Establishing the genetic relationships between the wild and cultivated olives using a nuclear intron from nitrate reductase (*nia*-i3)

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Abstract. In Oleaceae the most outstanding biological issue is to clarify the taxonomic relationships of cultivated and wild olives. To establish the genetic relationships between the wild (Olea europaea subsp. europaea var. sylvestris (Mill.) Lehr.), the cultivated olive (Olea europaea subsp. europaea var. europaea), and other taxa of the genus Olea (Olea europaea subsp. cuspidata (Wall. ex G. Don) Cif., Olea europaea subsp. cerasiformis G. Kunkel & Sunding, Olea paniculata R. Br.) and other Oleaceae (represented by Ligustrum vulgaris) we carried out the amplification by polymerase chain reaction (PCR) and the sequencing of the third nuclear intron of the nitrate reductase gene (nia-i3). Sequence analyses showed the presence of two different functional variants of the intron (nia1 and nia2) in the Oleaceae, in addition to a shorter non-functional one. Notably, while the shortest and the nial variants were present in all the taxa analysed, the nia2 variant was present only in the wild and the cultivated olive. These data confirm the close phylogenetic relationship between wild and cultivated olives and suggest that this gene could be duplicated in these two taxa after its divergence from the remaining Oleaceae. The presence of a target for AfIII enzyme in nia2 and its absence in nia1 variant enables easy distinction by PCR-RFLP between, on the one hand, wild and cultivated olive, and on the other the remaining subspecies of the *Olea europaea* L. complex (*O. e.* subsp. *cuspidata* and *O. e.* subsp. *cerasiformis*) as well as other Oleaceae (*O. paniculata*, *L. vulgaris* L.). Additionally, *nia*1 sequences provide useful information about phylogeny of the wild and cultivated olives inside the genus *Olea*.

Keywords: Nuclear intron; nitrate reductase; wild and cultivated olives; *Olea europaea* complex; Oleaceae; phylogenetic analyses; gene duplication

Introduction

In Oleaceae the most outstanding taxonomic issue concerns the relationships of the wild and the cultivated olives (Green 1965). This issue not only has taxonomic implications but is also useful to elucidate the origin of this important crop, to understand the history of its domestication, and to design strategies for its improvement and conservation.

Taxonomically, wild and cultivated olives have been classified at different levels, from

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different species (*Olea oleaster* Hoffm. et Link and *Olea europaea* L., respectively) to different subspecies of the same species (*O. europaea* L. subsp. *sylvestris* (Mill.) Rouy and subsp. *europaea*, respectively) (Turrill 1951, Morettini 1972). Other authors (Amaral and Rocha 1972, Pignatti 1982, Green 2002) considered the wild and the cultivated olive two different varieties (*O. euroapea* L. var. *sylvestris* Brot. and *O. europaea* var. *europaea*, respectively).

The genus Olea contains about 20 species native to warm temperate and tropical regions of southern Europe, Africa, southern Asia, Australia, New-Zealand, and Polynesia (Taylor 1945, Johnson 1957, Green and Wickens 1989, Green 2002). This genus is divided into two different subgenera, Olea and Paniculatae. The subgenus Paniculatae includes only the species O. paniculata. On the other hand, the subgenus Olea is separated into two sections, Olea and Ligustroides. The monotypic section Olea includes the Olea europaea L. complex, grouping wild and cultivated forms of the olive crop (Green and Wickens 1989). In addition to the subsp. europaea itself, three more subspecies have been traditionally described in this complex: a) subsp. laperrinei (Batt. & Trab.) Cif., corresponding to O. laperrinei of the Sahara; b) subsp. cuspidata, grouping taxa from southeastern Africa and Asia such as O. cuspidata Wall., O. indica Burm.f., O. ferruginea Royle. and O. africana Mill.; c) subsp. cerasiformis corresponding to the wild form of Macronesia. Two more subspecies were recently incorporated in the O. europaea complex: subsp. maroccana (Greuter & Burdet) P. Vargas et al. from Morocco and subsp. guanchica Vargas et al. from the Canary Island (Medail et al. 2001, Vargas et al. 2001). Finally, there exist a number of species included in section Ligustroides (O. capensis L., O. perrieri A. Chev. ex H. Perrier, *O. exasperata* Jacq., *O. woodiana* Knobl., etc).

With respect to the phylogeny of the genus *Olea*, in a general analysis including 76 species of Oleaceae of which four species were of the genus *Olea*, Wallander and Albert (2000), have presented a revised phylogenetic classification based in two noncoding chloroplast loci, the *rps*16

intron and the *trn*L-F region and concluded that the Olea species (O. europaea, O. capensis and O. paniculata, with the two former more closely related) form a monophyletic group. Notwithstanding, species such as O. brachiata (Lour.) Merr. (formerly placed in the separate genus Tetrapilus Lour.) appears in a separate different group (more related to another genus: Chionanthus L.). On the other hand, many molecular analyses using different markers (isozymes, RAPDs, ITSs, SCARs, AFLPs, SSRs, chloroplast and mitochondrial RFLPs and sequences) together with other data (morphological, chemical, etc.) support the idea that O. europaea should be considered a complex of subspecies, and grouped the wild and the cultivated olive as two closely related varieties (Besnard and Berville 2000, De Caraffa et al. 2002, Rallo et al. 2003, Lumaret et al. 2004, Reale et al. 2006). Finally, with respect to the geographic origin of the cultivated olives, Baldoni et al. 2006, have reported, for example, that cultivated olives of some Italian islands are clustered with local wild forms, suggesting that they originated either by selection from wild trees or by direct introduction from these areas. However, the available information is still unsuccessful in offering definitive solutions about the origin of the edible olive.

In this paper, we analyse the possibility of distinguishing, on one hand, between wild and cultivated olives and, on the other hand, between the olives and other Oleaceae members, with the aim of establishing their phylogenetic relationships using a genetic marker: the third intron from the nitrate reductase gene (*nia*-i3). This molecular marker is not frequently employed in the taxonomic analyses, but it has been shown to be useful in investigating the history and population biology of closely related plant species or complexes (Zhou et al. 1995; Zhou and Kleinhofs 1996; Howarth and Baum 2002, 2005).

Materials and methods

Plant material and DNA extraction. Oleaceae samples used in this study and their provenances are given in Table 1. Genomic DNA was isolated from

Species/subspecies	Provenance	Individuals	Genomic Clones/GB ^a
Olea europaea var. europaea (Arbequina)	Granada, Spain	2	A1, A2, A3, A4 (EF 113347-EF113350)
Olea europaea var. europaea (Hojiblanca)	Granada, Spain	2	H1, H2, H3, H4 (EF113351-EF113354)
Olea europaea var. sylvestris (W ^b)	Medina Sidonia, Cádiz, Spain	2	W1, W2, W3, W4 (EF113355-EF113358)
· · ·	4		W5, W6, W7, W8 (EF113359-EF113362)
Olea europaea var. sylvestris (Y ^b)	Alcalá de los Gazules, Cádiz, Spain	7	Y1, Y2, Y3, Y4 (EF113363-EF113366
•	•		Y5, Y6, Y7, Y8 (EF113367-EF1133670)
Olea europaea subsp. cerasiformis	Canary Islands, Spain	1	1, 2 (EF113372, EF113374)
Olea europaea subsp. cuspidata	Kew, UK (Chase 5705 ^c)	1	1, 2, (EF113375, EF113378)
Olea paniculata	Kew, UK (Chase 3882 ^c)	1	1, 2, (EF113379, EF113381)
Ligustrum vulgaris	Granada, Spain	1	1, 2, (EF113383, EF113384)
^a GenBank TM /EMBL accession numbers of <i>svlvestris</i> from Medina Sidonia (Cádiz) an	f <i>nia</i> -i3 sequences obtained, ^b W and Y and A Alcalá de los Gazules (Cádiz), respe	correspond to ctively, ^c DNA	two different populations of <i>O. europaea</i> var. bank voucher. <i>nia2</i> sequences are marked in

boldface at difference of nia ones

100 mg of leaf tissues from the different samples using Plant Dnazol kit (Invitrogen), following the manufacture's instructions.

PCR amplification of *nia***-i3.** Degenerated PCR primers were designed using Exon-primer introncrossing (EPIC) (Palumbi and Barker 1994), consisting of the search for conserved exons that flank variable introns. The primers used here were designed from multiple alignment of *nia* genes from many plant species, and were specific to exon 3-intron 3exon 4 sequence. 5'-CGGAACCAGCARTTRTTC ATCAT-3' was used as forward and 5'-CAATTA CTGGTGTTGGTGYTTYTGGTC-3' as reverse.

PCR was performed on a GeneAmp PCR system 2700 (Applied Biosystems). The reaction was carried out in 50 μ l using FastStart high-fidelity PCR system (Roche diagnostics), with a final concentration of 2.5 mM MgCl₂, 0.1 mM dNTPs and 0.5 μ M of each primer. The amplification conditions were as follows: denaturing at 94°C for 3 min, 35 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 1 min. The final elongation step was performed at 72°C for 10 min.

Cloning and sequencing. The PCR products obtained were gel-purified by GFXTM PCR DNA and Gel purification Kit (Amersham) and then cloned in Topo TA vector (Invitrogen), according to the manufacturer's indications. Several clones were sequenced from each taxa using SP6 and T7 primers with BigDye terminator v3.1 kit (Applied Biosystems) and run on a 3100-Avant Genetic Analyzer (Applied Biosystems). We sequenced a total of 32 cloned plasmid inserts. Of the 32 sequences: a) 16 were obtained from two individuals from each of two different populations (Medina-Sidonia -W- and Alcalá de los Gazules -Y-) of wild olive; b) Eight were obtained from two individuals from each of two different varieties (Arbequina -A- and Hojiblanca -H-) of cultivated olive. The other eight sequences belonged to the other taxa analysed (O. europaea. subsp. cerasiformis, O. europaea subsp. cuspidata, O. paniculata, and L. vulgaris). All these sequences of nia-i3 were submitted to Genbank and their corresponding accession numbers are given in Table 1.

The search of homology between the genomic sequences obtained and other sequences from GenBankTM/EMBL database was performed by the BLAST program (Altschul et al. 1990). Multiple alignment analyses were deduced using ClustalX 8.1 software (Thompson et al. 1997).

Phylogenetic relationships among taxa were estimated using three different methods: maximum parsimony (MP), maximum likelihood (ML) and neighbour-joining (NJ) methods. MP and ML were implemented by the PAUP^{*} v4b10 program (Swofford 2002) and the trees displayed with Treeview32 (Page 1996), while NJ was implemented by the MEGA program (Kumar et al. 2004). Gaps were treated as missing data. For MP, heuristic searches were run with 1000 random taxon-addition replicates using the TBR algorithm and the Multrees option. For the selection of the DNA-substitution model for ML and NJ, the aligned sequences were subjected to analysis using Modeltest v.3.6 (Posada and Crandall 1998), which performs a hierarchical test of likelihood (hLRT) under 56 different models of DNA substitution. Bootstrap support values were calculated on 1000 replicates in PAUP* v4b10 (Swofford 2002).

PCR-RFLP analysis. In order to demonstrate the presence/absence of the *nia*2 variant of the third intron of the nitrate reductase (*nia*) in the Oleaceae, gel purified PCR products of *nia* of all the taxa studied were digested by the restriction enzyme AfIII (Roche diagnostics), which digests in C/TTAAG, present exclusively in *nia*2 variant, and then separated on 1% agarose gel.

Results

Genomic clones-sequence analyses. Using the primers mentioned above and by PCR, we obtained two PCR-amplified products in all the Oleaceae examined, one (A) of about 900 bp and other (B) of about 250 bp (Fig. 1 a). Cloning and sequencing of the last one from O. paniculata indicated that corresponds to the NIA gene in the region of exon3 (132 bp)-exon4 (26 bp) with a reduced intron (only with 86 bp) (Fig. 1b). This sequence was submitted to Genbank (accession number: EF177404). On the other hand, the sequences determined from the 900-bp band correspond to the same region of this gene but with a longer intron 3 (670 bp). In both cases, the partial exon sequences displayed an identity ranging from 88 to 82% with exon 3 and exon 4 sequences of others NIA genes from the Genbank database: 88% with Nicotiana taba*cum* L. (GenBankTM/EMBL, access number X14058) (Vaucheret et al. 1989), 87% with *Petunia hybrida* Vilm. (GenBankTM/EMBL, access number L13691) (Slanoubat and Ha 1993), 83% with *Solanum lycopersicum* L. (GenBankTM/EMBL, access number X14060) (Daniel-Vedele et al. 1989) and 82% with *Phaseolus vulgaris* L. (GenBankTM/EMBL, access number U01029) (Jensen et al. 1994). Nonetheless, no significant homology was detected between the sequence obtained for the 3rd intron and other database sequences.

Among the sequences obtained in our analysis for the amplified product with the large intron, that is to say from the band of 900 bp, two distinct size variants of intron 3 were detected and these were termed nial and nia2 (Fig. 2). All the Oleaceae studied (O. europaea var. europaea, O. europaea var. sylvestris, O. europaea subsp. cuspidata, O. europaea subsp. cerasiformis, O. paniculata, L. vulgaris) had the nial variant. However, the other variant, nia2, was detected only in wild and cultivated olives sequences. The intron-size estimation showed that nia2 is about 50 to 70 bp larger than *nia*1. Nonetheless, the main outstanding character in nia2 concerns the exclusive presence of the CTTAAG motif at the 3' end of all the nia2 variant sequences cloned (Fig. 2). It bears noting that this last motif corresponds to the restriction site of AfIII enzyme (see above).

To confirm the exclusive presence of the nia2 variant in wild and cultivated olive, we designed a PCR-RPLP assay. For this, we amplified the region in a total of 10 individuals of Oleaceae and then digested the PCR product with the AfIII restriction enzyme. Figure 3 illustrates the result of digestion by AfIII after amplification of the nia intron in several of the individuals analysed. The gel showed the presence of three bands in the two olive cultivars (Arbequina and Hojiblanca) and in the populations Medina Sidonia y Alcalá de los Gazules of the wild olive. The larger one of about 900 bp corresponds to the nial variant and the two other bands of 600 and 300 bp, respectively, to the restriction of the nia2 form.



241 TTCCGA

Fig. 1. PCR amplification and sequencing of *nia* in *O. paniculata* (a) Analysis in 1% agarose gel of PCR products corresponding to *nia* from *O. paniculata*. 1: negative control performed by omitting DNA sample in the PCR reaction, 2: *O. paniculata* (b) Nucleotidic sequence determined from the 250-bp band from *O. paniculata*. Exon3 and exon 4 partial sequences are underlined and intron 3 sequence is framed

In all the other Oleaceae, only the band of 900 bp was obtained due to the absence of the *nia2* variant.

An analysis of the *nia*1 intron variant by multiple alignment of the sequences from all the taxa examined revealed a high degree of variation between and within taxa. Nevertheless, strong homogeneity was found for exonic sequences, lacking any phylogenetic signal. However, we have found the variation to be due to polymorphism within the intron region. In fact, from each individual analysed we have found in most cases two different *nia*1 intron sequences differing by almost 1% of the nucleotide sites. In the case of wild and cultivated olives from which we analyzed several individuals, a total of 24 sequences were obtained (as explained above, 16 were from O. europaea var. sylvestris and eight from O. europaea var. europaea). These sequences could be grouped in about 12 *nia*1 sequence variants, differing between 1 and 3.6%, which were subsequently used for phylogenetic analysis. The mean intra-taxon variability (Table 2) was similar in wild and cultivated olives (0.020 and 0.021 respectively) but higher compared with *O. europaea* subsp. *cerasiformis* (0.012), *O. europaea* subsp. *cuspidata* (0.015) and *O. paniculata* (0.012). This was probably due to sampling.

Phylogenetic relationships among the Oleaceae studied. Inter-taxa sequence distances for *nia*1 intron variant revealed similar genetic differentiation between wild, cultivated olives and *O. europaea* subsp. *cerasiformis*. On the other hand, the three taxa showed similar degrees of sequence divergence when compared with *O. europaea* subsp. *cuspidata* and with *O. paniculata*, suggesting that the first three *O. europaea* subspecies are closely related, with

a	1	0	20	30	40	50	60	71)	80	90	100
H1	GTAAGCTCAT	TTACCCCTA	CCTATOGCA	TAATTTO	TTGGTTTA	TGACACAGT	CAAATTTA	AGTCACTTA	TATTTTAT	TTTAAGCT	AGGAGAAG	ATTTTAAAA
Ligustrum1	GTAAGCTCAT	TTACCCCTA	CCTATOGC	TAATTTG	CTTGGTTTA	TGACACAGT	CAAATTTA	AGTCACTTA	TATTTTAT	TTTAAGCT	AGGAGAAG	ATTTTAAAA
W5 cuspidata1	GTAAGCTCAT GTAAGCTCAT	TTACCCCTA	CCTATGGC/	ATAATTTGO	CTGGGTTTA	TGCCACAGT	CAAATTTA CAAATTTA	AGTECETTA	TATTTATT TTTTTAAT	TTTAAGCT) TTTAAGGT/	AGGAGAAG) AGGAGAAG)	ATTTTCAAA ATTTTAAAA
	110	120	13	ο.	140	150	160	170	180	. 1	190	200
H1	TATEGTTTE	TTGGTAGTO	ATTATGAG	ATTGATT	TTTTGATCG	TTATAATGA	AAAGTAGG	TTCCACCTG	TGAGTGAA	GAAGTAAA	AAATGACG	GCTGAAATA
Ligustrum1	TATEGTTE	TTGGTAGTO	ATTATGAGA	ATTGATT	TTTTGATCG	TTATAATGA	AAAGTAGG	TTCCACCTG	TGAGTGAA	GAAGTAAA	AAATGACG	GCTGAAATA
cuspidata1	TATEGTTE	TTEGTAGTO	ATTATAAGA	ATTGATT	TTTGATCA	TTATAGCGA	AAAGTAGG	TGCCACCTG	TAAGTGAA	GAAGTAAA	AAATGACG	GCTGAAATA
ouspidului	210	220	230	240	2!	50	260	270	280	290	30	0
LI1	RTASASTAS	GOTTADAAA	ATTAATTT	TTTGAATO	BAACAATTO	CTATCAAAC	0.00000	TATOTAACA	TTTTCTAC	TAAAATO	TADADATA	TTOGGAGAG
Liaustrum1	GTAAAATAAT	GGTTAAAAA	ATTAATTT	TTTTGAATO	BAACAATTG	CTATCAAAC	66666 ··· C	TATGTAACA	TTTTCTAC	TTAAAATC	ATGGACAT	TTGGGAAAA
W5	GTAAAATAAT	GGTTAAAAA	ATTATTA	TTTTGAATO	BAACAATTG	CTATCAAAC	66666 · · C	TATGTAACA	TTTTTCTACT	TTAAAATC	ATGGACAT	TTGGGAAAA
cuspidata1	GTAAAATAAT	IGATTAAAAA	ATTAATTI	TTTTGAATO	GAACAATTG	CTATCAAAC	GGGGGGGAC	TATGTAACA	TTTTCTAC	TTAAAATC	ATGGACAT	TTGGGAAAA
	310	320	330	340	350	360	37	0	380	390	400	410
H1	TAACTTTTA	TAACACACTT	ATTTTGACA	AAGATGTA	AGACTTAT	TTTAAACCA	ACTTACCT	ATTTATTT	AAGCGTGA	AAAATATA	GACAAAA	TTTCATTAC
Ligustrum1	TAACTITTA	TAACACACTI	ATTCTGAC	AAGATGT	AGACTTAT	TTTAAACCA	ACTTACCT	ATTTATTT	AAGCGTGA	AAAATATA	GAAAAAAA	TTTCATTAC
W5 cusnidata1	TAACTITIA	TAACACACTT	ATTTTGACA	AAGATGT	AGACTIAT	TETAAACCA	ACTTACCT	ΔΤΤΤΔΤΤΤΤ	AAGCGTGA	AAAATATA AAAATATA	GARARAAA Garaaaaaa	TTTCATTAC
cuspidatai	420	ANCACACIT	n 4	LIAN LIAN LIAN LIAN LIAN LIAN LIAN LIAN	450	480	470	490	20	mî	500	510
L1	TGACCACGT	TATATAT	GCCAATAT	CTOTOTOT	TATOOTTTT	TATOOTOO	A920220	00000000	GGGTCCAA	AATTGAAC	TRCCATAC	CATAAAATA
Ligustrum1	TGACCACGTO	GCTGGCACGT	GCCAATATI	CTCTGTT	TATEGTTTT	TATGGTGGG	G		GGTCCAA	AATTGAAC	TGCCATAC	CATAAAATA
W5	TGACCCCGT	SCTOBCACO	GCCAATATI	TCTCTGTT	TATEGTTTT	TATGGTGGG	GCGGAGG-	··· 660666	GGGTCCAA	AATTGAAC	TOCCATAC	CATAAAATA
cuspidata1	TGACCACGT	GCTGGCACGT	GCCAATAT	TCTCTGTT	TATGGTTTT	TATGGTGGG	<u>G</u>		GGTCCAA	AATTGAAC	TECCATAC	CATAAAATA
	520	530	540	55	0 .	560	570	580	590	600	. (310
H1	CAAAAACTCI	ACATTCAAG	TACAATTA	FGGCCACAT	TGAGAAAT	TTTGAACAC	GTGAATAT	CACATGATG	ATCACGTA	AGTTAATA	TTTATTT	TAGAAAATA
Ligustrum1	CAAAAACTCI	ACATTCAAG	TACAATTA	IGGCCACAT	TICCGAAAT	TTTGAACAC	GEGAATAT	CACATGATG	ATCACGTA	AGTTAATA	TTATTT	TAGAAA TA
vvo cuspidata1	CAAAAACTGI	ACAGTCAAG	TACAATTAT	IGGCCACAT	TIGCGAAAT	TTTGAACAC	GTGAATAT	CACATGATG	ATCACGTA	AGTTAATA	TTTATTTC	TAGAAATAA
	620	630	640	650	660	670		680	690	700	710	
H1	620 ATTTTTGTT	630 1111111111	640 TTTG666	650	660	670 ATTGATAAT	TGAAACAT	680 TAGTATCTC	690 CAAATAA	700 ATTTGTGA	710 AATTGTGC	AG
H1 Ligustrum1	620 ATTTTTGTTT ATTTTTGGTT	630 	640 TTG6661 TTTT-6661	650 TAAAAAGT/ TAAAAGGT/	660 AACTATGAA AACCATGAA	670 ATTGATAAT ATTGATAAT	TGAAACAT TGAAAAAT	680 TAGTATCTC AACTCTCCT	690 CAAATAA CTAATAA	700 ATTTGTGA ATTCTTGA	710 AATTGTGC AATTGTGC	AG AG
H1 Ligustrum1 W5	620 ATTTITGTT ATTTITGGT ATTTITGTT		640 TTG6660 TTTT-6660 TTTT-6660	650 TAAAAAGT/ TAAAAGGT/ TAAAAAGT/	660 AACTATGAA AACCATGAA AACTATGAA	670 ATTGATAAT ATTGATAAT ATTGATAAT	TGAAACAT TGAAAAAT, TGAAAAAT,	AGTATCTC AACTCTCCT AACTATCTC	690 CAAATAA CTAATAA AAAATAA	700 ATTTGTGA ATTCTTGA ATTTGTGA	710 AATTGTGC. AATTGTGC. AATTGTGC.	AG AG
H1 Ligustrum1 W5 cuspidata1	620 ATTTTTGTT ATTTTTGGT ATTTTTGTT ATTTTTGTT		640 TTTG6661 TTTT-6661 TTTT-6661	650 TAAAAAGT/ TAAAAGGT/ TAAAAAGT/ TAAAAAGT/	660 AACTATGAA AACCATGAA AACTATGAA AACTATGAA	670 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAAT	TGAAACAT TGAAAAAT, TGAAAAAT, TGAAAAAT,	AGTATOTO AACTOTOCT AAGTATOTO AAGTATOTO	690 - CAAATAA - CTAATAA - AAAATAA CAAAATAA	700 ATTTGTGA ATTCTTGA ATTTGTGA ATTTGTGA	710 AATTGTGC AATTGTGC AATTGTGC AATTGTGC	AG AG AG AG
H1 Ligustrum1 W5 cuspidata1 b	620 ATTTTTGTTT ATTTTTGGTT ATTTTTGTTT ATTTTTGTTT		640 TTTG6661 TTTT-6661 TTTT-6661 TTTTT6661	650 TAAAAAGT/ TAAAAGGT/ TAAAAAGT/ TAAAAAGT/ 30	660 AACTATGAA AACCATGAA AACTATGAA AACTATGAA 40	670 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAAT 50	TGAAACAT TGAAAAAT, TGAAAAAT, TGAAAAAT, 60	TAGTATCTC AACTCTCCT AAGTATCTC AAGTATCTC 70	690 - CAAATAA - CTAATAA - AAAATAA CAAAATAA	700 ATTIGTGA ATTCTTGA ATTIGTGA ATTIGTGA 80	710 AATTGTGC. AATTGTGC. AATTGTGC. AATTGTGC. 90	AG AG AG 100
H1 Ligustrum1 W5 cuspidata1 b W1 A4	620 ATTITIGIT ATTITIGIT ATTITIGIT ATTITITITI GTAACTCCAG	630 ITTTTTTTTTT ITTTTTTTTTT ITTTTTTTTTTTT	640 TTTG6661 TTTT-6661 TTTT-6661 TTTTT-6661 20 20 ATTTTT666 ATTTTT666	850 TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ 30 CTTAATATI CTTAATATI	660 AACTATGAA AACCATGAA AACTATGAA AACTATGAA 40 FCT66CC6T FCT66CC6T	670 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAAT 50 TTCATAATA TTGATAATA	TGAAACAT TGAAAAAT. TGAAAAAT. TGAAAAAT. GO TTTCGTAAJ TTTCGTAAJ	680 AACTCTCCT AAGTATCTC AAGTATCTC AAGTATCTC 7/ ATTTTTTAT ATTTTTTAT	690 - CAAATAA - CTAATAA - AAAATAA CAAAATAA CAAAATAA CTTTTGAAAA TTTTGAAAA	700 ATTTGTGA ATTCTTGA ATTTGTGA ATTTGTGA 80 ACTCATTTT ACTCATTT	710 AATTGTGC. AATTGTGC. AATTGTGC. AATTGTGC. 90 TCAACGGTG TCGACGGTG	AG AG AG 100 CACATTCTT CACATTCTT
H1 Ligustrum1 W5 cuspidata1 b W1 A4	620 ATTTITGTT ATTTITGGT ATTTITGTT ATTTITTT GTAACTCCAC GTAACTCCAC 110		640 TTG6661 TTTT-6661 TTTT-6661 TTTT-6661 20 20 ATTTT666 ATTTT666	650 TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ 30 CTTAATATI CTTAATATI 0	860 AACTATGAA AACCATGAA AACCATGAA AACTATGAA 40 FCT66CCGT FCT66CCGC 140	670 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAAT 50 TTCATAATA TTGATAATA 150	Т GAAACAT Т GAAAAAT, Т GAAAAAT, Т GAAAAAT, Т GAAAAAT, 60 Т Т Т С G Т AAA Т Т Т С С Т AAA 160	680 AACTCTCCT AAGTATCTC AAGTATCTC AAGTATCTC 70 ATTTTTTAT ATTTTTTAT 170	690 - CAAATAA - CTAATAA - AAAATAA CAAAATAA CAAAATAA TTTTGAAAA 180	700 ATTIGTGA ATTCTTGA ATTTGTGA ATTTGTGA 80 ACTCATTT ACTCATTT	710 AATTGTGC. AATTGTGC. AATTGTGC. AATTGTGC. 90 TCAACGGTG TCCACGGTG 190	AG AG AG CACATTCTT CACATTCTT 200
H1 Ligustrum1 W5 cuspidata1 b W1 A4 W1	620 ATTTTTGTT ATTTTTGGT ATTTTTGTT ATTTTTTTT	630 ITTTTTTTTTTT ITTTTTTTTTTT ITTTTTTTTTT	640 TTTG - 666 TTTT - 666 TTT	650 TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ 30 CTTAATATT CTTAATATT 0 ATAGTTTTT	660 AACTATGAA AACCATGAA AACTATGAA AACTATGAA 40 ICTEGCCCGT ICTGCCCGC 140 ICTATTTTT	870 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAAT 50 TTCATAATA 150 TTTGGTTAA	Т GAAACAT Т GAAAAAT, Т GAAAAAT, Т GAAAAAT, 60 Т Т Т С G T AAJ Т Т Т С С Т ААJ 160 С С А Т G C A T	680 TAGTATCTC AACICICCT AAGTATCTC AAGTATCTC ATTTTTTAT ATTTTTTAT 170 TCTTAAAAAA	690 - CAAATAA - CTAATAA - AAAATAA - AAAATAA CAAAATAA CAAAATAA - TTTTGAAAA 180 	700 ATTIGTGA ATTCTTGA ATTIGTGA ATTIGTGA 80 ACTCATTTI ACTCATTTI	710 AATTGTGC. AATTGTGC. AATTGTGC. 90 TCAACGGTG TCGACGGTG 190 AATGGGGGC	AG AG AG CACATTCTT CACATTCTT 200 TTATTCTCA
H1 Ligustrum1 W5 cuspidata1 b W1 A4 W1 A4	620 ATTTTTGTT ATTTTTGTT ATTTTTGTT ATTTTTTTT	630 ITTTTTTTTTTT ITTTTTTTTTTTTTTTTTTTTTTT	640 (TTG - 666) (TTTT - 666) (TTT	650 TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ 30 CTTAATATT CTTAATATT 0 ATAGTTTTT	660 AACTATGAA AACCATGAA AACTATGAA 40 FCTGGCCGGT FCTGGCCGGT 140 TCTATTTTT TTTTT	670 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAAT 50 TTCATAATA 150 TTTGTTAA	Т GAAACAT Т GAAAAAT, Т GAAAAAT, Т GAAAAAT, Т GAAAAAT, 60 Т Т Т С С Т АА, 160 С С АТ G С АТ С С АТ G С АТ	680 TAGTATCTC AACTCTCCT AAGTATCTC AAGTATCTC 7/ ATTTTTTAT ATTTTTTAT 170 TCTTAAAAAA	690 - CAAATAA - CTAATAA - AAAATAA - AAAATAA - AAAATAA - CAAAATAA - CAAAATAA - CAAAATAA - CAAAATAA - 130 - 1	700 ATTIGTGA ATTCITGA ATTIGTGA ATTIGTGA S0 ACTCATTT ACTCATTT TATCTCAA	710 AATTGTGC. AATTGTGC. AATTGTGC. 90 TCAACGGTG TCGACGGTG 190 AATGGGGCC AATGGGGCC	AG AG AG CACATTCTT CACATTCTT 200 TTATTCTCA TTATTCTCA
H1 Ligustrum1 W5 cuspidata1 b W1 A4 W1 A4	620 ATTTTTGTT ATTTTTGTT ATTTTTGTT ATTTTTTTT	630 ITTTTTTTTTT ITTTTTTTTTTTTTTTTTTTTTTTT	640 TTTG666 TTTT-666 TTTT-666 TTTT-666 20 20 21 21 21 22 23 23 230 230 230 230 230 2	850 TAAAAAGTJ TAAAAGGTJ TAAAAAGTJ TAAAAAGTJ TAAAAAGTJ TTAATATT O ATAGTTTTT ATAAATTTT 240 240	660 AACTATGAA AACCATGAA AACTATGAA 40 FCT6GCCGCT 140 FCTATTTTT TCTATTTTT 22 4	670 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAAT 50 TTCATAATA 150 TTTGGTTAA TTTGGTTAA 50	TGAAACAT TGAAAAAT TGAAAAAAT TGAAAAAAT TGAAAAAAT 60 TTTCCTAAA TTTCTTAAA 160 CCCATGCAT CCCATGCAT 260	680 TAGTATCTC AACTCTCCT AAGTATCTC AAGTATCTC 7/ ATTTTTTAT ATTTTTTAT 170 TCTTAAAAA 270	690 - CAAATAA - CTAATAA - AAAATAA - AAAATAA - AAAATAA - CAAAATAA - CAAAATAA - CAAAATAA - 180 - 1	700 ATTIGIGA ATTIGIGA ATTIGIGA ATTIGIGA SO ACTCATITI ACTCATITI TATCTCAA TATCTCAA 290	710 AATTGTGC. AATTGTGC. AATTGTGC. 90 TCAACGGTI 190 AATGCGGC AATGAGGC 30	AG AG AG CACATCTT CACATTCTT 200 ITATTCTCA ITATTCTCA 0
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H1 Ligustrum1 W5 cuspidata1 b W1 A4 W1 A4 W1 A4	620 ATTTTTGTT ATTTTTGGT ATTTTTGGT ATTTTTGTT ATTTTTTT GTAACTCCAG GTAACTCCAG GTAACTCCAG 110 TAATAAAAT TAATAAAAT 210 AAAAATCTAG AAAAATCTAG 310	630 1111111111 1111111111 1111111111 0 120 12	640 ITTG - 660 ITTT - 660 ITTG - 600 ITTG - 600 ITTT - 600 I	650 TAAAAAGT/ TAAAAGGT/ TAAAAAGT/ TAAAAAGT/ 30 CTTAATATI CTTAATATI 0 ATAGTTTTA TTTAATTT 240 ATTTACTT/ 340	660 AACTATGAA AACCATGAA AACTATGAA 40 ICT66CCGT ICT66CCGC 140 ICTATTTTT 	670 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAATA TTGATAATA 150 TTTGGTTAA 150 TTTGGTTAA 150 CAGTTGGTA CAGTTGGTA CAGTTGGTA 360	TGAAACAT TGAAAAAT, TGAAAAAT, TGAAAAAT, TGAAAAAT, 60 TTTCCTAA, TTTCTTAA, 160 CCATGCAT CCATGCAT CCATACAT 260 AAGACTTT AAGACTTT 37	680 TAGTATCTC AACTCTCCT AAGTATCTC AAGTATCTC 70 ATTTTTTAT ATTTTTAT 170 TCTTAAAAA 270 TATTAAGAA 270 TATTAAGAA 10	690 - CAAATAA - CTAATAA - AAAATAA CAAAATAA CAAAATAA CAAAATAA - 130 GTATCAAT - 380 GTGTTTGG - 380	700 ATTTGTGA ATTTGTGA ATTTGTGA ATTTGTGA 80 ACTCATTTT ACTCCAA TATCTCAA 290 AATTGAAA AATTGAAA 390	710 AATTGTGC. AATTGTGC. AATTGTGC. 90 TCAACGGTI TCGACGGTI 190 AATGGGGC 30 TCTAGCAAI TCTAGCAAI 400	AG AG AG CACATTCTT CACATTCTT 200 TTATTCTCA TTATTCTCA 0 CTTCTAAGA CTTCTAAGA 410
H1 Ligustrum1 W5 cuspidata1 b W1 A4 W1 A4 W1 A4 W1	620 ATTTTTGTT ATTTTGGT ATTTTTGTT ATTTTTGTT ATTTTTTT GTAACTCCAC GTAACTCCAC GTAACTCCAC 110 TAATAAAAT TAATAAAAT 210 AAAAATCTAC AAAAATCTAC 310 ATGCTAAAAA	630 TTTTTTTTTT TTTTTTTTTT 0 CCCCACCCGG 120 TATTTGATAG TATTTGATAG CAAACGTACC CAAACGTACC 320 SATGTCAGA	640 ITTG - 660 ITTT - 660 I	650 TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ 30 CTTAATAT 30 CTTAATAT 0 ATAGTTTATAA TTTACTT/ 340 GTTTATAAC	660 AACTATGAA AACCATGAA AACTATGAA 40 1CTGGCCGGT 140 1CTATTTTT 1	670 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAATA 150 TTTGATAATA 150 TTTGGTTAA 150 CAGTTGGTA CAGTTGGTA 380 TAAATAAGT	TGAAACAT TGAAAAAT TGAAAAAAT TGAAAAAAT TTTCCTAAA TTTCCTAAA 160 CCATCCAT CCATCCAT 280 AAGACTTT AAGACTTT 37	680 TAGTATCTC AACTCTCCT AAGTATCTC AAGTATCTC 7/ ATTTTTTAT ATTTTTTAT 170 TCTTAAAAA 270 TATTAAGAA 270 TATTAAGAA 70	690 - CAAATAA - CTAATAA - AAAATAA - AAAATAA - CAAAATAA - CAAAATAA - CAAAATAA - CAAAATAA - 130 -	700 ATTTGTGA ATTTGTGA ATTTGTGA ATTTGTGA 80 TACTCATTT TACTCATTT TACTCATTT ACTCATTT 280 AATTGAAA 390 TAATATAT	710 AATTGTGC. AATTGTGC. AATTGTGC. 90 TCAACGGTI 190 AATGGGGC AATGGGGC 30 TCTAGCAAI TCTAGCAAI 400 TAAGCGCT	AG AG AG CACATTCTT CACATTCTT 200 TTATTCTCA 0 CTTCTAAGA CTTCTAAGA 410 TTTAGTCGGG
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H1 Ligustrum1 W5 cuspidata1 b W1 A4 W1 A4 W1 A4 W1 A4 W1 A4	620 ATTTTTGTT ATTTTTGTT ATTTTTGTT ATTTTTTTT	630 ITTTTTTTTT ITTTTTTTTTT ITTTTTTTTTTT	640 ITTG - 660 ITTT - 660 ITTG - 600 ITTG - 600 ITTT - 600 I	650 TAAAAAGT/ TAAAAAGT/ TAAAAAGT/ 30 21 21 20 21 21 20 21 21 21 21 21 21 21 21 21 21	660 AACTATGAA AACCATGAA AACTATGAA 40 ICTGGCCGGT ICTGCCGCGC 140 ICTATTTTT CTATCGCCGC 140 ICTATTTTT 22 ATAAGTATG ATAAGTATG 350 GTTCACGAG GTTCACGAG 460 AATAATTAT	670 ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAAT ATTGATAATA TTGATAATA 150 TTTGATAATA 150 TTTGGTTAA 150 CAGTTGGTAA CAGTTGAA CAGTTGGTAA CAGTTGGTAA CAGTTGAA CAGTTGGTAA CAGTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTTGGTAA CAGTGGT	TGAAACAT TGAAAAAT. TGAAAAAAT. TGAAAAAAT. TGAAAAAAT. TGAAAAAAT. 160 CCATGCAT CCATGCAT CCATGCAT CCATGCAT 280 AAGACTTT AAGACTTT 37 AAGACTTT 37 AATTTTTA 470 ATTTTTA	680 TAGTATCTC AACTCTCCT AAGTATCTC AAGTATCTC AAGTATCTC 77 ATTTTTTT 170 TCTTAAAAA 270 TCTTAAAAAA 270 TATTAAGAA 170 TCTTAAAAAA 270 TATTAAGAA 170 TCTTAAAAAA 270 TATTAAGAA 170 TCTTAACTA 270 TATTAAGAA 170 TCTTAACTA 270 TATTAAGAA 170 TCTTAACTA 270 TATTAAGAA 170 TCTTAACTA 270 TATTAAGAA 170 TCTTAACTA 270 TATTAAGAA 170 TCTTAACTA 270	690 - CAAATAA - CTAATAA - CTAATAA - CTAATAA - CAAAATAA - CAAAATAA - CAAAATAA - CAAAATAA - CAAAATAA - 180 - 1	700 ATTTGTGA, ATTTGTGA, ATTTGTGA, ATTTGTGA, ATTGTGA, AATTGTCA, 290 AATTGAAA' AATTGAAA' 390 TAATATATT TAATATAT' TAATATAT' D TAATCACAI TAATCACAI	710 AATTGTGC. AATTGTGC. AATTGTGC. AATTGTGC. 90 TCAACGGTI 100 AATGCGGC 100 AATGCGGC 30 TCAGCAAI 100 TCAGCAAI 400 TAAGCCGTT 500 CATTTTATC CATTTTATC	AG AG AG AG CACATICIT CACATICIT 200 TIATICICA 0 CITICIAAGA CITICIAAGA 410 TITAGIGGG TITAGIAGG 510 CATITITAT
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Fig. 2. Multiple alignment of some representative *nia*-i3 sequences from different Oleaceae. (a) *nia*1 sequences from *O. e.* var. *europaea* (H1), *L. vulgaris*, *O. e.* var. *sylvestris* (W5) and *O. e.* subsp. *cuspidata* (b) *nia*2 sequences from *O. e.* var. *sylvestris* (W1) and *O. e.* var. *europaea* (A4). In (a) and (b) Identical Nucleotide bases are marked on a grey background and deletions are indicated by points. Restriction motifs of AFIII enzyme are framed in (b)



Fig. 3. Analysis on 1% agarose gel of digestion PCR products by AfIII. 1: negative control (undigested *nia*-i3 PCR product), 2: *O. e.* var. *europaea* (Arbequina), 3: *O. e.* var. *europaea* (Hojiblanca), 4 and 5: *O. e.* var. *sylvestris* population W, 6 and 7: *O. e.* var. *sylvestris* population Y, 8: *O. e.* subsp. *cerasiformis*, 9: *O. e.* subsp. *cuspidata*, 10: *O. paniculata*, 11: *L. vulgaris*

Table 2. Intra-taxon variability and inter-taxa divergence for *nia*1 intron sequences. Numbers in boldface (diagonal) correspond to intra-taxon sequence diversity. Values below diagonal correspond to inter-taxa sequence divergence

	O. e. europaea	O. e. sylvestris	O. e. cerasiformis	O. e. cuspidata	O. paniculata
O. e. europaea	0.021				
O. e. sylvestris	0.022	0.020			
O. e. cerasiformis	0.018	0.020	0.012		
O. e. cuspidata	0.039	0.044	0.036	0.015	
O. paniculata	0.057	0.060	0.047	0.053	0.006

O. paniculata being an outgroup species related to the *O. europaea* complex. In fact, we have tried to analyse the phylogenetic relationships between *Olea* taxa. The phylogenetic analysis was performed using only *nia*1 intron sequences because of the absence of phylogenetic signal within the exon 3 and exon 4 flanking regions. For this purpose, we used a multiple sequence alignment with 12 wild and cultivated olive *nia*1 sequence variants found. Additionally, we used each of two representative sequences found in *O. europaea* subsp. *cerasiformis*, *O. europaea* subsp. *cuspidata* and *O. paniculata*. The alignment dataset contained 670 characters,

68 of which were parsimony informative. Neighbour-joining (Tamura distance), MP strict consensus (CI=0.82 [0.95]; RI=0. 81 [0. 94]; 123 [91] steps; the six most parsimonious tree; in brackets, values when characters were weighted by maximum value of rescaled consistency indices) and maximumlikelihood (K81uf+G model of DNA substitution) trees of *nia*1 sequences reflected the same topology (Fig. 4). The 18 representative sequences included in this analysis are clustered in four groups. Group I and group II include sequences of *O. paniculata* and *O. europaea* subsp. *cuspidata*, respectively. Group III is divided in two sub-groups, both



Fig. 4. Maximum-likelihood tree for *nia*1 sequences. Bootstrap confidence levels (1000 replicates) are given next to all nodes

including sequences of wild and cultivated olives. Finally, group IV contains the sequences of *O. europaea* subsp. *cerasiformis* and some sequences from cultivated and wild olives.

Discussion

Intron sequences provide a potentially valuable method for establishing high-resolution phylogenetic analysis within closely related species and strains. However, they remain almost unexploited in phylogeny studies because of a frequent dispute regarding their origins (Hurst 1994, Long et al. 1995). Nevertheless, introns from glyceraldehydes-3phosphate dehydrogenase (G3PDH) offered for example good genetic information at very low taxonomic levels (Olsen and Schaal 1999). In this paper, we try to establish the genetic relationships of olives among various Oleaceae using a nuclear intron from a functional gene, nitrate reductase (*nia*). This gene involved in the reduction of nitrate to nitrite has been isolated from algae, fungi and various plants (Zhou and Kleinhofs 1996) and sequenced in many species of disparate genera (Petunia Sensu Jussieu, Lotus L., Cichorium L., Pistacia L., Solanum L.). In the majority of higher plants, three introns have been characterized in this gene in highly conserved positions (Slanoubat and Ha 1993). However, the reported gene in question shows high polymorphism, particularly in its copy numbers and introns sequences (Zhou et al. 1994, 1996; Wu et al. 1995; Howarth and Baum 2002, 2005). In fact, NIA genes are present in more than one copy in some species (Zhou et al. 1994, 1996; Howarth and Baum 2002) and exhibit different numbers and lengths of introns (Zhou et al. 1995).

Our results evidenced that in the Oleaceae in general and in particular in wild and cultivated olive the intron number 3 of the nitrate reductase gene (*nia*-i3) is very variable. First of all, sequence analysis of the 250-bp product

detected by PCR amplification of *nia* in all the Oleaceae suggested that it corresponds possibly to a nonfunctional copy of the gene with a much reduced intron 3. Howarth and Baum (2002) reported similar results in *Scaevola* L. (Goodeniaceae).

However, secondly and more importantly, our analyses revealed the presence of two different variants of nia intron3 (nia1 and nia2) in both the wild and the cultivated olive (Fig. 2). However, only one variant (nia1) was detected in the other taxa of Olea (O. europaea subsp. cerasiformis, O. europaea subsp. cuspidata, O. paniculata) and in Ligustrum. Whereas nial and nia2 seemed to be functional, nia2 differed from nia1 in size and sequence. The presence of nia2 variant in wild and cultivated olive appears to be the consequence of duplication of this gene after divergence of these two taxa from the other Oleaceae. In fact, these two variants are detected all together in all the wild and cultivated olive specimens not only by cloning and sequencing but also by PCR-RFLP analysis (Fig. 3). These results are consistent with other studies, which have demonstrated duplication of the *nia* gene in the diploid and in the hexaploid wheat as compared with other related species (barley) (Zhou et al. 1994). Furthermore, the duplication of nia gene in wild and in cultivated olives cannot be attributed to polyploidy because the number of chromosomes is the same among the Oleaceae examined (2n =46) (Green 2002).

These results have implications from two standpoints. First of all, the presence after amplification, cloning, and sequencing of two variants of *nia*-i3 (*nia*1 and *nia*2) in wild and cultivated olives and its absence in the remaining Oleaceae is a potentially valuable test in order to differentiate between these taxa. The confirmation of this observation after PCR-RFLP in turn confirms the validity of this test because the *nia*2 variant has a target for AfIII enzyme absent in *nia*1 variant.

On the other hand, this last observation and those derived from the tree based on *nia*1 sequences gives useful information concerning the phylogenetic relationships in the genus *Olea*. First of all, the fact that, in all the Oleaceae, only the wild and cultivated olives have the two variants of *nia*-i3, possibly due to a duplication phenomenon of the gene, indicates the close genetic relationship between the two, validating their treatment as varieties of the same subspecies. With respect to the phylogeny based on the *nia*1 variant (Fig. 4), wild and cultivated olive sequences shared the same groups (clades) supported by high bootstrap values. This again constitutes new evidence on their close genetic relationship.

With respect to the origin of cultivated olives, different authors have suggested a multilocal selection of cultivated olive from wild cross-bred genotypes (Besnard et al. 2001, Rotondi et al. 2003), whereas others, with arguments based on the relevant genetic distance between the wild and the cultivated olive, have contended that large fractions of local cultivars could have an allochthonous origin (Angiolillo et al. 1999). Our phylogenetic analysis reveals that the *nia*1 sequences of the cultivated olives are paraphyletic (Fig. 4), which suggests that there is still gene flow between the wild olive and olive cultivars. This data is in accordance with another study, which has suggested using AFLP markers that some cultivated olives could have originated by domestication of local wild trees (Baldoni et al. 2006).

Finally, the presence of *O. europaea* subsp. *cerasiformis* (endemic olive of Canary Islands) sequences in the same phylogenetic group as some sequences of wild and cultivated olives and their similar sequence divergence (Table 2), could be in accordance with the studies of Lumaret et al. (2000), who found that wild, cultivated olive and *O. europaea* subsp. *cerasiformis* share a similar chlorotype and may have a common origin. Also, the presence of the sequences of *O. europaea* subsp. *cuspidata* and *O. paniculata* in separate clades (Fig. 4) confirmed the molecular analyses (Lumaret et al. 2000, Rallo et al. 2003)

considering these taxa to be differentiated with respect to the wild and the cultivated olive, inside and outside the *Olea europaea* complex, respectively.

In summary, the analysis of the *nia*-i3 in Oleaceae provides an unexpected vision of the direct relationships between wild and cultivated olives (they are the only Oleaceae with two functional variants for this marker, possibly due to the existence of two different genes only in these two taxa) and constitutes a potential method to differentiate the two (not only by sequencing but also by PCR-RFLP) from all the remaining taxa. Finally, the presence of two different loci for *nia* in the olive crop opens the possibility for further research regarding for its functional advantage in the nitrogen assimilation metabolism.

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