Ann. For. Sci. 58 (2001) 869–875

Original article

Genetic diversity of *Pinus halepensis* Mill. populations detected by RAPD loci

Aránzazu Gómez*, Ricardo Alía and María Angeles Bueno

Dpt. Breeding and Biotechnology, INIA, Ctra. Coruña Km. 7,5, 28040 Madrid, Spain

(Received 22 February 2000; accepted 29 June 2001)

Abstract – Genetic diversity of *Pinus halepensis* Mill. was analysed in nine populations (six Spanish populations and one each from Tunisia, France and Greece). Twenty four RAPD loci were amplified with 60 megagametophyte DNA samples from each population. Populations' contribution to Nei gene diversity and to allelic richness were calculated. Results showed higher within population genetic variation but also a G_{ST} = 13.6% higher than those detected in previous studies with different nuclear markers. The results obtained suggests that RAPD markers are valuable for the estimation of genetic diversity in *P. halepensis* and for the study of the divergence among population, allowing to think that eastern Mediterranean populations of *P. halepensis* have undergone a different history from those of the western Mediterranean area.

***Pinus halepensis* / RAPD / allelic richness / genetic diversity**

Résumé – **Diversité génétique des populations de *Pinus halepensis* Mill. révélée par des loci RAPD.** La diversité génétique de *Pinus halepensis* Mill. a été analysée dans six populations espagnoles et trois de Tunisie, de France et de Grèce. Vingt-quatre marqueurs RAPD ont été amplifiés avec l'ADN de 60 endospermes échantillonnés dans chaque population. La contribution des populations à l'indice de diversité génétique de Nei et à la richesse allélique a été calculée. Les résultats ont démontré l'existence d'une variation génétique intra-population plus grande et une valeur de G_{ST} =13,6 % plus élevée que celles des études précédentes menées avec d'autres marqueurs. Les marqueurs RAPD se sont révélés efficaces pour l'évaluation de la diversité génétique chez le *P. halepensis* et pour l'étude de la différenciation entre populations. Les résultats obtenus suggèrent une histoire différente entre les populations de l'est et de l'ouest de la Méditerranée.

***Pinus halepensis* / RAPD / richesse allélique / diversité génétique**

1. INTRODUCTION

Pinus halepensis Mill. is a Mediterranean species, distributed all around the Mediterranean basin, principally along the coast and exceptionally inland in Spain and Tunisia. The studies on the genetic diversity of Aleppo pine indicate its low level of variability, independent of the marker used, as terpenes [4, 25], isozymes [5, 14, 24, 26] and cpSSR [18]. The last authors have performed a complete description of the variability among populations, and have explained the low genetic diversity detected as the result of a genetic bottleneck effect during past glacial episodes of the Holocene.

* Correspondence and reprints
Tel. +34 91 347 68 57; Fax. +34 91 357 22 93; e-mail: argomez@inia.es

The lack of polymorphism in nuclear markers has resulted in poor estimates of the level of genetic diversity within populations for use in genetic resource conservation programs. For this reason, it would be important to estimate the differentiation of the natural populations and the contribution of each of them to the total genetic diversity [21]. These parameters could be calculated using both the Nei genetic diversity and the allelic richness. The last one best reflects the differentiation and contribution of each population [8].

Within the natural range of Aleppo pine, the Iberian peninsula represents the most important area (more than 1,000,000 has.) of the species in the Mediterranean basin, with a significant genetic variation [1, 9] and a clinal variation in isozyme loci [3] and climatic factors and morphological traits [2]. Studies of the total natural range seem to reflect that this area is not highly affected by human intervention and shows a divergence with respect to other populations of the species as happened with other studied species from the same area [4, 13, 18, 22, 23].

In order to obtain estimates of the within population genetic diversity and the contribution to the total diversity of each population, twenty-four polymorphic RAPD loci have been studied in megagametophytes from 6 populations from Spain and 3 covering the natural range of Aleppo pine. To avoid dominance, DNA has been extracted from haploid tissue of maternal origin, the seed megagametophyte. RAPD bands have been chosen by their repeatability, obtained by the optimization of reaction conditions, polymorphism and Mendelian inheritance [9].

2. MATERIALS AND METHODS

2.1. Plant material and DNA extraction

Plant material was collected from 9 populations (*table I*), 6 populations from Spain one from Greece (located in the area with the known higher genetic diversity of the species [18]), one population from Tunisia and the other one from France representing South European and North African groups of the species [24]. A pool of seeds were collected from 25 trees in each population with 30 meters between trees, and stored at 4 ºC until the DNA isolation. For RAPD analysis DNA was extracted from 60 megagametophytes isolated by removing the coats and the embryo after keeping the seeds from each population 24 hours in water. DNA extractions were performed by using the Doyle and Doyle (1990) protocol [6].

Table I. Geographic location and ecological conditions of the populations of *Pinus halepensis* analysed by RAPDs.

No.	Code	Population	Latitude	Longitude	Altitude (m)
1	SP-Cab	Cabanellas	42º1 4' N	2º 47' E	210
2	SP-Zue	Zuera	41º 55' N	0º 55' W	575
3	SP-Vil	Villa de Ves	39º 10' N	1º 14' W	850
4	SP-Ric	Ricote	38º 08' N	1º 25' W	780
5	SP-Car	Carratraca	36º 50' N	4º 50' W	650
6	SP-Sav	S'Avall	39º 17' N	3º 02' E	10
7	TU-Tab	Tabarka	36º 56' N	8º 39' E	50
8	FR-Gem	Gemenos	43º 25' N	5º 40' E	150
9	GR-Kas	Kassandra	40º 02' N	23º 02' E	50

2.2. Amplification

DNA amplifications by polymerase chain reaction (PCR) were carried out in a Perkin Elmer 9600 Thermocycler with the following profile: 1 cycle with 2 min denaturing at 94 ºC, 1 min annealing at 42 ºC and 2 min extension step of 72 ºC, 4 cycles with 45 s denaturing at 94 ºC, 1 min annealing at 42 ºC and 2 min extension step of 72 ºC and 40 cycles with 30 s denaturing at 94 ºC, 45 s annealing at 36 ºC and 1 min 30 s extension step of 72 ºC with a final extension step of 72 ºC for 8 min The volume of the reaction mixture was 25 µl and contained: four dNTPs (each 0.2 mM), 2.5 mM of $MgCl_2$, 0.2 µM, 1 × reaction buffer (Pharmacia), 2.5 ng of template DNA and 0.5 U of *taq*-polymerase (Pharmacia). Sixty oligonucleotide primers (Operon kits A, N and P) were tested in amplification, polymorphisms and Mendelian inheritance with DNAs from seeds that belong to the Spanish populations [9], The chosen 10 (OPA01, OPA07, OPA11, OPA18, OPA19, OPN06, OPN12, OPP01, OPP04 and OPP10) were used in the amplifications of DNAs from the 9 analysed populations.

The reactions were visualized under UV light, after running in 1.5% agarose gels in 1 × TAE buffer and ethidium bromide staining.

2.3. Data analysis

Usually, products below 300 bp or above 3 kb gave faint and non-reproducible bands, hence most of the scored products are in the range 0.4–2.5 kb (*table II*).

Table II. List of the primers used in the RAPD analysis, their sequence, number of bands and size range.

Primer	Sequence (5' →3')	Total No. of bands	No. of scorable bands	Size range of scorable bands (bp)
OPA01	CAGGCCCTTC	9	2	520–750
OPA07	GAAACGGGTG	7	2	510–700
OPA11	CAATCGCCGT	14	3	680–2500
OPA18	AGGTGACCGT	6	1	780
OPA19	CAAACGTCGG	9	3	530–1150
OPN06	GAGACGCACA	9	3	420–690
OPN12	CACAGACACC	7	2	300–450
OPP01	GTAGCACTCC	13	1	600
OPP04	GTGTCTCAGG	6	4	400–1200
OPP10	TCCCGCCTAC	7	3	600–1000

Diverse parameters were calculated [27]: Genetic diversity for each locus (H_{SP}), Gene frequency, Allele number, Effective allele number [11], Number of polymorphic loci, Nei gene diversity and G_{ST} [20].

The populations' contribution to Nei gene diversity and to allelic richness were calculated following [7] and [8] respectively. In both cases frequency of the most common allele (band presence or absence) was used. The contribution (C_T) of a population to the total diversity is calculated as the relative variation of the diversity when this population is added to the other ones. Similarly contribution to total diversity of a population due to its own diversity (C_S) and to its divergence (C_D) are calculated. The contribution of a population to the total allelic richness (C_T^r) is calculated following the rarefaction method of Hulbert [12] and contribution of this population due to its own diversity and due to its divergence are obtained from the partitioning of total allelic richness in similar way of contribution to total diversity [8].

Coefficients of genetic distance, *D*, were calculated for paired comparisons of the 9 populations, based on the normalised identity of all loci between each of the populations [20].

Genetic diversity parameters were calculated by the software POPGENE 1.21 [27].

3. RESULTS

3.1. Genetic diversity parameters

The ten selected primers were used to examine the level of polymorphism detectable in the nine populations of *P. halepensis*. Allele frequencies in each population are shown in *table III*. All 24 loci scored were polymorphic within the Aleppo pine genotypes tested, although some loci are monomorphic in some of the populations.

A summary of the genetic diversity parameters is given in *table IV*. Mean values of the effective number of alleles (1.55), percentage of polymorphic loci (90.3) and the gene diversity, *H* (0.32) for the Spanish populations are higher than the mean values if populations of FR-Gem, TU-Tab and GR-Kas are added (1.52, 87.5% and 0.30 respectively) These values are quite high and show the high degree of polymorphism detected by means of the RAPDs markers used in this study. The range of variation in gene diversity (*H*) between the Aleppo pine populations was large, 0.2249–0.3615.

Diversity parameters based on allele frequencies: effective number of alleles and genetic diversity have the highest values in the island population of SP-Sav since the lowest values correspond to the populations of TU-Tab, FR-Gem and SP-Vil (*table IV*). The proportion of percentage of polymorphic loci was the lowest in the Greek population (70.83%).

High *H* values (0.3475 and 0.3291) were also obtained for the SP-Cab and SP-Car populations. In contrast, the population from France (FR-Gem) was characterised by the lowest diversity with *H* value of 0.2249.

Estimates of diversity within populations and between populations were obtained. Estimates of genetic diversity (H_{SP}) and the coefficient of differentiation between populations are given for each locus and averaged over all loci (*figure 1*) with no relationships between both parameters.

Analysis of diversity, G_{ST} = 16.5% if only Spanish populations are considered and 13.6% if the nine populations are studied together, revealed a high proportion of variability between populations.

Table III. Band presence frequencies of RAPD loci in 9 Aleppo pine populations.

Loci	SP-Cab	SP-Zue	SP-Vil	SP-Ric	SP-Car	SP-Sav	TU-Tab	FR-Gem	GR-Kas
OPA01-1	0.817	0.867	0.967	1.000	0.833	0.733	0.233	0.000	0.800
OPA01-2	0.600	0.450	0.283	0.133	0.333	0.167	0.267	0.150	0.700
OPA07-1	0.333	0.250	0.617	0.833	0.933	0.167	0.767	1.000	0.400
OPA07-2	0.983	0.867	0.900	0.983	1.000	0.733	0.767	1.000	0.583
OPA11-1	0.550	0.383	0.167	0.217	0.833	0.150	0.017	0.250	0.400
OPA11-2	0.417	0.767	0.717	0.933	0.567	0.550	0.867	0.950	0.800
OPA11-3	0.483	0.500	0.900	0.983	0.517	0.883	0.883	0.900	0.000
OPA18-1	0.167	0.417	0.750	0.217	0.117	0.583	0.000	0.400	0.650
OPA19-1	0.567	0.200	0.000	0.367	0.167	0.233	0.200	0.050	0.500
OPA19-2	0.383	0.717	0.750	0.633	0.767	0.450	0.350	0.083	1.000
OPA19-3	0.567	0.700	0.817	0.300	0.617	0.467	0.233	0.117	0.100
OPN06-1	0.867	1.000	0.883	0.467	0.967	0.867	0.600	0.850	0.467
OPN06-2	0.883	1.000	0.700	0.983	0.900	0.800	0.717	1.000	0.000
OPN06-3	0.917	1.000	0.367	0.817	0.817	0.617	0.983	0.117	0.550
OPN12-1	0.567	0.717	0.867	0.817	0.683	0.583	0.783	0.650	1.000
OPN12-2	0.633	0.800	0.533	0.500	0.500	0.450	0.283	0.117	1.000
OPP01-1	0.633	0.433	0.267	0.367	0.617	0.533	0.733	0.567	1.000
OPP04-1	0.717	0.250	0.167	0.383	0.217	0.150	0.367	0.117	0.750
OPP04-2	0.900	0.533	0.767	0.433	0.650	0.650	0.683	0.933	0.650
OPP04-3	0.700	1.000	0.817	0.817	0.750	0.917	0.617	0.750	0.400
OPP04-4	1.000	0.467	1.000	0.650	0.617	0.983	0.983	0.800	1.000
OPP10-1	0.817	0.583	1.000	0.267	0.833	0.383	0.983	0.217	0.583
OPP10-2	0.867	0.950	1.000	0.600	0.817	0.700	1.000	0.583	0.550
OPP10-3	0.567	1.000	1.000	0.983	0.817	0.850	0.817	0.950	0.417

Table IV. Summary of genetic variation, based on 24 RAPD loci in 9 populations of *P. halepensis*.

Population	Percentage of polymorphic loci (*P*)	Gene diversity *H*	Effective number of alleles
SP-Cab	95.83	0.3475	1.6110
SP-Zue	79.17	0.3110	1.5505
SP-Vil	79.17	0.2576	1.4141
SP-Ric	95.83	0.3101	1.5355
SP-Car	100	0.3291	1.5514
SP-Sav	91.67	0.3615	1.6271
Mean	**90.30**	**0.3195**	**1.5482**
FR-Gem	83.33	0.2249	1.4697
TU-Tab	91.67	0.2857	1.3514
GR-Kas	70.83	0.3088	1.5714
Mean	**87.5**	**0.3040**	**1.5202**

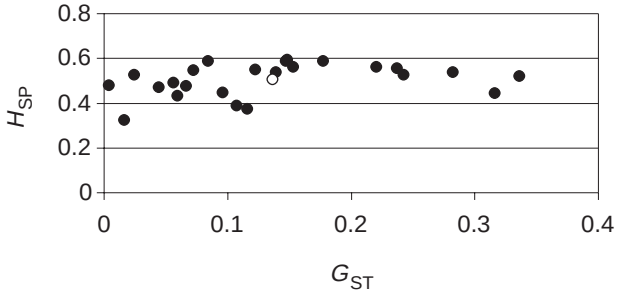

Figure 1. Estimates of genetic diversity (H_{SP}) and G_{ST} for each loci (black points) and averaged over all loci (white point, H_{SP} = 0.506; G_{ST} = 0.136).

Table V. Coefficients of Genetic identity (above diagonal) and genetic distances (below diagonal) (Nei, 1978) between 9 populations of *P. halepensis.*

Population	SP-Cab	SP-Zue	SP-Vil	SP-Ric	SP-Car	SP-Sav	FR-Gem	TU-Tab	GR-Kas
SP-Cab		0.882	0.850	0.855	0.936	0.862	0.883	0.773	0.786
SP-Zue	0.125		0.903	0.898	0.929	0.923	0.838	0.758	0.738
SP-Vil	0.162	0.102		0.871	0.893	0.924	0.854	0.807	0.723
SP-Ric	0.157	0.107	0.138		0.894	0.908	0.880	0.850	0.715
SP-Car	0.066	0.074	0.113	0.112		0.870	0.873	0.808	0.737
SP-Sav	0.149	0.080	0.080	0.096	0.139		0.878	0.866	0.740
FR-Gem	0.125	0.177	0.157	0.128	0.136	0.130		0.867	0.726
TU-Tab	0.257	0.277	0.215	0.163	0.214	0.144	0.142		0.607
GR-Kas	0.241	0.304	0.324	0.336	0.305	0.301	0.321	0.499	

The geographic structuration of the diversity can be analysed by the Nei genetic distance among populations (*table V*). Population of GR-Kas is the most distant population. FR-Gem population is closer to the Spanish popu lations than the other non-Spanish populations. The highest genetic distance (0.499) was found between the Tunisian population (TU-Tab) and the Greek one (GR-Kas). The most similar are two distant Spanish populations (SP-Cab and SP-Car) with a genetic distance of 0.066.

3.2. The populations' contribution to Nei gene diversity and to allelic richness

The contribution of each population to Nei diversity and allelic richness is shown in *figure 2*. The mean contribution was 0 for Nei diversity, whereas it was 0.022 for allelic richness, indicating a larger differentiation component for the measure in this last parameter.

Negative values for total gene diversity, due to a negative contribution for both diversity and differentiation components, are found in populations of SP-Vil, SP-Ric, TU-Tab and FR-Gem. The last two have the most important negative contribution (–0.20 and –0.22 respectively), but for different reasons. It results from a lack of diversity in TU-Tab and a lack of divergence from the other populations in FR-Gem.

Populations from SP-Car and SP-Sav, the southernmost and an island population respectively, show negative contributions for their own diversity, as the contribution to their divergence is higher. The population from Greece (GR-Kas) contributes more than the other studied populations to the total gene diversity (C_T = 0.39). This is due to the own diversity (C_S = 0.22) but also to a high divergence (C_D = 0.18).

The results based on the contribution of allelic richness (*figure 2*) are similar to those based on gene diversity. Although contributions to total allelic richness are

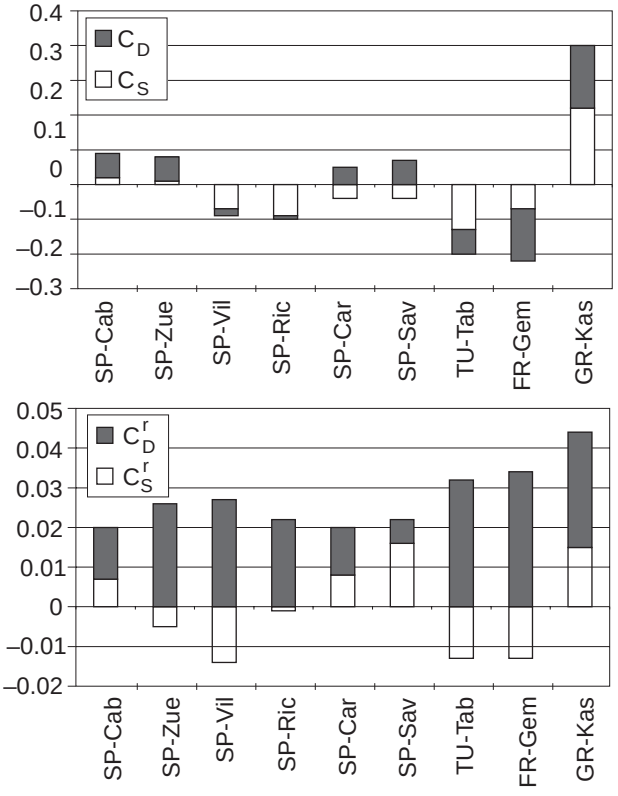

Figure 2. Total contribution to genetic diversity and allelic richness for each *P. halepensis* population subdivided in their two components: C_S: Contribution due to its own diversity; C_D: Contribution due to its divergence. C_S^r: Contribution to the total allelic richness due to its own diversity; C_D^r: Contribution to the total allelic richness due to its own divergence.

positive for all populations. The population of GR-Kas shows the highest values ($C_T^r = 0.044$) but is, on this occasion, followed by SP-Sav population with half its contribution ($C_T^r = 0.022$).

4. DISCUSSION

RAPDs markers from megagametophytes DNAs have been shown to be a powerful tool for analysing genetic variation in Aleppo pine populations. The high number of polymorphic loci analysed (24) allows us to have good relative estimates of variation within and between populations to use in breeding and genetic resource conservation programmes. The values obtained, however, are relative in the sense that they can be used for comparison among populations in this study as a result of the selection process of the primers. The selected loci were tested for polymorphism only in Spanish samples. However, other markers like microsatellites [17] and AFLPs [16] could be useful for genetic diversity analysis in *Pinus halepensis* as RAPD markers have demonstrated.

The majority of genetic variation detected in *Pinus halepensis* by RAPD markers ($G_{ST} = 13.6\%$) occurred within populations and the diversity of genotypes within populations is very high as evidenced by Nei gene diversity. The DNA analysis by RAPD markers shows a higher than expected genetic diversity found in natural populations of *P. halepensis* in Spain if the genetic differentiation values previously reported by other authors using allozyme markers [24] and chloroplast microsatellites [18] are considered. A number of studies involving outbreeding species have compared G_{ST} values from RAPDs to those from allozymes. Although there are very few studies in Gymnosperms, these results are congruent showing complete agreement between RAPD and allozyme diversity profiles [1, 3]. However, RAPD yielded much higher values of diversity than allozymes.

The patterns of variation detected, in terms of polymorphism and genetic diversity are in accordance with previous results in the same populations. There are only some differences when comparing some of the populations that can be due to the different meaning and mode of inheritance of the marker used.

Allelic richness is considered to be the most relevant criterion when studying population diversity [8, 15, 21]; some authors considered the preservation of alleles more important than the maintenance of allelic frequencies. So, for example, the Greek population showed the lower level of polymorphism (70.83%) but it is possible to detect a strong contribution to allelic richness in this population, since higher values for both measures have been obtained in other cases (e.g. SP-Sav population). In fact, selection of polymorphic bands based in results obtained with DNAs from the Spanish populations, is the cause of these results for distant populations from Spain like the Greek one.

These results may then be a signal of the divergence among the two groups, western and eastern Mediterranean populations of Aleppo pine, which might reflect the presence of two groups of Aleppo pine with different histories from the center of expansion after the last glaciation in Greece [18].

Migration is expected to result in a similarity between alleles; this prediction is observed in this study. The higher contribution to allelic richness in the non-Spanish populations must be viewed in the light of the fact that the bands studied had been selected for their polymorphisms only testing Spanish samples. The selection of RAPD bands acquires importance if we consider that the SP-Sav population shows a low allelic richness due to its divergence, that is, its “uniqueness”, but the highest gene diversity of the Spanish populations, something similar occurring with the TU-Tab and FR-Gem populations which show a negative contribution to diversity but high values for allelic richness due to their diversity.

The Nei genetic distance does not allow the quantification of the divergence for populations [21] but the results are congruent with the contribution to diversity of each population. The GR-Kas population is differentiated from the other populations more clearly than those of TU-Tab and FR-Gem, and these are the three non-Spanish populations. GR-Kas population is the more oriental of the studied populations, located more apart from the Iberian Peninsula populations.

The result obtained suggests that RAPD markers are valuable for the estimation of genetic diversity in *P. halepensis* and for the study of the divergence among populations which is not easy to find in forest trees [7, 11, 19]. This is specially true if populations that are located far apart are analyzed [10, 21]. These results and the genetic distance allow us to think that eastern Mediterranean populations of *P. halepensis* have undergone a different history from those of the western Mediterranean area.

Aknowledgements: We acknowledge the support given by the INIA (Spain) through a grant to the first author. Thanks given to M.T. Sevilla for who assisted in the

laboratory work; I. Trunkova who corrected the English and Dirección General de Conservación de la Naturaleza (DGCN) for sample collection. The study was founded by the European Commission, Brussels, Project FAIR CT95-0097.

REFERENCES

[1] Agúndez D., Degen B., Wuehlisch G., Alía R., Genetic variation of Aleppo pine (*Pinus halepensis* Mill.) in Spain, For. Genet. 4 (1997) 201–209.

[2] Agúndez D., Alía R., Estudio de la variación genética de *Pinus halepensis* Mill, Cuadernos de la SECF 5 (1997) 233–236.

[3] Agúndez D., Degen B., Wuehlisch G., Alía R., Multilocus analysis of *Pinus halepensis* Mill. from Spain: Genetic Diversity and Clinal Variation, Silvae Genetica 48 (1999) 173–178.

[4] Baradat P., Michelozzi M., Tognetti R., Khouja M.L., Geographical variation in the terpene composition of *Pinus halepensis* Mill., in: Baradat Ph., Adams W.T., Müller-Starck G. (Eds.), Populations genetics and genetic conservation of forest trees, SPB Academic Publishing, The Hague, 1995, pp. 141–158.

[5] Conkle M.T., Schiller G., Grunwald C., Electrophoretic analysis of diversity and phylogeny of *Pinus brutia* and closely related taxa, Systematic Bot. 13 (1988) 411–424.

[6] Doyle J.J., Doyle J.L., Isolation of plant DNA from fresh tissue, Focus 12 (1990) 13–15.

[7] El-Kassaby Y.A., Ritland K., Genetic variation in low elevation Douglas-fir of British Columbia and its relevance to gene conservation, Biodiv. Conserv. 5 (1996) 779–794.

[8] El Mousadik A., Petit R.J., High level of genetic differentiation for allelic richness among populations of the argan tree (*Argania spinosa* (L.) Skeels) endemic to Morocco, Theor. Appl. Genet. 92 (1996) 832–839.

[9] Gómez A., Análisis de la variabilidad genética de *Pinus halepensis* en España mediante el uso de marcadores de ADN: RAPDs y Cp-microsatélites, (Genetic Diversity Analysis of *Pinus halepensis* in Spain through DNA markers: RAPDs and Cp-Microsatellites). Ph.D. Thesis, UPM, Madrid, 1998 (not published).

[10] Hamrick J.L., Godt M.J.W., Effects of life history traits on genetic diversity in plant species, Philos. Trans. Roy. Soc. London Ser. B 189 (1996) 133–148.

[11] Hartl D.L., Clark A.G., Principles of population genetics, Sinauer Associates, Sunderland, 1989.

[12] Hulbert S.H., The nonconcept of species diversity: approach and alternative parameters, Ecology 52 (1971) 577–586.

[13] Jiménez P., Alía R., Agúndez D., Gil L., Genetic variation in central and marginal populations of *Quercus suber* L., Silvae Genetica 48 (1999) 278–284.

[14] Korol L., Schiller G., Relations between native Israeli and Jordanian Aleppo pine (*Pinus halepensis* Mill.) based on allozyme analysis: a note, For. Genet. 3 (1996) 197–202.

[15] Kremer A., Diversité génétique et variabilité de caractères phénotypiques chez les arbres forestiers, Gen. Sel. Evol. 26 (1994) 105–123.

[16] Lerceteau E., Szmidt A., Properties of AFLP markers in inheritance and genetic diversity studies of *Pinus sylvestris* L., Heredity 82 (1999) 252–260.

[17] Mariette S., Chagné D., Decroocq S., Vendramin G.G., Lalanne C., Madur D., Plomion C., Microsatellite markers for *Pinus pinaster* Ait., Ann. For. Sci. 58 (2001) 203–206.

[18] Morgante M., Felice N., Vendramin G. G., Analysis of hypervariable chloroplast microsatellites in *Pinus halepensis* reveals a dramatic genetic bottleneck, in: Karp A., Isaac P.G., Ingram D.S. (Eds.), Molecular tools for screening biodiversity, Chapman and Hall, London, 1998, pp. 407–412.

[19] Mosseler A., Egger K.N., Innes D.J., RAPD (Random Amplified Polymorphic DNA) confirm low levels of molecular genetic diversity in red pine (*Pinus resinosa* Ait.), in: Pardos J.A., Ahuja M.R., Elena Roselló R. (Eds.), Biotechnology of trees. Proceedings of the IUUFRO Working Party S2.0407, Somatic Cell Genetics, 18–23 October 1993, Valsain, Spain, p. 167.

[20] Nei M., Estimation of average heterozygosity and genetic distance from a small number of individuals, Genetics 89 (1978) 583–590.

[21] Petit R.J., El Mousadik A., Pons O., Identifying populations for conservation on the basis of genetic markers, Conserv. Biol. 4 (1998) 844– 855.

[22] Salvador L., Alía R., Agúndez D., Gil L., Genetic variation and migration pathways of Maritime pine (*Pinus pinaster* Ait.) in the Iberian Peninsula, Theor. Appl. Genet. 100 (2000) 89–95.

[23] Sánchez N., Grau J.M., Alba N., Manzanera, J.A., Bueno M.A., Genetic characterizazation of *Populus tremula* Regions of Origin in Spain using RAPD fingerprint, Silvae Genetica 49 (2000) 66–71.

[24] Schiller G., Conkle M.T., Grunwald C., Local differentiation among Mediterranean populations of Aleppo pine in their isoenzymes, Silvae Genetica 35 (1986) 11–19.

[25] Schiller G., Grunwald C., Resin monoterpenes in range-wide provenance trials of *Pinus halepensis* Mill. in Israel, Silvae Genetica 36 (1987) 109–114.

[26] Teisseire H., Fady B., Pichot Ch., Allozyme variation in five French populations of Aleppo pine (*Pinus halepensis* Miller), For. Genet. 2 (1995) 225–236.

[27] Yeh F.C., Yang R.C., Boyle T., POPGENE Version 1.21. Microsoft Window-based Freeware for Population Genetic Analysis, 1997.