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High Genetic Diversity and Clonal Growth in Relict Populations of *Olea europaea* subsp. *laperrinei* (Oleaceae) from Hoggar, Algeria

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• *Background and Aims* The Laperrine's olive (*Olea europaea* subsp. *laperrinei*) is an endemic tree from Saharan massifs. Its populations have substantially regressed since the Pleistocene and are presently distributed in a fragmented habitat. Long-term persistence of this taxon is uncertain and programmes of preservation have to be urgently implemented. To define a conservation strategy, the genetic diversity and breeding system of this tree have to be investigated.
• *Methods* One hundred and eleven ramets were prospected in the *laperrinei* populations from the Tamanrasset region, southern Algeria. Genetic polymorphism was revealed at nuclear and chloroplast DNA (cpDNA) microsatellite loci allowing a comparative assessment of the genetic diversity of *laperrinei* and Mediterranean populations based on bi-parental and maternal markers. Additionally, nuclear microsatellite markers enabled the genotypes to be identified unambiguously.
• *Key Results* Based on nuclear microsatellite data, the total diversity was high ($H_t = 0{\cdot}61$) in *laperrinei* populations and similar to that observed in western Mediterranean populations. A substantial cpDNA diversity ($H_t = 0{\cdot}19$) was also observed. Genetically identical ramets originated from the same stump (which can cover >80 m^2) were identified in each population. Sixteen per cent of genets exhibited more than one ramet. In addition, several cases of somatic mutations were unambiguously revealed in distinct ramets stemming from the same stump.
• *Conclusions* These data show that highly isolated and small *laperrinei* populations are able to maintain a high genetic diversity. This supports the existence of relict trees persisting for a very long time (probably since the last humid transition, 3000 years ago). It is proposed that the very long persistence associated with an asexual multiplication of highly adapted trees could be a strategy of survival in extreme conditions avoiding a mutational meltdown due to reproduction in reduced populations.

Key words: Chloroplast DNA, clonal structure, conservation, microsatellite, olive tree, Saharan desert, threatened species, wild genetic resources.

INTRODUCTION

The olive (*Olea europaea* subsp. *europaea*; Oleaceae) is an emblematic species that represents one of the most important fruit trees in the Mediterranean basin (Loumou and Giourga, 2003). Its primary genetic resources are taxonomically classified in the *Olea europaea* complex in which six subspecies are recognized (Green, 2002). Wild olive populations are distributed on a large area covering southern Asia, a great part of Africa and southern Europe (Green, 2002). Some populations are presently affected by several threats, due to human activities and/or climatic vicissitudes, so that programmes of evaluation and conservation should be urgently implemented (e.g. Médail *et al*., 2001). The genetic diversity in this complex has been analysed in a few studies (e.g. Hess *et al*., 2000; Besnard *et al*., 2001), but more specific research is needed to investigate the evolution of threatened taxa such as the subspecies *maroccana* (High Atlas, Morocco) or *laperrinei* (Saharan mountains).

The Laperrine's olive (*Olea europaea* subsp. *Laperrinei*) is a relict taxon restricted to massifs of central-southern Sahara and eastern Sahel (Wickens, 1976; Quézel, 1978; Maley, 1980; Médail *et al*., 2001; Green, 2002). This tree is present at high altitudes [from (1000) 1400 to 2800 m] on volcanic or eruptive rocks, generally in cliffs and banks of canyons (Fig. 1). This taxon is adapted to very dry conditions and in Hoggar (southern Algeria) it persists in areas reaching a mean rainfall of about 20–100 mm per year (Quézel, 1965). In a recent study (Besnard and Bervillé, 2002), maternally inherited genetic markers have attested that this subspecies is closely related to the eastern Mediterranean olive (*O. e. europaea*). However, this result was not supported by nuclear data which highlighted closer relationships between the *laperrinei* populations and north-western African taxa (Hess *et al*., 2000; Besnard *et al*., 2001). These genetic relationships probably indicate the existence of relatively recent exchanges between sub-tropical African and Mediterranean populations (during the Pleistocene) but the importance of such events has still to be assessed. This wild olive tree also displays traits of potential interest for the cultivated olive. It could be an important genetic resource for drought adaptation and, for instance, could be used as rootstock in dry areas.

Olea e. laperrinei populations have substantially regressed with desert expansion during the Pleistocene (Wickens, 1976; Quézel, 1978; Maley, 1980). As a consequence, its present distribution is highly fragmented and populations are generally composed of a few individuals (<100), and sometimes of only one isolated individual. Present populations of *O. e. laperrinei* endure extreme dry conditions and insufficient rainfall during several

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FIG. 1. Individuals belonging to the subspecies *laperrinei* in the Ti-n-Aleo canyon at Tanarouatine. The ramets are numbered 1 to 8. Based on molecular characterization, only three genets were distinguished: T-in-Aleo (1 and 2), T-in-Aleo (3) and T-in-Aleo (4–8). The stump of the last one covers about 80 m^2.

consecutive years could seriously alter their local persistence. Thus, this taxon has disappeared from Tibesti, Chad, during the Pleistocene (Wickens, 1976; Quézel, 1978). In addition, camels and cattle browse young shoots of this tree, thus limiting its development and possibilities of regeneration. A preservation programme should allow protection of present populations or reforestation of some zones where *O. e. laperrinei* is seriously declining or has recently disappeared (Baali-Cherif *et al*., 2000).

In desert conditions, the survival of *O. e. laperrinei* is greatly dependent on its particular traits. For instance, this taxon has the capacity to harness water from a great depth (due to an adapted root system) and its reduced leaf surface (lanceolate leaves) limits water loss. Moreover, this taxon is also considered to have great persistence ability. The natural vegetative multiplication (by development of shoots from stump) should favour locally adapted genotypes and/or regeneration of individuals after long unfavourable periods. In small populations, propagation of adapted genotypes via an asexual mechanism should limit inbreeding (which could be associated with inbreeding depression; e.g. Wang *et al*., 1999) and therefore confer a selective advantage in relict populations (e.g. *Cupressus dupreziana*; Pichot *et al*., 2001). Moreover, *O. e. laperrinei* trees rarely fructify in their natural environment and their regeneration is null (Maire, 1933; Quézel, 1965). For conservation purposes, it will be necessary to understand the reproductive biology of *O. e. laperrinei*. The survey of the genetic diversity in populations should help to reach this objective and to propose a strategy of preservation (e.g. El Mousadik and Petit, 1996; Pichot *et al*., 2001).

In this context, the genetic diversity was evaluated and a search was carried out to see if a clonal reproductive strategy occurs in populations of *O. e. laperrinei* from Hoggar. Genetic polymorphism was investigated at nuclear microsatellite (SSR) loci which are reported to be highly polymorphic and are suitable markers to discriminate different genotypes. These loci allowed the presence of clones in populations to be tested. Additionally, the genetic diversity in *O. e. laperrinei* in comparison to a few western Mediterranean populations was investigated using both nuclear and chloroplast DNA markers.

MATERIALS AND METHODS

Plant material

One hundred and eleven ramets of *Olea europaea* subsp. *laperrinei* (Batt. & Trab.) Ciferri were sampled in the east and north-east of Tamanrasset in four distinct mountain massifs (Adjelella, Adrar Heggueghene, Issekrâm and Tanarouatine; Table 1 and Figs 1 and 2). These mountains are located in an area covering about 2500 km^2. It was attempted to prospect all ramets present in each massif but, on some massifs (i.e. Adjelella and Adrar Heggueghene), a few ramets growing in cliffs were not accessible and were not prospected. A few leaves were collected and desiccated in silica gel for each ramet sampled. Furthermore, in Issekrâm, four distinct ramets (named Ilennanene 8a, b, c and d) originated from the same old stump were sampled on an area of about 20 m^2. These ramets have each a limited development and are intensively grazed by camels and cattle. They were characterized in order to test their genetic identity.

Additionally, to compare the genetic diversity of *laperrinei* populations with that of populations belonging to the *europaea* subspecies, wild Mediterranean olives (oleasters) were characterized. Thirty-four ramets were prospected in the south-east of Algiers (on two sites separated by 10 km: Birkhadem and Gué de Constantine; Table 1). Oleasters from Tamanar, Morocco (six) and Mt Belloua, Algeria (seven), previously characterized using RAPDs and cytoplasmic markers (Besnard *et al*., 2001, 2002), were also considered in this study (Table 1).

All individual DNAs were extracted from leaves using a CTAB method (Besnard *et al*., 2000).

Genetic markers

To characterize all individuals, nuclear microsatellites [or single strand repeats (SSR)] were chosen because such genetic markers should reveal a higher level of polymorphism compared with other markers (e.g. cytoplasmic DNA, AFLP or RAPD; Belaj *et al*., 2003) and allow a direct estimation of the heterozygosity. Nine SSR loci were selected from the literature: DCA1, DCA3, DCA8, DCA9, DCA14, DCA15 (Sefc *et al*., 2000; Bandelj *et al*., 2004), GAPU45 (Carriero *et al*., 2002), PA(ATT)2 (Saumitou-Laprade *et al*., 2000; Khadari *et al*., 2003) and EMO3 (de la Rosa *et al*., 2002). Their choice was based on three criteria: (1) loci displaying polymorphism (from 4 to 11 alleles) were selected in the cultivated olive; (2) only loci with an observed heterozygosity (H_o) similar to the expected heterozygosity (H_s) were used to eliminate all loci with null alleles; and (3) in order to perform multiplexing of loci, allele size range was considered. For each locus, one primer was labelled with a fluorochrome. Two distinct fluorochromes were used: FAM (blue) or HEX (green). PCR amplification of each locus was done separately. The PCR reaction mixture contained 10 ng of DNA template, 1× reaction buffer, 2 mM $MgCl_2$,

TABLE 1. *Geographic origin of the wild populations characterized in the present study*

Subspecies	Geographic origin	Altitude (m)	Latitude	Longitude	N_R	N_G
Subsp. *laperrinei*	Adjelella, south-east Tamanrasset, Algeria	1530	22°38′N	5°3′E	10	9
	Adrar Heggueghene, east Tamanrasset, Algeria	1390–1600	22°47′N	5°37′E	65	57
	Issekrâm, north Tamanrasset, Algeria	1525–1605	22°55′N	5°33′E	23	20
	Tanarouatine, north-east Tamanrasset, Algeria	1580–1600	23°06′N	6°03′E	13	8
Subsp. *europaea*	Gué de Constantine, south-east Algiers, Algeria	30	36°42′N	3°33′E	20	20
	Birkhadem, south-east Algiers, Algeria	30	36°42′N	3°39′E	14	14
	Mount Belloua, Tizi Ouzou, Algeria	790	36°47′N	4°04′E	7	7
	Tamanar, Essaouira, Morocco	~25	32°00′N	9°33′W	6	6

N_R corresponds to the number of ramets prospected at each site, and N_G corresponds to the number of genets identified among these ramets.

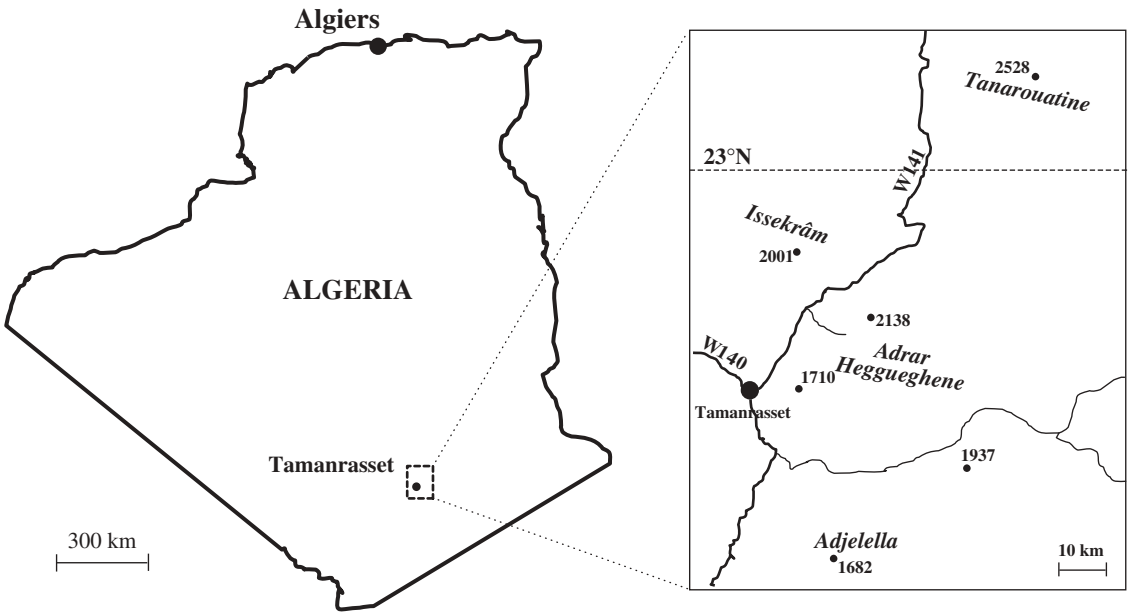

FIG. 2. Geographical location of the four mountain massifs (near Tamanrasset; 22°47′N, 5°31′E) in which populations of *O. e.* subsp. *laperrinei* were prospected in the present study.

0·2 mM dNTPs, 0·2 μmol of each primer, and 0·75 unit of DNA polymerase (Invitrogen) in a total volume of 25 μL. Reaction mixtures were incubated in a thermocycler (T1; Biometra) for 4 min at 94 °C followed by 36 cycles consisting of 1 min at 94 °C, 1 min at the defined annealing temperature (50 or 55 °C) and 1 min at 72 °C. The last cycle was followed by a 10-min extension at 72 °C. Electrophoresis of PCR products was directly carried out on a denaturing 5 % polyacrylamide gel using an automated sequencer (ABI 377; Applied Biosystems).

Twenty polymorphic sites (i.e. nine microsatellite or indel sites and 11 PCR-RFLPs) of the chloroplast DNA (cpDNA) were also investigated in the *laperrinei* and western Mediterranean *europaea* populations: ccmp5, ccmp7 (Weising and Gardner, 1999), the 14 polymorphic loci developed by Besnard *et al*. (2003), a microsatellite locus present in the *trn*T–*trn*L intergenic spacer and three additional restriction sites in the *mat*K spacer, which have recently been identified to be polymorphic in the olive complex (Table 2). The new loci (Table 2) were PCR amplified at an annealing temperature of 53 °C using conditions described before. For the other loci, PCR conditions reported in Weising and Gardner (1999) and Besnard *et al*. (2003) were used. PCR or restricted-PCR fragments were electrophoresed on a 5 % acrylamide gel as previously indicated. A sub-sample of five individuals per massif was characterized for each locus. The polymorphic loci were then used to characterize the whole sample.

Statistical analyses

Based on nuclear SSR data, the genetic relationships between ramets of the subspecies *laperrinei* were assessed. Shared allele distances, defined as 1 – proportion of shared alleles (Jin and Chakraborty, 1993), were computed between pairs of all individuals using the software Populations 1.2·28 (Langella, 1999). A phenetic tree was built using the neighbour-joining algorithm (Nei, 1987). Bootstrap values were computed using 1000 re-samplings on loci to evaluate support of the branches in both phenetic analyses.

When identical genotypes were identified on several independent ramets, it was necessary to evaluate the

TABLE 2. *Primer pairs for PCR amplifications of three chloroplast DNA regions displaying polymorphism in the* Olea europaea *complex (G. Besnard, R. Rubio de Casas and P. Vargas, unpubl. res.)*

cpDNA region	Primer sequence (5′–3′)	Fragment name	*T* (°C)	Polymorphism type	Fragment size (bp)
*trn*T-L	F: CATTCCTCCGCTTTCATTCG R: TATGTCTCTCTTCCTGCCAC	*trn*T-L-poly-A	53	LP; Poly A	106, 107
*mat*K	F: ATATCCACTTATCTTTCAGGAG R: TGGATTTATTGTCATAACCTGG	*mat*K-2-*Taq*I/*Mse*I	53	RS; *Taq*I	101 → 101 (–) → 63 + 38 (+)
				RS; *Mse*I	101 → 101 (–) → 54 + 47 (+)
*mat*K	F: AGATAGTAAAATCTCATAATTTT**T**C* R: GGGGTATTAGTATATCTAACAC	*mat*K-3-*Taq*I	53	RS; *Taq*I	102 → 102 (–) → 24 + 78 (+)

The fragment names, size variants, polymorphism types and annealing temperatures (*T*) are given.

* The bold nucleotide indicates a nucleotide change comparatively to the reference sequences (A→T) to create an absence–presence polymorphism of a *Taq*I restriction site.

LP, Length polymorphism; RS, restriction site polymorphism (nucleotide substitution); (–), absence of the restriction site; (+), presence of the restriction site.

TABLE 3. *Number of alleles (*N_a*), observed and expected heterozygosities (*H_o *and* H_s*), total genetic diversity (*H_t*) for each locus for pooled data from each of the two subspecies,* europaea *and* laperrinei

	europaea					*laperrinei*				
Locus	Allele size range	N_a	H_o	H_s	H_t	Allele size range	N_a	H_o	H_s	H_t
Nuclear SSR										
DCA1	208–244	10 (5)	0·44	0·45	0·45	222–280	24 (5)	0·91	0·92	0·92
DCA3	231–255	6 (3)	0·52	0·45	0·45	229–253	8 (3)	0·73	0·69	0·68
DCA8	127–165	16 (5)	0·83	0·83	0·89	119–147	11 (5)	0·88	0·83	0·83
DCA9	167–207	16 (9)	0·88	0·82	0·84	169–193	11 (9)	0·58	0·61	0·63
DCA14	153–192	13 (0)	0·91	0·85	0·88	144–165	12 (0)	0·83	0·77	0·78
DCA15	247–271	5 (1)	0·59	0·57	0·61	251–258	2 (1)	0·20	0·19	0·19
PA(ATT)2	106–124	5 (2)	0·66	0·61	0·66	106–109	2 (2)	0·13	0·12	0·12
GAPU45	183–185	2 (1)	0·04	0·04	0·04	185–193	5 (1)	0·62	0·58	0·59
EMO3	212–226	12 (5)	0·92	0·87	0·89	195–224	14 (5)	0·84	0·79	0·79
Total	–	85 (31)	0·64	0·61	0·63	–	89 (31)	0·64	0·61	0·61
cpDNA*	–	5 (0)	–	–	0·67	–	4 (0)	–	–	0·19

Nuclear and cpDNA data were analysed separately.

For N_a, the number of alleles shared between *laperrinei* and western *europaea* populations is in brackets.

* Based on the combination of the 20 investigated characters (see Table 4).

possibility that they represented the same genet. The probability that identical genotypes could result from independent formation of zygotes was estimated (e.g. Parks and Werth, 1993; Setsuko *et al*., 2004). The probability that a zygote acquires a given diploid genotype, P_{gen}, was calculated, following Parks and Werth (1993). $P_{gen} = (\Pi\, p_i)2^h$ where p_i is the frequency in the population of each allele represented in the genotypes and h is the number of loci that are heterozygous. The P_{gen} value represents the probability that two sampled ramets belonging to different genets would have the same genotype by chance.

Observed (H_o) and expected (H_s) heterozygosity was computed at each nuclear SSR locus independently in the *laperrinei* and western Mediterranean *europaea* populations using the Fstat software (Goudet, 1995). These estimations were based on the different genets and enabled the detection of a putative excess of homozygosity at a locus due to null alleles. The total genetic diversity (H_t; Nei, 1987) was also estimated.

RESULTS AND DISCUSSION

Marker data

Nuclear SSR markers showed a very good transferability from *europaea* to *laperrinei* confirming previous observations (Rallo *et al*., 2003). The nine nuclear SSR loci were polymorphic in both subspecies (Table 3). The total number of alleles revealed in these taxa was similar [85 for western Mediterranean (on 47 individuals) and 89 for *laperrinei* populations (on 94 genets from Hoggar; see below); Table 3]. The total gene diversity was also comparable in both subspecies (H_t = 0·63 and 0·61; Table 3). Only 31 alleles (21·7 %) were common to both *europaea* and *laperrinei* samples analysed indicating a great differentiation between these two taxa. Moreover, in the populations studied, the observed heterozygosity was very similar to the expected heterozygosity at each nuclear SSR locus, suggesting no excess of homozygosity in populations. In addition, the investigated loci should not display null alleles (or at a

very low frequency; <2 %) and should be suitable markers for a genetic population study in wild olive relatives. Such an absence of null alleles is probably due to the choice of nuclear SSR loci which have revealed very low deviation in the observed heterozygosity from the expected heterozygosity in previous studies on cultivated olives (Sefc *et al*., 2000; de la Rosa *et al*., 2002; Khadari *et al*., 2003; Bandelj *et al*., 2004). It is recommended that this choice criterion be used to avoid loci displaying null alleles being included.

Four cpDNA haplotypes were revealed in the subspecies *laperrinei* (Table 4): CL1 (86 %), CL2 (3 %), CL3 (9 %) and CL4 (2 %). The *laperrinei* haplotypes are related to the haplotype CE1 from the Mediterranean basin (Table 4) and all share a deletion in *trn*S-G-indel-1 specific to the lineage E1 (Besnard *et al*., 2002, 2003). This sustains the hypothesis of a single maternal origin of present populations in Hoggar probably originating in northern Africa (Besnard *et al*., 2002). The four haplotypes were present in the Adrar Heggueghene massif, while only one haplotype (CL1) was revealed in the Adjellela and Tanarouatine massifs. Furthermore, in the sample of the subspecies *europaea*, cpDNA haplotypes CE1 (4.3 %), COM1 (13·1 %), COM2 (65·2 %), CCK (15·2 %) and CCK2 (2·2 %) were identified. These haplotypes belong to three distinct cpDNA lineages (E1, E2 and E3; Table 4) as described in Besnard *et al*. (2002). The haplotype CCK2 is closely related to CCK and is newly described in this study (Table 4). The total cpDNA genetic diversity was higher in western Mediterranean populations (H_t = 0·67) than in *laperrinei* populations (H_t = 0·19), but this is probably a consequence of multiple maternal origins in the *europaea* populations from the western Mediterranean (Besnard *et al*., 2002).

Evidence of clones in laperrinei *populations*

The genetic similitude between all ramets of the subspecies *laperrinei* was assessed using a phenetic approach. In the phenogram, most branches were very poorly supported and there is no obvious individual cluster corresponding to the four massifs prospected in the Tamanrasset area (data not shown). Furthermore, the present results indicate that several ramets display the same genotype based on the nine loci. In all cases, genetically undistinguished ramets were sampled on the same site at a relatively small distance (<15 m; for example, see Fig. 1). Thus, on the 111 ramets analysed, only 94 genetic profiles were distinguished in the subspecies *laperrinei*. In contrast, based on nuclear SSR markers, the *europaea* individuals from the Maghreb were all distinguished. In the *laperrinei* populations, 12 distinct genetic profiles were each assigned to several ramets. For these genets, the probability of obtaining the same genetic profile (P_{gen}) was extremely low (ranging from $2{\cdot}4 \times 10^{-11}$ for I-n-Tôunine 9 and 10 to $5{\cdot}7 \times 10^{-7}$ in Ti-n-Aleo1-2) and, therefore, two different genotypes are expected to be distinguished in the present analysis. Consequently, it was considered that two undistinguished ramets correspond to a clone. There is no doubt that the undistinguished ramets have resulted from the development of shoots from an old stump because, in all cases, undistinguished ramets were sampled on the same site at small distances from each other. These genets can cover >80 m^2 (Fig. 1) and should have originated from very old stumps (probably persisting for >1000 years). Clonal growth was similarly reported in the *europaea* subspecies based on RAPD data [Besnard, 1999; oleaster populations Al Asharinah (Syria), Torvizcon (Spain) and Mt Boron (France)]. Nevertheless, the importance of this feature was not assessed in Mediterranean populations but the observation of stumps with a surface superior to 20 m^2 was exceptional, maybe due to higher competition in Mediterranean populations.

In the Adrar Heggueghene population, the three ramets of the genet I-n-Toûnine 1-2-3 each display three alleles at the DCA1 locus (e.g. allele sizes 236, 240 and 244). Such a result was found only on this genet. Similar observations have been reported on SSR loci in other vegetative-reproducing species such as grape vine (Franks *et al*., 2002; Riaz *et al*., 2002). It can be supposed that the presence of a third allele is due to a somatic mutation and that chimerism has been maintained during the development of the individual (e.g. Franks *et al*., 2002; Rodríguez López *et al*., 2004). Another interesting feature concerns the individual Ilennanene 8 for which the four ramets are genetically very similar but different (Fig. 3). Indeed, each ramet can be distinguished from each other, based on one to three alleles at the loci DCA1 (genotypes 240–272, 240–274 or 240–276), DCA14 (genotypes 151–156 or 152–156) and DCA9 (genotypes 181–189 or 181–193). These results were verified by two independent characterizations. These observations demonstrate that mutations can accumulate along time on distinct ramets originating from the same ancestral genet, as already shown in cultivated olive during prolonged periods of vegetative reproduction (Garcia-Díaz *et al*., 2003; Lopes *et al*., 2004). This propensity for clonal variation in wild-growing trees probably indicates that some stumps are very old and/or persist in environmental conditions, such as an intense grazing, favouring an emergence of mutations present in meristematic tissues. Indeed, shoot damage can unlock somatic mutations by promoting axillary growth (e.g. Marcotrigiano, 2000). Furthermore, it is suspected that other ramets from the same stump were distinguished due to mutations. Such ramets should be genetically similar and sampled on the same site and at a small distance. Among similar ramets, only Ilennanene 6 and 7 are indicative of this pattern (Fig. 3). These ramets were sampled at a distance of 3 m and were distinguished by both alleles of the locus DCA1 (genotypes 246–270 and 240–272). Excluding the locus DCA1, the probability of obtaining the same genetic profile for the eight other loci for Ilennanene 6 and Ilennanene 7 was very low (P_{gen} = $7{\cdot}4 \times 10^{-5}$). Consequently, it is suspected that these ramets originated from the same stump.

Conclusion and further prospects

To summarize, approx. 16 % of genets with several ramets were found in the *laperrinei* populations. Only four individuals displayed more than two ramets (from 3 to 5). The mean number of ramets per genet was 1·23. The long survival in combination with clonal growth should be an important feature explaining the persistence of the

TABLE 4. *Chloroplast DNA haplotypes detected in the populations from western Mediterranean (*europaea*) and Hoggar (*laperrinei*) based on the combination of 20 loci*

Haplotype	Lineage*	Characters																			
		ccmp5†	ccmp7†	*trn* T-L-poly-T	*trn* T-L-poly-A	*psb* K-*trn* S-poly-T/A	*trn* G-poly-T	*trn* G-R-poly T	*trn* S-G-indel-1	*trn* S-G-indel-2	*Trn* T-L-*Taq*I	*Tm* T-L-*Acs*I	*mat* K-*Rsa*I	*mat* K-*Taq*I	*trn* S-G-*Mse*I	*trn* S-G-*Nde*I	*mat* K-2-*Taq*I	*mat* K-2-*Mse*I	*mat* K-3-*Taq*I	*trn* G-*Pst*I	*psb* K-*trn* S-*Mse*I
CE1	E1	105	120	81	106	109	87	60	105	60	+	–	+	+	+	+	–	–	+	–	–
CL1	E1	105	120	81	106	109	87	60	105	60	+	–	+	+	+	–	–	–	+	–	–
CL2	E1	105	120	81	106	108	87	60	105	60	+	–	+	+	+	–	–	–	+	–	–
CL3	E1	105	120	82	106	109	87	60	105	60	+	–	+	+	+	–	–	–	+	–	–
CL4	E1	104	120	81	106	109	87	60	105	60	+	–	+	+	+	–	–	–	+	–	–
CCK	E3	106	120	81	106	109	87	60	114	60	+	–	+	+	+	–	–	–	+	–	–
CCK2	E3	105	120	81	106	109	87	60	114	60	+	–	+	+	+	–	–	–	+	–	–
COM1	E2	106	121	81	106	108	87	60	117	52	+	–	+	+	–	–	–	–	+	–	–
COM2	E2	105	121	81	106	108	87	60	117	52	+	–	+	+	–	–	–	–	+	–	–

The haplotypes CE1, CCK, CCK2, COM1 and COM2 were detected in *europaea* populations while CL1 to CL4 were only detected in *laperrinei* populations.
Eight polymorphic loci were observed.
For the nine first fragments, size variants in base pairs are given. For the next ones, restriction site presence (+) or absence (–) are indicated.
* Named according to Besnard *et al.* (2002; unpubl. res.).
† For ccmp5 and ccmp7, size of the variants was corrected comparatively to the previous estimation (Besnard *et al.*, 2002).

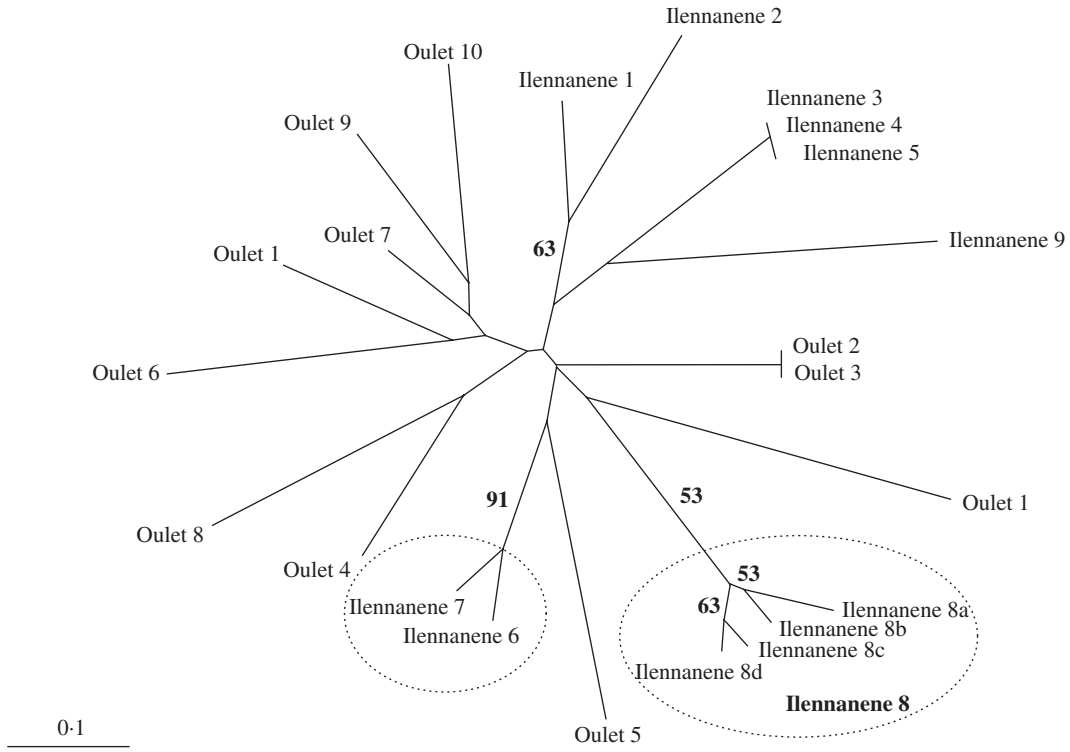

FIG. 3. Un-rooted phenetic tree showing the genetic similitude between ramets of the Issekrâm population using nine nuclear SSR loci. This analysis is based on shared allele distances (Jin and Chakraborty, 1993) and the neighbour joining algorithm (Nei, 1987). The bootstrap values superior to 50 % are indicated on each corresponding branch. The two genets Oulet 2-3 and Ilennanene 3-4-5 display two and three ramets, respectively. The surrounded clusters of ramets correspond to two groups of genetically similar ramets thought to be distinguished based on mutations.

laperrinei populations in Saharan mountains (Honnay and Bossuyt, 2005). This probably explains why this olive subspecies is able to maintain a high genetic diversity in very small populations over a very long period. It remains to clarify if the taxon could have sexually reproduced since the last humid transition, about 3000–3500 years ago (Quézel, 1965). The future persistence of these populations should not be dependent on strong genetic erosion (e.g. genetic drift due to sexual reproduction in reduced populations) but could be affected by serious environmental changes.

The authors can advise on the strategy to use for genetic resources management and the reforestation of zones where *O. e. laperrinei* is seriously declining or has recently disappeared. The vegetative propagation of existing individuals in different massifs or the production of novel plants stemming from seed germination can be proposed. The first alternative has the advantage of employing individuals which are highly adapted to very dry conditions since these trees have been naturally selected over a very long period. Such trees should be preserved in collections and characterized for their drought resistance.

The analysis of other populations is still needed to give a more exhaustive inventory of the genetic resources of this subspecies. Population genetic studies should bring new insights into the reproductive strategy of this taxon. Prospects, particularly from Niger and Tassili n'Adjer, are in progress to attain these objectives.

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LITERATURE CITED

Baali-Cherif D, Kara-Mostefa-Khelil L, Zemit O, Meghaoui A, Sahki A, Bouguedoura N. 2000. Etude de quelques aspects biologiques de l'olivier de Laperrine (*Olea laperrini*) en vue de la mise en place d'une banque ex situ. In: Ilizi W, ed. *Redecouvrir et réinventer une sylviculture en zones arides*. Djanet: CRSTRA-INRF, 82–89.

Bandelj D, Jakše J, Javornik B. 2004. Assessment of genetic variability of olive varieties by microsatellite and AFLP markers. *Euphytica* **136**: 93–102.

Belaj A, Satovic Z, Cipriani G, Baldoni L, Testolin R, Rallo L, *et al.* 2003. Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and of their effectiveness in establishing genetic relationships in olive. *Theoretical and Applied Genetics* **107**: 736–744.

Besnard G. 1999. *Etude de la diversité génétique de l'olivier cultivé et de ses formes sauvages apparentées à l'aide de marqueurs moléculaires: applications pour l'identification variétale et pour la gestion des ressources génétiques*. PhD Thesis, Université Montpellier II.

Besnard G, Bervillé A. 2002. On chloroplast DNA variations in the olive (*Olea europaea* L.) complex: comparison of RFLP and PCR polymorphisms. *Theoretical and Applied Genetics* **104**: 1157–1163.

Besnard G, Baradat P, Chevalier D, Tagmount A, Bervillé A. 2001. Genetic differentiation in the olive complex (*Olea europaea*) revealed by RAPDs and RFLPs in the rRNA genes. *Genetic Resources and Crop Evolution* **48**: 165–182.

Besnard G, Khadari B, Baradat P, Bervillé A. 2002. *Olea europaea* (Oleaceae) phylogeography based on chloroplast DNA polymorphism. *Theoretical and Applied Genetics* **104**: 1353–1361.

Besnard G, Khadari B, Villemur P, Bervillé A. 2000. Cytoplasmic male sterility in the olive (*Olea europaea* L.). *Theoretical and Applied Genetics* **100**: 1018–1024.

Besnard G, Rubio de Casas R, Vargas P. 2003. A set of primers for length and nucleotide-substitution polymorphism in chloroplastic DNA of *Olea europaea* L. (Oleaceae). *Molecular Ecology Notes* **3**: 651–653.

Carriero F, Fontanazza G, Cellini F, Giorio G. 2002. Identification of simple sequence repeats (SSRs) in olive (*Olea europaea* L.). *Theoretical and Applied Genetics* **104**: 301–307.

El Mousadik A, Petit RJ. 1996. High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. *Theoretical and Applied Genetics* **92**: 832–839.

Franks T, Botta R, Thomas MR, Franks J. 2002. Chimerism in grapevines: implications for cultivar identity, ancestry and genetic improvement. *Theoretical and Applied Genetics* **104**: 192–199.

Garcia-Díaz A, Oya R, Sánchez A, Luque F. 2003. Effect of prolonged vegetative reproduction of olive tree cultivars (*Olea europaea* L.) in mitochondrial homoplasmy and heteroplasmy. *Genome* **46**: 377–381.

Goudet J. 1995. Fstat (version 1.2): a computer programme to calculate *F*-statistics. *Journal of Heredity* **86**: 485–486.

Green PS. 2002. A revision of *Olea* L. (*Oleaceae*). *Kew Bulletin* **57**: 91–140.

Hess J, Kadereit JW, Vargas P. 2000. The colonization history of *Olea europaea* L. in Macaronesia based on internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and inter simple sequence repeats (ISSR). *Molecular Ecology* **9**: 857–868.

Honnay O, Bossuyt B. 2005. Prolonged clonal growth: escape route or route to extinction? *Oikos* **108**: 427–432.

Jin L, Chakraborty R. 1993. Estimation of genetic distance and coefficient of gene diversity from single-probe multilocus DNA fingerprinting data. *Molecular Biology and Evolution* **11**: 120–127.

Khadari B, Breton C, Moutier N, Roger JP, Besnard G, Bervillé A, *et al*. 2003. The use of molecular markers for germplasm management in a French olive collection. *Theoretical and Applied Genetics* **106**: 521–529.

Langella O. 1999. Populations 1.2.28 software (http://www.pge.cnrs-gif.fr/bioinfo/populations).

Lopes MS, Mendonça D, Sefc KM, Gil FS, da Câmara Machado A. 2004. Genetic evidence of intra-cultivar variability within Iberian olive cultivars. *HortScience* **39**: 1562–1565.

Loumou A, Giourga C. 2003. Olive groves: ''The life and identity of the Mediterranean''. *Agriculture and Human Values* **20**: 87–95.

Maire R. 1933. Études sur la flore et la végétation du Sahara central. *Mémoires de la Société d'Histoire Naturelle de l'Afrique du Nord* No. 3, Mission du Hoggar II, 166–168.

Maley J. 1980. Les changements climatiques de la fin du Tertiaire en Afrique: leur conséquence sur l'apparition du Sahara et de sa végétation. In: Williams MAJ, Faure, H, eds. *The Sahara and the Nile*. Rotterdam: AA Balkema, 63–86.

Marcotrigiano M. 2000. Herbivory could unlock mutations sequestered in stratified shoot apices of genetic mosaics. *American Journal of Botany* **87**: 355–361.

Médail F, Quézel P, Besnard G, Khadari B. 2001. Systematics, ecology and phylogeographic significance of *Olea europaea* L. subsp. *maroccana* (Greuter & Burdet) P. Vargas *et al*., a relictual olive tree in south-west Morocco. *Botanical Journal of the Linnean Society* **137**: 249–266.

Nei M. 1987. *Molecular evolutionary genetics*. New York: Columbia University Press.

Parks JC, Werth CR. 1993. A study of spatial features of clones in a population of bracken fern, *Pteridium aquilinum* (Dennstaedtiaceae). *American Journal of Botany* **80**: 120–126.

Pichot C, El Maataoui M, Raddi S, Raddi P. 2001. Surrogate mother for endangered Cupressus. *Nature* **412**: 39.

Quézel P. 1965. *La végétation du Sahara. Du Tchad à la Mauritanie*. Stuttgart: Gustav Fischer Verlag.

Quézel P. 1978. Analysis of the flora of Mediterranean and Sahara Africa. *Annals of the Missouri Botanical Garden* **65**: 479–534.

Rallo P, Tenzer I, Gessler C, Baldoni L, Dorado G, Martín A. 2003. Transferability of olive microsatellite loci across the genus *Olea*. *Theoretical and Applied Genetics* **107**: 940–946.

Riaz S, Garrison KE, Dangl GS, Boursiquot JM, Meredith CP. 2002. Genetic divergence and chimerism within ancient asexually propagated winegrape cultivars. *Journal of the American Society for Horticultural Science* **127**: 508–514.

Rodríguez López CM, Wetten AC, Wilkinson MJ. 2004. Detection and quantification of *in vitro*-culture induced chimerism using simple sequence repeat (SSR) analysis in *Theobroma cacao* (L.). *Theoretical and Applied Genetics* **110**: 157–166.

de la Rosa R, James CM, Tobutt KR. 2002. Isolation and characterization of polymorphic microsatellites in olive (*Olea europaea* L.) and their transferability to other genera in the Oleaceae. *Molecular Ecology Notes* **2**: 265–267.

Saumitou-Laprade P, Vassiliadis C, Epplen JT, Hardt C. 2000. Isolation of microsatellite loci for paternity testing in *Phillyrea angustifolia* L. (Oleaceae). *Molecular Ecology* **9**: 112–114.

Sefc KM, Lopes MS, Mendonça D, Rodrigues dos Santos M, Laimer da Câmara Machado M, da Câmara Machado A. 2000. Identification of microsatellite loci in olive (*Olea europaea*) and their characterization in Italian and Iberian olive trees. *Molecular Ecology* **9**: 1171–1173.

Setsuko S, Ishida K, Tomaru N. 2004. Size distribution and genetic structure in relation to clonal growth within a population of *Magnolia tomentosa* Thunb. (Magnoliaceae). *Molecular Ecology* **13**: 2645–2653.

Wang J, Hill WG, Charlesworth D, Charlesworth B. 1999. Dynamics of inbreeding depression due to deleterious mutations in small populations: mutation parameters and inbreeding rate. *Genetical Research* **74**: 165–178.

Weising K, Gardner RC. 1999. A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. *Genome* **42**: 9–19.

Wickens GE. 1976. The flora of Jebel Marra (Sudan) and its geographical affinities. *Kew Bulletin Additional Series* **5**: 1–368.