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Chloroplast DNA variations in Mediterranean olive

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SUMMARY
Over ten years, several European teams have characterised polymorphism in the olive chloroplast genome (cpDNA). A particularly low level of cpDNA polymorphism was detected; but, in a recent study based on 14 Mediterranean trees, an intergenic (*trn*D-*trn*T) spacer was reported to be sufficiently variable to distinguish at least six haplotypes in cultivated olive. Compared to previous studies, the level of polymorphism described (nine substitutions and seven short indels on a segment of approx. 1 kbp) was particularly high. In order to enlarge upon the sample analysed, new *trn*D-*trn*T sequences were generated from 18 olive trees originating from the Mediterranean Basin, Macaronesia, S. Algeria, Kenya, Yemen, Reunion Island, Hawaii, Iran, and China. Only two base substitutions were detected in this enlarged sample, one of which discriminated between *Olea europaea* subsp. *cuspidata* and other olive sub-species. Length variation at a (poly-T) microsatellite motif was also found. The relatively low variation revealed between distant populations of olive suggests that sequencing problems might be the origin of the high polymorphism previously reported in the *trn*D-*trn*T spacer. Furthermore, length polymorphism at the poly-T motif was investigated in 55 Mediterranean olive trees which had previously been analysed for other cpDNA polymorphisms. Two alleles were found in Mediterranean olive, but they did not permit detection of new cpDNA haplotypes. It is proposed that proper analyses of cpDNA polymorphism in Mediterranean olive should use a combination of seven markers in order to detect its correct phylogeographic structure.

Olive is one of the oldest cultivated trees. Its domestication is supposed to have occurred in different locations in the Mediterranean Basin, leading to the selection of numerous local varieties (Zohary and Spiegel-Roy, 1975; Terral and Arnold-Simard, 1996; Besnard and Bervillé, 2000). Different methodologies, based on morphological traits and, more recently, on genetic polymorphisms, have been proposed to distinguish between cultivars. Numerous studies have focussed on the nuclear genome, using DNA fingerprinting methods, microsatellites or single nucleotide polymorphisms (e.g., Belaj *et al*., 2003; Consolandi *et al*., 2007), and a few investigations have addressed polymorphism in the chloroplast genome (cpDNA). A particularly low level of polymorphism was found in olive cpDNA (e.g., Vargas and Kadereit, 2001; Baldoni *et al*., 2002; Besnard and Bervillé, 2002; Besnard *et al*., 2007c). However, in a recent study based on 14 Mediterranean olive trees (*Olea europaea* subsp. *europaea*), an intergenic (*trn*D-*trn*T) spacer was reported to be sufficiently variable to distinguish at least six haplotypes in cultivated olives from Italy (Intrieri *et al*., 2007). Compared to previous studies, the level of polymorphism described (i.e., nine substitutions and seven indels of 1 bp) was particularly high. The *trn*D-*trn*T spacer was thus considered a promising marker for genetic studies in the olive complex, as reported for other plant species (Shaw *et al*., 2007). Indeed, such plastid markers could have utility for cultivar identification (Intrieri *et al*., 2007), parentage, and phylogeographic analyses (Arroyo-García *et al*., 2007), and also reveal common maternally-inherited features (e.g., cytoplasmic male sterility; Besnard *et al*., 2000).

In order to enlarge upon the sample previously analysed, new *trn*D-*trn*T sequences were analysed from 18 accessions belonging to the six olive sub-species, originating from a very large geographic area. Sequence polymorphism was investigated, and a procedure was proposed to analyse polymorphism at a microsatellite motif. The present data do not confirm the high variability of the *trn*D-*trn*T spacer in the olive complex.

MATERIALS AND METHODS

Sequencing of the trn*D*-trn*T intergenic spacer*

Eighteen olive trees displaying various cpDNA haplotypes according to Besnard *et al*. (2007c) were analysed. These individuals belonged to the six olive sub-species (Table I; Green, 2002). Amplification of the intergenic *trn*D-*trn*T spacer was performed using the primers *trn*D-F and *trn*T-R (Demesure *et al*., 1995). PCR was conducted in 50 µl reaction volumes as described. Direct sequencing was performed using the Big Dye 3.1 Terminator cycle-sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, with an ABI Prism 3100 genetic analyser (Applied Biosystems). Fragments were sequenced with the same forward and reverse primers used for amplification. All sequences were deposited in GenBank (Table I). Sequence chromatograms are available, upon request, from the author.

Characterisation of polymorphism at a microsatellite motif

A variable microsatellite motif [poly(T)$_{10–13}$] was found in the *trn*D-*trn*T intergenic spacer (see Results). In order to investigate polymorphism at this motif in

TABLE I
List of individuals analysed for sequence variation in the trn*D*-trn*T intergenic spacer*

Individual	EMBL accession	Haplotype number
Olea europaea L. subsp. *europaea*		
- Cultivar 'Arbequina', Spain	AM690457	1
- Cultivar 'Toffahi', Egypt	AM690546	2
- Harem no 1, Syria	AM690459	1
- Serra da Arrabida no 5, Portugal	AM690458	2
- Mt Belloua no 9, Algeria	AM690462	9
- Alger no 1, Algeria	AM690461	12
- Tetouan no 3, Morocco	AM690460	13
O. e. subsp. *laperrinei* (Batt. & Trab.) Cif.		
- Jabbârene no 1, Tassili n'Ajjer, Algeria	AM690464	3
O. e. subsp. *guanchica* P. Vargas *et al.*		
- Tenerife no 1, Canary Islands, Spain	AM690466	14
- La Palma no 1, Canary Islands, Spain	AM690467	15
O. e. subsp. *maroccana* (Greut. & Burd.) P. Vargas *et al.*		
- Immouzzer no 1, High Atlas, Morocco	AM690463	20
O. e. subsp. *cerasiformis* G. Kunkel & Sunding		
- Funchal no 1, Madeira, Portugal	AM690465	23
O. e. subsp. *cuspidata* (Wall. ex G. Don) Cif.		
- Kerman no 3, Iran	AM690469	24
- Almhiwit no 5, Yemen	AM690470	27
- Guangzhou no 1, China	AM690468	34
- Timau no 1, Kenya	AM690471	36
- La Reunion no 1, Reunion Island, France	AM690472	38
- Maui no 1, Hawaii, USA	AM690473	39

EMBL accession and haplotype number detected with the methodology used by Besnard *et al.* (2007c) are given.

Mediterranean olive, 55 individuals [43 oleasters (wild forms) and 12 cultivars] were characterised (Table II). These trees had previously been analysed for cpDNA polymorphism as reported in Besnard *et al.* (2007c) and displayed seven different haplotypes [haplotype no 1 (23 trees), no 2 (6 trees), no 9 (10 trees), no 10 (1 tree), no 11 (9 trees), no 12 (4 trees), and no 13 (2 trees)].

Two primers were designed specifically to amplify the microsatellite region of the *trn*D-*trn*T spacer (*trn*D-T-polyT-For: 5'-TGAACAAATCCTATCCCTCA-3'; and *trn*D-T-polyT-Rev: 6-FAM-5' ATATTAGAATCGCC ACACTC-3'). Each PCR reaction (25 µl) contained 10 ng DNA template, 1X reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µmol of each primer, and 0.75 Units *Taq* DNA polymerase (Promega, Madison, WI, USA). Reaction mixtures were incubated in a thermocycler (T1; Biometra, Göttingen, Germany) for 4 min at 94ºC, followed by 36 cycles of 30 s at 94ºC, 30 s at 53ºC, and 30 s at 72ºC. The last cycle was followed by a 10 min extension at 72ºC. Electrophoresis of PCR products was carried out in denaturing 5% (w/v) polyacrylamide gels using an automated ABI 377 sequencer (Applied Biosystems).

TABLE II
List of Mediterranean olive trees (43 oleasters and 12 cultivars) analysed for length polymorphism at the trn*D-T-polyT locus*

Individual/Origin	Haplotype no.	*Trn*D-T-polyT (bp)	Individual/Origin	Haplotype no.	*Trn*D-T-polyT (bp)
Harem no 1/Syria	1 (E1)[a]	128	Tamanar no 1/Morocco	11 (E2)	128
Harem no 2/Syria	1 (E1)	128	Tamanar no 2/Morocco	11 (E2)	128
Harem no 3/Syria	1 (E1)	128	Tamanar no 11/Morocco	11 (E2)	128
Al Ascharinah no 1/Syria	1 (E1)	128	Serra da Arrabida no 3/Portugal	2 (E1)	128
Al Ascharinah no 2/Syria	1 (E1)	128	Serra da Arrabida no 4/Portugal	9 (E3)	127
Al Ascharinah no 3/Syria	1 (E1)	128	Serra da Arrabida no 5/Portugal	2 (E1)	128
Mont Carmel no 1/Israel	1 (E1)	128	Torvizcon no 4/Spain	1 (E1)	128
Mont Carmel no 2/Israel	1 (E1)	128	Torvizcon no 8/Spain	11 (E2)	128
Mont Carmel no 3/Israel	1 (E1)	128	Torvizcon no 12/Spain	11 (E2)	128
Adana no 1/Turkey	1 (E1)	128	Cala Grande no 1/Spain	12 (E2)	128
Urla no 1/Turkey	1 (E1)	128	Cala Grande no 2/Spain	11 (E2)	128
Urla no 2/Turkey	1 (E1)	128	Cala Grande no 3/Spain	11 (E2)	128
Urla no 3/Turkey	1 (E1)	128	Ali no 1/Italy	12 (E2)	128
Kineta no 1/Greece	2 (E1)	128	Ali no 10/Italy	12 (E2)	128
Cyrenaica no 4/Libya	11 (E2)	128	Ali no 11/Italy	1 (E1)	128
Cyrenaica no 8/Libya	1 (E1)	128			
Cyrenaica no 10/Libya	11 (E2)	128	cv. 'Kaissy'/Syria[b]	1 (E1)	128
Zaghouan no 1/Tunisia	9 (E3)	127	cv. 'Zaity'/Syria	2 (E1)	128
Zaghouan no 2/Tunisia	9 (E3)	127	cv. 'Toffahi'/Egypt	2 (E1)	128
Zaghouan no 4/Tunisia	9 (E3)	127	cv. 'Zarazi'/Tunisia	9 (E3)	127
Mt Belloua no 4/Algeria	9 (E3)	127	cv. 'Taksrit'/Algeria	1 (E1)	128
Mt Belloua no 7/Algeria	9 (E3)	127	cv. 'Galega'/Portugal	2 (E1)	128
Mt Belloua no 9/Algeria	9 (E3)	127	cv. ' Arbequina'/Spain	1 (E1)	128
Alger no 1/Algeria	12 (E2)	128	cv. 'Cornicabra'/Spain	1 (E1)	128
Alger no 10/Algeria	9 (E3)	127	cv. ' Hojiblanca'/Spain	1 (E1)	128
Alger no 19/Algeria	1 (E1)	128	cv. ' Lechin de Sevilla'/Spain	13 (E2)	128
Alger no 30/Algeria	10 (E3)	127	cv. 'Olivière'/France	9 (E3)	127
Tetouan no 3/Morocco	13 (E2)	128	cv. 'Frantoio'/Italy	1 (E1)	128

[a]cpDNA sub-lineage is given in brackets.
[b]cv. = cultivar
The haplotype number detected with the methodology used by Besnard *et a*l. (2007c) and the size (in bp) of *trn*D-T-polyT alleles are given.

RESULTS AND DISCUSSION

Eighteen new sequences in the *trn*D-*trn*T intergenic spacer were obtained from geographically distant populations of the olive complex. No high level polymorphism was observed, contrary to that reported by Intrieri *et al*. (2007). Only two substitutions were found. The first (position 99) allowed discrimination between *O. europaea* subsp. *cuspidata* and other olive sub-species, thus recognising the two olive lineages I and II identified by Besnard *et al*. (2007c). The second polymorphism (at position 396) showed a common G shared by trees displaying haplotypes 1 or 2 (sub-lineage E1), while all other trees displayed an A. This polymorphism was also detected by Intrieri *et al*. (2007) between cultivars and an Italian oleaster. In addition, length variation at a microsatellite motif (poly-T) was found in our sample. Consequently, these results indicate that the *trn*D-*trn*T intergenic spacer is not highly variable. Problems in sequencing may be responsible for the lack of agreement between the data in the present study and those reported by Intrieri *et al*. (2007). The results presented here confirm earlier studies stating that the level of polymorphism in the cytoplasmic genomes of olive is low, and less discriminating than the polymorphism found in the nuclear genome (e.g., Besnard *et al*., 2001; Belaj *et al*., 2003).

Polymorphism in the *trn*D-T-polyT microsatellite was investigated on a larger sample of Mediterranean olives (55 trees from different countries; Table II). Two length-variants were detected, but no new cpDNA haplotype was found when compared to the study by Besnard *et al*. (2007c). Indeed, allele 127 characterised all trees displaying haplotypes 9 or 10 (sub-lineage E3), while all other trees with haplotypes of sub-lineages E1 and E2 displayed allele 128 (Table II). The utility of the *trn*D-T-polyT locus to analyse cpDNA variation in other sub-species of the olive complex should also be investigated.

In order to analyse cpDNA polymorphism of oleaster populations, or in cultivated olive trees, the use of three combined loci [*ccmp5* (Weising and Gardner, 1999), *trn*S-G-indel-1, and *trn*S-G-indel-2 (Besnard *et al*., 2003)] is recommended. These allowed identification of the seven cpDNA haplotypes detected in the Mediterranean Basin by Besnard *et al*. (2007c). However, the use of other variable loci such as *trn*D-T-polyT (this study), *ccmp7* (Weising and Gardner, 1999), *psb*K-*trn*S-polyT/A, and *trn*T-L-polyT (Besnard *et al*., 2003) should also be helpful for detecting new cpDNA haplotypes not yet described. Such polymorphisms may be useful in phylogeographic studies, in population genetics (e.g., Besnard *et al*., 2007a, b), and for archaeological characterisation (e.g., Elbaum *et al*., 2006); but their application may still be of limited use for forensic analyses (e.g., Busconi *et al*., 2003) due to the poor discriminating power of the plastid genome.

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