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The genus *Olea*: molecular approaches of its structure and relationships to other *Oleaceae*

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ABSTRACT: Ribosomal DNA RFLP and RAPD polymorphisms were examined in taxa belonging to *Oleaceae* genera. The objective of this study was to determine the taxonomic position of the genus *Olea* and of its components: subg. *Olea* section *Olea*, subg. *Olea* section *Ligustroides*, subg. *Paniculatae* and subg. *Tetrapilus*. Ribosomal DNA polymorphisms supported that *O.* subg. *Tetrapilus* was separated from other *Olea* species and appeared close to other *Oleaceae* genera as *Nestegis, Chionanthus* and *Phillyrea*. Moreover, RAPDs enabled us to distinguish the four groups of the genus *Olea*. Despite of limited number of informative RAPDs, *O.* subg. *Tetrapilus* was also shown closer to *Nestegis* and *Chionanthus* than to the other *Olea* species. Thus, *Tetrapilus* should be considered as a genus. Our results sustained the common origin of the sections *Ligustroides* and *Olea*.

Key words: *Olea* - *Oleaceae* - Olive - taxonomy - *Tetrapilus* - ribosomal DNA – RAPD

RÉSUMÉ : Nous avons révélé des RFLP de l'ADN ribosomique et des marqueurs RAPD dans différents genres appartenant à la famille des Oléacées. L'objectif de cette étude était de déterminer la position taxonomique du genre *Olea* et de ses composantes : sous-genre *Olea* section *Olea*, sous-genre *Olea* section *Ligustroides*, sous-genre *Paniculateae* et sous-genre *Tetrapilus*. Les polymorphismes de l'ADN ribosomique soutiennent que le sous-genre *Tetrapilus* est séparé des autres espèces du genre *Olea*, et apparaît plus proche des genres *Nestegis*, *Chionanthus* et *Phillyrea*. De plus, les marqueurs RAPD nous permettent de distinguer les quatre groupes du genre *Olea*. Bien que le nombre de marqueurs RAPD informatifs soit limité, il apparaît que le sous-genre *Tetrapilus* est plus apparenté à *Nestegis* et *Chionanthus* que des autres espèces du genre *Olea*. Ainsi, *Tetrapilus* devrait être considéré comme un genre. Par ailleurs, nos résultats soutiennent l'origine commune des sections *Ligustroides* et *Olea*.

Mots-clés : ADN ribosomique - *Olea* - *Oleaceae* - Olivier - taxonomie - *Tetrapilus* – RAPD

I. INTRODUCTION

The family *Oleaceae* contains 25 genera and around 500 to 600 species which are separated in two sub-families: *Jasminoideae* and *Oleideae* (review of the classification in Rohwer, 1996). *Olea* is classified in the sub-family *Oleideae* and in the tribe *Oleeae*. The genus *Olea* includes around 30 to 40 species that are distributed in Oceania, in Asia, in Africa, and in the Mediterranean Basin. Olive tree (*Olea europaea* L. subsp. *europaea*) is an important crop in the Mediterranean basin. At the present time, many interests are focused on its genetic resources and on the elucidation of domesticated olive origins (Besnard *et al.*, 2001a), but little is known about the relationships between the different species of the genus *Olea*. The species closely related to the Mediterranean olive have been particularly studied by botanists and are grouped in the subgenus *Olea* section *Olea* which is currently named "*O. europaea* complex" (Green & Wickens, 1989). The other *Olea* species are less known and some (notably *O. capensis*) are supposed to be incompatible with *O. europaea* (Dyer, 1991). Apart from the olive tree, several *Olea* species could prove of economic importance. In Africa, several taxa such as

O. capensis subsp. *macrocarpa* and *O. perrieri* produce fruits rich in oil, and can be used in human alimentation (Palmer & Pitman, 1972; Perrier de la Bathie, 1952). Lastly, *O. paniculata* wood is used in fine carving or for flooring blocks in Australia (Kiew, 1979).

Several *Olea* classifications have been proposed, notably by De Candolle (1844), Bentham & Hooker (1876) and Knoblauch (1895). Confusions between the different genera have sometime occurred (Altamura *et al.,* 1987). Within the genus *Olea*, a group of species from South Eastern Asia has been distinguished and classified in the genus *Tetrapilus* by Johnson (1957) who has drawn it nearer to *Linociera* (presently considered a synonym of *Chionanthus*) based on the length of the corolla tube. Furthermore, *Tetrapilus* species were distinguished from the other *Olea* using pollen morphology (Nilsson, 1988) and flavonoid composition (Harborne & Green, 1980). Nevertheless, *Tetrapilus* as a genus has not been generally retained by botanists but considered as being part of the genus *Olea* (Kiew, 1979). A complete taxonomy of the described *Olea* species is in preparation (Green, in preparation). The characters retained as the most informative are displayed in Table I. This enabled us to distinguish three subgenera and two sections. The subgenus *Tetrapilus* is the most distinct group and presents a greater variability. Subgenus *Olea* is separated into two sections: *Olea* (comprising cultivated olive trees and its wild relatives) and *Ligustroides*. The main characters used to distinguish these two sections are the indumentum and the panicle position. Lastly, the subgenus *Paniculatae* is distinguished from the subgenus *Olea* notably by the presence of domatia on the undersides of the leaves and of both axillary and terminal panicles. The relationships between these different groups of *Olea* species remain unclear.

Currently, molecular approaches are routinely used in taxonomy and phylogeny studies. Comparison of restriction maps of genes (Olmstead & Palmer, 1994) - as ribosomal genes (rDNA), cytoplasmic DNA - and gene sequences (APG, 1998) have been used in plant phylogeny studies. Within the family *Oleaceae*, a phylogeography of *Fraxinus* has been proposed based upon sequences of ITS (Jeandroz *et al.,* 1997), a phylogeny of *Syringa* has been proposed on chloroplast polymorphisms (Kim & Jansen, 1998) and a phylogeny of most of the genera of the family has been proposed based upon chloroplast DNA sequences (Wallander & Albert, 2000). Use of RAPDs or RFLPs presents the advantage of covering a greater number of genome regions. Thus, these tools make it possible to study polymorphisms in different genomic regions. In *Oleaceae*, RAPDs have been used to detect interspecific hybridization in *Fraxinus* (Jeandroz *et al.,* 1996a), or to study the relationships between related taxa belonging to *Olea europaea* (Besnard *et al.,* 2001b). Nevertheless, these markers could be too variable when distant taxa are compared, and this should lead to a low level of informative characters.

The study of the genetic relationships between the species of *Olea* is required to determine the relationships of *Olea* with other *Oleaceae* genera and to understand the phylogeny of *Olea* species leading to the present structure of its diversity. Moreover, this will show the species complexes and will make it possible to define the history of cultivated olive. We analyzed different taxa of the genus *Olea* and related genera of *Oleaceae* with restriction maps of ribosomal RNA genes and with RAPDs to check the position of *Olea* within the family and to propose a molecular taxonomy of the genus in comparison with the morphologic classification.

II. MATERIAL AND METHODS

A. Plant material

The plant material was collected in the wild, in the collections of botanical gardens, and in those of the *Instituto di Ricerche sulla Olivicoltura* (*C.N.R.,* Perugia) and of the *Institut Nationale de Recherche Agronomique* (*INRA,* Montpellier). Forty-eight samples belonging to the different *Olea* subgroups (Table II), and fifteen other *Oleaceae* (Table III) were studied.

B. Ribosomal DNA study

The DNA extraction protocol has been described by Besnard *et al.,* (2000). Three µg of total DNA were restricted (8 U/µg, 37 ° C, 4-5 h) separately by *Bam*HI, *Eco*RI, *Eco*RV and *Sac*I plus their pairwise combinations *Bam*HI-*Eco*RI, *Eco*RI-*Eco*RV, and *Sac*I-*Eco*RI. The restriction fragments were electrophoresed onto 0.8 % agarose gel at 1.8 V/cm for 16 h. The Southern transfers were successively

Table I. Geographical distribution and discriminant morphological characters of the subgenus and sections of *Olea*.

	Groups of *Olea*			
	Subgenus *Tetrapilus*	**Subgenus *Olea* sect. *Olea* (*O. europaea* complex)**	**Subgenus *Olea* sect. *Ligustroides* Benth. & Hook.**	***Olea paniculata* Subgenus *Paniculatae***
Geographical distribution	South Eastern Asia	From China at East Southern Africa, Saharan mountains, Mediterranean basin, Canaria.	Central and Southern Africa	From India at Australia
Flower characters				
Corolla tube	longer than the corolla lobe	equal or shorter than the corolla lobe	equal or shorter than the corolla lobe	equal or shorter than the corolla lobe
Stigma	shortly bilobed or capitate	capitate	capitate	capitate
Dioecy	+/-	-	-	-
Calyx tube		+/- membranous	+/- coriace	
Panicles position	axillary	axillary (or subterminal)	terminal (and sometime axillary)	axillary and terminal
Leaf characters				
Leaf blade margins	entire or serrate	entire	entire	entire
Peltate scales	-	+	+	+
Domatia in axils of veins	-	-	-	+

Table II. Origin and code of the studied *Olea* accessions. VS = Voucher samples deposited at the herbarium of the Botanical Institute from the University Montpellier II (MPU); NA = Number of studied accessions for a taxon; Y = characterized for rDNA restriction sites; N = not characterized for rDNA restriction sites; * means that only one individual was characterized for rDNA analyses. B. G. = Botanical Gardens.

Taxa	Origin country	Locality	Botanical Gardens, collections, herbarium	N	rDNA analyses	Code
Subgenus *Tetrapilus* (Lour.)						
O. tsoongii (Merr.) Green	China		Edinburgh B. G. accession: 19 931 835	1	Y	T.TSO1
O. tsoongii (Merr.) Green	China	Yunnan	Edinburgh B. G. accession: 19 697 316	1	Y	T.TSO2
O. brachiata (Lour.) Merr.	Indonesia	Bangka, Sumatra	Bogor Botanical Garden	1	Y	T.BRA
Subgenus *Olea* section *Olea* (= *O. europaea* complex)						
[- subspecies *cuspidata* (Wall. ex DC) Ciferri]						
O. cuspidata Wall.	China		CNR Perugia collection	1	Y	E.CC
O. cuspidata Wall.	India		CNR Perugia collection	1	Y	E.CI
O. cuspidata Wall.	Iran	Guéno, Bandar Abas	VS. H Hosseinpour F2	1	Y	E.CR1
O. cuspidata Wall.	Iran	Marzondar, Ahmady	INRA Montpellier collection	1	N	E.CR2
O. chrysophylla Lam.	Yemen	Almhiwit	INRA Montpellier collection	2	Y*	E.CH1-2
O. africana Mill.	Kenya	Timau, Kenya Mount	INRA Montpellier collection	2	Y*	E.AK1-2
O. africana Mill.	South Africa	Kirstenbosch, Cape Town	Madrid Botanical Garden	2	Y*	E.AS1-2
O. africana Mill.	Reunion	Sentier de la Providence, Reunion	INRA Montpellier collection	2	Y*	E.AR1-2
[- subspecies *europaea*]						
var. *sylvestris* (Miller) Lehr.	Syria	Härim, Oronte Valley	INRA Montpellier collection	2	Y*	E.ES1-2
var. *sylvestris* (Miller) Lehr.	France	Ostricone, Corsica		2	Y*	E.EC1-2
[- subspecies *laperrinei* (Batt. & Trab.) Ciferri]						
O. laperrinei Batt. & Trab.	Algeria	La Source, Hoggar	INRA Montpellier collection	1	Y	E.LA
O. maroccana Greut. & Burd.	Morocco	Immouzzer, Atlas	INRA Montpellier collection	1	Y	E.MA1
O. maroccana Greut. & Burd.	Morocco	Mentaga, Atlas	VS. A Bervillé 1	1	N	E.MA2
[- subspecies *cerasiformis* (Webb & Berth.) Kunk. & Sund.]						
O. cerasiformis Webb & Berth.	Canary Islands	Santa Rosalia, La Palma		2	Y*	E.CE1-2
Subgenus *Olea* section *Ligustroides* Benth. & Hook.						
O. lancea Lam.	Mauritius		VS. L Forget 1 & 2	2	Y*	L.LM1-2
O. lancea Lam.	Reunion	Cap Noir, Reunion	INRA Montpellier collection	2	Y*	L.LR1-2
O. lancea Lam.	Madagascar	Tsinjoarivo	VS. RNF 016 & RNF 017	2	N	L.LA1-2
O. exasperata Jacq.	South Africa	Betty's Bay, Western Cape	VS. A Costa 01	1	N	L.EX
O. woodiana Knobl.	South Africa	Umzimkulu River, Natal	VS. A Costa 02	1	Y	L.WOS
O. woodiana Knobl.	Kenya	Kilifi, Indian Ocean coast	VS. H Sommerlate 1	1	N	L.WOK
O. welwitschii (Knobl.) Gilg & Schellenb.	Kenya	Kakamega Forest, Mt Elgon	Montpellier collection	2	N	L.CW1-2
O. capensis L. subsp. *capensis*	South Africa	Kirstenbosch, Cape Town	VS. A Costa 03	1	Y	L.CC
O. capensis subsp. *macrocarpa* (Wright) Verd.	South Africa	Bloukranspas, Tsitsikama, Southern Cape	VS. A Costa 04	1	Y	L.CMS
O. capensis subsp. *macrocarpa* (Wright) Verd.	Zimbabwe	Pungwe River, Inyangani	Harare Botanic Garden accession: 6041	1	Y	L.CMZ
O. capensis subsp. *madagascariensis* (Boiv.)	Madagascar	Montagne d'Ambre	VS. RNF 008, 009 & O11	3	N	L.MA1-3
O. capensis subsp. *madagascariensis* (Boiv.)	Madagascar	Ambatovy	VS. RNF 030 & 031	2	N	L.MA4-5
O. capensis subsp. *madagascariensis* (Boiv.)	Madagascar	Ambohitantely	VS. ROR 193	1	N	L.MA6
O. perrieri Chev.	Madagascar	Andasibe	VS. RNF 045	1	Y	L.PE1
O. perrieri Chev.	Madagascar	Marojejy	VS. RNF 006	1	N	L.PE2
Subgenus *Paniculatae*						
O. paniculata R.Br.	Australia		Kew B. G. accession: 19 66 67 111	1	Y	P.KEW
O. paniculata R.Br.	Australia	Brisbane, Queensland	VS. C Lambrides 1	1	N	P.BRI

Table III. List and origin of the studied *Oleaceae* accessions. VS = Voucher samples deposited at the herbarium of the Botanical Institute from the University Montpellier II (MPU); B. G. = Botanical Gardens. ENSAM: Ecole Nationale Supérieure Agronomique from Montpellier.

Species	Origin country	Locality (for wild prospection)	Botanical Gardens, Parks and Herbarium
Nestegis sandwichensis (A.Gray) Deg.	Hawaii (USA)		Kokee State Park, Hawaii – VS. T Flynn 6329
Osmanthus fragrans Lour.	India		Madrid Botanical Garden accession – VS. P Villemur 03
Phillyrea latifolia L.	Morocco	Immouzzer, Atlas	VS. A Moukhli 01
Ligustrum vulgare L.	France	Collias, Gard	
Noronhia emarginata (Lam.) Thouars	Madagascar		Olu Pua Gardens, Hawaii – VS. T Flynn 6331
Chionanthus ramiflorus Roxb. [sect. *Linociera* (Sw.)]	USA		Kauai National Tropical Botanical Garden accession: 75 094 70 01 - VS
Chionanthus virginicus L.	USA		Kew B. G. accession: 19 76 292
Syringa vulgaris L.	France		ENSAM park, Ornamental plant
Forestiera neomexicana A.Gray	USA		Madrid Botanical Garden accession – VS. P Villemur 04
Fraxinus angustifolia Vahl. subsp. *oxycarpa* (M.Bieb. ex Willd.) Franco & Rocha	France	Montpellier, Hérault	ENSAM park
Fontanesia phillyreoides Labill.	Turkey		Montpellier Botanical Garden accession
Jasminum officinale L.	-		Montpellier Botanical Garden accession
Jasminum fruticans L.	France	Montpellier, Hérault	
Forsythia x intermedia Zabel	-		ENSAM park, Ornamental plant
Schrebera alata (Hochst.) Welw.	-		Kew B. G. accession: 19 69 188 26

hybridized in a 7 % SDS, 0.25 M Na_2HPO_4 and 1 mM EDTA solution at 65 ° C for 18 h with the 18S rRNA gene from sunflower (Choumane & Heizmann, 1988), the 25S rRNA gene and the entire unit from flax (Goldsbrough & Cullis, 1981) as a probe. The membranes were rinsed three times at 65 °C in 2X SSC, 0.1 % SDS and in 0.2X SSC, 0.1 % SDS for 25 min and were exposed to Hyperfilm MP (Amersham) for 6-18 h. The entire *Oleaceae* sample was studied with this approach (Table III), whereas, only a sub-sample of plants of the genus *Olea* was analyzed (Table II).

C. RAPD

The RAPD amplification and electrophoresis procedures were described by Quillet *et al.,* (1995). Eight primers (Bioprobe, France), previously selected (Besnard *et al.,* 2001b) were used on the DNA's from all individuals: A1, A2, A9, A10, C9, C15, E15, O8. After electrophoresis, gels were placed in a 0.25 N HCl solution for 30 min. Then, the DNA was transferred by a 0.4 N NaOH solution onto a Nylon membrane Hybond N+ (Amersham) with a transblotter (Life Technologies) under a depression of 60 Pa for 1 h. The membranes were rinsed in 2X SSC solution (300 mM NaCl, 30 mM sodium citrate) and then baked to 80 ° C for 2 h. Some RAPD fragments were used as a probe. These were picked up on agarose gels and then purified with Wizard plus Minipreps (Promega). Recovered DNA was labeled using 74 mBq of $\alpha[^{32}P]$dCTP (111 Tbq/mmol). The hybridization conditions were those previously described.

The well separated and intense fragments were noted. Hybridization profiles enabled us to verify specificity of some fragments or the homology of sequence of fragments present in different species. In addition, hybridization enabled us to read some fragments without ambiguity. All individuals were characterized with this method except *Jasminum fruticans*.

D. Data analysis

- Ribosomal DNA data

Ribosomal DNA restriction maps were constructed. A matrix of presence/absence of each polymorphic site was established. Wagner parsimony phylogenetic trees (Farris, 1970) were constructed with these data using the phylogenetic inference package (PHYLIP, version 3.4) written by Felsenstein (1989).

- RAPD data

A matrix of presence/absence of fragments was established for *Olea* species. We computed Jaccard similarity (Jaccard, 1908) indexes: $S_{ij} = a / (a + b + c)$

where a is the number of common bands between i and j, and b and c the bands present in one individual (i or j respectively). We used both the Neighbor Joining Method (Nei, 1987) and the UPGMA algorithm (Benzécri, 1973) to construct phenetic trees.

III. RESULTS

A. Ribosomal DNA restriction maps

Seventeen rDNA restriction maps of *Oleaceae* taxa were obtained. Five were exhibited by *Olea* species (Fig. 1). All the polymorphisms were found located in the internal gene spacer (IGS) and in the internal transcribed spacers (ITS). Firstly, *O. tsoongii* and *O. brachiata* (subgenus *Tetrapilus*) were distinguished from the other *Olea* species by the presence of an additional *Sac*I site (S5), the absence of the *Bam*HI site (B1), and the 4 kb *Eco*RV fragment (V4-V7) instead of the 4.5 kb fragment (V3-V7) in *O. africana*, *O. woodiana* and *O. capensis* subsp. *capensis*. Furthermore, *O. capensis* subsp. *macrocarpa*, *O. lancea* and *O. perrieri* were distinguished from *O. africana*, *O. woodiana* and *O. capensis* subsp. *capensis* by a 5 kb *Eco*RV fragment (V2-V7) instead of the 4.5 kb fragment. An additional *Eco*RI fragment of 1 kb, only hybridized with the entire unit, was present in the section *Olea*, except in *O. africana*. The corresponding additional *Eco*RI site (E5) cannot be placed on the map because it was not revealed with the 18S and 25S probes. Lastly, *O. paniculata* also exhibited a similar additional fragment of 1.2 kb. A diagnostic fragment (B1-B2) of 0.6 kb enabled us to recognize the genus *Noronhia* and most of the *Olea* species, but it was absent in the subgenus *Tetrapilus*.

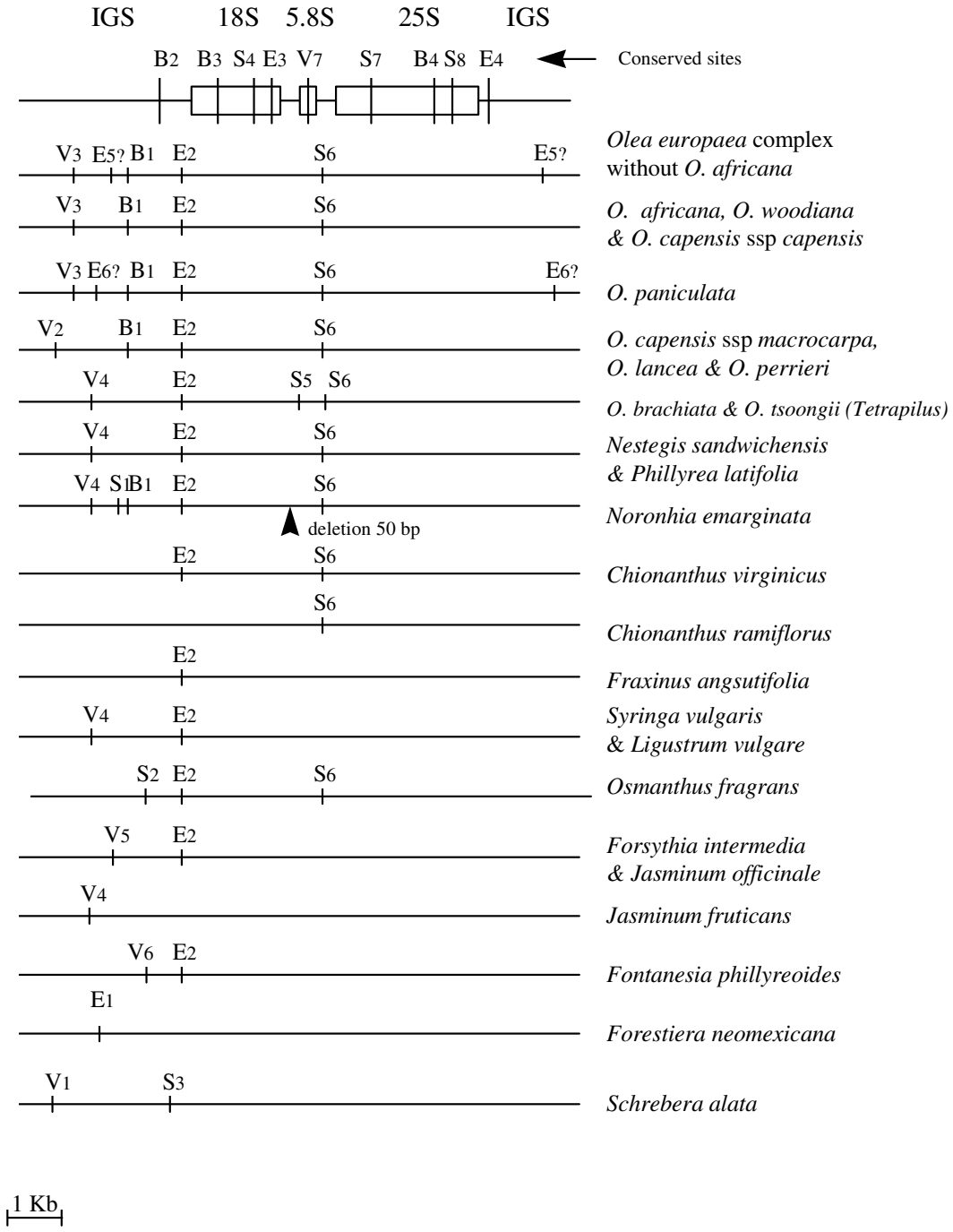

Fig. 1. Ribosomal DNA restriction maps of the different species of *Olea* and of *Oleaceae*.

B. Phylogenetic reconstruction based on rDNA restriction sites

Thus, 17 characters were considered of which 5 were informative (E2, V3, V4, S6, B1). Wagner parsimony based on these data enabled us to detect two homoplasic sites: E2 and V4. For these sites, we can suppose that at least two mutational events have led to their appearance and disappearance. E2 has likely appeared in *Jasminoideae,* for which it is polymorphic, and it has likely disappeared in *Chionanthus ramiflorus* (but it is present in *Chionanthus virginicus*). In addition, *Forestiera*, which did not display E2, displayed another *Eco*RI site (E1) in the IGS which could derive from E2 by a rearrangement (insertion) of the IGS region. Secondly, the *Eco*RV sites (V1-6) have likely been moved by insertion-deletion in the IGS. These sites should have disappeared several times and this might explain the homoplasy observed for the V4 site. Using all the information, 94 most

parsimonious trees were obtained. All these trees supported the separation of *Tetrapilus* from the other species of *Olea*. The rDNA consensus phylogenetic tree supported a separation of *Oleeae* from *Jasminoideae*, *Fraxinae* and *Forestiera neomexicana* (data not shown). Nevertheless, the nodes were not well supported due to a high frequency of homoplasic characters. Thus, we constructed and presented a tree only on the tribe *Oleeae* based on 12 characters of which 3 were informative (B1, V3 and V4) (Fig. 1 and 2). One homoplasy (V4) was detected in this data sample. *O. brachiata* and *O. tsoongii* (*Tetrapilus*) were not grouped with the other *Olea* species (this is supported by B1, V2 and V3 sites), but appeared related to *Nestegis* and *Phillyrea*.

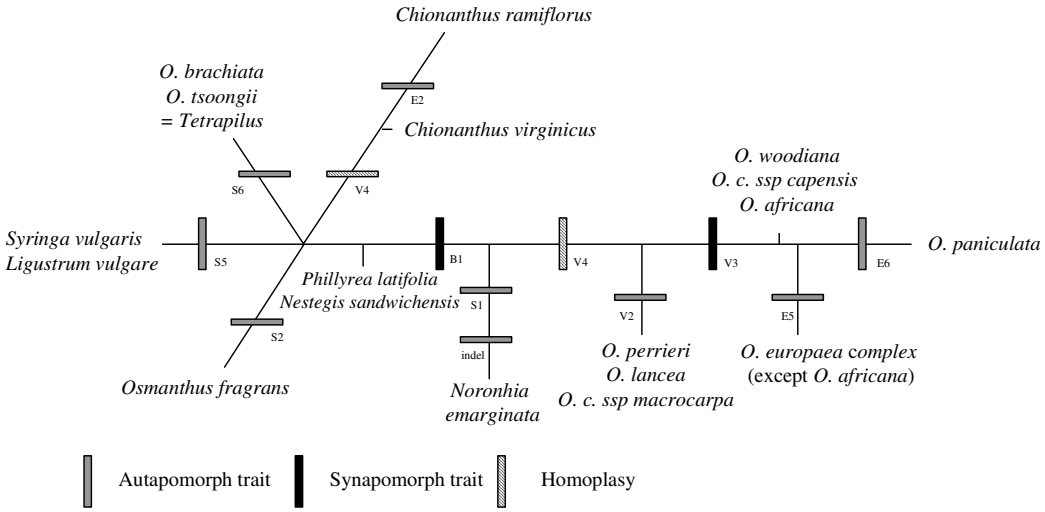

Fig. 2. Most parsimonious tree of the tribe *Oleeae* based on rDNA restriction sites.

C. RAPD analysis

The number of common bands according to their size between the different genera was very low, and we can consider that these were not informative without sequence homology verification. Consequently, we noted only the bands in the genus *Olea*. These were coded: primer-size in bp. Fifty-one *Olea* fragments were used as a probe (Table IV). A1-975 probe hybridized another fragment of approximately 925 bp (A1-925) and A10-1400 probe hybridized another fragment of approximately 1200 bp (A10-1200). Thus, 53 markers were verified for their sequence homology. The verification of the sequence homology of two fragments did not confirm the lecture based on the size homology. Thus, we considered such bands separately: A1-200a/A1-200b, A2-425a/A2-425b. In addition, 39 other intense and well-separated fragments were noted without sequence homology verification leading to a total of 92 markers.

In the genus *Olea*, most of the markers (79/92 = 86 %) were unique to a subgenus or a section: fourteen markers for the section *Olea*, twenty-nine for the section *Ligustroides*, sixteen for the species *O. paniculata* and twenty for the subgenus *Tetrapilus*. Three non-polymorphic markers were found in the genus *Olea* and these were found again in other genera of *Oleaceae* (Table V). Several genera displayed common bands with the different groups of the genus *Olea,* in particular *Nestegis* and *Chionanthus* (Table V). Based on these data, the subgenus *Tetrapilus* appeared more closely related to *Chionanthus* and *Nestegis* than to the other species of *Olea* (Table V).

Table IV. List of the picked fragments, which were used as a probe. Each fragment was coded: primer - size in bp - accession code of origin (see Table II).

Fragment	Individual code	Fragment	Individual code
A1-200a	T.TSO1	A1-450	L.LR1
A1-675	T.TSO1	A1-480	L.WOS
A2-425a	T.TSO1	A1-970	L.LR1
A2-675	T.TSO2	A2-480	L.WOS
A9-200	T.TSO2	A2-1050	L.WOS
A9-225	T.GRA	A10-525	L.WOS
A9-465	T.TSO1	A10-750	L.WOK
A9-525	T.GRA	A10-775	L.WOS
A9-700	T.GRA	A10-1400	L.LR1
A9-725	T.GRA	C9-975	L.PE1
C9-800	T.TSO2	C15-1000	L.WOS
C15-450	T.TSO1	E15-350	L.PE1
C15-775	T.TSO1	E15-650	L.MAS
E15-800	T.TSO2	O8-450	L.MAZ
A1-200b	E.LA	O8-600	L.LM1
A1-400	E.MA1	A1-750	P.KEW
A1-510	E.LA	A1-950	P.KEW
A1-975	E.MA1	A2-200	P.KEW
C9-475	E.CR2	A9-475	P.KEW
C9-900	E.MA1	C9-450	P.KEW
C15-800	E.ES1	C9-525	P.KEW
C15-1350	E.MA1	C9-850	P.KEW
E15-475	E.ES1	C15-500	P.KEW
E15-600	E.ES1	C15-1100	P.KEW
O8-350	E.ES1	O8-1200	P.KEW
O8-1100	E.ES1		

The phenetic trees constructed on these data (Fig. 3) show a clear separation between four groups in accordance with the morphological classification of the genus (P.S. Green, in preparation). However, *O. woodiana* from Kenya is related to the section *Olea* although it should be related to the section *Ligustroides*. This individual displayed no specific markers of *Ligustroides*. The identification of this species (performed by H. Sommerlatte C/O GTS Nairobi, Kenya) should be incorrect. The phenetic tree based on the Neighbor joining method allows revealing that the sections *Ligustroides* and *Olea* have proximal positions in comparison to *O. paniculata* and the subgenus *Tetrapilus*. This is due to a sample too limited for the latter species and to their high genetic divergence in comparison to the subgenus *Olea*. The genetic proximity of the two sections of the subgenus *Olea* are supported by the phenetic tree constructed using the algorithm UPGMA.

In each section, the relationships between species deduced from morphologic data were not confirmed here. In the section *Ligustroides*, the different taxa were well recognized except for two species from Madagascar, *O. c*. subsp. *madagascariensis* and *O. perrieri*, which are mixed. The *O. capensis* complex (subsp. *macrocarpa*, subsp. *capensis*, subsp. *madagascariensis*) is not supported by RAPD and ribosomal data. The four subspecies of the section *Olea* are not found. Three groups only can be distinguished based on RAPD data: *O. africana-O. chrysophylla*, *O. cuspidata-O. laperrinei* and *O. europaea-O. cerasiformis-O. maroccana*.

IV. DISCUSSION

A. Position of subgenera and sections of genus *Olea* within the *Oleaceae*

Our rDNA data are based mainly on IGS polymorphisms, but a high level of reorganization exists in this sequence, as already shown in *Fraxinus* (Jeandroz *et al.,* 1996b). Consequently, we have to be prudent in the interpretation of these data. Nevertheless, our results support the evidence that species

Table V. *Olea* markers revealed by hybridization in *Oleaceae* taxa. 1 = presence of the fragment; - = absence of the fragment; (-) means that the fragment is polymorphic in the group of considered taxa; * marker with a lower intensity. In brackets, the size of the revealed fragments is indicated when it is different of the used probe.

	A1-400	**A10-525**	**E15-475**	**C15-1000**	**08-1100**	**C15-800**	**A1-200a**	**A2-675**	**A9-700**	**C15-775**
O. **subg.** *Olea* **sect.** *Olea*	**1**	**1**	**1**	**1**	**1 (-)**	**1**	**-**	**-**	**-**	**-**
O. **subg.** *Olea* **sect.** *Ligustroides*	**1**	**1**	**1**	**1**	**1**	**1 (-)**	**-**	**-**	**-**	**-**
O. **subg.** *Paniculatea*	**1**	**1**	**1**	**1**	**1**	**-**	**-**	**-**	**-**	**-**
O. **subg.** *Tetrapilus*	**1**	**1**	**1***	**-**	**-**	**-**	**1 (-)**	**1 (-)**	**1**	**1**
Nestegis sandwichensis	**1***	**1**	**1* (500 bps)**	**1**	**1**	**1***	**-**	**1 (525 bps)**	**1 (500 bps)**	**1***
Chionanthus virginicus	**-**	**1**	**1***	**1**	**1**	**-**	**1**	**1 (500 bps)**	**1***	**1***
Chionanthus ramiflorus	**1 (450 bps)**	**1**	**-**	**-**	**-**	**-**	**1**	**-**	**-**	**-**
Noronhia emarginata	**1**	**1**	**-**	**-**	**1***	**-**	**-**	**-**	**-**	**-**
Osmanthus fragrans	**1***	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**1***
Phillyrea latifolia	**1 (450 bps)**	**-**	**-**	**1***	**1***	**-**	**-**	**-**	**-**	**1***
Ligustrum vulgare	**-**	**1**	**-**	**1**	**-**	**1**	**-**	**-**	**-**	**1* (750 bps)**
Syringa vulgaris	**-**	**-**	**-**	**1**	**-**	**1**	**-**	**-**	**-**	**1***
Fraxinus angustifolia	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**1***
Forestiera neomexicana	**-**	**1**	**-**	**1**	**1* (1050 bps)**	**-**	**-**	**-**	**-**	**1***
Schrebera alata	**1***	**-**	**1***	**1**	**1***	**-**	**-**	**-**	**-**	**1***
Jasminum officinale	**-**	**1**	**1***	**1**	**-**	**-**	**-**	**-**	**-**	**-**
Forsythia x intermedia	**-**	**-**	**-**	**-**	**-**	**1***	**-**	**-**	**-**	**-**
Fontanesia phillyreoides	**-**	**-**	**-**	**-**	**-**	**1**	**-**	**-**	**-**	**-**

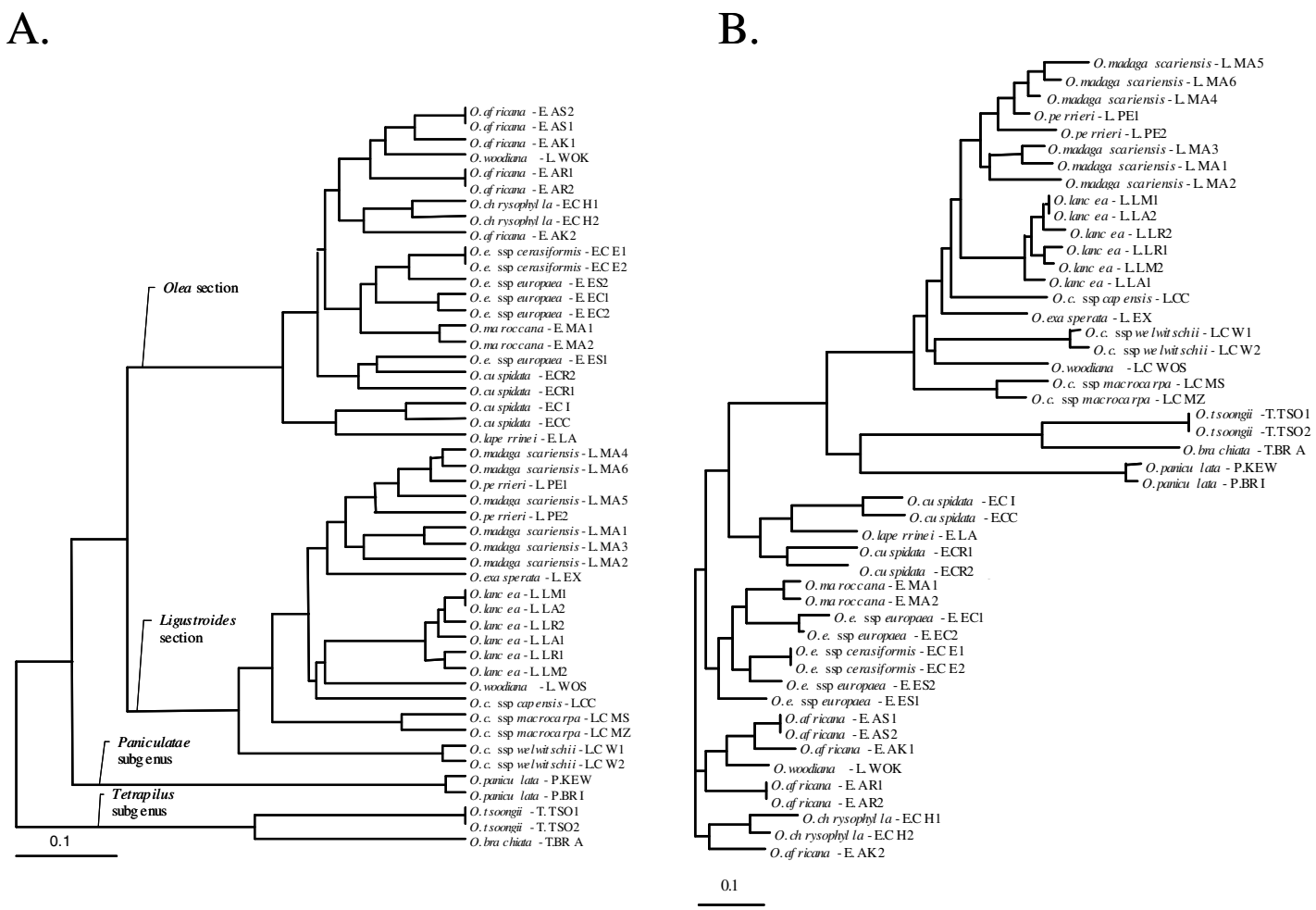

Fig 3. Phenetic trees of the species of *Olea* based on RAPD data: **A.** Phenetic tree based on Jaccard similarity (1908) and constructed with UPGMA algorithm. **B.** Phenetic tree based on Jaccard similarity (1908) and constructed with Neighbor Joining algorithm.

of the subgenus *Tetrapilus* species are more closely related to *Nestegis, Chionanthus* and *Phillyrea* than to the other *Olea* species. Such result was also supported by cpDNA information (Wallander & Albert, 2000). Furthermore, RAPD technology has led to a low level of informative characters (14 % of markers) when different genera, subgenera or sections were compared. Nevertheless, RAPD data also support that *Tetrapilus* species are related to *Chionanthus* and *Nestegis*. Combining rDNA and RAPD data, the genera *Nestegis* and *Chionanthus* appeared in an intermediary position between *Tetrapilus* and the other *Olea* species. Thus, *Tetrapilus* should be considered as a genus as proposed by Johnson (1957): *Tetrapilus* Lour. Nevertheless, an exhaustive study of the subgenus *Tetrapilus* is necessary to accumulate evidences. Moreover, we suggest that sequencing of ribosomal DNA ITS should lead to more informative characters and should avoid homoplasy.

B. Structure of the genus *Olea* revealed by molecular markers

In a recent study, AFLPs have led to the distinction of *O. lancea* and *O. paniculata* from the section *Olea* (Angiolillo *et al.,* 1999), but this work was limited to a few accessions of *Olea*. In our work, we analyzed a larger number of accessions and these belong to the different sections and subgenera of *Olea*. The distinction of four groups of *Olea* obtained with RAPD data is in accordance with the taxonomy based on morphological characters, except possibly for *O. woodiana* subsp. *disjuncta* from Kenya, if the material was correctly named.

We suspect that the molecular proximity is correlated to the cross-ability between the species as already shown for *Syringa* (Kim & Jansen, 1998). Thus, the four *Olea* groups could correspond to four species complexes.

Within section *Ligustroides*, the three subspecies of *O. capensis* studied were as clearly separated as all the other taxa, except *O. c.* subsp. *madagascariensis* and *O. perrieri*. The taxa of this section have a discontinuous distribution but some are probably sympatric in some areas (notably in Central Africa or in South Africa). From the great genetic proximity between the different taxa of this section and the sympatric distribution, we can suspect that gene flow occurred or could occur between some taxa.

Within the section *Olea*, the relationships deduced from rDNA RFLP and RAPD data were not in accordance with the morphological classification (Green & Wickens, 1989). Firstly, the *O. laperrinei* individual did not appear related to *O. maroccana* and *O. cerasiformis*, two other northern African taxa considered as relic forms of an ancient northern African population. This led us to suppose that the Northwestern African taxa and Saharan populations could derive from different ancestral populations. This hypothesis is also supported by cytoplasmic markers, which enabled to clearly separate *O. maroccana-O. cerasiformis* from *O. laperrinei* (Besnard & Bervillé, 2000). Otherwise, *O. chrysophylla* from Yemen and *O. africana* from Eastern and Southern Africa were grouped together with RAPDs but displayed a different rDNA unit. These two taxa displayed also different chlorotypes indicating their distinct origins (Besnard & Bervillé, 2000). These observations lead us to suspect gene flows between the different taxa during favorable periods. Thus, gene flow could have contributed to the evolution of the Mediterranean olive as suggested by Green & Wickens (1989) and Besnard *et al.* (2001b).

C. Origin of the genus *Olea*

We cannot deduce the geographical origin of the genus *Olea* with our molecular data. Nevertheless, *O. paniculata* is well separated from the subgenus *Olea* and this supports an ancient separation of the genus *Olea* between Asia-Oceania (subgenus *Paniculatae*) and Africa (subgenus *Olea*).

The distribution of the subgenus *Olea* is mainly throughout the African continent. The common origin of the sections *Ligustroides* and *Olea* is supported by our molecular study. Verdoorn (1956) supposed that the axial and terminal flowering exhibited in *O. woodiana* could be an ancestral character. Thus, this author considered that *O. woodiana* was in an intermediary position between *O. capensis* and *O. europaea*. This is partially supported by rDNA restriction maps: *O. africana* and *O. woodiana* from Africa were not distinguished with rDNA restriction maps. The absence of the E5 restriction site may be an ancestral state. Thus, we can suppose that the subgenus *Olea* originated in Africa rather in Asia where it is also present. Quézel (1978) and Maley (1980) have already suggested an African origin for *O. europaea* species in the Rand-Flora (indigenous African flora adapted to Mediterranean climate). Consequently, the section *Olea* and the section *Ligustroides* probably originated from the Rand-Flora about 10 to 20 millions years ago. At the present time, we cannot determine whether the differentiation of the subgenus *Olea* occurred before, during or after the Rand-Flora formation.

Dyer (1991) has reported an incompatibility between *O. capensis* subsp. *capensis* (section *Ligustroides*) and *O. africana* (section *Olea*). In contrast, cross-ability between *O. africana* and *O. europaea* was evidenced (Besnard *et al.,* 2001b), and this leads us to consider the *O. europaea* complex as a primary genetic resource of the cultivated olive tree. A study of the evolution of the wild and domesticated olive has to be performed on the whole *O. europaea* complex.

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Appendix 1: Classification and geographical distribution of species and subgenera of *Olea* with their main synonyms according to morphological data (Green, in preparation).

Subgenus	Sections	Species	Subspecies	Main synonyms	Geographical distribution
Olea L.					
	Olea				
		O. europaea L.			
			subsp. *europaea*		
				var. *sylvestris* (Mill.) Lehr.	Mediterranean Basin
				var. *europaea*	Mediterranean Basin
			subsp. *cuspidata* (Wall. ex G. Don) Ciferri		
				O. cuspidata Wall. ex G. Don	Iran, Pakistan, India, China
				O. chrysophylla Lam.	Arabia, Eastern Africa
				O. africana Mill.	Eastern and SouthernAfrica
			subsp. *laperrinei* (Batt. & Trab.) Ciferri		
				O. laperrinei Batt. & Trab.	Saharan Mountains
				O. maroccana Greut. & Burd.	Southern Morocco
			subsp. *cerasiformis* Kunk. & Sund.		
				O. cerasiformis Webb & Berth.	Canary Islands
				O. maderensis Lowe	Madeira
	Ligustroides				
	Benth. & Hook.	*O. capensis* L.	subsp. *capensis*		Southern Africa
			subsp. *enervis* (Harv.) Verd.		Southern Africa
			subsp. *macrocarpa* (Wright) Verd.		Southern Africa
			subsp. *madagascariensis* (Boiv. ex Perr.) ined.		Madagascar
		O. hochstetteri Bak.			Central Africa
		O. welwitschii (Knobl.) Gilg & Schellenb			Southern Africa
		O. schliebenii Knobl.			Tanzania (Tanganyika)
		O. perrieri Chev. ex Perr.			Madagascar
		O. woodiana Knobl.	subsp. *woodiana*		Southern Africa
			subsp. *disjuncta* ined.		Kenya, Tanzania
		O. ambrensis Perr.			Madagascar
		O. lancea Lam.			Mascareignes, Madagascar
		O. exasperata Jacq.			South Africa
		O. chimanimani Kupicha			Zimbabwe, Mozambique
Paniculatae ined.		*O. paniculata* R Br.		*O. bournei* Fyson, *O. glandulifera* Desf., *O. thozetii* Panch. & Seb.	India, Nepal, Northern Oceania, Australia, New Caledonia

Appendix 1, continued

Subgenus	Sections	Species	Subspecies	Main synonyms	Geographical distribution
Tetrapilus (Lour.) ined.		*O. borneensis* Boer.			Indonesia, Malaysia (Borneo)
		O. brachiata (Lour.) Merr.		*O. maritima* Wall. ex G. Don *O. graciliflora* Koor. & Val.	China, Cambodia, Thailand, Malaysia, Indonesia (Java, Sumatra)
		O. caudatilimba Chia			China (Yunan)
		O. cordatula Li			Vietnam
		O. decussata (Heine) Kiew			Indonesia, Malaysia (Borneo)
		O. dentata DC.		*O. gagnepainiana* Knobl *O. guangxiensis* Miao *O. penengiana* Ridl. *O. rubrovenia* (Elm.) ined.	Burma, Malaysia (Penang), Vietnam, Philippines, China
		O. dioica Roxb.		*O. heyneana* Wall. ex DC.	India
		O. gamblei Clarke			India
		O. hainanensis Li			China (Hainan)
		O. javanica (Blume) Knobl.			Indonesia, Malaysia
		O. laxiflora Li			China (Yunan)
		O. neriifolia Li			China (Hainan)
		O. obovata (Merr.) ined.			Philippines
		O. palawanensis Kiew			Philippines
		O. parvilimba (Merr. & Chun) Miao			China (Hainan)
		O. polygama Wight		*O. gardneri* Thw.	India
		O. rosea Craib.		*O. densiflora* Li *O. oblanceolata* Craib.	Cambodia, Laos, Vietnam, China, Thailand
		O. salicifolia Wall.			China (Xizang), India, Burma
		O. tetragonoclada Chia			China, India, Burma
		O. tsoongii (Merr.) Green		*O. yuennanensis* Hand.-Mazz	China (Guangdong, Sichuan,Yunan)
		O. wightiana Wall. ex G. Don			India