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Combination of chloroplast and mitochondrial DNA polymorphisms to study cytoplasm genetic differentiation in the olive complex (*Olea europaea* L.)

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Abstract Four hundred and four individuals belonging to the species *Olea europaea* were characterised using mitochondrial DNA (mtDNA) RFLPs. Twelve mitotypes were distinguished. The combination of mtDNA information with cpDNA polymorphism (characterised in a previous study) led us to recognise 20 cytoplasmic lineages of which seven were found in the Mediterranean area (oleasters, cultivars and *O. e.* subsp. *maroccana*). In the olive complex, strong cytoplasm genetic differentiation was revealed (F_{st} = 0.73). Very strong linkage disequilibrium between cpDNA and mtDNA polymorphisms was observed, particularly in the Mediterranean subspecies *europaea*. This high congruence between genetic structure based on cpDNA or mtDNA sustains a low level of recurrent mutation in both organelle DNAs and, thus, the polymorphisms used in this study were pertinent to reconstruct olive phylogeography. In the Mediterranean area, genetic drift due to population regression during Quaternary glaciations, and founder effects associated with the postglacial seed dissemination, have probably contributed to the existence of a high genetic linkage disequilibrium between cpDNA and mtDNA polymorphisms. Thus, four Mediterranean cytoplasmic lineages, clearly distinguished both by cpDNA and mtDNA polymorphisms, most likely reflect four distinct relic populations during Quaternary glaciations. Finally, *O. e.* subsp. *maroccana* from South Morocco, which also displayed specific cytoplasmic lineages, should be considered as another relic Mediterranean population.

Keywords CpDNA · Glacial refugee · MtDNA · Olive · Organellar genome association · RFLP

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Introduction

Since Greek Antiquity, and until now, several theories have been proposed about the origins of olive (*Olea europaea* L. subsp. *europaea*) (Chevalier 1948; and Moazzo 1994). However, during the last decades, molecular and archaeological data have given new insights about olive history and its domestication. It is now well established that oleaster [wild olive, *O. e.* subsp. *europaea* var. *sylvestris* (Mill.) Lehr.] is a native of the Mediterranean area (Zohary and Spiegel-Roy 1975; Terral and Arnold-Simard 1996; Besnard et al. 2001b). During the Quaternary glaciations, numerous species survived in favourable regions, and patterns of genetic differentiation between extant populations are often due to survival in different refugia zones combined with drift and founder effects during re-colonisation. With respect to genetic resource management, it appears necessary to identify original olive populations, which survived in these refuges during Quaternary glaciations and from which cultivated forms have been derived (Besnard et al. 2001a, 2002b).

MtDNA is not often used in phylogeographic studies of related plant species because of its very high level of sequence conservation (Wolfe et al. 1987; Laroche et al. 1997) and because of frequent rearrangements (Palmer and Herbon 1988). Nevertheless, more and more tree genetic studies based on mtDNA (using RFLP or PCR) have been performed to trace migration routes of forest species (Tomaru et al. 1998; Sinclair et al. 1999; Guguerli et al. 2001) for ecological purposes (Dawson et al. 1996; Latta and Mitton 1997; Tsumura and Suyama 1998; Wu et al. 1998) and to study domestication bottlenecks (Luo et al. 1995). Furthermore, the simultaneous studies of both maternally inherited mtDNA and cpDNA are still infrequent but are of great interest to check recurrent evolution of these genomes (Dumolin-Lapègue et al. 1998; Desplanque et al. 2000). We have already shown in olive that organelle DNAs are maternally inherited (Besnard et al. 2000). Recently, both mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA)

variations have been independently characterised (Besnard and Bervillé 2000; Besnard et al. 2002b). A combination of this information should enable us to check the pertinence of the polymorphism used to reconstruct a scenario of olive history.

Here, we describe mtDNA RFLPs in the *O. europaea* complex and report on a comparative analysis of both cpDNA and mtDNA variation. High linkage disequilibrium is shown between the polymorphisms of each organellar DNA. The reasons and the potential interest of these features for phylogeography reconstruction are discussed.

Materials and methods

Plant material

A sub-sample of the trees analysed for cpDNA polymorphism by Besnard et al. (2002b) was characterised using mtDNA RFLPs (Table 1). We examined 143 cultivated forms, 186 oleasters, and 75 trees belonging to *O. europaea* subspecies from Africa and Asia. *Olea woodiana* Knobl. was also characterised. This species belongs to the section *Ligustroides* Benth. & Hook., which is the *Olea* section closest to the *O.europaea* complex (Besnard et al. 2002a). For species denomination we followed the taxonomy proposed by Green and Wickens (1989), as revised by Vargas et al. (2001).

RFLP procedure to reveal mitochondrial polymorphism

The protocol to reveal RFLPs is described by Besnard et al. (2000). Five probes were used: *cox3* from *Oenothera* (Hiesel et al. 1987), *atp9* from maize (Dewey et al. 1985), *nad6* from wheat (Haouazine et al. 1993), *rpl2* from tobacco (Vitard et al. 1992) and 26S from *Oenothera* (Manna and Brennicke 1985). *Cla*I, *Hin*dIII, *Xba*I and *Xho*I were used separately to restrict DNA.

Diversity and linkage disequilibrium between cpDNA and mtDNA

We computed the haplotype H_e genetic diversity parameter (Nei 1987), of the species or a subspecies, for both mtDNA and cpDNA. This value was also computed for all the identified lin-

Table 1 List of the plant material analysed. N is the number of studied individuals for both mtDNA polymorphism (this study) and cpDNA polymorphism (Besnard et al. 2002b). Country of origin populations is indicated in brackets: Al = Algeria; Mo = Morocco; Sp = Spain; Fr = Continental France; Frc = Corsica, France; SA = South Africa; Re = Reunion Island. IRO P = "Institute for Olive Research", CNR, Perugia, Italy

Taxa	Provenance	N	Taxa	Provenance	N	Taxa	Provenance	N
Olea europaea L. subsp. *europaea*			*Olea europaea* L. subsp. *europaea*			*Olea europaea* L. subsp. *guanchica* Vargas et al.		
Cultivars*	Near East-Egypt	22	Oleasters	Torviczon (Spain)	4		La Palma (Canaria)	9
	Greece-Turkey	10		Sierra Crevillente (Sp)	1			
	Iran	5		Messine (Sicily, Italy)	17	subsp. *cuspidata* (Wall.) Ciferri		
	Algeria-Tunisia	10		Ali (Sicily, Italy)	3			
	Morocco	16		Ostricone (Frc)	6	"*O.africana* Mill."		
	Iberian Peninsula	11		Bonifacio (Frc)	3		Nairobi (Kenya)	2
	Italy-Yugoslavia	19		Filitosa (Frc)	1		Elgon Mount (Kenya)	4
	France	50		Mont Boron (Fr)	22		Timau (Kenya)	3
				Cap des mèdes (Fr)	3		Stellenbosch (SA)	3
Oleasters	Mont Carmel (Israel)	18		Repentence (Fr)	3		Morgenster (SA)	1
	Al Ascharinah (Syria)	4		La Gardiole (Fr)	1		Amalundu (Zimbabwe)	1
	Harim (Syria)	3		Montpeyroux (Fr)	5		La Providence (Re)	4
	Balcah (Turkey)	1		Pignan (Fr)	4			
	Urla (Turkey)	5		St Paul et Valmalle (Fr)	3	"*O.chrysophylla* Lam."		
	Cyrenaique (Libya)	8		Rivesaltes (Fr)	3		Almihwit (Yemen)	5
	Tizi Ouzou (Algeria)	12						
	Chefchaouen (Mo)	6	subsp. *laperrinei* (Batt. & Trab.) Ciferri			"*O.cuspidata* Wall."		
	Taounate (Morocco)	4		La Source, Hoggar (Al)	1		Kerman (Iran)	3
	Moulay Idriss (Mo)	5		Adriane Mt, Hoggar (Al)	21		IRO P (India)	1
	Khenifra (Morocco)	9					IRO P (China)	1
	Bin El Ouidane (Mo)	10	subsp. *maroccana* (Greut. & Burd.) Vargas et al.					
	Asni (Morocco)	6		Immouzzer (Morocco)	11	*Olea woodiana* Knobl.		
	Argana (Morocco)	6		Argana (Morocco)	2		Umzimkulu River (SA)	1
	Tamri (Morocco)	3		Mentaga (Morocco)	3			
	Tamanar (Morocco)	7						

eages by combining cpDNA and mtDNA information. For all these computations, we considered only the populations with at least three individuals. In addition, linkage disequilibrium between two organellar genomes was assessed using Lewontin's normalised D' (Lewontin 1964, 1988).

Overall genetic differentiation of *O. europaea*

The genetic differentiation among the different taxa was assessed using F_{st} values, calculated over the cytoplasmic lineages present in wild populations, according to the Weir and Cockerham method adapted to haploid data (Weir 1990) and extending the algorithm to a three-way nested statistical model with unbalanced data (Searle et al. 1992). Such extensions are implemented in the OPEP software (Baradat and Labbé 1995), which was used for the computations. Computations were done for all the five subspecies (*europaea*, *laperrinei*, *guanchica*, *cuspidata* and *maroccana*; Table 1), including the three subdivisions: subspecies, region and population. Computations were performed as described by Besnard et al. (2002b), except that geographic zone subdivision was replaced by subspecies subdivision.

Results

MtDNA polymorphism

Selection of the probe/enzyme combination showing polymorphism was performed on the same subset of samples used in cpDNA polymorphism screening (see Besnard et al. 2002b). Among the 20 pairwise probe/enzyme combinations, 18 revealed 51 mtDNA-RFLPs in the *O. europaea* complex. For each probe/enzyme combination, RFLP profiles can be defined. Considering all RFLP profiles, 12 mitotypes were identified in the *O. europaea* complex (Table 2). Two enzymes (*Hind*III and *Xba*I) associated with one probe (*atp9*) were sufficient to distinguish the mitotypes identified in the subspecies *europaea*, *laperrinei*, *guanchica* and *maroccana*. These two probe/enzyme combinations were used to analyse 376 individuals belonging to the Mediterranean and North African olives. In contrast, 28 individuals belonging to the subspecies *cuspidata* were characterised using the 20 probe/enzyme combinations.

Diversity and linkage disequilibrium between cpDNA and mtDNA

On the same tree sample, 15 chlorotypes were identified in the *O. europaea* complex (Besnard et al. 2002b). Taking together the cpDNA and mtDNA polymorphisms, 20 lineages were identified (Table 3). Based on both cytoplasmic data, H_e was 0.747 for the *O. europaea* complex, 0.591 for the subspecies *europaea* and 0.980 for the subspecies *cuspidata* (Table 4). Similar results were obtained using separately either cpDNA or mtDNA polymorphisms, but H_e values were always lower using mtDNA than with cpDNA (Table 4). Complete linkage disequilibrium was revealed between some chlorotypes and mitotypes (Table 3). In the subspecies *europaea* and *laperrinei*, CE1, CE2 and CCK are always associated

Table 2 Mitochondrial DNA RFLP profiles for each pairwise probe/enzyme combination enabling definition of mitotypes. For each probe/enzyme combination, a number was arbitrary assigned to each distinct profile

Mitotype	Hybridisation pattern																			
	atp9				*cox*III				26S				*nad6*				*rpl2*			
	*Xba*I	*Hind*III	*Cla*I	*Xho*I	*Xba*I	*Hind*III	*Cla*I	*Xho*I	*Xba*I	*Hind*III	*Cla*I	*Xho*I	*Xba*I	*Hind*III	*Cla*I	*Xho*I	*Xba*I	*Hind*III	*Cla*I	*Xho*I
ME1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ME2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MOM	2	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	2	1	1	1
MCK	3	3	2	1	2	2	1	3	2	2	2	1	1	1	2	3	3	2	3	3
MCE	4	4	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	2	2	2
MMA	4	1	1	2	1	1	1	1	1	1	1	1	1	1	1	3	3	2	2	2
MAK	5	1	1	1	1	1	1	4	1	1	1	2	1	1	1	3	4	1	2	1
MAS	5	1	1	1	1	1	1	4	1	1	1	2	1	1	2	3	3	2	2	3
MAR	5	1	1	1	1	1	1	4	3	1	2	2	1	1	2	3	3	2	2	3
MCIR	5	1	1	1	1	1	1	1	1	3	1	1	1	1	1	3	3	2	2	3
MCIN	6	1	1	3	1	3	1	2	1	3	1	1	1	1	1	3	5	2	2	3
MCC	6	1	1	3	1	3	1	2	1	3	1	1	1	1	1	4	5	3	2	1
MWO	7	5	3	4	1	1	1	5	1	4	3	3	2	1	1	3	6	4	2	4

Table 3 Cytoplasmic lineage definition based on cpDNA and mtDNA polymorphism and corresponding taxa

Cytoplasmic lineage	Mitotype	Chlorotype[a]	Taxa	N[b]
1	ME1	CE1	*O. e.* subsp. *laperrinei*, *O. e.* subsp. *europaea* (cultivars and oleasters)	185
2	ME2	CE2	*O. e.* subsp. *europaea* (cultivars)	12
3	MOM	COM1	*O. e.* subsp. *europaea* (cultivars and oleasters)	123
4	MOM	COM2	*O. e.* subsp. *europaea* (oleasters from South Sicily)	2
5	MCK	CCK	*O. e.* subsp. *europaea* (cultivars and oleasters)	29
6	MMA	CCE2	*O. e.* subsp. *maroccana*	10
7	MMA	CCE3	*O. e.* subsp. *maroccana*	6
8	MCE	CCE1	*O. e.* subsp. *guanchica*	4
9	MCE	CCE2	*O. e.* subsp. *guanchica*	4
10	MCE	CCE3	*O. e.* subsp. *guanchica*	1
11	MAK	CA1	*O. africana* (Kenya: Timau and Nairobi)	3
12	MAK	CA2	*O. africana* (Kenya: Elgon Mount and Nairobi)	4
13	MAK	CA3	*O. africana* (Kenya: Elgon Mount and Timau)	2
14	MAS	CA1	*O. africana* (South Africa)	4
15	MAS	CA4	*O. africana* (Zimbabwe)	1
16	MAR	CA5	*O. africana* (*La Réunion*)	4
17	MCIR	CC2	*O. chrysophylla* (Yemen)	5
18	MCIR	CC1	*O. cuspidata* (Iran)	3
19	MCIN	CC1	*O. cuspidata* (India)	1
20	MCC	CC1	*O. cuspidata* (China)	1

[a] From data described in Besnard et al. (2002b)
[b] N is the number of individuals for which the given combination chlorotype–mitotype was observed

Table 4 Genetic diversity (H_e) and cpDNA–mtDNA linkage disequilibrium (D') assessed for different taxa. H_e was computed from cpDNA, mtDNA, and cpDNA–mtDNA haplotype frequencies

Taxa	H_e			D'
	CpDNA[a]	mtDNA	cpDNA–mtDNA combination	
Subsp. *cuspidata*	0.956	0.822	0.980	0.81
Subsp. *europaea*	0.591[b]	0.578[b]	0.591[b]	0.98
O. europaea complex	0.745[b]	0.737[b]	0.747[b]	0.96

[a] From data described in Besnard et al. (2002b)
[b] Computed on wild sample data only

Table 5 Olive cytoplasmic DNA differentiation according to taxa, region or population

Item	Overall differentiation		
	Due to the taxon	Due to the region and the taxon	Due to all factors
F_{st}	0.256***	0.579***	0.728***
Confidence interval (95% level)	(0.209–0.303)	(0.516–0.642)	(0.677–0.779)

***$P < 0.001$

with ME1, ME2 and MCK, respectively. In the subspecies *cuspidata*, MAR is associated with CA5. In some cases, one mitotype is associated with several chlorotypes: MOM is associated with either COM1 or COM2, MCIR with CC1 or CC2, MAK with CA1, CA2 or CA3, MMA with CCE2 or CCE3, MCE with CCE1, CCE2 or CCE3, and MAS with CA1 or CA4. Inversely, one chlorotype can be linked to several mitotypes: CCE2 and CCE3 are associated with MMA or MCE, while CA1 is associated with MAK or MAS, and CC1 is associated with MCIR, MCIN or MCC. Lastly, it is noticeable that cpDNA–mtDNA linkage disequilibrium was higher in the subspecies *europaea* ($D' = 0.98$) than in the subspecies *cuspidata* ($D' = 0.81$) (Table 4).

Overall genetic differentiation of *O. europaea*

We detected 19 cytoplasmic lineages in wild *O. europaea* populations. The overall differentiation of the five taxa combining all factors was high (Table 5; $F_{st} = 0.73$). The values of the other two *F*-statistics (Table 5) showed that this differentiation could be quite equally attributed to the taxa and to the region, whereas the between-population variation was less important (Table 5).

Discussion

Linkage disequilibrium between cpDNA and mtDNA

Before interpreting the biogeographic significance of our results, we shall discuss the association between cpDNA and mtDNA haplotypes. Compared to cpDNA polymorphism resulting from point mutations (restriction sites) and insertions/deletions, the mtDNA polymorphism detected in our study results from rearrangements because of intragenomic recombinations involving small repeated sequences (Palmer and Herbon 1988). A high value for haplotypic disequilibrium was shown between cpDNA and mtDNA although different mechanisms of evolution have been involved. Moreover, complete associations between chlorotype and mitotype were observed. When one mitotype is associated with several chlorotypes (i.e. MOM, MMA, MCE), the chorotype distinction was of-

ten based on microsatellite polymorphism. This kind of polymorphism probably reflects a quite high rate of sequence variation. Moreover, homoplasic characters have most likely appeared in these DNA regions due to stepwise mutations. Indeed, homoplasy in the cpDNA microsatellite motif "ccmp5" was detected (Besnard et al. 2002b). This motif enabled us to distinguish all the chlorotypes associated with MOM, MCE and MMA. Nevertheless, the total linkage disequilibrium between several mitotypes and chlorotypes also showed that mutation events in these microsatellite motifs should be very infrequent.

Besnard et al. (2000) have shown that both cpDNA and mtDNA are maternally inherited in the olive. The high values for haplotypic disequilibrium observed between cpDNA and mtDNA associated with the maternal inheritance suggest that paternal leakage is not occurring. In consequence, both cytoplasm genomes are appropriate to study maternal lineages in olive. The strong congruence between cpDNA and mtDNA revealed, particularly in the Mediterranean populations, argued for a very low level of recurrent mutation in olive mtDNA. A similar result has also been revealed in *Beta vulgaris* L. (Desplanque et al. 2000). Consequently, the mtDNA polymorphism as detected in our study can be useful to analyse the geographic genetic structure of the cytoplasmic DNA diversity in olive, as in the preliminary work (Besnard and Bervillé 2000).

Cytoplasm genetic differentiation

A strong cytoplasmic genetic structure was revealed in the olive complex (F_{st} = 0.73). This value is higher than that computed from nuclear data (Besnard et al. 2001b; *phist* = 0.45), and this probably reflects the more reduced diffusion of cytoplasmic genes due to limited seed dissemination in comparison to pollen dissemination. Moreover, the differentiation pattern is in agreement with a mechanism of differentiation by distance since the genetic differentiation was mainly due to the taxa and the regions. This feature is similar to the structure revealed using nuclear markers (Besnard et al. 2001b). Thus, the combination of cpDNA and mtDNA enables us to distinguish all the studied regions in the subspecies *cuspidata* (South Africa, Zimbabwe, Kenya, Reunion, Yemen, Iran, India and China; Table 3).

Relic population in Mediterranean

The native origin of the olive in the Mediterranean is now well documented (Zohary and Spiegel-Roy 1975; Terral and Arnold-Simard 1996). We have already postulated that the subspecies *maroccana* constituted a Moroccan relic population (Médail et al. 2001; Besnard et al. 2002b). Furthermore, correlation between mtDNA and cpDNA is very strong in the other Mediterranean olive populations, in contrast with several taxa of the subspecies *cuspidata* and the North West African taxa. In the Mediterranean olive, similar high levels of genetic differentiation revealed by both cpDNA and mtDNA is probably due to genetic drift, which would have occurred with the contraction of the distribution during the Quaternary glaciations and subsequent re-colonisation during Holocene. The effect of glaciations on East and South African populations was probably less dramatic, and hence could explain the lower levels of differentiation between lineages observed in Africa. Thus, the Mediterranean lineages probably mark the diffusion of the species from glacial refugia. Compared to the distribution of the COM1–MOM lineage, the CCK–MCK lineage is restricted to a more limited area of the Mediterranean Basin: from France to the Maghreb (Besnard and Bervillé 2000; Besnard et al. 2002b). The high frequency of the CCK–MCK lineage in Kabylie and Tunisia suggests that its origin may have been in Northwest Maghreb. The COM1–MOM and COM2–MOM lineages mark a Western relic population. Sicily, where the COM1 and COM2 chlorotypes both occur (Besnard et al.2002b), could be the refugia area of this lineage, although this has to be verified considering more cpDNA polymorphism. Sicily has been a refuge for other tree species during Quaternary glaciations as attested by the presence of endemic taxa in *Betula* or *Zelkova* (Quézel 1995). The CE1–ME1 lineage probably reflects the occurrence of a relic population from the Eastern Mediterranean (Besnard and Bervillé 2000). Finally, the CE2–ME2 lineage, which was not found in oleasters but which is characteristic of several typical Eastern cutlivars (*Zaity*, *Toffahi*, *Amygdalolia*), probably reflects another Eastern relic population. It is possible that this lineage characterises an oleaster from a region that was not analysed in our study (e.g. Crete, Cyprus, Southern Israel or Northeast Syria).

In conclusion, we propose the existence of at least five glacial refuges for olive in the Mediterranean. Their geographic situation is still not well established. To obtain a more complete diffusion and domestication scenario of the olive tree, it will be necessary to study a large sample of wild olives throughout the Mediterranean Basin and in Central and Eastern Africa. Moreover, nuclear codominant markers will be useful to study potential gene flow between the different ancestral lineages (Comps et al. 2001). Lastly, the precise localisation of glacial refugia would be of great interest for the preservation of genetic diversity in wild olives.

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