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On chloroplast DNA variations in the olive (*Olea europaea* L.) complex: comparison of RFLP and PCR polymorphisms

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Abstract Previous papers have dealt with olive chloroplastic DNA (cpDNA) variation revealed using several methods (RFLPs, PCR-RFLPs and microsatellites) and have led to different conclusions. This paper aims to reconsider these divergences. A Southern approach was applied to reveal polymorphism. We used chloroplast DNA of *Phillyrea media* as a probe. Based on these data, only four chlorotypes were identified in the olive complex. The number of detected lineages was lower than reported in the literature using a direct cpDNA RFLP approach, and was insufficient to distinguish the North African subspecies *europaea*, *maroccana*, *guanchica* and *laperrinei*. Furthermore, one individual considered belonging to the subspecies *laperrinei* was questionable. Using other cpDNA and mitochondrial DNA (mtDNA) polymorphisms – based on PCR and RFLP methods, respectively – we showed that this individual displays the cytoplasmic lineage CE1-ME1 characteristic of most Eastern mediterranean cultivars and of *Olea europaea* subsp. *laperrinei* from Hoggar. However, based on RAPDs, this individual appeared as mislabelled and probably corresponded to a Mediterranean cultivar or a feral form. In addition, we checked *O. e.* subsp. *laperrinei* herbarium samples using two cpDNA microsatellites, which revealed polymorphisms. These also supported that both populations from Niger and Algeria displayed a chlorotype related to CE1. Consequently, based on cpDNA, the relationships of *O. e.* subsp. *laperrinei* from Hoggar with a Mediterranean lineage appeared well supported, whereas the South West Moroccan and Macaronesian olives appeared in a different clade using both mtDNA and cpDNA polymorphisms. We conclude that methods based on PCR reveal more polymorphisms in the cpDNA and lead to more-reliable results that the classical RFLP method.

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Introduction

Recently, several molecular studies have dealt with the cytoplasmic DNA variation in the olive (*Olea europaea* L.) complex using different methods to reveal DNA polymorphisms (Amane et al. 1999; Besnard and Bervillé 2000; Besnard et al. 2002b; Lumaret et al. 2000). PCR-RFLPs in chloroplast DNA (cpDNA) spacer fragments, cpDNA microsatellites, and classical RFLPs led to contradictions. We therefore reconsidered all the available data and we propose a new interpretation to reconcile the results.

First, we have shown that *O. e.* subsp. *laperrinei* from Hoggar and some Mediterranean olives shared the same cytoplasmic DNAs (both chloroplast and mitochondrial) since they both displayed the cytoplasmic lineage CE1-ME1 (Besnard and Bervillé 2000; Besnard et al. 2002b). This conclusion was strongly supported based on PCR-RFLPs, microsatellite polymorphisms, and RFLPs of cpDNA and on mitochondrial DNA (mtDNA) RFLPs. Moreover, 21 trees belonging to *O. e.* subsp. *laperrinei* from two sites have been characterised. In contrast, Lumaret et al. (2000) have reported that *O. e.* subsp. *laperrinei* was related to taxa from the Canary Islands (*O. e.* subsp. *guanchica*) and from SW Morocco (*O. e.* subsp. *maroccana*). This conclusion was based on only one tree (here called "*O. laperrinei* La Taessa") and one RFLP marker. Furthermore, due to the difficulties to sample *O. e.* subsp. *laperrinei*, the tree called "*O. laperrinei* La Taessa" was sampled in the garden of Algiers University and there is a doubt dealing with its taxonomic position.

Second, the structure of the Mediterranean cytoplasmic DNA diversity was also shown to be very different according to Amane et al. (1999) and to Besnard et al. (2002b). According to Amane et al. (1999, 2000), five chlorotypes were distinguished in oleaster (wild olive), of which two were only present in Morocco, one was detected in the Balearic Islands, another was present in

Western Mediterranean, and one preponderant chlorotype was found in all Mediterranean areas. In contrast, we have shown a strong genetic structure of the diversity in oleaster between the East and West Mediterranean (Besnard et al. 2002b). Thus, five cytoplasmic lineages characterising the subspecies *europaea* were found using both mtDNA and cpDNA polymorphisms. A great disequilibrium linkage between polymorphisms of the two organelle DNAs has shown the deep pertinence of these results (Besnard et al. 2002c). Furthermore, cultivar dissemination from the East Mediterranean has been supported by these data.

Consequently, it is necessary to point out the source of these disagreements between both sampling and technical problems, and to point out the pertinence of these conclusions. The aim of this paper is to answer to this point. Here, we report results about the cpDNA variation in the *O. europaea* complex revealed using different methods: microsatellites, PCR-RFLPs and RFLP markers. Herbarium analyses from various origins using microsatellite markers are also reported. Lastly, using RAPD markers, we checked the labelling of doubtful samples.

Materials and methods

Vegetal material

Thirty one individuals belonging to five *O. europaea* subspecies were characterised (Table 1). We followed the classification proposed by Green and Wickens (1989) and Vargas et al. (2001). The individual "*O. laperrinei* La Taessa" was provided by Dr. M. Dahmani (URBT, Alger). This tree is cultivated in the garden of the Algiers University.

We analysed 15 herbarium samples of which seven belong to the subspecies *laperrinei* (Table 2). These samples were mostly

Table 1 List of the studied individuals, their origin and their chlorotypes based on RFLPs, and according to the data described in Besnard et al. (2002c). IRO Perugia = "Institute for Olive Research", Perugia, Italy; OGB Cordoba = "Olive Germplasm Bank", Cordoba, Spain; INRAM = "Intstitut National de Recherche Agronomique, Montpellier, France"

Taxa	Individual	Prospected localities or collections	Cytoplasmic lineage code	
			CpDNA RFLP	Besnard et al. 2002c
O. europaea L. subsp. *europaea* var. *europaea*	'Chemlal'[a]	OGB Cordoba (from Algeria)	CCK	CCK-MCK
	'Chetoui'[a]	OGB Cordoba (from Tunisia)	CNC	CE2-ME2
	'Lechin de Sevilla'[a]	OGB Cordoba (from Spain)	CNC	COM1-MOM
	'Moraiolo'	IRO Perugia (from Italy)	CNC	CE1-ME1
	'Sabina' (clone no. 2)	Corsica, France	CNC	COM1-MOM
	'San Felice'[a]	IRO Perugia (from Italy)	CNC	CE1-ME1
	'Sevillenca'[a]	OGB Cordoba (from Spain)	CNC	CE1-ME1
O. e. subsp. *europaea* var. *sylvestris* (Mill.) Lehr.	Urla no. 1	Urla, Izmir, Turkey (INRAM)	CNC	CE1-ME1
	Al Ascharinah no. 2	Al Ascharinah, El Ghab, Syria (INRAM)	CNC	CE1-ME1
	Mont Carmel no. 11	Mont Carmel, Haifa, Israel	CNC	CE1-ME1
	Kabylie no. 7	Tizi Ouzou, Kabylie, Algeria (INRAM)	CCK	CCK-MCK
	Moulay Idriss no. 5	Moulay Idriss, Morocco	CNC	CE1-ME1
	Torviczon no. 1	Torviczon, Sierra Nevada, Spain	CNC	COM1-MOM
	La Repentence no. 2	La Repentence, Porquerolles, France (INRAM)	CNC	COM1-MOM
O. e. subsp. *laperrinei* (Batt. & Trab.) Ciferri	La Source no. 1	La Source, Hoggar, Algeria (INRAM)	CNC	CE1-ME1
	O. laperrinei La Taessa[a]	Algiers University	CNC	CE1-ME1
O. e. subsp. *guanchica* Vargas et al.	La Palma no. 2	La Palma, The Canary Islands, Spain	CNC	CCE2-MCE
O. e. subsp. *maroccana* (Greut. & Burd.) Vargas et al.	Mentaga no. 3	Mentaga, High-Atlas, Morocco	CNC	CCE2-MMA
	Immouzzer no. 1	Immouzzer, High-Atlas, Morocco (INRAM)	CNC	CCE2-MMA
O. e. subsp. *cuspidata* (Wall. ex G. Don) Ciferri				
O. cuspidata Wall. ex G. Don	Kerman no. 1	Kerman, Iran (INRAM)	CC	CC1-MCIR
	Kerman no. 2	Kerman, Iran (INRAM)	CC	CC1-MCIR
	Kerman no. 5	Kerman, Iran (INRAM)	CC	CC1-MCIR
	India no. 1[a]	IRO Perugia (collected in India)	CC	CC1-MCIN
	China no. 1[a]	IRO Perugia (collected in China)	CC	CC1-MCC
O. chrysophylla Lam.	Almihwit no. 2	Almihwit, Yemen	CC	CC2-MCIR
	Almihwit no. 4	Almihwit, Yemen	CC	CC2-MCIR
O. africana Mill.	Nairobi no. 5	Nairobi collection, Kenya	CA	CA1-MAK
	Mt Elgon no. 5	Elgon Mount, Kenya (INRAM)	CA	CA2-MAK
	Timau no. 4	Timau, Kenya, Mount Kenya (INRAM)	CA	CA1-MAK
	Kirstenbosch no. 1	Kirstenbosch, Cape Town, South Africa	CA	CA1-MAS
	Reunion no. 8	Sentier de la Providence, Reunion, France (INRAM)	CA	CA5-MAR

[a]Individuals also characterised by Amane et al. (1999) or Lumaret et al. (2000)

Table 2 List of the studied herbarium samples with an indication of their geographic origin and their cpDNA-microsatellite characters

Taxa	Individual reference (date of collection)	Locality of collection	Herbarium origin	Microsatellite data[a]	
				ccmp5	ccmp7
O. e. subsp. *laperrinei*	R. Maire 779 (18/03/1928)	Atakor-n-Ahaggar, Hoggar, Algeria	Montpellier Botanical Institute	116	129
	J. Lauriol (30/01/1934)	Addra Haggerane, Hoggar, Algeria	Montpellier Botanical Institute	116	129
	P. Quézel (03/11/1956)	Teffedest in Acoulmou, Hoggar, Algeria	Montpellier Botanical Institute	116	129
	Amirouch (00/00/1990)	Teffedest, Hoggar, Algeria	Sample provided by Dr Amirouch	116	129
	D. Hammer 691 (09/11/1989)	Imi'n Farah, Air, Niger	Montpellier Botanical Institute	116	129
	B. de Miré (00/00/1960)	Jebel Marra' crater, Sudan Republic	Montpellier Botanical Institute	118	129
	Jackson and Ramsay 3 (14/01/1953)	Jebel Marra, Sudan Republic	Royal Kew Gardens	118	129
O. e. subsp. *europaea*	A. Dubuis (04/05/1986)	Bordj Cha, Algeria	Montpellier Botanical Institute	115	130
	A. Faure (19/05/1934)	Santa Cruz, Algeria	Montpellier Botanical Institute	116	129
	R. Maire (26/04/1927)	Ben Zireg, Algeria	Montpellier Botanical Institute	116	129
O. e. subsp. *cuspidata*	Collenete 281 (07/03/1977)	Jebel Ibrahim, Saudi Arabia	Royal Kew Gardens	114	128
	A. Radcliffe-Smith 4035 (26/03/1976)	Jebel Akhdar, Oman	Royal Kew Gardens	116	128
	K.H. Rechinger 29512 (15/05/1965)	Quetta, Baluchistan, Iran	Royal Kew Gardens	116	128
	Poldech 16769 (10/09/1969)	Barikot, Kunar, Afghanistan	Royal Kew Gardens	116	128
	Dieterlen 313 (00/00/1903)	Lesotho	Montpellier Botanical Institute	116	129

[a] Size of the fragment is given in bp

provided by the herbariums of Kew Botanical Garden and the Montpellier Botanical Institute (MPU).

Molecular characterisation of fresh material

Total DNAs were prepared according to the method described by Besnard et al. (2000). Living collection and herbarium sample consultations are available at the request of the authors.

CpDNA RFLPs were revealed using blot transfers according to a method described by Forcioli et al. (1994). DNAs were restricted using the restriction enzymes *Hind*III, *Xba*I, *Eco*RI, *Bam*HI, *Cla*I and *Xho*I. Gel-electrophoresis and transfer procedures have been previously described by Besnard et al. (2000). To reveal cpDNA fragments, we used the total cpDNA of *Phillyrea media* L. (provided by P. Saumitou-Laprade, Lille University) as a probe. The genus *Phillyrea* belongs to the family Oleaceae and to the tribe *Oleeae*, and it is therefore closely related to the genus *Olea* (Wallander and Albert 2000; Besnard et al. 2002a). We have previously shown that this method enabled us to reveal polymorphism in the chloroplast genome (Besnard et al. 2000). We verified the specificity of polymorphisms no. 10, 14, 17, 19 and C described in Lumaret et al. (2000) which have been detected using the restriction enzymes *Bam*HI and *Hind*III. Moreover, all the trees were also characterised for cytoplasmic DNA polymorphisms, which have been described in Besnard et al. (2002b and c).

In addition, the individual called "*O. laperrinei* La Taessa" from Algiers was also genotyped with eight decamers (bioprobes: A1, A2, A9, A10, C9, C15, E15, O8) to check its RAPD profile. This profile was compared to those obtained previously for the other trees (Besnard et al. 2001a). Based on RAPD markers, we computed Jaccard distances (Jaccard 1908) between each pair of individuals, and then constructed a phenetic tree of individuals using the UPGMA algorithm (Benzécri 1973).

DNA extraction and PCR-characterisation of herbarium samples

Approximately 100 to 200 mg of leaves of a herbarium sample were ground in liquid nitrogen. The powder was mixed in 2 ml of 2 × CTAB (100 mM Tris-HCI pH8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB) solution containing 30 mg of sodium disulphite. This mix was incubated at 65 °C for 30 min. Then, 1 ml of chloroform/isoamylic alcohol (24/1) was added and mixed during 15 min. This mix was centrifuged at 4,500 rpm for 20 min. The supernatant was recovered and mixed with 2 ml of isopropanol and stored at –20 °C overnight. Then, it was centrifuged at 4,500 rpm for 30 min. The pellet was recovered in 100 µL of 1×TE. This solution was purified using a PCR-prep column (Promega) using the provider's recommendations. The DNA fragments were finally recovered in 50 µL of 1×TE. The DNA solutions from the herbarium were used directly for PCR amplification.

To characterise each herbarium DNA, we used two primer pairs to specifically amplify short chloroplast fragments with microsatellite motifs ccmp5 and ccmp7, according to the PCR procedure described by Weising and Gardner (1999). PCR products were separated on a 6% PAGE gel at 60 W for 1 h 30 min. These microsatellite markers have shown polymorphism in the *O. europaea* complex (Besnard et al. 2002b), enabling us to distinguish *O. e.* subsp. *laperrinei* from *O. e.* subsp. *maroccana* and *O. e.* subsp. *guanchica*.

Results

CpDNA polymorphisms revealed using RFLPs

Only six variants were detected in the *O. europaea* complex (Table 3, Fig. 1). These enabled us to recognise four chlorotypes (Table 1): the RFLP chlorotype of male-fertile (normal) cultivars, CNC (Mediterranean, Hoggar, SW Morocco, The Canary Islands), CCK (West Mediterranean), the RFLP chlorotype of *O. africana*, CA, and the CC RFLP chlorotype of *O. cuspidata* and *O. chrysophylla* from Arabia. The whole of these polymorphisms has been briefly referenced in Besnard and Bervillé

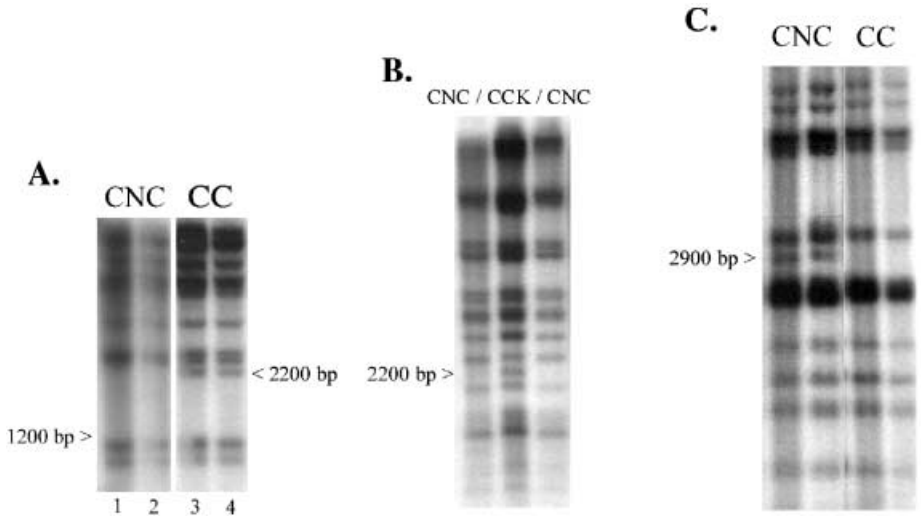

Fig. 1A–C Examples of cpDNA RFLPs revealed with the *Phillyrea* cpDNA as a probe and using different restriction enzymes: **A** using *Hind*III; **B** using *Xba*I; **C** using *Bam*HI. For the *Hind*III pattern, the individual codes are as follows: *1* = *O. e.* subsp. *guanchica* La Palma no. 2; *2* = *O. e.* subsp. *maroccana* Mentaga no. 3; *3* = *O. chrysophylla* Yemen no. 4; *4* = *O. cuspidata* Iran no. 1. The approximate size of the polymorphic fragments is indicated

Table 3 Chloroplast polymorphisms revealed in the *O.europaea* complex using RFLP markers. The polymorphism names are coded by the restriction enzyme employed and the approximate size in bp of the polymorphic fragment

Chlorotype	*Hind*III-2,200[a]	*Xba*I-2,200	*Bam*HI-800	*Bam*HI-2,900[b]	*Eco*RI-1,200	*Eco*RI-3,300/3,600[c]
CNC	0 [d]	0	1	1	1	0/1
CCK	0	1	0	1	1	1/0
CC	1	0	1	0	0	0/1
CA	0	0	1	1	0	0/1

[a] Equivalent to polymorphism no. 14 reported by Lumaret et al. (2000)
[b] Equivalent to polymorphism no. 10 reported by Lumaret et al. (2000)
[c] Equivalent to polymorphism A reported by Amane et al. (1999)
[d] 0: absence of fragment; 1: presence of fragment

(2000), but has not been published. This level of polymorphism in the *O. europaea* complex is lower than reported by Lumaret et al. (2000) using the same restriction enzymes. This is also lower than reported by Besnard et al. (2002b, c) using mtDNA RFLPs or cpDNA PCR polymorphisms (Table 1).

Forcioli et al. (1994) have shown a high congruence between RFLP patterns using Southern or direct cpDNA restriction. We can assume that such congruence should be obtained by us. In this way, the *Eco*RI pattern was identical using the two methods (Fig. 1; Amane et al. 1999). However, in our study, the polymorphisms named no. 17, 19 and C (Lumaret et al. 2000) were not detected, whereas polymorphisms no. 10 and 14, plus the CCK specific polymorphisms described in Amane et al. (1999), were found again. The CCK chlorotype is a well-distinguished lineage and this is probably due to the presence of an indel of about 300 bp. Furthermore, the non-detection of several characters described in Lumaret et al. (2000) is surprising because our electrophoresis conditions should enable us to distinguish shifts of about 60 to 100 bp on fragments with an approximate size of 1,000 bp. Thus, *O. e.* subsp. *maroccana* and *O. e.* subsp. *guanchica* were not distinguished from other samples based on the fragment of about 1,200 bp revealed using the restriction enzyme *Hind*III (Fig. 1A), although this fragment has been considered as diagnostic to distinguish about 20 accessions from North-Western Africa according to Lumaret et al. (2000).

Position of "*O. laperrinei* La Taessa"

The characterisation of "*O. laperrinei* La Taessa" using all cpDNA PCR-RFLPs and mtDNA RFLPs described in Besnard et al. (2002b, c), led us to conclude that this individual displayed the cytoplasmic lineage of Eastern Mediterranean olives and *O. e.* subspecies *laperrinei* from Hoggar, CE1-ME1 (data not shown). Consequently, this individual displayed a cytoplasmic lineage characteristic of Eastern Mediterranean olives and of *O. e.* subsp. *laperrinei* from Hoggar (Besnard et al. 2002c).

Based on RAPDs, "*O. laperrinei* La Taessa" displayed markers, which are all found again in Mediterranean individuals (data in Besnard 1999: individual no. 555). The phenetic dendrogram showed that it was not related to Saharan, SW Moroccan and Macaronesian wild olives (Fig. 2). In contrast, "*O. laperrinei* La Taessa" was related to cultivars and Mediterranean oleasters. This provided strong evidenced that this individual has been mislabelled and was probably derived from the subspecies *europaea*.

CpDNA-characterisation of herbarium samples

Table 2 reports results of the characterisation of herbarium samples. It appeared that all samples from Hoggar (Algeria) and from Air (Niger) displayed the microsatellite polymorphisms ccmp5-116 and ccmp7-129.

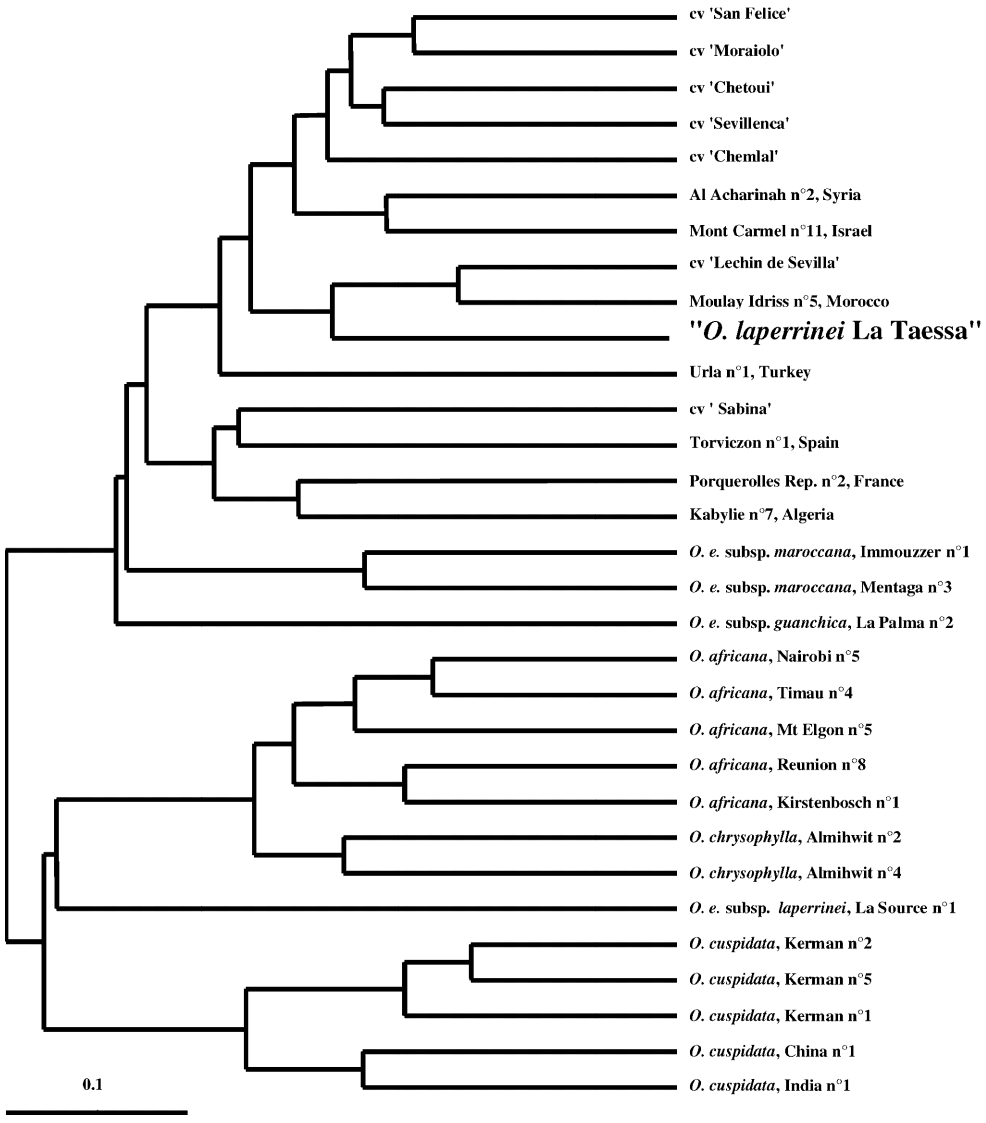

Fig. 2 Phenetic dendrogram based on 75 RAPD markers showing relationships between the studied individuals. This dendrogram was based on Jaccard distances (Jaccard 1908) and was constructed using a UPGMA algorithm (Benzécri 1973). *cv* = cultivar

Based on these polymorphisms, these individuals displayed a chlorotype related to CE1 (see Besnard et al. 2002b), but not to chlorotypes characteristic of *O. e.* subsp. *maroccana* and *O. e.* subsp. *guanchica* [CCE1, CCE2, or CCE3: see Besnard et al. 2002b]. In contrast, the two individuals from Jebel Marra (Sudan Republic) displayed the microsatellite characters ccmp5-118 and ccmp7-129. Based on these polymorphisms, these individuals displayed a chlorotype related to CCE2 (characteristic of the subspecies *maroccana* and *guanchica*: Besnard et al. 2002b). In addition, the five other individuals belonging either to subspecies *europaea* or *cuspidata* displayed polymorphisms which were characteristic of their region of origin, Mediterranean and Southern Africa or Southern Asia, respectively (see Besnard et al. 2002b).

Discussion

In this study, we verified the correspondence of the polymorphisms described by different authors. We pointed out the sources of the disagreements dealing with both tree identification and methods to reveal polymorphisms.

Technical aspect

Congruence between the RFLP pattern using Southern blotting or direct cpDNA restriction has been demonstrated in beet by Forcioli et al. (1994). However, using our RFLP approach, we did not confirm some of the polymorphisms (no. 17, 19, C) described by Lumaret et al. (2000). This indicates that our RFLP data did not support the distinction of *O. e.* subsp. *maroccana* from Mediterranean olives as described by Lumaret et al. (2000). In contrast, cpDNA PCR-RFLPs and microsatellites have enabled us to clearly distinguish them

(Besnard et al. 2002b). Furthermore, the polymorphism found in the Moroccan olive (Amane et al. 1999, 2000) was not confirmed by the general phylogeographic structure found in the Mediterranean Basin (Besnard and Bervillé 2000; Besnard et al. 2002b). In Amane et al. (1999), the polymorphisms enabling us to distinguish specific Moroccan chlorotypes corresponded to shifts of approximately 20 bp on fragments of about 1,000 to 3,000 bp. The separation of such fragments on a 1% agarose gel is questionable, and we considered that these polymorphisms are not detectable under our conditions. Moreover, all polymorphisms on the drawing patterns presented by Amane et al. (2000) concerned faint bands and enquired about their robustness. Heterogeneous DNA quality between samples could deeply influence the interpretation of the gel. Consequently, we suggest that technical problems were responsible for the previously enumerated disagreements. Moreover using PCR-RFLPs, Besnard et al. (2002b) have detected a deletion of about 300 bp in the *trn*K2–*trn*Qr cpDNA region. This deletion was specific of olive taxa from Arabia to China (clade C). Surprisingly, this has not been detected by Lumaret et al. (2000) using RFLPs. This may mean that polymorphisms revealed using the RFLP method cannot be attributed to a specific region unless a specific probe is used. We suppose that the polymorphisms no. 2, 14 and 2(7) (Lumaret et al. 2000) reflect such a difficulty and may correspond to this deletion. Phylogenetic studies based on these characters should be biased. In conclusion, we consider that methods based on PCR (i.e. PCR-RFLP and microsatellite markers) reveal more polymorphisms and lead to more reliable results that those based on the classical RFLP method.

Vegetal material sampling

The individual "*O. laperrinei* La Taessa" appeared to be mislabelled according to our data (Besnard 1999). Consequently, we wonder which markers enabled Lumaret et al. (2000) to distinguish it from the Mediterranean olive. Based on cytoplasmic DNA polymorphism, the true *O. e.* subsp. *laperrinei* from Hoggar was shown to be related to some Mediterranean olives (Besnard and Bervillé 2000; Besnard et al. 2002b). This feature appeared to be very important to understand the origins of the Mediterranean olive. Moreover, we supported this feature by analysing five herbarium samples (from Algeria and Niger) using the cpDNA microsatellites ccmp5 and ccmp7. Indeed, these trees displayed the characters ccmp5-116 and ccmp7-129 (Table 2) which characterise the cytoplasmic lineage CE1-ME1 (but also the *O. africana* cytoplasmic lineages). In contrast, the two individuals from Jebel Marra (Sudan Republic) displayed the characters ccmp5-118 and ccmp7-129, and could be related to SW Moroccan and Macaronesian olives. Nevertheless, this will have to be confirmed using more discriminating polymorphisms.

Summarising the results, we can conclude that *O. e.* subsp. *laperrinei* is related by the cytoplasm DNA polymorphism to oleasters and olive cultivars, but not to the subspecies *guanchica* and *maroccana*. Second, the *O. e.* subsp. *laperrinei* used as reference by Lumaret et al. (2000) is probably an oleaster, thus making their conclusion doubtful. Thirdly, the RFLP cpDNA pattern is too complex to detect faint variations (20 bp) whereas larger variations (300 bp) cannot be revealed. It is therefore more realistic to use the PCR-RFLP method, both cheaper and more efficient, to reveal reliable polymorphisms in different *Olea* taxa.

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