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Obtainment of inter-subspecific hybrids in olive (*Olea europaea* L.)

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Abstract To enrich the source of germplasm of cultivated olive (*Olea europaea* subsp. *europaea* L.), inter-subspecific hybrid plants have been produced by experimental crosses between several varieties of cultivated olive and Asian and African accessions of the wild related subspecies *cuspidata*. Germination of putative hybrid seeds was enhanced by using in vitro embryo culture. The genetic make-up of germinated seedlings was assayed with the aid of both AFLP and SSR molecular markers and their hybrid nature was proved by the presence of male-specific alleles in their molecular patterns. Most of the parent specific alleles showed segregation among F_1 progenies indicating high heterozygosity content of the parental lines. The majority of the hybrids derived from crosses in which an African accession of *cuspidata* was used as female parent. The overall morphological aspect of hybrids resembled that of the female parent. The production of inter-subspecific hybrid plants in *Olea* is discussed in relation to the genetic improvement of cultivated olive.

Keywords Inter-subspecific crosses · Embryo culture · AFLP · SSR · *Olea europaea* subsp. *europaea* · Subsp. *cuspidata*

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Introduction

The cultivated olive (*Olea europaea* subsp. *europaea* L.) is one of the oldest, most widespread and economically important crops of the Mediterranean basin. Taxonomically, it belongs to the *O. europaea* complex, which includes five additional non cultivated subspecies widely distributed in Asia and Africa (Green and Wickens 1989; Green 2002). It is generally assumed that olive domestication began in the Near East around 6,000 years ago and spread to the western Mediterranean region following human migrations. The modern cultivars originated from recurrent hybridisation events between wild and cultivated Mediterranean genotypes (Besnard et al. 2013 and references therein). Although the autoctonous cultivars are well adapted to their environment and suited for long term established cultivation practices, in many cases these fail to respond adequately to the requirements of modern oliviculture (Lavee 2013).

Furthermore, most of them are not resistant to insect and fungal diseases (Rugini 1986). For these reasons there is a need for new varieties characterized by resistance to major pests and abiotic stresses, early production without alternation, suitability for intensive cultivation and mechanized harvesting, and production of high quality oil and fruits (Bellini et al. 2008).

Implementation of olive breeding programs via inter-subspecific crosses might represent a useful strategy to exploit the enormous gene pool represented by the wild *O. europaea* subspecies (Rugini and Gutiérrez 2006; Rugini et al. 2011). As an example, the geographical isolation and adaptation to particular environmental conditions of some wild *O. europaea* subspecies led them to evolve an extent of inbreeding that might be a useful trait to be introgressed into cultivated olive trees in the perspective to develop self compatible new cultivars (Lavee 2013). Finally, the use of partially inbred plants belonging to other subspecies as parents in olive cross-breeding programs might exploit heterosis, leading to vigorous hybrids, as already reported in cultivated olive (Biton et al. 2012). *O. europaea* subsp. *cuspidata*, named Brown, or African or Indian olive, widespread in China, India as well as in North, East and South Africa, appears a promising candidate as source of useful traits for olive cultivars. It is adaptable to different climates, ranging from semi-arid to meso-humid ones, and shows resistance to fungal diseases (Hannachi et al. 2009). The high adaptability of *O. cuspidata* is witnessed in Australia where, from its introduction (in the mid of the 19th century) for horticultural purposes, is now considered an invasive and potentially dangerous weed (Spennemann and Allen 2000; Cuneo and Leishman 2006; Besnard et al. 2014). On the other hand, its wood is much appreciated in Africa for multiple purposes (Negash 2003).

Genetic affinity between cultivated *O. europaea* and *cuspidata* subspecies has been witnessed by the occurrence of spontaneous hybridisation events arisen in sympatrically grown natural populations or in the nearness of olive groves (Costa 1998; Belaj et al. 2001, 2004; Besnard et al. 2007; Omrani-Sabbaghi et al. 2007; Hannachi et al. 2009; Besnard et al. 2014). However, experimental evidences of sexual compatibility between the two subspecies are still lacking. The only documented experimental hybrids between olive and a wild form of the same genus were reported by Besnard et al. (2001). These authors characterised by means of low efficient RAPD markers, 14 putative hybrids between *O. europaea* subsp. *europaea* and *O. africana* (actually *O. europaea* subsp. *cuspidata*, Green and Wickens 1989) obtained in 1984 by Villemur from unspecified parents (cf. Besnard et al. 2001). On the other hand, hybrids between cultivated olive and any of the other subspecies of the same genus have never been found (Hannachi et al. 2009 and references therein).

On account of the preceding, we carried out experimental crosses by using cultivars of *O. europaea* and subspecies *cuspidata* plants as parents, with the dual purpose of i) verifying the genetic affinity between the two entities and ii) exploiting new sources of genetic variability for the genetic improvement of olive cultivars. Furthermore, it has been recently shown that some multigene characters are dominantly inherited from female parents in inter-varietal crosses (Lavee and Avidan 2011). Therefore, the direction of the crosses is crucial if a particular multigene trait should be transferred from one donor to a specific receiver parent. On this basis, several parental combinations in different cross directions were undertaken. The hybrid nature of the seeds obtained was corroborated with the aid of robust molecular markers. To overcome the known difficulties of olive seed germination (Cañas et al. 1987; Garcia et al. 2002), putative hybrid seeds were subjected to embryo culture that allows to obtain an higher percentage of germination in shorter time, thereby avoiding loss of potentially valuable hybrid genotypes (Istambouli and Neville 1977; Rugini 1986).

Materials and methods

Plant material and cross procedure

The plants of the cultivars and subspecies of *O. europaea* used in the crossing program were bred in pots according to the routine practices in greenhouse conditions at ISAFOM-CNR (Perugia, Italy). Genotypes of the subspecies *cuspidata* came from the areas of Himachal Pradesh (India), Sichuan (China), and from South Africa (accession A from Cape Town, and B from Morgenster). Olive varieties commonly cultivated in Umbria (Italy) chosen as parents, were Dolce Agogia, Frantoio, Leccino and Fs 17 (Fontanazza et al. 1998). Parent combinations and cross directions are reported in Table 1. Flowers of maternal parents were

Table 1 Plant material studied: crosses carried out according to different parent combinations and directions, and plants used in self and open pollination studies

Plant material	Code
Cross	
subsp. *cuspidata* South Africa A (♀) × subsp. *europaea* cv. Dolce Agogia (♂)	C1
subsp. *cuspidata* South Africa A (♀) × subsp. *europaea* cv. Fs 17 (♂)	C2
subsp. *cuspidata* South Africa A (♀) × subsp. *europaea* cv. Frantoio (♂)	C3
subsp. *cuspidata* South Africa A (♀) × subsp. *europaea* cv. Leccino (♂)	C4
subsp. *cuspidata* South Africa B (♀) × subsp. *europaea* cv. Dolce Agogia (♂)	C5
subsp. *cuspidata* South Africa B (♀) × subsp. *europaea* cv. Fs 17 (♂)	C6
subsp. *cuspidata* South Africa B (♀) × subsp. *europaea* cv. Leccino (♂)	C7
subsp. *cuspidata* South Africa B (♀) × subsp. *europaea* cv. Frantoio (♂)	C8
subsp. *cuspidata* China (♀) × subsp. *europaea* cv. Dolce Agogia (♂)	C9
subsp. *cuspidata* China (♀) × subsp. *europaea* cv. Fs 17 (♂)	C10
subsp. *cuspidata* China (♀) × subsp. *europaea* cv. Leccino (♂)	C11
subsp. *cuspidata* China (♀) × subsp. *europaea* cv. Frantoio (♂)	C12
subsp. *cuspidata* India (♀) × subsp. *europaea* cv. Dolce Agogia (♂)	C13
subsp. *cuspidata* India (♀) × subsp. *europaea* cv. Fs 17 (♂)	C14
subsp. *cuspidata* India (♀) × subsp. *europaea* cv. Leccino (♂)	C15
subsp. *europaea* cv. Dolce Agogia (♀) × subsp. *cuspidata* South Africa A (♂)	C16
subsp. *europaea* cv. Dolce Agogia (♀) × subsp. *cuspidata* China (♂)	C17
subsp. *europaea* cv. Dolce Agogia (♀) × subsp. *cuspidata* India (♂)	C18
subsp. *europaea* cv. Fs 17 (♀) × subsp. *cuspidata* South Africa A (♂)	C19
subsp. *europaea* cv. Fs 17 (♀) × subsp. *cuspidata* India (♂)	C20
Self pollination	
subsp. *cuspidata* South Africa A	S1
subsp. *cuspidata* South Africa B	S2
subsp. *cuspidata* China	S3
subsp. *cuspidata* India	S4
subsp. *europaea* cv. Dolce Agogia	S5
subsp. *europaea* cv. Fs 17	S6
Open pollination	
subsp. *cuspidata* South Africa A	OP1
subsp. *cuspidata* South Africa B	OP2
subsp. *cuspidata* China	OP3
subsp. *cuspidata* India	OP4
subsp. *europaea* cv. Dolce Agogia	OP5
subsp. *europaea* cv. Fs 17	OP6

Their code in the text is reported

emasculated with the aid of fine scissors and their pistils were dusted with pollen collected at maturity from the paternal parent. Pollinated flowers were then bagged with plastic membranes (DuPont™Tyvek®; Smith and Mehlenbacher 1994) and allowed to set seeds in isolation. Open and self pollination seeds from both African and Asian *cuspidata* accessions, and from Fs 17 and Dolce Agogia cultivars, were collected as control (cf. Table 1).

Embryo culture and growth conditions

Immediately after collection, the stones were separated from flesh of mature fruits and kept in 2 %

sodium hydroxide for 4 h, rinsed in H_2O and left in tap water for about 2 h, then allowed to dry.

For embryo isolation, seeds isolated from sclerified endocarp were sterilized by dipping in 70 % ethanol for 3 min and then in 20 % sodium hypochlorite for 20 min. Finally, they were rinsed several times in sterile distilled water and stored in sterile Petri dishes containing filter paper imbibed with 5 ml of sterile distilled water. The number of stones containing more than one seed was recorded. After 2 days of imbibition at room temperature, embryos were dissected aseptically under stereomicroscope by cutting off two lateral sections of the endosperm and freeing the embryo from the remaining seed tissues (Acebedo et al. 1997; Germanà et al. 2009).

The embryos were then cultured individually in sterile test tubes containing 10 ml of culture medium according to Rugini (1988), closed with plastic caps and sealed with Parafilm®. To avoid germplasm loss, even the embryos that had lost hypocotyls during handling were subjected to in vitro culture. The cultured embryos were incubated in a controlled environment growth chamber at 23 ± 1 °C under a 16/8-h (day/night) photoperiod with fluorescent light at the intensity of 40 μE m^{-2} s^{-1}, and weekly monitored. The opening of cotyledons and emergence of epicotyl were taken as evidence of germination, whereas the development of the hypocotyl and primary roots were not deemed essential to consider the embryo geminated. The shoots were cultured in OM medium according to Rugini (1984) and then transferred on a rooting medium (Mencuccini 2003). The hardening phase was carried out as described by Rugini (1984). Surviving plantlets were transplanted into pots and bred in the greenhouse according to the routine culture practices. The left stones derived from self and open pollination fruits not used for embryo culture (see Table 2) were mechanically scarified, sowed on trays containing potting soil and bred in the greenhouse.

DNA isolation, AFLP and SSR procedures

Genomic DNA was purified from leaf tissue (100 mg) using the DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. DNA concentration, purity and integrity were assessed by both spectrophotometric (Cary 100 Scan, Varian) and electrophoretic analyses. DNA concentrations were adjusted to 100 ng/μl in all samples.

AFLP procedure was as described by Labombarda et al. (2002) with minor modifications. Four hundreds (instead of 500) ng from each of 20 DNA samples (15 putative hybrids and 5 related parental lines) were restriction-ligated using 50 pmol (instead of 30) of *Mse*I adapters. Pre- and selective amplifications were carried out as reported (Labombarda et al. 2002) using the combinations F*Eco*CAA/*Mse*CCA and F*Eco*CAC/*Mse*CGC as selective primers. AFLP amplicons were separated on a 6 % denaturing polyacrylamide gel and visualized in the Genomyx SC Scanner (Beckman). The pictures were collected as a TIFF image and the grey levels were adjusted manually by using the software Adobe Photoshop CS5 version 12.0 to enhance the sharpness of the images. Only clear and reproducible bands were considered and scored as 1 or 0 (for band presence or absence, respectively).

Three simple sequence repeat (SSR) markers, DCA5, DCA9 and GAPU103A developed in *O. europaea* subsp. *europaea* (Sefc et al. 2000; Carriero et al. 2002) and tested for their effectiveness (Baldoni et al. 2009) were used. PCR amplifications were performed in a volume of 25 μl containing 25 ng of template DNA, 5 μl of 5 × Colorless Go Taq® Flexi Buffer, 2 mM $MgCl_2$, 0.3 μM of 5′ fluorescein-labelled primer, 0.3 μM of 3′ primer, 0.5 mM of each dNTPs and 1.25 U of Go Taq® Hot Start Polymerase (Promega). Amplifications were performed on a 2720 Thermal Cycler (Applied Biosystems) using the following profile: an initial denaturation step of 95 °C for 5 min; 35 cycles of 20 s at 95 °C, 20 s at annealing temperature specific of each locus, 30 s at 72 °C; a final elongation step of 1 h at 72 °C. Amplicon lengths were detected loading 0.5 μl of each fluorescent sample mixed with 0.25 μl of LIZ 500 size standard (Applied Biosystems) up to a final volume of 10 μl with formamide. The DNA fragments were denatured and size fractioned using capillary electrophoresis on an ABI 310 Genetic Analyzer (Applied Biosystems). The software GeneMapper 4.0 (Applied Biosystems) was used to estimate allele size.

All the molecular analyses were repeated twice.

Table 2 Results of the experimental crosses, and self and open pollination studies

Code	Number of flowers	Number of fruits obtained	Fruit set (%)	Number of stones used for embryo culture	Number of seeds obtained	Number of germinated embryos
Cross						
C1	173	2	1.16	2	2	2
C2	449	0	0	0	0	0
C3	204	6	2.94	6	10	10
C4	308	4	1.30	4	7	7
C5	374	28	7.49	18	11	0
C6	51	6	11.76	6	7	0
C7	267	3	1.12	2	3	0
C8	126	0	0	0	0	0
C9	21	1	4.76	1	1	1
C10	43	0	0	0	0	0
C11	12	0	0	0	0	0
C12	49	0	0	0	0	0
C13	209	1	0.48	1	1	0
C14	100	0	0	0	0	0
C15	12	0	0	0	0	0
C16	47	3	6.38	3	3	2
C17	28	4	14.29	4	4	3
C18	20	1	5.00	1	1	1
C19	22	0	0	0	0	0
C20	198	6	3.03	6	6	4
Total	2,713	65		54	56	30
Self pollination						
S1	709	4	0.56	2	2	1
S2	362	1	0.28	1	1	0
S3	656	0	0	0	0	0
S4	332	1	0.30	0	0	0
S5	74	7	5.18	2	2	2
S6	112	6	5.36	1	1	1
Total	2,245	19		6	6	4
Open pollination						
OP1	2,720	293	10.77	10	17	9
OP2	365	23	6.30	2	2	0
OP3	1,424	26	1.83	3	3	3
OP4	100	2	2.00	0	0	0
OP5	120	12	10.00	3	3	3
OP6	336	40	11.90	6	6	4
Total	5,065	396		24	31	19

The number of flowers is referred to those emasculated for the different crosses, or sampled for self and open pollination studies

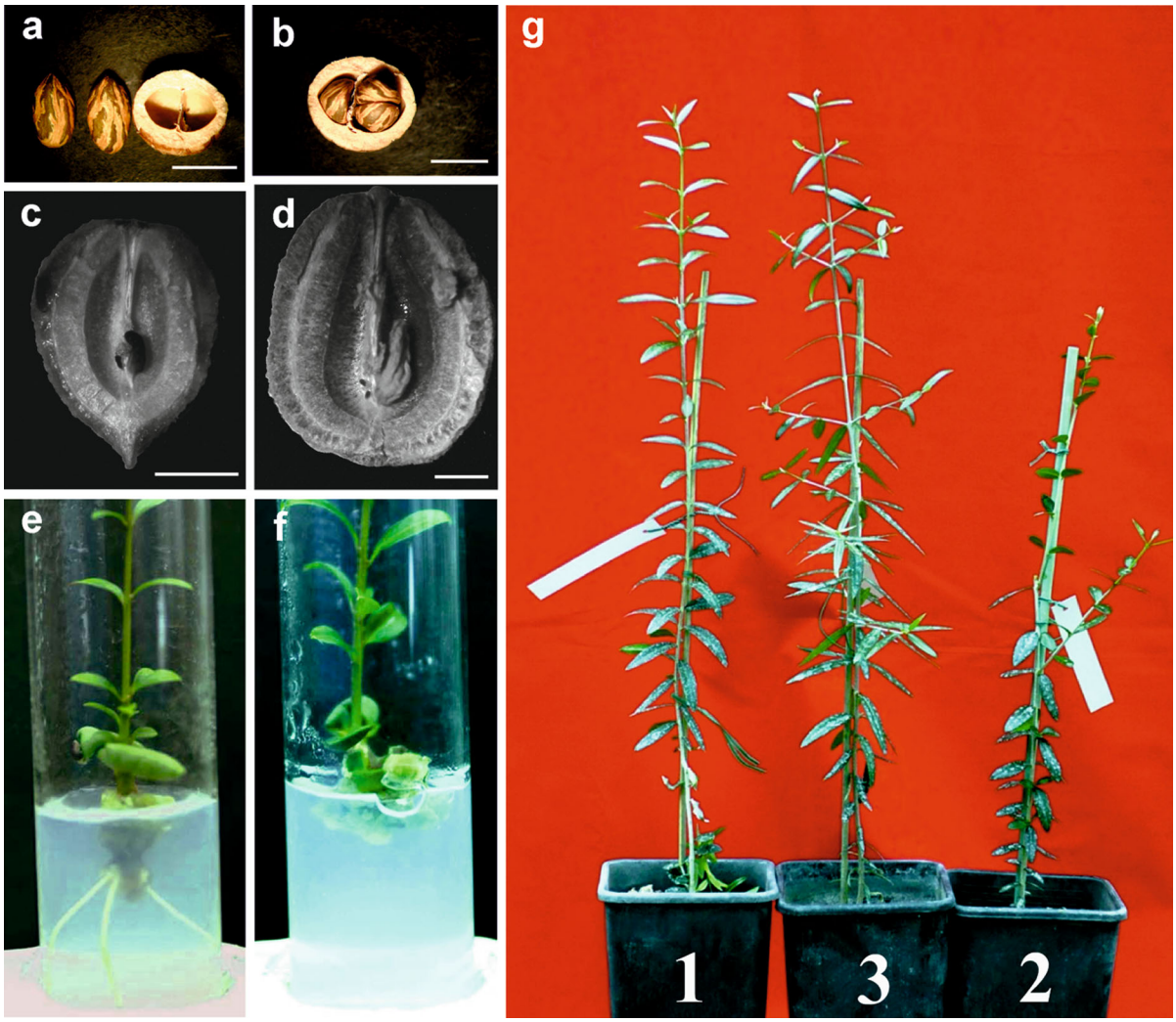

Fig. 1 Transversal sections of stones containing two (**a**) or three (**b**) seeds, produced by crosses C3 and C4, respectively. *Bar* 5 mm. Longitudinal median sections of two fruits produced by cross C5 showing a parthenocarpic (**c**) or abnormally developed (**d**) seed. *Bar* 2.5 mm. **e** C3 well rooted seedling after 40 days of embryo culture. **f** C1 non rooted seedling after 40 days of embryo culture. **g** Eighteen month old plants in pots after the hardening phase. Dolce Agogia (1) and subsp. *cuspidata* A (2) plants from seeds self pollination-obtained; hybrid C16-1 obtained by crossing Dolce Agogia as maternal parent and subsp. *cuspidata* accession A as paternal parent (3)

Results and discussion

Crossings

The results of 20 experimental crosses each involving an olive cultivar and a *cuspidata* accession as parents (cf. Table 1) are reported in Table 2. The limited pollen availability from poorly flowering *cuspidata* plants hampered to carry out some reciprocal crosses, thereby forcing us to use some genotypes instead of others as seed parent. From a total of 2,713 cross pollinated flowers, 65 fruits were obtained corresponding to an average fruit set of 2.47 %. Eight cross combinations did not yield any fruit. In most of the unsuccessful crosses, the female parent was a plant either from India or China *cuspidata* accessions. This could be due to the high rate of ovule abortion observed in the same accessions (data not shown). Conversely, the highest percentages of fruit set were obtained when the female parents were the African *cuspidata* accession B (C5 and C6) and cv. Dolce Agogia (C16 and C17). However, the seeds produced

Table 3 Results of AFLP analysis on 15 individuals obtained from four inter-subspecific crosses. The number of individuals analysed for each cross is reported in brackets

Primer combination F*Eco*CAA/*Mse*CCA			Cross			
			C1 (1)	C3 (6)	C4 (5)	C17 (3)
Scored bands						
Total	223		56	61	56	50
Monomorphic	83					
	Not inherited		9	1	0	1
	Segregating		–	11	17	11
	Not segregating		17	6	3	7
Polymorphic	136					
	Female specific					
		Not inherited	4	2	4	6
		Segregating	–	21	23	13
		Not segregating	6	0	0	5
	Male specific					
		Not inherited	14	1	1	2
		Segregating	–	16	5	3
		Not segregating	6	2	2	0
Non parental	4		0	1	1	2
Primer combination F*Eco*CAC/*Mse*CGC						
Scored bands						
Total	240		53	62	70	55
Monomorphic	78					
	Not inherited		10	3	2	0
	Segregating		–	8	16	9
	Not segregating		12	5	6	7
Polymorphic	150					
	Female specific					
		Not inherited	12	2	6	7
		Segregating	–	20	26	21
		Not segregating	7	0	0	0
	Male specific					
		Not inherited	8	2	1	5
		Segregating	–	18	9	4
		Not segregating	2	0	0	0
Non parental	12		2	4	4	2

in C5, C6 and C7 cross combinations contained not viable embryos probably due to post-zygotic incompatibility. Moreover, a strict pre-zygotic genome incompatibility between African *cuspidata* accession A and cv. Fs 17 (C2 and C19) was recorded, as in neither parent directions fruit set was obtained. The cross combination *cuspidata* India and cv. Fs 17 was carried out in both directions too (C14 and C20). A nuclear-cytoplasmic incompatibility does exist between the two genomes because fruits were obtained only when cv. Fs 17 was used as maternal parent. Similar explanation could be given for the cross combinations involving African *cuspidata* accession A and cv. Dolce Agogia (C1 and C16). As a matter of fact, when *cuspidata* accession A was used as maternal plant, a hybrid was obtained from one of the two viable embryos (the other did not survive beyond the seedling stage), while a single individual arisen from self fertilization was obtained in the reciprocal cross (as showed by the molecular analyses, see below).

A poor rate of fruit set was obtained through self pollination in African accessions A and B (0.56 and 0.28 %, respectively), and in Asian accessions (China 0 %, India 0.3 %; Table 2) of *cuspidata*. In cultivars Dolce Agogia (S5) and Fs 17 (S6), both characterized by a variable extent of self compatibility (Fontanazza and Baldoni 1990; Fontanazza et al. 1998) this parameter raised up to over 5 % as expected.

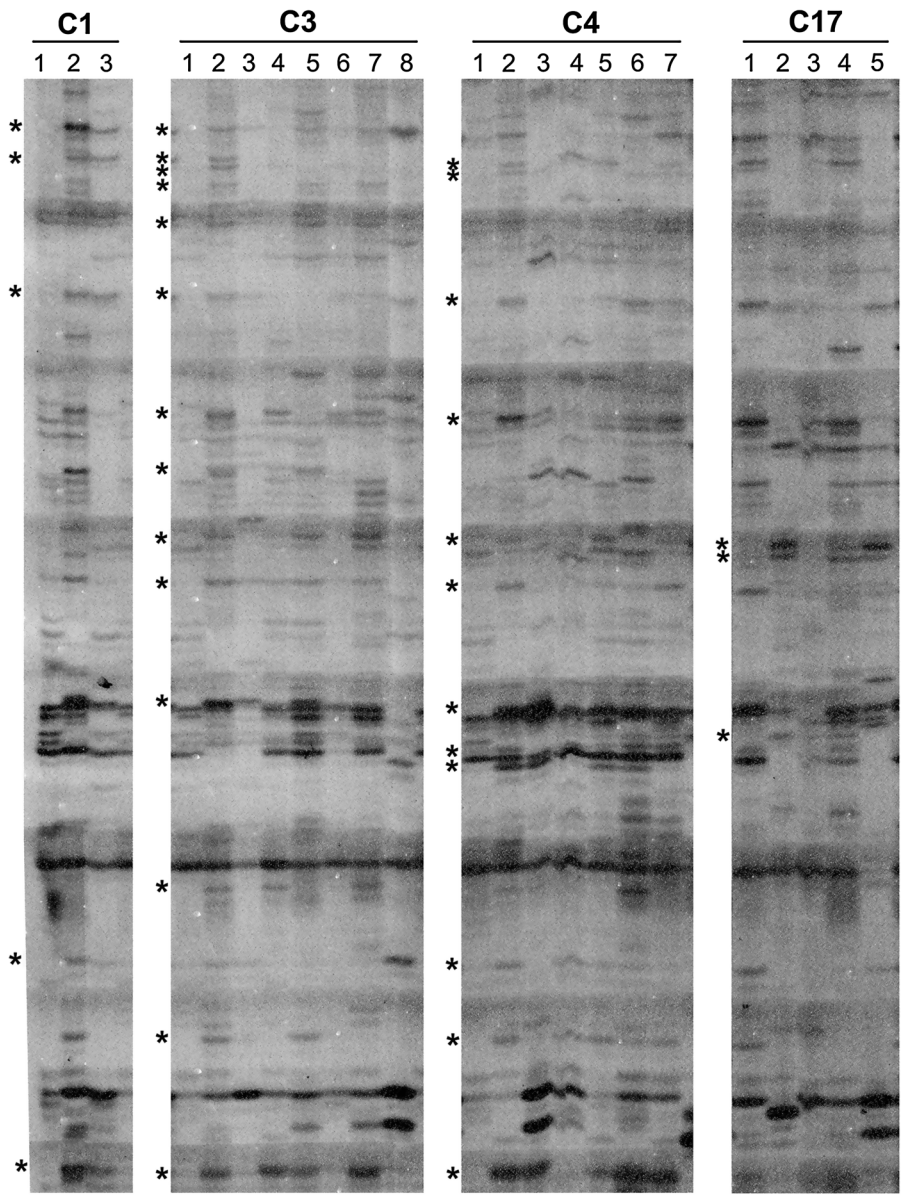

Fig. 2 AFLP band patterns of female (1) and male (2) parents, and individuals forming their progenies (from 3 to 8) in the *C1*, *C3*, *C4*, and *C17* crosses. The AFLP patterns were obtained by using the primer combination F*Eco*CAA/*Mse*CCA. *Asterisks* indicate male-specific bands transferred to the progenies

Overall, higher percentage of fruit set was obtained through open pollination (Table 2). An average of 8.54 % was shown by both African *cuspidata* accessions (OP1 and OP2), 1.92 % by Asian accessions (OP3 and OP4), 10.95 % by cultivars Dolce Agogia (OP5) and Fs 17 (OP6). Either African or Asian accessions yielded very poor fruit set from self pollination, that could be due to a common high rate of self incompatibility expressed in the environment in which the crosses have been carried out. However, differently from the African accessions, the Asian ones showed also poor open pollination fruits, due to the high rate of observed ovule abortion (see above) likely depending on its scarce environmental adaptation.

Stones containing more than one seed were frequently produced by crosses C3, C4, C6, and C7, involving both A and B African *cuspidata* accessions

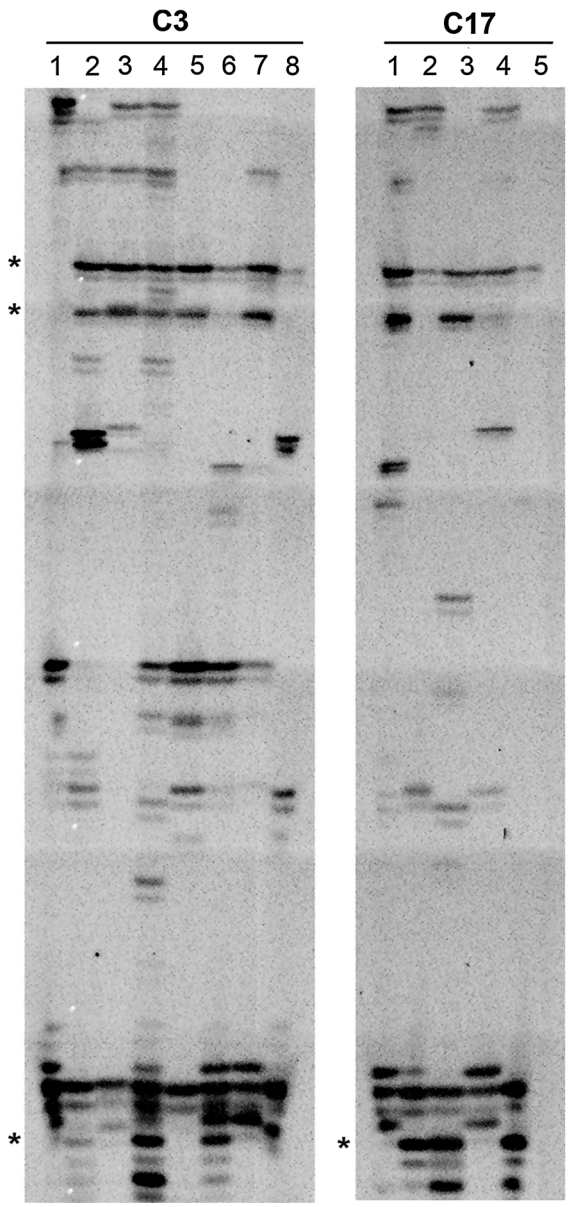

Fig. 3 AFLP band patterns of female (1) and male (2) parents, and individuals forming their progenies (from 3 to 8) in the *C3* and *C17* crosses. The AFLP patterns were obtained by using the primer combination F*Eco*CAC/*Mse*CGC. *Asterisks* indicate the male-specific bands inherited by C3-3, C3-6, and C17-3 plants showing their hybrid nature

as female parent (Table 2; Fig. 1a, b). In olive cultivars, only one out of the four ovules, each containing an embryo sac, is usually fertilized whereas the others degenerate (Altamura Betti et al. 1982). As cyto-embryological studies in *cuspidata* are lacking, we assume that the multiple seeds observed in the stones analysed were likely arisen from fertilization of more than one embryo sac. Furthermore, about one third of the fruits produced by cross combination C5 (including African accession B as maternal parent) were parthenocarpic or characterised by an anomalous seed development (Table 2; Fig. 1c, d).

To sum up, although variable rates of anomalies were observed on fruits derived from crosses involving African accessions, on the basis of the high rate of viable hybrid embryos obtained (see below) we are in the position to recommend these accessions as suitable female parents in crosses with cultivated olive.

Some stones obtained from open pollination were sowed in soil to analyse the germination in *cuspidata*, a subspecies for which this information is scarce. The seed sample OP1 showed a percentage of germination equal to 0.35 %, while seeds derived from OP2, OP3 and OP4 did not germinate at all. About 10 % of seeds derived from cvs. Dolce Agogia and Fs 17 (OP5 and OP6, respectively) showed active germination: a value similar to that reported for other olive cultivars (Acebedo et al. 1997).

Embryo culture

All the cross-derived seeds were subjected to embryo culture. Few seeds obtained from open- and self-pollination involving both African and Asian *cuspidata* accessions as well as cultivars Dolce Agogia and Fs 17 were also subjected to embryo culture as control (Table 2).

Thirty, 4 and 19 actively growing embryos were obtained after 15 days of culture from 56, 6 and 31 cultured embryos which were extracted from seeds derived from cross, self and open pollinations, respectively (Table 2). The majority (63.33 %) of the 30 cross-derived viable embryos arose from crosses in which African *cuspidata* accession A was used as female parent. After 40 days, six embryos out of 30 showed a well-developed root system together with a vigorous shoot (Fig. 1e), whereas the others underwent a delayed development (Fig. 1f). After 90 further days of culture, nineteen well-developed plantlets from cross-derived seeds (C3, C4, C9, C16, C17, C18, and C20) were obtained. These were transferred in pots and maintained in the greenhouse for further growth, along with some self and open pollination-derived individuals as control. The remaining putative hybrids were considered not viable

Table 4 Alleles length (bp) at each of three SSR *loci* (DCA5, DCA9, GAPU103A) amplified in 19 individuals from seven crosses, and in their parents

Code	Genotype			
		DCA5	DCA9	GAPU103A
C3	subsp. *cuspidata* A (♀)	196–196	167–169	Null
	cv. Frantoio (♂)	194–202	183–206	160–172
	C3-1	196–202	169–206	172
	C3-2	194–196	169–183	160
	C3-3	196–202	169–183	172
	C3-4	196–202	169–183	160
	C3-6	194–196	169–206	172
	C3-7	196–202	169–183	172
	C3-10	196–202	169–183	172
C4	subsp. *cuspidata* A (♀)	196–196	167–169	Null
	cv. Leccino (♂)	194–202	163–206	172-184
	C4-6	196–202	163–169	184
	C4-7	196–202	163–169	184
C9	subsp. *cuspidata* China (♀)	198–198	185–187	Null
	cv. Dolce Agogia (♂)	192–204	173–187	172–176
	C9-1	198–204	185–187	172
C16	cv. Dolce Agogia (♀)	192–204	173–187	172–176
	subsp. *cuspidata* A (♂)	196–196	167–169	Null
	C16-1	196–204	167–187	172
C17	cv. Dolce Agogia (♀)	192–204	173–187	172–176
	subsp. *cuspidata* China (♂)	198–198	185–187	Null
	C17-1	198–204	185–187	172
	C17-2	198–204	173–185	172
	C17-3	192–198	185–187	172
C18	cv. Dolce Agogia (♀)	192–204	173–187	172–176
	subsp. *cuspidata* India (♂)	198–198	185–187	Null
	C18-1	192–198	173–187	172
C20	cv. Fs 17 (♀)	202–204	206–208	173–173
	subsp. *cuspidata* India (♂)	198–198	185–187	Null
	C20-1	198–204	185–206	173
	C20-2	198–204	187–208	173
	C20-3	198–204	187–208	173
	C20-4	202–204	206–206	173–173

because they never reached a full development stage. This could be due to post-zygotic barriers, or even to the use of a culture medium unsuitable for subspecies *cuspidata*. Nine (47.37 %) out of the 19 successfully obtained plantlets derived from crosses involving African *cuspidata* accession A as female parent (cf. Table 4). This percentage value allows us to suggest that, among the accessions analysed, subsp. *cuspidata* A is the best candidate to cross with subsp. *europaea* in order to obtain viable embryos and plantlets.

AFLP analysis

Table 3 shows the results of AFLP analysis carried out on 15, early germinated, putative hybrids by using DNA extracted from leaflets of six-week-old

plants. Two primer combinations were tested on hybrids belonging to the C1 (1 hybrid), C3 (6 hybrids), C4 (5 hybrids) and C17 (3 hybrids) cross combinations together with their related parental lines. A total of 463 scorable bands, with an average of 115.75 bands per parent combination, were obtained. Of these, 161 (34.77 %) were considered not informative because monomorphic between parents. Out of 286 polymorphic bands, 185 female- and, in particular, 101 male-specific bands were highly informative to assess the hybrid nature of cross-derived plants. Of the male-specific bands, 34 were not transmitted to any of the progenies, 8 were inherited by the unique putative hybrid of the cross C1, 4 were inherited by the whole progeny (not segregating, Table 3) of each of the two crosses C3 and C4, and 55 bands segregated in all crosses for which more than one putative hybrid was considered. The majority of parent specific bands (62.59 %) showed segregation in crosses C3, C4 and C17 indicating high level of heterozygosity of parental lines. Part of the AFLP profile obtained with the primer combination F*Eco*CAA/*Mse*CCA in all the tested individuals is reported in Fig. 2: bands with asterisks are male-specific alleles transmitted to the progenies. All the putative hybrids showed one or more male-specific bands with the exception of individuals C3-3, C3-6 and C17-3 for which some bands, although present, appeared very faint. Such uncertainty on the hybrid nature of these individuals was ruled out by the results showed in Fig. 3, in which the male inherited bands were clearly detectable (asterisks). Moreover, 16 non parental bands were observed in the AFLP patterns of hybrid plants (Table 3). Non parental, PCR-amplified bands were frequently observed in interspecific crosses in plants (Yin et al. 2002; Luo et al. 2002) and in segregating populations of *Populus* (Wu et al. 2000), *Pinus* (Cato et al. 1999) and *Phaseolus* (Muñoz et al. 2004). Most of these non parental bands are believed to originate from heteroduplex molecules formed between sequences showing partial matching coming from both parents (Ayliffe et al. 1994), thereby constituting a further evidence of hybridity.

SSR analysis

SSR analysis was undertaken to confirm the hybrid nature of the plants screened with AFLP and to analyse additional putative hybrids that developed with delay compared to the previous ones. Three SSR markers, DCA5, DCA9 and GAPU103A, were used and the microsatellite profiles of the hybrids were compared with those of their related parental lines. Eleven hybrids previously analysed by means of AFLP, together with 7 hybrids generated from 4 new cross combinations (C9, C16, C18, and C20) and an additional delayed hybrid from the C3 cross, were subjected to SSR analysis (Table 4). The allele sizes were compared to those reported in Oleadb (www.oleadb.it), a database collecting information about SSR markers in olive cultivars. Minor variations were recorded between our material and the database. The most remarkable difference concerned the *locus* GAPU103A, showing a biallelic pattern in cv. Dolce Agogia (172–176 bp) as analysed by us, instead of the reported monoallelic one (174 bp). This discrepancy, like others in allele length that we consider less significant, could be addressed to the different resolution power of the instrument and the standard size used, as already stated by Baldoni et al. (2009). Anyway, the same *locus* showed a null allele in all the *cuspidata* accessions, thereby rendering this marker highly informative for hybridity test when these accessions were used as maternal parent.

SSR analysis thereby confirmed the hybrid nature of the individuals previously analysed with AFLP. Furthermore, seven (C3-1, C9-1, C16-1, C18-1, C20-1, C20-2, C20-3) out of the additional cross-derived individuals tested were also considered hybrid on the basis of the presence of paternal specific SSR alleles, whereas the individual C20-4 was probably arisen from self pollination.

Morphological analysis

A preliminary evaluation of the morphological aspect of the hybrids was carried out after 18 months of their maintenance in greenhouse. The overall morphological habit, and in particular plant height and leaf morphology, did not differ from that of the maternal parent while branching was more similar to the paternal one (Fig. 1g). Other morphological traits, including those presumably related to heterotic effects, have not been noticed because the hybrid plants are still in juvenile phase.

Conclusions

The use of the molecular markers allowed us to assess the hybrid nature of 23 genotypes obtained by crossing *O. europaea* subsp. *europaea* with subsp. *cuspidata*. Four of them did not survive the hardening phase. Among all the cross derived seedlings analysed, only one resulted a self pollination product. The remaining eighteen plants are vigorously and actively growing. They represent the first example of intraspecific hybrids obtained from different subspecies of *O. europaea* and for which a robust evidence of hybridity, based on highly efficient molecular markers of different nature, was provided. The obtainment of these plants demonstrated the feasibility of inter-subspecific crosses in *O. europaea*. Moreover, the possibility to obtain hybrids in multiple cross directions opens interesting opportunities to exploit alternative nucleus-cytoplasm interactions aimed at the production of specific superior genotypes. Hybrids are under observation to assess the inheritance of traits from the progenitors.

The possibility to use these hybrids in olive breeding will depend on their genetic stability in terms of both male and female gametophyte development. The formation of balanced gametes (gametes with the appropriate haploid chromosome set) is a key factor influencing hybrid fertility and therefore the transfer of traits to the progenies. These parameters, together with morphological traits related to fruit production, will be analysed as soon as the hybrids will reach maturity.

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