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

A. M. Hamman-Khalifa[1], R. Navajas-Pérez[1], R. de la Herrán[1], M. Ruiz Rejón[1], M. A. Garrido-Ramos[1], C. Ruiz Rejón[1], J. L. Rosúa[2]

[1]Departamento de Genética, Facultad de Ciencias, Universidad de Granada, Spain
[2]Departamento de Botánica, Facultad de Ciencias, Universidad de Granada, Spain

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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 (*nia*1 and *nia*2) in the Oleaceae, in addition to a shorter non-functional one. Notably, while the shortest and the *nia*1 variants were present in all the taxa analysed, the *nia*2 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 AflII enzyme in *nia*2 and its absence in *nia*1 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

Correspondence: Carmelo Ruiz Rejón, Departamento de Genética, Facultad de Ciencias, Universidad de Granada, 18071, Spain
e-mail: carmelo@ugr.es

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 south-eastern 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

Table 1. Oleaceae taxa employed and their provenance

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) W5, W6, W7, **W8** (EF113359-EF113362)
Olea europaea var. *sylvestris* (Y[b])	Alcalá de los Gazules, Cádiz, Spain	2	**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™/EMBL accession numbers of *nia*-i3 sequences obtained, [b]W and Y correspond to two different populations of *O. europaea* var. *sylvestris* from Medina Sidonia (Cádiz) and Alcalá de los Gazules (Cádiz), respectively, [c]DNA bank voucher. *nia2* 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 intron-crossing (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 3-exon 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 µl using FastStart high-fidelity PCR system (Roche diagnostics), with a final concentration of 2.5 mM $MgCl_2$, 0.1 mM dNTPs and 0.5 µM 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 GFX™ 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 GenBank™/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).

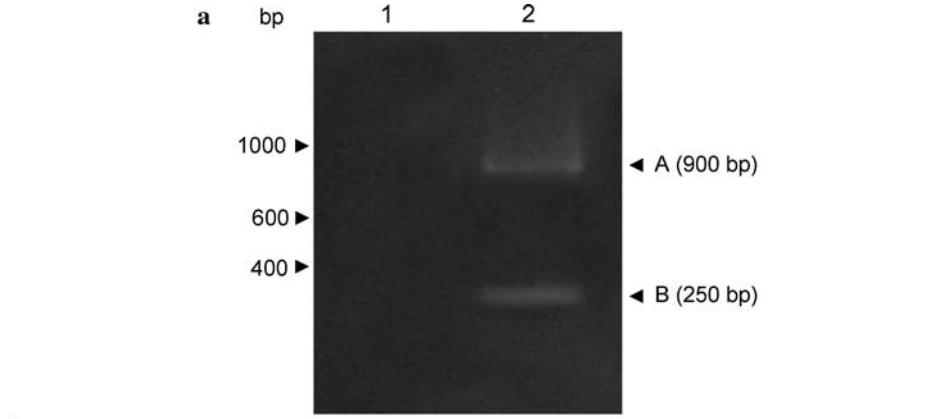
PCR-RFLP analysis. In order to demonstrate the presence/absence of the *nia*2 variant of the third intron of the nitrate reductase (*nia*) in the Oleaceae, gel purified PCR products of *nia* of all the taxa studied were digested by the restriction enzyme AflII (Roche diagnostics), which digests in C/TTAAG, 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 tabacum* L. (GenBank™/EMBL, access number X14058) (Vaucheret et al. 1989), 87% with *Petunia hybrida* Vilm. (GenBank™/EMBL, access number L13691) (Slanoubat and Ha 1993), 83% with *Solanum lycopersicum* L. (GenBank™/EMBL, access number X14060) (Daniel-Vedele et al. 1989) and 82% with *Phaseolus vulgaris* L. (GenBank™/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 *nia*1 and *nia*2 (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 *nia*1 variant. However, the other variant, *nia*2, was detected only in wild and cultivated olives sequences. The intron-size estimation showed that *nia*2 is about 50 to 70 bp larger than *nia*1. Nonetheless, the main outstanding character in *nia*2 concerns the exclusive presence of the CTTAAG motif at the 3′ end of all the *nia*2 variant sequences cloned (Fig. 2). It bears noting that this last motif corresponds to the restriction site of AflII enzyme (see above).

To confirm the exclusive presence of the *nia*2 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 AflII restriction enzyme. Figure 3 illustrates the result of digestion by AflII 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 *nia*1 variant and the two other bands of 600 and 300 bp, respectively, to the restriction of the *nia*2 form.



b

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  1 AATTACTGGTGTTGGTGTTTTTGGTCACTCGAGGCGGAGGTGTTGGACTTACTTGGTGCC

 61 AAAGAAATTTGTGTTCGAGCTTGGGATGAGACTCTCAACACACAACCCGAGAAGCTCATT

121 TGGAATGTCATGGTAAGAGTCAAGGATGAGTAGATCTAAATTTTTAGGCGAGGTACCGAT

181 AATTTGTTGACTAATTATTATTGTGTTATAATTATTCAGGGAATGATGAACAACTGCTGG

241 TTCCGA
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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 *nia*2 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

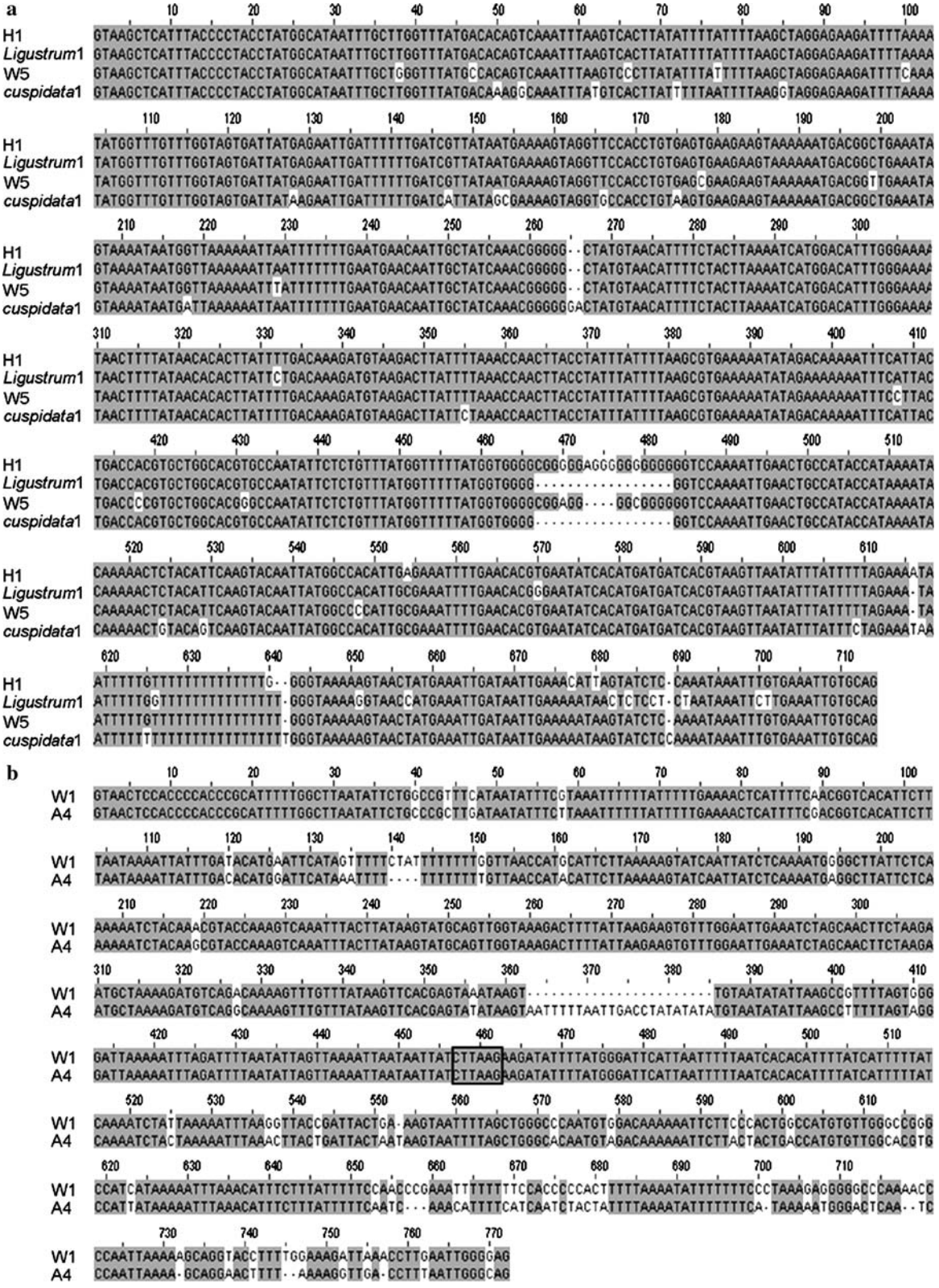
a
H1
Ligustrum1
W5
cuspidata1
b
W1
A4

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 AFlII enzyme are framed in (b)

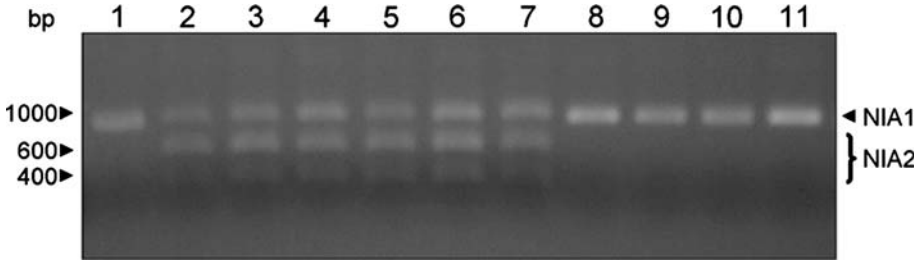

Fig. 3. Analysis on 1% agarose gel of digestion PCR products by AflII. 1: negative control (undigested *nia*-i3 PCR product), 2: *O. e.* var. *europaea* (Arbequina), 3: *O. e.* var. *europaea* (Hojiblanca), 4 and 5: *O. e.* var. *sylvestris* population W, 6 and 7: *O. e.* var. *sylvestris* population 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 maximum-likelihood (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

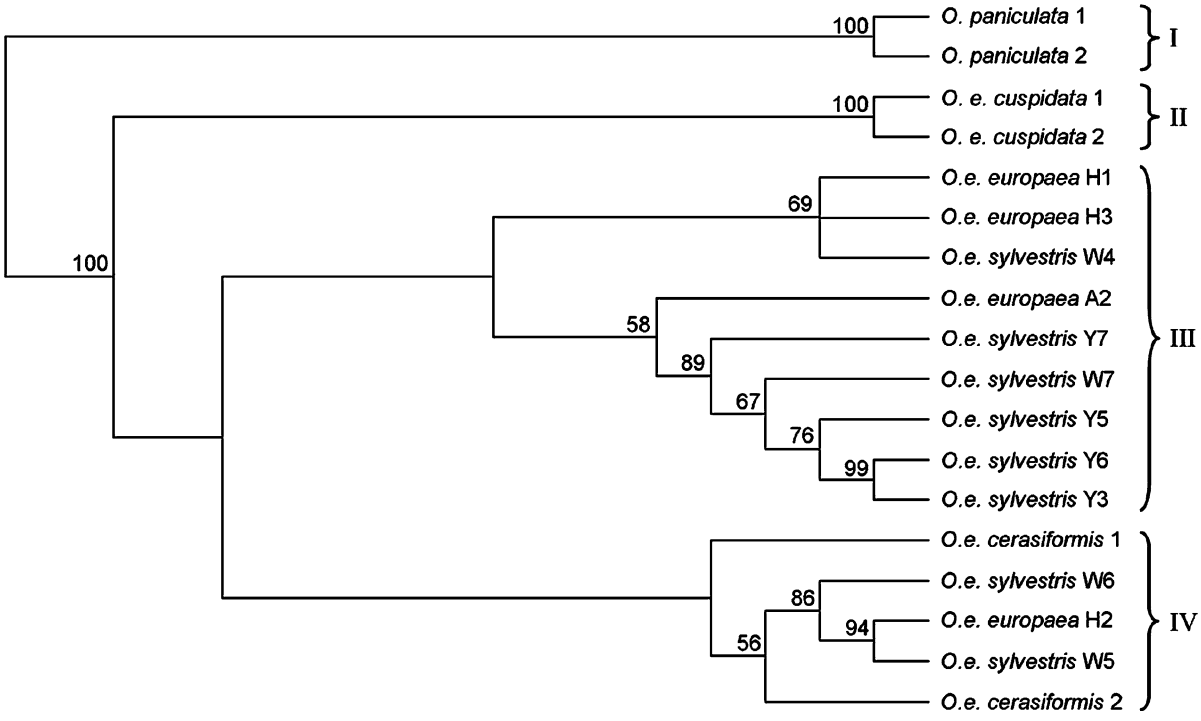

Fig. 4. Maximum-likelihood tree for *nia*1 sequences. Bootstrap confidence levels (1000 replicates) are given next to all nodes

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-3-phosphate 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 (*nia*1 and *nia*2) in both the wild and the cultivated olive (Fig. 2). However, only one variant (*nia*1) was detected in the other taxa of *Olea* (*O. europaea* subsp. *cerasiformis*, *O. europaea* subsp. *cuspidata*, *O. paniculata*) and in *Ligustrum*. Whereas *nia*1 and *nia*2 seemed to be functional, *nia*2 differed from *nia*1 in size and sequence. The presence of *nia*2 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 AflII 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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