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The colonization history of *Olea europaea* L. in Macaronesia based on internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and intersimple sequence repeats (ISSR)

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Abstract

Phylogenetic relationships in the *Olea europaea* complex and the phylogeography of 24 populations of the Macaronesian olive (*O. europaea* ssp. *cerasiformis*) were assessed by using three molecular markers: nuclear ribosomal internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and intersimple sequence repeats (ISSR). Parsimony analysis of the ITS-1 sequences and Neighbour-joining (NJ) analyses of RAPD and ISSR banding variation revealed four major lineages in the *O. europaea* complex: (1) ssp. *cuspidata*; (2) ssp. *cerasiformis* from Madeira; (3) ssp. *laperrinei*; and (4) ssp. *cerasiformis* from the Canary Islands plus ssp. *europaea*. These results provide unequivocal support for two independent dispersal events of *Olea* to the Madeira and Canary Islands. Molecular and morphological evidence led to recognition of two separate olive taxa in Macaronesia, to date included in ssp. *cerasiformis*. NJ analyses of the combined RAPD and ISSR data suggest that the colonization of the Canaries by *O. europaea* may have followed an east to west stepping-stone model. An interisland dispersal sequence can be recognized, starting from the continent to Fuerteventura, Gran Canaria, Tenerife, La Gomera, and finally La Palma. High dispersal activity of the lipid-rich *Olea* fruits by birds in the Mediterranean region is congruent with multiple dispersal of olives to Macaronesia and successive colonization of the archipelagos. The observation of strong genetic isolation between populations of different islands of the Canary Islands suggests, however, that subsequent interisland dispersal and establishment has been very rare or may not have occurred at all.

Keywords: ISSRs, ITS-1 sequences, Macaronesia, *Olea europaea*, phylogeography, RAPDs

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Introduction

Oceanic islands provide a 'natural laboratory' to study the potential of organisms to colonize and establish. Molecular evidence can yield essential information on the number of introductions of a particular plant group, time since dispersal through the use of a molecular clock, and dispersal patterns within archipelagos (Baldwin *et al*. 1998). Traditional and molecular studies of various angiosperm groups (Carlquist 1965, 1980; Kim *et al*. 1996; Sakai *et al*. 1997; Baldwin *et al*. 1998) have revealed similar characteristics with respect to colonization of oceanic islands: (i) the success of long-distance dispersal is inversely proportional to island remoteness; (ii) different diaspore types succeed in dispersal; (iii) introductions to distant archipelagos took place once primarily; (iv) ecological diversity in oceanic islands favours the reception of disparate immigrants; and (v) return to continents appears to be rare.

Molecular evidence is particularly important at the populational level when morphological variation contains little information. This is the case of *Olea europaea* L. in Macaronesia. Based on few morphological features, four subspecies have been recognized in the *O. europaea* complex (Green & Wickens 1989): ssp. *europaea* (Mediterranean Basin), ssp. *laperrinei* (Batt. & Trabut) Ciferri (Sahara), ssp.

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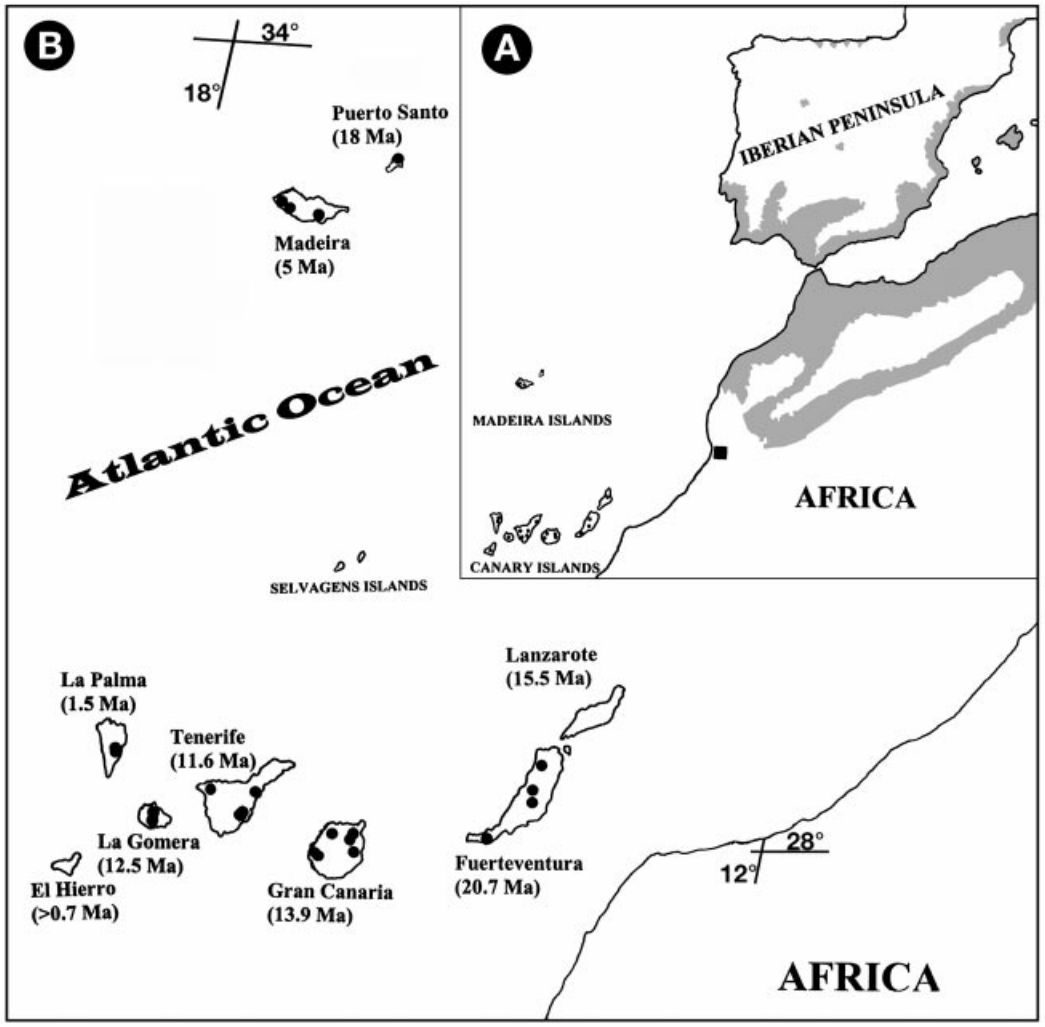

Fig. 1 (A) Geographical distribution of *Olea europaea* ssp. *cerasiformis* in Macaronesia (●) and *O. europaea* ssp. *europaea* in the West Mediterranean Basin (shaded area). One population of *O. europaea* ssp. *laperrinei* is also indicated (■). (B) Populations sampled of *O. europaea* ssp. *cerasiformis* (●) and island names and ages (Galopim de Carvalho & Brandão1991, Carracedo 1994).

cuspidata (Wall. ex DC.) Ciferri (south and east Africa, south-west Asia, and Asia), and ssp. *cerasiformis* (Webb & Bernth.) Kunkel & Sundig (Macaronesia). Subspecies *cerasiformis* is an endemic to the Madeira and Canary Islands, being absent in the Azores and Cape Verde Islands (Hansen & Sundig 1993) (Fig. 1). Allozyme variation in *O. europaea* has demonstrated the existence of an exclusive genotype of this subspecies from Madeira (Ouazzani *et al*. 1993). Lack of molecular evidence for representatives of *Olea* from the Canary Islands, however, does not allow the assessment of genetic relationships both between and within the Macaronesian archipelagos. In the present paper, we investigate the colonization history of *O. europaea* in Macaronesia by using three molecular markers successfully used in *Olea* and other angiosperms: randomly amplified polymorphic DNAs (RAPD) (Fabri *et al*. 1995), intersimple sequence repeats (ISSR) (Wolfe & Liston 1998; Vargas & Kadereit submitted), and internal transcribed spacer 1 (ITS-1) sequences of nrDNA (Baldwin *et al*. 1995). More specifically, we addressed the following issues: (i) identification of major lineages of the *O. europaea* complex; (ii) assessment of frequency of *Olea* introduction to Macaronesia; and (iii) determination of the dispersal modes between and within the Madeiran and Canarian archipelagos.

Materials and methods

Plant material

Single individuals of five populations belonging to the *Olea europaea* complex were sequenced for ITS-1: *O. europaea* ssp. *cerasiformis* (two populations), *O. europaea* ssp. *cuspidata* (one population), and *O. europaea* ssp. *laperrinei* (two populations) (Table 1). Unfortunately, we

Table 1 List of accessions used for the ITS-1 sequencing and RAPD and ISSR PCR amplifications of the *Olea europaea* complex providing subspecies, variety or cultivar names; country or island of origin; location; and collector's name, collection number or DNA source. Abbreviations: DL, Dieter Lüpnitz; F, collection number from France (INRA, Montpellier); GN, Gonzalo Nieto; JC, Juan Castro; JH, Jochen Hess; PV, Pablo Vargas; UH, Ulrich Hecker

Subspecies and variety	Country or island of origin	Location	Collection number	Collector DNA source	ISSRs	RAPDs	ITS-1
cerasiformis	Gran Canaria	Tafira, Botanic Garden	1JH798	J. Hess	x	x	
cerasiformis	Gran Canaria	Tafira, Lentiscal	4JH798	J. Hess	x	x	
cerasiformis	Gran Canaria	Ingenio, Barranco de Guayadeque	32JH798	J. Hess	x	x	
cerasiformis	Gran Canaria	Valsequillo, Barranco de los Cernícalos	38JH798	J. Hess	x	x	
cerasiformis	Gran Canaria	Tafira, carretera de Bandama	48JH798	J. Hess	x	x	
cerasiformis	Gran Canaria	Tasarte	50JH798	J. Hess	x	x	
cerasiformis	Gran Canaria	Degollada de Tasartico	52JH798	J. Hess	x		
cerasiformis	Gran Canaria	El Sao, Los Berrazales	53JH798	J. Hess	x	x	
cerasiformis	Fuerteventura	Morro Jable, Barranco de Vinamar	13JH798	J. Hess	x		
cerasiformis	Fuerteventura	Tuineje	23JH798	J. Hess	x		
cerasiformis	Fuerteventura	Betancuria, Morro de la Cruz	25JH798	J. Hess	x	x	
cerasiformis	Fuerteventura	Montana Tindaya	29JH798	J. Hess	x	x	
cerasiformis	La Gomera	Las Rosas	31PV798	P. Vargas	x	x	
cerasiformis	La Gomera	Agulo-Vallehermoso	30PV798	P. Vargas	x	x	
cerasiformis	Tenerife	Güimar	76PV97	P. Vargas	x		
cerasiformis	Tenerife	Villa de Arico	77PV97	P. Vargas	x	x	
cerasiformis	Tenerife	El Río	78PV97	P. Vargas	x		
cerasiformis	Tenerife	Los Silos, Tierra del Trigo	1DL98	D. Lüpnitz	x	x	
cerasiformis	La Palma	Breña Baja, 'Finca Amado'	JC2	J. Castro	x	x	
cerasiformis	La Palma	San Antonio	JC3	J. Castro	x	x	
cerasiformis	La Palma		F47	INRA Montpellier			x
cerasiformis	Madeira	Madalena do Mar	104PV(1)98	P. Vargas	x		
cerasiformis	Madeira	Paul do Mar	106PV(1)98	P. Vargas	x	x	x
cerasiformis	Madeira	San Gonçalo	118PV98	P. Vargas	x	x	
cerasiformis	Puerto Santo	Pico Juliana	117PV98	P. Vargas	x	x	
europaea var. *sylvestris*	Spain	Pechón, Asturias	25PV98	P. Vargas	x	x	
europaea var. *sylvestris*	Spain	Cádiz, Caños de Meca	27PV98	P. Vargas	x		
europaea var. *sylvestris*	Spain	Castellón	3903GN	G. Nieto	x	x	
europaea var. *sylvestris*	Morocco	Anti Atlas	1UH98	U. Hecker	x		
europaea var. *sylvestris*	Israel	Mont Carmel	F46	INRA Montpellier	x	x	
europaea var. *sylvestris*	Morocco	Taounate	F22	INRA Montpellier		x	
europaea var. *sylvestris*	Morocco	Tamanar, west coast	F24	INRA Montpellier	x	x	
europaea var. *sylvestris*	Algeria	Kabylie	F26	INRA Montpellier	x	x	
europaea var. *sylvestris*	Corsica	Ostricone	F38	INRA Montpellier	x	x	
laperrinei	Morocco	Agadir	no. 53704	Davis (RNG, Reading)	x	x	x
laperrinei	Morocco	Imounidane	F48	INRA Montpellier	x	x	
laperrinei	Algeria	Hoggar, La Source	F49	INRA Montpellier	x	x	
laperrinei	Algeria	Hoggar, Asselkrem	no. 33373	Podlech (MJG, Mainz)			x
cuspidata	China		F57	INRA Montpellier			x
cuspidata	Iran		F56	INRA Montpellier	x	x	
cuspidata	South Africa	Kistenbosh	F53	INRA Montpellier	x	x	
cuspidata	Kenya	Kimakia Forestry Station	no. 220699	Kew Gardens greenhouse	x		

Table 1 *continued*

Subspecies and variety	Name of cultivar	Origin of individual	Main distribution of cultivar	Collection number	Collector or DNA source	ISSRs	RAPDs
europaea var. *europaea*	?	Puerto Santo, Pico do Castelo	?	111PV98	P. Vargas	x	x
europaea var. *europaea*	'Leccino'	Perugia Collection	Italy	F14	INRA Montpellier	x	x
europaea var. *europaea*	'Souri'	Cordoba Collection	Syria/ Lebanon	F19	INRA Montpellier	x	x
europaea var. *europaea*	?	Gran Canaria, Barranco Guayadeque	?	33JH798	J. Hess	x	x
europaea var. *europaea*	?	Gran Canaria, Barranco Arguineguin	?	60JH798	J. Hess	x	x
europaea var. *europaea*	'Cornicabra'	Mora de Toledo, Spain	Spain	999PV98	Family Gomez-Delgado	x	x

were unable to amplify ITS-1 of *O. europaea* ssp. *europaea*. Single individuals from the following 45 populations of *O. europaea* were included in the ISSR and/or RAPD studies (Table 1): three of ssp. *cuspidata*, three of ssp. *laperreini*, nine of ssp. *europaea* var. *sylvestris* (wild olive), six of ssp. *europaea* var. *europaea* (cultivars), and 24 of ssp. *cerasiformis*. Because of limited data availability, eight individuals were used only in the ISSR analysis and not in the RAPD analysis, and one individual was used in the RAPD analysis and not in the ISSR analysis (see Table 1). For the Madeira and Canary Islands, at least two geographically distant populations from each major island were included in the study (Table 1; Fig. 1). Subspecies *cerasiformis* could not be found on El Hierro or Lanzarote where it is extremely rare. Voucher specimens and DNA extractions are deposited at the Johannes Gutenberg-University/Mainz (Germany) and INRA (Montpellier, France). Identification of accessions collected in the field is based on the morphological characters provided by Green & Wickens (1989), and identification of olive collections by P. Villemur (Montpellier) were accepted as correct.

DNA extraction

Leaf material was collected in the field, and total genomic DNA was extracted from ≈ 0.5 g of silica dried material using the Dneasy-kit (QIAGEN Inc.) following the protocols provided by the manufacturer. DNA samples received from INRA (Montpellier, France) were extracted with CTAB and purified in a caesium chloride gradient (G. Besnard, personal communication).

ITS-1 amplification and sequencing

Polymerase chain reaction (PCR) amplifications were performed using the oligonucleotide primers 17SE and 26SE (Sun *et al*. 1994). Double bands were frequently obtained. Amplified products were cleaned using the QIAGEN QIAquick TM PCR Purification kit (QIAGEN Inc., Chatsworth, CA) following the protocols provided by the manufacturer. Cleaned products were directly sequenced using the DyeDeoxy Terminator cycle sequencing premix kit (Applied Biosystems, Foster City, USA). For cycle sequencing, nested primers ITS4 and ITS5 (White *et al*. 1990) were used. Unincorporated dye terminators were removed using phenol–chloroform, sodium acetate, and absolute alcohol precipitation. Samples were loaded on a 5.75% polyacrylamide gel, analysed on an ABI 377 DNA Sequencer, and sequence data were collected on a Macintosh platform. Sequence data were edited and assembled using the program 'Sequencer' (Gene Codes Corporation, Miami). Overlapping peaks were found throughout the resulting chromatograms. Double bands were also obtained when using a combination of both external (17SE and 26SE) and internal primers (ITS5, ITS4, ITSA, ITSB, Hungerer & Kadereit 1998). Finally, we managed to obtain a chromatogram for the ITS-1 region with few ambiguities by using two consecutive PCR amplifications: one external (with primers 17SE and 26SE), and one internal (with primers 17SE or ITSA and ITSC), followed by gel isolation of the ITS-1 fragment (~200–250 bp) using the QIAGEN QIAquick TM PCR Gel Isolation kit (QIAGEN Inc.). For cycle sequencing, nested primers ITSA and ITSC were used. The boundaries of the ITS-1 and rDNA coding regions were identified by comparison with *Daucus carota* L. and *Vicia faba* L. (Yokota *et al*. 1989). Despite the success in sequencing the ITS-1 region in the *O. europaea* complex following the above procedure, we did not manage to amplify ITS-1 in *O. europaea* ssp. *europaea* var. *europaea*.

RAPD amplification and electrophoresis

PCR amplifications were performed in 25 μL reaction mixtures containing 1 unit DNA polymerase (Genecraft, Münster/Germany), 1× enzyme buffer (16 mM $(NH_4)2SO_4$, 67 mM Tris-HCl pH 8.8, 0.01% Tween 20), 2.5 mM $MgCl_2$, 10 pM primer, 0.2 mM each dNTP, and 12.5 ng template DNA. After 3 min pretreatment at 94 °C, the PCR program was: 35 cycles of 20 s at 94 °C, 30 s at 40 °C, and 1 min at 72 °C, followed by extension of 8 min at 72 °C. Of the 70 primers tested (Operon kits A, AB, C, D, E, T, and U), 15 were used (A7: GAAACGGGTG; AB1: CCGTCGGTAG; C9: CTCACCGTCC; C16: CACACTCCAG; D12: CACCGTATCC; E7: AGATGCAGCC; E10: CACCAGGTGA; E16: GGTGACTGTG; E17: CTACTGCCGT; E19: ACGGCGTATG; T9: CACCCCTGAG; T18: GATGCCAGAC; T19: GTCCGTATGG; U11: AGACCCAGAG; U17: ACCTGGGGAG). Amplification products of 36 samples were electrophoresed in 1.2% (w/v) agarose gels, and banding patterns were visualized under UV and photographed. Negative controls were included and each primer was replicated on the 36 samples to verify repeatability of results.

ISSR amplification, electrophoresis, and silver staining

A set of 15 ISSR primers, commercialized by the University of British Columbia Biotechnology Laboratory, were tested in three samples to find out suitable repeats and anchors. As a result, three primers of three dinucleotide repeat motifs were used: 887, DVD $(TC)_7$; 834, $(AG)_8$ YT; 855, $(AC)_8$ YT. PCR testing reactions were performed from high (55 °C) to low (50 °C) annealing temperatures to reduce reaction stringency to as little as possible. PCR reaction mixtures (20 μL) contained the following components/concentrations: 0.25 units *Taq* polymerase (Boehringer, Mannheim/Germany), 1× reaction buffer (10 mM Tris-HCl, 50 mM KCl), 3.125 mM $MgCl_2$, 0.2 mM each dNTP, 0.3 μM primer, and 25 ng template DNA. The final thermocycler program was: 2 min at 95 °C; 35 cycles of 1 min at 95 °C, 1 min at 50 °C, 1.5 min at 70 °C, followed by extension of 10 min at 70 °C. Negative controls and replicates were included to verify repeatability of results.

Amplification products from primer tests were first characterized on 1.5% (w/v) agarose gels immersed in 1× TBE buffer. Gels with ethidium bromide were run until the bromophenol blue front had reached ≈ 12 cm from start. Once the primers were selected, the above PCR conditions were extended to the set of 44 samples. Eventually, amplification products were resolved on precast polyacrylamide gels (Cleangel 48S, Pharmacia Biotech, Sweden) rehydrated in 75 mM Tris-acetate (pH 6.4) with 0.2 M Tris base, 0.2 M tricine, 0.55% (w/v) SDS, pH 8.0, as electrode buffer. Flat-bed electrophoresis was conducted at 100 V max, 20 mA max, 10 W max for 20 min, and then at 250 V max, 30 mA max, 20 W max for 3 h.

ISSR fragments were visualized using the method of Bassam *et al*. (1991) as modified by Charters *et al*. (1996): (i) 30 min fixing in 250 mL of 10% acetic acid; (ii) 3 × 2 min washings in 250 mL of distilled water; (iii) 40 min silvering in 200 mL of freshly prepared 0.1% (w/v) $AgNO_3$, with 200 μL of 40% w/v formaldehyde added immediately prior to use; (iv) 20 s rinse in 500 mL of distilled water; (v) about 15–20 min developing in 200 mL of freshly prepared 2.5% (w/v) Na_2 CO_3 with 200 μL of 2% (w/v) Na_2SO_3 and 200 μL of formaldehyde added immediately prior to use at 10 °C in a prechilled dish; (vi) 10 min stop/de-silver in 200 mL of 2% (w/v) glycine, 0.5% (w/v) EDTA-Na_2; and (vii) 30 min gel impregnation in 250 mL of 5% (v/v) glycerol.

Analysis of ITS-1 sequences, RAPD and ISSR data

ITS-1 sequences. Five ITS-1 sequences were examined from populations of the *O. europaea* complex (Table 1). One sequence of *O. europaea* ssp. *cuspidata* from China was chosen as an outgroup based on results of previous analyses of the *mat*K sequences and ISSR banding patterns (Vargas & Kadereit submitted) which indicated that ssp. *cuspidata* is sister to sspp. *cerasiformis*, *laperrinei*, and *europaea*. Sequences were aligned by visual inspection, and ambiguous characters were treated as polymorphic using IUPAC ambiguity symbols. Phylogenetic analyses were conducted using Fitch parsimony (as implemented in PAUP, Swofford 1998) with equal weight-ing of all characters and of transitions/transversions. The branch-and-bound analysis was performed using stepwise addition, mulTrees, and furthest addition sequence in effect. Reliability of lineages was assessed by bootstrapping (1000 replicates) with the same options as above.

RAPD, ISSR, and combined data. RAPD and ISSR bands were scored across the 36 (RAPD) and 44 (ISSR) samples by visual inspection, and fragment sizes were estimated with a 100-bp size ladder (PHARMACIA). ISSR bands larger than 1700 bp generally were discarded for accuracy of scoring the shorter fragments of presumably identical size (for discussion see Dowling *et al*. 1996; Vargas & Kadereit submitted). Thus, the scored ISSR bands ranged from 270 to 1700 bp. Band intensity was not considered in scoring either ISSR or RAPD bands. Presence/absence of bands was scored as diallelic for each assigned locus (1 = band present; 0 = band absent) and compiled into a matrix. In the combined data analysis, we used bands from 35 samples of which RAPD and ISSR data were available. Pairwise comparisons between samples were performed using Dice and Jaccard similarity coefficients with the NTSYS-pc program (Rohlf 1993). These distances

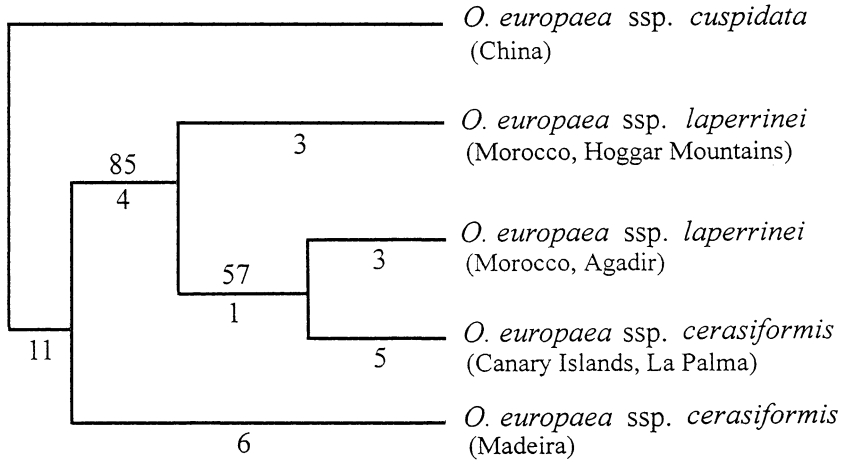

Fig. 2 The single branch-and-bound tree of 33 steps (CI: 0.73; RI: 0.62, excluding uninformative characters) from the analysis of the ITS-1 region in the *Olea europaea* complex. Tree branch numbers are bootstrap values (above) and nucleotide substitutions (below).

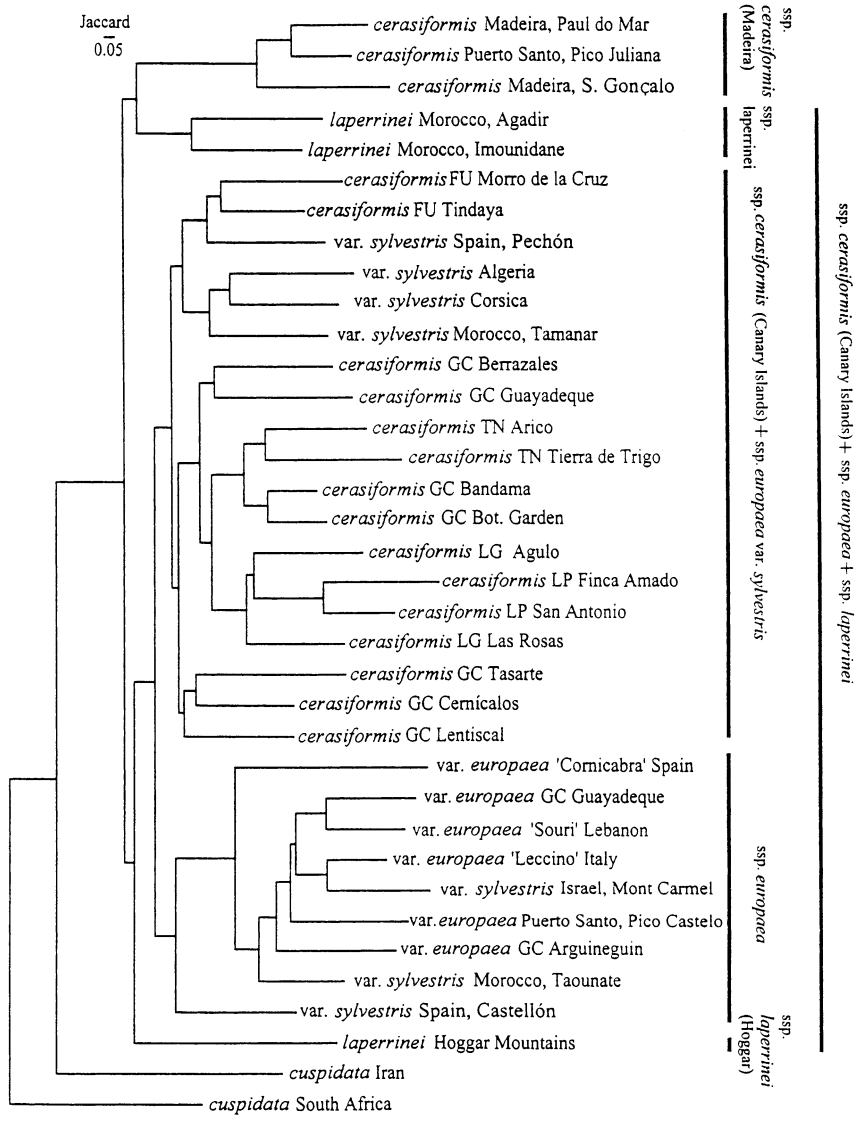

Fig. 3 Neighbour-joining analysis of 36 plants (105 RAPD phenotypes) of the *Olea europaea* complex generated from the Jaccard distance matrix. Population and taxon names as in Table 1. Canary Islands names abbreviated as follows: FU, Fuerteventura; GC, Gran Canaria; TN, Tenerife; LG, La Gomera; LP, La Palma.

were analysed using the Neighbour-joining (NJ) algorithm (in PAUP, Swofford 1998). NJ trees generated from Dice or Jaccard distances were very similar in the separate data sets and identical (except for one rearrangement in the La Palma/La Gomera cluster) in the combined data. As a consequence, we will only show and discuss trees based on Jaccard distances. A Mantel test (Mantel 1967) was performed to calculate the degree of positive correlation between genetic distance (Dice) and geographical distance among the 15 individuals of ssp. *cerasiformis* from the Canary Islands and one individual of ssp. *europaea* var. *sylvestris* from Morocco (Tamanar). Significance of the matrix correlation was evaluated by comparing the observed Mantel test statistic, Z, with its random distribution obtained after 9000 permutations. For these calculations the computer program NTSYS-pc (Rohlf 1993) was used.

Results

ITS-1 sequences

ITS-1 sequences of the five individuals analysed could be aligned unambiguously (accession no. forthcoming). The ITS-1 region in the entire *Olea europaea* complex is 209 bp in length. The aligned sequence matrix includes 39 variable sites, of which 10 are parsimoniously informative. Overlapping nucleotide peaks in *O. europaea* ssp. *laperrinei* (Agadir) were found at one informative site (119). No indels were found. Pairwise comparisons of the ITS-1 sequences using the Kimura 2-parameter model of sequence evolution yielded high infraspecific divergences ranging from 3.45% (between the two ssp. *laperrinei* accessions) to 11.22% (between ssp. *cuspidata* and ssp. *cerasiformis* from the Canary Islands). The branch-and-bound analysis found a single most parsimonious tree of 33 steps (C.I. 0.73; R.I. 0.62, excluding uninformative characters). This tree showed a well-supported clade (four synapomorphies, 85% bootstrap support) containing the two accessions of ssp. *laperrinei* plus that of ssp. *cerasiformis* from the Canary Islands (Fig. 2). Subspecies *cerasiformis* from Madeira is sister to this clade. Nucleotide comparison of ssp. *cerasiformis* sequences from Macaronesia revealed 16 nucleotide substitutions between the plants from La Palma (Canary Islands) and Madeira (Fig. 2).

RAPDs

A total of 105 reproducible bands were scored when using 15 RAPD primers. Invariable bands were present but not considered. Primers generated between one (E10) and 14 (E17) reproducible bands (7.1 bands/primer average). No exclusive bands to taxa of the *O. europaea* complex were found. The NJ tree showed three major clusters, where subspecies accessions do not form clear clusters (Fig. 3): (1) ssp. *cerasiformis* from Madeira; (2) ssp. *laperrinei* from Morocco (Agadir and Imounidane); and (3) ssp. *cerasiformis* from the Canary Islands, ssp. *europaea*, and ssp. *laperrinei* from the Hoggar. In the third cluster, ssp. *laperrinei* from Hoggar comes out first, and then two subclusters can be recognized: one with six accessions of the cultivated olive (var. *europaea*) plus three accessions of the wild olive (var. *sylvestris*); and a second one with four accessions of var. *sylvestris* and 15 of ssp. *cerasiformis* from the Canary Islands.

ISSRs

The three ISSR primers generated a total of 89 scored bands (887: 37; 834: 32; 855: 20). No invariable bands were found. The NJ tree showed three major clusters, where subspecies accessions do not form clear clusters (Fig. 4): (1) ssp. *laperrinei* plus one accesssion of ssp. *cuspidata*; (2) ssp. *cerasiformis* from Madeira; and (3) ssp. *cerasiformis* from the Canary Islands plus ssp. *europaea*. Within the third cluster, two subclusters were found: one with six accessions of ssp. *europaea* var. *europaea* plus one of var. *sylvestris*; and the other subcluster with all accessions (20) of ssp. *cerasiformis* from the Canary Islands plus seven ssp. *europaea* var. *sylvestris*. The latter subcluster includes a group of 20 accessions of Canarian plants (ssp. *cerasiformis*) plus one accession of ssp. *europaea* var. *sylvestris* from Morocco (Anti Atlas).

Combined RAPD and ISSR data

The NJ analysis of the combined 105 RAPD and 89 ISSR bands for 35 individuals showed three major clusters (Fig. 5): (1) all three individuals of ssp. *laperrinei*; (2) the three Madeiran accessions of ssp. *cerasiformis*; and (3) ssp. *cerasiformis* from the Canary Islands plus ssp. *europaea*. In the third cluster, one subcluster contains all accessions of the cultivated olive (var. *europaea*) plus two of var. *sylvestris*, and a second subcluster contains two groups: one with the 15 accessions of ssp. *cerasiformis*; and the other with four accessions of ssp. *europaea* var. *sylvestris*. The placement of the accession of *O. europaea* ssp. *laperrinei* (Agadir) in the ISSR–RAPD (Fig. 5) and the ITS-1 analyses (Fig. 2) is not congruent. The lack of strong support (57% bootstrap value) for the clade formed by *O. europaea* ssp. *laperrinei* and ssp. *cerasiformis* from Canary Islands suggests that further investigation of this relationship is needed.

Mantel test

The results of the Mantel test indicated that the correlation between genetic distance and geographical distance is highly significant ($r = 0.6541$, $P = 0.0001$).

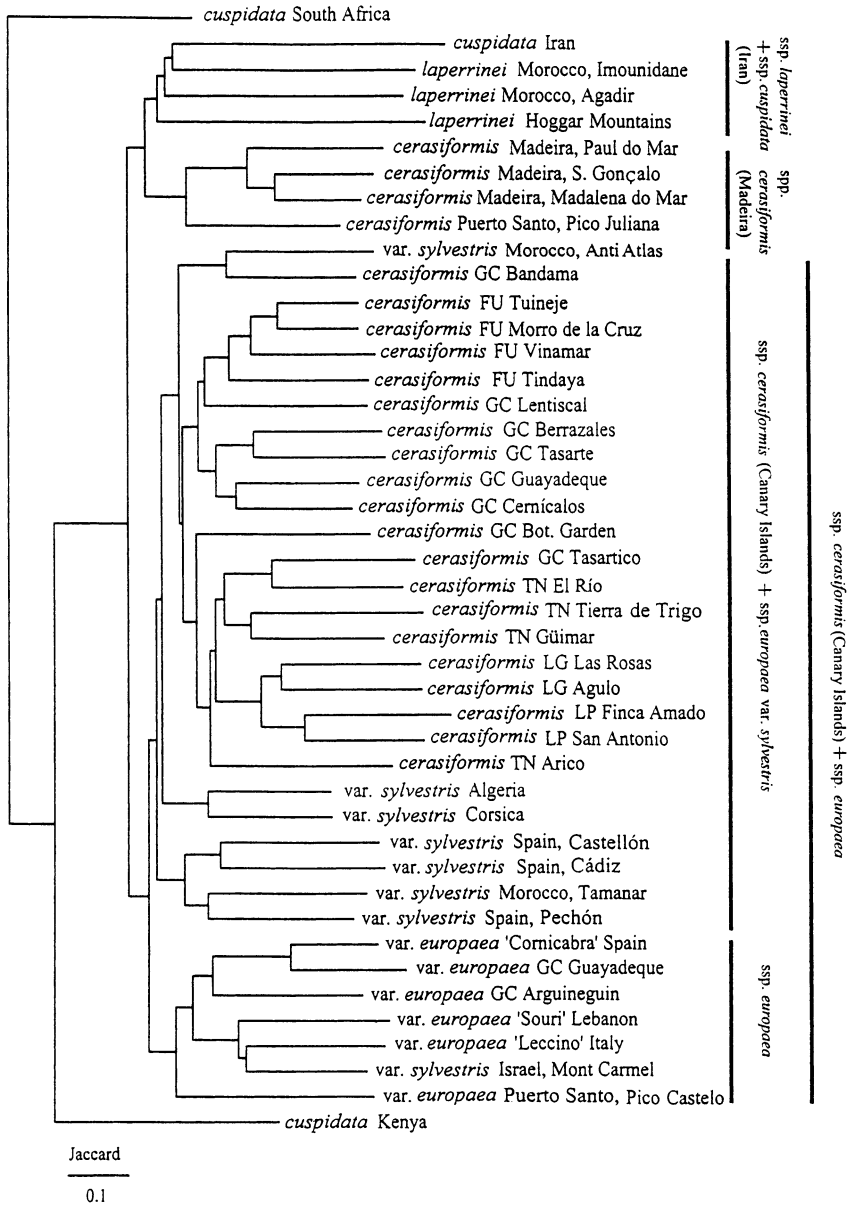

Fig. 4 Neighbour-joining analysis of 44 plants (89 ISSR phenotypes) of the *Olea europaea* complex generated from the Jaccard distance matrix. Population and taxon names as in Table 1. Canary Islands names abbreviated as follows: FU, Fuerteventura; GC, Gran Canaria; TN, Tenerife; LG, La Gomera; LP, La Palma.

Discussion

Major lineages in the Olea europaea *complex*

Chloroplast sequences of the 5′ region of *mat*K recognized two major lineages within the *Olea europaea* complex: ssp. *cuspidata* on the one hand, and sspp. *laperrinei*, *cerasiformis*, and *europaea* on the other (Vargas & Kadereit submitted). *mat*K sequences were identical in the latter three subspecies. Our nuclear data are in agreement with this basic division of the *O. europaea* complex, and provide further resolution to evaluate phylogenetic relationships among populations of sspp. *cerasiformis*, *laperrinei*, and *europaea*. In the ITS-1 tree, ssp. *cerasiformis* from Madeira is sister to a well-defined clade (85% bootstrap) of ssp. *laperrinei* from Agadir and Hoggar plus ssp. *cerasiformis* from La Palma (Fig. 2). The populational analysis based on the combined RAPD–ISSR data recognized three major lineages: (1) ssp. *laperrinei*; (2) ssp. *cerasiformis* from Madeira; and (3) ssp. *cerasiformis* from the Canaries plus ssp. *europaea* (Fig. 5). The genetic distinctiveness of the Madeiran ssp. *cerasiformis* evident from our analyses had previously been suggested by the occurrence of a unique allele at the phosphoglucose isomerase locus (Ouazzani *et al*. 1993). Within the third lineage, 15 Canarian populations of ssp. *cerasiformis* form an independent sublineage which is most similar to four accessions of ssp. *europaea* var. *sylvestris*, and in the

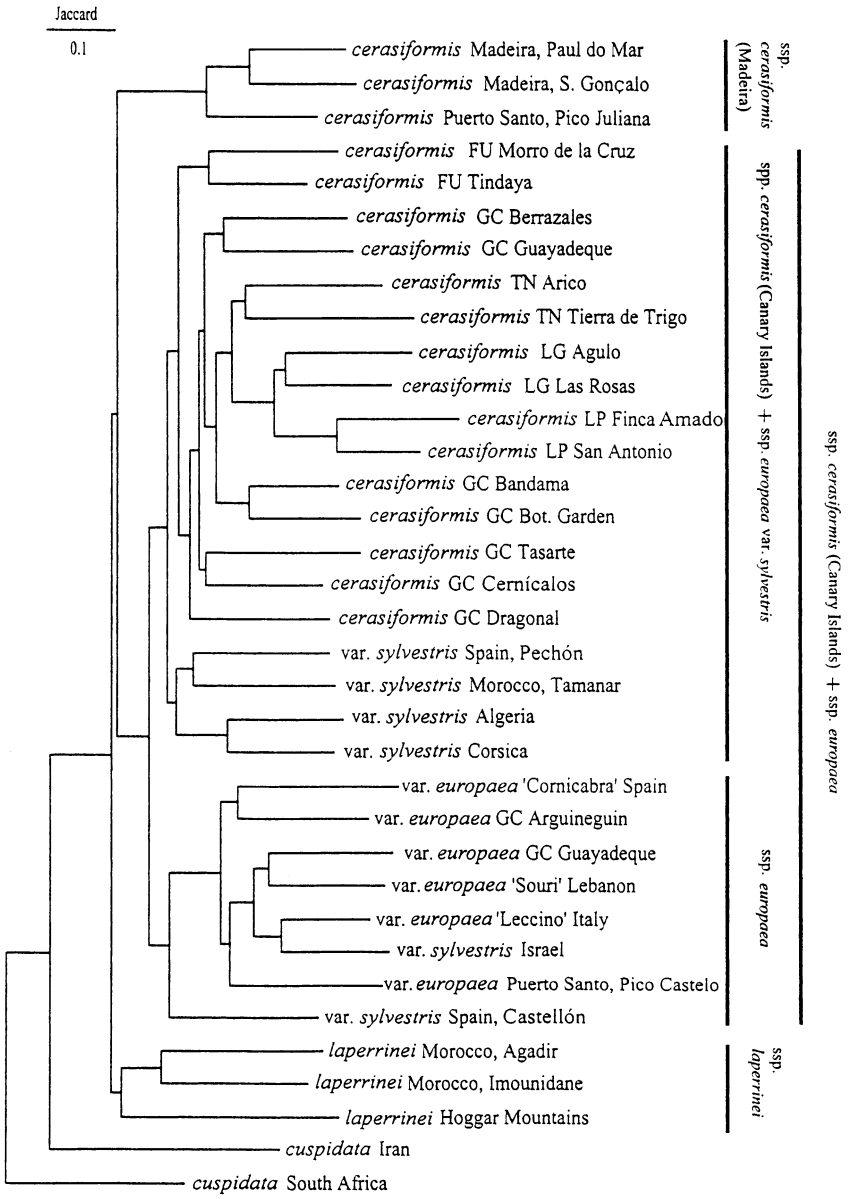

Fig. 5 Neighbour-joining analysis of 35 plants from the combined data (194 RAPD and ISSR phenotypes) of the *Olea europaea* complex generated from the Jaccard distance matrix. Population and taxon names as in Table 1. Canary Islands names abbreviated as follows: FU, Fuerteventura; GC, Gran Canaria; TN, Tenerife; LG, La Gomera; LP, La Palma.

second sublineage individuals of the cultivated olive (var. *europaea*) and wild olive (var. *europaea*) are intermingled. This latter finding may support previous reports about hybridization between var. *europaea* and var. *sylvestris*, or may illustrate the multiple domestications of the olive tree (Ouazzani *et al*. 1993; Zohary 1994; Lumaret *et al*. 1997; Vargas & Kadereit submitted). The three cultivars (var. *europaea*) from Madeira and the Canary Islands fall into a RAPD–ISSR subcluster containing cultivated and wild olives (ssp. *europaea*) from the continent (Fig. 5). This may indicate that little or no gene flow takes place between the native and cultivated olives in Madeira and the Canary Islands. However, a larger sample is necessary to assess the potential risk of genetic erosion of the locally endangered ssp. *cerasiformis* through hybridization with cultivars.

Accessions of the wild olive (var. *sylvestris*) are included in clusters of ssp. *cerasiformis* from the Canary Islands in the RAPD, ISSR, and RAPD–ISSR combined analyses. Thus, close genetic similarity between ssp. *cerasiformis* from the Canary Islands and ssp. *europaea* may indicate a close phylogenetic relationship between them.

In conclusion, molecular evidence clearly indicates that Madeiran and Canarian populations of ssp. *cerasiformis* do not form a monophyletic group. A principal component analysis of leaf and fruit characters of ssp. *cerasiformis* (Hess 1999) showed that Madeiran plants usually have narrower leaves and larger fruits than Canarian plants. Although these morphological characters are not sufficient to distinguish Madeiran and Canarian populations unambiguously, molecular and morphological evidence

suggest the recognition of two separate taxa in the interest of a more natural classification of the *O. europaea* complex. An unusually high degree of infraspecific ITS-1 divergence and lack of molecular support for the monophyly of ssp. *cerasiformis* suggest that the taxonomy of the *O. europaea* complex should be re-examined.

Dispersal of O. europaea *to Macaronesia*

Both ITS-1 sequence and RAPD–ISSR evidence clearly suggest that the colonization of the Madeira and Canary Islands by *O. europaea* took place twice. Although similar results have been reported in *Lavatera* (Ray 1995) and *Hedera* (Vargas *et al*., 1999), the majority of angiosperm radiations in Macaronesia started from single founders (*Echium*, Böhle *et al*. 1996; Sempervivoideae, Mes *et al*. 1996; *Argyranthemum*, Francisco-Ortega *et al*. 1997; *Cheirolophus*, Susanna *et al*. 1999; *Saxifraga*, Vargas *et al*. 1999). An early dispersal event may have occurred first to the Madeira Islands, as suggested by the basal position of the Madeiran plant in the ITS-1 tree (Fig. 2), followed by a second dispersal to the Canary Islands. As the Canary Islands (0.7–20.7 Ma; Carracedo 1994) are older than the Madeira Islands (5–18 Ma; Galopim de Carvalho & Brandão 1991), the colonization of the oldest Canarian island (Fuerteventura, 20.7 Ma) may have taken place at least several million years after its formation (Fig. 1). Alternatively, two more interpretations can be considered. First, the colonization by *Olea* occurred immediately after the formation of the islands, but the Madeira lineage had existed somewhere on the continent (or on other islands) where it became extinct after dispersal; a similar interpretation has been advocated by Sytsma *et al*. (1991) to explain the presence of *Fuchsia cyrtandroides* (estimated age: 10 Ma) on Tahiti (2 Ma). Second, the Macaronesian floras contain several plant taxa, including other species of Oleaceae, which are relicts of Miocene and Pliocene continental floras (Takhtajan 1969; Bramwell 1976). In view of this, it may be possible that the Madeiran ssp. *cerasiformis* represents a relict of an ancient ancestor of ssp. *cerasiformis*, *laperrinei*, and *europaea* which spawned a new line of evolution in the Mediterranean Basin, from where it colonized the Canary Islands. A palaeobotanical analysis of the flora of Mediterranean and Saharan African, conversely, suggested that *Olea* evolved in sclerophyllous forests in the Miocene (Quézel 1978). This may have occurred much earlier than the formation of the Macaronesian islands.

Our ISSR–RAPD data reveal a close relationship between ssp. *cerasiformis* from the Canary Islands and ssp. *europaea* and suggest a single introduction to the Canary Islands (Fig. 5). The internal position of a wild plant of ssp. *europaea* from the Anti Atlas (Morocco), only used in the ISSR analysis (Fig. 4), among ssp. *cerasiformis* from the Canaries is intriguing and may suggest an African origin of the Canarian olive. A larger sample is, however, necessary to pinpoint the potential source area of the Canarian olive.

Colonization pattern in the Canary Islands

The colonization of the Canary Islands by *O. europaea* may have followed a stepping-stone model in which an east–west pattern can be recognized. The two populations of the youngest island studied (La Palma, 1.5 Ma) are most closely related to the two populations from the nearby La Gomera (12.5 Ma; Figs 1, 5). This lineage is most similar to the two populations from Tenerife (11.6 Ma), located further east. The accessions of these three islands form one cluster with seven populations from Gran Canaria (13.9 Ma), and this entire lineage is related to two populations from the eastern Fuerteventura (20.7 Ma) (see Carracedo 1994 for island ages). The east–west arrangement of the Canary Islands (Fig. 1) may have been decisive for the formation of this clear pattern of colonization from the continent (closest point at $\approx$ 100 km east). Interisland dispersal between similar habitats has been described for other Canarian plants such as *Argyranthemum*, *Sonchus*, *Aeonium*, and *Crambe* (see Baldwin *et al*. 1998 for revision). The Canarian olive grows between 200 and 1000 m (Rodrigo Pérez & Montelongo Parada 1984) where conditions are similar to those in the Mediterranean region (Ceballos & Ortuño 1951).

The highly significant correlation between geographical and genetic distances ($r = 0.6541$, $P = 0.0001$) in our Canary Islands study illustrates that the populations in the different islands are well isolated at present, and that apparently little or no gene flow takes place. Studies on the reproductive biology of plants in the Mediterranean region have shown that the wild olive is one of the most actively bird-dispersed species among fleshy-fruited plants (Jordano 1987; Herrera 1995). Dispersal by animals of olive drupes is mainly caused by their lipid-rich composition. Olive drupes ripen from autumn to winter when extensive consumption of *Olea* fruits and bird migration take place (Biebach *et al*. 1986; Bairlein & Gwinner 1994). Although seed passage through guts is not necessary for seed germination of ssp. *cerasiformis* from Madeira and the Canary Islands (Hess, Kadereit, and Vargas, unpublished data), ingestion by birds promotes germination efficiency (Ridley 1930). Interestingly, *Hedera* and *Olea* are two of the three documented angiosperm genera dispersed to Macaronesia more than once which possess fleshy fruits and over 30% lipid contents (Kay 1992). These characteristics may have been crucial for successful bird ingestion and long-distance dispersal. The remarkable dispersal potential of the olive fruits may explain the repeated introduction of *O. europaea* to Macaronesia and successive colonization of the archipelagos. The observation

of strong genetic isolation between populations of different islands of the Canary Islands suggests, however, that subsequent interisland dispersal and establishment has been very rare or may not have occurred at all.

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