

Leaf morphology displays no detectable spatial organisation in the relict *Quercus afares* Pomel compared to the co-occurring parental species *Q. canariensis* Willd. and *Q. suber* L

Samah Mhamdi · Oliver Brendel · Pierre Montpied ·
Hanah Ghoul-Amimi · Ibrahim Hasnaoui · Erwin Dreyer

Received: 4 February 2013 / Accepted: 1 July 2013 / Published online: 23 July 2013
© INRA and Springer-Verlag France 2013

Abstract

• **Context** Oak species display a large within-species diversity combined with significant gene flow between species. *Quercus afares* Pomel is an endemic species that grows as a small relict population in Northern Tunisia, together with *Quercus suber* L. and *Quercus canariensis* Willd. It is considered to be a fixed hybrid between the two latter.
• **Aims** We tested (1) whether this population of *Q. afares* is declining with respect to the neighbouring populations of the

two other species and (2) whether the spatial distribution of leaf morphology is random among individuals within this small population of *Q. afares* or influenced by the neighbouring species.

• **Methods** Spatial distribution and diameter at breast height were recorded in the population of *Q. afares*. We identified two perpendicular transects across the population reaching from areas dominated by *Q. canariensis* to areas dominated by *Q. suber* and defined 20 plots along each. Eleven traits

Handling Editor: Bruno Fady

Contribution of the coauthors Samah Mhamdi did all the measurements, sampling and data analyses and wrote a first version of the manuscript. Erwin Dreyer supervised the PhD, co-designed the study and coordinated the preparation of the manuscript. Pierre Montpied designed the data analysis model and contributed to the writing of the manuscript. Oliver Brendel co-supervised the research and contributed to writing the manuscript. Ibrahim Hasnaoui co supervised the PhD, suggested the topic, identified the population of *Q. afares* and contributed to writing the study. Hana Ghoul contributed to the design of the study and to sampling and data analysis.

Electronic supplementary material The online version of this article (doi:10.1007/s13595-013-0312-x) contains supplementary material, which is available to authorized users.

S. Mhamdi · O. Brendel · P. Montpied · E. Dreyer (✉)
INRA, UMR1137 Ecologie et Ecophysologie Forestières,
54280 Champenoux, France
e-mail: dreyer@nancy.inra.fr

S. Mhamdi
e-mail: samehmhamdi2006@yahoo.fr

O. Brendel
e-mail: brendel@nancy.inra.fr

P. Montpied
e-mail: montpied@nancy.inra.fr

O. Brendel · P. Montpied · E. Dreyer
Université de Lorraine, UMR 1137 Ecologie et Ecophysologie
Forestières, 54280 Champenoux, France

S. Mhamdi · I. Hasnaoui
Laboratoire des Ressources Sylvo-Pastorales,
Institut Sylvo Pastoral de Tabarka,
IRESA, Tunis, Tunisia

I. Hasnaoui
e-mail: hasnaoui.brahim@iresa.agrinet.tn

H. Ghoul-Amimi
Faculté des Sciences de Bizerte, Université de Carthage,
Tunis, Tunisia
e-mail: hanaghouil@yahoo.fr

were assessed on 10 leaves collected from the upper crown of trees from the three species. A multiple correspondence analysis and an ANOVA were used to test for within and between-species diversity in leaf morphology.

• **Results** *Q. afares* individuals occurred at a low frequency among the two other species. Nevertheless, *Q. afares* showed no particular evidence of decline with respect to the two other species. Leaf traits displayed a large inter-specific variability with very little overlap between *Q. afares* and *Q. suber* and some between *Q. afares* and *Q. canariensis*. A high level of diversity was detected for these traits within *Q. afares*, but we were unable to unravel any spatial organisation of this diversity; in *Q. afares*, leaf traits varied independently of the presence of neighbouring *Q. suber* or *Q. canariensis*.

• **Conclusion** *Q. afares* seems to be a fixed species with specific leaf traits that are independent of that of the species of the neighbouring trees. This population is not suffering a detectable trend towards decline except for the lack of any visible regeneration below any of the three species.

Keywords Endemic oak species · Hybridization · Leaf morphology · Mixed oak stands

1 Introduction

In plant populations, interspecific hybridization is a common feature, and may include backcrosses and complex hybrid forms. Its occurrence depends on genetic, physiological and environmental characteristics (Howard et al. 2003). Such processes are very commonplace in the genus *Quercus*, in which the precise delineation of the large number of species is still a matter of fierce debate (Howard et al. 1997; Grant 1981; Nixon 1993; Rusthon 1993; Petit et al. 2003; Muir and Schlötterer 2005). Indeed, oaks represent an example of a taxonomic group in which individual species maintain distinct morphological and ecological identities despite extensive hybridization and introgression (Grant 1981; Curtu et al. 2007; Lepais and Gerber, 2010). The hybrids, detected from molecular markers and morphological traits, remain morphologically distinct in their allopatric and sympatric distributions like in the case of *Quercus crassifolia* and *Quercus crassipes* Humb. (Tovar-Sanchez and Oyama 2004) or of *Quercus affinis* Scheidw. and *Quercus laurina* Humb. and Bonpl. (González-Rodríguez et al. 2004).

The most important timber-producing oak species have been studied in detail with respect to their ecology, genetics and productivity. There are nevertheless still many minor, endemic or hybrid species that received much less attention although they may provide interesting insights into the processes of hybridisation, speciation and the occurrence of reproductive barriers among species. Moreover, the small habitat range of endemic species often leads to a high risk

of extinction (Rodrigues et al. 2006) while they contribute significantly to biodiversity hotspots and protected areas (Myers et al. 2000; Zurlini et al. 2002).

This is particularly true for endemic species growing in tightly mixed stands where major gene flow may be expected through pollen dispersal. *Q. afares* Pomel is one such endemic oak species. It is distributed in the North African Atlas mountains, with very localised populations in Northern Tunisia (approximately 750 individuals) and in North Eastern Algeria (12,000 ha; Quezel and Santa 1962). It usually grows in mixed stands with zeen oak *Quercus canariensis* Willd. and cork oak *Quercus suber* L. A few monospecific populations were described between 1,000 and 1,300 m elevation in areas that had been burnt in the Akfadou mountains in Algeria (Boudy 1959). The distribution of *Q. afares* is severely constrained both in Algeria and Tunisia, in a poorly recognised biodiversity hotspot, made of forests, mountains and coastal ecosystems and threatened by human activity (Véla et al. 2007).

Q. afares is defined as displaying a similar albeit thinner bark than cork oak, and deciduous but smaller and narrower leaves than zeen oak. It is therefore rather easy to distinguish from the co-occurring species (Maire 1961; Tutin et al. 1993, see Electronic supplementary material (ESM) Fig. 1). It was frequently hypothesised that *Q. afares* is a fixed hybrid between *Q. suber* and *Q. canariensis*. This was recently confirmed by a genetic analysis combining cpDNA structure and several allozymes (Mir et al. 2006; Welter et al. 2012). Moreover, the two populations of *Q. afares* in Algeria and in Tunisia consistently displayed similar alleles which confirm that the species is probably stabilised and no longer exchanging genes with the parent species. Another important observation was that the alleles from *Q. suber* dominated and that the chlorotype of *Q. afares* was the same than that of *Q. suber*, which would imply that *Q. suber* was the mother species. The terpene emission patterns of adult trees in the three species showed a clear divergence in *Q. afares* with respect to the two parent species, with a smaller monoterpene emission than in *Q. suber* and no isoprene emission while *Q. canariensis* emits large amounts (Welter et al. 2012).

In Tunisia, the presence of *Q. afares* is currently restricted to a unique population in the forest of Ain Zena (36°43'N, 8°51'E, 950 m elevation) with about 750 trees remaining in a mixed coppice with a few high standards together with *Q. canariensis* and *Q. suber* (Hasnaoui 1992). Little is known about this very small population, except that the stand was heavily logged during the 40 s to provide timber wood. No specific management was done since then, and the stand, although protected as a natural reserve, is still grazed by cattle and only very little regeneration is visible (unpublished observations). This population was part of the samples used by Mir et al. (2006) for genetic analyses. The small number

of *Q. afares* individuals in Tunisia makes it a taxa in need of a priority conservation program.

Initially, hybridization was detected in oaks on the basis of morphological traits (Cottam et al. 1982; Rusthon 1993). Given that oaks bear fruits at rather late stages, leaf morphology is commonly used as a tool for the analysis of phenotypic variability within and among species to identify morphological units, particularly when combined with multivariate statistical analyses (Jensen 1988, 1989). Leaf morphology was in particular used to demonstrate the occurrence of clear phenotypic differences between *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. on a sample of 2,000 trees across Europe (Dupouey and Badeau 1993; Kremer et al. 2002). Although the ranges of single variables widely overlapped between taxa, multivariate analyses with a combination of traits resulted in two clusters with very little overlap. This suggests the occurrence of only a small number of hybrids. At the same time, at molecular level, differences in allelic frequencies lead to significant inter-specific differentiation only when a large number of nuclear markers are taken into account (Kremer et al. 2002).

Leaf morphology has been frequently used to evaluate the patterns of relatedness among populations across geographical gradients. This was done for instance in two Mexican red oaks (*Q. affinis* Scheidw. and *Q. laurina* Humb.) in which leaf morphology combined with RAPD markers revealed an introgression at the contact zone (González-Rodríguez et al. 2004; González-Rodríguez and Oyama 2005). Likewise, a multivariate analysis of morphological traits revealed a clear distinction and small overlap between *Quercus alnifolia* Poech and *Quercus coccifera* L. in a mixed stand on Cyprus. These two species were also clearly separated in terms of chloroplast DNA haplotypes (Neophytou et al. 2011). Leaf morphology in a mixed oak stand of south-western France was able to provide clear differences among four co-existing species (*Q. petraea*, *Q. robur*, *Quercus pubescens* Willd. and *Quercus pyrenaica* Willd.) that had been unambiguously identified by nuclear markers (Viscosi et al. 2009).

Our aim was therefore to investigate the status of this small population of *Q. afares* relative to their parent species, in order to find out whether it is a declining population subjected to the competition of the dominant *Q. canariensis* and *Q. suber*, and whether the leaf traits of individual trees in this population are homogenous, randomly distributed or influenced by the neighbouring species.

To document these two questions, we mapped the whole *Q. afares* population. It was spreading along a slope from a low area dominated by *Q. canariensis* to an upper area dominated by *Q. suber*. We therefore identified two perpendicular gradients across the population, one along the slope and the second perpendicular to the first and defined 20 plots along each. In each plot, we assigned trees to one of the three species based on trunk and leaf phenology. We recorded the

absolute frequency of the three species and the diameter at breast height of each tree. We sampled 10 leaves on a subsample of 27–62 trees per species, described their morphology with 11 variables and used a multivariate analysis to describe the inter- and intra-specific variability. We finally tested whether there was a correlation between (1) spatial position of the *Q. afares* individuals with respect to neighbouring species and (2) leaf morphology.

2 Material and methods

2.1 Study area

The study site is located in the forest of Ain Zena, Tunisia (47 ha, 36°43'N, 8°51'E, 950 m elevation) at the southern edge of the large forest of Ain Draham, dominated by cork and zeen oaks. The site is currently managed as a natural reserve, after heavy logging during the mid-twentieth century. Most of the existing trees are coppiced stems. The soil is in general very acidic, developed on Oligocene ferruginous sandstone.

2.2 Field data collection

Field identification of species was established from the stem shape, the shape of acorn, and bark texture (ESM Fig. 1): (1) *Q. suber* has a rather short trunk with a very thick bark with deep longitudinal cracks, ovoid acorns with a short point in a large-scaled cup; (2) *Q. canariensis* has a dark grey rugged bark, thinner than cork oak, cylindrical acorns in an almost sessile cup showing short, thick coalescent scales (Pottier-Alapetite 1979); (3) *Q. afares* has slender stems, a thinner dark red–brown bark with thin cracks, sub-cylindrical light red-brown and longitudinally stripped acorns in two to five groups of pedunculate cups covered with spreading to curling scales (Maire 1961). As the stand was long managed as a coppice, “tree” is to mean a group of stems growing from a common stump among which the dominant one, i.e., with the greatest diameter at breast height (DBH), was retained. Each tree was mapped with a GPS (Garmin 12, Garmin, Kansas City, KS, USA) with a precision of about 10 m and reported on a geographic information system Arcview 3. 2 (ESRI; www.esri.com). The whole population covered about 10 ha.

Figure 1 shows the precise spatial distribution of the 750 individuals of *Q. afares* along a 100 m elevation gradient. Two perpendicular transects were defined across the population, (1) along the main slope (roughly along an east–west gradient) and (2) along a mid-elevation line (roughly along a north–south gradient). Twenty and 19 circular plots were selected along each gradient. They were about 30 m apart, covered 500 m² each and centred on *Q. afares* trees. They contained on the average 35 trees, all three species

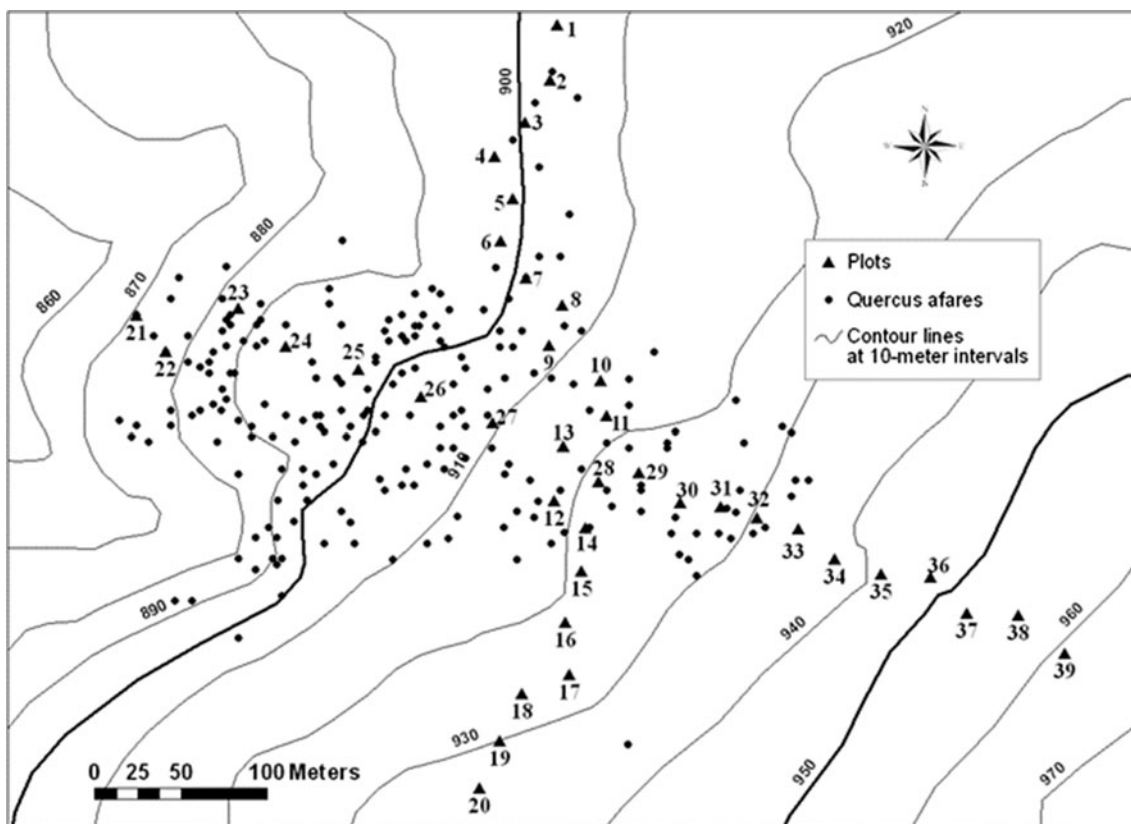


Fig. 1 Spatial distribution of (1) all *Q. afares* trees in the investigated stand (dots) and (2) the 39 observation plots (triangles) along two transects. The background originates from the topographical map of Tunisia, Zahrat Madyane 17 SW

confounded and a complete census of these trees was used to compute a presence frequency for each species in each plot.

For the analysis of the frequency distribution of diameter at breast height, *Q. canariensis* and *Q. suber* trees were sampled only in the transect plots, whereas *Q. afares* individuals were sampled over the whole stand. Measured trees were ascribed to one of the three species based on the phenotypic criteria defined above.

2.3 Leaf morphology

Between eight to ten well-developed and intact sun leaves were harvested on two to three twigs per tree during June 2009. We sampled 50 *Q. afares*, 62 *Q. canariensis* and 27 *Q. suber* trees. Leaves were digitised with a flat scanner (HP scan jet 5400c, Hewlett-Packard) at a resolution of 300 dpi. Digitised pictures were treated with the Image J software

Table 1 List of the morphological traits recorded from the leaves of *Q. afares*, *Q. suber* and *Q. canariensis*. See also Fig. 1 for a graphical display of the traits

Morphological trait	Symbol in Fig. 2.	Units
Leaf area	D	cm ²
Lamina length	LL	cm
Feret: longest distance between any two points along the leaf	F	cm
Maximal lamina width	LW	cm
Length/maximal width	LL/LW	
Aspect ratio: major axis/minor axis of fitted ellipse	Aspect	
Distance between lamina base and the largest width, relative to lamina length	RHei	
Solidity: area/convex area (area of the convex polygon including the leaf)	D/C	
Circularity: 4π (area/perimeter) ² ; 1, perfect circle; 0, straight segment	Circ	
Roundness: $4 \times \text{area} / (\pi \text{ perimeter}^2)$	Round	
Symmetry	Q/R	

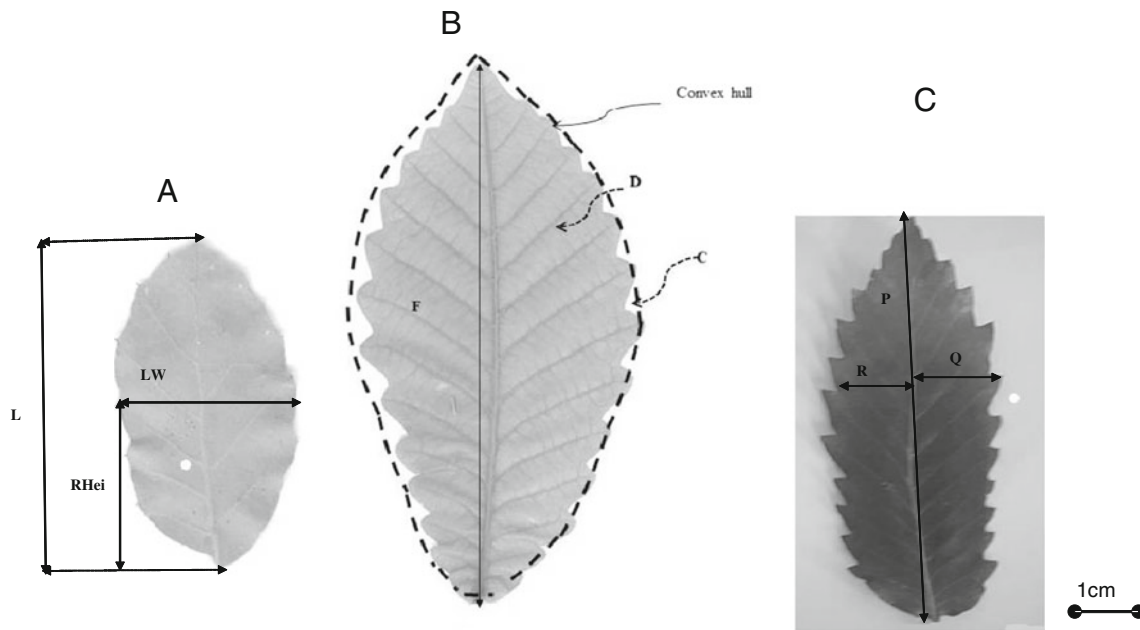


Fig. 2 Typical leaves of *Q. suber* (a), *Q. afares* (b) and *Q. canariensis* (c). The leaf trait used for morphological measurements are indicated. See Table 1 for a detailed description of the traits

(version 1. 43, <http://rsbweb.nih.gov/ij/features.html>) and the traits reported in Table 1 and Fig. 2 were measured and computed to describe form and dimensions.

2.4 Statistical analyses

Morphology traits were compared individually among species with an ANOVA analysis followed by a post hoc Tukey's HSD test (R software, version 2. 14. 0). Variables were normalised, when required, using logarithm, arcsinus (square root) or exponential transformations (Table 2). When

present, heretoscendasticity was modelled as a power function of predicted values.

The morphology traits were then jointly analysed with a multiple correspondence analysis (MCA) in order to characterise their joint within- and among-species variations. Each variable was divided into 15 classes of nearly equal weight (Kremer et al. 2002).

Spatial structure of *Q. afares* leaf morphology along each transect was assessed by Mantel tests of correlation between the geographical distance matrix of the trees and morphological Euclidian distance matrices calculated from either

Table 2 Confidence intervals of the mean (CI 95 %) and results of a one-way ANOVA (*F* statistics and *p* value) for the leaf traits recorded on 10 leaves of individuals of *Q. canariensis*, *Q. suber* and *Q. afares*. Values followed by the same letter are not significantly different at the 5 % level (*n*=139 trees)

Variable	Transformation	<i>Q. canariensis</i>	<i>Q. afares</i>	<i>Q. suber</i>	<i>F</i> (1, 116)	<i>p</i>
Leaf area	log	31.4–35.6 a	16.4–19.15 b	8.2–10.1 c	264, 2	<0.0001
Length	log	90.1–97 a	73.5–80.2 b	44.5–50.1 c	193, 3	<0.0001
Feret	log	89.8–96.9 a	73.4–80 b	44.6–50 c	193, 1	<0.0001
Width	log	51.3–55.2 a	32.1–34.8 b	25.9–28.8 c	325, 2	<0.0001
Length/width	log	1.7–1.8 b	2.2–2.4 a	1.64–1.8 b	75, 18	<0.0001
Aspect ratio	log	1.75–1.9 b	2.3–2.5 a	1.64–1.83 b	69, 43	<0.0001
RHei	log	0.48–0.52 ns	0.46–0.5 ns	0.47–0.52 ns	0, 78	0.45
Solidity	exp	0.92–0.93 c	0.93–0.94 b	0.95–0.97 a	56, 1	<0.0001
Circularity	exp	0.42–0.46 b	0.42–0.46 b	0.58–0.63 a	64, 51	<0.0001
Roundness	log	0.53–0.57 a	0.4–0.44 b	0.54–0.61 a	69, 47	<0.0001
Symmetry	arcsin (square root)	0.86–0.89 a	0.82–0.85 b	0.85–0.88 a	13, 75	<0.0001

raw mean morphological traits per tree or mean first three dimensions coordinates from MCA.

3 Results

3.1 Structure of the *Q. afares* population

The population of *Q. afares* expands from the bottom of the slope, where it is mixed with a dense population of *Q. canariensis*, up to the top of the hill where it is mixed with *Q. suber* (Fig. 1). The 39 plots comprised 106 individuals of *Q. afares*, 1,026 of *Q. canariensis* and 256 of *Q. suber* (total, 1,388 individuals). The relative frequencies of the species in the plots along the two gradients (Fig. 3) show that (1) *Q. afares* is always severely dominated, in terms of frequencies, by the companion species (it never represents more than 30 % of the individuals in a plot); (2) the transition from the *Q. canariensis* dominated to the *Q. suber*-dominated area is very steep. *Q. suber* is absent from the bottom plots, and the plots on top of the hills contain a less dense mix of *Q. suber* and *Q. afares*.

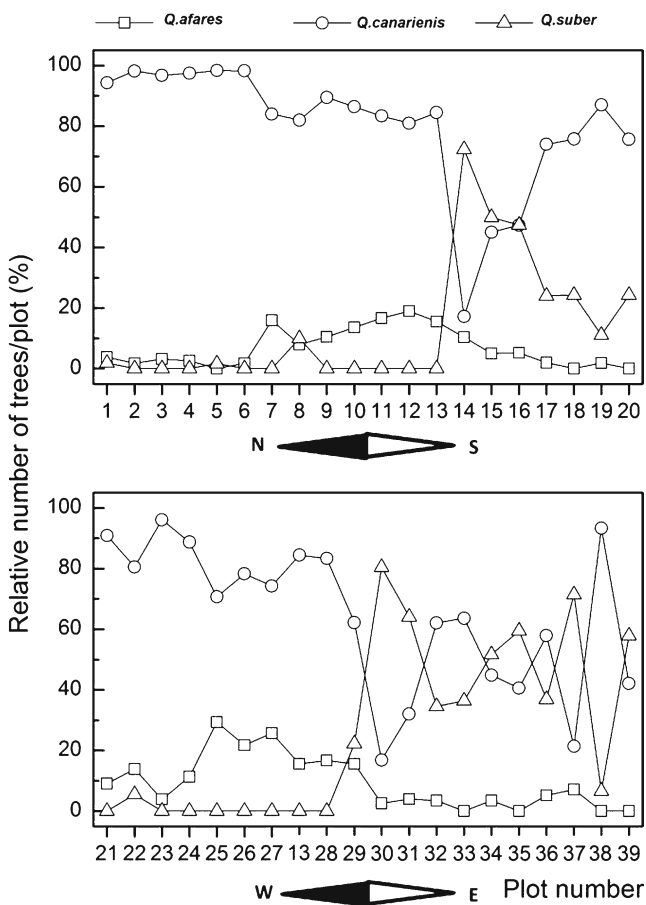


Fig. 3 Relative frequency of trees from the three species (*Q. afares*, *Q. canariensis* and *Q. suber*) in the 39 plots along two transects (along the slope, E–W and at mid elevation, N–S) across the Ain Zena forest. Successive plots were about 30 m apart

The distribution of the diameters at breast height was uni-modal and very similar in the three species (Fig. 4) with a mode between 10 and 15 cm. This confirms that (1) the stand is a rather young coppice with a very small number of older standards with large diameters (there are a few large *Q. afares* trees) and (2) the distribution is very similar among species, which shows that *Q. afares* is not dominated by the two other species, and that the three species probably share a similar management history. The almost complete lack of small trees shows also that the stand in general is not undergoing any significant regeneration whatever the species.

3.2 Leaf morphology

We found a large morphological variability among species. The leaves of *Q. canariensis* and *Q. afares* differed with respect to leaf size (Table 2). *Q. canariensis* displayed the

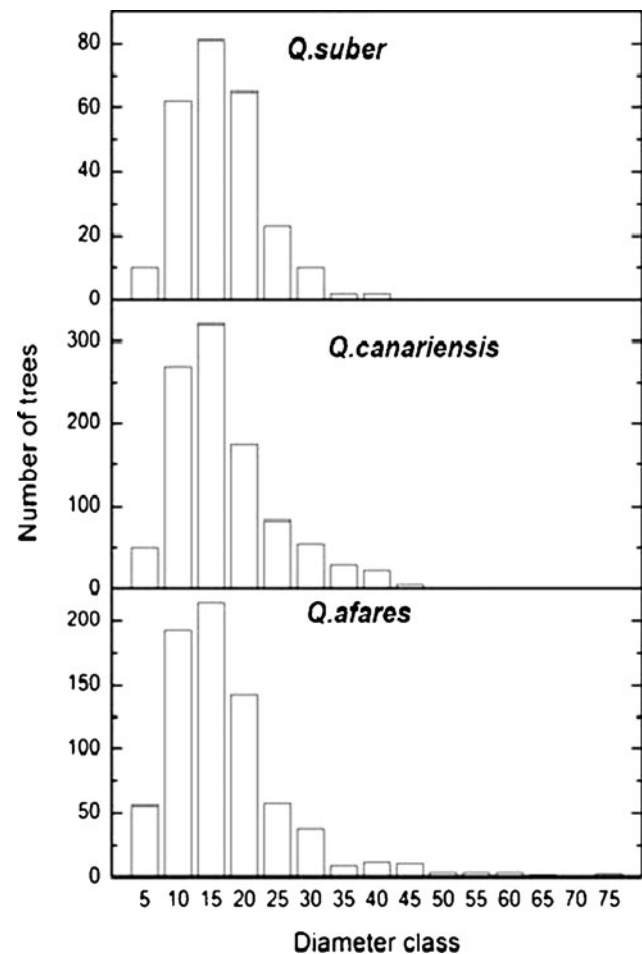


Fig. 4 Frequency distribution of the diameter classes (in centimeter) for trees from the three species (*Q. afares*, *Q. canariensis* and *Q. suber*) in the 39 plots sampled along the transect. In the case of *Q. afares*, the whole population was taken into account to reach similar sample sizes than in the two other species

largest leaves with higher leaf area, length, longest Feret distance, width and solidity than the others and *Q. suber* the smallest ones, while *Q. afares* was intermediate with respect to these traits. For shape traits like roundness, symmetry, aspect ratio and length/width, *Q. afares* differed from the two other species that were similar to each other (with smaller or larger values). Only a single trait (relative height) did not differ at all among the three species.

The projections of the individual tree centroids along with the species centroids in the first factorial plan of the multiple correspondence analysis (MCA) are shown in ESM Fig. 2. The distribution of discrete values (15 classes for each trait) along the two first axes of the MCA (ESM Fig. 2) shows that axis 1 separates mainly the individuals according to leaf size (from small to intermediate), to roundness and to the occurrence of small lobes. Axis 2 discriminates individuals with large leaves and a slightly elongated silhouette at the lowest end.

The projection of individual trees into the first factorial plane of the MCA (Fig. 5) yielded three groups of distribution of individuals corresponding to the three species with a clear distinction between them. Thus, *Q. suber* and *Q. canariensis* differed widely and were completely disjointed from each other. *Q. afares* to some extent bridged the two other species, few individuals being closer to *Q. suber* and others to *Q. canariensis*, with a rather large variability. The morphological variability in *Q. suber* was lower than in the two other species.

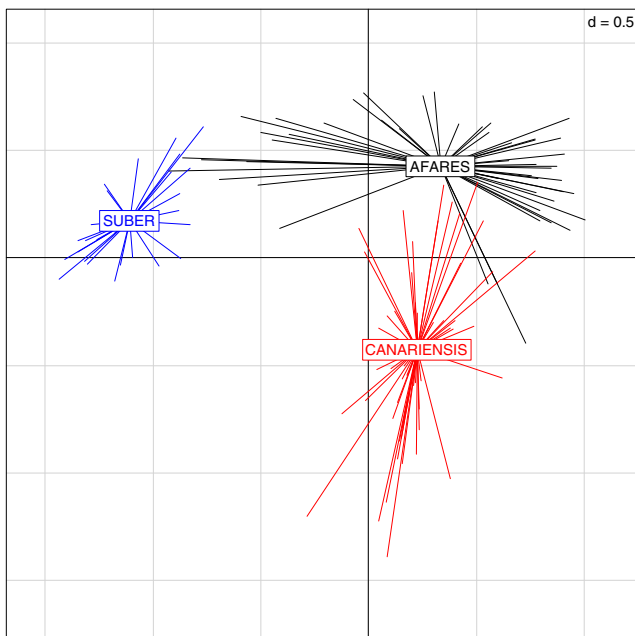


Fig. 5 Distribution of individual trees from the three species along the two first axes of the multiple correspondence analysis. Trees (average of 10 leaves) are represented by a vector pointing from each species centroid to each tree centroid

None of the Mantel tests evidenced any significant spatial structure of *Q. afares* leaf morphology along both transects ($r=0.03$, $p=0.29$). That is, the morphological closeness of *Q. afares* to either neighbouring other two species, which are distributed at each end of the transects was not established.

4 Discussion

4.1 Status of the *Q. afares* population

The relict population of *Q. afares* analysed here in northern Tunisia is spread over a rather small area in a forest dominated by *Q. canariensis* at the bottom of the slope and *Q. suber* at the top. It displays some very consistent features. *Q. afares* individuals were mostly dispersed among *Q. canariensis*, and much less among *Q. suber* individuals. *Q. afares* was mainly present in the bottom of the slope where soils are deeper with a larger water reserve. *Q. afares* individuals were always a minority and did never build homogeneous population patches.

The stand consists mostly of small coppiced stems with DBH below 25 cm in the whole area. The fact that DBH distributions were very similar among species, and that only a few isolated individuals displayed larger diameters shows that most individuals were coppiced and re-sprouted and that only few standards were kept. The area was heavily logged in the 1940s and underwent severe fires at that time. As a result, the stumps of the three species re-sprouted simultaneously and the stand evolved into a rather even coppice with no dominant species. The three species probably underwent the same history. Moreover, the DBH distributions show that *Q. afares* is not currently undergoing a specific decline process and that its population displays a similar status than that of the co-occurring species.

The lack of natural regeneration in any of the three species is probably due to the lack of shrubs which would provide physical protection against predators, and a large anthropic pressure due to herds of cattle, sheep and goats that are repeatedly grazing the understory, despite the status of a natural park given to the area.

Our observations do not point to a decline process specific to *Q. afares*. This species is thought to display a smaller competitive ability with respect to the two other species; extensive studies in Algeria identified lower growth dynamics (i.e., smaller annual increment rings) for *Q. afares* with respect to *Q. canariensis* in different populations of deciduous oaks (Messaoudène and Tessier 1997). Also, at least at seedling stage, *Q. afares* displayed a larger sensitivity to drought compared to *Q. suber*, *Quercus faginea* Lam. and *Quercus ilex* L. (Acherar et al. 1991). As a conclusion, our data suggest that this population of *Q. afares* is not at a stage of acute decline. It nevertheless remains a very small

population under the form of an ageing coppice with no evident regeneration. Efficient management measures (fencing, thinning, soil preparation ...) would be needed as a first step, to enable regeneration and in situ conservation of this endangered population.

The other important question addressed here is whether the present population of *Q. afares*, a supposedly fixed hybrid between *Q. suber* and *Q. canariensis*, contains first generation hybrids. In other words, during the parental generation of the present trees, did *Q. afares* exchange genes with neighbouring individuals from *Q. suber* and *Q. canariensis*, or is the *Q. afares* population homogenous with features delineating a phenotype that significantly differs from that of the neighbouring ones?

4.2 Variability of leaf morphology among and within species

In our study, the multifactorial analysis of leaf size and shape traits produced a distribution along a parabolic gradient in the MCA planes, reaching from small and round leaves (*Q. suber*) and one end to large and lengthy ones (*Q. canariensis*) and the other end. *Q. afares* leaves in most cases displayed an intermediate morphology. Leaves of *Q. suber* and *Q. canariensis* could be unambiguously discriminated from each other, while those of *Q. afares* were in some cases overlapping with those of the two other species. This overlap was of limited extent, supporting the hypothesis that introgression was low, at least as detected from morphological traits.

These results are quite similar to those found for other oak species complexes. Tovar-Sanchez and Oyama (2004) have shown in Mexico that *Quercus dysophylla*, a hybrid between *Q. crassifolia* and *Q. crassipes*, presents leaves of intermediate morphology with respect to the parent species that are clearly distinct. Likewise, *Quercus crenata* Lam. is a putative hybrid between the sympatric *Quercus cerris* and *Q. suber*; nevertheless, its leaf morphology, although intermediate between that of the two parents, is closer to that of *Q. suber*. Interestingly, the molecular and biochemical evidence provided by Bellarosa et al. (2005) showed that *Q. crenata* is more closely related to *Q. suber*. *Q. crenata* is considered a relic of ancient hybridization (Cristofolini and Crema 2005).

Our results also suggest that *Q. afares* has maintained a large diversity in leaf morphology over successive generations and that morphological extremes might be trees resulting from more recent hybridization events between *Q. afares* and one of the parent species. However, it is interesting to note that a more detailed analysis (ESM Fig. 2) and Fig. 5 indicated that *Q. afares* was intermediate between the supposed parental species for leaf size, whereas it differed from the two parents for shape traits, for example with lengthier and narrower leaves than either of the parental species. Similar results have been reported by Gailing et al. (2012) in *Quercus rubra* L. and *Quercus ellipsoidalis* E. J.

Hill, that hybridise when co-occurring, or by Viscosi et al. (2012) in Italian oaks, for which the use of leaf shape allowed for an accurate separation between those species.

Mir et al. (2006) used Nei's distances of cpDNA and allozymes to state that *Q. afares* displays a larger genetic distance from *Q. canariensis* than from *Q. suber*, thereby identified as the maternal parent. Our assessment of leaf morphology resulted in similar phenotypic distances from both potential parents. In contrast, many other cases of hybrids with a leaf morphology closer to that of one of the parents have been reported, like in the case of controlled inter-specific crosses between *Q. petraea* and *Q. robur*; the morphology of the hybrids was much closer to that of the maternal parent (Kleinschmit et al. 1995). Similarly, Neophytou et al. (2007) showed that hybrids between *Q. alnifolia* and *Q. coccifera* displayed a leaf morphology closer to that of the maternal parent *Q. alnifolia*.

4.3 Spatial structuration of morphological leaf traits

The Mantel tests revealed no correlation of leaf morphology with spatial distribution of individual trees along the two transects. The fact that *Q. afares* trees were spatially closer to either *Q. suber* or *Q. canariensis* had no real impact on their leaf morphology. At a larger spatial scale, Tovar-Sanchez and Oyama (2004) showed that the spatial proximity of hybrid individuals to the allopatric areas of parental species increased their morphological and genetic similarity.

The lack of a structured spatial distribution of leaf morphology in *Q. afares* suggests also a lack of recent gene flow among the species in the previous generation. Several factors nevertheless have to be taken into account in this discussion: (1) the stand is a coppice and has probably been managed as such also for previous tree generations; albeit we have no precise historical records, we believe that the human pressure on these forests was quite ancient (Quezel and Bonin 1980; Hasnaoui 1992); this situation leaves little space for regeneration and therefore for phenotypic evidence of gene flow; nevertheless we cannot exclude the occurrence of an active gene flow. (2) The spatial distance between trees of the different species is small and certainly below the pollen dispersal distance, hybridization should therefore be possible; however other barriers to gene flow may occur. Flowering periods of the three species do not overlap; they follow usually the chronology *Q. canariensis* followed by *Q. afares* and finally by *Q. suber* (personal observations).

5 Conclusion

The risk of extinction of this small population of *Q. afares* at Tunisian scale seems limited at least in the near future, as the demographic status of the remaining individuals was very

close to that of the co-occurring *Q. suber* and *Q. canariensis*. The complete lack of regeneration for any of the three species nevertheless creates a real threat for the future of this relict species in Tunisia. Leaf morphology of *Q. afares* revealed a high level of phenotypic variability, similar to that in *Q. canariensis* and *Q. suber*, despite the small size of the population. There was only little overlap between species showing that three distinct phenotypes occurred and corresponded to the three species with a rather sharp delimitation. Finally, there was no spatial structuration of leaf morphology within the population, irrespective of the distance to the neighbouring species. This comes in support to the idea that gene flow during the previous generation between the parent species and *Q. afares* was limited and that there is no active hybridisation process underway, resulting in second- and higher-degree hybrids, which would gradually lead to the extinction of the *Q. afares* population. But a firm conclusion about this point would require an extensive molecular study to evaluate the hybrid status of the different *Q. afares* trees.

Acknowledgments The technical support of Institut National de Recherche du Génie Rural et des Eaux et Forêts and of Institut Sylvopastoral (Tunis, Tabarka) for field work and leaf collection is gratefully acknowledged. We also extend many thanks to Fadal Megdich and Abdnbi Nadderi for useful help in cartography. This work is part of the PhD dissertation of SM, who was partly supported by a bilateral cooperation project between France and Tunisia (Comité Ministériel de Coopération Universitaire). M Ksontini (INRGREF, Tunis) provided practical support to the project. Fruitful discussions with Jean Luc Dupouey (INRA Nancy) are gratefully acknowledged.

Funding Financial support for this study was provided by INRA and by INRGREF, as well as by a bilateral cooperation project between France and Tunisia (Comité Ministériel de Coopération Universitaire).

References

- Acherar M, Rambal S, Lepart J (1991) Évolution du potentiel hydrique foliaire et de la conductance stomatique de quatre chênes méditerranéens lors d'une période de dessèchement. *Ann Sci For* 54:347–358
- Bellarosa R, Simeone M, Papini A, Schirone B (2005) Utility of ITS sequence data for phylogenetic reconstruction of Italian *Quercus* spp. *Mol Phylogenet Evol* 34:355–370
- Boudy P (1959) Guide du forestier en Afrique du Nord. La Maison Rustique, Paris, pp 172–184
- Cristofolini G, Crema S (2005) A morphometric study of the *Quercus crenata* species complex (Fagaceae). *Bot Helv* 115:155–167
- Cottam WP, Tucker JM, Santamour FS (1982) Oak hybridization at the University of Utah. State Arboretum of Utah, Salt Lake City, Utah, USA
- Curtu AL, Gailing O, Finkeldey R (2007) Evidence for hybridization and introgression within a species-rich oak (*Quercus* spp.) community. *BMC Evol Biol* 7:218–233. doi:10.1186/1471-2148-7-218
- Dupouey JL, Badeau V (1993) Morphological variability of oaks (*Q. robur* L., *Q. petraea* (Matt) Liebl., *Q. pubescens* Willd.) in northeastern France: preliminary results. *Ann Sci For* 50:35–40
- Gailing O, Lind J, Lilleskov E (2012) Leaf morphological and genetic differentiation between *Quercus rubra* L. and *Q. ellipsoidalis* E.J. Hill populations in contrasting environments. *Plant Syst Evol* 298:1533–1545
- González-Rodríguez A, Arias DM, Valencia S, Oyama K (2004) Morphological and RAPD analysis of hybridization between *Quercus affinis* and *Q. laurina* (Fagaceae), two Mexican red oaks. *Am J Bot* 91:401–409
- González-Rodríguez A, Oyama K (2005) Leaf morphometric variation in *Quercus affinis* and *Q. laurina* (Fagaceae), two hybridizing Mexican red oaks. *Bot J Linn Soc* 147:427–435
- Grant V (1981) Plant speciation. Columbia Univ. Press, New York
- Hasnaoui B (1992) Chênaies du nord de la Tunisie: Ecologie et régénération. Thèse Université de Provence Aix –Marseille I, 202p
- Howard DJ, Preszler RW, Williams J, Fenchel S, Boecklen WJ (1997) How discrete are oak species? Insights from a hybrid zone between *Quercus grisea* and *Quercus gambelii*. *Evolution* 51:747–755
- Howard DJ, Britch SC, Braswell WE, Marshall JL (2003) Evolution in hybrid zones. In: Singh RS, Uyenoyama MK (eds) The evolution of population biology. Cambridge University Press, Cambridge, pp 297–314
- Jensen RJ (1988) Assessing patterns of morphological variation of *Quercus* spp. in mixed-oak communities. *Am Midl Nat* 120:120–135
- Jensen RJ (1989) The *Quercus falcata* Michx. complex in land between the lakes, Kentucky and Tennessee: a study of morphological variation. *Am Midl Nat* 121:245–255
- Kleinschmit JRG, Bacilieri R, Kremer A, Roloff A (1995) Comparison of morphological and genetic traits of pedunculate oak (*Q. robur* L.) and sessile oak (*Q. petraea* (Matt.) Liebl). *Silvae Genet* 44:256–269
- Kremer A, Dupouey JL, Deans JD, Cottrell J, Csaikl U, Finkeldey R, Espinel S, Jensen J, Kleinschmit J, Van Dam B, Ducouso A, Forrest I, de Heredia UL, Lowe AJ, Tutkova M, Munro RC, Steinhoff S, Badeau V (2002) Leaf morphological differentiation between *Quercus robur* and *Quercus petraea* is stable across western European mixed stands. *Ann For Sci* 59:777–787
- Lepais O, Gerber S (2010) Reproductive patterns shape introgression dynamics and species succession within the European white oak species complex. *Evolution* 65:156–170. doi:10.1111/j.1558-5646.2010.01101.x
- Maire R (1961) Flore de l'Afrique du Nord (Maroc, Algérie, Tunisie, Tripolitaine, Cyrénaïque et Sahara). Ed Paul Lechevalier 7:117–120, Paris
- Messaoudène M, Tessier L (1997) Relations cerne-climat dans des peuplements de *Quercus afares* Pomel et *Quercus canariensis* Willd. en Algérie. *Ann For Sci* 54:347–358
- Mir C, Toumi L, Jarne P, Sarda V, Di Giusto F, Lumaret R (2006) Endemic North African *Quercus afares* Pomel originates from hybridisation between two genetically very distant oak species (*Q. suber* L. and *Q. canariensis* Willd.): evidence from nuclear and cytoplasmic markers. *Heredity* 96:175–184
- Muir G, Schlötterer C (2005) Evidence for shared ancestral polymorphism rather than recurrent gene flow at microsatellite loci differentiating two hybridizing oaks (*Quercus* spp.). *Mol Ecol* 14:549–561
- Myers N, Mittermeier R, Mittermeier C, Da Fonseca G, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature* 403:853–858
- Neophytou C, Palli G, Dounavi A, Aravanopoulos F (2007) Morphological differentiation and hybridization between *Quercus alnifolia* Poech. and *Quercus coccifera* L. (Fagaceae) in Cyprus. *Silvae Genet* 56:271–277
- Neophytou C, Aravanopoulos F, Fink S, Dounavi A (2011) Interfertile oaks in an island environment. II. Limited hybridization between *Quercus alnifolia* Poech and *Q. coccifera* L. in a mixed stand. *Eur J Forest Res* 130:623–635

- Nixon KC (1993) Infrageneric classification of *Quercus* (Fagaceae) and typification of sectional names. *Ann Sci For* 50:25–34
- Petit RJ, Bodénès C, Ducouso A, Roussel G, Kremer A (2003) Hybridization as a mechanism of invasion in oaks. *New Phytol* 161:151–164
- Pottier-Alapetite G (1979) Flore de la Tunisie. Angiospermes-Dicotylédones. Apétales-Dialypétales. Imprimerie Officielle de la République Tunisienne, Tunis, 651 p
- Quezel P, Santa S (1962) Nouvelle flore de l'Algérie et des régions désertiques méridionales. Ed du Cent Nat de la Rech Sci (CNRS) 1:264–266, Paris
- Quezel P, Bonin G (1980) Les forêts feuillues du pourtour méditerranéen : constitution, écologie, situation actuelle, perspective. *Rev For Fr* XXXII:253–268
- Rodrigues A, Pilgrim J, Lamoreux J, Hoffmann M, Brooks T (2006) The value of the IUCN Red List for conservation. *Trends Ecol Evol* 21:71–76
- Rusthon BS (1993) Natural hybridization within the genus *Quercus* L. *Ann Sci For* 50:73–90
- Tovar-Sanchez E, Oyama K (2004) Natural hybridization and hybrids zones between *Quercus crassifolia* and *Quercus crassipes* in Mexico: morphological and molecular evidence. *Am J Bot* 91:1352–1363
- Tutin TG, Burges N A, Chater A O, Edmonson J R, Heywood V H, Moore D M et al. (eds) (1993) *Flora Europaea*, 2nd edn. Cambridge University Press: Cambridge. Vol 1, pp 72–75
- Véla E, Benhouhou S (2007) Évaluation d'un nouveau point chaud de biodiversité végétale dans le Bassin méditerranéen (Afrique du Nord). *CR Acad Sci Biol* 330:589–605
- Viscosi V, Lepais O, Gerber S, Fortini P (2009) Leaf morphological analyses in four European oak species and their hybrids: a comparison of traditional and geometric morphometric methods. *Plant Biosyst* 143:564–574
- Viscosi V, Antonecchia G, Lepais O, Fortini P, Gerber S, Loy A (2012) Leaf shape and size differentiation in white oaks: assessment of allometric relationships among three sympatric species and their hybrids. *Int J Plant Sci* 173:875–884
- Welter S, Bracho-Nuñez A, Mir C, Zimmer I, Kesselmeier J, Lumaret R, Schnitzler JP, Staudt M (2012) The diversification of terpene emissions in Mediterranean oaks: lessons from a study of *Quercus suber*, *Quercus canariensis* and its hybrid *Quercus afares*. *Tree Physiol* 32:1082–1091. doi:10.1093/treephys/tps069
- Zurlini G, Grossi L, Rossi O (2002) Spatial-accumulation pattern and extinction rates of Mediterranean flora as related to species confinement to habitats in preserves and larger areas. *Conserv Biol* 16:948–963