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Polyploidy in the Olive Complex (*Olea europaea*): Evidence from Flow Cytometry and Nuclear Microsatellite Analyses

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• *Background* Phylogenetic and phylogeographic investigations have been previously performed to study the evolution of the olive tree complex (*Olea europaea*). A particularly high genomic diversity has been found in north-west Africa. However, to date no exhaustive study has been addressed to infer putative polyploidization events and their evolutionary significance in the diversification of the olive tree and its relatives.
• *Methods* Representatives of the six olive subspecies were investigated using (*a*) flow cytometry to estimate genome content, and (*b*) six highly variable nuclear microsatellites to assess the presence of multiple alleles at co-dominant loci. In addition, nine individuals from a controlled cross between two individuals of *O. europaea* subsp. *maroccana* were characterized with microsatellites to check for chromosome inheritance.
• *Key Results* Based on flow cytometry and genetic analyses, strong evidence for polyploidy was obtained in subspp. *cerasiformis* (tetraploid) and *maroccana* (hexaploid), whereas the other subspecies appeared to be diploids. Agreement between flow cytometry and genetic analyses gives an alternative approach to chromosome counting to determine ploidy level of trees. Lastly, abnormalities in chromosomes inheritance leading to aneuploid formation were revealed using microsatellite analyses in the offspring from the controlled cross in subsp. *maroccana*.
• *Conclusions* This study constitutes the first report for multiple polyploidy in olive tree relatives. Formation of tetraploids and hexaploids may have played a major role in the diversification of the olive complex in north-west Africa. The fact that polyploidy is found in narrow endemic subspecies from Madeira (subsp. *cerasiformis*) and the Agadir Mountains (subsp. *maroccana*) suggests that polyploidization has been favoured to overcome inbreeding depression. Lastly, based on previous phylogenetic analyses, we hypothesize that subsp. *cerasiformis* resulted from hybridization between ancestors of subspp. *guanchica* and *europaea*.

Key words: Flow cytometry, hexaploidy, High Atlas, Macaronesia, *Olea europaea*, olive, SSR, tetraploidy.

INTRODUCTION

The cultivated olive (*Olea europaea* subsp. *europaea*) is one of the most important crop species in the Mediterranean Basin, and its early cultivation was reported in both the eastern and western Mediterranean regions about 6000 years ago (Zohary and Hopf, 2000; Terral *et al.*, 2004). In addition to the cultivated olive, the *O. europaea* complex (Green and Wickens, 1989) includes five non-Mediterranean subspecies: subsp. *laperrinei* distributed in Saharan massifs (Hoggar, Aïr, Jebel Marra); subsp. *cuspidata* from South Africa to south Egypt and from Arabia to north India and south-west China; subsp. *guanchica* in the Canary Islands; subsp. *maroccana* in the Agadir Mountains (Morocco); and subsp. *cerasiformis* in Madeira (Green, 2002). Phylogeographic studies on the olive complex have been performed recently to investigate the relationships between all subspecies and to determine the origins of the Mediterranean olives (e.g. Rubio de Casas *et al.*, 2006; Besnard *et al.*, 2007*b*). It was shown that *O. europaea* subsp. *cuspidata* has diverged early from North African and Mediterranean taxa. A high diversity was reported in north-west Africa where four subspecies occur (subspp. *europaea*, *maroccana*, *guanchica* and *cerasiformis*), because of geographic isolation and fragmentation of habitat in this area.

All the species of *Olea* that have been investigated previously by chromosome counts [i.e. *O. europaea* (subspp. *europaea* and *cuspidata*), *O. capensis*, *O. paniculata*, *O. dioica* and *O. salicifolia*; for a review see Green and Wickens (1989)] have been reported as $2n = 46$, except for an early count of $n =$ approx. 24 in *O. europaea* (Anderson, 1931). However, artificial triploids and tetraploids have been isolated from cultivars ‘Frantoio’ and ‘Leccino’ mutants (Rugini *et al.*, 1996). Although many genetic and cytogenetic studies have been performed in the last decade on *O. europaea* subsp. *europaea* (e.g. Katsiotis *et al.*, 1998; Bitonti *et al.*, 1999; Minelli *et al.*, 2000; Contento *et al.*, 2002), surprisingly no exhaustive studies were performed on the possible occurrence of polyploids in the genus *Olea* and particularly in some taxa of the *O. europaea* complex. Additionally, using highly variable multi-allelic loci, polyploidy was not found in a large sample of both wild and cultivated Mediterranean olives (e.g. Khadari *et al.*, 2003; Bandelj *et al.*, 2004; Belaj *et al.*, 2004; Breton *et al.*, 2006). However, polyploidy was recently suspected to occur in *O. europaea*

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subspp. *cuspidata* (from Iran) and *maroccana* based on microsatellites (Rallo *et al.*, 2003), but no specific investigations were performed to test levels of polyploidy.

Newly developed analytical tools such as flow cytometry and multiallelic molecular markers (microsatellites) can help to determine ploidy levels rapidly in closely related taxa in the absence of meristematic tissues. In the present work, a sample of trees of the six subspecies of the *O. europaea* complex was characterized using flow cytometry and highly polymorphic microsatellites.

MATERIALS AND METHODS

Plant material

Thirty-one wild trees belonging to the six subspecies of the *O. europaea* complex were sampled from ten locations (Table 1). Five distant populations across the native range (from Iran, Yemen, Kenya, Reunion and South Africa) were analysed to test polyploidy in subsp. *cuspidata* (Rallo *et al.*, 2003). Furthermore, three invasive trees (subsp. *cuspidata*) from Australia, two Spanish cultivars ('Arbequina' and 'Manzanilla'; subsp. *europaea*) and three controlled hybrids [subspp. *laperrinei* (Hoggar) × *cuspidata* (Iran)] were investigated. Genomic DNA from fresh leaves or leaves desiccated in silica gel was extracted using a 2X CTAB method (Besnard *et al.*, 2000) for the 39 individuals.

Flow cytometry analyses

The C-value of each olive sample was estimated using flow cytometry. For each individual, intact nuclei were extracted from approx. 0·2 cm^2 of leaf tissue in a Petri dish. The plant material was chopped for 30 s with a sharp razor blade in 200 μL of extraction buffer (Partec, Münster, Germany). Three hundred microlitres of extraction buffer were added and the mix was incubated for about 1 min. The nuclei suspension was then filtered in a 30-μm CellTrics® disposable filter (Partec). For each sample, the filtrate was mixed with 1 μL of chicken erythrocyte nuclei (CEN) or CEN Singlets (BioSure, Grass Valley, CA, USA) as an internal standard. Two millilitres of staining buffer (Partec; containing 10 mg mL^{-1} of RNase) were then added and the mix was incubated for at least 30 min in the dark. The samples were then randomly analysed using a CyFlow (Partec). Data acquisition and analysis were performed in real time with a standard Pentium PC connected to the CyFlow. The software used was FloMax Version 2·0 (Partec). For each individual, the 2C DNA content was estimated by comparing the mean peak intensity of its nuclei with the mean peak intensity of the chicken erythrocyte nuclei (2C DNA = 2·5 pg) following Doležel and Bartoš (2005). Only measurements with coefficients of variation smaller than 8% were accepted. To assess the significance of genome size difference between invasive and native olives of subsp. *cuspidata*, a Mann–Whitney *U*-test was performed using the JMP® software version 5·0 (SAS Institute Inc., 2002).

TABLE 1. *List of material characterized using flow cytometry and microsatellite analyses*

Taxon	Geographic origin	Collection	*N*	Ploidy level
Olea europaea L. subsp. *europaea*				
var. *sylvestris* (Mill.) Lehr	Trassierra, Spain (wild)	UCM	3	2*x*
var. *europaea*	'Arbequina', Spain (cultivar)	MA	1	2*x*
	'Manzanilla', Spain (cultivar)	MA	1	2*x*
O. europaea subsp. *laperrinei* (Batt. & Trab.) Cif.	La Source, Algeria	INRAM	1	2*x*
O. europaea subsp. *guanchica* P. Vargas *et al.*	Tenerife, Spain	CIT	3	2*x*
O. europaea subsp. *cerasiformis* G. Kunkel & Sunding	Funchal, Madeira, Portugal	UNIL	4	4*x*
O. europaea subsp. *maroccana* (Greuter & Burdet) P. Vargas *et al.*	Immouzzer, Morocco	UNIL	3	6*x*
O. europaea subsp. *Cuspidate* (Wall. ex G. Don) Cif.	Kerman, Iran	INRAM	3	2*x*
	Almihwit, Yemen	INRAM	3	2*x*
	Rift Valley, Kenya	INRAM	3	2*x*
	La Providence, La Réunion	INRAM	3	2*x*
	Grahamstown, South Africa	UNIL	5	2*x*
	Campbelltown, Australia	UNIL	3	2*x*
O. europaea subsp. *laperrinei* × subsp. *cuspidata*	Hybrids 'Hoggar × Iran'	UNIL	3	2*x*

The ploidy level of each individual was estimated using 2C DNA content (Fig. 1) and allelic variation at six microsatellite loci (see Supplementary information available online).

N, number of individual(s) per population analysed in the present study; CIT, Cabildo Insular de Tenerife; INRAM, Institut National de Recherche Agronomique de Montpellier; MA, Royal Botanic Garden of Madrid; UCM, Universidad Complutense de Madrid; UNIL, University of Lausanne.

Nuclear microsatellite polymorphism characterization

To characterize the 39 trees, six nuclear microsatellite (SSR) loci were used: ssrOeUA-DCA1, ssrOeUA-DCA3, ssrOeUA-DCA8, ssrOeUA-DCA9, ssrOeUA-DCA14 (Sefc *et al.*, 2000) and EMO03 (de la Rosa *et al.*, 2002). For these loci, absence or low frequency of null alleles was reported in subspp. *europaea*, *laperrinei* and *cuspidata* by Baali-Cherif and Besnard (2005) and Besnard *et al.* (2007*a*). Moreover, allele number and gene diversity revealed at the six loci were high in these taxa rendering sufficient variability to distinguish diploids (maximum of two alleles per locus) from polyploids (presence of more than two alleles per locus). PCR conditions and electrophoresis procedures were used as previously described (Baali-Cherif and Besnard, 2005). For each locus, all

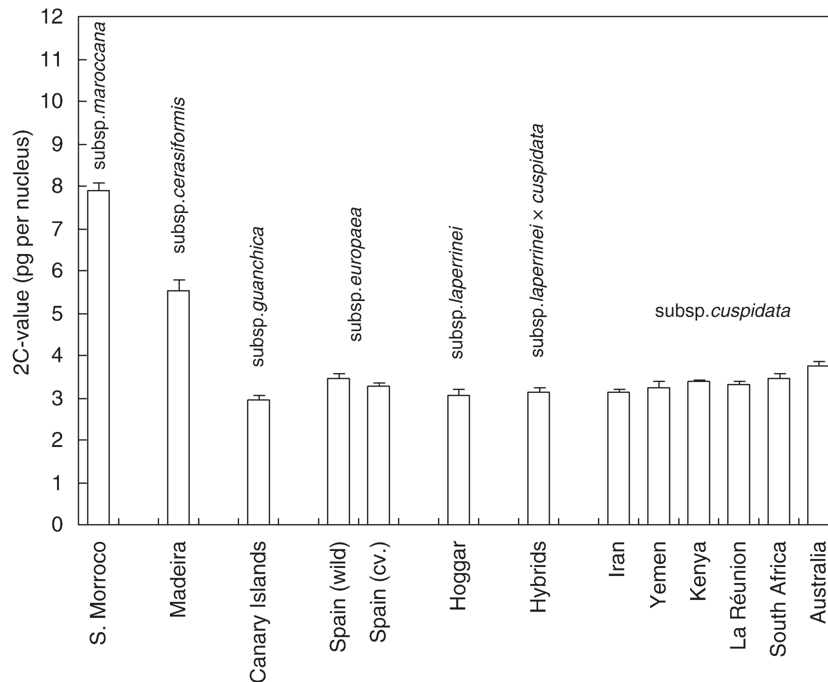

FIG. 1. Flow cytometric analysis of the olive complex: comparative genome content (in pg per nucleus) between different taxa and populations of the olive complex. For each provenance, the mean genome size was estimated from the individuals analysed. Standard deviation based on two to five individuals (see Table 1) is given for each provenance, except for the sample of *O. europaea* subsp. *laperrinei* for which the only individual sampled was independently characterized three times.

alleles revealed (based on their size in base pairs) from each individual were scored and a matrix of allelic phenotypes was constructed.

Genetic characterization of a subsp. maroccana *offspring*

To check for abnormalities in chromosome inheritance leading to aneuploid formation, offspring from a controlled cross between two individuals of subsp. *maroccana* (S1 and S2, from Immouzzer Ida-Outanane) we analysed. This cross was performed in a greenhouse at INRA, Montpellier. Nine F_1 plants were obtained (from 21 seeds) and analysed with the six highly variable microsatellite loci as previously described.

RESULTS

Flow cytometry analyses

The flow cytometry analyses of 39 olive tree samples showed that the genome size is variable in the *O. europaea* complex and three discrete DNA values were distinguished among infraspecific taxa (Fig. 1): subsp. *maroccana* (2C value = 7·88 ± 0·19 pg), subsp. *cerasiformis* (2C value = 5·52 ± 0·28 pg) and populations of the four remaining subspecies (2C value ranging from 2·93 to 3·75 pg).

Twenty genome sizes were estimated for subsp. *cuspidata* ranging from 3·06 pg to 3·86 pg (Fig. 1). DNA content was highest for the Australian (2C-value = 3·75 ± 0·12 pg) and lowest for the Iranian population (2C-value = 3·11 ± 0·08 pg). Genome size of the introduced Australian trees was significantly larger than those of native trees ($z = 2{\cdot}65$, d.f. = 1, $P = 0{\cdot}008$, $N = 20$). The chromosome number of one sample from Australia was, however, determined to be $2x = 46$, as for native trees (N. Galland, unpubl. data).

Nuclear microsatellite polymorphism characterization

The SSR allelic phenotypes for all samples analysed is given in the Supplementary Information (available online). A total of 146 alleles was revealed for the six loci (between 23 and 28 per locus). Three or more alleles in most loci were detected in single individuals for subspp. *maroccana* and *cerasiformis* (Supplementary information available online). Individuals of subsp. *maroccana* displayed a maximum of six alleles per locus whereas individuals of subsp. *cerasiformis* displayed a maximum of four alleles per locus.

Genetic characterization of offspring from a controlled cross in subsp. maroccana

Among the six loci used, ssrOeUA-DCA8 and ssrOeUA-DCA14 displayed a high level of polymorphism with five or six alleles in *maroccana* trees S1 and S2 (see Supplementary information available online). These two loci were then considered to check for abnormality in chromosome inheritance leading to aneuploid progenies. No abnormality was observed on ssrOeUA-DCA14 since all F_1 trees displayed three alleles from each parent.

In contrast, some irregularities in the allele inheritance of ssrOeUA-DCA8 were found since individuals m5 and m8 received only two of the six alleles found in parent S1 (Fig. 2).

DISCUSSION

Genome content in the olive complex

The flow cytometry analyses indicated unambiguously that *O. europaea* subspp. *maroccana* and *cerasiformis* have a larger genome than the other subspecies, which is, given the approximately multiplicative 2C values, likely to be related to polyploidy (Leitch and Bennett, 2004). The DNA content estimations for subsp. *cuspidata* from Kenya were similar to those previously reported in the literature (Bitonti *et al.*, 1999); the three Kenyan individuals had a 2C-value of 3·38 ± 0·04 pg whereas Bitonti *et al.* (1999) reported a 2C-value of 3·2 and 3·4 for two trees from the living collections at Kew, also collected in Kenya. The mean 2C-value estimation for Mediterranean samples (subsp. *europaea*) was 3·39 ± 0·13 for wild individuals and cultivars. This estimation was intermediate between those previously reported for Italian cultivars (2C-value between 3·90 pg and 4·66 pg; Rugini *et al.*, 1996; Bitonti *et al.*, 1999) and those recently reported for Portuguese trees (2C-value between 2·90 and 3·19 pg; Loureiro *et al.*, 2007). Such different results are likely to be due to comparisons of different genotypes and methodological differences in flow cytometry. The internal cytometry reference standards were different in these three investigations: *Sorghum bicolor* in Bitonti *et al.* (1999), *Pisum sativum* in Loureiro *et al.* (2007) and CEN in the present study. It is therefore not possible to determine if these differences are due to genome size variation between Mediterranean populations or to methodological reasons. We agree that DNA-content variation of small magnitude may be technique-related, as recently reported for several plant species (Greilhuber, 2005).

It was also shown in the present study that genome size of the introduced Australian trees was significantly larger than those of other trees of subsp. *cuspidata* sampled in the native range. Minor differences of genome size between introduced and native populations could be related to the presence of a variable content of repeated elements (such as tandem repeats or transposable elements; Bennetzen *et al.*, 2005; Piegu *et al.*, 2006). Such a correlation has already been described by Bitonti *et al.* (1999) in the olive complex but additional investigations are still needed to identify causes behind genome size variation in olive diploids. For more precise genome size comparisons between olive populations and subspecies, it is recommended that a larger sample of individuals is used and genome size estimations are repeated as recently suggested by Loureiro *et al.* (2007).

Agreement between cytometry and SSR analyses

Individuals of *O. europaea* subspp. *maroccana* and *cerasiformis* displayed three or more alleles at most loci.

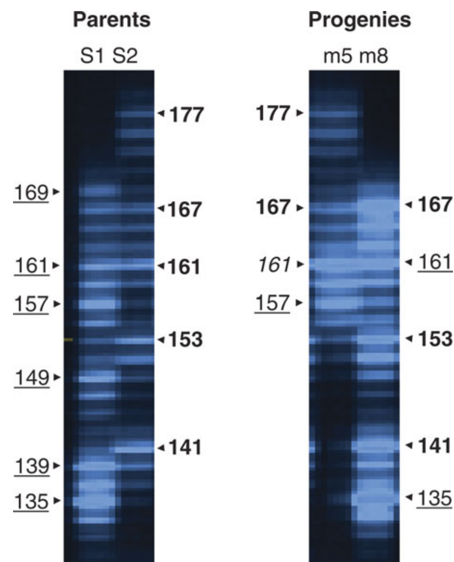

FIG. 2. Inheritance of alleles at locus ssrOe-DCA8 in two F_1 trees (m5 and m8) of the cross between individuals S1 × S2 of *O. europaea* subsp. *maroccana*. Electrophoresis of PCR products was carried out on a denaturing 5% acrylamide gel using an automated sequencer (ABI377; Applied Biosystems). For each individual, the size of each allele is indicated in the margin. The underlined sizes indicate alleles from the female (S1) and the sizes in bold-faced type indicate alleles from the male (S2). In individual m5, allele 161 is in italic because it was probably inherited from both parents. The F_1 trees m5 and m8 only received two of the six alleles revealed in parent S1 (m5: alleles 161 and 157; m8: alleles 135 and 161).

These SSR characterizations are in agreement with the DNA-ploidy levels measured with flow cytometry and suggest that subsp. *maroccana* is hexaploid and subsp. *cerasiformis* tetraploid. This agrees with the conclusions of Rallo *et al.* (2003) with regard to subsp. *maroccana*; however, the present results do not confirm polyploidy in Iranian populations of subsp. *cuspidata* as suggested from SSR variation by Rallo *et al.* (2003). Except for subspp. *maroccana* and *cerasiformis*, the remaining four subspecies of the olive complex showed a maximum of two alleles per locus, supporting a diploid level (see Supplementary information available online). Diploidy has also been suggested from previous studies of SSR variation for subspp. *laperrinei* (94 genets; Baali-Cherif and Besnard, 2005), *europaea* (157; Besnard *et al.*, 2007*a*), and *cuspidata* (115; Besnard *et al.*, 2007*a*). An extended sample (181 trees from additional populations of the six subspecies) characterized with the same (ssrOeUA-DCA3, ssrOeUA-DCA8) and additional nuclear SSR loci gave similar results (C. Garcia-Verdugo *et al.*, unpubl. data).

Abnormal chromosome inheritance leading to aneuploids has been reported in several polyploid taxa (Ramsey and Schemske, 2002; Comai, 2005). It is, however, difficult to count chromosomes accurately in polyploid olive trees because of their expected high number (i.e. $4x = 92$ or $6x = 138$). As an alternative method, highly variable microsatellites can be useful to test failures in regular chromosome inheritance in progenies. For one locus (ssrOeUA-DCA8; Fig. 2), allelic inheritance from parent S1 did not fit our

expectations (i.e. inheritance of three parental alleles) in two F_1 trees. This observation proves that inheritance of chromosomes in olive polyploids may have irregularities due likely to multivalent formation during meiosis leading to aneuploid formation (Ramsey and Schemske, 2002).

Origin of polyploidy in the olive complex

The present study is the first report of natural polyploids in *O. europaea*. Diversification of the Macaronesian and southwest Moroccan olives by at least two polyploidization events strongly supports the taxonomic treatment proposed by Vargas *et al.* (2001) and Green (2002). They distinguished three closely related subspecies in this area (subspp. *guanchica, cerasiformis* and *maroccana*), and each of these taxa is characterized by a specific ploidy level in the present study. Polyploidy is a prominent process in plant evolution which has already been reported in other genera of Oleaceae (*Fraxinus*, *Jasminum*; Taylor, 1945). In addition, tribe Oleeae (in which genus *Olea* is included) is derived from an ancient polyploid lineage (Wallander and Albert, 2000). The origins of polyploidy have been discussed by several authors (for reviews, see Soltis *et al.*, 2004; Comai, 2005). A high degree of genomic and biochemical flexibility may offer to polyploids some opportunities to colonize new habitats (Levin, 2002). In contrast to extensive polyploidization in arctic plants (Stebbins, 1984; Brochmann *et al.*, 2004), a relationship between polyploidy and narrow endemic taxa is observed here, since *O. europaea* subspp. *maroccana* (hexaploid) and *cerasiformis* (tetraploid) are only found in restricted areas. These two taxa are endemic to subtropical areas of the Agadir Mountains and Madeira, respectively. Moreover, the Agadir Mountains are considered as an important refugium since numerous endemic plants have been reported in this area (Médail and Quézel, 1999). Strong geographic isolation associated with episodic reduced population size could lead to strong genetic erosion provoking a mutational meltdown. Polyploidization may have thus been favoured to overcome inbreeding depression by providing the possibility of maintaining higher gene diversity than in diploids (Brochmann *et al.*, 2004; Soltis *et al.*, 2004). However, the populations of subspp. *cerasiformis* and *maroccana* are endangered (Hess *et al.*, 2000; Médail *et al.*, 2001) and presently occur in sympatry with the Mediterranean olive tree (subsp. *europaea*). Particularly, *O. europaea* subsp. *maroccana* is considered to be one of the ten most threatened trees in the Mediterranean Basin (Barbero *et al.*, 2001; Médail *et al.*, 2001). It is thus suspected that some disadvantages of polyploidy, including larger genome size and difficulties at both mitosis and meiosis (for a review, see Comai, 2005), may also affect the success of these taxa.

The precise origins of polyploids, via autopolyploidization or segmental allopolyploidization, have still to be determined. Based on phylogenetic data (from nuclear ribosomal and plastid DNA), historical processes of reticulation lead to the hypothesis that allopolyploidy occurs (Besnard *et al.*, 2007*b*). Wild plants of *O. europaea* subsp. *cerasiformis* from Madeira display a close genetic relationship with subsp. *guanchica* (Canary Islands) based on the plastid genome, whereas they appear to be closely related to Western Mediterranean olives (subsp. *europaea*) based on nuclear DNA (Hess *et al.*, 2000; Besnard *et al.*, 2007*b*). This could mean that subsp. *cerasiformis* originated from hybridization between ancestors of subspp. *guanchica* and *europaea*. In the case of the hexaploid subsp. *maroccana*, the available molecular data only support a close phylogenetic relationship with the diploid subsp. *guanchica*. The origin of a diploid endemic to an oceanic archipelago (subsp. *guanchica*) from ancestors related to hexaploid populations (subsp. *maroccana*; Hess *et al.*, 2000) deserves further investigation. Additional phylogenetic evidence using single-copy nuclear genes may also be useful in such a study (e.g. Fortune *et al.*, 2007).

SUPPLEMENTARY INFORMATION

Supplementary information is available online at http://aob.oxfordjournals.org/ and consists of a matrix of allelic phenotypes at the six microsatellite loci for all trees analysed in the present study.

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