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Chloroplast-DNA variation in cultivated and wild olive (*Olea europaea* L.)

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Abstract Polymorphism in the lengths of restriction fragments of the whole cpDNA molecule was studied in cultivated olive and in oleaster (wild olive) over the whole Mediterranean Basin. Seventy two olive cultivars, 89 very old trees cultivated locally, and 101 oleasters were scored for ten endonucleases. Moreover, maternal inheritance of cpDNA in olive was shown by analysing the progeny of a controlled cross between two parents which differed in their cpDNA haplotypes. In the whole species, three site- and three length-mutations were observed, corresponding to five distinct chlorotypes. The same chlorotype (I) was predominant in both oleasters and cultivated olive trees, confirming that these are closely related maternally. Three other chlorotypes (II, III and IV) were observed exclusively in oleaster material and were restricted either to isolated forest populations or to a few individuals growing in mixture with olive trees possessing the majority chlorotype. An additional chlorotype (V) was characterised by three mutations located in distinct parts the cpDNA molecule but which were never observed to occur separately. This chlorotype, more widely distributed than the other three, in both cultivated and wild olive, and occurring even in distant populations, was observed exclusively in male-sterile trees showing the same specific pollen anomaly. However, in the present study, no evidence was provided for a direct relationship between the occurrence of the cpDNA mutations and male sterility. It is suggested that the large geographic distribution of chlorotype V may be related to the high fruit production usually observed on male-sterile trees. These may be very attractive for birds which are fond of olive fruit and spread the stones efficiently. Probably for the same reason, people preserved male-sterile oleasters for long periods and, in several places, used male-sterile cultivars over large areas.

Key words Wild and cultivated Olive · (*Olea europaea* L.) · cpDNA RFLPs · Chlorotype geographic distribution · Male sterility

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Introduction

For millennia, cultivated olive [*Olea europaea* L. subsp. *europaea* (= *O. europaea* L. var. *sativa* Lehr.)] has been the main oleaginous crop of the Mediterranean Basin. Geographical, biological and archaeological evidence (Zohary and Spiegel-Roy 1975) support the assumption that cultivated olive has been derived from wild olive, namely oleaster, [*O. europaea* L. subsp. *sylvestris* (Miller) hegi]. Oleaster domestication began probably in prehistoric times (fourth and third millennia B.C.) in the eastern part of the Mediterranean Basin (Liphschitz et al. 1991; Zohary and Hopf 1993) by empirical selection of individual trees showing superior performance for fruit size and/or oil content. These individuals were propagated vegetatively as clones, using cuttings which were planted directly or, more recently, grafted onto indigeneous oleasters. Numerous introductions of olive accessions have probably been made around the Mediterranean area as the result of commercial exchanges and human invasions. At present, oleaster is not necessarily wild in origin: in many instances it originates as

a product of the genetic segregation of olive cultivars or as the progeny of crosses between cultivated olive and oleaster which are fully inter-fertile and constitute two closely connected components of the same species (Lumaret et al. 1997). According to Zohary and Spiegel-Roy (1975), wild-oleaster forms occupy niches not disturbed by cultivation and thrive as an important constituent of Mediterranean forests, whereas secondary feral forms occur in disturbed areas and sites of abandoned cultivation.

Recently, a partial classification of olive cultivars was obtained from allozyme studies using either pollen (e.g. Loukas and Krimbas 1983; Trujillo et al. 1995) or leaf extracts (Ouazzani et al. 1993, 1995, 1996). most of the genetic variation observed in these cultivars was not restricted to a particular area, suggesting that a substantial number of these cultivars may have a common geographic origin, located most probably in the eastern part of the Mediterranean Basin (Ouazzani et al. 1995).

Among available genetic molecular markers, restriction-site analysis of chloroplast DNA (cpDNA) a very conservative cytoplasmic molecule, inherited maternally in most angiosperms, has been shown to be a powerful tool for phylogenetic reconstruction at both inter- and intra-specific levels (Palmer 1987). In the present paper, we studied restriction-site variation (RFLP) of cpDNA in cultivated olive and in oleasters from the whole Mediterranean Basin. The objectives of this study were: (1) To check for the mode of inheritance of cpDNA in *O. europaea* using a controlled cross between two cultivars which showed distinct chlorotypes, and (2) To assess cpDNA variation in cultivated and oleaster forms and establish the cytoplasmic relationships between these two closely connected components of *O. europaea*.

Materials and methods

Origins of plant material

Chloroplast DNA variation was analysed in 72 olive cultivars distributed over the Mediterranean Basin. These are listed in Table 1 with an indication of their country of origin and the collection site. Analyses were also carried out using 89 very old trees cultivated locally but not identified formally as cultivars. They were collected in 24 distinct localities (two in the North of Israel, two in Cyprus, two in France and five, three and ten located in the North, Centre and South of Morocco, respectively). In addition, 101 oleaster trees (*O. europaea* subsp. *sylvestris*) were analysed from 40 localities (one in Algeria, two in Cyprus, four in the South of France, three in Israel, three in Italy, six, seven and seven in the North, Centre and South of Morocco respectively, one in Portugal, three in Spain, two in Tunisia and one in Turkey). Name, geographic coordinates and sample sizes of the localities may be obtained from the corresponding author. In 34 localities, oleaster forms occurred in disturbed areas and sites of abandoned cultivation and were probably of feral origin whereas in the six other localities (two in Spain, two in France, one in Italy and one in Tunisia) the very numerous oleaster trees were growing in open forests, far from cultivation areas, and were considered to be mostly of wild origin.

Table 1 Name, original country, and collection sites of the 72 Olive cultivars analysed for cpDNA RFLPs. Collecting site A: INRA collection, Mauguio, France; B: olive germplasm bank of Cordoba, Spain; C: CNR olive collection, Perugia, Italy; D: collected in cultivated areas by the authors of the present work

	Cultivar	Origin	Collecting site
1	Chemlal de Kabylie	Algeria	A
2	Sigoise	Algeria	B
3	Karydolia	Crete	A
4	Koronaiki	Crete	B
5	Oblica	Croatia	B
6	Kiti	Cyprus	D
7	Klirion	Cyprus	D
8	Toffahi	Egypt	B
9	Belgentier	France	A
10	Cailletier	France	A&D
11	Cayon	France	A
12	Lucques	France	A
13	Olivière	France	A
14	Picholine	France	A
15	Salonenque	France	A
16	Amygdalolia	Greece	A
17	Karolia	Greece	A
18	Kothreiki	Greece	A
19	Gaidourelia	Greece	A
20	Kalamon	Greece	B
21	Valanolia	Greece	B
22	Merhavia	Israel	B
23	Barnea	Israel	B
24	Cassanese	Italy	C
25	Cellina	Italy	C
26	Cipresino	Italy	A
27	Dolce Agogia	Italy	C
28	Frantoio	Italy	C
29	Giaraffa	Italy	C
30	I-77	Italy	C
31	Leccino	Italy	C
32	Leucocarpa	Italy	C
33	Nocellara del Belice	Italy	C
34	Pendolino	Italy	C
35	San Felice	Italy	C
36	Souri	Lebanon	B
37	Bouchouika	Morocco	D
38	Dahbia	Morocco	A
39	Hamrani	Morocco	D
40	Haouzia	Morocco	D
41	Meslala (Beldia)	Morocco	D
42	Meslala (Romia)	Morocco	D
43	Menara	Morocco	D
44	Picholine marocaine	Morocco	A
45	Branquita	Portugal	A
46	Galega	Portugal	B
47	Arbiquina	Spain	B
48	Chesna	Spain	B
49	Cornicabra	Spain	B
50	Ecijino	Spain	B
51	Empeltre	Spain	B
52	Lechin de Sevilla	Spain	A
53	Manzanilla	Spain	A
54	Oblonca	Spain	A
55	Picual	Spain	A&B
56	Pudriaco	Spain	B
57	Sevillenca	Spain	B
58	Villalonga	Spain	B
59	Zarza	Spain	B
60	Kaissy	Syria	B
61	Moui Stambouli	Syria	B

Table 1 (Continued)

Cultivar		Origin	Collecting site
62	Zaity	Syria	B
63	Barouni	Tunisia	A
64	Bidelhaman	Tunisia	A
65	Chemlali de Sfax	Tunisia	A
66	Chetoui	Tunisia	B
67	Meski	Tunisia	A
68	Ayvalik	Turkey	A&B
69	Domat	Turkey	A&B
70	Memecik	Turkey	A
71	Sofralik	Turkey	A
72	Uslu	Turkey	B

A single individual per cultivar was usually studied except in 18% of the cultivars for which 2–5 trees were analysed. With regard both to trees cultivated locally and oleasters, 1–8 individuals (3.0 on average) were studied per locality. This small sample size is consistent with the theoretical expectation which predicts very low variation occurring locally in cpDNA (Pons and Petit 1995).

Tree material for the study of cpDNA inheritance consisted of two parent trees (cultivars Nos. 13 and 47, see Table 1) which differed by their cpDNA haplotype, and of their adult progeny (100 individuals) which had been obtained from a controlled cross made in 1975. The tree used as female was male-sterile and had been pollinated using standard pollination bags. Parent and progeny trees were growing in the experimental orchard of the National Institute of Agronomy Research (INRA) near Montpellier (southern France).

From eight to ten small-leafed branches, were collected on each tree. The branches were placed inside a plastic bag and brought, or sent by Postage, to the laboratory.

Preparation and restriction endonuclease analysis of cpDNA

The leafed branches were placed in the dark for 8 days to de-starch the leaves before they were ground in liquid nitrogen and freeze-dried. Chloroplasts were isolated from aliquots of 2 g of freeze-dried powder using a non-aqueous procedure, and cpDNA was extracted from chloroplasts as described by Michaud et al. (1995). Aliquots of 20 μg chloroplast DNA were incubated for 5 h with six 6-cutter endonucleases (*Bam*H1, *Eco*RI, *Dra*I, *Xho*I, *Hin*dIII, and *Ava*I) and with four 4-cutter endonucleases (*Cfo*I, *Hae*III, *Hpa*II and *Nci*I), according to the recommendation of the suppliers (Appligene, Boehringer). These restriction enzymes provided regularly clear restriction patterns with a large number of fragments (usually over 40) except for *Hin*dIII and *Xho*I which generated fewer and larger-sized fragments. RFLP analyses of the cultivated olive and oleaster trees sampled were performed for all the restriction enzymes listed above except for *Xho*I which was used exclusively in cultivars 1, 13, 23, 27, 37, 40, 43, 55, 61 and 72 and in 30% of the oleasters, and for *Nci*I used to analyse cultivars 1, 10, 13, 14, 18, 27, 34, 36, 37, 43, 61 and 69, and 18% of the oleaster individuals. The digestion products were fractionated by electrophoresis on horizontal 0.85% and 1.2% agarose-slab gels in order to separate and identify a large range of fragment sizes. Gels were stained with ethidium bromide and photographed under UV light. Lambda DNA digested with *Hin*dIII and *Eco*RI, Raoul™ (Appligene) and a 1-kb Ladder DNA were used as size standards. Gels with 1.2% agarose were used more particularly for *Dra*I and the 4-cutter endonucleases which produced many small fragments. For each cpDNA restriction endonuclease pattern, DNA restriction fragment sizes were determined using "Bande" software (Duggleby et al. 1981).

In addition, several of the mutations (more particularly those which were found to be associated in the same specific individuals) were located on the cpDNA molecule by using Southern-blot hybridization of the cpDNA restriction patterns which were transferred to a nylon membrane (Biodyne A from Pall Filtration technik GmBH). Heterologous probes consisting of nine clones, pTBa1, pTBa2, pTBa5, pTB7, pTB13, pTB18, pTB25, pTB28 and pTX6, from *Nicotiana tabaccum* L. (Sigiura et al. 1986), and three additional clones (ctP4, ctP5 and ctP7) from *Hordeum vulgare* L. (Day and Ellis 1985), were used in order to cover the whole cpDNA molecule. The probes were labelled with dioxigenin-d-UTP (Non-radioactive DNA labelling and Detection Kit, Boehringer Mannheim west Germany) and were hybridized overnight at 68°C. Hybridized olive cpDNA fragments were revealed by immunological detection and chemiluminescence using CSPD from Tropix (Saumitou-Laprade et al. 1993).

Identification of cpDNA mutations

The cpDNA restriction endonuclease patterns of individual trees were scored for fragment-length differences. The cpDNA changes were identified as either length or site mutations (or even conversion, if any). The detection of specific changes, each revealed from an individual tree by several restriction enzymes, suggests that alterations in the length of the fragments may be due to DNA length mutations rather than site mutations. By scoring those length mutations arbitrarily as the same mutations (same letter), we avoided counting the same addition/deletion several times and, therefore, overestimating the number of distinct mutations.

In oleasters, Nei's (1987) genetic diversity statistics was adapted to small and unequal sample sizes, $H = n(1 - \Sigma pi^2)/(n - 1)$ where pi is the frequency of the ith allele and n is the population size, were calculated in each population (Hs) and over all populations (Ht), and the proportion of diversity resulting from genetic differentiation among populations (Gst) was estimated. Total genetic diversity (Ht) was also calculated separately over the cultivars and the trees cultivated locally.

Results

Chloroplast DNA variation in *O. europaea*

In the entire *Olea* material analysed by digestion with the ten endonucleases, 28 different banding patterns were observed giving a total of 475 different fragments, of which only 30 (6.3%) varied among the patterns. The restriction banding patterns obtained for *Cfo*I, *Dra*I, *Ava*I and *Eco*RI are illustrated in Fig. 1. In our study, 37% of the individual trees were analysed on at least two identical gels per enzyme and no variation was observed among replicate samples. Overall, the restriction endonucleases *Cfo*I, *Bam*HI, *Eco*RI, *Ava*I, *Dra*I, *Hin*dIII, *Xho*I, *Hae*III, *Hpa*II and *Nci*I generated an average of 53.5, 42.0, 48.5, 50.0, 45.5, 25.0, 26.7, 58.5, 58.0 and 54.0 fragments, respectively. Chloroplast-DNA molecular size was estimated by adding together the size of the fragments generated by each endonuclease, particularly those produced by *Xho*I and *Hin*dIII which provided fewer and larger-sized fragments. In *Olea*, the cpDNA size was estimated to range between 132 and 134 kb.

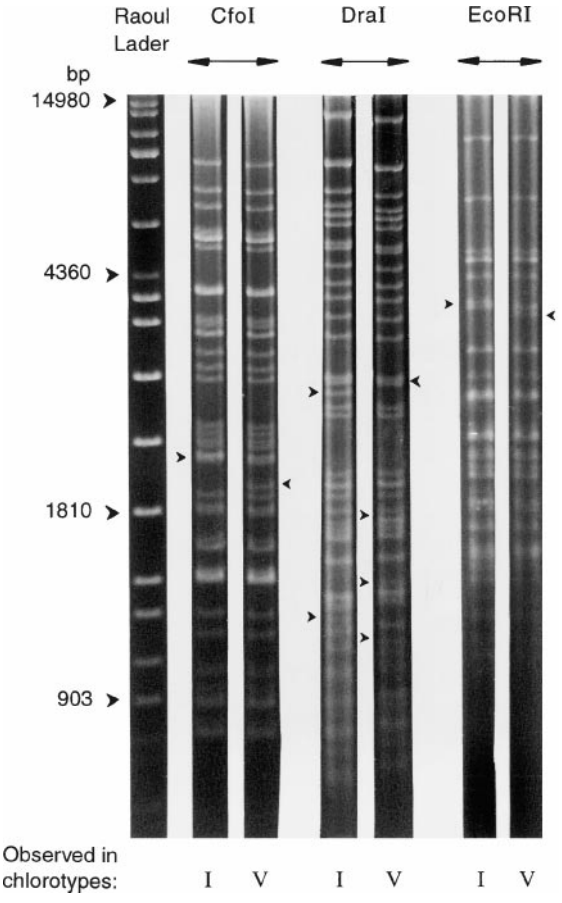

Fig. 1 Example of the restriction fragment patterns obtained by digestion of cpDNA with *Cfo*I, *Dra*I and *Eco*RI in *O. europaea*. Mutation A (see Table 2) can be observed from the patterns obtained using *Cfo*I, *Dra*I and *Eco*RI, and mutation 3 (Table 2) is shown by comparing the two restriction patterns obtained using *Dra*I. The agarose concentration in the original gels was 0.85

The mutations responsible for cpDNa variation, as compared to the most common restriction pattern observed in *O. europaea* for each of the ten restriction enzymes, could be identified and are listed in Table 2. Three site mutations and three distinct length mutations were found. The 220-bp deletion (A), the 20-bp deletion (C) and the 20-bp addition (B) were observed using seven, five and four distinct enzymes respectively. No cpDNA variation was detected using *Ava*I. Site mutations 1, 2 and 3 and the length mutation A were confirmed and were located on the cpDNA molecule using probes ctP5, pTB13, pTBa1 and either pTba5 or pTBx6, respectively. The mutations 1 and 3 were located in two distinct parts of the large single-copy region whereas the mutations A and 2, which both concerned a single fragment, were very close to the inverted repeat region IRb.

From all the mutations obtained with the ten endonucleases, five distinct haplotypes (chlorotypes) were obtained. Chlorotype I, which corresponds to the majority restriction patterns observed for the ten endonucleases, was found in 70 of the 72 olive cultivars studied, in the whole trees cultivated locally and in 87 of the 101 oleasters analysed in the present work, and can be considered therefore as the predominant chlorotype in *O. europaea*. Chlorotype II differs from chlorotype I by mutation B (20-bp addition) and was observed in a single oleaster tree out of six oleasters analysed from Zerhoun, a locality close to Volubilis, in the North of Morocco. As compared to chlorotype I, chlorotype III is characterised by mutation C (20-bp deletion) and was found exclusively in the six oleasters analysed from Tannant and Ouzoud (two close localities of the Middle Atlas, in the centre of Morocco), as well as in one of the nine oleasters studied from Ouazzane, in the North of Morocco. Chlorotype IV is due to the occurrence of site mutation 2 and was observed in the single oleaster tree analysed from Majorque, the Balearic islands, Spain. Chlorotype V is characterised by the occurrence of the two site mutations 1 and 3 and of the 220-bp deletion A. Although they are located in three distinct regions of the cpDNA molecules these mutations were never found to occur separately. Chlorotype V was observed in the French cultivar "Olivière" (No. 13 in Table 1), in the Algerian cultivar "Chemlal de Kabylie" (No. 1), in a millenary oleaster tree from Luras, in the North of Sardinia (Italy), in two out of the six oleaster trees analysed from Almoraima, a locality in the South of Andalusia (Spain), and in two out of the eight oleasters studied from Tabarka (North West of Tunisia).

Total genetic diversity was equal to 0.055, 0.000 and 0.256 in cultivars, in trees cultivated locally and in oleasters respectively. In the latter, 83.3% of the average total diversity was attributable to differentiation among populations.

Inheritance of cpDNA in olive

Chloroplast DNA RFLP was analysed in both parent trees and in the 100 progeny individuals collected from cultivar 13, using three endonucleases, namely *Cfo*I, *Bam*HI and *Dra*I, which very clearly discriminated the parental chlorotypes V (cultivar 13 used as the female tree) and I (cultivar 47 as the pollen tree). All the progeny individuals showed the maternal restriction patterns for the three enzymes, indicating maternal inheritance.

Discussion

Results from the controlled cross provided evidence for predominant (if not exclusive) maternal inheritance of cpDNA in *O. europaea*. This result is consistent with previous ultrastructural studies which showed an

Table 2 Restriction fragment length changes (kb) and type of mutation (site, length) in variant restriction patterns of *O. europaea* compared to the majority pattern observed in the species. Changes attributable to the same mutation are indexed with the same letter

Restriction enzyme	Mutation			
	Code	Site	Code	Length
		Majority pattern → Variant		Majority pattern → Variant
*Cfo*I			A	2090 → 1870
			B	2240 → 2260
			C	2180 → 2160
*Bam*HI	1	2960 + 870 → 3830		
*Eco*RI			A	4700 → 4480
			C	2220 → 2220
*Dra*I	2	8700 → 8200 + 500	A	1140 → 920
	3	2620 → 1550 + 1070		
*Xho*I			B	2360 → 2380
			C	880 → 860
*Hin*dIII			A	9160 → 8940
			B	1300 → 1320
			C	1130 → 1110
*Hae*III			A	2410 → 2190
			B	2040 → 2060
			C	2040 → 2020
*Hpa*II			A	2230 → 2010
*Nci*I			A	8690 → 8470

absence of plastids in the generative/sperm cells of that species (Hageman and Schroder 1989).

In oleaster, cpDNA variation is rather low as compared to that observed in other wild fruit-tree species of the Mediterranean area, e.g. in *Argania spinosa* which showed 11 distinct chlorotypes in Morocco (El Mousadik and Petit 1996), or in forest trees such as the European oaks (Dumolin-Lapègue et al. 1997). Low cpDNA variation in olive may be related to its very long life span (at least of several centuries and up to several millenia) which corresponds to a low generation turnover and, therefore, to a low probability for mutations to be fixed in later generations.

As in beech (Demesure et al. 1996), the same chlorotype is predominant over the whole geographical distributions of cultivated olive and the oleaster forms. As expected, more-numerous variant chlorotypes were observed in oleasters which reproduce sexually than in the cultivated olive, which can be considered as a subset of individuals maintained by vegetative propagation. All the variant chlorotypes observed in the oleasters were confined either to individuals of a single or of a few populations within a restricted area (e.g. chlorotype III was observed in two close oleaster populations isolated at high elevation in the Moroccan Middle Atlas) or to a few trees growing in mixture with many others which showed the predominant chlorotype. The occurrence of chlorotype III in a single tree located in the North of Morocco, 300 km from the Middle Atlas area and characterised by the occurrence of this chlorotype in all the oleasters analysed, may be due either to accidental introduction by birds, which are very fond of oleaster fruits, or to human introduction, because oleaster fruits, specifically those from high elevation areas, are used exclusively to produce a specific medicinal oil. As in many other angiosperms which showed maternal cpDNA inheritance, in oleaster, most of the cpDNA variation is observed among, rather than within, populations and the Gst value (83%) is similar to corresponding values obtained in the common European beech (Demesure et al. 1996) and in the several oak species studied to-date (Kremer and Petit 1993).

The wider distribution of chlorotype V, characterised by three mutations which were never observed separately, in trees from several distant oleaster populations and in two cultivars from different countries, is of particular interest. The trees possessing chlorotype V were all male-sterile and showed the same specific anomaly occurring at the microsporous stage after tetrad formation, as described in detail by Villemur et al. (1984) and by Ouksili (1988). However, other male-sterile cultivars, e.g. Lucques from France (No. 12 in Table 1) or Sevillenca from Spain (No. 57), analysed in the present study and which showed other anomalies as being responsible for their male sterility (Villemur et al. 1984), possessed the majority chlorotype (I). Although the presence of chlorotype V was restricted to male-sterile trees, and was never found in normal ones growing in the same oleaster populations, no decisive

evidence was obtained in our study for a direct relationship between the occurrence together in the same individuals of the three cpDNA mutations and a specific type of male sterility. The male-sterile individuals may also possess a specific mitochondrial DNA (mtDNA) molecule responsible for male sterility and which should be transmitted jointly with chlorotype V. However, the possibility that all or a part of the mutations which characterise chlorotype V may be responsible for male sterility cannot be completely ruled out. The contribution of cpDNA to male sterility has been shown in several other plant species, e.g. in cotton (Galau and Wilkins 1989) and in sorghum (Chen et al. 1990). Further analyses of both cpDNA and mtDNA variation in male-sterile and normal individuals are necessary to clarify this point.

The occurrence of chlorotype V in Olivière and Chemlal, two cultivars used in France and Algeria respectively to produce oil, indicates that they have the same maternal lineage, perhaps as the result of a long common human history between the two countries. Chlorotype V was also observed in male-sterile trees located in very distant areas and growing in mixture either with feral and/or cultivated normal olive trees (e.g. the Sardinian millenary oleaster tree) or with normal oleasters in very large populations which consisted of tens of thousands of individuals located in the humid forests of Southern Spain and Northern Tunisia. In these two areas, the large oleaster populations were shown to possess several allozymes which were never observed in feral and cultivated olive. These populations were considered therefore to be mainly of wild origin (Lumaret et al., unpublished data). In natural conditions, the maintenance of male-sterile trees may be due to their high average fruit production, as observed in situ, by comparison to that of the normal trees (A. Martin, personal communication). High fertility in male-sterile trees is usually attributed to higher vegetative development and to an outcrossing effect which was often related positively with fertility in olive (Griggs et al. 1975). Trees with high fruit productivity were shown to be more attractive for birds (Alcantara et al. 1997) so that male-sterile cytoplasm can be dispersed to diverse distances from the mother tree as long as the pollination rate is sufficient. For similar reasons, male-sterile trees may also have been scattered or maintained by humans. For instance, the exceptional fruit production of the Sardinian millenary male-sterile oleaster (S. Dettori, personal communication) may be responsible for its maintenance over a very long period, whereas many oleasters are usually eliminated in cultivated areas. Moreover, several male-sterile cultivars show wide geographic distributions and may have been selected for their ability to produce high fruit yield. Such an aptitude was reported experimentally when pollination by compatible pollen cultivars was sufficient (Chaux 1959; Ouksili 1988).

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