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Morphometric Analyses of Leaf Shapes in Four Sympatric Mediterranean Oaks and Hybrids in the Algerian Kabylie Forest

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Abstract: (1) Background: local morphological variation can provide useful information to clarify the role of hybridization in Mediterranean oaks. Accordingly, we have characterized putative hybrids and oak species coexisting in a highly diversified oak forest in Algeria with four native oak species (*Quercus suber* L., *Q. ilex* L., *Q. canariensis* Willd., and *Q. afares* Pomel). (2) Methods: sixteen plots, including 89 trees from the four native species and their putative hybrids were sampled. Leaves were scanned and their geometric morphometry analyzed by using 11 landmarks on the right side on their abaxial surface. Variation within and among species, and the relationship among oak species and their hybrids were analyzed, utilizing an ANOVA and a canonical analysis using morphoJ software. (3) Results: using the geometric morphometry analysis, we observed that *Q. afares* shape is intermediate between *Q. suber* and *Q. canariensis*, being *Q. ilex* very different from the others, and that there is no overlap among them. Putative hybrids are morphologically close to *Q. afares* and, to a lesser extent, to *Q. suber* and, finally, to *Q. ilex* and *Q. canariensis*. (4) Conclusions: the study opens the field for future molecular characterization of hybrids, and for determining their role in terms of adaptation to actual and predicted future climatic conditions. The morphological proximity of hybrids to *Q. afares* demonstrate this species advantage in the area of study and its importance for the future evolution of the species in the Mediterranean.

Keywords: hybrids; morphological variation; *Quercus*

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1. Introduction

Natural hybridization and introgression are two general evolutionary factors in plants with major evolutionary implications [1] related to the appearance of new entities [2] or new variants more adapted to environments different from those of the parental species [3], or even favoring the colonization of new environments [4,5]. Therefore, hybridization and introgression have practical implications in genetic resources conservation and breeding, as they determine the value of different individuals or populations in defining those activities [6,7]. Accordingly, hybrid zones can provide a wealth of novel genetic variation of value under future climatic conditions [8].

Oaks represent good models for hybridization studies, as interspecific hybridization is a frequent mechanism related to the presence of individuals morphologically and ecologically intermediate between different oak species [9–13] at different spatial scales (local vs. broad range). Hybridization in oaks is related to the extensive sharing of organelle and nuclear genes between different species in a given population [5,14,15]. Different factors influence the frequency and the distribution of hybrids in a population, mainly, reproductive barriers and the direction of introgression [12,16–19]. The extent and pattern of hybridization can also be influenced by environmental heterogeneity, and the spatial distribution [5,20,21] related to the selective advantages of the species and hybrids.

The study of hybrids in mixed stands could be of great interest in understanding the dynamics and evolution of oaks, and different studies have already characterized the extent of hybridization and the mechanisms involved [10,11]. Phenotypic differentiation between species can provide insights into understanding the mechanisms underlying species differentiation in a given area [22]. These are of special interest under climate change scenarios, as mixed oak forests present a large phenotypic variation in morphological and physiological traits related to adaptation [23,24], derived from the large level of intrapopulation variation of the different oak species [10,24,25], and its implications in local adaptation [26].

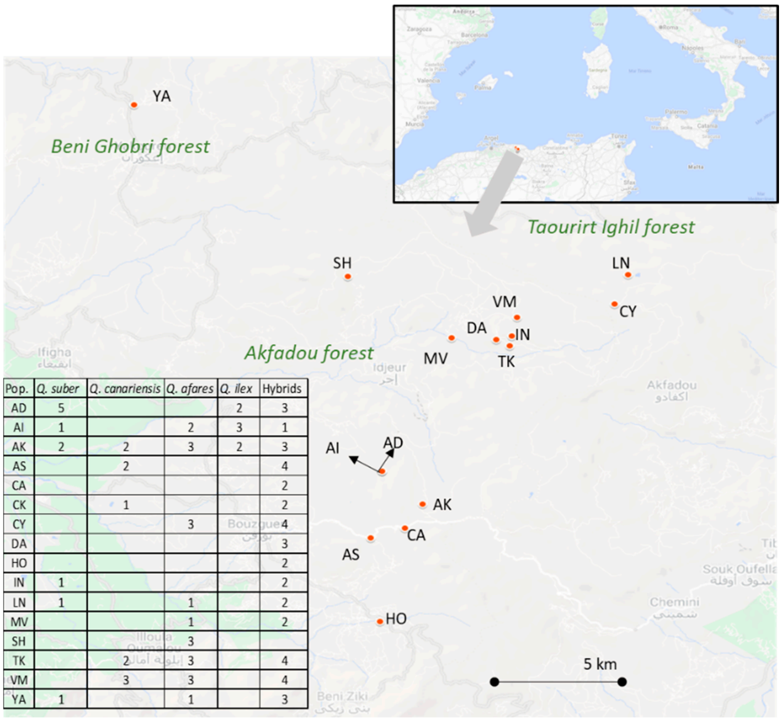
Oaks (*Quercus* spp.) are well represented throughout the Mediterranean basin, and have been described as a complex made of the species and its hybrids. High levels of intrapopulation variation and differentiation have been shown in Mediterranean oak forests [11,27]. Oaks represent an example of a Mediterranean taxonomic group in which individual species maintain morphological and ecological identities despite extensive hybridization and introgression [5,28]. The use of leaf morphology traits has allowed the analysis of the complex genetic structure in populations of this group of species [29–32]. Leaf morphological traits are highly variable among species [11,33], and can be used to discriminate among them [34]. It has been shown that a morphological classification [21,35,36] has a clear correspondence with the genetic assignment of individuals. These traits have also implications in the adaptation of the species and hybrids to different ecological conditions [37]. However, there is an ongoing critical revision at the taxonomic level, based on multidisciplinary approaches, demonstrating that extremely varied taxonomic frameworks often do not correspond to a real genetic and morphological diversity [38–41].

The Kabylie forests in Algeria is a mixed forest, which allows the analysis of leaf morphology diversity of oak species and hybrids. This particular forest consists of a mixture of sympatric species living in the same geographic area: *Quercus afares* Pomel (Afares oak, a stabilized hybrid [42]), *Q. canariensis* Willd. (Zéen oak), *Q. ilex* L. (Evergreen oak) and *Q. suber* L. (Cork oak). *Q. canariensis* is the dominant species, occupying about 45% of the wooded area; while the pure cork oak occupies 15% of the peripheral zone [43]. The endemic afares oak can be found on certain ridgelines, and is continuously distributed from the Kabylie region in Algeria to the Kroumiria region in Tunisia [43]. Additionally, pure stands occupy about 15% of the wooded area, in occasions mixed with hybrids, while mixed stands cover about 25% of the forested area of cork and afares oaks. Some *Q. canariensis* and *Q. afares* over 250 years old have been identified in many Kabylie forests sites, showing their ancestral origin [44].

Our study concerns the leaf characterization of four oak species and their hybrids in the Kabylie forest: *Q. afares*, *Q. canariensis*, *Q. ilex*, and *Q. suber*. Geometric morphometry [45] quantifies the size and shape of the leaves by analyzing the relative positions of anatomical landmarks and sets of points in them, and has a strong relationship with other morphological traits [46]. Our hypothesis is that mixed Mediterranean oak forests present a high level of inter- and intra-specific variability in leaf morphology, with hybrids increasing the level of phenotypic variation in the area. To study the pattern of phenotypic variation in these mixed stands, we analyze the geometric morphometry of the leaves of hybrids and individuals from the different oak species in the area. Finally, we discuss the implications of our results in the improvement of Mediterranean oaks management and conservation, given that phenotypic variation is the basis for natural selection operating under changing climatic conditions.

2. Materials and Methods

Sampling was carried out in 89 trees located in 16 plots in three Kabylie forests (Akfadou, Beni Ghobri and Taourirt Ighil forests) (Figure 1 and Figures S1–S6). The identification of species and hybrids was based on their taxonomical descriptions, using leaf morphological traits [34,47,48]. Leaf morphology has also been used in the characterization of hybrids in the studied area [49]. Bark has additionally been used to confirm the identification of *Q. suber* and its hybrids (Figures S1–S6). In total, we sampled *Q. afares* (20 trees), *Q. canariensis* (10), *Q. ilex* (7), *Q. suber* (11), and putative hybrids (41). The number of sampled trees differs from one plot to another depending on the presence of putative hybrid trees. From each tree, we collected 5 to 10 intact mature leaves at the same period—when leaves were fully developed (10/2015 to 11/2015)-, in the four directions of the crown and at the same height, to avoid dimorphism.



Pop.	*Q. suber*	*Q. canariensis*	*Q. afares*	*Q. ilex*	Hybrids
AD	5			2	3
AI	1		2	3	1
AK	2	2	3	2	3
AS		2			4
CA					2
CK		1			2
CY			3		4
DA					3
HO					2
IN	1				2
LN	1		1		2
MV			1		2
SH			3		
TK		2	3		4
VM		3	3		4
YA	1		1		3

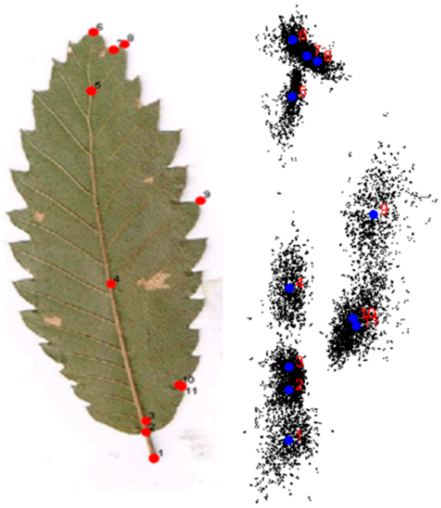
Figure 1. Geographical location of sampled populations and number of sampled trees. (Name of the locations: AD-Adrar, AI-Ait Aicha, AK-Akfadou, AS-Ait Salah, CA-Col Ait Salah, CK-Col Akfadou, CY-Cynegetic centre, DA-Damous, HO-Houra, IN-Intersection, LN-Lac noir, MV-Mehaga village, SH-Sidi El Hadi, TK-Tala Kittan, VM-Vide Mehaga, YA-Yakouren.

2.1. Morphometric Analysis

The leaves were pressed and dried (Figure S7), and the abaxial surface was scanned using a Brother scanner at a resolution of 300 DPI. The digitalized images were used to record 11 landmarks on the right half side of each leaf [45,50] (Figure 2). In this study, we assessed 532 leaves coming from the 89 sampled trees (a mean of 6 leaves per tree). We scanned two images per leaf (1064 images), and we made two assessments for each scanned image to minimize digitization errors [45,51]. These landmarks were converted into a configuration of 11 pairs of Cartesian coordinates of each leaf. The analysis was made by the software morphoJ [52].

2.2. Data Analysis

Cartesian coordinates of the landmarks were used for data analysis. We performed an outlier analysis based on the visualization of the cumulative distribution of distances of individual samples of the average form of the complete sample, showing no outliers in the data (Figure S8).



Landmark	Description
1	Start of petiole
2	Limb base
3	Intersection between the main rib and the first full secondary rib
4	Intersection between the main rib and the longest secondary rib
5	Intersection between the main rib and the last full secondary rib
6	Limb apex
7	Base of the first sinus between the apex and the last lobe(Landermark8)
8	Last lobe
9	Widest lobe
10	Base of the first sinus from the limb
11	First limb

Figure 2. Position and description of landmarks on the right side of a leaf in the Akfadou forest and generalized procrustean overlay of all samples. Average conformations (consensus) are represented by blue dots.

The data were used to test the morphological discrimination of the trees (pure and hybrid) present in the forest. Having carried out a limited genetic study in order to discriminate pure oak species in the first place, we checked the homogeneity of the leaf shape conformation in the individuals of the species and putative hybrids.

We used the variation of leaf shape hierarchically (procrustean ANOVA) distributed among leaves, trees, and species to create mean configurations at each of these levels. Leaf-level data were analyzed for leaf shape variation, tree-level data for species discrimination, and species-level data (including hybrid individuals) for multivariate analysis. Centroid size and conformation components of leaves were used for further analysis.

In a second step, a canonical analysis was applied to the individuals, excluding the hybrids, to check for differences among the species described in the Akfadou forest. Canonical analysis was used to find the morphological feature that best separates groups of specimens. The method describes, explains and predicts the membership of predefined groups (species) of a set of observations (trees) from a series of predictive variables (cartesian coordinates). The main purpose of the method is to reduce the number of data's dimensions, by looking for those where individuals are most separate. These new variables are linear combinations with the original ones. Factorial graphs in scatter plots are constructed enabling a distinction between data groups. A discriminant analysis of comparison (DA) was used to compare each of the two pairs of species.

In a third step, a canonical analysis and a discriminant comparison analysis were applied to all the individuals—including hybrids- to check for the distribution of hybrids in the morphometric space of the pure species in the forest.

3. Results

3.1. Morphological Variation in Oaks Species in the Akfadou Forest

The Procrustes overlay (Figure 2) indicates the dispersion of the landmarks in the samples. Each landmark is surrounded by a cloud of dots representing the different coordinates of all leaves, and the variability from the consensus (higher for landmarks 1, 4, 9, 10 and 11).

The results obtained by the ANOVA analysis (Table 1), indicates the importance of the species (46.77% of total variation in size and 22.11 in conformation or shape) in leaf morphology, followed by that of the plot (21.53 and 9.66% respectively) and, to a lesser extent, of the tree within species and plot (9.63 and 20.14% respectively).

Table 1. Hierarchical analysis of variance for the centroid size (size) and conformation (shape) of the leaves. (DF refer to degrees of freedom. Significance: ***: $p < 0.001$, **: $p < 0.01$).

	Size				**Shape**			
Effect	**DF**	**Sum of Squares**	**F**	**Percentage of Variation (%)**	**DF**	**Sum of Squares**	**F**	**Percentage of Variation (%)**
Plot	15	27,722,600.99	19.35 ***	21.53		270	6.71 ***	9.66
Species	35	60,215,876.60	18.02 ***	46.77	630	8.13	5.02 ***	22.11
Trees	81	12,391,290.78	1.6 **	9.63	1458	7.41	1.98 ***	20.14
Leaves	293	27,979,062.07	92.49 ***	21.73	5274	13.56	19.85 ***	36.87
Error	415	428,489.35		0.33	7470	0.97		2.63

3.2. Morphometric Leaf Variation between Species

The canonical analysis diagram (Figure 3) shows the segregation of groups according to the average conformations per tree of the same species. Along axes 1 and 2, the species are significantly discriminated, and these two axes explain 93.05 % of the total variation. We observe that the shape of *Q. afares* is intermediate between *Q. suber* and *Q. canariensis*, being *Q. ilex* very different from the other species, and without overlap among them.

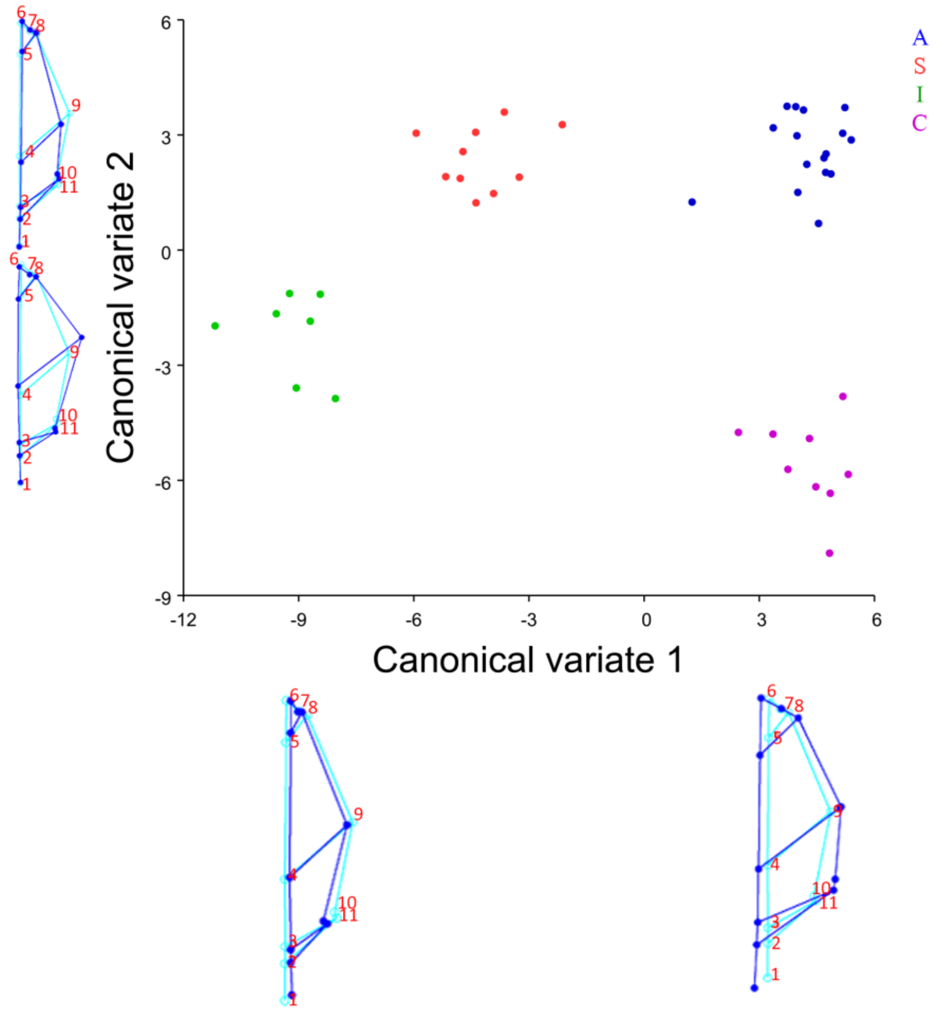


Figure 3. Canonical analysis of the four species in the Akfadou forest [Q. afares (A), Q. suber (S), Q. ilex (I), Q. canariensis (C)], and representation of leaf differences along the two canonical axes (CV1 = 66.93%, CV2 = 26.12%).

The discriminant analysis of comparison confirms the proximity between *Q. afares* and *Q. suber* (Figure 3), and the differences with the other species. Boxplots of the populations confirm the differences among the species (Figure S9).

3.3. Morphometric Variations of Hybrids

When including the putative hybrids in the analysis, the canonical analysis shows that those individuals are morphologically close to *Q. afares* and, to a lesser extent, to *Q. suber* and, finally, to *Q. ilex* and *Q. canariensis* (Figure 4).

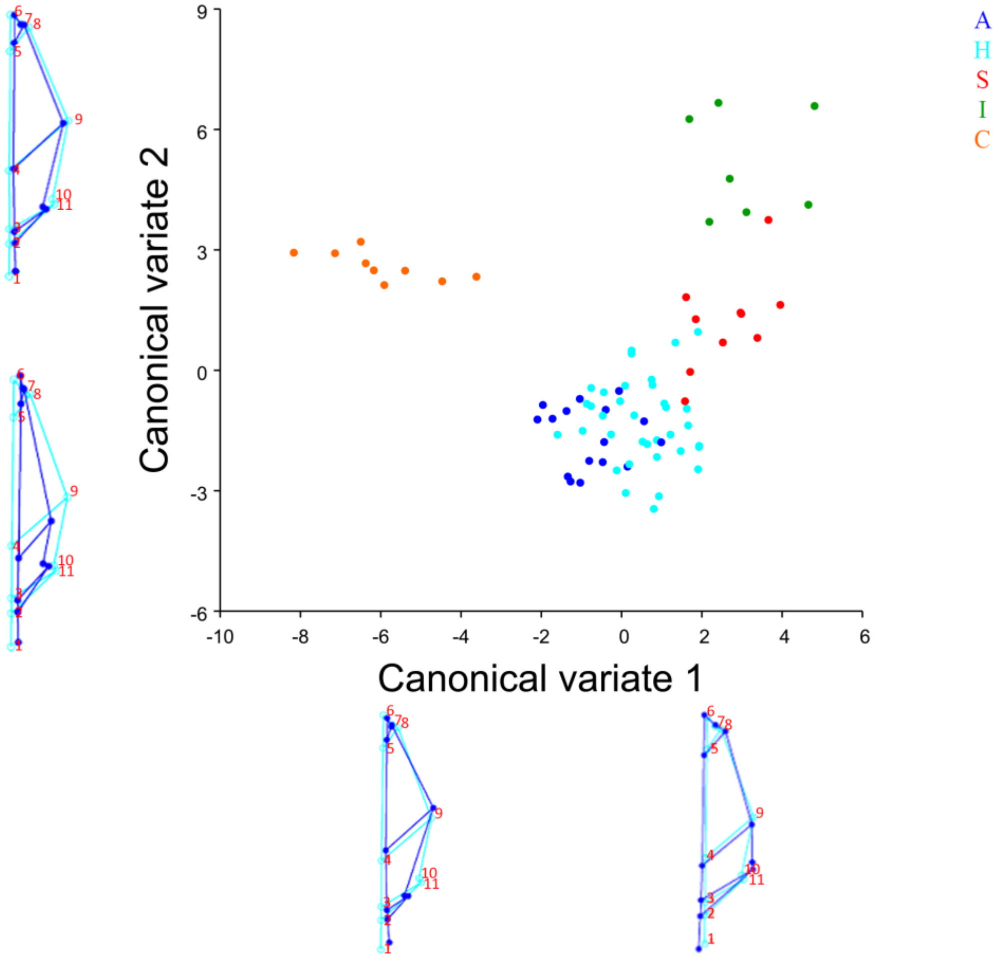


Figure 4. Canonical analysis of the four species and hybrids in the Akfadou forest [*Q. afares* (A), *Q. suber* (S), *Q. ilex* (I), *Q. canariensis* (C) and the hybrids (H)], and representation of leaf differences along the two canonical axes (CV1 = 47.08%; CV2 = 35.88%).

The Cross-validation of DA has revealed an overlap of hybrids with *Q. afares*, and to a lesser extent with *Q. suber* (T2 values significant, $p < 0.0001$). The overlaps with *Q. canariensis* and *Q. ilex* are non-significant (Figure 5).

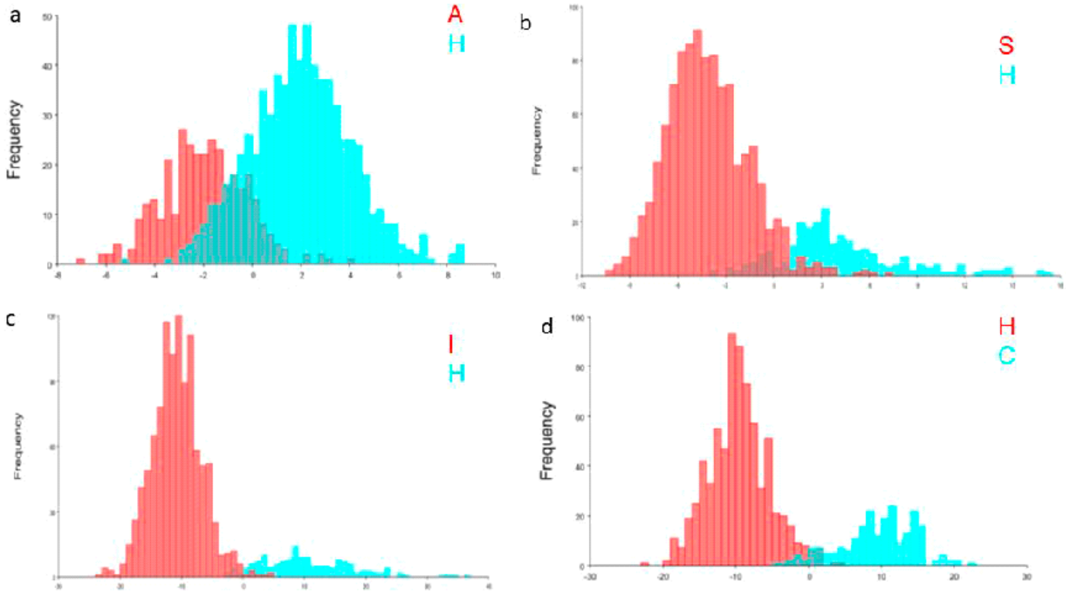


Figure 5. Discrimination analysis (DA) of leaf shape for pairs of species. (**a**) *Q. afares* (A) vs. Hybrids (H), (**b**) *Q. suber* (S) vs. Hybrids (H), (**c**) *Q.ilex* (I) vs. Hybrids (H), (**d**) *Q. canariensis* (C) vs. Hybrids (H).

4. Discussion

Our study shows that geometric morphometry can explain the pattern of variation among oak species and hybrid individuals at a reduced spatial scale. The results suggest that, in the studied area, the hybrids identified are close to *Q. afares* and *Q. suber*, but clearly differ from *Q. canariensis* and *Q. ilex* there.

Oak species variation in mixed forest stands has been an open topic for decades [35,36,50] and, in particular, for *Q. afares*, which has been described as a stabilized hybrid between *Q. suber* and *Q. canariensis* [42,44,49]. Our results confirm, after a discriminant analysis, the clear separation of *Q. afares* from the parental species. *Q. afares* is closer to *Q. suber* than to the other parental species (*Q. canariensis*) in leaf geometric morphometry. The pattern of variation in these morphological traits (a significant effect of site and trees indicative of within-species variation at the local scale), is consistent with other studies [49,51,53], and reflect the existence of standing variation for natural selection to act [54]. The significative within-tree effect is indicative of the phenotypic plasticity of these traits, an important evolutionary factor in Mediterranean oaks at different levels [55,56].

The existence of a significant number of hybrids allows the identification and the analysis of the relationship with the parental species using leaf morphological traits. Based on our results, the hybrids are similar to some of the *Q. afares* individuals, indicating a likely similar origin (*Q. suber* × *Q. canariensis* crosses), or a backcrossing of *Q. afares* with *Q. suber* individuals. In all the cases, the similarity with *Q. canariensis* or *Q. ilex* is quite low. The leaf morphology of the hybrids is similar to *Q. afares*, and it is predominant in the area. This leaf morphology could be related to the different sensitivity of this species to the climate [44], and to an absence of particular evidence of decline of the hybrids with respect to the parental species [49]. In our case, we lack a precise genetic characterization to determine the genetic background of *Q. afares* and the hybrids. In a preliminary genetic characterization of the parental species and the hybrids using five nSSR developed for *Q. suber* and *Q. ilex* (Annex 1), the genotypes of 4 out of the 5 hybrids analyzed are compatible with different types of hybrids (eg. *Q. afares* × *Q. suber* or *Q. suber* × *Q. canariensis*). However, we cannot discard *Q. ilex* as one of the maternal species, even if geometric morphology does not show any relationship with it. These results are concordant with the analysis of *Q. afares* and lack of spatial structure [42,49], and with the value of leaf morphology in the analysis of variation in oaks [31,57,58]. However, a complete genetic analysis is needed to discriminate the discrepancies of genetic assignment using morphological traits in a group as complex as that of Mediterranean oaks [29,59]. We also need to consider the necessary samples sizes to better understand this process, related to the inter- and intra-specific variability of Mediterranean oaks [35,53].

One question still open is the impact of hybrids on the genetic structure of the parental species in the long term. Hybrids of *Q. ilex* and *Q. suber* [17] are mainly derived from a crossing event, with no clear effects in the long term. However, our results suggest that *Q. afares*, as a stabilized hybrid, could have an impact on the future genetic structure of the populations, as some of the hybrids are increasing the leaf morphological space of oaks. This is an important aspect for local diversity, a pattern that has not previously been reported at the local scale. However, in some cases, interspecific gene exchanges with molecular markers have been detected in the absence of obvious morphologically intermediate forms [14,60]. In conclusion, we need further genetic research to address this important question.

The implications for management and conservation are related to the use of the different species and to how their hybrids can represent important genetic resources for the future in terms of adaptation or use of those species. At this moment, hybrids are not appreciated, as their main, production-related, characteristics are dissimilar to those of *Q. suber*, which can have a higher economic value. However, hybrids can have some role in the adaptation to intermediate ecological conditions with respect to the parental species. For instance, *Q. afares* and *Q. canariensis* present contrasting differences in relation to the area's climatic variability [44] and, therefore, we need to increase our knowledge of the role of these hybrids.

This study demonstrates the importance of hybridization in Mediterranean oaks at a local scale, opening the field for future studies analyzing the genetics of the hybrids in terms of molecular characterization and the implications for adaptation to future conditions in a changing global context. We also need to determine if hybridization at the local scales is a mass effect due to the high diversity of oak species and their extensive gene flow, with small effects in the short term in these mixed forests. This information will be essential for implementing efficient conservation measures [61].

5. Conclusions

Mediterranean oaks present a complex genetic structure among the species and populations. Mixed forests, such as the Argelian Kabylie are frequent, consisting of pure species of the genus *Quercus*; *Q. canariensis*, *Q. afares*, *Q. suber* and *Q. ilex* and natural hybrids. This study demonstrates the prevalence of hybrids with leaf morphology similar to that of *Q. afares*, a stabilized hybrid in the area. The new hybrids defined in this work are similar to *Q. afares* individuals, indicating a bias towards their morphology. In addition, it demonstrates the extensive importance of hybridization in the complex genetic structure at the local scale, and opens the field for future studies. We need to develop population and species genetic studies to further our understanding of the role of hybridization and introgression in the evolutionary history of this species, and to confirm the degree of hybridization in this forest. We also need to analyze the role of hybrids in the adaptation to future conditions. All this information is essential to improve the management and/or genetic conservation programs of the species in the Mediterranean.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f13040508/s1, Figure S1: Hybrid oaks (Location: CY-Cynegetic centre); Figure S2: *Quercus afares* (Location: SH- Sidi El Hadi); Figure S3: *Quercus afares*: a –tree, b-bark (Location: SH- Sidi El Hadi; Figure S4: *Quercus suber* (Location: AD-Adrar); Figure S5: Hybrids (a-Location: YA-Yakouren, b- VM- Vide Mehaga); Figure S6: Hybrids (Location: LN- Lac noir); Figure S7. Leaves from the different species analysed, Figure S8: Cumulative distribution of distances of individual samples of the average distribution of the complete sample; Figure S9: Size Centroid variation after average species within trees (box plot). [*Q. afares* (A), *Q. suber* (S), *Q. ilex* (I), *Q. canariensis* (C) and the hybrids (H)]. Annex 1. Genetic characterization.

Author Contributions: Conceptualization, A.A., K.R. and E.T.; Formal analysis, A.A. and R.A.; Funding acquisition, R.A. and E.T.; Methodology, Z.L. and E.T.; Project administration, E.T.; Supervision, K.R.; Writing—original draft, A.A. and R.A.; Writing—review & editing, A.A., Z.L., R.A., K.R. and E.T. All authors have read and agreed to the published version of the manuscript.

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