Genetic signals of ancient decline in Aleppo pine populations at the species' southwestern margins in the Mediterranean Basin

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Microsatellite markers were used to characterize the structure of genetic diversity in natural Moroccan Aleppo pine (*Pinus halepensis* Mill.) populations, the most southwesterly marginal populations of the species in the Mediterranean Basin. Twenty-two natural populations and one artificial population, located in four regions covering most of the natural range of *P. halepensis* in the country, were sampled. Across this range, towards the south and west (and towards high altitudes) the populations become increasingly discrete and discontinuous. The nuclear microsatellite marker analysis suggests that a large proportion of the Aleppo pines in Morocco have derived from a single genetic lineage, represented by a central group of 11 of the examined populations located in the High and Middle Atlas Mountains. In addition, two smaller groups, represented by the marginal southwestern High Atlas populations, and three still smaller north / northeastern groups of populations located in the Rif and northeast Middle Atlas Mountains, could be genetically distinguished. Further, coalescence analysis of historical demographic population patterns suggests that ancient bottlenecks occurred in all of the natural populations. However, the population differentiation and genetic diversity levels we found were good ($F_{st} = 15.47$), presumably because of the species' good potential for long-distance dispersal of seeds and high invasive capacity, which appear to have maintained a state of stable near-equilibrium, meta-population dynamics since ancient times.

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The central-marginal hypothesis postulates that genetic diversity of a species is likely to be lower, and genetic differentiation between its populations higher, at the limits of its range (WANG et al. 2002; SCHWARTZ et al. 2003). In a recent review of 134 studies covering 115 species, ECKERT et al. (2008) found some, but far from unanimous, support for this prediction, i.e. declines in intra-population diversity and higher population differentiation toward range edges in 64.2% and 70.2% of the studies, respectively. Successive founder events, repeated local extinctions, small effective population size and reductions in gene flow between populations are among the factors that can lead to a reduction in genetic diversity in marginal populations. These processes have often been overlooked in out-crossing, wind-pollinated tree species because analyses of neutral markers have generally revealed weak genetic partitioning among their populations (AUSTERLITZ et al. 2000). Some studies have found nearly random distributions of genetic variability in tree species (MUIR et al. 2004; ROBLEDO-ARNUNCIO et al. 2005; O'CONNEL et al. 2006), while others have detected local genetic structure over short distances (JUMP and PENUELAS 2006; TSUMURA et al. 2007). Within some pine populations, fine-scale

genetic structure has been detected, suggesting that once the trees colonized an empty site, little exchange with neighbouring sites occurred (OLINE and MITTON 2000; TROUPIN et al. 2006).

Aleppo pine *(Pinus halepensis* Mill.) is a common pine species of the Mediterranean Basin (BARBÉRO et al. 1998). Moroccan populations are at the southwestern margins of the species' distribution in the Mediterranean, and allozyme studies have shown that these populations have the lowest heterozygosities of all the Mediterranean populations (SCHILLER et al. 1986; KOROL et al. 2001). Throughout their range in Morocco, Aleppo pine populations gradually become increasingly discrete and discontinuous from sea level to an altitude of 2600 m. Aleppo pine has also undergone several episodes of extensive reduction in population size, related to human-induced disturbances, such as timber exploitation, fuel extraction, fires and overgrazing (NAHAL 1962; QUEZEL 1980; QUEZEL et al. 1980). In the Rif Mountains in northern Morocco, populations have also been historically threatened by deforestation, and hence regional extinction (MOORE et al. 1998; BENABID 2000). However, the species still persists in the landscape, possibly as a meta-population due to its good dispersal

potential. Aleppo pine is also one of the most invasive pine species, capable of rapidly colonizing disturbed habitats generated by fire or other factors; seed dispersal and successful establishment of the species can occur even in the absence of fire-disturbed habitats (NATHAN et al. 1999; NATHAN and MULLER-LANDAU 2000). Therefore, severe population contraction may be followed by rapid expansion. In the present study, microsatellite markers were used to characterize the structure of genetic diversity in natural Moroccan populations of *Pinus halepensis*, which grow in the most adverse ecological conditions for the species in the Mediterranean basin. Hence there have presumably been strong selective pressures on the species (PAPAGEORGIOU 1997), and accordingly variations that appear to be linked to both geographic and local ecological conditions in Aleppo pines have been described (PANETSOS 1981; BARADAT 1986; NAHAL 1986; QUEZEL 1986). In addition, two major groups (southwest and northeast), each containing two minor groups of populations of this species in Morocco have been identified by factorial component analysis (FCA) of morphological characters and hierarchical classification (BOULLI et al. 2001). Studies of genetic variation in this species have detected substantial within-population differences in both allozyme markers and quantitative traits (SCHILLER et al. 1986; SCHILLER and GRUNDWALD 1987).

We hypothesized that successive population contractions (due to natural or human-induced disturbances) followed by expansions (due to the good potential for pines to regenerate) could be expected to be reflected in the genetic and demographic structure of the Aleppo pine populations. In this study we tested this hypothesis, and specifically addressed the following series of questions regarding the population structure of the species, and the demographic history of the populations. Among populations how many distinct groups are present? To what extent have the populations diverged? Is there a relationship between geographic location and inter-population variation? Are there populations or regions that should be given high priority for gene conservation due to their genetic uniqueness? Is it possible to identify signs of population changes (ancient, recent or both)? What kinds of changes have occurred: reductions, expansions, or both, and are the changes related to anthropogenic effects? To address these questions we analyzed microsatellite markers, since they are particularly useful for detecting allelic variation in populations (BOYS et al. 2005).

MATERIAL AND METHODS

Plant material and laboratory methods

Twenty-two natural populations, located in four regions covering most of the natural geographic range of *Pinus halepensis* in Morocco, were sampled as follows: 13 from the Oued Tensift region (I), four from the Moulouya region (II), three from the Rif region (III), and two from the Oued Sebou region (IV). The Oued Sebou region includes the basin of the Sebou river, stretching from areas with extensive oak and cedar forests and scattered lakes between Morocco's Rif and Middle Atlas ranges to the Atlantic Ocean in Mehdia. The Moulouya region is located in the Middle Atlas Mountains. The Rif is a mainly mountainous region in northern Morocco. Finally, the Oued Tensift is on the southwestern slopes of Jbel Igdet and Jbel Toubkal in the High Atlas (Fig. 1). The distribution of Aleppo pine in Morocco is largely fragmented, consisting of small forest stands covering 20–75 ha. For this reason, although among-population statistical analyses were performed separately for each population in each region, for demographic statistical analyses some populations relatively close to each other within the same region (MA 1 and 20, MA 2 and 21, MA 7 and 23, MA 6 and 16, and MA 4, 17 and 22) were analysed together to meet minimum sample size requirements. Seeds were then collected from a minimum of 80 individuals in stands with ages varying from 60–80 years old at each locality and kept in the dark at 4°C until they germinated, as described below. From each locality, 36 seedlings were analysed.

The seeds collected from each population were placed on moist filter paper in Petri dishes and incubated at 26°C under a 14 h photoperiod for 10 days. Total DNA was then extracted from the germinating seedlings using a
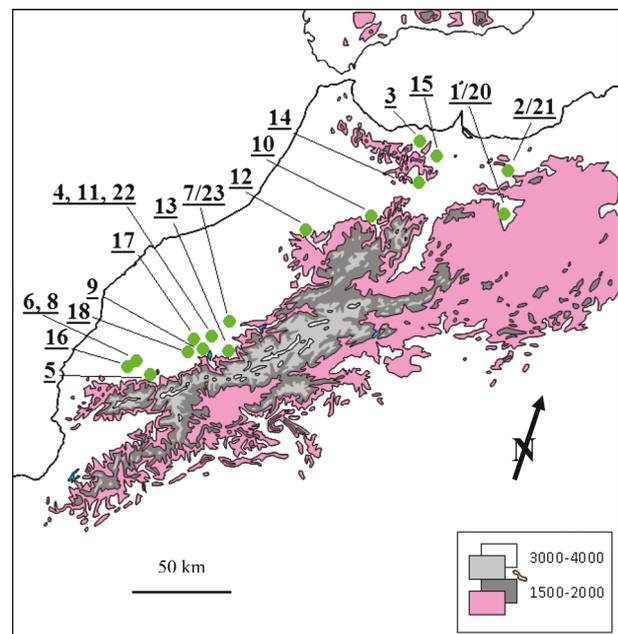
Fig. 1. Locations of the 22 natural *Pinus halepensis* Mill. populations studied in Morocco (MA 19* is artificial and is not shown in this figure).

Sigma mini-preparation kit, as described by NAYDENOV et al. (2006, 2007). Individuals were then genotyped by screening six microsatellite sequences (nuSSR) using six primer pairs that had been originally developed for other *Pinus* species (PtTX-3025, PtTX-2090, PtTX-3030, PtTX-3020, PtTX-3123 and Pde-7), fluorescently labelled with 6-FAM, VIC or NED (LIAN et al. 2000; AUCKLAND et al. 2002). Briefly, template DNA (25 to 35 ng) was used in a master mix of PCR reactants containing 6.6 µl HotMaster buffer (Eppendorf North America, Westbury, NY, USA), 100 µg µl⁻¹ gelatine, 1% DMSO, 50 µM of each dNTP, 100 nM of each forward and reverse primer, 0.325 U HotMaster Taq DNA Polymerase (Eppendorf North America) and 1.5–4.0 mM $MgCl_2$ in a 10 µl reaction volume. The target loci were amplified using a thermocycler (Perkin-Elmer 9700) and the following protocol: initial Taq activation at 95°C for 2 min 30 s; 30 cycles of 2.5 min denaturation at 94°C, 2.0 min annealing temperature and 1 min extension at 72°C; and a final extension for 3 min 30 s at 72°C. PCR analysis was similar to that described by DIEFFENBACH and DVEKSLER (1995) and WILLIAMS et al. (2000).

Fluorescently-labelled PCR products (0.4 µl) were added to 10 µl Hi-Di Formamide (Applied Biosystems, Westborough, MA, USA), an internal size standard and 0.4 µl ROX-1000 (MapMarker, BioVenture Inc., Murfreesboro, TN, USA) then denatured for 5 min at 95°C, placed on ice, and separated by capillary electrophoresis using a Prism 3130 Genetic Analyzer (Applied Biosystems).

Statistical analysis

Data acquired from the PCR products were analyzed using DataCOLLECTION ver. 3.0 software (Applied Biosystems), and genotypes of the samples were inferred from the individual size profiles of the nuclear DNA markers using GeneMAPPER ver. 3.7 software (Applied Biosystems). The MicroCHECKER ver. 2.2.3 program of VAN OOSTERHOUT et al. (2004) was used to test for null alleles and scoring errors caused by large allele dropout and stutter peaks. Allele frequencies were calculated using GENETIX ver. 4.2 (BELKHIR 2002). Unbiased expected heterozygosity (uH_e), private alleles (A_{pa}), effective number of alleles (A_e) according to KIMURA and CROW (1964), locally common alleles (less than 25% and 50%), symmetrical gene flow $N_m = (1 - F_{st}) / 4F_{st}$ (SLATKIN 1985), the Shannon-Weaver information index (I) and F_{st} and G_{st} coefficients (WRIGHT 1965; NEI 1975) were all calculated using GenAlEx ver. 6 (PEAKALL and SMOUSE 2006).

A preliminary BOTTLENECK ver. 1.2. software test (CORNUET and LUIKART 1996) showed that for all populations the distributions of molecular markers were consistent with

several mutation models, including the Infinite allele model (IAM), Two phase model (TPM) and Stepwise mutation model (SMM). At single population level, SMM assumptions showed the best results. Two models were tested to identify among-population structures: (1) the neighbour-joining algorithm (NJ) of SAITOU and NEI (1987) and ROGERS (1972), based on geometric distances rather than mutational distances; and (2) a frequency strategy – the Bayesian stochastic approximation 'non-admixing' algorithm implemented in BAPS ver. 5.1 software (CORANDER et al. 2006; CORANDER and TANG 2007).

The distance-based inter-population structure was analysed based on allelic frequency data with 10^3 nonparametric bootstrap replications, according to EFRON and TIBSHIRANI (1998), FELSENSTEIN (1985) and HOLMES (2003), using the 'ade4' package of R ver. 2.5.1. (IHAKA and GENTLEMAN 1996). BAPS software uses allele frequencies to resolve the number of genetically divergent groups in a population, which is considered as a random variable. To complete the analysis, AMOVA methods were applied to quantify the inter-population and inter-group genetic variation (ARLEQUIN ver. 2.1; EXCOFFIER et al. 2002).

Population size (Θ; *theta* $= 4N_e\mu$) and gene flow ($4N_m$; migration) between the studied populations were calculated using the Markov chain Monte Carlo (MCMC) algorithm implemented in MIGRATE ver. 2.6 (BEERLI and FELSENSTEIN 2001), with the following Markov chain settings: short/ long chains, 10/ 3; trees (short/ long) sampled, $10^4/10^5$; trees (short/ long) recorded, $5 \times 10^2/ 5 \times 10^3$; discard trees per chain, 10^4. To detect bottlenecks or effective population size expansion, we used the coalescent model-based method of BEAUMONT (1999) with the following conditions: rectangular prior limit of –8 to +8 with the linear model; 2×10^9 replications per run (10^5 steps times 2×10^4 thinned updates), the first 10% were discarded as burn-in, and remaining data were used to obtain the modes of 10%, 50% and 90% highest posterior densities (HPD) of the posterior distributions for each parameter using the module LOCFIT ver. 2.0 of the statistical package R (<www.r-project.org>). As a complementary test, we used the ratio of the mean number of alleles to allele size range to calculate *M*-ratios (GARZA and WILLIAMSON 2001). An *M*-ratio less than the critical value, M_c ($M_r < M_c$) is indicative of an important reduction in the effective population size. The M_r value was calculated for each population with 10^4 replicates. *M*-ratio analysis is only capable of detecting recent demographic events (i.e. $< 4N_e$), according to CORNUET and LUIKART (1996).

The contemporary effective population size (N_e) was calculated using the model-based algorithm of TALLMON et al. (2004), which is based on Bayesian simulation of a single population. We used lower and upper bounds

on the prior for N_e of 2 and 10^3, respectively. Generally, estimates of N_e are much lower than census estimates; therefore we also tested upper bounds of 25×10^3 and 125×10^3 to guide the choice of prior bounds to use in subsequent analysis.

RESULTS

Genetic diversity of Aleppo pine populations

A total of 28 alleles were detected at the six microsatellite loci, with numbers of alleles per locus ranging from two for PtTX-3025, three to PtTX-3030, four to PtTX-3020, five to PtTX-2090, six to PtTX-2123 and eight for Pde-7. The average number of alleles per population ranged from one at locus PtTX-3025 to seven at locus Pde-7, and the effective number of alleles (A_e) varied from 1.294 to 1.992. Between one and three private alleles (A_{pa}) were detected in the three most marginal populations studied (MA 3, MA 9 and MA 20; Table 1). No locally common alleles (N_{lca}; at less than 25% level) were found in eight of the populations investigated.

Population differentiation and genetic relationship among populations

Bootstrapping using 10^3 replications over individuals and loci in the among-population distance analysis showed clear structure, with eight groups of populations. The analysis clearly identified two 'NJ clusters' (MA 5; MA 6/16 and MA 8) in the southwestern region of the natural geographical distribution, and three (MA 1/20; MA 2/21; 3, 14, 15) in the northeastern area, connected to one (the biggest) consisting of 11 central populations in the watersheds of the Oued Tensifi and Oued Sebou rivers. Complementary sub-'NJ cluster' structures were observed in the northeastern and central areas (Fig. 2). The 'non-admixing cluster' Bayesian algorithm detected the presence of $K = 6$ clusters, with a similar among-population pattern: one large central cluster connected to between two and three peripheral clusters in both the northeastern and southwestern regions (Fig. 3). The artificial population MA 19 seems to originate from the NE Atlas lineage (i.e. 'non-admixing cluster 1').

Table 1. *Genetic diversity in the studied Morocco populations of Pinus halepensis Mill.*

Pop	Name	Region	N	Lat. (N)	Long. (W)	Alt. (m)	uH_e	A_{pa}	A_e	I	N_{lca} $\leq 25\%$	N_{lca} $\leq 50\%$
MA-1	Lalla Mimouna	II	18	34°01′	2°53′	1200	0.173	–	1.294	0.286	–	0.167
MA-2	Jbel lakhder	II	18	34°45′	2°41′	700	0.197	–	1.355	0.313	0.167	0.333
MA-3	Al Hoceima	III	36	35°22′	3°52′	300	0.267	3	1.445	0.473	–	0.167
MA-4	Aït Bouissa	I	12	31°41′	6°56′	1460	0.260	–	1.513	0.418	0.167	0.333
MA-5	Ijoukak	I	36	30°57′	8°07′	1660	0.374	–	1.608	0.594	0.500	0.667
MA-6	Imintala	I	18	31°07′	8°16′	1630	0.357	–	1.672	0.558	0.167	0.333
MA-7	Tamga	I	18	32°00′	6°08′	1490	0.379	–	1.704	0.594	0.333	0.500
MA-8	Tizgui	I	35	31°11′	8°12′	1460	0.351	–	1.682	0.560	0.500	0.667
MA-9	Aït Tamlil	I	36	31°29′	6°57′	1670	0.315	1	1.610	0.518	0.333	0.667
MA-10	Skoura	IV	36	33°27′	4°35′	1230	0.224	–	1.387	0.388	0.167	0.333
MA-11	Bouguemez	I	36	31°47′	6°27′	1630	0.248	–	1.479	0.405	–	0.167
MA-12	Zaouia Ifrane	IV	35	33°11′	5°31′	1400	0.194	–	1.359	0.314	–	0.167
MA-13	Ksiba	I	36	31°22′	5°42′	1550	0.254	–	1.470	0.412	–	0.167
MA-14	Aknoul	III	36	34°36′	3°50′	1100	0.279	–	1.468	0.451	–	0.333
MA-15	Oued Nekour	III	36	35°30′	3°49′	400	0.293	–	1.471	0.461	–	0.167
MA-16	Amezmez	I	18	31°09′	8°18′	1520	0.321	–	1.571	0.499	0.167	0.333
MA-17	Bouissa	I	12	31°44′	6°56′	1380	0.332	–	1.586	0.497	–	0.167
MA-18	Zerectene	I	36	31°24′	7°23′	1260	0.336	–	1.731	0.564	0.500	0.667
MA-19	Jbel Temsamane	art	36	35°21′	3°48′	1300	0.195	–	1.295	0.329	0.500	0.500
MA-20	Lalla Mimouna	II	18	34°03′	2°55′	1260	0.258	–	1.397	0.422	0.500	0.500
MA-21	Jbel lakhder	II	18	34°46′	2°41′	740	0.377	1	1.992	0.659	0.667	0.833
MA-22	Tifni	I	12	31°43′	6°58′	1370	0.377	–	1.719	0.561	0.167	0.333
MA-23	Tamga	I	18	32°02′	6°10′	1510	0.374	–	1.732	0.562	0.167	0.333
Mean							0.293	1.67	1.545	0.471	0.333	0.384

Note: The total number of trees analysed was 610. Abbreviations: I, the Oued Tensift river basin; II, eastern part of the Moulouya river valley; III, Rif; and IV, Oued Sebou river basin; N, sample size; uH_e, unbiased expected heterozygosity; A_{pa}, private alleles; A_e, effective number of alleles (KIMURA and CROW 1964); I, information index (LEWONTIN 1972); N_{lca}, locally common alleles ($\leq 25\%$ and $\leq 50\%$).
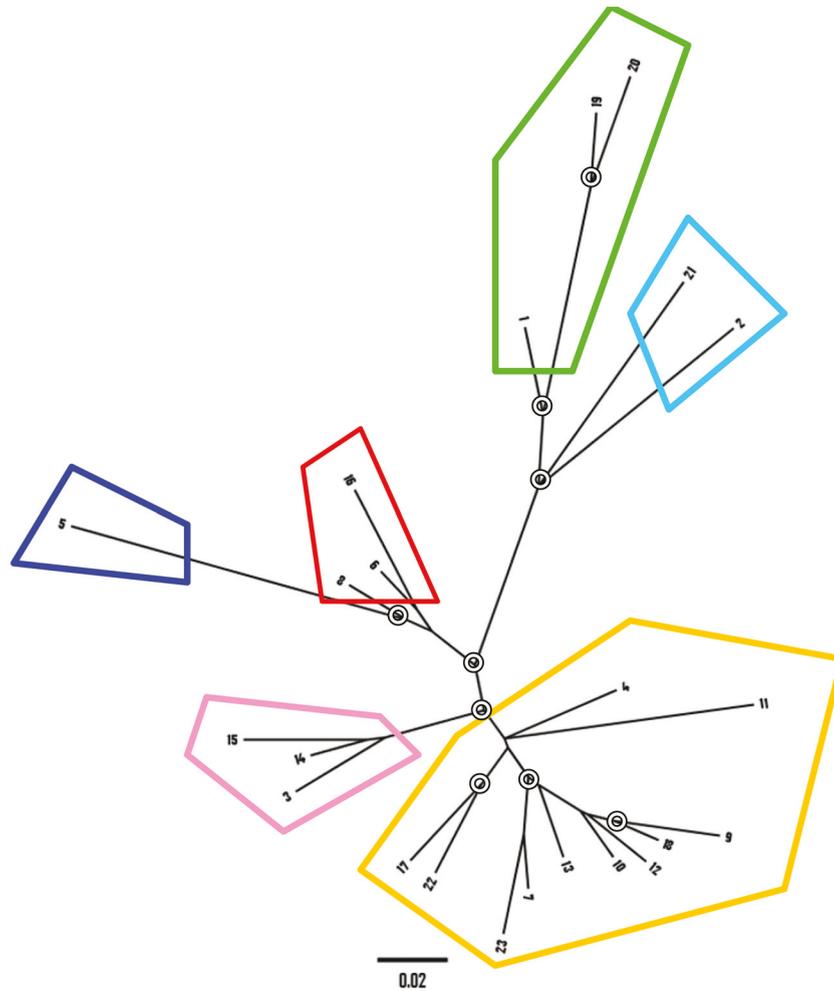
Fig. 2. The neighbour-joining (NJ) algorithm of ROGERS (1972) on a matrix of geometric distances of between 23 sampling locations. The rings indicate nodes with at least 50% bootstrap support (1000 replications).

The hierarchical AMOVA indicated that among-group variance accounted for 7.3% and 7.01% of the total 'NJ clustering' and 'non-admixing clustering' variance, respectively, and among-population genetic variance within the groups for 7.91% and 9.04%, respectively (unpubl.). The variations in percentage (PV) for the global AMOVA values, $G_{st(nc)}$ and F_{st} were 14.34, 13.80 and 15.47, respectively.

Historic demographic fluctuations

The average effective population size (N_e) was found to be 64.24 (CI 95%: 34.61–242.84). The coalescence analysis, following BEAUMONT (1999), detected signals of ancient demographic decline for all of the studied populations (Table 2), which ended (on average) 154 000 generations ago ($\log_{10}t_f = 3.08$ at 90% HPD; using the formula $2N_e \times$ atilog-$\log_{10}t_f$). The generation time for Aleppo pine, in terms of the time required for an individual to grow and mature sufficiently to flower consistently and abundantly, is usually about 15–16 years in the southeastern region of Spain (TAPIAS et al. 2001). The results indicate that a very sharp population decline in the ancient past has been followed by a long post-bottleneck period of population equilibrium, which started before the Last glacial depression (LGD) and the genetic diversity of the populations is still lower than it was prior to the decline. Demographic contractions had differing patterns at 10% HPD, ranging from sudden, discrete, very ancient events ($\log_{10}t_f \approx 6$; MA 2/21, 7/23, 8, 12, 6/16), through more recent but still very old discrete events $\log_{10}t_f \approx 4$; MA 1/20, 5, 9, 10, 14, 15, 18, 23) to multiple periodic waves (MA 3, 11, 13, 18, 4/17/22; Fig. 4). During the period of decline the populations lost at least 98.88% of their original size (average $\log_{10}r = -3.76$ at 90% HPD).

However, although the *M*-ratios (GARZA and WILLIAMSON 2001) for two markers (PtTX-2090, Pde-7) were lower than the critical ratio indicative of an important reduction
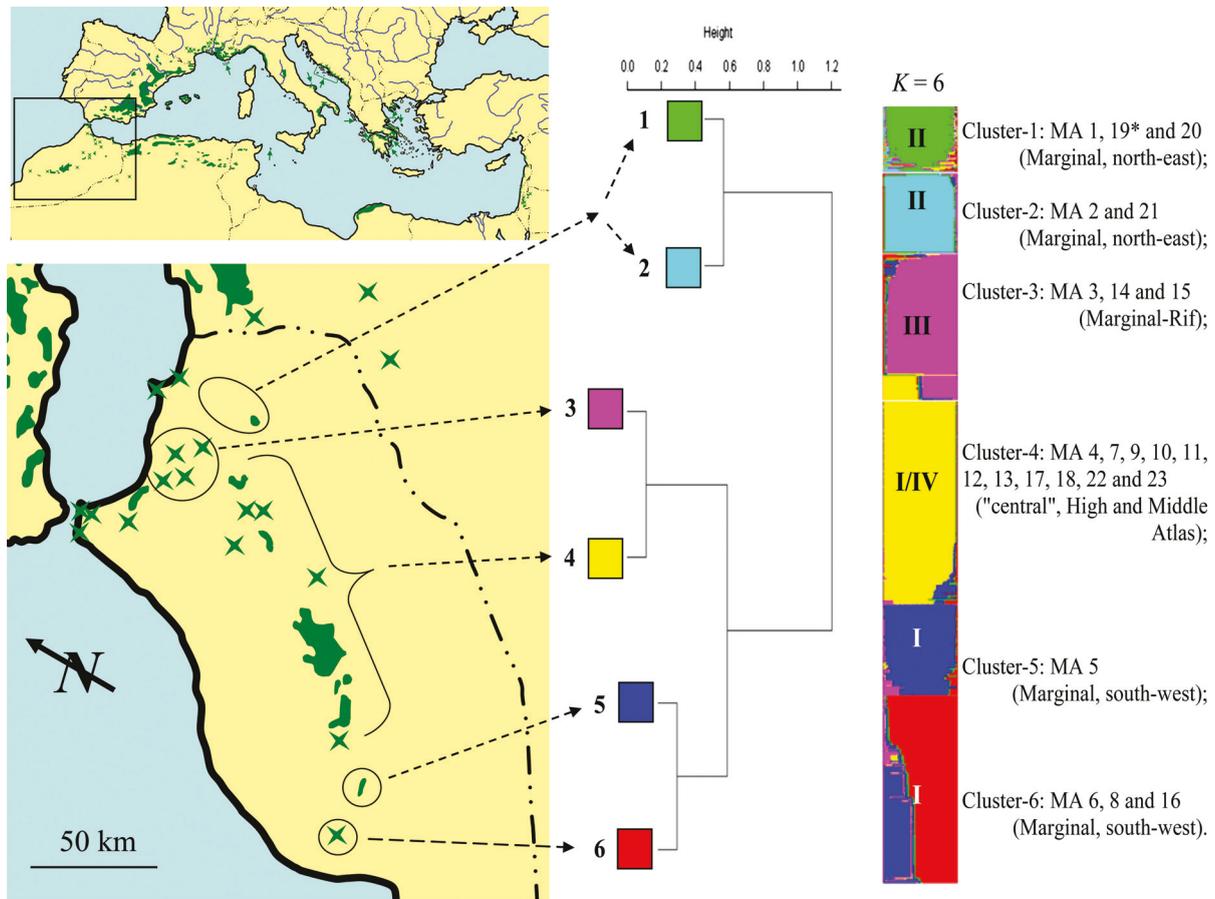
Fig. 3. Natural area of distribution of Aleppo pine in the Mediteraneene region (left/top) according to CRITCHFIELD and LITTLE (1966) and that of *Pinus halepensis* in Morocco with indications of the cluster assignments (bottom/left). Dominant membership of individuals (right) from the 23 populations in the six clusters ($K = 6$) identified by the 'without admixture' BAPS model for correlated allele frequencies. The Roman numerals indicate the following geographic regions: I, the Oued Tensift watershed; II, the eastern part of the Moulouya river valley; III, the Rif mountains; and IV, the Oued Sebou river watershed (*MA 19 is an artificial population).

in the effective population size, in each population, the ratios for all of the remaining markers exceeded the critical value (0.680). Further, the overall *M*-ratio (0.436) was significantly lower than *M-critical*, due to the large standard deviation (SD ≈ 0.25; Table 2). These results suggest that these populations have experienced significant bottleneck events in the distant past (i.e. not recent population decline), assuming mutation – drift equilibrium existed.

The number of migrants per generation, N_m, indicating average symmetrical gene flow, amounted to 1.36 individuals, while asymmetric migration amounted to 1.40 and 0.78 individuals per generation (for IAM and SMM respectively), similar to N_m values (unpubl.). Thus, the total exchange and the difference in exchange were 2.34 and 0.46 individuals per generation for IAM and 1.40 and 0.17 for SMM, respectively. The most intensive asymmetric gene flow was detected in the 11 populations located in the watersheds of the Oued Tensifi and Oued

Sebou rivers (i.e. 'Central BAPS non-admixing cluster 4'). The average population size (Θ; *theta* = $4N_e\mu$) was 0.231 (SD = 0.086) for SMM. The theta values under SMM were much higher for populations located in the watershed of the Oued Tensifi and the Rif Mountains (MA 3, 5, 9 and 15) than for populations in the other regions (unpubl.).

DISCUSSION

Our results show that the genetic diversity in *Pinus halepensis* in Morocco is lower than has been reported in other pine species investigated by nuclear microsatellite analyses. In the present study, mean diversity values in Aleppo pine ranged from 0.173 to 0.379 with an overall mean average of 0.293, compared with values between 0.620 and 0.773 (H_e) obtained for *P. sylvestris* (from Bulgaria); and compared with values between 0.382 and 0.490 obtained for *P. pinaster* from Morocco using the

Table 2. *10%, 50% and 90% highest posterior density (HPD) limits of the posterior distribution of historical population size ($\log_{10}r$) and timing ($\log_{10}t_f$) of ancient changes obtained using a linear model of change, based on the Bayesian coalescent-based approach to analyzing historical demographic patterns of* BEAUMONT *(1999); and results of M-ratio analysis (*GARZA *and* WILLIAMSON *2001) analysis of recent population genetic demographic history, up to on average 256.96 ; $4N_e$, where N_e 64.24) generations. The bold numbers indicate the presence of bottlenecks in the studied population (i.e. $\log_{10}r$ 1).*

| Pop	M-ratio (SD)	10% HPD		50% HPD		90% HPD	
		$\log_{10}r$	$\log_{10}t_f$	$\log_{10}r$	$\log_{10}t_f$	$\log_{10}r$	$\log_{10}t_f$
MA-1/20	0.392 (0.26)	**−5.58/−5.17**	4.54/4.94	**−6.54/−4.41**	3.80/5.93	**−7.69/−3.44**	2.88/7.11
MA-2/21	0.446 (0.26)	**−6.39/−6.05**	5.34/5.69	**−7.07/−5.30**	4.61/6.37	**−7.79/−4.24**	3.58/7.10
MA-3	0.473 (0.24)	**−4.74/−4.25**	3.60/4.16	**−6.14/−3.55**	2.89/5.66	**−7.34/−2.84**	2.17/7.18
MA-4/17/22	0.429 (0.27)	**−6.13/−5.78**	4.84/5.17	**−6.87/−5.06**	4.17/5.93	**−7.76/−4.03**	3.21/6.88
MA-5	0.464 (0.26)	**−5.51/−5.02**	4.30/4.78	**−6.63/−4.25**	3.55/5.94	**−7.75/−3.39**	2.73/7.12
MA-6/16	0.461 (0.24)	**−5.17/−4.81**	3.87/4.20	**−6.01/−4.14**	3.28/5.05	**−7.46/−3.27**	2.51/6.63
MA-7/23	0.427 (0.24)	**−6.32/−5.96**	5.28/5.65	**−7.05/−5.14**	4.50/6.37	**−7.78/−4.00**	3.40/7.13
MA-8	0.467 (0.24)	**−6.05/−5.65**	5.13/5.51	**−6.88/−4.85**	4.33/6.36	**−7.77/−3.80**	3.34/7.27
MA-9	0.461 (0.23)	**−6.27/−5.93**	4.80/5.11	**−6.97/−5.15**	4.11/5.74	**−7.73/−3.99**	3.08/6.55
MA-10	0.423 (0.27)	**−6.06/−5.71**	4.94/5.30	**−6.83/−4.96**	4.20/6.10	**−7.75/−3.93**	3.19/7.06
MA-11	0.446 (0.26)	**−6.47/−6.11**	5.36/5.70	**−7.14/−5.36**	4.63/6.38	**−7.82/−4.13**	3.50/7.10
MA-12	0.391 (0.26)	**−6.25/−5.88**	5.16/5.54	**−6.99/−5.12**	4.40/6.29	**−7.79/−4.07**	3.40/7.12
MA-13	0.446 (0.26)	**−6.36/−6.02**	5.23/5.58	**−7.06/−5.27**	4.51/6.28	**−7.81/−4.18**	3.47/7.09
MA-14	0.416 (0.26)	**−5.23/−4.84**	4.06/4.44	**−6.17/−4.19**	3.45/5.40	**−7.52/−3.39**	2.69/6.88
MA-15	0.475 (0.25)	**−5.16/−4.77**	3.97/4.35	**−6.09/−4.10**	3.34/5.32	**−7.52/−3.28**	2.58/6.93
MA-18	0.353 (0.25)	**−6.47/−6.13**	5.44/5.78	**−7.11/−5.34**	4.67/6.42	**−7.80/−4.16**	3.51/7.13
Mean	0.436 (0.25)	**−5.88/−5.50**	4.74/5.12	**−6.72/−4.76**	4.03/5.97	**−7.69/−3.76**	3.08/7.02

Note: M– stat: critical M values (M_c) were obtained from simulations with 10^4 replicates at $\triangle_g=3.5$ the average size of multi-step mutations and $p_s=20\%$ of the average proportion of one-step mutation; *M-ratios* (SD)$=k/r$ where k is the number of observed allele and r is the overall allelic size range (GARZA and WILLIAMSON 2001).

same nuSSR markers (WAHID et al. 2010; NAYDENOV et al. unpubl.). Low levels of H_e are commonly found in populations of *P. halepensis* across its geographical range (GOMEZ et al. 2001; KOROL et al. 2001), suggesting that low genetic diversity is a feature of the species. Further, the results presented here indicate that a substantial proportion of this low diversity originated from ancient events before the last glacial depression. VENDRAMIN et al. (2008) have similarly suggested recently that there is a widespread area in the Mediterranean region in which the genetic diversity of *P. pinea* (L.) is low, due to a sharp decline that predated the LGD.

Population differentiation

The AMOVA indicated that there is a 14.34% differentiation among Aleppo pine populations, and the clustering analyses clearly identified geographic structure in the differentiation. Essentially the same main clusters were detected by both the 'NJ' and 'BAPS non-admixing cluster' analyses, but the substructuring at the regional level detected by the two approaches differed somewhat. Use of the distance- and frequency-based methods together provided a better estimate of inter-group differentiation.

The nuclear microsatellite marker analysis suggests that Aleppo pines in Morocco have good, genetic structures that follow the mountain formations, with a main lineage represented by a group of 11 populations in the High and Middle Atlas mountains. Hence, Atlas Mountain populations comprise one of the main gene pools of *P. halepensis* in Morocco and materials originating from this region are used for reforestation and forest breeding programs. In some of these populations (MA 5, 8, 18, 20 and 21) the frequencies of common alleles (N_{lca}) are higher than average (Table 1). In addition to this main group, five smaller groups, represented by the southwestern High Atlas marginal populations (MA 5, 6/16 and 8); the Rif mountain populations (MA 3, 14, 15), and northeastern Atlas populations (MA 1/20 and 2/21) could be distinguished. Different private alleles (A_{pa}) were detected in the Rif and Atlas marginal groups, indicating that these are distinct from other groups (Table 1). The results indicate a geographic pattern with a clear northeast/ southwest divide. The High Atlas populations are the most southwesterly marginal populations of Aleppo pine in the Mediterranean basin, and they grow solely at high elevations in this region, probably because the precipitation and humidity are too low at lower elevations. Populations of the Middle
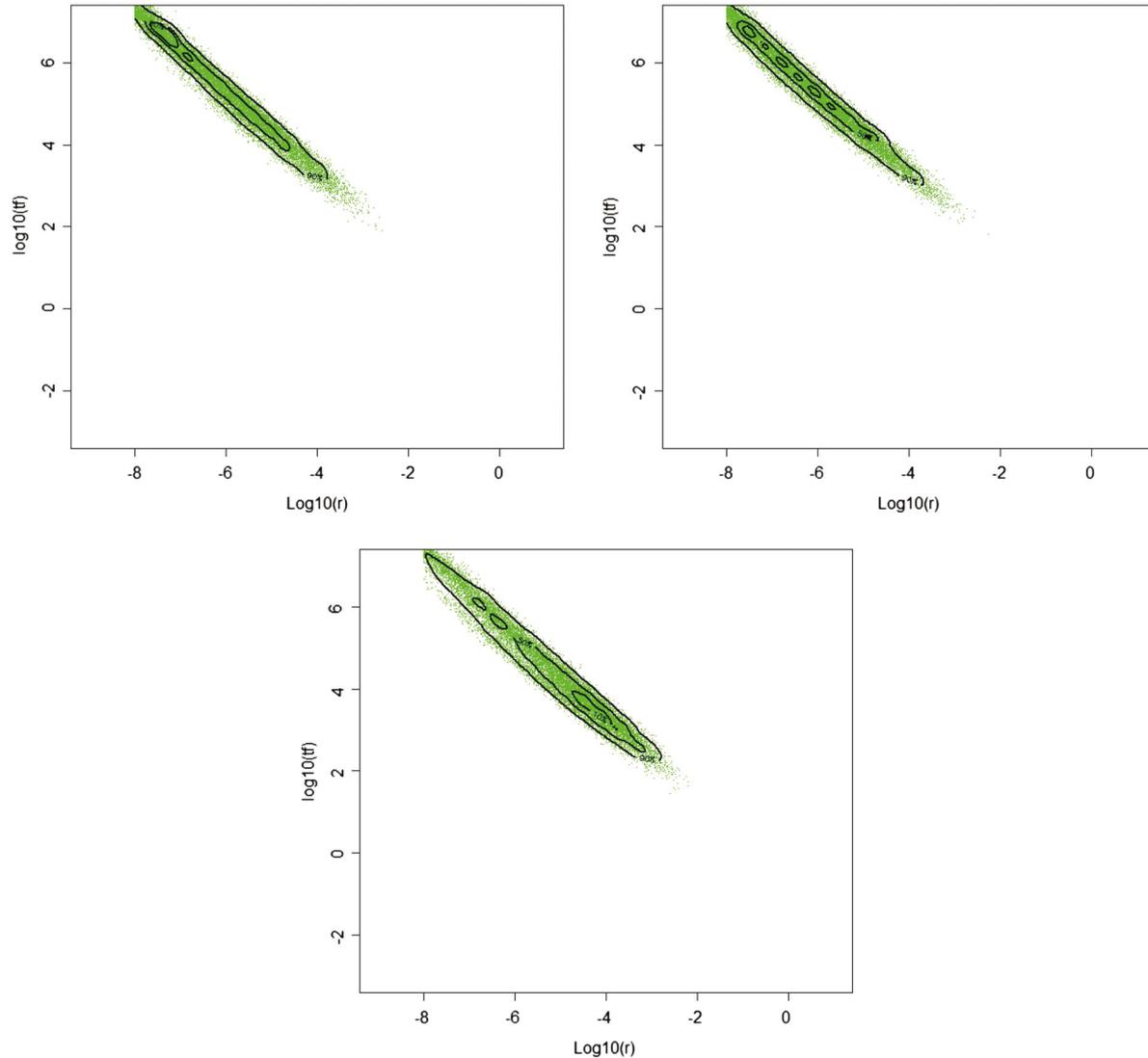
Fig. 4. Plots of 18 000 simulated points from the marginal posterior distribution of log10(r) and log10(t_f) for MA 12 (left/top), MA 13 (right/top) and MA 14 (bottom) populations of Aleppo Pine. The solid lines indicate 90%, 50% and 10% HPD limits estimated from microsatellite data. Note that log10(r) < 1 (and >1) indicate decline (and expansion) in population size, respectively, and log10(t_f)>0 is synonymous with the timing of demographic events in the past. The x- and y-axis show log10(r) and log10(t_f), respectively. The pattern of MA 12 is similar to that of MA 2/21, 6/16, 7/23 and 8; the pattern of MA 13 shows 5–6 waves of population size decline, and is similar to that of populations MA 3, 4/17/22, 11 and 18; the remaining populations have a similar pattern to MA 14, i.e. long genealogy, with a strong bottleneck ($\log_{10}$(r) < −1) and very ancient population decline to the left ($\log_{10}$(t_f) ≈ 6 at 10% HPD) and less ancient, but still old, population decline to the right (log10(t_f) ≈ 4 at 10% HPD).

Atlas and the Occidental Rif chain overlap to some degree and experience intermediate degrees of rainfall. Accordingly, variations in geographic and local ecological conditions have been suggested to contribute to the variations among Aleppo pines (PANETSOS 1981; BARADAT 1986; NAHAL 1986; QUEZEL 1986).

Genetic diversity and bottlenecks

Many authors, (BAZILE-ROBERT et al. 1980; BIGER and LIPHCHITS 1991; BARBERO et al. 1998) have studied various

aspects of the ecology and history of pines related to the LGD in the Mediterranean Basin. The Aleppo pine is a "thermophilic" pine that is particularly well adapted to dry conditions and forest fires – better adapted to such conditions than other pines from the same region according to the "etage" concept *sensu* QUEZEL (1974). According to examinations of material in the Iberian Peninsula, Aleppo pines begin flowering early for a tree species (at ages of 4–8 years), persist in the canopy for 5–20 years and produce seeds with high germination capacities (> 95%) for ca 20 years. They also produce high proportions

(40–80%) of serotinous cones, and their seeds are light, readily dispersing distances up to 100 m. These characteristics facilitate the species' tolerance of xeric conditions, and they differ (to varying degrees) from those of *P. pinea, P. pinaster, P. canariensis, P. nigra, P. sylvestris* and *P. uncinata* (TAPIAS et al. 2004). The comparisons between *P. halepensis* and six other pines of the Mediterranean region in the cited study indicate that Aleppo pine populations have high capacities to persist on marls, limestone, dolomite and dry volcanic soils in this region, and to maintain their genetic diversity if human activities are restricted or well managed. Furthermore, *M*-ratio analysis results do not provide any indications of recent ($< 4N_e$) reductions in the effective population size. However, contrary to our expectations, there is strong evidence of ancient bottlenecks in all of the Aleppo pine populations we examined. Population decline appears to have ended many generations ago, indicating that human activity in the last 25 000–30 000 years is not the main reason for the low genetic diversity of *P. halepensis* in the northwestern part of Africa. Rather, it is probably an additional factor with effects that are difficult to assess using currently available statistical tools. An early suggestion that bottlenecks were present in the natural populations of Aleppo pine (CONKLE et al. 1988; MORGANTE et al. 1998) was not derived with a model-based statistical method, but was based only on observations of genetic diversity (i.e. heterozygosity and effective number of haplotypes). The present analysis confirms that there has been a real population decline in the past and suggests that the last glacial depression was not the cause of an effective population contraction in the areas studied. However, climatic fluctuations have probably contributed to restricted contractions / expansions, in accordance with dynamic changes that have apparently occurred historically in forest communities in various regions of southern Europe and other parts of the world (QUEZEL and BARBERO 1992; COLINVAUX et al. 1996; RICHARDSON 2000; PETIT et al. 2008). Two thirds of the Aleppo pine populations examined here are small contemporary N_e, which is indicative of a small historic effective population size and moderate numbers of founders during many generations before the last 25 000–30 000 years of human activity. The majority of these populations are located in the Atlas Mountains which are watersheds of the Oued Tensift and Oued Sebou rivers.

To interpret the current pattern of genetic differentiation of Aleppo pine populations in Morocco, both historical and demographic factors need to be considered. Long-term persistence of tree populations in and around the Mediterranean basin is so common that the genetic structure of their species is believed to reflect population divergence that pre-dates the onset of the Mediterranean climate in the Pliocene (PETIT et al. 2005). Populations of *P. halepensis* in Morocco survive at intermediate or high altitudes, forming either continuous or isolated forest patches at sites that have been subject to both natural or anthropogenic disturbances and environmental conditions of varying favourability. A possible scenario is that long ago the Aleppo pine populations were subjected to a strong bottleneck (of uncertain causes) that severely reduced their genetic diversity. Then, according to the "shifting-balance" model of evolutionary change proposed by WRIGHT (1940), it can be speculated that natural selection quite rapidly eliminated homozygotes with maladaptive alleles following the unknown event that caused the bottleneck, without causing permanent damage to the new 'post-bottleneck' gene pool. The residual populations then passed through an 'adaptive valley' of reduced fitness due to genetic drift, which pushed them away from an initial equilibrium. It is possible that in the initial 'post-bottleneck' period there were moderate numbers of founders that were not reproductively isolated from the other *P. halepensis* populations. This may have prevented founder-effect speciation and sub-species diversification (and may be continuing to do so). Thus, the meta-population dynamics of Aleppo pine populations in isolated regions of Morocco with low anthropogenic pressures may be stable and in near-equilibrium. The homogenizing effect of seeds with high dispersal abilities on the genetic structure of meta-populations in scattered patches (BOHRER et al. 2005) may have played an important role in this inhibition of speciation and diversification. However, Aleppo pine in northwest Africa is clearly under great pressure due to its effective radius of distribution being less than 100 m, severe fragmentation (accompanied by local extinctions), and adverse human activities. Nevertheless, a sound management-conservation program, in combination with the high invasive capacity of the species, should help to maintain its population differentiation and total genetic diversity at present levels.

Conservation implications

A management strategy for Aleppo pine including conservation of its gene pool is needed to prevent genetic erosion of the species, which already has low diversity. It is, therefore, essential to preserve genetic variability within populations and to keep numbers of the breeding population as large as possible. Our results show the presence of good genetic substructures, which allow the delineation of clear conservation units for Aleppo pine in Morocco (i.e. *in situ* gene conservation). We can, however, recommend special efforts to protect the natural southwest High Atlas marginal population and the northeastern area population. These populations may contain certain co-adapted gene complexes specific to their local environments, and may represent vestigial fragments of a highly specific genetic structure that future forest breeding programs should try

to protect. In addition, samples should be collected for *ex situ* gene conservation, to maximize the potential for the conservation of genetic diversity and rare alleles. The development of an effective conservation strategy for *P. halepensis* that includes *ex situ* collection should cover multiple geographic areas, in order to maximize genetic diversity.

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