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Pollen-mediated gene flow in a highly fragmented landscape: consequences for defining a conservation strategy of the relict Laperrine's olive

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Abstract

In the present central Saharan conditions, the Laperrine's olive regeneration has never been observed and its populations are locally threatened. The production of plants originating from seeds was proposed as a multiplication strategy. In order to determine the impact of sexual reproduction, seeds issued from ten mothers (sampled from four locations in the Hoggar, Algeria) were genotyped using microsatellites. Compared to the initial population, a significant lost of allelic richness was revealed, indicating that our seed sampling was not representative of the local gene diversity. Paternity analyses allowed measurement of the effective pollen-mediated gene flow within patches. Preferential mating between some genotypes was revealed. A trend for a higher multipaternity on seeds collected on trees from relatively large patches was also observed. Lastly, seedlings issued from trees of small patches displayed low growth performance. The implications of our observations in the development of an efficient conservation strategy by seeds are discussed. ***To cite this article: G. Besnard et al., C. R. Biologies 332 (2009).***

Résumé

Flux de pollen dans un habitat hautement fragmenté : Conséquences pour la conservation des populations reliques d'olivier de Laperrine. La régénération naturelle de l'olivier de Laperrine n'a jamais été observée dans les montagnes sahariennes et certaines de ses populations sont menacées. Pour faire face à ce problème, la multiplication du taxon par graines a été proposée. Afin de déterminer l'impact de cette pratique, des graines provenant de dix arbres (échantillonnés sur quatre localités dans le Hoggar) étaient caractérisées avec des microsatellites. Une perte significative de richesse allélique était révélée par rapport à la population initiale, indiquant que notre échantillon de graines n'était pas représentatif de la diversité génétique locale. Les flux de pollen effectifs étaient ensuite mesurés par des analyses de paternité. Des croisements préférentiels entre génotypes étaient révélés. Une tendance pour une plus forte multi-paternité sur les graines collectées d'arbres provenant de peuplements relativement larges était observée. Finalement, les plantules issues d'arbres de petits peuplements montraient de faibles performances de croissance. L'importance de nos résultats est discutée pour développer une stratégie adaptée de multiplication par graines. ***Pour citer cet article : G. Besnard et al., C. R. Biologies 332 (2009).***

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1. Introduction

The high mountains of Hoggar, Aïr, Tibesti and Jebel Marra are considered as important refuges for plants and animals in the middle of the Saharan desert [1–3]. A few endemic species have been reported in these massifs [4]. However, this diversity is increasingly threatened, mainly due to an intensification of human activities in these areas [5]. Tree species are particularly vulnerable due to their limited regeneration in the present dry conditions occurring on the Sahara. They also represent an important component of the ecosystems and are a putative, but limited, wood resource for local human populations. Among the most representative woody species, we can cite *Acacia ehrenbergiana, A. tortilis, Balanites aegyptiaca*, *Calotropis procera*, *Cupressus dupreziana, Erica arborea, Ficus salicifolia*, *F. ingens*, *Maerua crassifolia*, *Myrtus nivellei*, *Olea europaea*, *Pistacia atlantica*, *Rhus tripartita*, *Salvadora persica*, *Tamarix articulata* or *Ziziphus lotus* [1]. The endemic *C. dupreziana* is particularly endangered [6], but several of the other taxa are distributed in a highly fragmented habitat making uncertain their long-term persistence [7]. All these trees have a potential utility for the fight against desertification and could constitute a source of genes for pharmacology and heat resistance. Moreover, some remarkable trees (particularly for the Laperrine's olive and Tassilian cypress) could also help the development of tourism in certain region of the Saharan mountains. For all these reasons, conservation programs should be implemented.

The Laperrine's olive [*Olea europaea* L. subsp. *laperrinei* (Batt. & Trab.) Ciferri] is the endemic Oleaceae of the Saharo-Sahelian Mountains from South Algeria to North West Sudan [1,2,8]. This emblematic tree is locally threatened by extinction [9,10] due to human activities, particularly in Niger and Algeria. This taxon grows rather at high altitudes, from 1400 to 2700 meters on volcanic or eruptive rocks, where mean rainfall reach about 50–100 mm per year. They are often found in cliffs and banks of canyon (or wadi) what made them frequently arduous to attain.

Desert expansion since the Upper Miocene has considerably diminished the distribution of the Laperrine's olive, which disappeared from Tibesti, Chad [2,7,11,12]. Only fragmented populations composed of patches with a few individuals (generally inferior to 100) are found in Niger and Algerian mountains [13]. Currently, camels and cattle are a supplementary threat for *O. e. laperrinei* because they limit their development and possibilities of regeneration by browsing young shoots [9,10]. In addition, this tree is a very important forage resource for endangered wild animals such as the Barbary sheep (*Ammotragus lervia*). Under present conditions of the South Algeria and Niger mountains, the Laperrine's olive is sometimes able to produce seeds (rarely with abundance), but young trees have never been observed [9,13] leading some authors to consider it as a relict taxon [8,14].

The genetic diversity of remnant Laperrine's olive populations has been recently characterized [15]. It was suggested that the Hoggar Mountains act as an important genetic reservoir because a higher allelic richness was observed compared to other populations from Central Saharan massifs (e.g. Tassili n'Ajjer, Tamgak, Bagzane). This olive tree displays an exceptional lifespan and a propensity to clonal growth, allowing it to avoid population regeneration via a sexual reproduction, and these life traits probably explain his ability to persist in very small patches [10,16]. Such biological characteristics may therefore make this tree more vulnerable to serious environmental changes than to genetic erosion [16].

To create nurseries, the Laperrine's olive can be multiplied either using a vegetative way or by seeds. The advantage of vegetative multiplication is the use of highly adapted individuals to dry conditions, which have been naturally selected over a very long period of time. However, the evolutionary potential of the Laperrine's olive tree may be affected by the absence of sexual reproduction [17]. As an alternative, the production of plants originating from seed germination was proposed and appeared to be a possible way to recreate populations [13,15]. Nevertheless, this strategy heavily depends on the possibility of seed collecting and on the number of individuals locally involved in sexual reproduction (flowering and fructifying trees). Recently, effective gene flows mediated by pollen have been assessed by the means of highly variable molecular markers (microsatellites) in numerous tree species (e.g. [18–21]). These studies have brought important insights about the factors limiting pollen gene flows (i.e. distance, topography, cross-incompatibility between genotypes), and

Table 1
Geographic origin and characteristics of the four Laperrine's olive patches studied. For each location, N_G is the number of genets identified with the SSR loci, while N_R is the number of ramets sampled. The list of the ten mother genets is also given. In brackets, N_S is the number of seeds analysed for each mother tree.

Location	Altitude (m)	Latitude	Longitude	Patch size	N_G/N_R	Mothers (N_S)
Tonget	1600	23°07′N	5°59′E	7	7/7	Tonget 1 (19) Tonget 2 (22) Tonget 3 (22)
T-in-Hamor	1500–1700	22°50′N	5°37′E	>150	39/48	T-in-Hamor 1 (21)
Adjellela	1500–1600	22°38′N	5°37′E	16	15/16	Adjellela 4-5 (19) Adjellela 6 (13) Adjellela 9 (21) Adjellela 10 (32)
Akerakar	1800–1850	23°04′N	5°43′E	>150	59/75	Akerakar 1 (8) Akerakar 3-4-5 (34)

such an information should be really relevant for conservation purposes particularly in a highly fragmented landscape [22]. It should notably allow an assessment of the impact of reproduction by seeds as a conservation strategy.

The olive tree is allogamous, with a system of gametophytic self-incompatibity [23,24], and anemophilous. Multipaternity is thus expected but the cross-compatibility between trees is also an important factor that leads to fertilisation with preferential fathers [19,25]. If only a few individuals are involved in the sexual reproduction, a multiplication by seeds could have some negative consequences on the genetic diversity of resulting plantlets compared to the initial population (e.g. genetic erosion). Moreover, one can predict that seedlings sampled from small patches could also display a low genetic diversity and reduced growth performance (Allee effect; [26]). Thus, the collection of individuals should be set in an area as wide as possible in order to keep an elevated genetic diversity and thus preserve the evolutionary potential of the species [15].

In this context, seeds issued from ten different mother Laperrine's olive trees were collected and characterized with eight nuclear microsatellite loci in order to perform paternity analyses. The father identity of each seedling allowed us measuring the effectiveness of gene flow within population. Growth performances of a few seedlings were also followed during the first five months to test for differences between studied provenances. The implications of our results are finally discussed to develop an efficient conservation strategy of the Laperrine's olive using a multiplication by seeds.

2. Materials and methods

2.1. Study sites and plant material

In the Hoggar Mountains (South Algeria), relatively favourable water conditions for flowering and fructification of the Laperrine's olive have occurred in 2007 compared to the ten previous years [13]. Flowering trees were briefly observed in April 2007 at four distant locations near Tamanrasset (Tonget, T-in-Hamor, Adjellela and Akerakar; Table 1). Seeds were then collected on ten accessible mother trees in August 2007 for paternity analyses (Table 1). All individuals (or ramets) in a perimeter of about 1500 m of each mother tree were sampled, excepted on the T-in-Hamor locality (see below). Position of trees in the landscape is given for the Adjellela and Akerakar sites (Fig. 1), but not for Tonget and T-in-Hamor, because this information was not very useful to interpret results for these two latter sites (see below). For each ramet, leaves were desiccated in silica gel. Topography and individual number (patch size) varied between each location:

- 1 – Tonget is a very isolated patch of seven individuals located at the basis of a cliff (no other tree patches have been observed at less than five kilometres). Three individuals (Tonget 1, 2 and 3) flowered and then produced mature seeds in 2007. Sixty-three seeds were collected on these three ramets (between 19 and 22 per ramet);
- 2 – Sixteen ramets (of which 12 have reached flowering) were sampled in two fissures of a cliff at Adjellela (Fig. 1A). Eighty-six mature fruits were sampled on four genets (Adjellela 4-5, 6, 9 and 10; Fig. 1A). Adjellela is probably the most southern massif where the

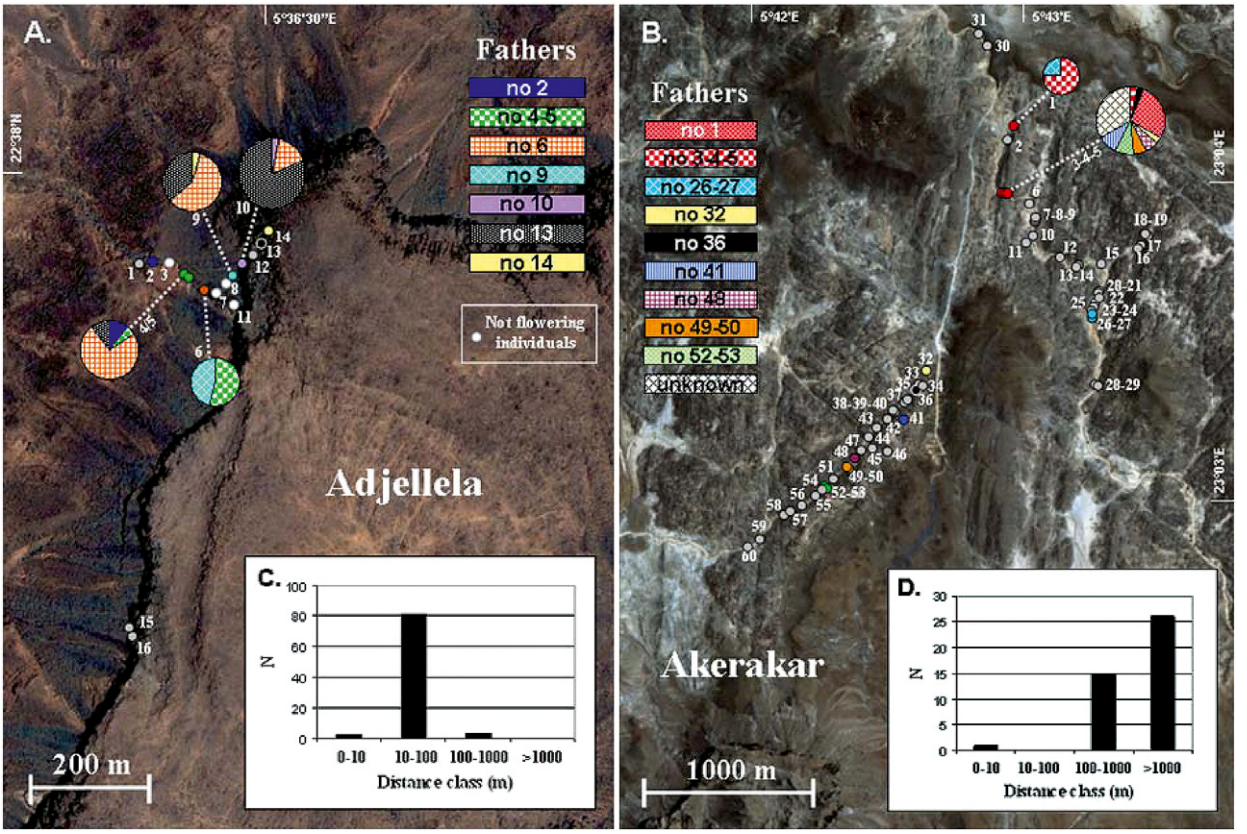

Fig. 1. Assessment of pollen-mediated gene movement between Laperrine's olive trees at two locations in the Hoggar Mountains. For seeds of each mother genet, fathers were identified with SSR loci. Contribution of each father is indicated in the pie-charts by a different colour and/or texture. **A**. Adjellela locality (all individuals were sampled and characterized); **B**. Akerakar locality (all individuals occurring in a 1500-m perimeter around the two mother genets were sampled and characterized). Fifteen additional trees (Akerakar 61 to 75), sampled in the south and north parts of this locality, are not represented on the picture (because they were not involved in the pollination of the two mother trees). For the mother genet Akerakar 3-4-5, no fathers were identified for 11 seeds. A minimum of six fathers were involved in the fertilisation of these seeds; **C–D**. Distance distribution of effective pollen dispersal events in both locations, Adjellela and Akerakar, respectively. N = Number of seeds.

Laperrine's olive still occurs in the Hoggar Mountains. No other massifs with olive trees are known in less than 15 km. However, we recently observed (on November 15th, 2008) at least 20 additional trees in the southern slope of the Adjellela massif. This patch is located at about 1000 m of the other trees. These trees were difficult to attain and were unfortunately not sampled;

• 3 – The location T-in-Hamor is a favourable situation for the Laperrine's olive. It corresponds to an abrupt valley and the number of individuals is estimated to more than 150. Only 48 ramets were sampled because some of them were very difficult to attain at the top of the mountain. Twenty-one mature fruits were sampled on only one tree (T-in-Hamor 2) at the basis of the mountain, and the number of flowering trees during the springtime was not estimated;

• 4 – Lastly, the Akerakar locality is also characterized by a relatively large patch size. Contrary to other locations studied, the trees are dispersed in the bed or on borders of temporary rivers (wadi). More than 150 ramets were observed in a network of wadis. Forty-two incompletely mature fruits were sampled on two genets (Akerakar 1 and 3-4-5), and we then sampled the 60 ramets occurring in a perimeter of 1500 m (Fig. 1B). Thirteen additional ramets more in the south (at about four km of the mother trees) and two others in the North (at about three km) were also collected, leading to a total of 75 sampled ramets. The number of flowering trees on this locality was not estimated.

2.2. *Seed germination and seedling growth*

On October 14th, 2007, germination of 170 seeds from Tonget, Adjellela and T-in-Hamor was induced by first removing the endocarp and by depositing them on filter paper humidified with deionised water. Seeds were

then placed in the darkness at the laboratory temperature (20 °C). Fifteen days later, the germinated seeds (72 seedlings) were transferred into a mix of compost and sand. When the seedling was sufficiently developed, half of a cotyledon was taken. Furthermore, the embryo of the seeds, which did not germinated after 15 days (98), were also retrieved. Cotyledon and seed materials were frozen for further DNA extraction. For Akerakar, 42 incompletely mature embryos were isolated and directly used for DNA isolation.

Among the 72 seedlings, six (three from Tonget and three from T-in-Hamor) did not display chlorophyll ("white seedlings"). Their growth was of course very limited at two or three weeks but material was collected for DNA extraction before degeneration. The growth of the remaining 66 seedlings (27 from Tonget, 22 from Adjellela and 17 from T-in-Hamor) was followed during five months. The height of each individual and the number of leaf pairs were measured after 90 and 150 days of growth. The survival of individuals during this period was also noted. Growth differences between progenies from the three provenances were tested using a Wilcoxon test with a Bonferroni correction.

2.3. Genetic characterization and paternity analyses

For all the plant material (embryos, cotyledons or leaves), DNA extraction was realised using a CTAB method [27]. The complete sample of 146 ramets and 212 seeds/seedlings was then genotyped with eight microsatellite loci selected from the literature: ssrOeUA-DCA1, ssrOeUA-DCA3, ssrOeUA-DCA5, ssrOeUA-DCA8, ssrOeUA-DCA9, ssrOeUA-DCA15, ssrOeUA-DCA18 [28] and GAPU71A [29]. Five of them were already used in previous investigations [15,16] and three new loci were considered (i.e. ssrOeUA-DCA5, ssrOeUA-DCA18, GAPU71A). These loci were chosen for their clear and polymorphic amplification patterns and their allele size range was also considered for the multiplexing of loci. Polymerase chain reaction (PCR) and electrophoresis procedures were previously described [16]. To avoid mistakes in the dataset, electrophoregrams were read independently twice by two different persons. All ambiguous data were removed and the characterization was run again. With this procedure, we consider that a very low error rate should persist in our data.

Allele frequencies, allelic richness (R_S), expected and observed heterozygosities (H_T and H_O), Nei's gene diversity (H_S) were calculated separately on the initial population, seeds and seedlings (surviving after five months) using Fstat [30]. A paired t-test (one-sided) was then used to compare the values of R_S and H_T of the initial population with both seeds and seedlings. In addition, the global genetic differentiation between patches was estimated using Fstat. Pairwise genetic differentiations (F_{ST}) between the four sampled patches, and between the initial population and the sampled seeds or seedlings were also estimated. Significance of pairwise differentiation was assessed using a permutation test (6000 randomisations).

Exclusion and identity probabilities [31,32] were generated on the initial population using FaMoz (http://www.pierroton.inra.fr/genetics/labo/Software/Famoz/index.html; [33]). Paternity analyses were then performed with the same software. In this program, the statistical analysis is based on a maximum likelihood method [34]. The log of the odds ratio (LOD) score represents the likelihood of a particular genotype being the father compared to all other genotypes. The genotype with the highest LOD score was considered as the most likely father. We did not take in account possible typing errors because lot of precautions was done to avoid them (see above). For each mother tree, we additionally used Gerud v2.0 [35] to determine the minimum number of fathers involved in the fertilisation of seeds not assigned to a father. Only the five more variable loci (ssrOeUA-DCA1, ssrOeUA-DCA3, ssrOeUA-DCA5, ssrOeUA-DCA8 and ssrOeUA-DCA18) were used in the analysis due to some limitations of the software [35]. The software uses a reconstructed genotype method based on the multilocus genotype known (the mother) and the multilocus genotype of offspring. By subtracting maternal alleles from the offspring genotype for each locus, it is possible to deduce the paternal alleles. Combining all pairwise paternal alleles for each locus, all single-locus genotypes are then combined to create all the possible multilocus genotypes. Through an exhaustive search, the minimum number of fathers necessary to explain the offspring genotypes is estimated. Lastly, the number of seeds per mother being comprised between 8 and 34, it was needed to standardize our observations to perform comparisons. Using the rarefaction index of Hurlbert [36], a standardized value of the number of fathers involved in fertilisation of a mother (N_{PS}) was calculated. This estimate was thus determined for eight seeds per mother.

Table 2
Characteristics of the eight nuclear microsatellite loci: allele size range (in base pairs), number of alleles (N_a), expected and observed heterozygosities (H_S and H_O), and exclusion and identity probabilities for the parental genotypes are given.

Locus	Allele size range	N_a	H_S	H_O	Exclusion probability	Identity probability
ssrOeUA-DCA1	226–278	24	0.88	0.87	0.819477	0.289028
ssrOeUA-DCA3	229–255	10	0.68	0.72	0.453554	0.482094
ssrOeUA-DCA5	199–251	21	0.86	0.88	0.720006	0.034784
ssrOeUA-DCA8	119–149	13	0.84	0.86	0.662646	0.247654
ssrOeUA-DCA9	169–193	12	0.60	0.58	0.382580	0.444280
ssrOeUA-DCA15	251–258	2	0.25	0.25	0.099158	0.915784
ssrOeUA-DCA18	152–187	11	0.80	0.81	0.601374	0.098477
GAPU71A	217–225	4	0.59	0.61	0.389704	0.257045
All loci	–	97	0.69	0.70	0.998739	0.000012

3. Results

3.1. Microsatellite polymorphism, genotype discrimination and gene diversity

The eight loci used were polymorphic and the number of alleles per locus ranged from two to 24, with an average of 12.1 (Table 2). Among the 146 ramets analysed of the four locations, 120 genotypes were identified (Table 1). The high cumulative exclusion probability (0.999) and the low cumulative identity probability (0.000) of the loci used (Table 2) showed that the set of markers may be able to adequately discriminate all genets as previously reported in other studies on the olive tree (e.g. [16,19,25,37]). There is thus no doubt that the undistinguished ramets have resulted from the development of shoots from an old stump (leading to clones; see also [10,16]), since in all cases, they were sampled on the same site at small distances from each other (less than 25 m). Consequently, only one ramet was then considered for each clone for further analyses, and all different genotypes were named genets. In addition, more than two alleles at a locus was found for some genets; Akerakar 61 displayed three alleles at locus ssrOeUA-DCA9 (169/181/185); and surprisingly one genet of the T-in-Hamor locality (T-in-Hamor 9-10), displayed three alleles on the four more discriminating loci (ssrOeUA-DCA1, ssrOeUA-DCA5, ssrOeUA-DCA8 and ssrOeUA-DCA18). Such a complex genetic pattern (i.e. at least three alleles at several loci) has recently been revealed in the olive complex for polyploid trees [38] and the diploid status of clone T-in-Hamor 9-10 can thus be questioned. The genets Akerakar 61 and T-in-Hamor 9-10 were removed for further analyses but we verified manually that they were not involved as fathers on seed genotypes.

In the initial population, the expected and observed heterozygosities were not different, suggesting a very level (or a total absence) of null alleles in the loci used (Table 2). A significant, weak global differentiation between patches was observed in our sample ($F_{ST} = 1.70\%$; $p < 0.001$). Similarly, a significant and weak pairwise differentiation was observed between the four patches sampled (Table 3). This low differentiation is in concordance with our previous study [15], and even if the different patches are presently isolated from each other in different massifs, we considered them as belonging to the same ancient meta-population [15]. A significant lost of allelic richness (R_S; Table 4) was found between this parental population and the produced seeds (of 29%; $t = 2.70$, $p = 0.015$) or surviving seedlings (of 32%; $t = 2.91$, $p = 0.011$), while no difference was found in gene diversity. In addition, a significant differentiation was also revealed between the parental population and all seeds ($F_{ST} = 1.63\%$; $p < 0.01$) or surviving seedlings ($F_{ST} = 1.70\%$; $p < 0.01$), attesting of a genetic drift related to our sampling.

Table 3
Pairwise genetic differentiation (F_{ST}) between the four studied Laperrine's olive patches based on eight nuclear microsatellite loci. The number of genets for each patch is given in brackets. Significance of patch differentiation was tested using 6000 permutations.

	Tonget (7)	Adjellela (15)	T-in-Hamor (38)
Adjellela (15)	0.035*		
T-in-Hamor (38)	0.035*	0.014**	
Akerakar (58)	0.029*	0.009**	0.015**

* $p < 0.05$, ** $p < 0.01$.

3.2. Paternity analyses and pollen dispersal in the Laperrine's olive population

The high cumulative exclusion probability of the loci used (Table 2) also indicates that, for paternity analyses, there is a very low probability of wrongly attributing a

Table 4
Allelic richness (R_S) and gene diversity (H_T) for the initial population (four localities), the analysed seeds (from the ten mother parents) and the seedlings (surviving after five months; 54 individuals issued from eight mother trees of three localities).

Locus	Initial population (118)		Seeds (212)		Seedlings (54)	
	R_S	H_T	R_S	H_T	R_S	H_T
ssrOeUA-DCA1	20.64	0.91	11.86	0.85	12.00	0.87
ssrOeUA-DCA3	8.42	0.68	6.83	0.68	7.00	0.75
ssrOeUA-DCA5	16.24	0.86	9.88	0.85	10.00	0.85
ssrOeUA-DCA8	11.39	0.83	8.94	0.84	8.00	0.80
ssrOeUA-DCA9	10.31	0.58	6.50	0.61	5.00	0.56
ssrOeUA-DCA15	2.00	0.22	2.00	0.28	2.00	0.26
ssrOeUA-DCA18	9.19	0.79	8.24	0.81	8.00	0.80
GAPU71A	4.00	0.65	3.93	0.54	4.00	0.57
Total	82.19	0.69	58.18	0.68	56.00	0.68

genotype as the father [18]. The paternity assignment was thus performed by allocating pollen donors to the seeds sampled from the maternal trees. In our analysis, there was no ambiguity when paternity was successfully assigned to a seed; indeed, we only detected one possible father, which always belonged to the same patch than the mother tree. LOD score to assign a father to a seed varied from 3.7 to 18.7.

• In the Tonget locality, only three identified pollen donors (Tonget 1, 2 and 3) were responsible for the fertilisation of the 63 seeds analysed. Genet Tonget 1 was involved as a parent in the 63 seeds. In contrast, no embryos resulted from the combination between Tonget 2 and 3 (which are distant of 25 m) suggesting a cross-incompatibility between these two genets. The standardized numbers of fathers (N_{PS}) were 1.98, 1.00 and 1.00 for Tonget 1, 2 and 3, respectively;

• In Adjellela, seven identified pollen donors are responsible for the fertilisation of the 86 seeds, and two selfings were observed (Fig. 1A). For each mother, one or two fathers were more frequently involved in pollination probably as a consequence of cross-compatibilities and incompatibilities between the genets of the patch. For instance, there was no evidence of crossing between Adjellela 4-5, 9 and 10, while Adjellela 6 and 13 largely contributed to the pollination of these mothers genets (55/62 seeds; Fig. 1A). The standardized numbers of fathers (N_{PS}) were 2.58, 2.00, 2.30 and 2.04 for Adjellela 4-5, 6, 9 and 10, respectively;

• In T-in-Hamor, only one identified pollen donor (T-in-Hamor 11) is responsible for the fertilisation of one seed. No pollen donors were found for the 20 remaining seeds, because our tree sampling was insufficient due to the inaccessibility of a lot of individuals. Using Gerud, we determined that a minimum of five fathers were involved in the unassigned seeds [one father genotype (further named T-in-Hamor 49) involved in the fertilisation of eleven seeds was unambiguously identified]. Based on these results, the standardized number of fathers was estimated to a minimum of 3.95;

• Lastly, in the Akerakar locality, nine identified pollen donors are responsible for the fertilisation of 31 seeds (one selfing occurred), while no pollen donors were found for the 11 remaining seeds, all from Akerakar 3-4-5 (Fig. 1B). Using Gerud, we determined that a minimum of six fathers were involved in the unassigned seeds. The standardized numbers of fathers (N_{PS}) were 2.00 and min. 6.14 for Akerakar 1 and 3-4-5, respectively. A cross-compatibility was clearly evidenced between the two mother genets analysed since 36% of seeds were issued from the reciprocal crossings between Akerakar 1 (6/8) and 3-4-5 (9/34).

To summarize, the mean standardized numbers of fathers (for eight seeds) was of 1.84 (SD = 0.61) for locations with less than 15 genets (Tonget and Adjellela), while it was of 4.03 (SD = 2.07) for those composed by more than 150 individuals (T-in-Hamor and Akerakar), but regarding on the low number of observations, this difference was of course not significant.

In Tonget, only pollination at short distance (< 50 m) was evidenced since the three genets Tonget 1, 2 and 3 are in a 50-m perimeter (data not shown). In Adjellela, a similar situation is observed and pollination occurred at a maximum of 140 m (between Adjellela 4-5 and 13; Fig. 1A). For T-in-Hamor, our results were inconclusive since we were not able to identify more than one father. Lastly for Akerakar, distances of pollination were relatively well estimated (Fig. 1B and 1D) and if a quite large proportion of fathers (38%) are located at proximity of each mother (about 380 m between Akerakar 1 and 3-4-5), longer pollen dispersals (>1000 m) were evidenced; for instance Akerakar 52-53 contributed to the fertilisation of three seeds (9%) of Akerakar 3-4-5 although it is located at 1980 m. In addition, since all

trees occurring in a 1500-m perimeter were sampled, the 11 seeds with no identified fathers were considered to be fertilised by trees at more than 1500 m. In conclusion, 26 seeds (62%) were issued of pollination from fathers located at more than 1000 m in this location.

3.3. Seedling growth and inbreeding depression

In both localities Tonget and T-in-Hamor, paternity analysis indicated that the individuals without chlorophyll ("white seedlings") were issued from the same parents (i.e. three from Tonget 1×3 or 3×1, and three from T-in-Hamor 2×49). Among the 11 germinated seeds of each of these two crossings, the proportion of white seedlings (27%) is compatible with the presence of a deleterious recessive gene in both parents, but for a confirmation, we need to study a larger seedling sample. This phenomenon could be interpreted as due to a crossing between two related individuals from the same population, which thus leads to an inbreeding depression. To bring new arguments for this assumption, we also followed the growth of a few seedlings from three localities with different patch size during five months (Fig. 2). A lower viability after five months was observed for Tonget since nine seedlings on 27 (33%) died, compared to only three (on 22) and zero (on 17), for Adjellela and T-in-Hamor, respectively. Moreover, our observations indicated that seedlings from the bigger patch (T-in-Hamor) have the better growth performances (e.g. for individual height and number of leaves), while the worst performances were observed for seedlings originating from the smallest one (Tonget). All these differences were significant, even after 150 days when nine unviable seedlings of Tonget were eliminated (Fig. 2). Growth performances of Adjellela seedlings were intermediate between those of T-in-Hamor and Tonget. These observations thus suggest that performances of our seedlings are positively related to the patch size of origin, but it is not excluded that other reasons having influenced seed development, such as variable water conditions between locations, can be responsible for such differences.

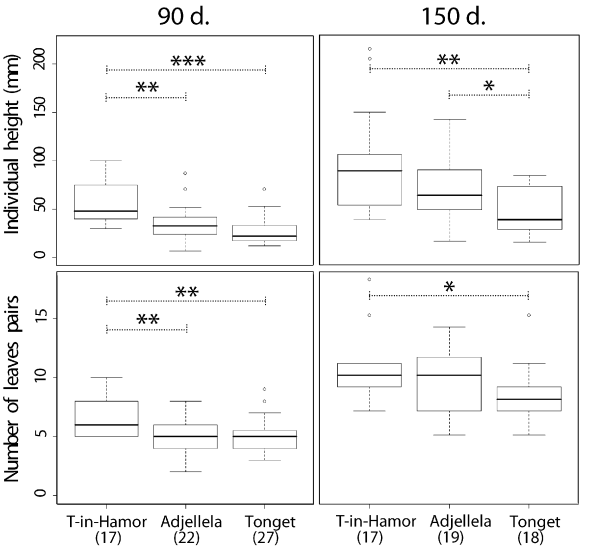

Fig. 2. Growth performances of seedlings originating from three different locations: T-in-Hamor (large-size patch; > 150 trees), Adjellela (small-size patch; 15 genets) and Tonget (small-size patch; 7 genets). Measures were done at 90 and 150 days after seed germination, for the individual height and the number of leaves pairs. The number of seedlings considered at each measurement is given in brackets. Significance of differences between locations of origin were assessed by a Wilcoxon test, and are indicated when significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Note that, for the individual height, Wilcoxon tests were applied on data after transformation (i.e. reciprocal of height) resulting in a constancy of variance between locations. Between 90 and 150 days, zero, three, and nine seedlings died for localities T-in-Hamor, Adjellela, and Tonget, respectively.

4. Discussion

It is now well accepted that the Laperrine's olive populations from Algeria and Niger did not regenerate efficiently since a long time [9,10,16]. This lost of sexual reproduction should be due to the climatic rigor combined with other factors as for example the anthropic activities and browsing by animals [10]. In this context, the multiplication of this taxon could be an important issue to maintain the present populations and to create nurseries in order to preserve this important genetic resource of the cultivated olive (particularly for drought adaptation). The strategy of multiplication (via seeds vs. vegetative propagation) remains an open question. The understanding of pollen flow in the populations is an important step in order to evaluate the utility of the multiplication by seeds and to optimize the strategy of sampling. We thus performed a paternity analysis on seeds from four localities in the Hoggar Mountains. This study is also the first report on pollen-mediated gene flows within a wild population of olive trees (but for the cultivated olive see also [19,25]).

4.1. Effective pollen-mediated gene flow and multipaternity in the Laperrine's olive populations

In Akerakar and T-in-Hamor, a trend for higher multipaternity was found (up to a minimum of 15 fathers/34 seeds for the mother Akerakar 3-4-5) than in Tonget and Adjellela. This supports the prediction that multipaternity should greatly depends on the number of flowering

trees occurring in a location. In Akerakar, pollen flow occurred between relatively close trees (one case of selfing, and crossing between Akerakar 1 and 3-4-5) but can reach up to at least 2000 m (Fig. 1B). Our study thus demonstrated that effective wind pollen dispersals at relatively long distance are possible in the olive tree, as recently shown in another Oleaceae species (*Fraxinus excelsior*; [21]) and numerous trees (for a review see [20]). Of course, such long-distance flow strongly depends on the habitat fragmentation and the occurrence of pollen sources in the perimeter around mother trees [39], but the landscape topology probably also influences pollen movement. The Akerakar and T-in-Hamor olive patches are located in valleys with several interconnected wadis, which should allow a relatively huge effective dissemination of pollen by wind. In contrast, the small patches of Adjellela and Tonget survive in massif cliffs and this situation should limit the possibility of effective pollen flow at relatively long distance (>500 m; Fig. 1A, C).

Cross-compatibilities also appeared as another important factor limiting the multipaternity [18,25]. Indeed, some reciprocal cross-incompatibilities between flowering genets (in small patches of Tonget and Adjellela) were strongly suggested by our paternity analyses. In contrast, preferential fathers were also found for mother genets (Fig. 1A). Such phenomena, also reported in the cultivated olive [18,25], are probably due to gametophytic compatibilities between some genets (avoiding or not reciprocal crossings) but could also be due to some shifts of blooming periods.

4.2. *Effect of seed multiplication on gene diversity*

The impact of seed multiplication was measured by the analysis of genetic diversity between the initial population and the produced seeds, and this analysis revealed a significant loss of allelic richness (of 29% in seeds, and 32% in surviving seedlings). In contrast, H_S was not affected, but compared to R_S, this parameter is known to be less sensitive to stochastic effects as reported in *Argania spinosa* [40] and also in olive populations after successive bottlenecks associated to the recolonisation [41,42]. The observed decrease in allelic richness is of course mainly due to the relatively low number of trees involved in the sexual reproduction for the seeds collected (a minimum of 31 parents has been involved either as a mother or a father, but with a high variance in the reproductive output) and this result clearly demonstrates that our seed and seedling samples are far to be fully representative of the genetic diversity of the initial population [43]. Similar results were recently obtained in restoration programs of forest tree species (e.g. [44–46]). To avoid this phenomenon, collecting of fruits on a larger number of mother trees from different locations is required [43].

As previously mentioned, we did not reveal pollen migrants in the two isolated, small patches of Tonget and Adjellela, since the 149 seeds were issued from pollination at short distance. This means that such a break of connectivity should rapidly create pattern of patch differentiation when sexual regeneration occurs (due to genetic drift), and could potentially lead to the appearance of inbreeding depression, as suggested by our growth observations in the Tonget seedlings (Fig. 2). Isolation in small patches should also contribute to increase the spatial genetic structure (SGS) at the population scale as revealed in the Niger Mountains [15]. In our previous study, no significant relationships between genetic and geographic distances were revealed in the Adrar Heggueghene massif, Hoggar [15]. This massif includes several large valleys (of whom T-in-Hamor) with a relatively high number of Laperrine's olive trees (probably more than 500 individuals in an area of about 80 km^2), and the maintenance of relatively big patches should partially explain the absence of a significant SGS on this massif [15]. It is likely that the Laperrine's olive populations have regressed during arid periods on the Sahara and then re-expanded during interglacial periods, and especially during the Holocene [47,48]. These populations have probably again regressed during the last millennia. We can thus suspect that the connectivity between close patches has been affected relatively recently, and SGS should have not been particularly increased due to a considerably limited sexual regeneration during this period.

Even if this study was limited to a relative low number of mother trees, it brings some important insights on the conservation strategy of the Laperrine's olive based on seed multiplication. It may be important to maximise the genetic diversity of seedlings particularly to limit the genetic drift associated to the population regeneration and thus to preserve their evolutionary potential. This could be realised by sampling seeds in several large populations with numerous trees reaching sexual reproduction (e.g. Akerakar, T-in-Hamor or Ilamane). Favourable flowering and fructifying conditions (e.g. at least as in 2007) leading to high numbers of mothers and pollen donors as well as adequate landscape conditions (e.g. valleys) are necessary for an optimal sexual reproduction. Small patches have to be avoided since the number of parents can be very limited (e.g. Tonget) and an Allee effect should probably affect the progeny performances issued from such places.

4.3. Further prospects

Several perspectives can be given to this study. First, our analysis should be expanded to a higher number of mother trees and seeds in order to better estimate the distance of pollen dissemination in the Laperrine's olive populations. Similar patches (for individual number and density) located in different conditions (e.g. wadis network, cliffs) should be considered to assess the impact of the topography. It should also be interesting to prospect seeds on several successive years on trees located in favourable locations. The effect of the year on the multipaternity could be assessed. Moreover, the cross-incompatibilities should be investigated and the characterization of *S*-alleles [49] should help to disentangle this phenomenon. Based on these different parameters (e.g. multipaternity, distance of pollen gene flow, location topography, year of flowering ...), it could be possible to maximise genetic diversity of seedlings by modelling the seed sampling [43]. Lastly, the comparison of pollen-mediated gene flows between species located in the same landscape (for instance *Rhus tripartita*, *Pistacia atlantica*, *Ficus salicifolia* and/or *Acacia ehrenbergiana* on the Akerakar or T-in-Hamor localities) could allow a generalization of conclusions on the conservation strategies of other trees. Such approaches could be really relevant in order to manage endangered woody species of the high Saharan Mountains.

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