classification analysis through DFA with stoma characters showed that

Table 2. Glandular and non-glandular foliar trichomes found in the abaxial surface through SEM in individuals of *Quercus crassifolia*, *Q. crassipes* and *Q.* × *dysophylla* in Mexico.

Taxa	Glandular trichome	Non-glandular trichome				
	Simple uniseriate	Solitary unicellular	Fasciculate sessil	Fasciculate stipitate	Multirradiate	Simple stellate
Q. crassifolia	×	×	×	×	×	
Q. crassipes	×			×		×
Q. × dysophylla	×			×	×	×

Table 3. Mean ± standard error and ANOVA results for all micro- and macromorphological characters for *Quercus crassipes*, *Q. crassifolia* and *Q.* × *dysophylla*. Different letters indicate significant differences between taxa (Tukey, $p < 0.05$).

Character	*Q. crassifolia*	*Q.* × *dysophylla*	*Q. crassipes*	F_{Taxa}	*Q.* × *dysophylla* phenotype
Micromorphological					
Stomata				$F_{(2, 1798)}$	
SL	26.53 ± 0.08[a]	26.05 ± 0.09[b]	24.32 ± 0.10[c]	144.50 *	Intermediate
SW	24.38 ± 0.08[a]	23.90 ± 0.10[b]	22.87 ± 0.09[c]	65.60 *	Intermediate
SD	58.72 ± 2.21[a]	59.28 ± 2.66[a]	66.05 ± 3.42[a]	0.19 ns	Parental-like
SC	84,399.33 ± 943.83[a]	78,363.44 ± 977.65[b]	63,413.83 ± 948.36[c]	131.93 *	Intermediate
Trichomes				$F_{(2, 1941)}$	
TSL	136.98 ± 1.64[a]	116.59 ± 1.48[b]	99.47 ± 1.34[c]	162.58 *	Intermediate
RL	779.05 ± 11.36[a]	672.84 ± 8.75[b]	459.76 ± 5.64[c]	325.86 *	Intermediate
NR	2.66 ± 0.01[a]	2.80 ± 0.01[b]	3.12 ± 0.01[c]	308.90 *	Intermediate
TSL%	19.07 ± 0.24[a]	18.56 ± 0.31[a]	22.81 ± 0.49[b]	56.53 *	*Q. crassifolia*-like
Macromorphological				$F_{(2, 4909)}$	
FA	43.93 ± 0.46[a]	22.19 ± 0.28[b]	8.04 ± 0.09[c]	2,780.47 *	Intermediate
PA	0.64 ± 0.01[a]	0.25 ± 0.01[b]	0.11 ± 0.01[c]	2,131.98 *	Intermediate
VA	48.18 ± 0.17[a]	44.30 ± 0.21[b]	37.81 ± 0.23[c]	745.80 *	Intermediate
PA%	1.70 ± 0.03[a]	1.22 ± 0.02[b]	1.53 ± 0.07[c]	86.95 *	Intermediate

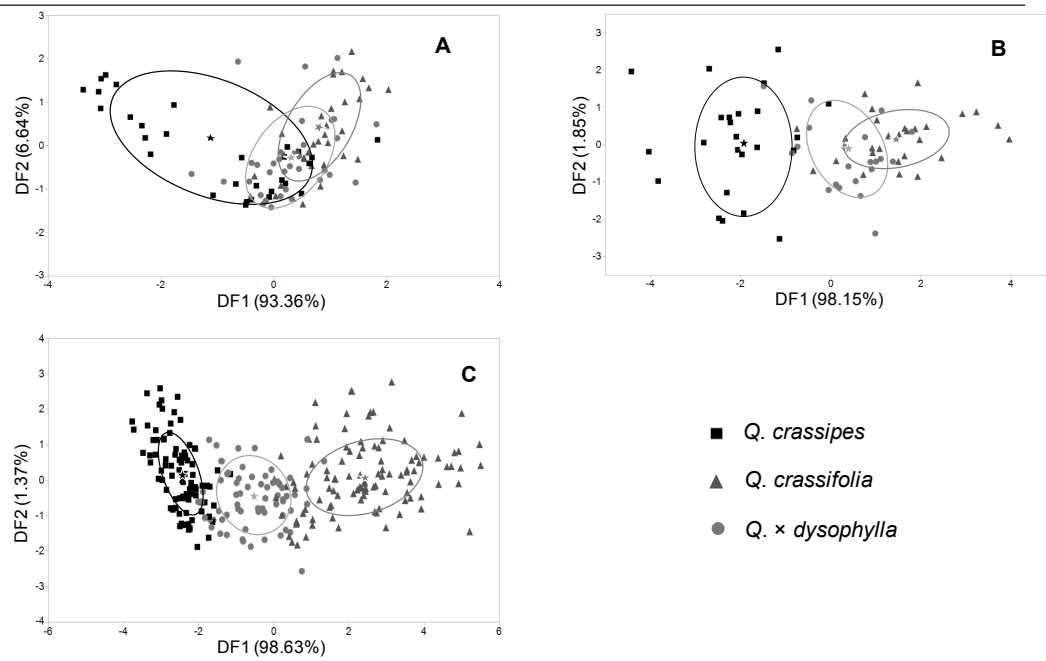

Figure 3. Plot of DF2 *vs*. DF1 extracted through DFA of stomata (**A**), trichome (**B**), and foliar macromorphological characters (**C**) of *Quercus crassifolia*, *Q. crassipes* and *Q.* × *dysophylla* in central Mexico. Stars indicate group centroids and ellipses depict the estimated zone that includes 50 % of population data for each taxa.

only 11.11 % of individuals of *Q. crassifolia* were misclassified as *Q.* × *dysophylla*, while a significant percentage of *Q. crassipes* and *Q.* × *dysophylla* were misclassified (Table 5).

In a similar fashion, DFA with trichome characters produced two DF that explained 100 % of the variation in the original data set (Figure 3B). Trichome stipe length and the relation between stipe length and ray length showed the highest standardized canonical coefficients in DF1, while the relation between stipe length and ray length (TSL%) and ray length showed the highest standardized canonical coefficients in DF2 (Table 4). The plot of DF1 *vs*. DF2 with trichome characters showed that *Q. crassifolia* and *Q. crassipes* show almost no overlap in the ordination space (Figure 3B). However, *Q.* × *dysophylla* individuals were placed in an intermediate space. Classification analyses showed that parental species were not misclassified between them. However, some individuals of the parental species were classified as *Q.* × *dysophylla*. Also, some *Q.* × *dysophylla* individuals individuals were classified as *Q. crassipes* or *Q. crassifolia* (Table 5).

Table 4. Standardized canonical coefficients of each micro and macromorphological variable derived from DFA of allopatric and hybrid zones between *Q. crassifolia* and *Q. crassipes*.

Morphological variable	DF1	DF2
Stomata		
SL	1.67	-9.62
SW	0.64	-8.55
SC	-1.23	17.19
Trichomes		
TSL	2.27	0.15
RL	-1.29	0.46
NR	-0.49	0.27
TSL%	2.17	0.95
Foliar macromorphological		
FA	0.63	0.07
PA	0.65	0.15
VA	0.37	-0.6
PA%	-0.27	0.75

Table 5. Classification analysis performed by DFA overall *Quercus* populations. The percentages of assignment to each category (taxa) are shown for stomata, trichome and foliar macromorphological characters

Genetic assignment	Predicted by DFA (%)			Percentage of misclassification
Taxa	*Q. crassifolia*	*Q. crassipes*	*Q. × dysophylla*	
Stomata				
Q. crassifolia	88.89	0	11.11	11.11
Q. crassipes	25.71	48.58	25.71	51.42
Q. × dysophylla	57.22	8.50	34.28	65.72
Trichome				
Q. crassifolia	71.43	0	28.57	28.57
Q. crassipes	0	87.50	12.50	12.50
Q. × dysophylla	9.52	4.76	85.72	7.14
Foliar macromorphological				
Q. crassifolia	90.75	0	9.25	9.25
Q. crassipes	0	96.66	3.34	3.34
Q. × dysophylla	5.79	2.89	91.30	4.34

DFA with leaf macromorphological character as dependent variables produced two DF that explained 100 % of the variation in the original characters data set (Figure 3C). Foliar area and petiole area showed the highest standardized discriminant coefficients in DF1, while vein angle and the relation between petiole and foliar area (AP%) did it in DF2. The plot of DF1 *vs*. DF2 revealed that individuals of all three taxa showed almost no overlap in the ordination space; *Q.*

Table 6. Percentages of transgressive, intermediate and parental like characters of *Quercus* × *dysophylla* in seven hybrid zones in central Mexico. For details refer to Appendix 1.

Q. × dysophylla phenotype	Locality							Mean
	Cantera	Canalejas	Tlaxco	Acajete	Esperanza	Agua Blanca	Palo Bendito	
Transgressive	8.33	0	16.67	0	16.67	16.67	16.67	10.72
Intermediate	33.33	33.33	50.00	25.00	41.67	25.00	50.00	36.9
Q. crassifolia-like	8.33	50.00	0	25.00	33.33	0	25.00	20.24
Q. crassipes-like	25.00	8.33	25.00	16.67	0	8.33	0	11.9
Parental-like	25.00	8.33	8.33	33.33	8.33	50.00	8.33	20.24

× *dysophylla* individuals were placed in an intermediate space between individuals of both parental species (Figure 3C). Classification analysis showed low percentages of misclassification in all taxa (Table 5).

Analysis of phenotypic expression of *Q.* × *dysophylla* in each of the seven hybrid zones revealed that transgressive, intermediate and parental-like characters show geographic variation (Table 6). In general, we recorded few transgressive and intermediate characters (10.71 % and 36.9 % respectively). However, characters not different to at least one parental species or parenta-like characters represented 52.38 % of all phenotypes recorded (Table 6). The complete phenotypic analysis per hybrid zone is shown in Appendix 1.

Lastly, regression analysis revealed that geographic distance (in a west-east direction) did not have significant effects in morphological characters of *Q.* × *dysophylla* except for three stomata-related characters (Table 7). Interestingly, a number of parental-species' macro and micromorphological characters were significantly affected by geographic distance. Regarding stomata characters, *Q. crassipes* showed significant negative correlations with distance in the length and width of stomata. That is, stomata tend to be smaller when *Q. crassipes* is in closer proximity with its allopatric stand in the SMOr. The same pattern arises with *Q. crassipes* foliar macromorphological characters and with the stipe length of trichomes (Table 7). However, stomatal density and the number or trichome rays tend to increase when *Q. crassipes* individuals are located in proximity to its allopatric site. On the other hand, stomata characters of *Q. crassifolia* were not significantly affected by the geographic distance. However, the stipe length of trichomes and the ray length of trichomes show significant associations with geographic distance. Both characters tend to increase its values when *Q. crassifolia* individuals are closer to its allopatric stand in the SMOc. The same pattern arises for two macromorphological characters. However, the trichome number of rays and the relation between stipe length and ray length of trichomes showed the opposite pattern (Table 7).

Table 7. Regression analysis between each macro and micromorphological character of *Quercus crassifolia*, *Q. crassipes*, and *Q.* × *dysophylla* and geographic distance. Only correlations significant at $p < 0.05$ are shown. See text for details.

Character	*Q. crassifolia*		*Q.* × *dysophylla*		*Q. crassipes*	
	r	r^2	*r*	r^2	*r*	r^2
Stomata						
SL	-	-	-	-	-0.67	0.49
SW	-	-	0.56	0.31	-0.54	0.29
SD	-	-	-0.56	0.32	0.72	0.52
SC	-	-	-0.43	0.19	-0.63	0.39
Trichomes						
TSL	-0.45	0.20	-	-	-0.53	0.28
RL	-0.63	0.39	-	-	-	-
NR	0.74	0.55	-	-	0.51	0.26
TSL%	0.42	0.18	-	-	-0.51	0.26
Foliar macromorphological						
FA	-	-	-	-	-	-
PA	-0.45	0.21	-	-	-0.52	0.27
VA	-	-	-	-	-	-
PA%	-0.51	0.26	-	-	-0.52	0.27

Discussion

Phenotype expression of Quercus crassifolia, Q. crassipes and Q. × *dysophylla*. In this paper, we explored the morphological variation of *Q.* × *dysophylla* and its parental species, *Q. crassipes* and *Q. crassifolia*. Identification of oak species and their hybrids is usually difficult due to the continuous morphological and genetic variation. In this regard, detailed morphometric studies arise as an important approach to identify diagnostic characters that enable the discrimination between parental and hybrid categories. Results show that, overall populations, *Q.* × *dysophylla* shows an intermediate phenotype of all the micro- (except for SD and TSL%) and macromorphological characters evaluated. However, leaf macromorphological characters

clearly discriminate between taxa. This result is in accordance with other studies that report hybrid phenotypes in several *Quercus* species. For instance, Tovar-Sánchez & Oyama (2004) found that hybrid individuals between *Q. crassipes* and *Q. crassifolia* exhibit an intermediate phenotype in macromorphological characters. Also, Song *et al*. (2015) explored the phenotype between *Q. austrochinchinensis* and *Q. kerri* in China. The authors found that 13 out of 14 characters showed an intermediate phenotype in the hybrid. Similar results have been found between several *Quercus* species (Borazan & Babaç 2003, González-Rodríguez *et al*. 2004, Albarrán-Lara *et al*. 2010, Fortini *et al*. 2015), suggesting that quantitative characters in several *Quercus* species may have a polygenic control with additive effects.

Despite that intermediate phenotypes are found when all populations of *Q. crassipes* and *Q. crassifolia* are analyzed together, the phenotype of *Q.* × *dysophylla* seems to show geographic variation. When hybrid zones are analyzed individually, *Q.* × *dysophylla* shows a mosaic of intermediate, transgressive and parental-like characters. Results show that, when hybrid zones are analyzed individually, only 36.9 % of characters showed an intermediate phenotype, while a significant portion (52.38 %) were characters not different to one or both parental species. This result highlight the fact that hybrids are not uniform; they may exhibit a wide array of phenotypes depending on the hybrid class analyzed (F1, backcrosses). Also, selection pressures acting at each site may differ between hybrid zones. This may partially explain the differential phenotypes found between hybrid zones. Future studies should analyze the hybrid phenotypes for each hybrid class (F1, F2, backcrosses) under uniform conditions in order to know the contribution of phenotypic plasticity to hybrid phenotypes.

Quercus crassipes and *Q. crassifolia* may be easily differentiated through leaf macromorphological characters. These characters separated parental individuals into discrete clusters (Figure 2C) and > 90 % of individuals of both parental species were correctly classified to their pre-established category (Table 5). Micromorphological trichome characters showed the same pattern (Figure 2B, Table 5). This finding is in accordance with other studies that support the use of leaf macromorphological and trichome characters to discriminate between *Quercus* species (Fortini *et al*. 2015, Song *et al*. 2015). However, these characters were not informative when *Q.* × *dysophylla* was brought into analysis. When *Q.* × *dysophylla* was included, the individuals were placed in an intermediate space between parental species through DFA. However, up to 28.57 % of individuals of parental species were misclassified as *Q.* × *dysophylla*. This suggests that trichome characters may be initially informative when discriminating between hybrid and parental categories but other characters may be take into account. In this sense, qualitative rather than quantitative characters may be useful for *Q.* × *dysophylla* identification. In accordance with previous work (Valencia-Ávalos & Delgado-Salinas 2003, Vázquez 2006, Scareli-Santos *et al*. 2013), we found that parental species can be differentiated by trichome types. A diagnostic character for *Q. crassipes* is the presence of simple stellate trichomes, while for *Q. crassifolia* solitary unicellular, fasciculate sessile and multirradiate non-glandular trichomes were its diagnostic features. However, these diagnostic features recombine in *Q.* × *dysophylla*. The hybrids exhibit both multirradiate and simple stellate non-glandular trichomes. This characteristic may be considered as diagnostic features for *Q.* × *dysophylla*. In a previous study, Scareli-Santos *et al*. (2013) identified multirradiated trichomes in *Q. crassipes*, however we failed to recognize this trichome type in this species. So, we suggest that the presence of multirradiated trichomes in this species is not a constant character in *Q. crassipes*. However, future studies should analyze micromorphological variation employing more individuals of *Q. crassifolia*, *Q crassipes* and their hybrid *Q.* × *dysophylla* in order to evaluate if the trends found in this study can be generalized across these taxa.

Spatial morphological variation of Quercus × dysophylla *and its parental species*. Previous genetic studies in hybrid zones between *Q. crassipes* and *Q. crassifolia* in Mexico found that geographic proximity to a species' allopatric stand increases the genetic similitude of hybrid individuals to that species (Tovar-Sánchez & Oyama 2004). We hypothesized that the same pattern would occur when micro- and macromorphological characters were studied. However, we did not find an effect of geographic distance in the morphological characters of *Q.* × *dysophylla* except for three stomata micromorphological characters. Several authors have found a strong correlation of foliar morphological characters with geographic distance or ecological gradients

(Bruschi *et al*. 2000, Bruschi *et al*. 2003, Albarrán-Lara *et al*. 2010, Fortini *et al*. 2015). However, this was not the case for *Q*. × *dysophylla*. Despite several authors have recorded a strong effect of environmental gradients in the hybrid micromorphological phenotypic expression (*e.g*. Bruschi *et al*. 2003), our analyses show that the environment is not a driver of the morphological expression of *Q*. × *dysophylla*, suggesting that hybrids show increased morphological variation due to their recombinant origin (Wei *et al*. 1995). Nonetheless, individuals included into analysis are both probable F1 individuals and backcrosses toward both parental species. The inclusion of several hybrid classes obscure the effects of geographic distance over the macro- and micro morphological characters due to differential phenotype expression between hybrid classes.

Although *Quercus* × *dysophylla* phenotype did not exhibit an effect of geographic distance, some parental species' characters did show a significant effect of geographic distance. For instance, 9 out of 12 stomata, trichome and leaf macromorphological characters of the parental species were significantly affected by geographic distance. Meanwhile, all trichome characters and two foliar macromorphological characters of *Q. crassifolia* showed an effect of geographic distance. The fact that both parental species show the same pattern of geographical variation in trichome and macromorphological phenotype expression may be the result of an ecological or environmental gradient along FVT. Future studies should correlate the morphology of *Q. crassipes* and *Q. crassifolia* with environmental conditions as done elsewhere (*e.g*. Bruschi *et al*. 2003).

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Appendix 1. Phenotype characterization of leaf macromorphological and stoma and trichome micromorphological characters for seven hybrid zones between *Quercus crassipes* and *Q. crassifolia* in Mexico. - = transgressive negative character, + = transgressive positive character.

Character	*Q. crassifolia*	*Q.* × *dysophylla*	*Q. crassipes*	Phenotype
CANTERA				
Macromorphological				
VA	53.06 ± 6.55[a]	45.68 ± 5.92[b]	41.08 ± 7.59[c]	Intermediate
FA	53.04 ± 6.54[a]	45.85 ± 5.88[b]	40.96 ± 7.5[c]	Intermediate
PA	0.68 ± 0.3[a]	0.28 ± 0.12[b]	0.16 ± 0.09[c]	Intermediate
PA %	1.38 ± 0.78[a]	1.09 ± 0.46[b]	1.94 ± 1.1[c]	-
Micromorphological				
RL	718.61 ± 239[a]	456.31 ± 75.32[b]	573.91 ± 185.2[c]	Intermediate
TSL	125.28 ± 39.8[a]	106.77 ± 21.49[a]	128.75 ± 21.49[b]	*Q. crassifolia*-like
NR	2.66 ± 0.2[a]	2.9 ± 0.29[b]	2.92 ± 0.3[b]	*Q. crassipes*-like
TSL%	18.68 ± 6.7[a]	23.78 ± 6.93[b]	23.79 ± 5.1[b]	*Q. crassipes*-like
SL	26.53 ± 2.3[a]	26.34 ± 2.08[a]	26.76 ± 2.18[a]	Parental-like
SW	24.96 ± 2.2[a]	24.37 ± 2.07[b]	24.12 ± 1.99[b]	*Q.crassipes*-like
SD	54.8 ± 12.9[a]	47.4 ± 9.65[ab]	45.6 ± 1.14[b]	Parental-like
SC	2091.57 ± 270[a]	2027.8 ± 243.03[a]	2041.8 ± 266.84[a]	Parental-like
CANALEJAS				
Macromorphological				
VA	49.02 ± 6.43[a]	46.07 ± 7.62[b]	34.24 ± 9.8[c]	Intermediate
FA	48.34 ± 22[a]	22.78 ± 9.62[b]	7.71 ± 2.8[c]	Intermediate
PA	0.74 ± 0.26[a]	0.38 ± 0.19[b]	0.22 ± 0.1[c]	Intermediate
PA %	1.74 ± 0.77[a]	1.84 ± 0.99[a]	3.45 ± 7.19[b]	*Q. crassifolia*-like
Micromorphological				
RL	755.43 ± 222[a]	761.3 ± 228.26[a]	541.27 ± 194.46[b]	*Q. crassifolia*-like
TSL	134.75 ± 30.6[a]	119.63 ± 31.27[b]	113.46 ± 25.2[b]	*Q.crassipes*-like
NR	2.6 ± 0.28[a]	2.78 ± 0.28[b]	3.01 ± 0.36[c]	Intermediate
TSL%	18.92 ± 5.67[a]	16.61 ± 4.83[a]	27.28 ± 30.22[b]	*Q. crassifolia*-like
SL	27.2 ± 2.33[a]	26 ± 2[a]	24.88 ± 2.74[b]	*Q. crassifolia*-like
SW	24.5 ± 2.16[a]	24.42 ± 2.44[ab]	23.55 ± 2.63[b]	Parental-like
SD	42.2 ± 9.44[a]	38.2 ± 5.01[a]	58.4 ± 9.31[b]	*Q. crassifolia*-like
SC	2109.16 ± 285[a]	2109.2 ± 282.39[a]	1851.2 ± 265.64[b]	*Q. crassifolia*-like
TLAXCO				
Macromorphological				
VA	46.09 ± 5.65[a]	41.16 ± 7.75[b]	42.3 ± 7.85[b]	*Q. crassipes*-like
FA	48.05 ± 19.1[a]	18.2 ± 9.92[b]	7.55 ± 2.72[c]	Intermediate
PA	0.37 ± 0.15[a]	0.1 ± 0.09[b]	0.08 ± 0.07[b]	*Q. crassipes*-like
PA %	0.91 ± 0.58[a]	0.61 ± 0.47[b]	1.06 ± 1.4[c]	-
Micromorphological				
RL	795.79 ± 295[a]	663..01 ± 160.4[b]	336.59 ± 102.27[c]	Intermediate
TSL	150.62 ± 40.8[a]	95.69 ± 18.29[b]	60.26 ± 17.29[c]	Intermediate
NR	5.3 ± 1.46[a]	4.7 ± 1.04[b]	8 ± 1.07[c]	-
TSL%	20.66 ± 7.46[a]	15.09 ± 3.93[b]	15.88 ± 4.65[b]	*Q. crassipes*-like
SL	27.37 ± 1.67[a]	26.02 ± 2.11[b]	21.53 ± 1.56[c]	Intermediate
SW	25.44 ± 3.05[a]	24.2 ± 2.02[b]	20.53 ± 1.4[c]	Intermediate
SD	61.6 ± 13.6[a]	70 ± 9.13[ab]	85 ± 31.05[b]	Parental-like
SC	704.29 ± 103[a]	636.5 ± 83.43[b]	471.74 ± 53.15[c]	Intermediate
ACAJETE				
Macromorphological				
VA	47.85 ± 5.12[a]	46.28 ± 7.2[a]	29.49 ± 10.7[b]	*Q. crassifolia*-like

Appendix 1. Continuation.

Character	*Q. crassifolia*	*Q.* × *dysophylla*	*Q. crassipes*	Phenotype
FA	35.31 ± 11.5[a]	20.24 ± 8.73[b]	9.02 ± 3.04[c]	Intermediate
PA	0.24 ± 0.13[a]	0.1 ± 0.05[b]	0.05 ± 0.07[c]	Intermediate
PA %	0.74 ± 0.43[a]	0.57 ± 0.38[b]	0.67 ± 0.76[b]	*Q. crassipes*-like
Micromorphological				
RL	749.17 ± 306[a]	558.1 ± 138.5[b]	439.65 ± 93.74[c]	Intermediate
TSL	141.68 ± 59.4[a]	110.68 ± 23.49[b]	102.23 ± 18.22[b]	*Q. crassipes*-like
NR	2.73 ± 0.3[a]	2.76 ± 0.25[a]	2.86 ± 0.29[a]	Parental-like
TSL%	19.86 ± 7.15[a]	20.75 ± 5.7[a]	24 ± 5.51[b]	*Q. crassifolia*-like
SL	26.52 ± 2.29[a]	27.12 ± 2.21[a]	26.08 ± 2.49[b]	*Q. crassifolia*-like
SW	23.41 ± 2.24[a]	24.3 ± 2.22[a]	24.32 ± 2.81[a]	Parental-like
SD	48.8 ± 77.1[a]	55.2 ± 6.22[ab]	61 ± 10.72[b]	Parental-like
SC	1968.98 ± 291[a]	2086.2 ± 275.06[ab]	2012 ± 379.44[b]	Parental-like
ESPERANZA				
Macromorphological				
VA	47.32 ± 7.86[a]	43.89 ± 7.21[b]	33.43 ± 6.53[c]	Intermediate
FA	46.17 ± 18.2[a]	20.45 ± 10.51[b]	7.8 ± 3.14[c]	Intermediate
PA	0.75 ± 0.29[a]	0.3 ± 0.13[b]	0.1 ± 0.04[c]	Intermediate
PA %	1.75 ± 0.7[a]	1.72 ± 0.85[a]	1.38 ± 0.63[b]	*Q. crassifolia*-like
Micromorphological				
RL	713.84 ± 232[a]	742.12 ± 194.2[a]	536.4 ± 114.64[b]	*Q. crassifolia*-like
TSL	142.31 ± 26.8[a]	133.02 ± 27.08[ab]	137.63 ± 24.51[b]	Parental-like
NR	2.91 ± 1.34[a]	2.78 ± 0.33[b]	3.33 ± 0.26[c]	-
TSL%	21.33 ± 5.5[a]	18.89 ± 5.18[b]	26.32 ± 5.16[c]	-
SL	26.72 ± 2.07[a]	26.04 ± 1.79[a]	24.9 ± 1.39[b]	*Q. crassifolia*-like
SW	25.24 ± 2.1[a]	24.27 ± 2.32[b]	23.42 ± 1.37[c]	Intermediate
SD	66.4 ± 6.87[a]	69.6 ± 5.85[a]	48.64 ± 0.41[b]	*Q. crassifolia*-like
SC	2,130.28 ± 290[a]	1,996.8 ± 246.25[b]	1,837.4 ± 140.85[c]	Intermediate
AGUA BLANCA				
Macromorphological				
VA	44.93 ± 7.86[a]	43.944 ± 7.57[a]	42.94 ± 8.04[a]	Parental-like
FA	36.77 ± 15.2[a]	23.74 ± 99.8[b]	9.89 ± 3.77[c]	Intermediate
PA	0.37 ± 0.14[a]	0.32 ± 0.13[b]	0.15 ± 0.07[c]	Intermediate
PA %	1.57 ± 0.66[a]	1.58 ± 0.81[b]	1.79 ± 1.19[c]	Intermediate
Micromorphological				
RL	558.1 ± 139[a]	679.81 ± 208.59[b]	471.83 ± 21.25[c]	+
TSL	110.68 ± 23.5[a]	118.13 ± 29.53[ab]	120.87 ± 29.53[b]	Parental-like
NR	2.76 ± 0.2[a]	2.84 ± 0.3[ab]	2.91 ± 0.34[b]	Parental-like
TSL%	20.73 ± 5.7[a]	17.98 ± 5.12[b]	26.73 ± 7.76[c]	-
SL	25.78 ± 2[a]	24.86 ± 1.83[a]	24.09 ± 2.39[a]	Parental-like
SW	24.01 ± 1.5[a]	22.66 ± 1.69[a]	22.61 ± 2.59[a]	Parental-like
SD	74.8 ± 8[a]	71.6 ± 13.04[a]	76.2 ± 0.44[a]	Parental-like
SC	1954.42 ± 229[a]	1780.5 ± 205.38[b]	1722.3 ± 233.55[b]	*Q. crassipes*-like
PALO BENDITO				
Macromorphological				
VA	45.9 ± 5.94[a]	43.58 ± 6.18[b]	39.93 ± 6.2[c]	Intermediate
FA	60.43 ± 26.2[a]	24.8 ± 11.08[b]	8.83 ± 3.98[c]	Intermediate
PA	0.61 ± 0.31[a]	0.19 ± 0.07[b]	0.09 ± 0.06[c]	Intermediate
PA %	1.14 ± 0.76[a]	0.89 ± 0.52[b]	1.14 ± 0.84[c]	-
Micromorphological				
RL	829.28 ± 295[a]	73.61 ± 234.55[b]	399.82 ± 119.94[c]	Intermediate

Appendix 1. Continuation.

Character	*Q. crassifolia*	*Q.* × *dysophylla*	*Q. crassipes*	Phenotype
TSL	128.36 ± 35[a]	110.24 ± 51.21[b]	84.6 ± 22.5[c]	Intermediate
NR	2.77 ± 0.24[a]	2.9 ± 0.28[b]	3.1 ± 0.33[c]	Intermediate
TSL%	17.29 ± 7.51[a]	16.8 ± 13.02[a]	21.88 ± 5.15[b]	*Q. crassifolia*-like
SL	25.2 ± 1.94[a]	25.93 ± 2.98[a]	24.18 ± 1.76[b]	*Q. crassifolia*-like
SW	23.08 ± 1.71[a]	23.14 ± 3.34[a]	23.21 ± 1.52[a]	Parental-like
SD	55.2 ± 6.3[a]	63 ± 20.82[ab]	62.6 ± 10.94[b]	+
SC	1837.09 ± 217[a]	1916.4 ± 385.54[a]	1769.7 ± 190.86[b]	*Q. crassifolia*-like