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Phylogenetics of *Olea* (Oleaceae) based on plastid and nuclear ribosomal DNA sequences: Tertiary climatic shifts and lineage differentiation times

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• *Background and Aims* The genus *Olea* (Oleaceae) includes approx. 40 taxa of evergreen shrubs and trees classified in three subgenera, *Olea*, *Paniculatae* and *Tetrapilus*, the first of which has two sections (*Olea* and *Ligustroides*). Olive trees (the *O. europaea* complex) have been the subject of intensive research, whereas little is known about the phylogenetic relationships among the other species. To clarify the biogeographical history of this group, a molecular analysis of *Olea* and related genera of Oleaceae is thus necessary.
• *Methods* A phylogeny was built of *Olea* and related genera based on sequences of the nuclear ribosomal internal transcribed spacer-1 and four plastid regions. Lineage divergence and the evolution of abaxial peltate scales, the latter character linked to drought adaptation, were dated using a Bayesian method.
• *Key Results Olea* is polyphyletic, with *O. ambrensis* and subgenus *Tetrapilus* not sharing a most recent common ancestor with the main *Olea* clade. Partial incongruence between nuclear and plastid phylogenetic reconstructions suggests a reticulation process in the evolution of subgenus *Olea*. Estimates of divergence times for major groups of *Olea* during the Tertiary were obtained.
• *Conclusions* This study indicates the necessity of revising current taxonomic boundaries in *Olea*. The results also suggest that main lines of evolution were promoted by major Tertiary climatic shifts: (1) the split between subgenera *Olea* and *Paniculatae* appears to have taken place at the Miocene–Oligocene boundary; (2) the separation of sections *Ligustroides* and *Olea* may have occurred during the Early Miocene following the Mi-1 glaciation; and (3) the diversification within these sections (and the origin of dense abaxial indumentum in section *Olea*) was concomitant with the aridification of Africa in the Late Miocene.

Key words: Internal transcribed spacer (ITS), relaxed molecular clock, olive tree, leaf peltate scales, plastid DNA, Tertiary climatic shifts, systematics.

INTRODUCTION

Oleaceae comprise about 600 species and 24 genera (Johnson, 1957; Rohwer, 1996; Wallander and Albert, 2000; Green, 2004). Within this family, *Olea* and ten other (extant) genera constitute the subtribe *Oleinae* within the tribe *Oleeae* (Wallander and Albert, 2000). Thirty-three species and nine subspecies of evergreen shrubs and trees have been circumscribed in *Olea* based on morphological characters (Green, 2002). In addition, these taxa are classified in three subgenera, *Olea*, *Paniculatae* and *Tetrapilus*, the first of which has two sections (*Olea* and *Ligustroides*). Section *Olea* is formed exclusively by the olive complex (*Olea europaea*), in which six subspecies are recognized (Vargas *et al.*, 2001; Green, 2002). This subgenus is distributed from South Africa to China, across the Saharan mountains, Macaronesia and the Mediterranean basin. *O. europaea* is also found outside of its native range as a result of human-mediated dispersal; it has been repeatedly introduced in the New World and has become naturalized and has invaded numerous areas in Australia, New Zealand and the Pacific islands (Green, 2002; Besnard *et al.*, 2007*b*). Section *Ligustroides* includes eight species from central and southern Africa, displaying numerous similarities in morphological and biochemical traits with section *Olea* (Harborne and Green, 1980; Green, 2002). Key morphological characters discriminating these two sections are the inflorescence position (axillary in section *Olea* vs. terminal and sometimes axillary in section *Ligustroides*), the density of peltate scales (densely covered abaxial leaf surface in section *Olea* vs. leaves with no or scattered scales in section *Ligustroides*) and the structure of the calyx tube ($\pm$ membranous in section *Olea* vs. $\pm$ coriaceous in section *Ligustroides*; Green, 2002). Subgenus *Paniculatae* includes only one taxon (*Olea paniculata*) distributed from Pakistan to New Caledonia. This species is characterized by leaf domatia in the axils of the midrib and primary veins (Green, 2002). Lastly, subgenus *Tetrapilus* contains 23 species from south-eastern Asia. Limited flower shape variability is found in this subgenus, whereas variable vegetative and reproductive traits are observed (e.g. leaf morphology, hermaphrodite vs. dioecious species; Green, 2002). Key characters defining subgenus *Tetrapilus* are a corolla tube longer than corolla lobes and the absence of peltate scales.

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Olea taxa are found in a wide range of habitats. Most species are distributed in subtropical and tropical areas where they can be important vegetation components (Green, 2002). Some taxa in both sections of subgenus *Olea* occur in arid environments, although representatives can also be found in other habitats. The olive complex (section *Olea*) is present in open forests of the Mediterranean and subtropical regions of the Old World, from Macaronesian cloud forests (subspp. *cerasiformis* and *guanchica*) to extremely arid Saharan mountains (subsp. *laperrinei*). Members of section *Ligustroides* occur in various habitats in subtropical and equatorial Africa (Green and Kupicha, 1979; Green, 2002), such as dry brush on coastal dunes (e.g. *O. exasperata* and *O. woodiana*), scrub vegetation among quartzite crags (*O. chimanimani*), upland forests with low rainfall (e.g. *O. capensis* subsp. *macrocarpa*) and altitudinally transitional and humid upland forests (e.g. *O. schliebenii* and *O. welwitschii*). Stomata protected by dense abaxial peltate scales (a trait considered to be linked to dry habitats; Bongi *et al.*, 1987) are only found in section *Olea* (Green, 2002). In contrast, other traits associated with arid environments, such as the presence of a thick cuticle and lanceolate leaves, are found in both sections (Green, 2002). Subgenus *Paniculatae* is present in coastal scrub and rain forests (Kiew, 1979). Lastly, subgenus *Tetrapilus* is found in a variety of habitats (Kiew, 1979; Chang *et al.*, 1996; Green, 2002), from xeric sandstone in open rocky country (e.g. *O. dentata*) to dense and moist lowland tropical forests (e.g. *O. guangxiensis* and *O. rosea*).

Some attempts have been made to determine the affinities and phylogenetic relationships between *Olea* species and related genera of Oleaceae using biochemical and molecular data (e.g. Harborne and Green, 1980; Angiolillo *et al.*, 1999; Wallander and Albert, 2000; Vargas and Kadereit, 2001; Baldoni *et al.*, 2002; Besnard *et al.*, 2002; Jensen *et al.*, 2002). However, molecular studies conducted to date have lacked suitable representation of subgenera and sections. Polyphyly of the genus *Olea* was suggested by Wallander and Albert (2000) and Besnard *et al.* (2002) based on plastid DNA sequences and nuclear ribosomal DNA (nrDNA) restriction fragment-length polymorphisms (RFLPs), respectively. In particular, subgenus *Tetrapilus* was proposed as a separate genus by Besnard *et al.* (2002), as previously considered by Johnson (1957) based on morphology. However, these conclusions lacked strong support, as the number of taxa of *Olea sensu lato* included in those studies was limited and most studies published to date have been focused on section *Olea*. Reticulation events appear to have played an important role in the evolution of this section (e.g. Angiolillo *et al.*, 1999; Rubio de Casas *et al.*, 2006; Besnard *et al.*, 2007*c*) and polyploidy also contributed to its diversification in North-West Africa [e.g. subspp. *maroccana* (6×) and *cerasiformis* (4×); Besnard *et al.*, 2008; Brito *et al.*, 2008; García-Verdugo *et al.*, 2009]. Little is known about the biogeography and evolutionary history of other *Olea* taxa (i.e. *Tetrapilus*, *Ligustroides* and *Paniculatae*), but a better understanding of *Olea* taxonomy and diversification may be of great importance for the future management of olive genetic resources and for the *in situ* conservation of genetically differentiated entities (Forest *et al.*, 2007). Additional investigations, including both extended sampling and additional polymorphic markers, are thus needed to reconstruct a robust phylogeny to test for polyphyly and to infer the origin and centres of diversification of the genus *Olea*. Moreover, the use of palaeobotanical data in phylogenetic analyses helps in dating major lineage divergence times and then in identifying differentiation events (e.g. Magallón and Sanderson, 2001).

Contrasting between plastid and nuclear analyses is useful in interpreting the biogeographical history of taxa and the evolution of phenotypic traits (e.g. Maurin *et al.*, 2007; Wang *et al.*, 2007; Figueroa *et al.*, 2008). Incongruence between classifications based on both genomes can reveal evolutionary events such as reticulation or incomplete lineage sorting (Linder and Rieseberg, 2004). However, if methodologies to generate plastid DNA sequences are generally simple in plants, the phylogenetic use of nuclear markers can be more challenging (Álvarez and Wendel, 2003). The internal transcribed spacers (ITS) of nrDNA have been useful in resolving phylogenetic relationships in three genera of Oleaceae (*Fraxinus*: Jeandroz *et al.*, 1997; Wallander, 2008; *Ligustrum* and *Syringa*: Li *et al.*, 2002), although some limitations of such markers have been encountered in the analysis of angiosperm phylogenies (Baldwin *et al.*, 1995; Álvarez and Wendel, 2003; Nieto Feliner and Rosselló, 2007). Typically, ITS sequences are subject to concerted evolution, display a rapid rate of evolution compared with most plastid loci, and can be readily amplified and sequenced even from poorly preserved material (Mort *et al.*, 2007). However, technical difficulties have been encountered when sequencing ITS regions in the olive complex due to the presence of numerous pseudogenes (Besnard *et al.*, 2007*c*). As a consequence, this set of markers has to be used cautiously and with improved techniques to isolate functional ITS sequences from nrDNA units (see Nieto Feliner and Rosselló, 2007).

In the present study, we used maternally inherited (plastid DNA) and nuclear (functional ITS-1) sequences to address four main objectives: (1) to reconstruct phylogenetic relationships in the genus *Olea* with a focus on subgenus *Olea*, which contains the African members related to the cultivated olive; (2) to evaluate congruence of plastid and nuclear phylogenetic trees with current taxonomic groupings; (3) to date and to interpret the main splits leading to the diversification of the genus *Olea* using palaeobotanical data and a relaxed molecular clock approach; and (4) to infer the evolution of a key character linked to drought adaptation (i.e. protection of stomata by peltate scales).

MATERIALS AND METHODS

Plant material and molecular characterization

Sixty-one accessions of *Olea* were used for phylogenetic analyses (Appendix) as follows: 35 of subgenus *Olea* section *Olea* (the monotypic *O. europaea* and its six subspecies); 17 of subgenus *Olea* section *Ligustroides* (all eight species); seven of subgenus *Tetrapilus* (seven species of 23, including the type species; Green, 2002); and two of subgenus *Paniculatae* (the only species). Because of difficulties in obtaining samples of subgenus *Tetrapilus*, the present sample was limited to seven species representing the geographical distribution of this group [from south-west China (Yunan and Hainan), Thailand, Sumatra and the Philippines] and some morphological variation (including leaf shape, petiole hairiness, abaxial

indumenta of leaves and inflorescence size; Green, 2002). In subgenus *Olea*, only one subspecies (*O. woodiana* subsp. *disjuncta*; section *Ligustroides*) was not sampled. The placement of the rare Madagascan species *O. ambrensis* (section *Ligustroides*) was of particular interest, given that its morphological description is incomplete and lacks an appropriate account of the reproductive organs (Perrier de la Bâthie, 1952; Green, 2002). DNA from each individual was extracted using a 2× CTAB method (Besnard *et al.*, 2000), except for 12 specimens from herbarium collections (Appendix). For these samples DNA was extracted from about 10–20 mg of plant material using the DNeasy Plant Mini Kit (Qiagen, GmbH, Hilden, Germany). Outside the genus *Olea*, 17 samples of Oleaceae were also considered in our analyses (*Chionanthus broomeana*, *C. retusus*, *Fraxinus americana*, *F. excelsior*, *F. quadrangulata*, *Ligustrum vulgare*, *Nestegis sandwicensis*, *Noronhia emarginata*, *N. longipedicellata*, *N. luteola*, three samples of *Noronhia* spp., *Osmanthus fragrans*, *O. heterophyllus*, *Phillyrea latifolia* and *Syringa vulgaris*; see Appendix). Three species (*Fraxinus americana*, *F. quadrangulata* and *Syringa vulgaris*; from GenBank) were only used in the ITS-1 analyses (see below). All these 17 species belong to the monophyletic tribe *Oleeae*, and 14 are placed in the subtribe *Oleinae* (members of genera *Chionanthus*, *Nestegis*, *Noronhia*, *Osmanthus* and *Phillyrea*) and are phylogenetically relatively close to the genus *Olea* (Wallander and Albert, 2000).

All individuals were characterized using four plastid DNA regions (*trnL-trnF*, *matK*, *trnT-trnL* and *trnS-trnG*). DNA amplification of each region was performed using the PCR protocol described by Guzmán and Vargas (2005). Standard primers were used for amplification of *matK* (Johnson and Soltis, 1994), *trnL-trnF* and *trnT-trnL* (Taberlet *et al.*, 1991), and new primers were designed to amplify *trnS-trnG* (see Supplementary Data 1, available online). PCR amplification of herbarium DNA generally failed for fragments of size greater than 300 bp. Consequently, several overlapping fragments (between 200 and 320 bp) were generated to obtain a complete consensus sequence of the four spacers for the 12 herbarium samples (Appendix). Two fragments were generated for *trnL-trnF*, three for *trnT-trnL* and five for *matK* and *trnS-trnG*. The complete list of primers used is given in Supplementary Data 1. The PCR reaction mixtures contained 5 μL of DNA solution, 1× PCR buffer, 2·5 or 5 mM $MgCl_2$ (see Supplementary Data 1), 0·2 mM dNTPs, 0·2 μmol of each primer and 0·75 units of DNA polymerase (GoTaq, Promega, Madison, WI, USA) in a total of 25 μL. After 3 min at 94 °C, the PCR thermocycler programme (T1, Biometra, Göttingen, Germany) was: 36 cycles of 30 s at 94 °C, 30 s at the defined annealing temperature (50 or 53 °C; see Supplementary Data 1) and 90 s at 72 °C. The last cycle was followed by a 10-min extension at 72 °C. PCR products were cleaned using spin filter columns (PCR Clean-up, MoBio Laboratories, Carlsbad, CA, USA) and then directly sequenced using a Big Dye 3·1 Terminator cycle sequencing kit (Applied Biosystems, Little Chalfont, UK) according to manufacturer's instructions and an ABI Prism 3100 genetic analyser (Applied Biosystems, Foster City, CA, USA).

To compare the information contained in the plastid and nuclear genomes, sequences of a nuclear region were also generated. nrDNA polymorphism was analysed because of its informativeness in angiosperms (Baldwin *et al.*, 1995; Nieto Feliner and Rosselló, 2007) and because it previously showed reliable molecular variation for *Olea* phylogenetics (Besnard *et al.*, 2007*c*). Moreover, the poor preservation of the DNA extracted from the 12 herbarium specimens prevented effective use of single-copy nuclear genes on these samples. New primers were designed to specifically generate sequences of functional ITS-1 units. The functional units (i.e. AJ585193 and AM403099) and different pseudogenes isolated from the olive complex (see Besnard *et al.*, 2007*c*) were aligned with functional ribosomal units from various members of Oleaceae from which pseudogenes have not been isolated (e.g. Jeandroz *et al.*, 1997; Li *et al.*, 2002). Two primers located in the 18S and 5·8S genes (18Sf: 5′-CAAGGTTTCCGTAGGTGAACC-3′ 5·8Sr: 5′-TCGCA TTTTGCTGCGTTCTTC-3′) were designed in the conserved regions of functional units, and the forward primer (18Sf) was designed to avoid amplification of all pseudogenes. The PCR reaction mixture contained 1–10 ng DNA template, 1× AccuPrime™ PCR Buffer II (with 0·2 mM dNTPs and 2 mM $MgSO_4$), 2 μL dimethyl sulfoxide (DMSO), 0·2 μmol of each primer and 0·75 U DNA polymerase (AccuPrime Taq, Invitrogen, Carlsbad, CA, USA) in a total of 25 μL. After 2 min at 94 °C, the PCR thermocycler programme (T1, Biometra) was: 36 cycles of 30 s at 94 °C, 30 s at 58 °C and 45 s at 68 °C. The last cycle was followed by a 10-min extension at 68 °C. Direct sequencing was performed as previously described for plastid DNA fragments. Unreadable chromatograms due to co-occurrence of different ITS-1 copies with indels (leading to frame shifts and generating chromatogram mismatches) were observed in two individuals (see below). For these samples, ITS-1 haplotypes were isolated using the InsT/Aclone™ PCR product cloning kit (Mbi Fermentas, Vilnius, Lithuania) and sequenced as described by Besnard *et al.* (2007*c*).

Phylogenetic reconstructions

Each of the four plastid DNA regions was first aligned using Clustal W (Thompson *et al.*, 1994) and then combined. Manual alignment was necessary in segments where indels were observed. Indels were coded using Seqstate v. 1·32 (Müller, 2005). The aligned matrix is available from the authors upon request. Parsimony-based analyses were conducted using a heuristic search strategy with 1000 random addition replicates followed by tree-bisection-reconnection (TBR) branch swapping, with the options Mulpars and Steepest Descent in effect (as implemented in Paup 4·0b10; Swofford, 2001). Support values were assessed from 1000 bootstrapping (bs) pseudo-replicates, with the maximum number of rearrangements set at 100 000 000 to avoid excessive computation time. Coded indels were then excluded for further analyses.

Phylogenetic relationships among haplotypes were also evaluated using Bayesian inference with MrBayes 3·1·2 (Ronquist and Huelsenbeck, 2003). The best-fit model was obtained with MrModeltest 2·0 (Nylander, 2004) for each of the four plastid DNA fragments according to the Akaike Information Criterion (*trnL-trnF*: HKY + I; *matK* and

trnT-trnL: GTR + G; and *trnS-trnG*: GTR + I + G). Two parallel runs, each of four chains, were run for 10 000 000 generations and a tree was sampled every 1000 generations after a burn-in period of 3000 000 generations. All model parameters were optimized separately for each DNA region.

Before reconstructing phylogenetic trees based on the ITS-1 data sample, we tested whether the sequences displayed characteristics of functional units (e.g. G + C content, conserved motifs) following the procedures described by Besnard *et al.* (2007*c*). To help identify pseudogenes, minimum-energy secondary structure (Δ) of ITS-1 of each sequence was estimated with MFOLD (http://mfold.burnet.edu.au; Zuker, 1989) using the default temperature of 37 °C. Data were then analysed as previously described for plastid DNA both for maximum parsimony (MP) and Bayesian inference (BI) analyses, except that MP analyses were conducted without limiting the number of rearrangements per bootstrap replicate. BI analyses were performed using the GTR + I + G nucleotide substitution model, which was the model with the best fit for ITS-1 sequences.

Molecular dating

Using a Bayesian method implemented in the software BEAST v. 1·4·8 (Drummond *et al.*, 2006; Drummond and Rambaut, 2007), a tree was inferred and simultaneously calibrated based on both nuclear and plastid markers. In this analysis, only accessions for which both plastid and ITS-1 data were available were considered. The two individuals for which two divergent ITS-1 sequences were isolated by cloning (see below) were excluded. In addition, two other olive individuals of subspp. *cuspidata* and *laperrinei* from Egypt and Hoggar, respectively, were also removed because they displayed deeply incongruent placements in plastid and ITS-1 phylogenetic trees (see below), probably as a consequence of hybridization (see Besnard *et al.*, 2007*c*). All markers were considered simultaneously and were analysed under a GTR + I + G nucleotide substitution model. Substitution rate was unfixed and a relaxed molecular clock with uncorrelated lognormal rates was used. The tree prior was set to a birth–death speciation process. Other priors remained unchanged except time constraints on three nodes of the phylogeny. According to fossil evidence, the divergence between *F. excelsior* and the ingroup occurred before 37·2 Mya (Suzuki, 1982; Call and Dilcher, 1992). This node was calibrated using a normal distribution with a mean of 40 Mya and a standard deviation of 3 Mya. In addition, the divergence of subgenus *Olea* occurred at least 23 Mya (Muller, 1981; Palamarev, 1989; Terral *et al.*, 2004), and the crown of subgenus *Olea* was constrained between 23 and 30 Mya using a uniform distribution. Finally, the olive complex is known to be older than 3·2 Mya (Palamarev, 1989; Terral *et al.*, 2004) and was therefore constrained between 3·2 and 10 Mya, following a uniform distribution. Descendants of the three nodes corresponding to calibration points were forced to be monophyletic. However, this had no effect as these nodes were also strongly supported in unconstrained Bayesian analyses (data not shown). Bayesian analyses were run for 10 000 000 generations, sampling parameters every 1000 generations. The burn-in period was set to 1000 000 generations. Results were visualized in TRACER v. 1·4 (Drummond and Rambaut, 2007) to check that the analysis converged and they were summarized using TREEANNOTATOR (Drummond and Rambaut, 2007). Ages of nodes were estimated by the node mean heights.

Morphological and scanning electron microscopy analysis

Scanning electron microscopy (SEM) analyses of the leaf abaxial surfaces of 22 taxa were performed with a Hitachi S-3000 N scanning electron microscope. All the samples were also initially observed with an Olympus Bx 60 optical microscope to identify structures of interest. Only fully mature leaves of herbarium specimens of reproductive individuals were analysed. Leaf fragments for microscopic observations were taken from the central quarter of the leaf blades, between the midrib and the margin. All subgenera and sections were represented by at least one sample. Several outgroup species were also included (Appendix). The evolution of trichome morphology considered as a qualitative character (i.e. glabrous leaves, scattered scales or dense indumenta) was analysed in the light of phylogenetic relationships.

RESULTS

Plastid sequence characteristics and phylogenetic reconstructions

The four plastid DNA regions (*trnL-trnF*, *trnT-trnL*, *trnS-trnG* and *matK*) were sequenced on 71 samples of tribe *Oleeae*. The total aligned matrix was 3509 bp long, and had 261 variable sites and 121 potentially parsimony-informative characters (Table 1). The number of indels detected in the alignment was 55, of which 26 were potentially parsimony-informative. Five multi-state microsatellite motifs were initially excluded from the analysis. When using only *O. paniculata* (Australia) and *O. europaea* subsp. *europaea* ('Toffahi'), our plastid DNA data provided more polymorphisms (22 substitutions and five indels) than in a previous study based on *rps16* and *trnL-trnF* (Wallander and Albert, 2000), with five substitutions and three indels. In subgenus *Olea* (sections *Olea* and *Ligustroides*; excluding *O. ambrensis*), 57 variable sites and 13 indels were detected, of which 33 and five were potentially parsimony-informative, respectively (Table 1). Maximum sequence divergence in this subgenus was low (0·8 %).

Plastid phylogenetic reconstructions are shown in Fig. 1. Only the BI tree of the plastid DNA data set is shown because the topology obtained via MP was the same. The analyses did not support the monophyly of the genus *Olea*. First, *O. ambrensis* was placed in a clade formed by accessions of *Noronhia* (endemic to Madagascar). Second, a clade comprising two *Osmanthus* species, *Chionanthus retusus*, *Nestegis sandwicensis*, *Osmanthus* spp., *Phillyrea latifolia* and *Olea* subgenus *Tetrapilus*, was sister to the other *Olea* species. A monophyletic group of three well-supported clades corresponding to *Olea* subgenus *Paniculatae*, *Olea* subgenus *Olea* section *Ligustroides* and *Olea* subgenus *Olea* section *Olea* was recovered. In section *Ligustroides*, phylogenetic reconstructions supported one cluster containing only accessions from southern Africa (*O. exasperata* and *O. capensis* subspp. *macrocarpa*, *capensis* and *enervis*) and one cluster including *O. woodiana*, *O. schliebenii* and *O. welwitschii*. However,

TABLE 1. *Summary of phylogenetic characteristics obtained from the analysis of nuclear ribosomal (nr)DNA (ITS-1) sequences, and plastid* trnL-trnF, trnT-trnL, trnS-trnG *and* matK *sequences of the full* Oleeae *sample and of subgenus* Olea *(i.e. section* Olea *and* Ligustroides*; excluding* O. ambrensis*)*

	nrDNA	*trnL-trnF*	*trnT-trnL*	*trnS-trnG*	*matK*	cpDNA
Substitution model	GTR + I + G	HKY + I	GTR + G	GTR + I + G	GTR + G	–
Full *Oleeae* sample						
Length range (bp)	256–288 (222–254)*	327–343	566–672	1066–1108	1221–1229	–
Aligned length (bp)	304	353	724	1200	1232	3509
No. of variable/potentially informative characters	147/121	23/6	55/28	94/55	89/32	261/121
Maximum sequence divergence (K-2-p[$]) (%)	38·1	3·5	3·9	3·9	3·1	3·4
No. of variable/potentially informative indels‡	28/21	5/2	17/5	26/13†	7/6	55/26
Mean G + C content (%)	68	35	27	31	32	–
Subgenus *Olea*						
No. of variable/potentially informative characters	57/42	3/2	10/5	27/16	17/10	57/33
Maximum sequence divergence (K-2-p) (%)	9·9	0·9	0·9	1·1	0·8	0·8
No. of variable/potentially informative indels‡	12/11	1/1	4/0	8/4	0/0	13/5

To estimate the number of variable sites or indels, maximum sequence divergence and G + C content we only considered species for which all five regions were available.

* The length range of the ITS-1 spacer (excluding 5·8S and 18S segments) is given in parentheses.

[$] Kimura-2-parameter.

† The number of variable indels in *trnS-trnG* includes a 41-bp inversion specific to *O. neriifolia*.

‡ Excluding multi-state microsatellite motifs.

accesions of *O. capensis* did not form a monophyletic group. In section *Olea*, a well-supported, biphyletic resolution was obtained, in which *O. europaea* subsp. *cuspidata* was sister to the other five subspecies of the olive complex. However, the *cuspidata* accession from southern Egypt was related to the Mediterranean subspecies as previously detected with plastid markers (Besnard *et al.*, 2007*c*).

ITS-1 sequence characteristics

Forty-six *Olea* ITS-1 sequences (including short flanking segments of 18S and 5·8S) were analysed (alignment available in Supplementary Data 2, available online). Two sequences were isolated by cloning from individuals 'Tassili n'Ajjer' (*O. europaea* subsp. *laperrinei*) and 'Tenerife' (*O. europaea* subsp. *guanchica*), for which chromatograms were not readable because of indels between different ITS-1 copies. A few double peaks (a maximum of three sites per sequence) were also observed on 14 chromatograms by direct sequencing and sites were coded with International Union of Pure and Applied Chemistry (IUPAC) symbols (see Supplementary Data 2). Using this approach, ITS-1 sequences were also generated from 13 other members of Oleaceae including outgroup sequences for *Fraxinus excelsior* and *Ligustrum vulgare*. A sequence identical to that published by Li *et al.* (2002; EMBL accession no. AF361298) was obtained for the latter, whereas the *F. excelsior* sequence of our analysis displayed a high sequence similarity (98·3 %) with the one from Jeandroz *et al.* (1997; EMBL accession no. U82866). The ITS-1 sequences were between 222 and 254 bp in length (Table 1). In subgenus *Olea*, the length of this spacer ranged from 243 bp (for most samples of the olive complex) to 254 bp (for one Madagascan sample of *O. capensis* subsp. *macrocarpa*; RNF08). The shortest sequences were found in samples of *Noronhia* spp. and *O. ambrensis*, which displayed indels in the 5′ part of ITS-1. Sequence characteristics supported that our ITS-1 sequences are part of functional nrDNA units (i.e. group 4; Besnard *et al.*, 2007*c*): (1) the G + C content varied between 55·4 % (*Ligustrum vulgare*) and 73·4 % (*O. capensis* subsp. *enervis*), with a mean value of 68 % (Table 1); (2) all the ITS-1 sequences analysed had TCGA at the 5′ end, except for two sequences of *O. capensis* subsp. *macrocarpa* from Madagascar (ROR193 and RFN015), which had CCGA; (3) the highly conserved ITS-1 motif of flowering plants, GGCRY-(4-7 n)-GYGYCAAGGAA (Liu and Schardl, 1994), was also present in all our sequences as GGCGC-GRRRA-GCGYCAAGGAA; and (4) minimum energy values of the secondary structure (Δ) of ITS-1 ranged from −72·6 to −106·4 kcal mol^{-1} and thus had a lower value than those reported for olive pseudogenes (−47·8 to −62·2 kcal mol^{-1}; Besnard *et al.*, 2007*c*). In addition, a 5·8S gene segment of 28 bp was also sequenced in all accessions and was shown to be highly conserved.

ITS-1 phylogenetic reconstructions

The ITS-1 sequences provided 147 variable sites (Table 1). In ITS-1 sequences (304 sites after alignment), the same number of potentially informative characters (121) were found as in the four plastid DNA regions (with a total sequence length of 3509 bp). Only the BI tree is presented in Fig. 2, as the same topology was obtained in the MP analysis. These analyses show the polyphyletic pattern for the genus *Olea* as observed in the plastid DNA analyses. *Olea* subgenus *Tetrapilus* again appeared as a separate lineage, but it was placed in a different position in the ITS-1 phylogeny. *Olea ambrensis* was again closely related to *Noronhia*. Furthermore, *Olea* subgenus *Paniculatae* was sister to subgenus *Olea* (topology not resolved in the plastid DNA tree). In this latter clade, two subclades with high support values defined sections *Olea* and *Ligustroides* (Fig. 2), in agreement with the plastid phylogeny. Within each section, the ITS-1 tree, however, depicted groupings of accessions with only low congruence with groups of the plastid tree (see above). In section *Ligustroides*, samples of *O. capensis* (particularly those of subsp. *macrocarpa*) did not form a

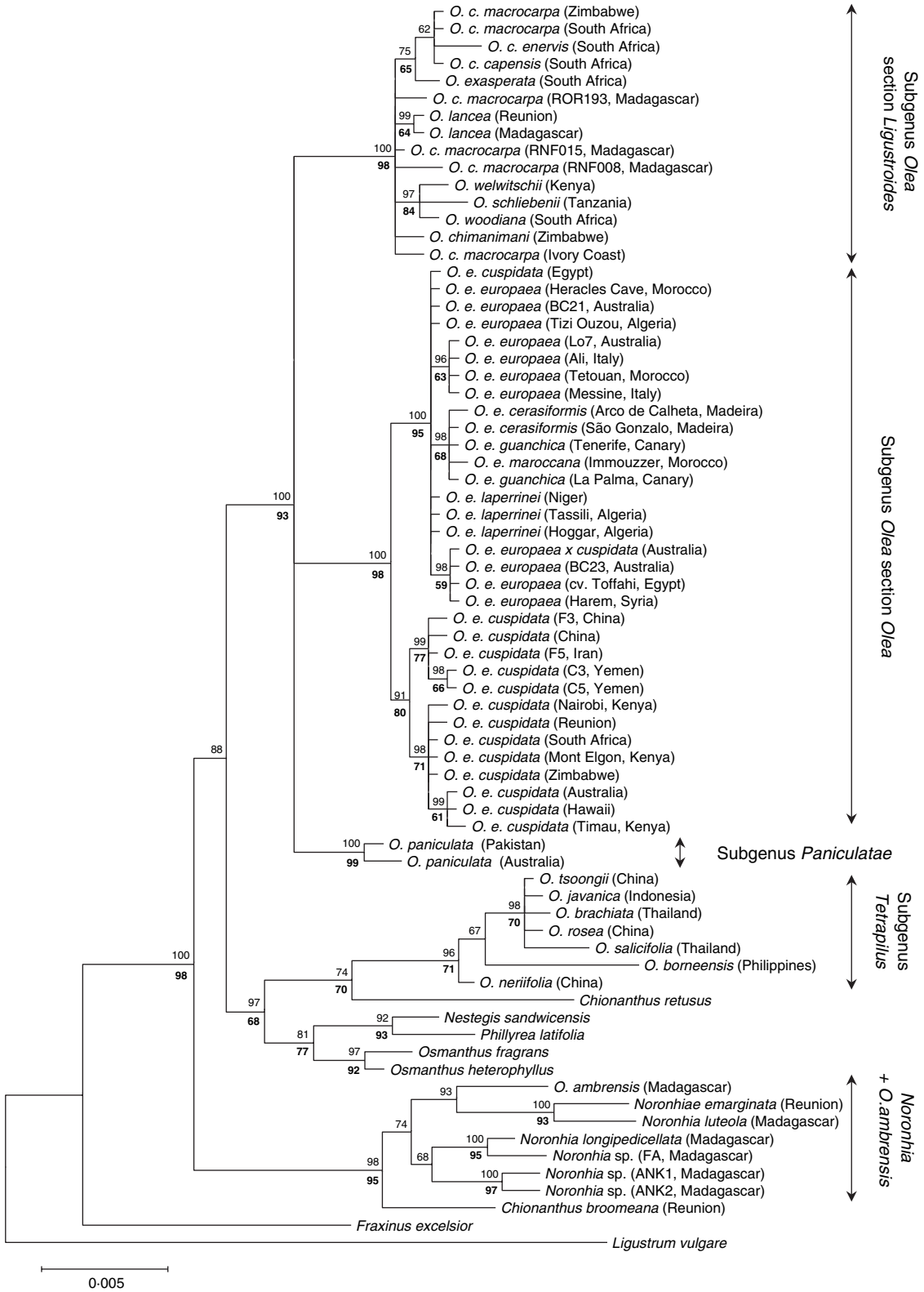

FIG. 1. Phylogenetic tree for *Olea* species based on four plastid DNA regions (*trnT-trnL*, *trnL-trnF*, *trnS-trnG* and *matK*). Majority-rule consensus tree of the Bayesian inference (BI) analysis. BI support values (posterior probability) are indicated on tree branches. *Ligustrum vulgare* served as the outgroup to root the tree. Numbers in bold below branches indicate maximum parsimony node support (bootstrap value). Abbreviations: *O. c.*, *Olea capensis*; *O. e.*, *Olea europaea*.

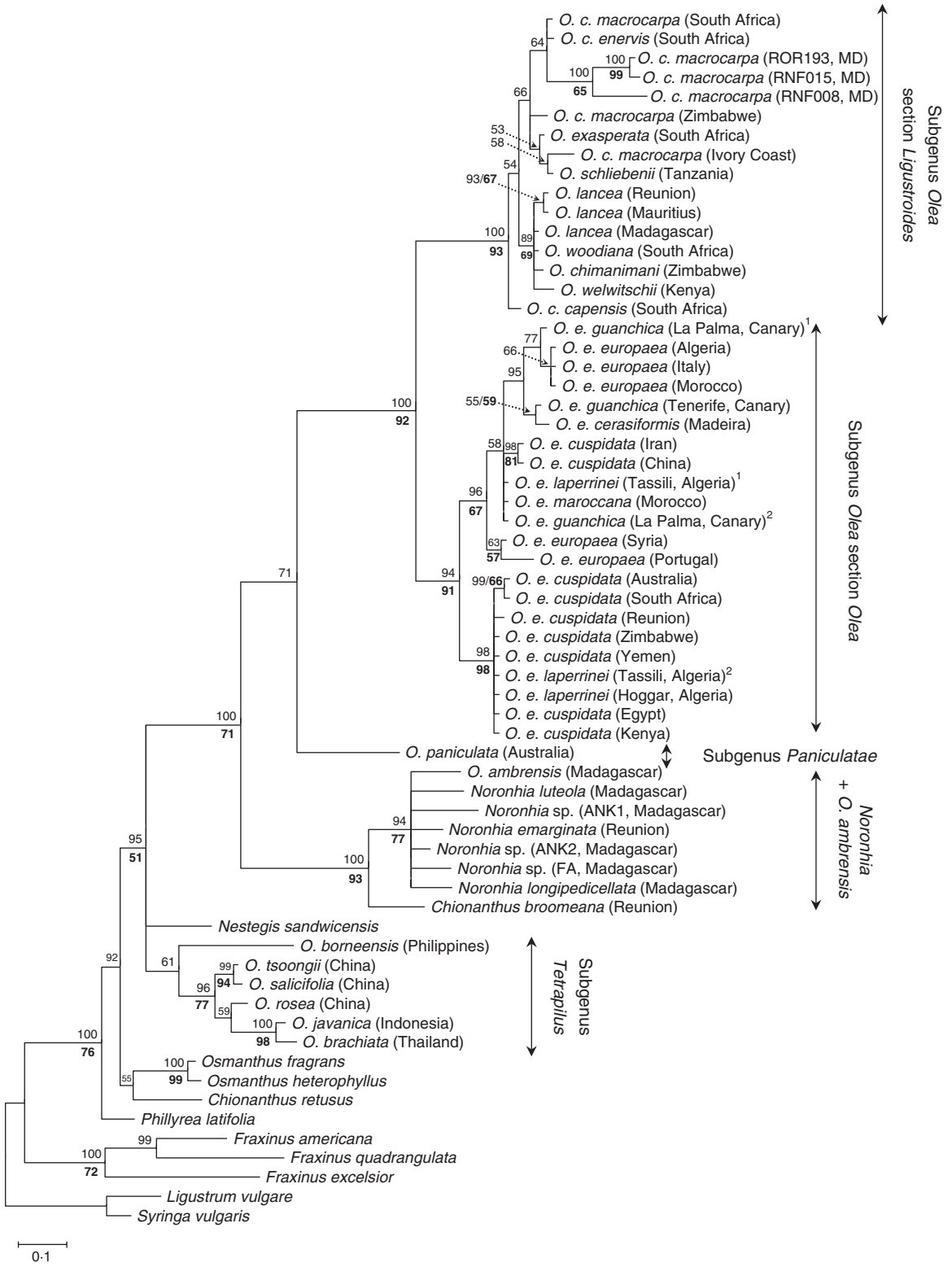

FIG. 2. Majority-rule consensus tree of nuclear ribosomal DNA (ITS-1) sequences using Bayesian inference. Bayesian support values are indicated above branches. Numbers in bold below branches indicate maximum-parsimony node support (bootstrap value). Divergent haplotypes found in a single tree (from Tassili n'Ajjer and Tenerife) are numbered 1 and 2. The tree was rooted using *Ligustrum vulgare* and *Syringa vulgaris*. Abbreviations: *O. c.*, *Olea capensis*; *O. e.*, *Olea europaea*; MD, Madagascar.

monophyletic group, as already observed in the plastid tree. In section *Olea*, samples of subsp. *cuspidata* from Africa and Arabia were clearly divergent from other accessions, and did not form a monophyletic group with other *cuspidata* accessions. Two ITS-1 copies were also detected in one individual of subsp. *laperrinei* and one individual of subsp. *guanchica*, which belonged to two different clades. This may be caused by inter-subspecific hybridizations, as already suggested (Besnard *et al.*, 2007*c*; García-Verdugo *et al.*, 2009). In addition, the positions of subsp. *laperrinei* from the Hoggar (Algeria) and subsp. *cuspidata* from Egypt were incongruent in the ITS-1 and plastid trees, pointing to reticulate evolution.

Molecular dating and peltate scale occurence in Olea

Both plastid DNA and ITS-1 sequences were used for the combined analysis (Supplementary Data 3, available online). A compressed tree with the main supported nodes is presented in Fig. 3. Ages are given with limits of the 95 % of the posterior distribution of heights (in parentheses). According to our molecular dating analysis, divergence times of the ingroup from the outgroup (*Ligustrum*) took place 59·2 (75·6–42·9) Mya (Fig. 3). The *Noronhia* lineage (including *C. broomeana* and *O. ambrensis*) branched off first [35·8 (40·8–30·7) My] in subtribe *Oleinae*. The split of subgenera *Olea–Paniculatae* from a group including *Osmanthus*, *Phillyrea*, *Nestegis*, *C. retusus* and *Olea* subgenus *Tetrapilus* occurred 32·6 (37·8–28·5) Mya. The most common recent ancestor of subgenera *Paniculatae* and *Olea* was in existence 24·4 (26·9–23·0) Mya. In subgenus *Olea*, sections *Ligustroides* and *Olea* diverged from each other 17·7 (21·7–13·8) Mya. Diversification of section *Ligustroides* began 7·6 (10·2–5·3) Mya, whereas the first divergence of two subgroups in section *Olea* was estimated to be 6·1 (8·3–4·0) Mya. Lastly, North African *O. europaea* subspecies diverged from Asian subsp. *cuspidata* 4·4 (5·9–3·3) Mya.

SEM observations of abaxial leaf surfaces confirmed that peltate scales were totally absent in most taxa of subtribe *Oleinae* analysed in the present study (*Noronhia*, *Chionanthus*, *Nestegis*, *Olea* subgenus *Tetrapilus*), although in some cases they had glandular structures, and in one case (*O. rosea*) linear trichomes (Fig. 4A–J). Scattered peltate scales were only observed in *O. paniculata* and taxa of section *Ligustroides* (Fig. 4K–R), whereas leaves of section *Olea* were densely covered by larger peltate scales. Scale coverage is so dense that leaves of *Olea europaea* (section *Olea*) had a multilayered indumentum with overlapping scales (Fig. 4S–V), particularly in the case of subsp. *laperrinei*, where the stomata were completely protected and never directly visible.

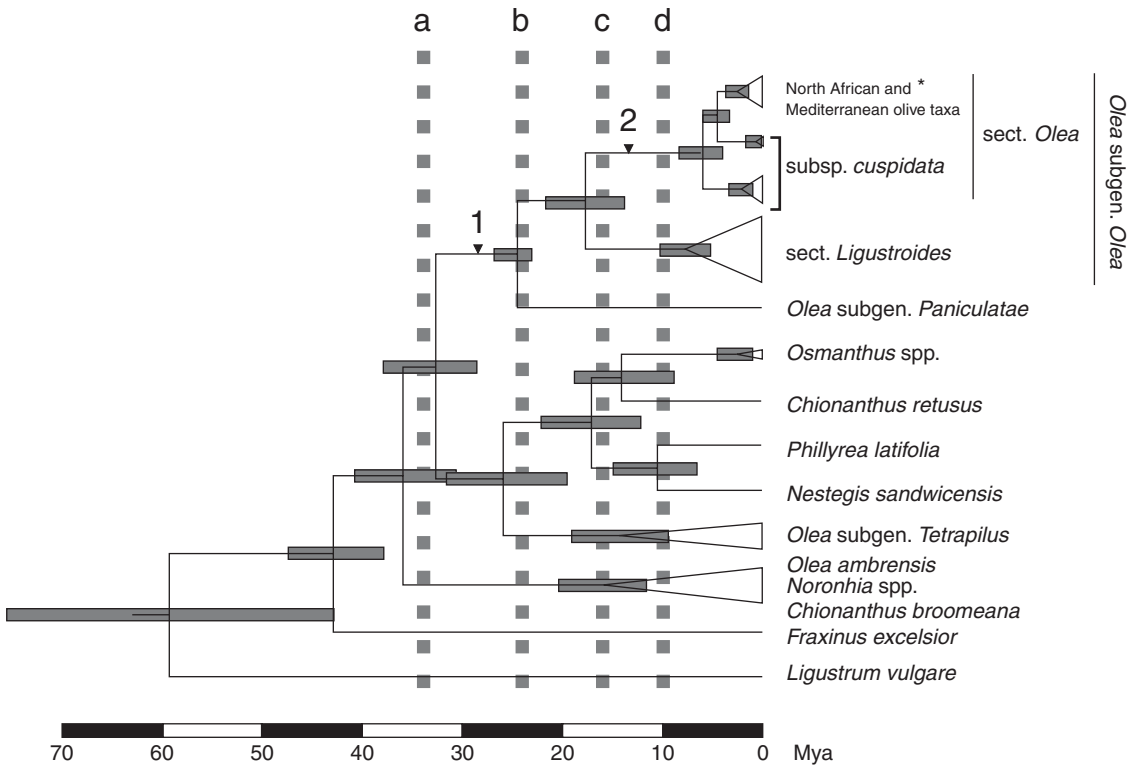

FIG. 3. Divergence time estimates based on both ITS-1 and plastid DNA sequences for major *Olea* lineages. The scale is in millions of years (Mya). Horizontal rectangles on nodes represent standard deviations of divergence times. Vertical dotted lines indicate major Tertiary bioclimatic events: (a) Oi-1 glacial maximum (34 Mya; Zachos *et al.*, 2001); (b) Mi-1 glaciation at the Miocene–Oligocene boundary (24 Mya; Zachos *et al.*, 2001); (c) marked global cooling and subsequent aridification of Africa (16–2·8 Mya; Zachos *et al.*, 2001), increase of the East Antarctica Ice Sheet and the meridional temperature gradient (Flower and Kennett, 1994); (d) desertification of the Sahara (approx. 10–7 Mya; Flower and Kennett, 1994), establishment of the Benguela current and aridification of southern Africa (Linder, 2003). The appearance of scattered peltate scales (solid triangle 1) and indumenta with large lobed peltate scales (solid triangle 2) on the abaxial leaf surface is indicated on the corresponding branches. See Supplementary Data 3 for more details on the phylogenetic tree topology. **Olea europaea* subspp. *cerasiformis*, *europaea*, *guanchica* and *maroccana*.

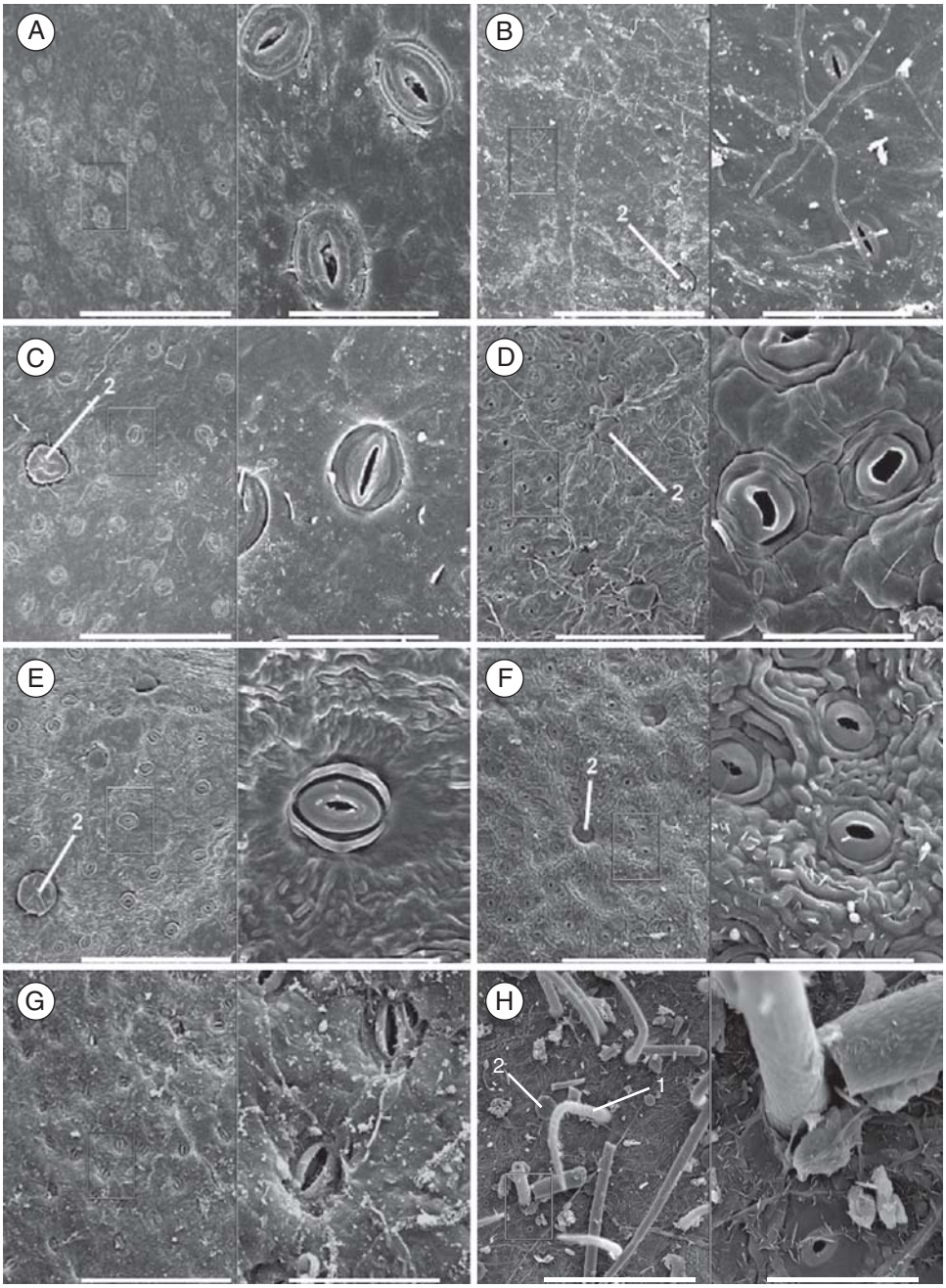

FIG. 4. SEM photographs of the abaxial surface of several members of *Olea* and related taxa. Stomata protected by dense scales (4) are only observed in samples of subgenus *Olea*, section *Olea*, although scattered peltate trichomes (3) are observed in other species of *Olea*. Specimens of subgenus *Tetrapilus* and other genera do not have scales, although in some cases they possess glandular structures (2) and, in the case of *O. rosea*, linear trichomes (1). Detailed views of stomata are shown, except for *O. europaea* subsp. *laperrinei*, for which stomata could not be observed because of the density of the scales. The orifices (5) seen in this image are sectioned stems of missing trichomes. (A) *Noronhia* sp. (ANK1); (B) *Noronhia* sp. (ANK2); (C) *Chionanthus ramiflorus*; (D) *Nestegis sandwicensis*; (E–K) subgenus *Tetrapilus*: (E) *Olea tsoongii*, (F) *O. neriifolia*, (G) *O. wightiana*, (H) *O. rosea*, (I) *O. hainanensis*, (J) *O. brachiata*, (K) *O. paniculata* (subgenus *Paniculatae*); (L–R) subgenus *Olea* section *Ligustroides*: (L) *O. schliebenii*, (M) *O. exasperata*, (N) *O. capensis* subsp. *capensis*, (O) *O. capensis* subsp. *macrocarpa*, (P) *O. welwitschii*, (Q) *O. lancea*, (R) *O. woodiana*; (S–V) subgenus *Olea* section *Olea*: (S) *O. europaea* subsp. *europaea*, (T) *O. europaea* subsp. *cuspidata*, (U) *O. europaea* subsp. *guanchica*, (V) *O. europaea* subsp. *laperrinei*. Scale bars are 200 μm and 40 μm for the left-hand and right-hand images in each pair, respectively. The scale bar in (V) is 200 μm.

DISCUSSION

Informativeness of DNA markers

The four plastid DNA regions screened in the present study showed a higher amount of polymorphisms within the genus *Olea* than the *rps16* and *trnL-trnF* sequences used in a previous study (Wallander and Albert, 2000). The *trnS-trnG* intergenic spacer was the most variable region (Table 1) and is highly recommended for phylogenetic reconstructions of Oleaceae. This result agrees with recent reports on the

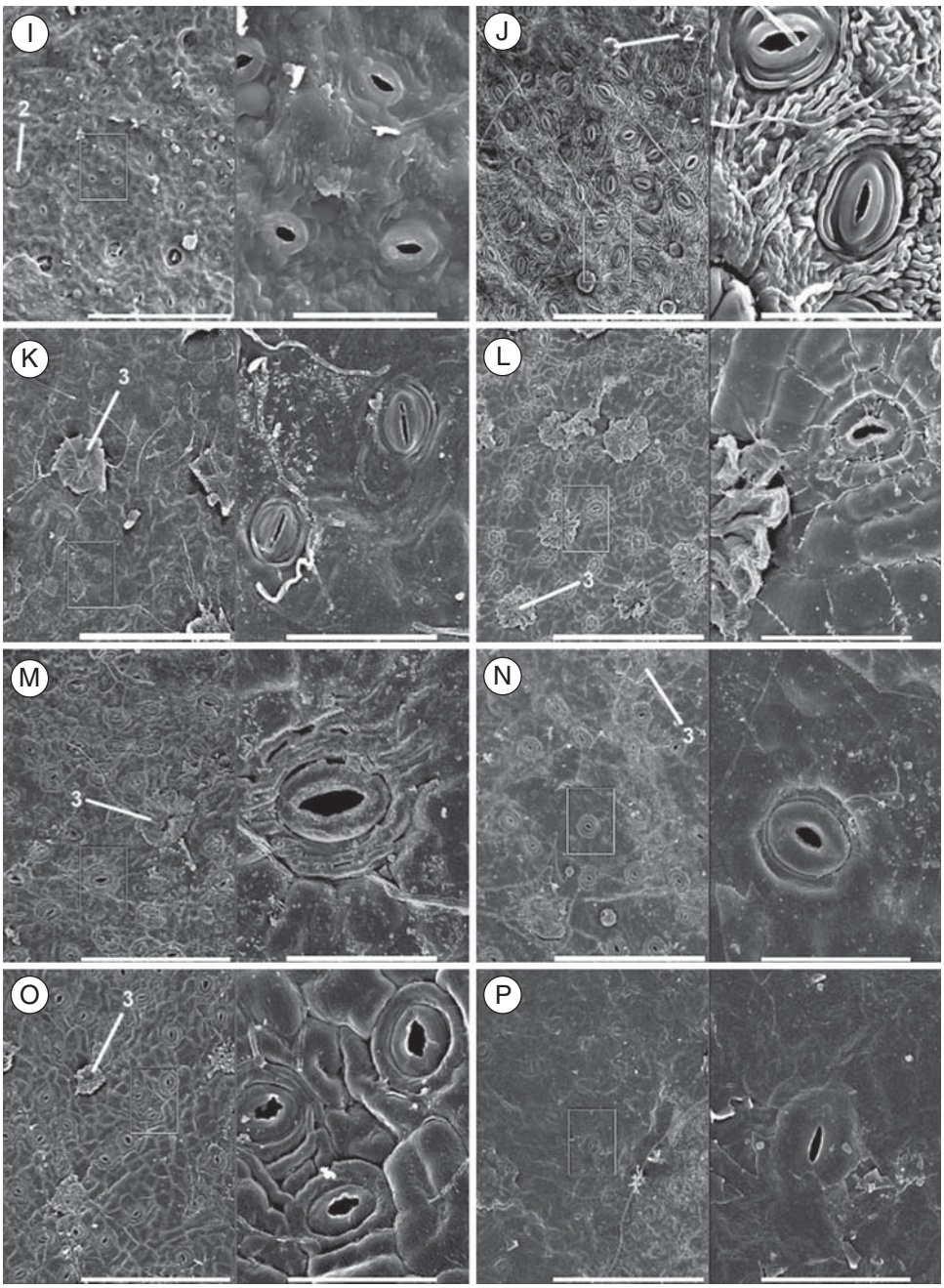

FIG. 4. Continued

phylogenetic utility of this plastid DNA fragment in plants at different taxonomic levels (Shaw *et al.*, 2005; Parisod and Besnard, 2007). Furthermore, the present study provides for the first time a phylogenetic reconstruction of ITS-1 sequences from presumed functional nrDNA units on a complete sample of the olive complex and related taxa. Indeed, previous studies using ITS-1 sequences of the olive complex were based on nrDNA pseudogenes that exhibited higher variability, but were less amenable to conventional phylogenetic analyses (Hess *et al.*, 2000; Elbaum *et al.*, 2006; Besnard *et al.*, 2007*c*). The present results confirmed that the ITS-1 sequences, even in their functional form, are far more variable than any plastid DNA intergenic spacer (Table 1), as already shown in *Fraxinus* (Jeandroz *et al.*, 1997) and many other angiosperms (Mort *et al.*, 2007).

Systematic implications

The validity of genus *Olea*, as a natural group, has long been discussed. Indeed, Johnson (1957) proposed the recognition of *Tetrapilus* as an independent genus based on morphology. This possibility was suggested more recently in studies based on plastid sequences (Wallander and Albert, 2000), nrDNA RFLPs (Besnard *et al.*, 2002) and biochemical and

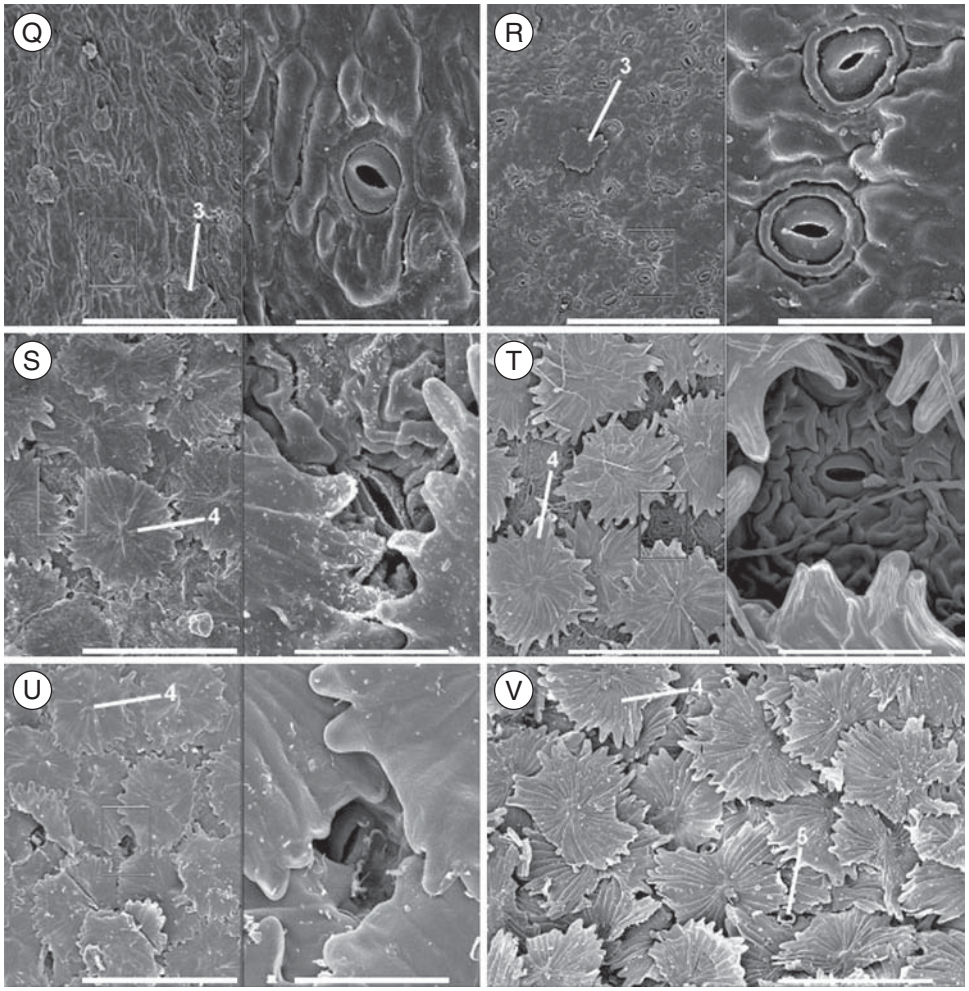

FIG. 4. Continued

morphological data (Harborne and Green, 1980; Nilsson, 1988; Jensen *et al.*, 2002). The current phylogenetic analyses supported the polyphyletic hypothesis inasmuch as accessions of *Chionanthus*, *Osmanthus*, *Nestegis*, *Norhonia* and *Phillyrea* were intermingled with those of *Olea* in all analyses (Figs 1–3). Indeed, *Olea* subgenus *Tetrapilus* appeared as a natural group distant from the main core of *Olea* (i.e. subgenera *Olea*–*Paniculatae*), as Johnson (1957) had predicted. In addition, a close relationship between *Noronhia* spp., *Chionanthus broomeana* and *Olea ambrensis* was retrieved in all analyses (Figs 1–3), suggesting that *O. ambrensis* is incorrectly classified in *Olea*. Unfortunately, key characters, such as the corolla, stamen and ovary, are missing from the original description (Perrier de la Bâthie, 1952; Green, 2002). As flower parts are of paramount importance for the classification of Oleaceae, further taxonomic research is necessary to verify whether this species belongs to *Noronhia* (as already suggested by R. Capuron; cf. hand writing on the type specimen of *O. ambrensis* in the herbarium at the Museum d'Histoire Naturelle, Paris). Alternatively, this taxon could be related to tropical African species of *Chionanthus*. An extended sample is also needed to test for the polyphyly of *Chionanthus* (*sensu* Stearn, 1976), which is suggested by the distant placement of the two samples included in the present study (*C. retusus* and *C. broomeana*).

Subgenera *Olea* and *Paniculatae* formed a well-defined monophyletic group (Figs 1 and 2). In the light of these results, we propose that a more natural (monophyletic) taxonomy of *Olea* should restrict the 'true' genus *Olea* to the type species (*O. europaea*) and closely related taxa (i.e. subgenera *Olea* and *Paniculatae*). Phylogenetic reconstructions also enabled a clear distinction between the two sections of subgenus *Olea*: section *Ligustroides* (excluding *O. ambrensis*) and section *Olea*. Poor resolution within section *Ligustroides* in the plastid DNA phylogenetic tree hinders conclusive hypotheses on the evolution of this monophyletic group. Despite a significant sampling of *O. capensis*, accessions of the three subspecies did not form a monophyletic group. The lack of agreement between morphological and molecular results, the poor congruence between plastid DNA and ITS-1 phylogenetic trees and the similar plastid DNA sequences found in geographically close taxa (i.e. south-eastern African species of section *Ligustroides*) suggest that reticulate evolution in section *Ligustroides* may have been extensive (Besnard *et al.*, 2002; Green, 2002), as already documented in section *Olea* (Besnard *et al.*, 2007*c*).

The phylogenetic reconstructions obtained with both nuclear and plastid DNA sequences show a clear differentiation between subsp. *cuspidata* and the other subspecies of section *Olea*. However, the molecular data sets for *O. europaea* [e.g. from amplified fragment length polymorphisms (AFLPs) (Angiolillo *et al.*, 1999; Rubio de Casas *et al.*, 2006), plastid DNA sequences (Besnard *et al.*, 2007*c*) and ITS (Besnard *et al.*, 2007*c*)] indicate that reproductive isolation of subspecies *cuspidata* may have not been complete. The lack of key taxonomic characters in this section (Green, 2002), the presence of fertile hybrids between subspecies (Besnard *et al.*, 2007*b*, 2008) and the close relationship of accessions of multiple subspecies are in agreement with the long-standing subspecific treatment of the *O. europaea* complex (Ciferri and Breviglieri, 1942; Green and Wickens, 1989; Vargas *et al.*, 2001; Green, 2002).

The evolutionary history of Olea

Fossils that can be attributed to specific nodes in the phylogeny of Oleaceae are rare. This low number of calibration points, together with their conservative usage, has led to wide confidence intervals in the estimation of node ages (Fig. 3). These high standard deviations are inherent in molecular clock analyses and cannot be avoided with the amount of palaeobotanical data currently available. Nevertheless, the statistical support for time estimates presented in this study is high and can thus be considered as indicative of the most likely divergence times in *Olea*.

Concordance between lineage appearance and major climatic events in the Tertiary (Zachos *et al.*, 2001; Pälike *et al.*, 2006) suggests links between climate shifts and clade divergences in the genus *Olea*. The Oi-1 glaciation (about 34 Mya) is recognized as one of the most remarkable palaeoclimatic events in the Oligocene (Zachos *et al.*, 2001). This glaciation could have played a role in the separation of subgenera *Olea* and *Paniculatae* from closely related genera of *Oleinae* (including *Tetrapilus*) 32·6 (37·8–28·5) Mya (see also dating for *Olea* in Lee *et al.*, 2007). According to the present dating results, the Mi-1 glaciation at the Miocene–Oligocene boundary (Zachos *et al.*, 2001) coincides in time with the split [24·4 (26·9–23·0) Mya] between subgenera *Olea* and *Paniculatae*, which together form 'true' *Olea*. These results suggest that lineage differentiation may have taken place multiple times after recurrent contractions of wet and dry tropical forests during major climatic shifts (Zachos *et al.*, 2001).

SEM photographs revealed a total absence of peltate scales on the abaxial leaf surface in accessions other than those of subgenera *Paniculatae* and *Olea* (Fig. 4). In contrast, *Nestegis*, *Chionanthus* and samples from subgenus *Tetrapilus* exhibit glandular structures that were not observed in subgenera *Olea* and *Paniculatae* (Fig. 4). Our observations indicate that peltate scales may have appeared in a most recent common ancestor of subgenera *Paniculatae* and *Olea*. Leaves of section *Olea* (*O. europaea*) are densely covered by large peltate lobed scales. In contrast, the abaxial scales observed in *O. paniculata* and some taxa of section *Ligustroides* are smaller, have a different shape and are much more scattered, perhaps playing a role in defence against herbivores (e.g. Rudgers *et al.*, 2004). The characteristic indumentum of *O. europaea* might thus be the result of a further modification of previously existing structures to face the dryer environments in the Middle and Late Miocene (Zachos *et al.*, 2001; Sepulchre *et al.*, 2006), and in that case they can be interpreted as a preaptation (*sensu* Gould and Vrba, 1982). This morphological character, together with leaf surface reduction and cuticle thickness, has traditionally been considered as an adaptation to arid environments (Uzunova *et al.*, 1997), as trichomes protect stomata by creating favourable micro-environmental conditions for gas exchange (Bongi *et al.*, 1987). Indeed, all taxa of *O. europaea* display a dense abaxial cover of peltate scales and linear–lanceolate leaves and inhabit dry or arid environments (Fig. 4). This set of characters is extreme in subsp. *laperrinei*, which grows under desert-like conditions in the Sahara and has a multi-layered indumentum (Besnard *et al.*, 2007*a*).

According to the relaxed molecular clock results, the separation of section *Olea* and section *Ligustroides* took place during the Miocene, at 17·7 (21·7–13·8) Mya. It coincides with the Early Miocene warming that followed the Mi-1 glaciation at the Miocene–Oligocene boundary (approx. 24 Mya) leading to the Mid-Miocene Climate Optimum (approx. 16 Mya; Zachos *et al.*, 2001). Aridification of continental Africa, as revealed by grassland expansion 16–2·8 Mya (Retallack, 1992; Cerling *et al.*, 1997) and the contraction of forest ecosystems to refugia (Bobe, 2006; Sepulchre *et al.*, 2006), appears to be related to further differentiation within sections *Olea* and *Ligustroides*. These environmental changes had significant consequences for several plant groups [e.g. *Ehrharta* (Verboom *et al.*, 2003) and *Nemesia* (Datson *et al.*, 2008)] and triggered the diversification of arid-adapted taxa (Fiz *et al.*, 2008). The new xeric environments might have favoured the establishment of the leaf morphotypes observed in section *Olea*.

Molecular clock results also show that the further split of the lineage formed by the subsp. *cuspidata* populations primarily distributed in eastern and southern Africa from the lineages including the remaining populations appears to have occurred at approximately 6·1 (8·3–4·0) Mya. This date coincides with the aridification of African midlatitudes that followed the expansion of the East Antarctic ice sheet and the topographic uplift of eastern African mountains (Flower and Kennett, 1994; Zachos *et al.*, 2001; Sepulchre *et al.*, 2006). This climatic event triggered the desertification of the Sahara, potentially promoting vicariance, as already described for other organisms (e.g. elephant shrews; Douady *et al.*, 2003). Some degree of gene flow between subsp. *cuspidata* and the other subspecies, notably subsp. *laperrinei*, might have taken place following secondary contacts. Recurrent isolation and hybridization events may largely have been caused by geographical barriers and bridges (such as the Sahara). Waxing and waning processes caused by climatic changes during the Pleistocene may have brought about reticulation in the *O. europaea* complex (for a more detailed discussion, see Besnard *et al.*, 2007*c*).

Concluding remarks

The present results indicate the necessity of revising current taxonomic boundaries in *Olea* (particularly for *O. ambrensis*

and *Olea* subgenus *Tetrapilus*). The long evolutionary history of this group, which spans most of the Tertiary, appears to have been shaped by the major climatic events of that period. Further research should focus on disentangling the phylogeny and biogeography of subgenus *Olea*, as reticulation and recent geological events (formation of the Sahara, emergence of Macaronesian islands and establishment of the Mediterranean regime) may have been crucial in the evolution of *Olea* (Suc, 1984; Terral *et al.*, 2004).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org/ and consist of the following files. Supplementary Data 1: List of primers used for the PCR amplification and sequencing of plastid DNA fragments. Supplementary Data 2: ITS-1 alignment of the Oleaceae accessions used for phylogenetic reconstructions. Supplementary Data 3: Consensus calibrated phylogenetic tree inferred from plastid and ITS-1 sequences using BEAST.

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APPENDIX

Taxa analysed in the present study using plastid (trnL-F, trnT-L, trnS-G and matk) *and nuclear (ITS-1) sequences, and/or SEM photographs. For each sample, corresponding information (geographical origin, voucher sample, living collection and EMBL DNA accession numbers) is given*

Taxa	Geographical origin	Plastid DNA	ITS-1	SEM	Voucher sample	Collection	EMBL accession no(s)
Subgenus *Olea* sect. *Olea*							
Olea europaea L. subsp. *europaea*	Harem, Syria	X	–	–	–	INRA-M	AM931472, AM933032, AM933176, AM933379
	Al Ascharinah, Syria	–	X	–	–	INRA-M	AM933436
	'Toffahi', Egypt	X	–	–	–	OGB-C	AM931473, AM933033, AM933177, AM933380
	Tizi Ouzou, Algeria	X	X	–	–	INRA-M	AM931474, AM933034, AM933178, AM933381, AM933437
	Ali, Sicily, Italy	X	X	–	–	IRO-P	AM931475, AM933035, AM933179, AM933382, AM933438
	Messine, Sicily, Italy	X	–	–	–	IRO-P	AM931476, AM933036, AM933180, AM933383
	Tetouan, Morocco	X	X	–	–	–	AM931477, AM933037, AM933181, AM933384, AM933439
	Heracles Cave, Morocco	X	–	–	–	–	AM931478, AM933038, AM933182, AM933385
	Lonsdale (ind. Lo7), Australia	X	–	–	–	–	AM931479, AM229542, AM229548, AM229554
	Brownhill Creek (BC21), Australia	X	–	–	–	–	AM931480, AM229540, AM229546, AM229552
	Brownhill Creek (BC23), Australia	X	–	–	–	–	AM931481, AM229541, AM229547, AM229553
	Cantabria, Spain	–	–	X	MA611446 (MA)	–	–
	Serra da Arrábida, Portugal	–	X	–	–	–	AJ585193
O. e. subsp. *cuspidata* (Wall ex G. Don) Cif.	Guangzhou (ind. CH1), China	X	X	–	–	–	AM931482, AM933039, AM933183, AM933386, AM933440
	Kerman (ind. F3), Iran	X	X	–	–	INRA-M	AM931483, AM933040, AM933184, AM933387, AM933441
	Kerman (ind. F5), Iran	X	–	–	–	INRA-M	AM931484, AM933041, AM933185, AM933388
	Almhiwit (ind. C3), Yemen	X	–	–	–	INRA-M	AM931485, AM933042, AM933186, AM933389
	Almhiwit (ind. C5), Yemen	X	X	–	–	INRA-M	AM931486, AM933043, AM933187, AM933390, AM933442
	Gebel Elba, Egypt	X	X	–	Fahmy & Hassib s.n. (K)*	–	FM208235, FM208227, FM208243, FM208251, FM208217
	Mt Elgon (ind. K6), Kenya	X	X	–	–	INRA-M	AM931487, AM933044, AM933188, AM933391, AM933443
	Nairobi, Kenya	X	–	–	–	–	AM931488, AM933045, AM933189, AM933392
	Timau (ind. K12), Kenya	X	–	–	–	INRA-M	AM931489, AM933046, AM933190, AM933393
	Amalundu, Zimbabwe	X	X	–	–	-	AM931490, AM933047, AM933191, AM933394, AM933444
	Kirstenbosch, South Africa	X	X	X	MA690609 (MA)	RJB-M	AM931500, AM933049, AM933193, AM933396, AM933445
	St Denis, Reunion	X	X	–	–	INRA-M	AM931491, AM933048, AM933192, AM933395, AM933446

Continued

TABLE *Continued*

Taxa	Geographical origin	Plastid DNA	ITS-1	SEM	Voucher sample	Collection	EMBL accession no(s)
	Maui (ind. Ma1), Hawaii, USA	X	–	–	–	–	AM229535, AM229537, AM229543, AM229549
	Sydney (ind. Ca1), Australia	X	X	–	–	UNIL	AM229536, AM229538, AM229544, AM229550, AM933447
O. e. subsp. *europaea* × *cuspidata*	Sydney (ind. Ca21), Australia	X	–	–	–	–	AM931501, AM229539, AM229545, AM229551
O. e. subsp. *laperrinei* (Batt. & Trab.) Cif.	Hoggar (ind. L1), Algeria	X	X	–	–	INRA-M	AM931492, AM933050, AM933194, AM933397, FM208219
	Hoggar, Algeria	–	–	X	MA381126 (MA)	–	–
	Tassili n'Adjer (ind. LT), Algeria	X	X	–	–	–	AM931493, AM933051, AM933195, AM933398, AM933448/9
	Bagzane (ind. O81), Niger	X	–	–	–	–	AM931494, AM933052, AM933196, AM933399
O. e. subsp. *maroccana* (Greut. & Burd.) P.Vargas *et al.*	Immouzzer, Morocco	X	X	–	–	UNIL	AM931495, AM933053, AM933197, AM933400, AM933450
O. e. subsp. *cerasiformis* G.Kunkel & Sunding	Arco de Calheta, Madeira	X	X	–	–	–	AM931496, AM933054, AM933198, AM933401, AM933451
	São Gonzalo, Madeira	X	–	–	–	–	AM931497, AM933055, AM933199, AM933402
O. e. subsp. *guanchica* P.Vargas *et al.*	La Palma, Canary Islands	X	X	–	–	–	AM931498, AM933056, AM933200, AM933403, AM933452/3
	El Río, Tenerife, Canary Islands	X	X	X	MA651540 (MA)	–	AM931499, AM933057, AM933201, AM933404, FM208218
Subg. *Olea* sect. *Ligustroides* Benth. & Hook.							
O. woodiana Knobl. subsp. *woodiana*	Umzimkulu River, Natal, South Africa	X	X	X	A. Costa 02 (MPU)	–	AM931502, AM933058, AM933202, AM933405, AM933454
O. ambrensis H.Perrier	Fenerive, Madagascar	X	X	–	Schatz *et al.* no. 3405 (K)*	–	AM931503, AM933059, AM933203, AM933406, AM933455
O. lancea Lam.	St Denis, Reunion	X	X	–	–	St Denis University	AM931504, AM933060, AM933204, AM933407, AM933456
	Chamarel, Mauritius	–	X	X	L. Forget 01 (MPU)	–	AM933457
	Tsinjoarivo, Madagascar	X	X	–	R.N.F. 016 (MPU)	–	AM931506, AM933061, AM933205, AM933408, AM933458
O. exasperata Jacq.	Betty's Bay, Western Cape, South Africa	X	X	X	A. Costa 01 (MPU)	–	AM931507, AM933063, AM933207, AM933410, AM933460
O. chimanimani Kupicha	Mt Chimanimani, Zimbabwe	X	X	–	Charpin 24660 (G)*	–	AM931505, AM933062, AM933206, AM933409, AM933459
O. schliebenii Knobl.	Uluguru Mts, Tanzania	X	X	X	Schlieben 3553 (MA)*	–	AM931508, AM933064, AM933208, AM933411, AM933461
O. capensis L. subsp. *capensis*	Kirstenbosch, Cape Town, South Africa	X	X	X	A. Costa 03 (MPU)	–	AM931509, AM933065, AM933209, AM933412, AM933462
O. c. subsp. *enervis* (Harv.) I.Verd.	Transvaal, South Africa	X	X	–	Schlieben 10647 (G)*	–	AM931510, AM933066, AM933210, AM933413, AM933463
O. c. subsp. *macrocarpa* (C.H.Wright) I.Verd.	Tsitsikama, Southern Cape, South Africa	X	X	–	A. Costa 04 (MPU)	–	AM931511, AM933067, AM933211, AM933414, AM933464

Continued

TABLE *Continued*

Taxa	Geographical origin	Plastid DNA	ITS-1	SEM	Voucher sample	Collection	EMBL accession no(s)
	Inyangani, Zimbabwe	X	X	–	–	HBG, no. 6041	AM931512, AM933068, AM933212, AM933415, AM933465
	Montagne d'Ambre, Madagascar	X	X	–	R.N.F. 008 (MPU)	–	AM931513, AM933069, AM933213, AM933416, AM933466
	Ambohitantely, Madagascar	X	X	–	R.O.R. 193 (M)	–	AM931514, AM933070, AM933214, AM933417, AM933467
	Andasibe, Madagascar	X	X	–	R.N.F. 015 (MPU)	–	AM931515, AM933071, AM933215, AM933418, AM933468
	Mt Momy, Ivory Coast	X	X	–	J. Maley s.n. (ISEM)*	–	AM931516, AM933072, AM933216, AM933419, AM933469
	Piedra Nzas, Equatorial Guinea	–	–	X	MA621700 (MA)	–	–
O. welwitschii (Knobl.) Gilg & Schellenb.	Kakamega Forest, Kenya	X	X	X	G. Besnard 01–2008 (G)	INRA-M	AM931517, AM933073, AM933217, AM933420, AM933470
Subg. *Paniculatae* P.S.Green							
O. paniculata R.Brown	Rawalpindi, Pakistan	X	–	–	Podlech 20046 (G)*	–	AM931518, AM933074, AM933218, AM933421
	Brisbane, Australia	X	X	X	C. Lambrides 01 (MPU)	–	AM931519, AM933075, AM933219, AM933422, AM933471
Subg. *Tetrapilus* (Lour.) P.S.Green							
O. borneensis H.L.Li	Mt Kitangland, Philippines	X	X	–	N.R. Ingle 437 (A)*	–	FM208232, FM208224, FM208240, FM208248, FM208215
O. brachiata (Lour.) Merr.	Ku Chum, Narathiwat, Thailand	X	X	–	Niyomdham 1726 (K)*	–	AF231864, AM933078, AM933222, AM933425, AM933473
	Tanah Merah, Kelantan, Malaysia	–	–	X	E. Soepadmo Q66O3938–R/52–I (A)	–	–
O. hainanensis H.L.Li	Janfengling, Hainan, China	–	–	X	K.S. Chow 78347 (A)	–	–
O. javanica (Blume) Knobl.	Sumatra, Indonesia	X	X	–	–	Bogor BG	AM931521, AM933077, AM933221, AM933424, AM933472
O. neriifolia H.L.Li	Sam-Tui Kai, Hainan, China	X	–	X	S.K. Lau 28388 (A)*	–	FM208231, FM208223, FM208239, FM208247
O. rosea Craib	Bubeng, Yunnan, China	–	–	X	Li Yan–Hui 31758 (A)	–	–
	Yunnan, China	X	X	–	C.W. Wang 79171 (A)*	–	FM208233, FM208225, FM208241, FM208249, FM208216
O. salicifolia Wall. ex G.Don	Puntay Bay, Tarutao, Thailand	X	X	–	G. Congdon 804 (A)*	–	FM208234, FM208226, FM208242, FM208250, FM208214
O. tsoongii (Merr.) P.S.Green	Yunan, China	X	X	X	K.S. Walter s.n. (MPU)	EBG, no. 19931835	AM931520, AM933076, AM933220, AM933423, AJ938148
O. wightiana Wall. ex G.Don	Western Ghats, India	–	–	X	MHN 7817 (A)	–	–
Outgroup species							
Chionanthus ramiflorus Roxb.	Hawaii, USA	–	–	X	T. Flynn 6332 (MPU)	KNTBG, no. 750947001	–
C. retusus Lindl. & Paxton	China	X	X	–	–	–	AF231811, DQ120723
C. broomeana (Horne ex Oliver) A.J.Scott	Marelongue, Reunion	X	X	–	–	–	AM931522, AM933079, AM933223, AM933426, AM933474

Continued

TABLE *Continued*

Taxa	Geographical origin	Plastid DNA	ITS-1	SEM	Voucher sample	Collection	EMBL accession no(s)
Fraxinus americana L.	–	–	X	–	–	–	U82908
F. excelsior L.	Lausanne, Switzerland	X	X	–	G. Besnard 01–2007 (G)	–	AM931523, AM933080, AM933224, AM933427, AM933475
F. quadrangulata Michx.	–	–	X	–	–	–	U82882
Ligustrum vulgare L.	Lausanne, Switzerland	X	X	–	G. Besnard 02–2007 (G)	–	AM931524, AM933081, AM933225, AM933428, AM933476
Nestegis sandwicensis (A.Gray) Deg.	Hawaii, USA	X	X	X	T. Flynn 6329 (MPU)	–	AM931525, AM933082, AM933226, AM933429, AM933477
Noronhia emarginata (Lam.) Thouars	St Philippe, Reunion Island	X	X	–	–	UNIL	AM931526, AM933083, AM933227, AM933430, AM933478
N. longipedicellata H. Perrier	Ankarana RS, Madagascar	X	X	–	G. Besnard 53–2006 (G)	–	AM931527, AM933084, AM933228, AM933431, AM933479
N. luteola H. Perrier subsp. *ankaranensis* H.Perrier	Ankarana RS, Madagascar	X	X	–	G. Besnard 51–2006 (G)	–	AM931528, AM933085, AM933229, AM933432, AM933480
Noronhia sp. (ANK1)	Ankarana RS, Madagascar	X	X	X	G. Besnard 49–2006 (G)	–	AM931529, AM933086, AM933230, AM933433, AM933481
Noronhia sp. (ANK2)	Ankarana RS, Madagascar	X	X	X	G. Besnard 50–2006 (G)	–	AM931530, AM933087, AM933231, AM933434, AM933482
Noronhia sp. (FA)	Montagne d'Ambre, Madagascar	X	X	–	G. Besnard 46–2006 (G)	–	AM931531, AM933088, AM933232, AM933435, AM933483
Osmanthus heterophyllus (G.Don) P.S.Green	Cultivated (RBG-M)	X	X	–	–	RJBM 261-82	FM208238, FM208230, FM208246, FM208254, FM208222
Osmanthus fragrans Lour.	Cultivated (RBG-M)	X	X	–	–	RJBM 46-83	FM208237, FM208229, FM208245, FM208253, FM208221
Phillyrea latifolia L.	Cultivated (RBG-M)	X	X	–	–	RJBM 27-95	FM208236, FM208228, FM208244, FM208252, FM208220
Syringa vulgaris L.	–	–	X	–	–	–	DQ184479

Abbreviations: A, Herbarium of the Arnold Arboretum, Harvard University; G, Herbarium of the Geneva Botanical Gardens; EBG, Royal Botanic Garden Edinburgh; HBG, Harare Botanical Garden; ISEM, Institut des Sciences de l'évolution de Montpellier; KNTBG, Kauai National Tropical Botanical Garden; K, Herbarium of the Royal Botanic Gardens, Kew; MA, Herbarium of the Royal Botanical Garden of Madrid; MPU, Herbarium of the Botanical Institute of the Montpellier University; RBG-M, Royal Botanic Garden of Madrid; INRA-M, Institut National de Recherches Agronomiques de Montpellier; OGB-C, Olive Germplasm Bank, Cordoba; IRO-P, Institute for Olive Research, CNR, Perugia; Harare BG, Harare Botanical Garden.

* DNA directly prepared from a herbarium sample.