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PRIMER NOTE

A set of primers for length and nucleotide-substitution polymorphism in chloroplastic DNA of *Olea europaea* L. (Oleaceae)

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Abstract

Chloroplastic DNA (cpDNA) variation at five microsatellite motifs, two insertion-deletion sites, and eight nucleotide substitution sites was investigated in the *Olea europaea* complex. Primers were designed for flanking regions of these sites to amplify short cpDNA regions. They provided polymorphism when polymerase chain reaction (PCR) products from a representative sample of 128 *O. europaea* individuals were either resolved by size into polyacrylamide gels (length polymorphism) or digested with restriction enzymes (nucleotide-substitution polymorphism). These polymorphisms serve to distinguish most of the cytoplasmic haplotypes previously recognized. Potential application of these markers in *O. europaea* includes phylogeography, conservation and germplasm identification, even when using poorly preserved material from herbarium specimens or forensic and archaeological materials.

Keywords: chloroplastic DNA, microsatellite, *Olea*, olive, single nucleotide polymorphism

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The *Olea europaea* L. complex consists of the olive tree (ssp. *europaea*) and five closely related siblings (Green 2002): ssp. *laperrinei* (Batt. & Trabut) Ciferri, ssp. *cuspidata* (Wall. ex G. Don) Ciferri, ssp. *guanchica* P. Vargas *et al.*, ssp. *maroccana* (Greuter & Burdet) P. Vargas *et al.*, and ssp. *cerasiformis* (Webb & Berth.) Kunkel & Sundig. Phylogeographic reconstructions have been addressed by using chloroplastic DNA (cpDNA), but low variation was observed within this species (Vargas & Kadereit 2001; Baldoni *et al.* 2002; Besnard *et al.* 2002). Five Mediterranean cpDNA haplotypes were distinguished by Besnard *et al.* (2002) using microsatellite motifs (ccmp5 and ccmp7; Weising & Gardner 1999) and insertion-deletion (indel) variation in the *trn*Q-*trn*R fragment by polymerase chain reaction–restriction fragment length polymorphisms (PCR–RFLPs) (Dumolin-Lapègue *et al.* 1997). The PCR–RFLP technique usually needs the amplification of large DNA fragments (i.e. from 2000 to 5000 bp), and consequently requires DNA templates of suitable quality. Lack of resolution in certain cases led us to pursue the finding of more variable molecular markers to distinguish populations in the *Olea europaea* complex. A second objective is to improve previous techniques, so they can be applied on herbarium specimens (Besnard & Bervillé 2002).

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Olea europaea sequences for three cpDNA regions (i.e. *mat*K, *trn*T-*trn*L and *trn*Q-*trn*R) are now available in EMBL databanks (see Table 1 for Accession nos). Considering these cpDNA regions, we defined a set of primer pairs (Table 1) enabling PCR-amplification of polymorphic cpDNA fragments in the *O. europaea* complex. Length polymorphism was investigated at five single-nucleotide microsatellite motifs (four in *trn*Q-*trn*R and one in *trn*T-*trn*L) and two indel sites of 8 bp and 10 bp in *trn*Q-*trn*R, whereas eight nucleotide substitutions were analysed using the PCR–RFLP procedure (two in *mat*K, four in *trn*Q-*trn*R and two in *trn*T-*trn*L; Table 1). Primers were designed for the flanking regions of each polymorphism (Table 1). Each PCR reaction mixture contained 10 ng of DNA template, 1 × reaction

Table 1 Primer pairs for cpDNA amplifications of 128 individuals from the *Olea europaea* complex. Chloroplast DNA regions, GenBank Accession nos, fragment names and size variants, polymorphism types and annealing temperatures (*T*) are also indicated

cpDNA region	GenBank Accessions nos	Fragment name	Polymorphism type	Fragment size (bp)	*T* (°C)	Primer sequence (5′–3′)
*trn*T-L	AY040806 to AY040816	*trn*T-L-polyT	LP; Poly T	79, 80, 81	56	F: *AGGAAGAAATCAAAGAAGGG R: ATACTGGAACCCTTGAATTC
*trn*Q-R	AJ577575 to AJ577578	*psb*K-*trn*S-polyT/A	LP; Poly T – Poly A	108, 109, 110, 111	53	F: *AAACCTCATTTCTTGGTGTC R: GTAAGCATTACACAATCTCC
*trn*Q-R	AJ577575 to AJ577578	*trn*G-polyT	LP; Poly T	86, 87, 88	52	F: *GGATTTAATCCTTTACCTCTC R: CACTTTCTAAATGACTCATG
*trn*Q-R	AJ577575 to AJ577578	*trn*G-R-polyT	LP; Poly T	60, 61	52	F: AATTTCTCACATACAATCCG R: *CGTATTTTGACTTTTCTACC
*trn*Q-R	AJ577575 to AJ577578	*trn*S-G-indel-1	LP; 10-bp indel + Poly T	105, 114, 115, 117	55	F: *GATAAAGGAAGGGCTCGAAC R: AGGCCATCAGAATAAGAAGG
*trn*Q-R	AJ577575 to AJ577578	*trn*S-G-indel-2	LP; 8-bp indel	52, 60	53	F: CAAATCAGGAATTTCTTTTAG R: *TAGGCTCGTTCGAGCCCTTC
*trn*T-L	AY040806 to AY040816	*trn*T-L-*Taq*I	RS; *Taq*I	118 → 118 118 → 95 + 23	50	F: GTGTCTATAGAATATAGAAAAGG R: ATTAGAATTGTGATAAAGAAATC
*trn*T-L	AY040806 to AY040816	*trn*T-L-*Acs*I	RS; *Acs*I	81 → 81 81 → 37 + 44	53	F: *CCATCTATATTGAATTGCCG R: TTTCTTCCTTATCGGAAACC
*mat*K	AF359497 to AF359504	*mat*K-*Rsa*I	RS; *Rsa*I	109 → 109 109 → 42 + 67	55	F: CAACGGAGAGTTCTGAGTC R: *CCTATCCATTTATTCATTTGACCC
*mat*K	AF359497 to AF359504	*mat*K-*Taq*I	RS; *Taq*I	91 → 91 91 → 43 + 48	55	F: *GAAAAAGCAACCAGCTTCTGTTC R: GTCCTTGCCGTATCAGGCAC
*trn*Q-R	AJ577575 to AJ577578	*trn*S-G-*Mse*I	RS; *Mse*I	80 → 32 + 48 80 → 32 + 5 + 43	53	F: *GCTATCAAAACTTCTTCAGC R: ATAAGATTCTGAAAGGAGGG
*trn*Q-R	AJ577575 to AJ577578	*trn*S-G-*Nde*I	RS; *Nde*I	78 → 78 78 → 55 + 23	55	F: *CTTTAGTCCACTCAGCCATC R: AAAGAAAGACYCCTTACAT**C**AT†
*trn*Q-R	AJ577575 to AJ577578	*trn*G-*Pst*I	RS; *Pst*I	63 → 63 63 → 44 + 19	52	F: *CAAGACTTCCAATTGAATAAG R: GAAATCTACTTTCTATCT**G**CA†
*trn*Q-R	AJ577575 to AJ577578	*psb*K-*trn*S-*Mse*I	RS; *Mse*I	79 → 64 + 15 79 → 37 + 27 + 15	55	F: CTATTCCACACGTTTAACTAAG R: *TCTTTCCGGTTCCGTTGATG

*Primer labelled with the fluorochrome IRD800.

†Bold nucleotides indicate nucleotide changes comparatively to the reference Accessions (T → C for *trn*S-G-*Nde*I and T → G for *trn*G-*Pst*I) to create an absence–presence polymorphism of a restriction site (*Nde*I or *Pst*I, respectively).

LP, length polymorphism; RS, restriction site polymorphism (nucleotide substitution).

buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μmol of each primer (one of each primer pair was 5′-labelled with the IRD800 fluorochrome; MWG-Biotech), and 0.75 units of DNA polymerase (Invitrogen) in a total volume of 25 μL. Reaction mixtures were incubated in a thermocycler (I-cycler, Bio-Rad), first for 4 min at 94 °C, and then for 36 cycles consisting of 1 min at 94 °C, 1 min at the optimized annealing temperature (Table 1) and 1 min at 72 °C. The last cycle was followed by a 6 min extension at 72 °C. Digestions of eight PCR fragments (Table 1) were performed using restriction enzymes *Taq*I, *Rsa*I, *Acs*I, *Pst*I, *Mse*I (Invitrogen) or *Nde*I (BioLabs Inc.) according to the manufacturer's recommendations. Electrophoresis of DNA fragments was carried out on a denaturing 8% polyacrylamide gel using a 4200 LiCor automated sequencer (LiCor Inc.).

Each polymorphism was used to characterize 128 individuals chosen to represent the six subspecies of the *O. europaea* complex and the cytoplasmic diversity previously reported (Besnard *et al.* 2002). The same DNAs of 126 individuals were previously analysed for other chloroplast markers by Besnard *et al.* (2002): 31 individuals of ssp. *cuspidata* from South Africa to China (11 populations), five individuals of ssp. *maroccana* (Morocco, two populations), six individuals of ssp. *guanchica* (Canary Islands, three populations), two individuals of ssp. *laperrinei* (southern Algeria, two populations), 82 individuals of ssp. *europaea* from the Mediterranean Basin including 70 oleasters (17 populations) and 12 cultivars. In addition, DNAs from two herbarium samples of ssp. *cerasiformis* (Funchal, Madeira, J. Gonzalés 368; San Gonçalo, Madeira, Newberry 2) were extracted using DNeasy Plant Mini Kit (Qiagen) and analysed.

The 14 investigated fragments of the three cpDNA regions were easily PCR amplified using the above conditions. Thirty-four polymorphic variants were recognized (Table 1). The use of the molecular markers herein presented, together with those published in Besnard *et al.* (2002), allowed the identification of 22 cpDNA haplotypes. Length polymorphism variation in the *trn*Q-*trn*R fragment obtained by Besnard *et al.* (2002) using PCR–RFLP was not fully congruent with the polymorphism described in the present study. DNA fragment sizes were incorrectly estimated by Besnard *et al.* (2002) because two or more mutations affecting nucleotide variation yielded similar PCR–RFLP fragments. This has been tested further by sequencing the *trn*Q-R region (unpublished data). In the *trn*S-G-indel-1 fragment, the 10-bp deletion was present in one variant size (105 bp; Table 1) and was found only in samples from the Mediterranean Basin and Sahara. Thus, in the *trn*S-G-indel-1 fragment, two characters should be individually considered (the 10-bp indel and single nucleotide variations in the poly T stretch). In the *trn*S-G spacer, an 8-bp indel (*trn*S-G-indel-2) and a restriction site (*trn*S-G-*Mse*I) were both detected in two western Mediterranean haplotypes (namely COM1 and COM2; Besnard *et al.* 2002). A restriction site (*psb*K-*trn*S-*Mse*I) was specifically detected in the subspecies *maroccana*, *cerasiformis* and *guanchica*. Polymorphisms found in fragments *mat*K-*Rsa*I, *mat*K-*Taq*I, *trn*G-*Pst*I and *trn*G-polyT serve to identify ssp. *cuspidata*. Furthermore, Asian and African samples of this subspecies were segregated by PCR–RFLP size variants of the *trn*T-L-*Taq*I fragment and by length variants in the *trn*G-R-polyT microsatellite motif. However, length variation in *psb*K-*trn*S-polyT/A microsatellite motifs was incongruent with both previous data and taxa identification, suggesting probable occurrence of homoplasy at this site.

DNAs from herbarium samples were successfully amplified using all primers (Table 1), as previously reported for ccmp5 and ccmp7 primers (Besnard & Bervillé 2002). It is possible to identify 20 of the 22 haplotypes so far designated by using the molecular markers herein described coupled with two cpDNA microsatellites (ccmp5 and ccmp7). Only two related haplotypes from Kenya (namely CA2 and CA3) can not be distinguished. Potential application of the above-mentioned primers and techniques in *Olea* includes phylogeography, conservation and germplasm identification, even when using poorly preserved material from herbarium specimens or forensic and archaeological materials.

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