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Natural hybridisation between kermes (*Quercus coccifera* L.) and holm oaks (*Q. ilex* L.) revealed by microsatellite markers

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ABSTRACT

Hybridisation between species of the genus *Quercus* is a common phenomenon as a result of weak reproductive isolation mechanisms between phylogenetically close species that frequently co-occur in mixed stands. In this study, we use microsatellite markers to analyse introgression between kermes (*Quercus coccifera* L.) and holm (*Q. ilex* L.) oak, two closely related taxa that frequently dominate the landscape in extensive areas in the Mediterranean region. All tested microsatellites amplified and were polymorphic in both kermes and holm oaks. Bayesian admixture analyses showed a good correspondence between each species and one of the two inferred genetic clusters. Five sampled individuals were *a priori* tentatively identified as hybrids on the basis of intermediate morphological characteristics, and it was confirmed that they also presented mixed genotypes. However, we also detected different levels of genetic introgression among morphologically pure individuals, suggesting that successful backcrossing and/or reduced phenotypic expression of genetic variance in certain individuals may have resulted in strong convergence towards a single species phenotype.

INTRODUCTION

Hybridisation is an interesting phenomenon with important evolutionary implications, which can range from modulating genetic variation within species to new species formation (Arnold 1997, 2006). Hybridisation between species of the genus *Quercus* (oak) is widespread as a result of weak reproductive isolation mechanisms between phylogenetically close species that frequently co-occur in mixed stands (Williams *et al.* 2001; Curtu *et al.* 2007a; Valbuena-Carabaña *et al.* 2007; Lepais *et al.* 2009; Salvini *et al.* 2009). Although morphology can provide valuable information for hybrid identification within the genera *Quercus*, the wide variability of leaf and acorn morphology frequently limits the utility of morphological characters for hybridisation diagnosis (Curtu *et al.* 2007a). Genetic markers such as microsatellites offer an interesting complementary tool to study introgression in oaks (*e.g.*, Curtu *et al.* 2007a; Burgarella *et al.* 2009; Lepais *et al.* 2009).

The Mediterranean evergreen kermes (*Q. coccifera* L.) and holm (*Q. ilex* L.) oaks are two closely related taxa that frequently dominate the landscape in extensive areas in the Mediterranean region. These species frequently co-occur in mixed stands where hybridisation may take place (de Casas *et al.* 2007). The relative dominance of the two species in these mixed stands is highly variable, probably associated with the prevalence of the different environmental requirements of each species. Although gene exchange between the two species appears to be so frequent as to lead to complete sharing of cpDNA haplotypes (Jiménez *et al.* 2004; López de Heredia *et al.* 2007), morphological hybrids between them have rarely been described (de Casas *et al.* 2007). Here, we use

microsatellite markers to study introgression between kermes and holm oak in three areas differing in the relative abundance of these two oak species.

MATERIAL AND METHODS

Study area and plant material

Leaves of 26 kermes and 40 holm oaks were randomly collected in three different areas from Toledo and Madrid Provinces differing in the relative dominance of the two species: (i) Huecas (39°59′N, 4°13′W), a holm oak-dominated area with some isolated kermes oaks; (ii) Aranjuez (40°1′N, 3°35′W), a kermes oak-dominated area with some scattered holm oaks; and (iii) Puebla de Montalbán (39°49′N, 4°16′W), a mixed forest where the frequency of the two species is similar. In this last area we identified *a priori* some individuals (n = 5) as hybrids on the basis of intermediate morphological characteristics. We did so using a simple criterion based on leaf pubescence: kermes oaks are completely glabrous whereas the lower surface of leaves of holm oaks is densely covered with persistent hairs. Individuals with intermediate morphology are slightly hairy; the hair is less persistent and can be easily detached from the leaf surface. Leaves of five individuals showing such intermediate morphological characteristics were also collected for genetic analyses.

Genetic analyses

We used nine polymorphic microsatellite markers (previously developed for other *Quercus* species) to genotype both kermes and holm oaks (Table 1). We used NucleoSpin Plant II kits (Macherey-Nagel, Düren, Germany) to extract and purify genomic DNA from leaf samples. Approximately 5 ng of template DNA was amplified in 10-μl reaction volumes containing 1 × reaction buffer (EcoStart reaction buffer; Ecogen, Barcelona, Spain), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, PET, VIC or NED) and 0.1 U of *Taq* DNA EcoStart polymerase (Ecogen). The PCR programme used was 9 min denaturing at 95 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at the annealing temperature (Table 1) and 45 s at 72 °C, ending with a 5 min final elongation stage at 72 °C. Amplification products were electrophoresed using an ABI 310 genetic analyser (Applied Biosystems, Foster City, CA, USA) and genotypes were scored using GeneMapper 3.7 (Applied Biosystems).

Data analyses

We used Arlequin 3.1 to test for linkage equilibrium within each pair of loci and population/species using a likelihood ratio statistic, whose distribution was obtained by a permutation procedure (Excoffier *et al.* 2005). We assessed the diagnostic power of each marker by calculating allele frequency differentials (δ; Shriver *et al.* 1997). F_{ST} values were calculated using the program Fstat 2.9.3.2 (Goudet 2001). We also used the Hedrick's (2005) standardized population differentiation statistic (G'_{ST}). G'_{ST} was obtained using Recodedata 0.1 (Meirmans 2006) and Fstat (Goudet 2001) following the procedure described by Meirmans (2006). We used a Bayesian

Table 1. Polymorphism characteristics of microsatellite loci used to type 25 kermes and 34 holm oaks showing pure genotypes based on a preliminary clustering analysis with Structure software. The Table shows number of alleles (K) and private alleles (K_p), allele frequency differentials (δ), F_{ST} and G'_{ST} values, range size of alleles, annealing temperature (T_a, in °C), expected heterozygosity (H_E) and observed heterozygosity (H_O) at each locus.

locus	species	K	K_p	δ	F_{ST}	G'_{ST}	range	T_a	H_E	H_O	source/source species
MSQ4	*Quercus ilex*	3	1	0.05	–0.018	–0.014	193–197	50	0.25	0.19	Dow *et al.* 1995
	Q. coccifera	2	0				195–197	50	0.15	0.08	(*Q. macrocarpa*)
MSQ13	*Q. ilex*	9	2	0.41	0.035	0.220	197–213	50	0.82	0.94	Dow *et al.* 1995
	Q. coccifera	10	3				191–211	50	0.82	0.88	(*Q. macrocarpa*)
QpZAG9	*Q. ilex*	10	5	0.38	0.031	0.143	219–255	55	0.79	0.95	Steinkellner *et al.* 1997
	Q. coccifera	7	2				233–255	55	0.79	0.70	(*Q. petraea*)
QpZAG15	*Q. ilex*	14	2	0.41	0.056	0.367	108–140	50	0.85	0.76	Steinkellner *et al.* 1997
	Q. coccifera	16	4				106–142	50	0.84	0.92	(*Q. petraea*)
QpZAG36	*Q. ilex*	12	6	0.76	0.348	0.772	197–225	55	0.70	0.54	Steinkellner *et al.* 1997
	Q. coccifera	6	0				201–215	55	0.55	0.52	(*Q. petraea*)
QpZAG46	*Q. ilex*	6	2	0.88	0.237	0.937	181–195	53	0.67	0.40	Steinkellner *et al.* 1997
	Q. coccifera	10	6				179–225	53	0.84	0.88	(*Q. petraea*)
QrZAG7	*Q. ilex*	3	1	0.27	0.070	0.068	114–118	57	0.23	0.26	Kampfer *et al.* 1998
	Q. coccifera	5	3				110–122	57	0.44	0.46	(*Q. robur*)
QrZAG11	*Q. ilex*	16	6	0.36	0.018	0.150	246–304	50	0.88	0.95	Kampfer *et al.* 1998
	Q. coccifera	11	1				246–282	50	0.87	0.74	(*Q. robur*)
QrZAG20	*Q. ilex*	18	12	0.54	0.083	0.662	159–191	57	0.89	0.83	Kampfer *et al.* 1998
	Q. coccifera	12	6				159–188	57	0.86	0.96	(*Q. robur*)

model-based clustering method (STRUCTURE 2.1, Pritchard *et al.* 2000; Falush *et al.* 2003) to assign individuals to K populations (species in this case) based on multilocus genotype data. This allowed us to analyse the correspondence between morphologically based species/hybrids and inferred genetic structure. We ran STRUCTURE assuming correlated allele frequencies and admixture, and conducted five independent runs of K = 1–5 to estimate the true number of clusters with 10^6 MCMC cycles, following a burn-in period of 100,000 iterations (Pritchard *et al.* 2000; Falush *et al.* 2003). In STRUCTURE, the posterior probability (q) describes the proportion of an individual genotype originating from each cluster (K categories). We used a threshold value (Tq) of 0.90 to classify each individual as purebred or hybrid (Vähä & Primmer 2006; *e.g.*, Burgarella *et al.* 2009). Thus, we considered that a value of q higher than or equal to the threshold (Tq) indicates a purebred genotype, and a value of q lower than the threshold indicates an introgressed genotype (Burgarella *et al.* 2009).

RESULTS AND DISCUSSION

All tested microsatellites, previously proved to be useful in holm oak (Soto *et al.* 2007), were also amplified and found to be polymorphic in kermes oak (Table 1). Previous studies revealed a tight linkage between the microsatellites QpZAG36 and QpZAG46 (Barreneche *et al.* 1998). However, after applying Bonferroni corrections for multiple comparisons, we found no consistent pattern of linkage disequilibrium within each pair of loci across species–populations. Such apparent lack of linkage disequilibrium between the microsatellites QpZAG36/QpZAG46 could have resulted from the relatively low sample sizes analysed. Therefore, we repeated STRUCTURE analyses twice, each time excluding one of the two markers (QpZAG36 or QpZAG46) supposedly located in the same linkage group. STRUCTURE analyses showed a maximum Pr(X|K) for K = 2 that revealed a good correspondence between each oak species and one of the two inferred genetic clusters (Fig. 1), and the obtained results were almost identical when the marker QpZAG36 was excluded from the analyses (see Supporting Information). However, the assignment scores changed slightly when microsatellite QpZAG46 was not considered, probably due to the higher diagnostic power of the QpZAG46 marker, as suggested by its higher allele frequency differential value in comparison with the QpZAG36 locus (Table 1). Overall, these results suggest that these two morphological oak species have maintained their genetic identity in spite of relatively frequent hybridisation processes (de Casas *et al.* 2007; López de Heredia *et al.* 2007; see also Curtu *et al.* 2007b). However, both allele frequency differentials and genetic differentiation estimates were much smaller than those obtained between cork and holm oak (Table 1; Burgarella *et al.* 2009), further supporting previous studies based on nuclear and chloroplast DNA markers that also revealed relatively low genetic differentiation between kermes and holm oak (Jiménez *et al.* 2004; de Casas *et al.* 2007; López de Heredia *et al.* 2007).

Bayesian admixture analyses also revealed that all five individuals tentatively identified as hybrids on the basis of intermediate morphological characteristics also presented mixed genotypes (Fig. 1). On the other hand, the 26 kermes and 40 holm oak samples were apparently pure individuals on the basis of morphological criteria. The deviation from pure genotypes, calculated using the assignment scores provided by STRUCTURE analyses, strongly differed between hybrid and non-hybrid individuals classified on the basis of their morphological appearance (one-way ANOVA: $F_{1,\ 69} = 49.97$; $P < 0.001$; Fig. 1). Different levels of genetic introgression have also been detected among some individuals tentatively identified as morphologically pure using the above-described criterion (Fig. 1). Considering a threshold value of 0.90 to classify each individual as purebred or hybrid, genetic analyses revealed the presence of seven additional individuals with different levels of genetic introgression, six of which were apparently purebred holm oak individuals and one a purebred kermes oak (Fig. 1). Thus, all morphologically intermediate individuals showed intermediate genotypes but some apparently purebred individuals also showed signatures of genetic introgression. The most plausible explanation is that such individuals come from crosses between hybrids or backcrosses between hybrids and

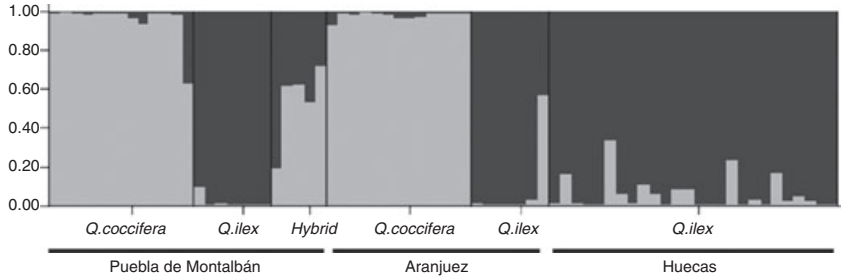

Fig. 1. Results of genetic assignment based on Bayesian method implemented in the program STRUCTURE. Individuals are grouped in the three study areas and according to their morphological appearance (*Quercus coccifera*, *Q. ilex* or hybrids). Each individual is represented by a thin vertical line, which is partitioned into two coloured segments that represent the individual's probability of belonging to the cluster with that colour.

either of the parental species, resulting in individuals with most of their genome from one of the parental species and an apparently purebred morphology (Fig. 1). The presence of these individuals is very interesting in evolutionary terms, as it suggests that F1 hybrids are not sterile or unfit and that backcrosses may be relatively frequent and result in long-lasting introgression and effective gene exchange between the two species (Burgarella *et al.* 2009). Another possibility is that reduced phenotypic expression of genetic variance in some apparently F1 hybrid individuals (with observed q close to 0.5) ultimately resulted in the expression of only one of the original parental phenotypes (de Casas *et al.* 2007). It is also noteworthy that a high proportion of introgressed holm oaks were detected in the Huecas study area where the kermes oak is very scarce. As suggested for other study systems, this may indicate past local extinction of one of the two parental species or long-distance pollen dispersal (*e.g.*, Lepais *et al.* 2009).

Hybridisation seemed to be more frequent in the La Puebla de Montalbán area, a mixed forest where the relative abundance of both oak species is similar. However, we were not able to identify any morphological hybrid in the two other stands dominated by a single oak species. This preliminary finding suggests that bidirectional introgression is particularly important when both parental species are equally represented, although further studies are required to properly disentangle the impact of the relative abundance of the two oak species on their rates of interspecific gene flow (Currat *et al.* 2008; *e.g.*, Burgarella *et al.* 2009; Lepais *et al.* 2009). The fact that six out of the seven morphologically purebred individuals with signatures of genetic introgression showed holm oak morphotypes suggests that directionality of backcrosses and introgression differ between the two studied species (Fig. 1). Hence, extensive sampling covering a more significant proportion of the species range should help to resolve these questions in the future (*e.g.*, Burgarella *et al.* 2009; Lepais *et al.* 2009).

Overall, we found that hybridisation between kermes and holm oak seems to be a relatively common phenomenon that often results in detectable individuals with intermediate morphology. Several apparently purebred individuals showed signatures of genetic introgression, suggesting that the analysed molecular markers are more appropriate to study hybridisation and introgression between kermes and holm oaks than morphological criteria. This study indicates that holm and kermes oak offer an interesting model system to study hybridisation dynamics and opens the possibility to address a number of interesting questions on the ecology and evolution of these two taxa, which often dominate vast areas of the Mediterranean landscape. Future experimental pollinations, detailed monitoring of species phenology, and assessment of pollination direction through extensive paternity analyses may help to identify relevant barriers to interspecific gene flow (Williams *et al.* 2001; Varela *et al.* 2008; Salvini *et al.* 2009). Further empirical research should also focus on studying the expression of differential life history traits in hybrids (*e.g.*, 2-year *versus* 1-year maturation of acorns in kermes and holm oak, respectively) and hybrid performance under contrasting environmental conditions (Arnold 1997; Gugerli *et al.* 2007).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Results of genetic assignment based on Bayesian method implemented in the program STRUCTURE (a) excluding the QpZAG36 marker and (b) excluding the QpZAG46 marker.

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