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Intra-specific morphological and molecular diversity in brown olive (*Olea cuspidata*) of Iran

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ABSTRACT: Brown olive thrives in diverse environmental conditions in Iran suggesting the possible occurrence of genetic diversity in these populations. Moreover in some regions, they occur close to cultivated olive suggesting the possibility of natural hybridization among them. The goal of the present study was to investigate these possibilities. Morphological and RAPD analyses were performed on 8 brown olive populations of Iran using 24 morphological characters. ANOVA test showed significant difference in leaf length and leaf width among different populations and PCA analysis showed that the leaf characteristics (venation, width, trichome, colour in the ventral and dorsal surfaces), number, and distribution of grooves in the endocarp and fruit characteristics (apex, base, and shape) are the most variable characters among the brown olive populations studied. The 38 RAPD primers used produced 541 reproducible bands (loci) out of which 515 bands were polymorphic and 26 bands were common in the populations studied. The Anveh population showed the highest level of polymorphic loci (78%) and the Jareh population showed the lowest value (28%). The highest mean genetic diversity and Shannon information indices occurred in the Anveh population (0.21 and 0.34, respectively) and the lowest values of the same occurred in the Jareh population (0.11 and 0.16, respectively), indicating the presence of a high genetic diversity among the populations studied. The Homag population showed the highest number of specific bands (8 bands). Both morphological and molecular analyses suggested the presence of intra-specific variations.

KEYWORDS: morphometry, natural hybrids, RAPD, wild olive

INTRODUCTION

The olive belongs to the genus *Olea* (Oleaceae), which contains about 40 species, subspecies, and varieties, distributed in Africa, Asia, Europe, and Oceania[1–3]. The olive plant (*O. europaea* subsp. *europaea* var. *europaea*)[1,4,5], is one of the most ancient horticultural plants used for oil and fruit, whose wild and cultivated forms are considered as an important botanical research subject. In spite of extensive efforts to clarify the taxonomic limits among the different forms of *O. europaea*[1,6,7], taxonomic treatment of the genus remains controversial and problematic possibly due to the limited geographic barriers among *Olea* taxa, the long history of olive cultivation, and the extensive hybridization between wild and crop trees[8–10].

Mediterranean wild olive (oleaster, *O. europaea* subsp. *europaea* var. *sylvestris* (Miller)) shows close affinity to the cultivated olive (*O. europaea* subsp. *europaea* var. *europaea*) and possibly is the progenitor of the cultivated form olive[10–12]. Oleaster has smaller fruit and a lower oil content than the cultivated olive[13]. Non-Mediterranean wild olive forms grow in different regions and have been geographically isolated from the Mediterranean oleaster[10,14,15]. These wild forms are adapted to new environmental conditions showing different morphological characters and have therefore been treated as separate species, subspecies, or varieties by different authors[5,10,14]. However, Green[1] in a recent revision of *Olea*, due to minor morphological differences available among wild olives occurring in South to North-East Africa and South-West Asia, considered them as members of a single species aggregate and proposed the name *Olea europaea* subsp. *cuspidata* for this aggregate.

Controversy also exists about the number of olive species and subspecies occurring in Iran. Parsa[16] reported the occurrence of two *O. europaea* L. and *O. europaea* subsp. *cuspidata*, while Murray[17] in Flora Iranica reports *O. aucheri* (Chev.) Ehrendf., in

addition to the other two species. Recently, Azadi[18] followed the Green taxonomy of the genus[1] and considered *O. europaea* subsp. *cuspidata* as the only wild olive subspecies growing in Iran.

There have been reports on the occurrence of natural hybrids between cultivated and brown olive plants in other parts of the world and its possibility in Iran as well[2,3,19]. It therefore seems important to look for any natural hybrid trees in Iran.

Different molecular markers including RAPD markers have been used to study olive genetic diversity and cultivated olive identification[20–23]. Therefore, the present study was carried out to reveal brown olive intra-specific diversity present in the country and to verify the eventual occurrence of hybrids between these two subspecies by using morphological and RAPD markers.

MATERIALS AND METHODS

Plant materials

For morphological studies, plant materials including fresh leaves, inflorescences, and fruits were collected randomly from brown olive (*O. europaea* subsp. *cuspidata* Green[1]). We sampled plants thriving in eight wild populations of four provinces, namely: (1) Hormozgan (Pahtak, Geno, Homag, Bokhoon, and Anveh populations), (2) Khoozestan (Jareh population), (3) Charmahal-Bakhteyari (Khersan population) and (4) Kerman (Kerman population, Fig. 1). There were only two trees in Jareh population whereas more trees could be found in other localities. Some cultivated olive trees grown in the north and south of Iran were included in the present study as an outgroup to the brown olive plants collected.

For morphometric analysis, 24 characters (Table 1) were selected based on wild olive ecotypes characters[13] and IOC (International Olive Council) descriptors[24]. Morphological characters (vegetative and floral characteristics) were coded as binary or multistate characters accordingly (Table 1). Analysis of variance (ANOVA) followed by the least significant difference (LSD) test were performed for quantitative morphological characters among the geographical regions studied. UPGMA (unweighted paired group using arithmetic average) and neighbour joining (NJ) clustering as well as ordination plots based on principal components analysis (PCA) and principal coordinate analysis (PCO) were used for grouping of the trees. Factor analysis was used to identify the most variable morphological characters among populations studied. For clustering, morphological data were standardized (mean = 0, variance = 1) and used to determine taxonomic and Euclidean distances[25]. Similar morphological studies were performed in the cultivated olive cultivars available in Iran and data obtained were compared with those of brown olives.

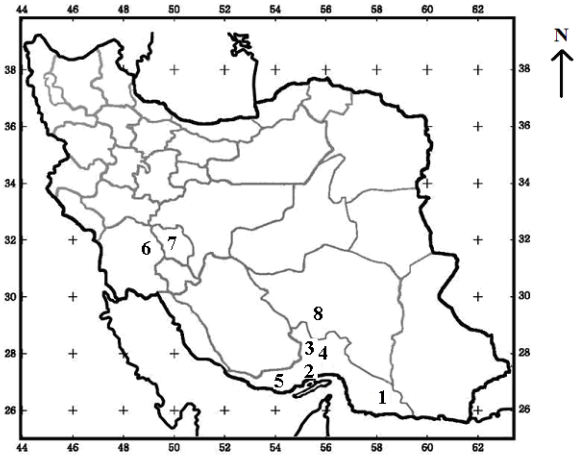

Fig. 1 Distribution map and localities of the brown olive populations studied. Population codes: 1–5: Pahtak, Geno, Homag, Bokhoon, and Anveh populations of Hormozgan province; 6: Jareh population of Khoozestan province; 7: Khersan population of Charmahal-Bakhteyari province; 8: Kerman population of Kerman province.

RAPD analysis

For RAPD study, brown olive plants were selected from 6 populations which showed morphological differences (Sikhoran village close to Homag village had 1 plant which was included in the RAPD but not in the morphological analysis). 38 decamer RAPD primers of Operon technology (Alameda) belonging to OPA and OPH sets were used in the molecular study of the brown olives. DNA extraction was done by using the CTAB method[26] with modifications[27]. DNA was extracted from 10 randomly selected leaves in each tree and pooled together for further study.

The PCR reaction mixture consisted of 20–40 ng template DNA, 1 × PCR buffer (10 mM Tris-HCl pH 8.8, 250 mM KCl), 200 µM dNTPs, 0.80 µM 10-base random primers and 1 unit of *Taq* polymerase, in a total volume of 25 µl. DNA amplification was performed on a palm cycler GP-001 (Corbet). Template DNA was initially denatured at 92 °C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 1 min at 92 °C, primer annealing at 36 °C for 1 min, and primer extension at 72 °C for 2 min. A final incubation for 10 min at 72 °C was performed to ensure that the

Table 1 Morphological characters in brown olive populations studied.

Characters	States
Leaf trichome	slender / powdery / non-pubescent
Leaf venation	evident / not evident
Colour of leaf in ventral surface	orange-green / yellow-orange / silver
Colour of leaf dorsal surface	green silver / leathery pale green / leathery dark green
Leaf length	short / medium / long
Leaf width	narrow / medium / broad
Leaf shape	elliptic / elliptic-lanceolate / lanceolate
Longitudinal curvature	flat / hyponastic / helicoid
Fruit weight	low / medium / high / very high
Fruit symmetry	symmetric / asymmetric
Fruit shape	spherical / ovoid / elongate
State of colour change	from the base / uniformly across the whole epidermis / from the apex
Position of maximum transverse diameter in fruit	towards base / central / towards apex
Fruit apex	pointed / round
Fruit base	truncated / pointed / round
Position of maximum transverse diameter in endocarp	towards base / central / towards apex
Number of grooves in endocarp	low / medium / high
Endocarp base	truncated / pointed / rounded
Endocarp shape	spherical / ovoid / elliptic / elongated
Endocarp width	low / medium / high / very high
Endocarp apex	pointed / rounded
Endocarp symmetry (position A)	symmetric / asymmetric
Endocarp symmetry (position B)	symmetric / asymmetric
Distribution of regular grooves in endocarp	regular / irregular

primer extension reaction proceeded to completion. The PCR amplified products were separated by electrophoresis on a 2% agarose gel using 0.5 $\times$ TBE buffer (44.5 mM Tris/Borate, 0.5 mM EDTA, pH 8.0) or 6% polyacrylamide gels. The gels were stained with ethidium bromide and visualized under UV light. A 100-bp DNA ladder (GeneRuler, Fermentas) was used as the molecular standard in order to confirm the appropriate RAPD markers. RAPD markers were named by primer origin, followed with the primer number and the size of the amplified products in base pairs. The experiment was repeated 3 times and those bands present in all of them were used for further analysis.

RAPD bands were treated as binary characters and coded accordingly (presence = 1, absence = 0). Jaccard similarity[25] as well as Nei's genetic distance[28] were determined among the cultivars studied and used for grouping of the genotypes by clustering methods and ordination based on principal coordinate analysis (PCO)[25]. The fit of dendrograms obtained were checked by cophenetic correlation.

To describe the level and distribution of variation, intra-population genetic diversity of brown olives studied was determined by Nei's gene diversity[29] as well as Shannon information index (H)[30]. H was calculated at the population level ($H_{\rm pop}$) and species level ($H_{\rm sp}$). The proportion of variation found within population was determined from $H_{\rm pop}/H_{\rm sp}$, whereas the proportion of variation distributed among populations was determined by $(H_{\rm sp} - H_{\rm pop})/H_{\rm sp}$[31]. SPSS 9 was used for ANOVA and LSD tests, NTSYS 2.02 and DARWIN 5 were used for clustering and PCO analyses. Bayesian clustering was performed by MRBAYES 3.1. Genetic diversity was determined by POPGENE 1.32.

RESULTS

ANOVA test showed significant differences in leaf length and leaf width among the populations studied. PCA analysis showed that the first 3 components comprise about 84% of the total variation, with the first factor contributing to 51% of the variation. In this factor, characters like leaf venation, colour of leaf in the ventral surface, and the number of grooves in the endocarp show the highest positive correlation ($>$0.90). Characters like colour of leaf in the dorsal surface, distribution of the grooves in endocarp, and fruit apex show the highest negative correlation ($-$0.80). In the second factor with about 22% of the total variation, characters like leaf trichomes and fruit base show the highest positive correlation ($>$0.80), whereas leaf width and fruit shape show the highest negative correlation ($-$0.70). Therefore, these are the most variable morphological characters separating the brown olive populations studied.

Clustering and ordination plots of morphological characters produced similar results separating the cultivated olive (outgroup) from the brown olive populations studied (Fig. 2). Trees of the populations Bokhoon and Homag from Hormozgan province

showed more similarity and were placed close to each other, and trees of Jareh population from Khoozestan province join them. Trees of the Anveh population also from Hormozgan province form a distinct cluster and show similarity to populations of Bokhoon and Homag.

Trees of the two populations of Pahtak and Geno from Hormozgan province showed similarity and were placed together, and trees of Kerman population join them at some distance. Trees of Khersan population stood far from other brown olive populations and were placed closer to the cultivated olives in an intermediate position.

RAPD analysis showed that all 38 primers used produced reproducible bands. Out of a total of 541 bands (loci), 515 bands were polymorphic and only 26 bands were common in brown olive populations (Fig. 3). Anveh showed the highest level of polymorphic loci (77.7%), the highest mean genetic diversity, and Shannon information indices (0.21 and 0.34, respectively, Table 2). Shannon index determined within and among populations (Table 2) showed the highest value of within genetic variation in Anveh and Khersan populations. Jareh and Homag populations showed the highest values of among populations variation (0.85 and 0.84, respectively), differing the most from other populations.

Some specific RAPD bands were observed in the populations studied. Bands of OPH-07 (350 bp), OPC-01 (200 bp), and OPI-18 (300 bp) were specific for Kerman trees, while bands OPC-08 (2500 bp) and OPR-15 (320 bp) were specific for Khersan trees. The Homag population showed the highest number of specific bands (8 bands). Bands OPC-10 (3000 bp), OPC-11 (2750 bp), OPC-12 (2400 bp), OPR-01 (2500 bp), OPI-12 (3100 bp), OPI-13 (3000 bp), OPA-18 (3500 bp), and OPH-14 (2000 bp) occurred only in Homag trees. Other brown olive populations also showed some specific bands too.

Nei's genetic identity and genetic distance[31] showed the highest similarity between Kerman and Jareh populations and between Kerman and Khersan populations. The lowest similarity occurred between Khersan and Homag (Table 3).

NJ and Bayesian clustering (not shown) as well as PCO ordination plot (not shown) of brown olive trees based on RAPD data produced similar results. All of these analyses showed quite good bootstrap and clustering occurrence values. Therefore only the NJ tree is shown here (Fig. 4). Trees from each of the populations studied were grouped together forming a cluster and separated from trees of the other brown olive populations. Trees from Kerman population show more genetic affinity with trees of Khersan population and are placed in a cluster close to each other. Anveh trees show close genetic affinity with Homag trees.

Cultivated olives form a distinct cluster separated from brown olive populations. The Dakal and Ghavi cultivars show morphological differences from the others and are far from the other cultivars.

DISCUSSION

Putative hybrids

In clustering and ordination plot of morphological characters, clustering of Khersan population between cultivars and brown olive may give rise to a question about the hybrid nature of this population. Indeed, trees of this population show somewhat intermediate morphological characters between cultivated and brown olives based on size of the leaf, colour of the upper surface of leaf, size of fruit, and number of fruit groves.

The location of this population is in the vicinity of cultivated olives. Therefore we suggest that these trees are formed either by hybridization between cultivated and brown olive trees of the neighbouring localities or they have escaped from cultivation and have become adapted to the new environmental conditions showing morphological changes as feral trees[32]. Unfortunately, we could not perform RAPD analysis of brown and cultivated olive trees together to check if the Khersan population stands intermediate between brown and cultivated olive trees as in the morphological analysis. However, as will be discussed further, RAPD analysis of brown populations studied grouped the Khersan population in a separate cluster further supporting their genetic difference from the other populations studied.

Intra-specific variations

The presence of 515 polymorphic bands in brown olive trees studied and the presence of only 26 common bands among these trees suggests extensive genetic difference among the populations studied. This is also supported by the presence of private RAPD bands in each population. Moreover, the occurrence of specific bands only in some of the trees collected in each population suggests intra-population genetic variations of the populations studied.

In both morphological and RAPD analyses, trees from different populations form almost separate distinct clusters indicating the presence of different morphological and genetic entities in *O. europaea* subsp. *cuspidata* of Iran. Khersan trees show a close

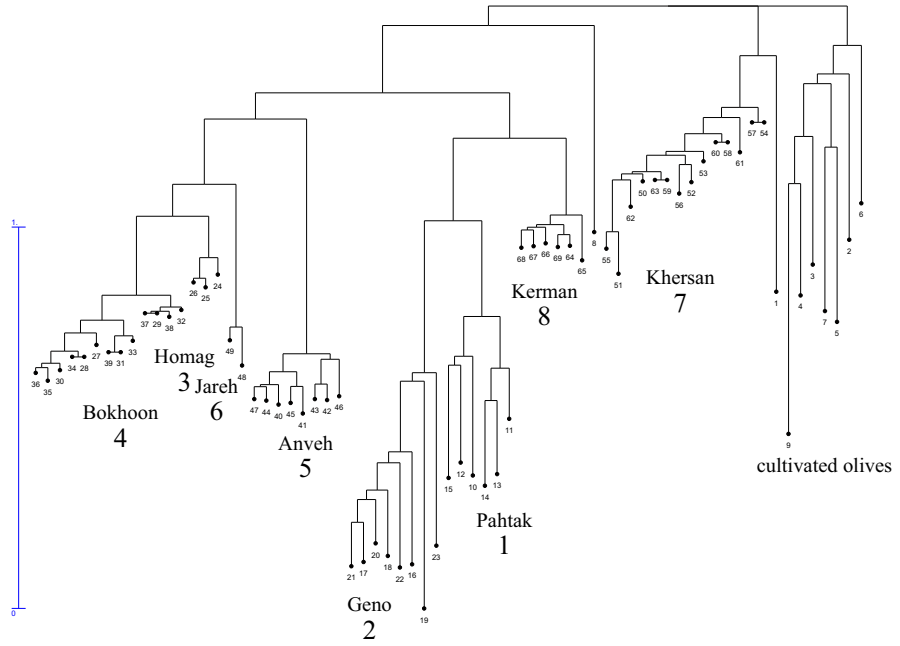

Fig. 2 NJ dendrogram of brown olives based on all morphological characters.

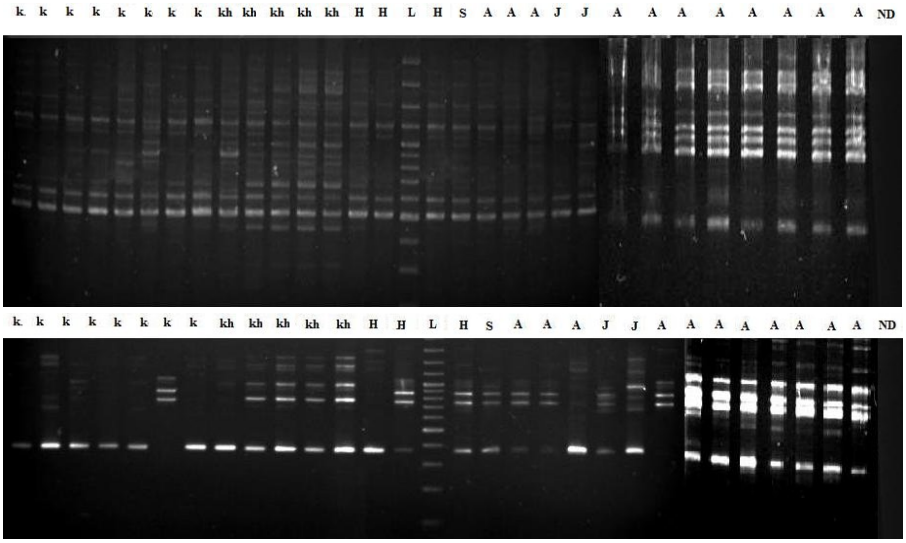

Fig. 3 RAPD profile of primers OPA-11 (top) and OPM-10 (bottom) in brown olive populations studied. K = Kerman, Kh = Khersan, H = Homag, S = Sikhoran, J = Jareh, A = Anveh, L = molecular ladder, ND = no DNA.

Table 2 Shannon index and Nei's genetic diversity in wild olive populations studied.

Population	Polymorphic %	Shannon's index	Nei's genetic diversity	$H_{\mathrm{pop}}/H_{\mathrm{sp}}$	$(H_{\mathrm{sp}} - H_{\mathrm{pop}})/H_{\mathrm{sp}}$
Kerman	44.6	0.23 (0.28)	0.15 (0.19)	0.19	0.81
Khersan	49.0	0.27 (0.29)	0.18 (0.21)	0.22	0.77
Homag	31.9	0.18 (0.28)	0.13 (0.19)	0.15	0.84
Anveh	77.7	0.34 (0.24)	0.22 (0.17)	0.28	0.71
Jareh	28.0	0.17 (0.27)	0.12 (0.18)	0.14	0.85

Table 3 Nei's genetic distance among wild olive populations. Values above the diagonal are Nei's genetic identities and below the diagonal genetic distance values.

Pop	1	2	3	4	5
1	—	0.85	0.73	0.85	0.86
2	0.16	—	0.73	0.82	0.81
3	0.31	0.32	—	0.79	0.81
4	0.16	0.20	0.24	—	0.84
5	0.15	0.21	0.22	0.18	—

Pop: 1 = Kerman, 2 = Khersan, 3 = Homag, 4 = Anveh, 5 = Jareh

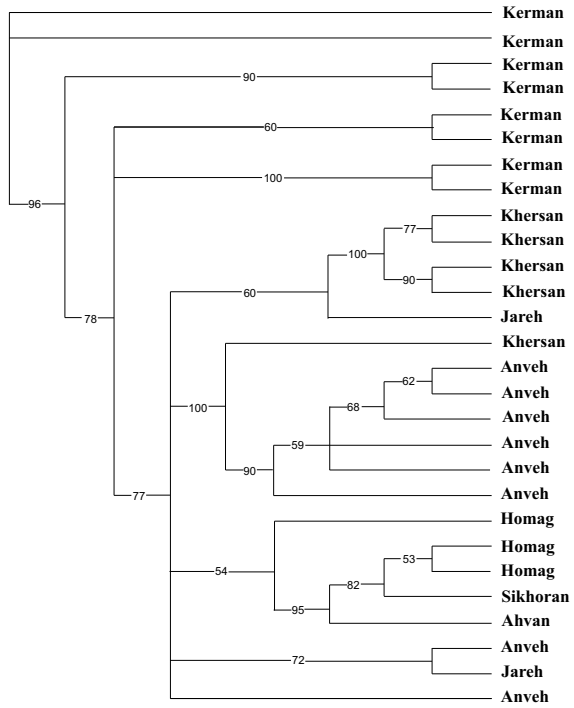

Fig. 4 NJ dendrogram of brown olive trees based on RAPD data. The numbers above branches indicate bootstrap values.

relationship to Kerman trees. As stated by other researchers, extensive morphological variations exist in wild olive mainly due to the limited geographic barriers among olive taxa, the long history of olive cultivation and the extensive hybridization between wild and crop trees[5,6,10]. Therefore, morphological and genetic studies suggest the presence of intra-specific variations in *O. cuspidata* subsp. *cuspidata* of Iran and possibly indicate the existence of intra-specific forms. Rallo et al[33], in their molecular studies of olive species, included also 3 samples from Iran which were treated as 3 different species (*O. cuspidata*, *O. chrysophylla*, and *O. ferruginae*). Dendrograms obtained based on simple sequence repeats markers placed these 3 taxa in a single cluster separated from the other olive taxa studied showing their close affinity. However these taxa showed similarity difference and joined each other with genetic distance indicating that they may be different olive forms, supporting the results of present morphological and molecular study. Besnard et al[19], while studying genetic diversity of wild olives, have stated that the classification of trees based on RAPDs enabled them to distinguish more taxa than the taxonomy based on morphology[6]. They recognized two taxa in the Mediterranean Basin (East and West), two for the subspecies *laperrinei* (*O. laperrinei* and *O. maroccana*), and the three taxa of the subspecies *cuspidata* (*O. africana*, *O. chrysophylla*, and *O. cuspidata*). This latter distinction was not possible using the morphologic traits indicated by Green and Wickens[6]. The geographic isolation of these taxa certainly explains their molecular differentiation.

Azadi[18] in his taxonomic treatment of the genus *Olea* in Iran, separated brown olive (*O. europaea* subsp. *cuspidata* from the cultivated olive (*O. europaea* subsp. *europaea*) based on the size of fruits (smaller in brown olive), size of endocarp (smaller in brown olive), and colour of the leaf lower surface which is not at all stable morphological characters among brown olive populations studied. Moreover, Murray[17] in Flora Iranica describes *O. ferruginae* (synonym = *O. europaea* subsp. *cuspidata*) and *O. aucheri* (Chev.) as two wild olive subspecies growing in Iran based on type and size of inflorescence (paniculate with 3–5 cm length in *O. ferruginae* and racemose with 1 cm length in *O. aucheri*). These characters are also variable in the populations studied.

The present study showed that brown olive populations differ by morphological characters used in taxonomy. Moreover, we also showed that they differed by molecular markers. We may therefore agree at present with the idea of Besnard et al[19], also suggested by Zohary[10], that brown olive forms which thrive in different regions and show different morphological characters could be considered as separate species or subspecies (subspecies is preferred by the authors). Moreover, our data suggest that natural hybridization between subsp. *cuspidata* and the olive will eventually occur.

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