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Genetic differentiation in the olive complex (*Olea europaea*) revealed by RAPDs and RFLPs in the rRNA genes

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Abstract

We assessed the genetic differentiation of the Mediterranean olive from its wild relatives found in different geographic areas (Mediterranean, Asia, Africa) using eighty RAPDs revealed with eight primers. Variance analysis (AMOVA) enabled us to estimate the overall genetic differentiation parameters between wild populations. Oleasters from the Near East and Turkey were discriminated from the other Mediterranean populations. *Olea laperrinei*, *O. maroccana* and *O. cerasiformis* were the taxa the most related to the Mediterranean olive. In contrast, *O. africana* was shown to be the most genetically distant taxa from the Mediterranean olive. However, we characterised hybrid trees between these two taxa. Significant trends between genetic and geographic distances were met within the subspecies *cuspidata* and within the Mediterranean olive. A genetic diversity gradient was observed in both subspecies *europaea* and *cuspidata*. These results are in agreement with a mechanism of differentiation by distance in the *O. europaea* complex, but another non-exclusive mechanism could also be gene flow between differentiated taxa. Furthermore, we characterised the discriminating power of each RAPD to recognise the different taxa using intraclass correlation coefficients. Lastly, IGS-RFLPs enabled us to assess rDNA polymorphisms on a sub-sample of individuals. On the basis of these data, a low interspecifc differentiation was found. This suggests a recent genetic divergence between the different taxa of the *O. europaea* complex or the occurrence of gene flow during favourable periods or because human displacements. All the olive cultivars were genetically related to the oleaster populations supporting that Mediterranean is the olive domestication area.

Introduction

The spatial organisation knowledge of the genetic diversity for both cultivated plants and their wild relatives has proved to enlighten the study of plant domestication and adequate sampling for the genetic diversity conservation. In few cultivated tree species, this has led notably to recognise the wild stock from which cultivated plants were selected (Lashermes et al. 1993; de la Cruz et al. 1995; Luo et al. 1995). This has also enabled to detect events of hybridisation (or introgression) leading to an increase of the genetic diversity basis of cultivated trees (Durham and Korban 1994; Lashermes et al. 2000). Lastly, this information has enabled to show the influence of humans in the displacements of cultivated plants (de la Cruz et al. 1995; El Mousadik and Petit 1996; Besnard and Bervillé 2000).

Olive (*Olea europaea* subsp. *europaea* var. *europaea*) is an emblematic Mediterranean cultivated tree. Oleaster [wild Mediterranean olive = *O. e.* subsp. *europaea* var. *sylvestris* (Miller) Lehr.] has been considered as the genetic stock from which present cultivars were issued (Zohary and Spiegel-Roy 1975). Domestication has been supposed to occur during the Chalcolithic period (5700–5500 years B.P.) in the Near-East (Zohary and Hopf 1994) but olive exploitation was also reported in Spain during Neolithic (Terral and Arnold-Simard 1996). Moreover, cultivated olive introduction into the Mediterranean Basin from the Middle-East or Eastern-Africa, has been suggested by botanists (Chevalier 1948; Turrill 1951; Green and

Wickens 1989). Thus, the origin of cultivars remains unclear and is certainly complex.

The *O. europaea* complex represents the taxa which are closely related to the Mediterranean olive tree. Green and Wickens (1989) have proposed the classification of the different taxa according to their morphological traits and their geographic origins. They recognised four subspecies: (1) subspecies *europaea* in the Mediterranean Basin; (2) subspecies *cerasiformis* (Webb & Berth.) Kunk. & Sund., in The Canary and Madeira Islands; (3) subspecies *laperrinei* (Batt. & Trab.) Ciferri, in the Saharan Mountains (*O. maroccana* Greut. & Burd. in Moroccan Atlas, and *O. laperrinei* Batt. & Trab. in Hoggar, Aïr and Jebel Marra); (4) subspecies *cuspidata* (Wall.) Ciferri, which comprises several taxa: *O. africana* Mill. in Eastern and Southern Africa, *O. chrysophylla* Lam. in Arabia and Abyssinia, and *O. cuspidata* Wall. in Asia. All these taxa were supposed to be sexually intercompatible; but this fact was not proved. In a recent study of *Olea*, we recognised the *O. europaea* complex from other *Olea* sections and subgenera with molecular markers, suggesting a common origin of the four subspecies previously presented (Besnard et al., submitted). *O. europaea* may be derived from the Rand-Flora in Southern Africa from which it had diffused in Northern Africa, Mediterranean Basin and in Asia (Quézel, 1978; Maley, 1980). The genetic relationships between the present *O. europaea* taxa are unknown. The origin of the Mediterranean olive is still unclear and two main hypotheses have been invoked. First, one supposed that the subspecies *europaea* could have affinities with the subspecies *laperrinei*. Quézel (1978) and Maley (1980) have suggested that the Mediterranean olive could derive from North-African populations before the Saharan desert formation starting at the Pliocene. Furthermore, during the Pliocene, favourable periods have likely enabled gene flows between *Olea* from the Mediterranean Basin and from Tropical Africa. Second, Mediterranean olive could have affinities with the subspecies *cuspidata* from Asia. This last subspecies is sometimes exploited in India (Browicz and Zielinski 1990). The occurrence of olive displacements by humans from Middle East (Iran, Afghanistan, or India) into Mediterranean across the Fertile Crescent has been supposed (Green and Wickens 1989). Thus, different subspecies of the complex could have contributed to the evolution of the Mediterranean olive and of the cultivated olive (Green and Wickens 1989; Zohary 1994). This led us to study the genetic diversity structure of the *O. europaea* complex to unravel the origins of wild oleaster and cultivated Mediterranean olive.

For assessing the genetic diversity, two approaches were chosen to reveal polymorphisms in the nuclear DNA. We used the RAPD technology on a main sample of individuals belonging to the four subspecies of the *O. europaea* complex, and then, we revealed RFLPs in the rRNA genes on a subsample of individuals representing the extremes of the RAPD diversity. The rDNA variation is commonly used to distinguish related taxa (Rogers & Bendich, 1987; Kabbaj et al., 1995). The olive rRNA gene diversity was previously shown to be low with RFLPs (Besnard et al., submitted). Thus, to reveal more polymorphisms than with the RFLP classical approach using ribosomal genes as probes, we studied the rDNA variation using an olive IGS as a probe. The Inter Gene Spacer (IGS) has been reported to be the most variable part of the rRNA unit (Rogers and Bendich 1987).

RAPDs led to a different structure than the morphological classification and were shown to be efficient to recognise the different *O. europaea* taxa. Furthermore, IGS-RFLPs suggested a low differentiation of the rRNA genes between Northern African, Asian and Mediterranean taxa.

Material and methods

Vegetal materials

We collected 501 individuals belonging to the different subspecies of the *O. europaea* complex, fourteen of which supposed hybrids between *O. africana* and *O. e.* subsp. *europaea*; these hybrids were obtained by P. Villemur in 1984 (Villemur unpublished data) (Table 1). Five wild oleaster populations (four from France and one from Morocco), located at proximity of orchards, were prospected. They could be considered as escaped from the cultures (feral forms), and thus, they were excluded from the samples of natural populations. But these populations were used as supplementary data to compare their RAPD profiles to those of Eastern and Western Mediterranean natural populations. Moreover, 121 cultivars were included.

RAPD procedure

The DNA preparation methods have been previously described by Besnard et al. (2000). The RAPD procedure has been described by Quillet et al. (1995). Eight previously screened decamers (Besnard 1999) were

Table 1. List of vegetal material and codes of the individuals analysed for the IGS polymorphism. N = Number of individuals. The population considered as feral are indicated by their names in italic.

Taxa	Prospected localities or collections	N	Individuals analysed for IGS polymorphism	Code
O. europaea subsp. *europaea* var. *europaea*	Cultivars collected in reference collections and in orchards	121 genotypes	'Chemlal', Algeria	C-1
			'Chetoui', Tunisia	C-2
			'Lechin de Sevilla', Spain	C-3
			'Moraiolo', Italy	C-4
			'Sabina', Corsica, France	C-5
			'San Felice', Italy	C-6
			'Sevillenca', Spain	C-7
O.e. subsp. *europaea* var. *sylvestris*	Urla, Izmir, Turkey	6	Urla n^o 1	O-3
	Harim, Oronte Valley, Syria	13		
	Al Ascharinah, El Ghab, Syria	12	Al Ascharinah n^o 2	O-1
	Mont Carmel, Haifa, Israel	18	Mont Carmel n^o 11	O-2
	Cyrenaique, Libya	15		
	Zaghouan, Tunisia	6		
	Tizi Ouzou, Kabylie, Algeria	7	Kabylie n^o 7	O-4
	Moulay Idriss, Morocco	10	Moulay Idriss n^o 5	O-5
	Tamanar, Morocco	10		
	Immouzzer, Atlas, Morocco	5		
	Torviczon, Sierra Nevada, Spain	15	Torviczon n^o 1	O-6
	Asturias, Spain	5		
	Messine, Sicily, Italy	17		
	Ali, Sicily, Italy	12		
	Cap des Mèdes, Porquerolles, Var, France	22		
	La Repentence, Porquerolles, Var, France	10	La repentence n^o 2	O-7
	Ostricone, Corsica, France	11		
	Filitosa, Corsica, France	11		
	Ogliastro, Corsica, France	10		
	Bonifacio, Corsica, France	6		
	Mont Boron, Alpes Maritimes, France	22		
	La Gardiole, Hérault, France	8		
	Montpeyroux, Hérault, France	15		
	Saint Paul et Valmalle, Hérault, France	18		
	Pignan, Hérault, France	8		
O. cuspidata (*O. e.* subsp. *cuspidata*)	Kerman, Iran	5	Kerman n^o 1, 2 & 5	CU-1, CU-2, CU-3
	Perugia collection (collected in India)	1		
	Perugia collection (collected in China)	1		
O. chrysophylla (*O. e.* subsp. *cuspidata*)	Almihwit, Yemen	9	Almhihwit n^o 2 & 4	CHR-1, CHR-2
O. africana (*O. e.* subsp. *cuspidata*)	Nairobi collection, Kenya	5	Nairobi n^o 5	AF-6
	Elgon Mount, Kenya	5	Mt Elgon n^o 5	AF-4
	Timau, Kenya, Mount Kenya	4	Timau n^o 4	AF-5
	Kirstenbosch, Cape Town, South Africa	6	Kirtenbosch n^o 1	AF-2
	Morgenster, South Africa	3		
	Harare Botanical Garden (Zimbabwe)	1	Amalundu	AF-3

Table 1. Continued.

Taxa	Prospected localities or collections	N	Individuals analysed for IGS polymorphism	Code
	Sentier de la Providence, Reunion	10	Reunion n^o 8	AF-1
	Kew Gardens collection (from Kenya)	2		
O. maroccana	Mentaga, Atlas, Morocco	3	Mentaga n^o 3	MA
(*O. e.* subsp. *laperrinei*)	Immouzzer, Atlas, Morocco	4		
	Davis Collection (Atlas, Morocco)	1		
O. laperrinei	La source, Hoggar, Algeria	1	Hoggar n^o 1	LA
(*O. e.* subsp. *laperrinei*)	Davis Collection (Hoggar, Algeria)	10		
O. cerasiformis	La Palma, The Canary Islands, Spain	10		
(*O. e.* subsp. *cerasiformis*)	La Gomera, The Canary Islands, Spain	1		
	Tenerife, The Canary Islands, Spain	1		
Hybrids *O. africana* × *O. e.* subsp. *europaea*	INRA Montpellier collection	14		

used: Al, A2, A9, A10, C9, C15, E15, O8 (Bioprobe). A presence/absence matrix of RAPDs was constructed (not shown). The same size between two fragments revealed in two different individuals was assimilated to a sequence homology. For the eight used primers, such a consideration has been shown to be acceptable by Besnard et al. (submitted).

Statistical analyses

AM0VA analyses

From RAPD data, a global index of population differentiation using a one-way MANOVA model, ϕ_{st}, was determined from a Molecular Analysis of Variance (AMOVA) (Excoffier et al., 1992). A differentiation index for each pair of populations, ϕ_{stp}, was also computed, and a dendrogram based on these parameters was built with the Neighbor Joining Algorithm (Saitou and Nei 1987). The feral populations were not considered in these computations. The significance levels of the ϕ_{st} and the ϕ_{stp} were computed using permutations of individuals between populations. Thus, $\phi_{st}{}'$ and $\phi_{stp}{}'$ were computed on a matrix of individuals randomly selected and the significance level was determined from the number of occurrences where $\phi_{st}{}'$ or $\phi_{stp}{}'$ were greater than ϕ_{st} and ϕ_{stp} on the total number of permutations.

Discriminant analyses

The following analyses were performed with the OPEP software (Baradat and Labbé 1995). A Discriminant Analysis on Qualitative Data (DISQUAL procedure: Saporta 1990; Lebart et al. 1997) was performed to precise the relationships between populations. The principle of the DISQUAL procedure is to use, as input data of the Discriminant Analysis, the coordinates of the individuals for the most significant axes of a Multiple Correspondence Analysis. We used this method in a geographical area restricted to the Mediterranean Basin, only using the RAPDs showing polymorphism within this zone. The sizes of these populations ranged from 5 to 22 trees. The same analysis was done for the subspecies *cuspidata* for which the sizes of the populations ranged between 2 and 10 trees. Moreover,we used this method to compare the genetic proximities between a set of 121 cultivars and the 8 main groups of the *O. europaea* complex displayed by the ϕ_{stp} differentiation indices of the AMOVA and the associated dendrogram described above. We used at this step the complete set of RAPDs, which were polymorphic overall natural area. The RAPD profiles of cultivars were used as supplementary data since the first step of the DISQUAL procedure.

One-marker differentiation parameters

In order to compare the different polymorphic markers for an overall measure of the differentiation between populations from the eastern and western parts of Mediterranean, we computed the corresponding intra-class correlation coefficients. What we call further 'regions' are entire countries or parts of them (e.g.: Corsica or Sicily). Differentiation parameters were computed from the coordinates of individuals on the first axis of a Multiple Correspondence Analysis, which gives the most significant pattern for the complete RAPD profile of each individual. The computed parameters were based on a two-way nested ANOVA non-orthogonal model which was performed to estimate the parameters of genetic differentiation between regions, between populations within a given region, or between regions and populations within regions:

$$y_{ijk} = \mu + Ci + p_{ij} + e_{ijk}$$

where y_{ijk} is the k^{th} value of the j^{th} population in the i^{th} region, μ is the overall mean, c_i is the effect of the i^{th} country, p_{ij} is the effect of the j^{th} population within the i^{th} region and e_{ijk} is the random individual deviation from the population average. The variances corresponding to these effects are σ_c^2, $\sigma_{p|c}^2$ and σ_e^2, respectively.

Using suffix symbols analogous to those of Excoffier et al. (1992) for the 'ϕ parameters' of the AMOVA, computed with a different algorithm, we defined the three intra-class correlation coefficients, θ:

$$\theta_{st} = \frac{\sigma_c^2 + {}^2_{p|c}}{\sigma_c^2 + \sigma_{p|c}^2 + \sigma_e^2}$$

(Overall differentiation due to region and population within region)

$$\theta_{ct} = \frac{\sigma_c^2}{\sigma_c^2 + \sigma_{p|c}^2 + \sigma_e^2}$$

(Overall differentiation due to region alone)

$$\theta_{sc} = \frac{\sigma_{p|c}^2}{\sigma_{p|c}^2 + \sigma_e^2}$$

(Differentiation of populations within a given region)

For the subspecies *cuspidata*, only a simpler one-way ANOVA was done using ten populations of which the sizes ranged from two to ten trees. The corresponding model was:

$$y_{ij} = \mu + p_i + e_{ij}$$

where p_i is the effect of the i^{th} population and, with variance σ_p^2 and e_{ij} is the j^{th} deviation from this population. It allowed the estimation of a unique θ_{st} value for the overall differentiation of populations:

$$\theta_{st} = \frac{\sigma_p^2}{\sigma_p^2 + \sigma_e^2}$$

These computations were done only for the RAPDs with an average frequency comprised between 0.125 and 0.875. It led to 36 retained RAPDs for the subspecies *europaea* and 30 for the subspecies *cuspidata*.

The significance of all these parameters from two-way and one-way ANOVA was assessed by the Jackknife method (Shao & Tu, 1995), deleting one individual at a time from the complete data set.

Relationship between genetic and geographic distances

Lastly, we measured within these two sets of populations (Mediterranean olive and subspecies *cuspidata*) the relationships between the population differentiation and their physical separation by studying the trends between the genetic and the geographical distances. The genetic distances were measured by the Mahalanobis distances from the Discriminant Analyses on Qualitative data described above.

IGS analysis

Isolation of olive rRNA genes

We isolated clones carrying rRNA genes from an olive genomic bank in *Lambda Fix II* phages from leaves of 'Moraiolo' cultivar provided by L. Baldoni (IRO, Perugia). Phages were mixed with bacteria in multiplication [XL1-Blue MRA (P2) (Stratagene)], and then seeded on LB-Top agarose plate (20 × 25 cm). This was incubated at 37 °C one night. A replicate of the phage culture was made by a contact of the culture with Hybond N^+ membrane (Amersham) during 2 min. The membrane was washed in a denaturing solution (NaCH 0.5 M, NaCl 1.5 M) for 2 min, and then, in a neutralising solution (NaCl 1.5M, Tris-HCl 0.8 M, pH 8) for 5 min, and finally, in a rinsing solution (2x SSC, Tris-HCl 0.2 M, pH 8). The membrane was dried. The entire ribosomal unit from flax (Goldsbrough and Cullis 1981) was used as a probe using hybridisation conditions described by Besnard et al. (2000). The positive clones were picked up and incubated in a SM solution during one hour. Purification with chloroform was performed. The positive

clones were isolated after repetitions of the last steps as far as the phage plates were sufficiently isolated to avoid the mix of several clones. Some positive isolated clones were amplified in liquid culture using the method described by Sambrook et al. (1989) and conserved at 4 °C. Then, the inserts were isolated and ligated in pUC18 (BRL) according to provider recommendations.

Use of the olive-IGS as a probe

The physical maps of the clones were constructed with 4 restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, and *Sac*I). From the clone n^o 2, a *Bam*HI digestion enabled to liberate the fragment carrying most of the IGS region and it was separated on agarose gel (1%). This fragment was picked up and purified by the freeze squeeze method. Southern transfer carrying DNAs from one oleaster from Corsica, one oleaster from Porquerolles, *O. laperrinei*, *O. africana* from The Reunion Island, and *O. cuspidata* from Iran, separately restricted with 10 different restriction enzymes: *Bam*HI, *Bg*/II, *Cla*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Sma*I, *Xba*I and *Xho*I, was hybridised with the IGS fragment as a probe. We therefore screened those profiles for a clear readability.

To reveal RFLPs in the IGS, we chose the pairwise enzyme combination *Bam*HI-*Eco*RI. Three μg of total DNA were restricted using 24 U of each restriction enzyme (Boehringer) according to provider recommendation. The fragments were electrophoresed onto agarose gel (0.8 %) at 1.8 V/cm for 18 h. The conditions of Southern transfer and of hybridisation have been described previously (Besnard et al. 2000). Twenty-seven individuals belonging to the different taxa, except *O. cerasiformis* were characterised for RFLP in the IGS (Table 1). We constructed a matrix of presence/absence of RFLP fragments, which were sufficiently separated. Nei and Li (1979) distances were calculated between the different individuals. We used the UPGMA algorithm (Benzécri, 1973) to build the corresponding dendrogram.

Results

Level of RAPD polymorphism

The eight primers led to eighty RAPDs and eight conserved fragments. The Mediterranean olive and the subspecies *cuspidata* displayed 56 and 52 polymorphic markers, respectively. Some markers were unique or specific to a taxon (Table 2). The Mediterranean olive displayed markers common with the different subspecies. A mosaic structure was supported by these data. Based on the number of polymorphic RAPD, the intrapopulation genetic diversity was variable and the lowest values were found in the population of the Reunion Island displaying only 4 polymorphic markers while the higher was found in the 'Cap des Mèdes' population displaying 43 polymorphic markers (data not shown). The hybrid origin of 14 individuals from a cross between *O. africana* and *O. e.* subsp. *europaea* was pointed out with RAPDs, since they combined specific or unique markers of the two taxa. Any of these combinations was found in other individuals.

From the AMOVA analysis, the global ϕ_{st} computed on the whole sample was 0.448, but this parameter computed only on the Mediterranean populations was lower: ϕ_{st} = 0.247. The comparison of the one-marker differentiation parameters (θ parameters) within the Mediterranean and in the subspecies *cuspidata* shows different patterns according to the RAPDs and to the group:

In the Mediterranean (Table 3), the overall differentiation parameter (θ_{st}) including region and population within region was significant for 31 markers out of the 36 retained. The region contribution was usually greater than the population within region contribution: θ_{st} was found usually close to θ_{ct} and only 9 θ_{sc} values were significant. The genetic differentiation based on the complete RAPD profile was logically more accurately estimated and the three parameters were all significant with similar values for θ_{st} (0.773, $P < 0.001$) and θ_{ct} (0.701, $P < 0.001$) and a much lower θ_{sc}, (0.240, $P < 0.01$), confirming the general tendency observed for the one-marker structure. Ranking the frequencies of RAPD types respectively to regions clearly shown a non-independent co-occurrence of many of them. For instance, two markers: A9-460 and A10-625 were simultaneously absent in the most eastern part of the prospected geographic area (Syria, Israel and Turkey).

For the subspecies *cuspidata*, 29 RAPDs over 30 gave a significant structure with often high θ_{st} values, over 0.5 (Table 4). This meant an average differentiation greater for the subspecies *cuspidata* than for the Mediterranean olive. The very high value of the parameter for the RAPD profile (θ_{stg} = 0.988, $P < 0.001$) confirmed these observations. In this subspecies, we found the same systematic between-maker associations than for the Mediterranean group. For in-

Table 2. Number of markers unique or specific for each taxon. A minimum frequency of 5% was considered. *For the subspecies *europaea*, we did not consider the feral populations and the cultivars, and thus only 20 populations were considered.

Taxa	Number of studied individuals	Number of studied populations	Unique marker	Frequency in the taxa
O. e. subsp. *europaea*	235 (oleasters*)	20	A1-275	0.52
			A1-300	0.30
			A1-850	0.17
			C15-425	0.08
			C15-1100	0.07
O. maroccana	8	2	A1-250	0.88
			O8-500	0.25
			C15-1350	1.00
O. cerasiformis	12	3	A9-500	0.83
			E15-1050	1.00
O. laperrinei	2	1	–	–
O. chrysophylla	9	1	O8-1080	0.67
O. africana	52	9	A9-450	0.81
			A9-460	0.14
			C9-1075	0.94
O. cuspidata	7	3	A9-250	0.72
			A10-800	0.43

Table 3. Differentiation parameters for individual RAPDs of the subspecies *europaea*.

RAPD	A1-225	A1-275	A1-300	A1-525	A1-800	A1-825	A1-850	A1-1000	A1-1200
θ_{st}	0.526 ***	0.385 ***	0.265 *	0.120 *	0.365 ***	0.537 ***	0.282 ***	0.446 ***	0.372 ***
θ_{ct}	0.491 ***	0.164 *	0.103 NS	0.002 NS	0.254 **	0.337 ***	0.099 NS	0.304 ***	0.211 ***
θsc	0.068 NS	0.264 **	0.181 *	0.119 NS	0.149 NS	0.302 ***	0.203 NS	0.204 *	0.204 *
RAPD	A2-450	A2-480	A2-500	A2-650	A9-225	A9-275	A9-650	A9-675	A9-700
θ_{st}	0.417 ***	0.136 *	0.203 **	0.670 ***	0.209 **	0.406 ***	0.304 ***	0.132 NS	0.177 *
θ_{ct}	0.411 ***	0.117 NS	0.000	0.670 ***	0.206 **	0.286 ***	0.190 **	0.125 NS	0.000
θ_{sc}	0.010 NS	0.022 NS	0.203 **	0.000	0.003 NS	0.168 NS	0.41 *	0.008 NS	0.177 *
RAPD	A9-950	A10-400	A10-625	A10-875	A10-1050	A10-1250	C15-400	C15-675	C15-950
θ_{st}	0.153 ***	0.306 ***	0.204 ***	0.132 NS	0.118 *	0.297 ***	0.099 NS	0.178 NS	0.184 *
θ_{ct}	0.153 ***	0.231 ***	0.102 NS	0.000	0.118 *	0.297 ***	0.085 NS	0.000	0.103 NS
θ_{sc}	0.000	0.098 NS	0.114 NS	0.132 NS	0.000	0.000	0.015 NS	0.178 NS	0.090 NS
RAPD	E15-700	C9-450	C9-500	C9-1000	C9-1050	O8-200	O8-550	O8-1025	O8-1050
θ_{st}	0.135 NS	0.161 *	0.423 ***	0.074 NS	0.116 *	0.232 ***	0.261 ***	0.165 **	0.306 ***
θ_{ct}	0.000	0.115 NS	0.203 **	0.009 NS	0.013 NS	0.181 *	0.132 NS	0.145 *	0.300 ***
θ_{sc}	0.135 NS	0.052 NS	0.275 **	0.066 NS	0.105 NS	0.062 NS	0.149 NS	0.023 NS	0.008 NS

**: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; NS: not significant.

Table 4. Differentiation parameters for individual RAPDs of the subspecies *cuspidata*.

RAPD	A1-825	A2-450	A2-475	A2-480	A2-500	A2-650	A9-450	A9-475	A9-675	A9-700
θ_{sc}	0.722 ***	0.834 ***	0.918 ***	0.822 ***	0.393 *	0.576 **	0.723 ***	0.710 ***	0.536 ***	0.794 ***
RAPD	A9-950	A10-1050	A10-1250	C15-650	C15-950	C15-1000	E15-950	C9-400	C9-475	C9-500
θ_{st}	0.413 *	0.000	0.449 *	0.808 ***	0.731 ***	0.894 ***	0.810 ***	0.489 **	0.895 ***	0.650 ***
RAPD	C9-850	C9-900	C9-925	C9-1075	C9-1100	O8-300	O8-850	O8-1080	O8-1100	O8-1125
θ_{st}	0.415 **	0.597 **	0.383 NS	0.931 ***	0.517 *	0.851 ***	0.875 ***	0.559 *	0.469 *	0.447 **

***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.995$; NS: not significant.

stance, O8-300 and O8-1100 were met together in Iran and Yemen and could not be found in other regions (Eastern and Southern Africa). On the opposite side, A1-825, A9-450 and C9-1075 where both absent in these two regions and represented in all other regions. These two regions were shown as significantly separated from the others on the basis of the first axis of the Correspondence Analysis at the 0.1% level (data not shown).

Delineation of relationships between populations and taxa

Overall pattern of relationships

The matrix of differentiation indices between populations and taxa from AMOVA analysis (ϕ_{stp}) and the corresponding dendrogram (Figure 1) enabled us to distinguish all the groups defined by morphological observations and their geographical origin: *O. e.* subsp. *europaea, O. cuspidata, O. africana, O. chrysophylla, O. laperrinei, O. maroccana* and *O. cerasiformis*. Central and North-Western African taxa (*O. cerasiformis, O. maroccana* and *O. laperrinei*) were the most related to the Mediterranean olive.

We can notice that the two populations from the Porquerolles Island ('La Repentence' and 'Cap des Mèdes' separated by about 1500 m) were significantly different ($\phi_{stp} = 0.165$; $P < 0.01$). This indicates a likely different origin of these two groups of trees. Indeed, the morphology of some individuals from these two populations appeared different in the field collection.

Separate analysis of the subspecies europaea *and* cuspidata

The Qualitative Discriminant Analysis of the 20 Mediterranean populations showed a high resoluting power: over the 19 axes of the analysis, 11 were significantly discriminant (Wilks test) at a minimum level of 1 % and the 12th at the 2 % level. All the tests F of Mahalanobis distances (12 and 204 d.f.) between populations were significant, at least at the 0.1% level. Figure 2A shows the positions of the centroids of the 20 populations on the plane of the first two axes (42.79 % of the discrimination). A strong separation is clear on the first axis (32.38 % of the discrimination) between the four eastern populations and the other ones. The unique Libyan population of the sample (Cyrenaique) appeared as a transition between the eastern and western groups. The global consideration of both Figure 1 and Figure 2A shows that the subspecies *europaea* displayed a gradient of differentiation between East and West Mediterranean. The feral populations appeared clustered together, and they were also in an intermediate position between Eastern and Western populations (Figure 2A).

For the subspecies *cuspidata* (10 populations), Figure 2B gives the positions of the population centroids on the first two axes. These axes represented 93.67% of the discrimination and all the differences between populations from different taxa were significant at the 0.1% level. The first axis displayed a clear separation between *O. africana* populations and *O. cuspidata* from Iran. The subspecies *cuspidata* was thus not homogenous and a genetic gradient was suggested between Asia and South Africa. The *O. chrysophylla* population from Arabia was genetically intermediary between *O. africana* and *O. cuspidata*. Nevertheless, a greatest affinity of *O. chrysophylla* was found with *O. africana*, as shown by Figure 2B.

Relationships between genetic and geographic distances within the subspecies europaea *and* cuspidata

For the subspecies *europaea*, a significant trend between the genetic and geographic distances was found (Figure 3A). The genetic distance between populations in this zone appeared as an increasing func-

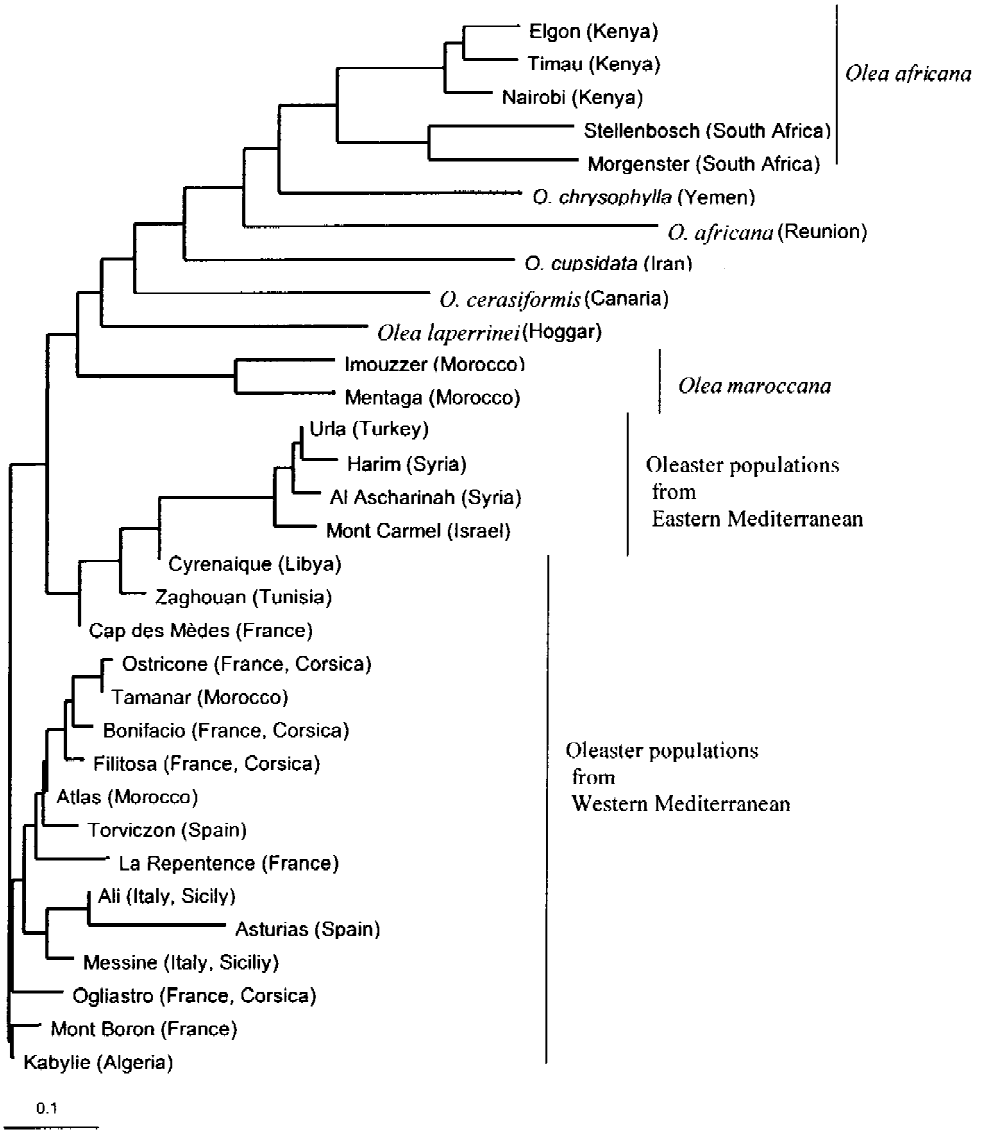


Figure 1. Dendrogram based on ϕ_{stp} and constructed with the Neighbor Joining algorithm for wild populations.

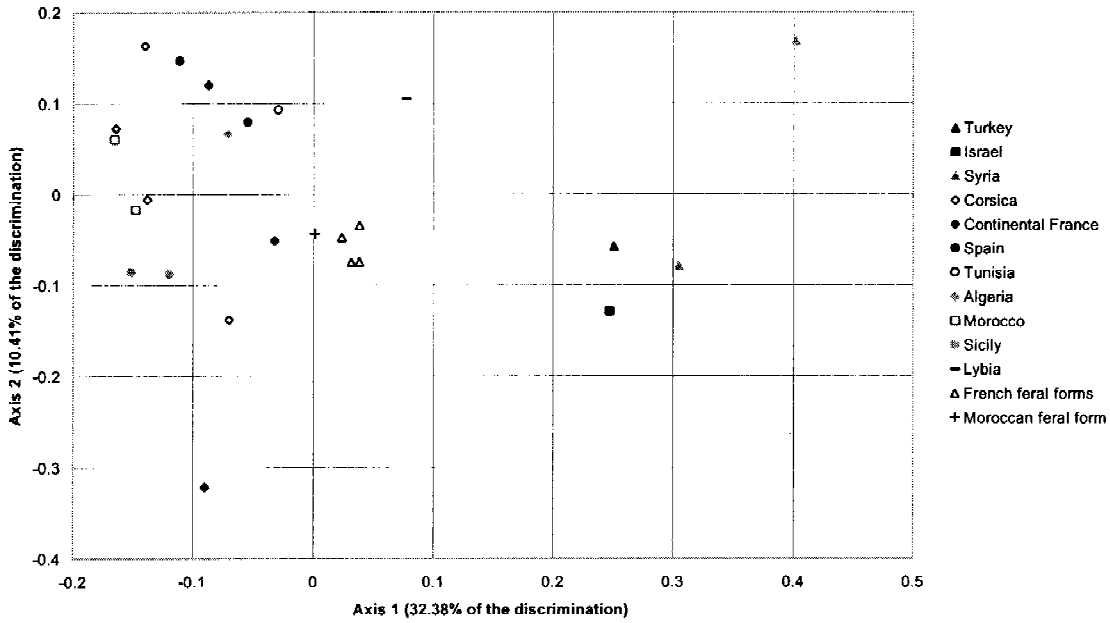
tion of the geographic distance. This highly significant relationship may be fitted by a third-order polynomial regression which gave a slightly better adjustment to data than a simple linear regression ($R^2 = 0.428$, $P < 0.001$ in place of 0.408, $P < 0.001$.

For the subspecies *cuspidata*, Figure 3B shows also a significant trend when considering separately the relationships within the *africana* populations or between this taxa and the *chrysophylla* or *cuspidata* populations. The distributions within each group are obviously not bivariate normal due to association of pairs of coordinates into clusters. The three regression lines are parallel and the Spearman's rank correlation coefficient of the two distances adjusted for group effect was highly significant: r = 0.92 for $P < 0.001$ 22 d.f.).

Genetic relationships between cultivars and wild olive

Concerning the relationships between the cultivars and the natural populations, we noted that the former display 45 RAPDs which were all found in oleasters. This shows the close relationship of cultivars with the wild Mediterranean olive. Figure 4 shows this prox-

A.



B.

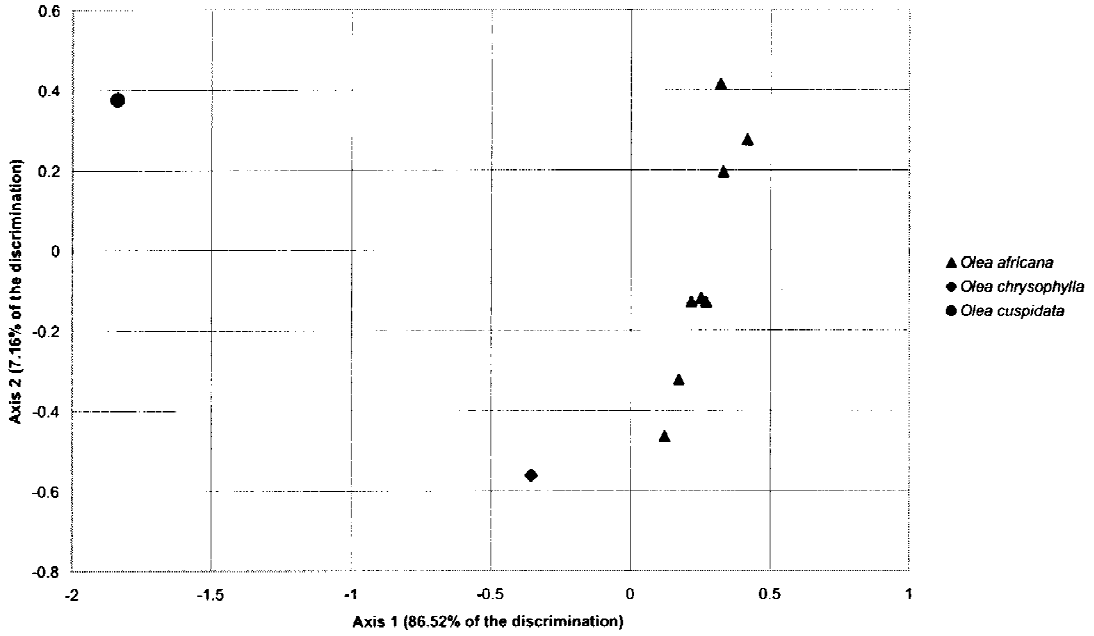
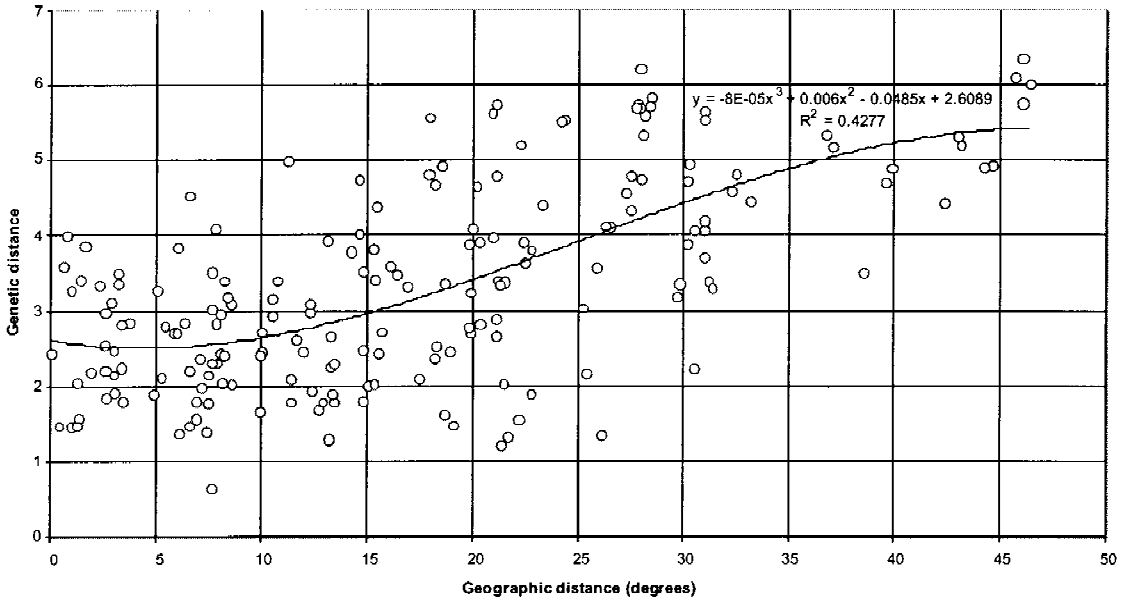


Figure 2. Qualitative Discriminant Analysis on the RAPD profiles: A. Coordinates of the 20 populations from East and West Mediterranean on the plane of the two first axes of a Qualitative Discriminant Analysis; B. Separation of the three taxa of the subspecies *cuspidata* on the plane of the first two axes of a Qualitative Discriminant Analysis.

A.



B.

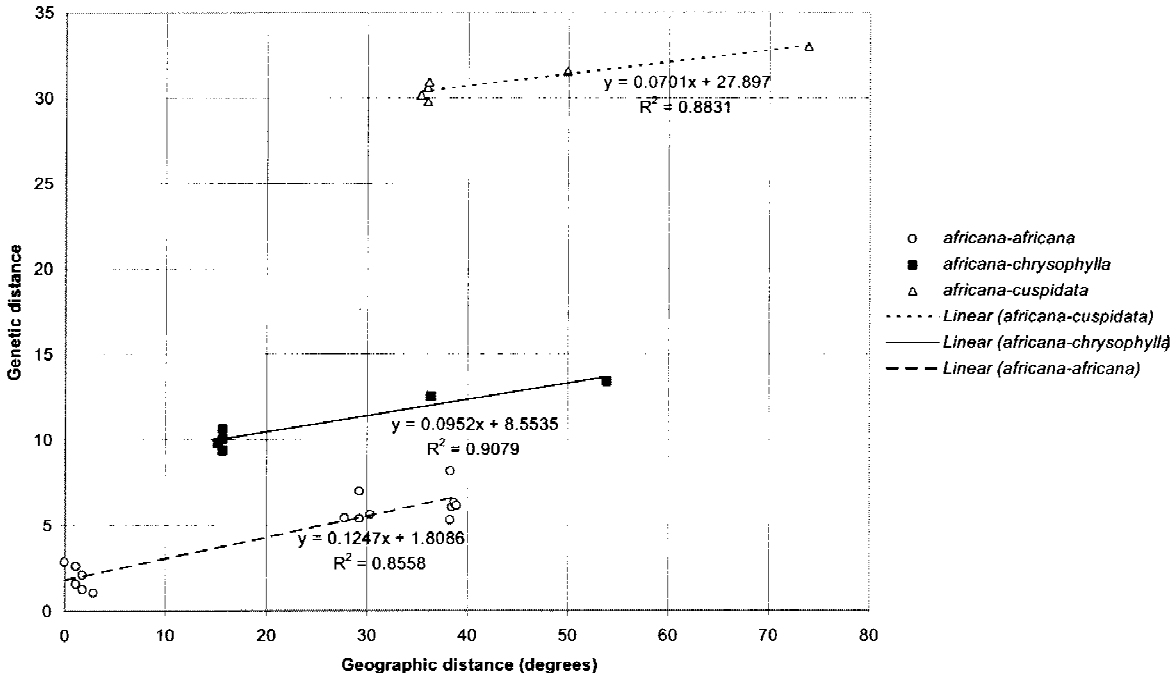


Figure 3. Relationship between genetic and geographic distances: A. for the wild Mediterranean olive; B. for the subspecies *cuspidata*.

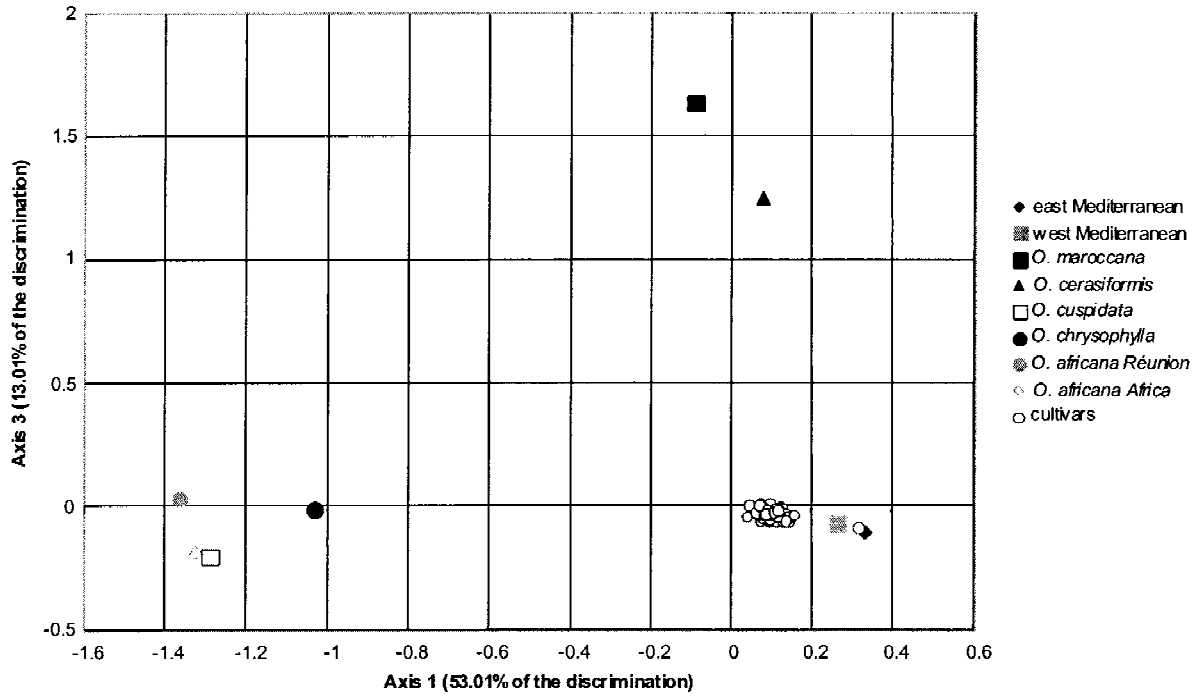


Figure 4. Genetic proximities between olive cultivars and different taxa of the *O. europaea* complex.

imity: the group of 121 cultivars is closely clustered with the oleasters. Taking into account the matrix of Mahalanobis distances between the 121 cultivars and the 8 taxa computed on the five significant axes, we confirmed that, without any exception, a given cultivar was always clustered into the Eastern or Western Mediterranean groups but never to another one (data not shown).

RFLPs in the rRNA genes

Four phage clones carrying a rDNA unit were isolated. These units were very similar but presented some differences in the IGS region. The restriction maps of these four clones (Figure 5) were in agreement with the restriction map of *O. europaea* established by Besnard et al. (submitted) using the RFLP method. In addition, a *Sac*I site (*S**) in the olive IGS was not detected by RFLP but we suggested that methylation of this site prevented the restriction in olive. From the clone n^o 2, we isolated the 5.2 kb fragment (B4-B1) carrying most of the IGS region. This fragment, used as a probe, hybridised weakly DNAs of the *Olea* species from the section *Ligustroides* (data not shown). In the twenty-seven individuals studied, the IGS probe enabled us to reveal 29 well separated RFLPs and led to intrataxa and intertaxa polymorphic fragments (Table 5). All the individuals were differentiated, excepted 'Moraiolo' (C4) and 'San Felice' (C6), but some bands were not exploited because insufficiently separated. Mediterranean olive displays common RFLPs with other sub-species. The phenetic tree based on these RFLPs showed the distinction of *O. africana* from the other taxa (Figure 6). One diagnostic fragment (F4 = E4-E5) was specific to *O. africana*. This fragment has been already shown by Besnard et al. (submitted).

Most of the studied individuals displayed probably several types of rDNA units because summing the size of all the IGS-RFLPs of a lane is much more higher than the expected 5.2 kb of the IGS region excepted in *O. africana* from The Reunion Island. Nevertheless, we can suppose that the methylation of the IGS sequence could lead to partial restriction and thus to several fragments for one rDNA unit.

Discussion

Classification of the O. europaea *taxa using RAPDs*

The classification of trees based on RAPDs enabled us to distinguish more taxa than the taxonomy based on

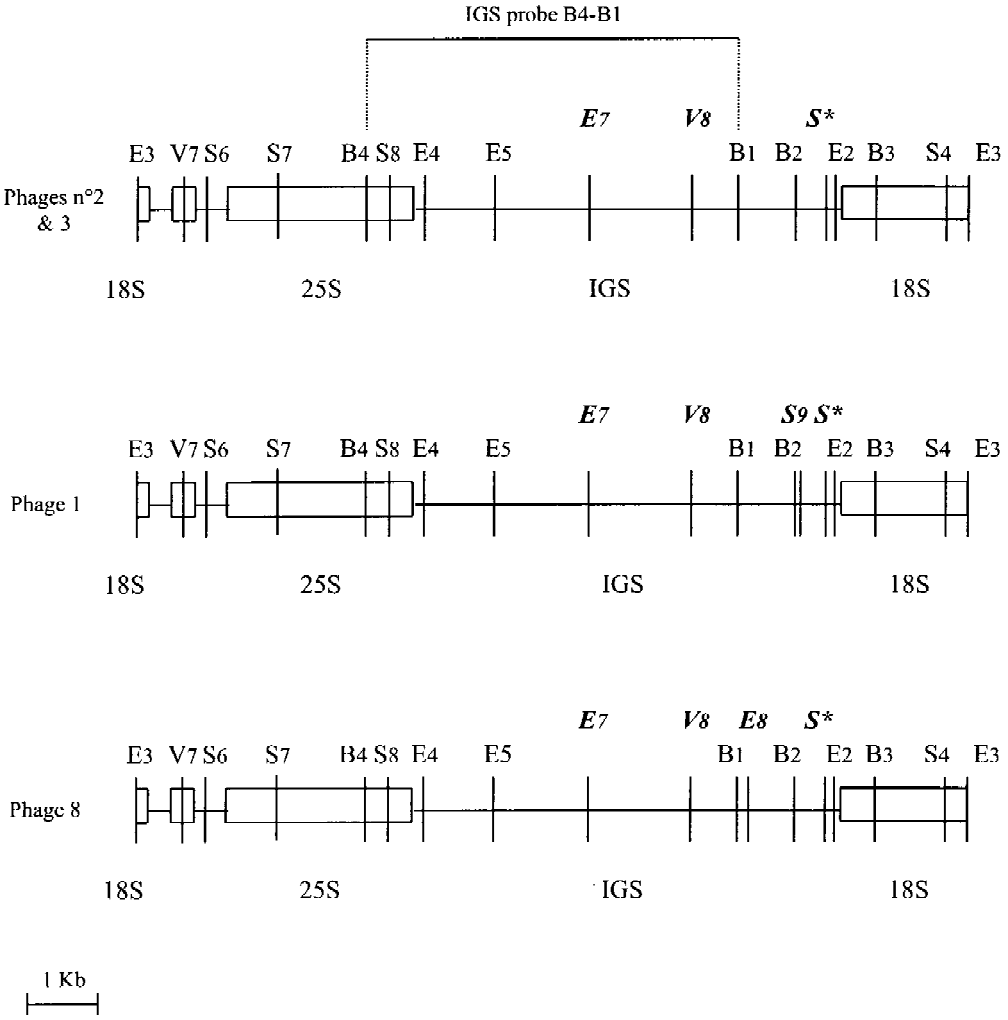


Figure 5. Restriction maps of the olive rDNA clones. The restriction sites are noted according to Besnard et al. (submitted). The restriction sites in bold type correspond to the sites not detected with the RFLP method. The no detection of *S** is attributed to methylation of the site.

morphology (Green and Wickens 1989). Thus, we distinguished two taxa in the Mediterranean Basin (East and West), two for the subspecies *laperrinei* (*O. laperrinei* and *O. maroccana*), and the three taxa of the subspecies *cuspidata* (*O. africana*, *O. chrysophylla*, and *O. cuspidata*) were clearly recognised. This latter distinction was not possible using the morphologic traits indicated by Green and Wickens (1989). The geographic isolation of these taxa certainly explains their differentiation. Moreover, we pointed out the sexual intercompatibility between two distant taxa: *O. africana* and *O. e.* subsp. *europaea*. This compatibility sustains the definition of the *O. europaea* complex and underlines the interest of all these taxa for olive genetic resources.

The Northern African subspecies *laperrinei* and *cerasiformis* were the taxa the most related to the Mediterranean olive, as already shown with AFLP markers on the same individuals (Angiolillo et al. 1999). As for numerous other species (Hewitt 1996), geographic isolation, population reduction and further expansion due to climatic changes have probably contributed to the divergence of the Northern African and Mediterranean taxa and to the geographic structure of their genetic diversity.

Furthermore, cultivars and oleasters were found to be closely related. The comparison of these two taxa should lead to reveal the origins of the genetic background of the cultivated olive (Besnard et al., in preparation).

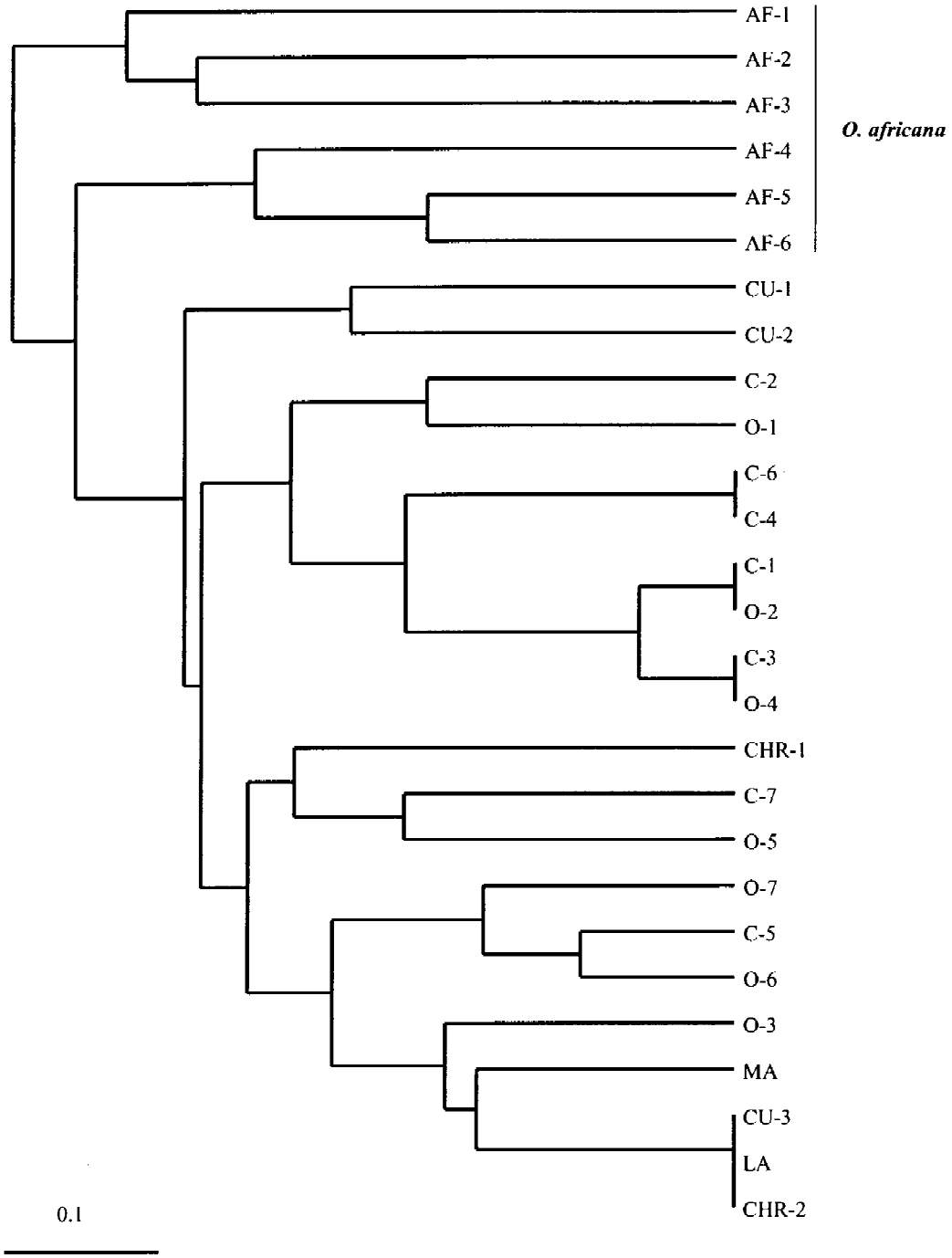


Figure 6. Dendrogram based on distances of Nei & Li (1979) from IGS RFLP data and constructed with the UPGMA algorithm.

Structure of the genetic diversity between the O. europaea *taxa*

A clear relationship between genetic and geographic distances was observed in two parts of the overall *O. europaea* repartition. The influence of distance seems higher within the subspecies *cuspidata* than within the Mediterranean olive. For the later group, this might reflect the human influence, which could generate gene flows towards various and opposite directions leading

Table 5. List of the IGS RFLPs for each tested taxon. The approximate size of each fragment is in brackets. The bold characters mean that the fragment is displayed both in the Mediterranean olive and in another taxon. UF = Number of unique fragment in a taxon; NF = Number of fragment displayed by a taxon.

RFLP	subsp. *europaea*	subsp. *laperrinei*	*O. cuspidata*	*O. chrysophylla*	*O. africana*
Fl (300)	8/14	–	–	–	–
F2 (600)	**7/14**	–	**2/3**	–	–
F3 (650)	2/14	–	–	–	–
F4 (900)	**14/14**	**2/2**	**3/3**	**2/2**	–
FS (950)	–	–	–	1/2	–
F6 (1400)	–	–	–	–	1/6
F7 (1500)	–	–	1/3	–	1/6
F8 (1700)	1/14	–	–	–	–
F9 (1800)	–	–	–	–	2/6
F10 (2000)	–	–	–	–	5/6
F11 (2200)	–	–	–	–	2/6
F12 (2400)	–	–	–	–	1/6
F13 (2500)	**1/14**	–	**1/3**	–	**1/6**
F14 (2800)	**6/14**	–	**1/3**	–	**1/6**
F15 (3000)	**4/14**	–	–	**1/2**	**1/6**
F16 (3200)	–	–	–	–	3/6
F17 (3300)	**1/14**	–	**1/3**	–	–
F15 (3400)	–	–	2/3	–	–
F19 (3500)	**14/14**	**2/2**	**3/3**	**2/2**	**3/6**
F20 (3600)	–	–	–	–	3/6
F21 (3900)	**2/14**	–	–	–	**1/6**
F22 (4000)	–	–	–	–	1/6
F23 (4100)	–	–	–	1/2	–
F24 (4200)	4/14	–	–	–	–
F25 (4300)	5/14	–	–	–	–
F26 (4400)	**12/14**	–	–	**1/2**	**2/6**
F27 (5000)	**1/14**	**1/2**	–	–	–
F28 (5500)	**2/14**	–	–	**1/2**	–
F29 (6000)	**7/14**	–	–	**1/2**	**2/6**
NF	17	3	8	8	16
UF	5	0	1	2	8

to dim the divergence. Epperson (1990) has reviewed the causes and the measures of spatial patterns of genetic variations. The general model relating the kinship coefficient, phi_{ij}, between two genes, i and j, probability that they originate from the same ancestral population, under gene flow limited by distance has the general form: $\phi_d = d^{-c}\ ae^{bd}$. In this expression, d is the distance between the two considered populations, and, a, b, c are positive constants depending on the gene flow characteristics. This model reduces into: $\phi_d = ae^{-bd}$ for a stepping stone model where gene flow only occurs between adjacent populations. Therefore, the genetic divergence between populations, measured by the probability to meet common alleles is always an increasing function of distance, whatever the values of the constants. But olive selection by human should heavily reduce the spatial correlation. Indeed, RAPDs, in spite of their neutrality, might be associated with non-neutral loci (linkage disequilibrium), resulting into a 'hitch-hiking' effect.

The wide geographic distribution of the subspecies *europaea* and *cuspidata* was well represented by our sample. In these two subspecies, a genetic diversity gradient was shown:

First, we suspected that the gradient of diversity between Asia and Africa might be due to a hybridisation between two differentiated taxa (*O. cuspidata* and *O. africana*) in the two continents (Besnard and Bervillé, 2000). This is sustained by the fact that *O. africana* and *O. cuspidata* displayed several unique markers and that *O. chrysophylla* combined markers only present in *O. africana* or in *O. cuspidata*. The taxa *O. chrysophylla* displayed only one specific marker. Thus, *O. chrysophylla* from Yemen was likely an hybrid taxa between *O. cuspidata* and *O. africana*.

Second, two taxa were distinguished in the Mediterranean Basin. This might reflect at least two independent refugial zones during the last glaciation (20,000–12,000 BP) or independent olive introductions from region adjacent to the Mediterranean. Within the Mediterranean wild olive, the differentiation between populations was also correlated to the geographic distances and this fact confirms the general importance of isolation by distance for the differentiation within the *O. europaea* complex. This pattern is still apparent in spite of dissemination by human of the species, which has occurred at least since the Holocene (Terral and Arnold-Simard 1996). The Libyan and the feral populations were found genetically intermediate between Eastern and Western populations. These populations did not display unique markers and combines Eastern and Western markers. Consequently, these populations likely derive from an hybridisation between east and west populations. The natural dissemination or the human displacement of cultivars from the Eastern Mediterranean (Zohary and Spiegel-Roy 1975) might explain the existence of such hybrid populations. We cannot exclude that some other western populations may be hybrids. This could be demonstrated by using codominant markers as microsatellites.

rDNA polymorphism insights

The use of one IGS as a probe to reveal rDNA polymorphism is not a common method but this has been already performed in radish (Tremousaygue et al. 1988). The evolution of IGS has been reported rapid in the several plant species and in particular in the genus *Fraxinus* (Jeandroz et al. 1996). This explains probably the low sequence homology between IGS of the species belonging to the section *Ligustroides* and this of the species belonging to the *O.europaea* complex. Consequently, this method enabled us to detect much more rDNA polymorphisms that the classical approach using the rDNA coding sequences as a probe (Besnard et al., submitted). In olive, we obtained a complex pattern, which reflected likely the existence of several rDNA unit types in a same individual. Furthermore, three rDNA loci have been detected by *in situ* hybridisation in the olive tree (Cionini et al., personal communication). This fact may explain why an intra-individual polymorphism is conserved in a species. Consequently, it is difficult to recognise each rDNA unit type. Nevertheless, the high level of IGS variation in olive brought some insights about the relationships between the different *O. europaea* taxa. Mediterranean olive shared common fragments with the different taxa. Moreover, *O. cuspidata* CU-3, *O. laperrinei* LA and *O. chrysophylla* CHR-2 were closely related, and this argues for a common origin of these taxa or gene flows between them. The trees may derive from an ancestral population covering Central Africa, Eastern Africa and Western Asia. Intra-population polymorphisms were also detected for *O. chrysophylla* and *O. cuspidata*. The homogenisation of rDNA unit types located in different arrays is a long process and has been modelled in different species: the main mechanisms are unequal crossing over and conversion (Santoni and Bervillé 1992). Consequently, in absence of gene flow between differentiated populations, we expected a low level of polymorphism in a population. Therefore, the intrapopulation polymorphisms, revealed in *O. chrysophylla* and *O. cuspidata*, sustain that gene flows between differentiated taxa have occurred. Furthermore, *O. africana* was distinguished from the other taxa. This present clear separation results likely from an ancient geographic isolation of this taxa from the others. The mixing of the different subspecies relatively to IGS polymorphism could be attributed to hybridisation between some taxa before the migrations or the isolation of the present taxa. This could explain why in the *O. europaea* complex, a mosaic structure is revealed using RAPDs, IGS polymorphisms but also AFLPs (Angiolillo et al. 1999) and cytogenetic data (Bitonti et al. 1999). Thus, Mediterranean olive could derive from an ancestral population introgressed between Northern African and Asian taxa. It is therefore clear that all these species represent an important source of genes for olive since they are located in different climatic zones with severe stresses, mainly drought and cold.

Conclusion

The long process of genetic differentiation of olive resulted in a wide divergence between populations, which gave the different interrelated taxa were described. We observed a close relationship between the conclusions of direct spatial structure measured by regression of genetic distance on geographic distance and the parameters of genetic differentiation computed from variance components (prevailing role of large-scale spatial structure over small-distance variability). The present structure of genetic polymorphism between the different regions of its wide geographic area, as countries or eastern-western parts of Mediterranean Basin, is therefore in favour of a large-scale geographic differentiation of olive tree. Community of origin and gene flow maintained a relative genetic uniformity between populations in limited areas.

The realised crosses between the different taxa suggest that gene flows between them could occur during more or less recent stages of their differentiation. Our results about trends between genetic and geographic distances agree with the existence of such gene flows limited by distance.

Other groups than Mediterranean olive could be used for the improvement of cultivated olive tree (resistance to disease, quality . . .). For example, the fruits of *O. chrysophylla* from Arabia and Eastern Africa are sweet (Zohary, 1994). Moreover, we observed a low bitterness in the fruits of the hybrids *O. africana* – Mediterranean olive. Consequently, prospection and conservation of the taxa related to Mediterranean olive is required to evaluate the agronomic value of such trees but also to study the organisation of the genetic diversity on a greater sample and then to elucidate the origins of the Mediterranean olive. Moreover, the analyses of genetic diversity with different markers (RAPDs, cytoplasmic DNA, microsatellites) will enable to examine the different ways to improve the discrimination of populations or individual trees among the *O. europaea* complex.

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