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Diversity Among Circum-Mediterranean Populations of Aleppo Pine and Differentiation from Brutia Pine in their Isoenzymes: Additional Results*

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Summary

Seed material from 20 circum-Mediterranean *Pinus halepensis* MILL (Aleppo pine) populations and 32 *Pinus brutia* TEN. Subsp. brutia populations of range-wide distribution in Turkey was used to analyze the genetic diversity and structure of the species, by applying the starch gel electrophoresis technique to isoenzymes extracted from the seed megagametophytes.

The results show that not all the enzyme systems were resolved equally in the two species, therefore, data for several loci are not available for one or the other species. Significant differences between the two species in the overall average of allele frequencies in many loci can be observed. The largest differences occurred in the Aco, Acp, Adh$_2$, Gdh, Got$_1$, Mdh$_1$, Pgm$_2$, Pgi$_2$, 6Pgd$_2$ and 6Pgd$_3$ enzyme systems and loci. In these enzyme systems allele frequencies alternated between the first and second locus in the two species.

In Aleppo pine, statistical analysis of allele frequency data revealed that the mean number of alleles per locus ranged between 1.3 and 2.0; the percentages of polymorphic loci at the 0.95 criterion ranged between 16.0 and 44.0; the direct count mean heterozygosity ranged between 0.025 and 0.144; and HARDY-WEINBERG expected heterozygosity ranged between 0.052 and 0.188. A UPGMA dendrogram based on the genetic distance-Wagner procedure aggregated the 20 Aleppo pine populations mainly into three geographically distinct groups, namely: a western Mediterranean group that includes populations from Morocco, Spain and France; an eastern European group comprising Greek and Italian populations; and an eastern Mediterranean group comprising populations from Turkey, Jordan and Israel. The four Tunisian Aleppo pine populations were divided, according to allele frequencies in several enzyme systems, between the eastern and the western Mediterranean groups. Significant differences among the groups were found also in several genotype frequencies but especially in those of the Aap 1-1, Lap 0-1 and 2-2 genotypes.

In *Pinus brutia* TEN. The results revealed that the number of alleles per locus ranged from 1.95 to 2.40 with an average of 2.16; the percentage of polymorphic loci, by the 95% criterion, ranged from 65% to 75% averaging 68%; the effective number of alleles per locus ranged from 1.41 to 1.60, with an average of 1.49. Consequently, the observed and expected heterozygosities were 0.191 (ranging from 0.144 to 0.267) and 0.271 (ranging from 0.233 to 0.312), respectively.

Key words: Pinus halepensis, *Pinus brutia*, Allozymes, Genetic diversity and structure.

Introduction

The availability of Aleppo pine (*Pinus halepensis* MILL.) seed material, collected within the framework of the IUFRO-FAO project on Mediterranean pine species (MORANDINI, 1976), was the basis of the genetic diversity analysis, done by means of the isoenzyme starch gel electrophoresis technique (CONKLE *et al.*, 1982), among 19 circum-Mediterranean populations of Aleppo pine and 10 *Pinus brutia* TEN. populations (SCHILLER *et al.*, 1986; CONKLE *et al.*, 1988). Subsequent studies that utilized the same technique concerned themselves only with the regional distribution of the genetic diversity in *Aleppo and Brutia pines* (e.g., GRUNWALD *et al.*, 1986; TEISSEIRE *et al.*, 1995; AGUNDEZ *et al.*, 1997; KOROL and SCHILLER, 1996; AGUNDEZ *et al.*, 1999; PUGLISI *et al.*, 1999; KARA, *et al.*, 1997; PANETSOS *et al.*, 1998; GANI-GULBABA and OZKURT, 2000). Several other studies used biochemical traits as genetic markers, such as resin monoterpene composition analyzed with the gas chromatography technique, to evaluate the genetic diversity among populations of Aleppo pine (e.g., SCHILLER and GRUNWALD, 1986, 1987; BARADAT *et al.*, 1989, 1995). Recently high levels of genetic diversity among circum-Mediterranean populations of Aleppo pine was revealed by means of the chloroplast microsatellites technique (e.g., BUCCI *et al.* 1998; MORGANTE *et al.*, 1998; VENDRAMIN *et al.*, 1998).

The aim of the present study was to extend our knowledge of the intra- and intergenetic diversity and the phylogenetic relationships among circum-Mediterranean occurrences (provenances) of Aleppo and Brutia pines by means of the isoenzyme technique. To compare the results echived on *P. halepensis*

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Mill. with those achieved in a different study on *P. brutia* Ten. The availability of several old and new seed material collections of *Pinus halepensis* and *Pinus brutia* served for new analysis of within and among species inter and intra provenances genetic diversity

This study was carried out within the framework of the European Union INCO-DC research project on “Global, physiological and molecular responses to climatic stresses of three Mediterranean conifers”.

Materials and Methods

a. Seed Materials

Aleppo pine seeds from bulked seed lots originated in overseas natural forest populations (provenances), and from non-bulked single-tree seed collections within the Israeli native populations were used to analyze the genetic diversity and structure of Aleppo pine (*Table 1*).

Table 1. – Geographic locations of the seed sources analyzed.

Seed Source	Country and FAO code		Longitude	Latitude	Altitude (m a.s.l)
1. Caireval	Fra		5^0 21’	43^0 40’	300
2. Istiaia	Gre	A-3	23^0 17’	38^0 56’	125
3. Kassandra	Gre	A-5	23^0 28’	40^0 02’	60
4. Velez Blanco	Spa	A-18	2^0 10’ W	37^0 40’	1200
5. Jarafuel	Spa	A-20	1^0 00’ W	38^0 55’	600
6. Selloum	Tun	A-10	8^0 40’	35^0 05’	-----
7. Birino	Tun	-----	8^0 37’	35^0 28’	950
8. Oum Jedour	Tun	A-9	8^0 57’	35^0 38’	-----
9. Takrouna	Tun	----	-------	--------	-----
10. Tamga	Mor	----	6^0 07’W	32^0 02’	1350
11. Ikherifene	Mor		4^0 35’W	35^0 07’	915
12. Lalla Mimouna	Mor	----	3^0 07’W	34^0 03’	1100
13. Chiavari	Ita	----	9^0 19’	44^0 19’	----
14. Vico del Gargano	Ita	A-27	16^0 00’	41^0 54’	225
15. Jerash	Jor	----	35^0 54’	32^0 17’	-----
16. Ajlun	Jor	----	35^0 46’	32^0 20’	----
17. Yirka	IL	----	35^0 10’	32^0 57’	430
18. Bet J’ann	IL	----	35^0 23’	32^0 53’	850
19. Turkey	Tur	seed orchard	-----	-----	-----
20. Mt. Carmel	IL	----	35^0 02’	32^0 43’	450

b. Electrophoresis

Aleppo pine seeds were germinated and enzymes were extracted from the megagametophyte (maternal) tissue that was homogenized in a griding plate together with 75 µl of 0.2 M phosphate buffer pH 7.5, 0.1 Triton x-100, 1% BSA, 0.1 b-mercatoethanol, for all enzyme systems (Kelly and Adams, 1977). Horizontal starch gel electrophoresis was performed according to Conkle *et al.* (1982). Four different electrophoresis buffer systems were used to analyze the 15 enzyme systems according to Korol and Schiller (1996) (*Table 2*). Analyses were performed with eight haploid megagametophytes per mother tree (probability of detecting a heterozygous tree is 0.992).

Table 2. – Enzyme systems analyzed, E.C. numbers and buffer systems used

ENZYMES	ABBREVIATION	LOCUS	E.C.No	BUFFER SYSTEM
Alanine aminopeptidase	AAP	1	3.4.11.2	I
Aconitase	ACO	1	4.2.1.3	III
Acid phosphatase	ACP	1	3.1.3.2	IV
Alcohol dehydrogenase	ADH	1, 2	1.1.1.1	I
Catalase	CAT	2	1.11.1.6	II
Glutamate dehydrogenase	GDH	1	1.4.1.3	II
Glutamate-oxaloacetate transaminase	GOT	1, 2, 3	2.6.1.1	II
Isocitric dehydrogenase	IDH	1	1.1.1.42	III
Leucine aminopeptidase	LAP	1	3.4.11.1	I
Malate dehydrogenase	MDH	1, 2, 3, 4	1.1.1.37	IV
Menadione reductase	MNR	1, 2	1.6.99.2	I
Phosphoglucose isomerase	PGI	1, 2	5.3.1.9	IV
Phosphoglucomutase	PGM	1, 2	2.7.5.1	I
6-Phosphogluconate dehydrogenase	6PGD	2, 3	1.1.1.44	II
Shikimate	SKDH			III

c. Data analysis

By applying the BIOSYS-1 computer program, version 1.7 (e.g., Swofford and Selander, 1981) calculations of intra- and interpopulation genetic diversity were performed.

Results

a. Description of isoenzyme patterns

15 enzyme systems were examined by means of starch gel electrophoresis (*Table 2*). In enzymes with more than one zone of activity, alleles were numbered according to their frequencies, the most common allele, being numbered as “1”. Exceptions in numbering were in the loci Mdh_2, Mdh_4, Mnr_1 and Pgm_2, which were numbered as in previous studies (Korol and Schiller, 1996). Of the 27 loci analyzed, Pgi_1, Pgm_1 and $6Pgd_1$ were monomorphic; the SKDH system did not resolved on the gels for *P. halepensis*. Several loci revealed the existence of a second, third or fourth allele in only one or a few of the 20 populations analyzed. Allele frequencies in each of the populations and enzyme systems analyzed are presented in *Table 3*.

b. Genetic variability

A UPGMA dendrogram based on genetic distance measures (Rogers, 1972) obtained with the distance-Wagner procedure, which, according to Westfall and Conkle (1992) is better for visualizing clines, is presented in *Figure 1*. Four main population assemblages are presented: *a.* western Mediterranean including Morocco, Spain and France; *b.* eastern Mediterranean including Israel, Jordan and Turkey; and *c.* eastern European including Italy and Greece. *d.* the four populations growing in Tunisia that are the first at the branching dichotomy, divided between the two branching directions: two populations are at the origin of the eastern European group, and two at the origin of the eastern Mediterranean group. The same phenomenon was found during the analysis of the data by means of Nei’s (1972) genetic distance or similarity procedures. The split of the Tunisian population is due to large differences in allele frequencies in the Aap, Adh_2, Cat_2, Mdh_3, Mdh_4 and Pgm_2 loci, although the geographical data indicate that the distances separating the four populations are not large. Allele frequencies in the loci mentioned above, in the Selloum and Takrouna

Table 3. – Allele frequencies in each of the enzyme systems and populations analyzed

Populations **Locus / allele**		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
(N)		43	76	45	36	72	43	72	65	57	17	34	16	108	108	168	56	37	27	30	239
AAP,	**1**	0.942	0.572	0.544	0.986	0.986	0.733	0.535	0.631	0.684	1.000	1.000	0.937	0.495	0.551	0.393	0.393	0.162	0.370	0.400	0.320
	2	0.035	0.060	0.078	0.014	0.014	0.267	0.465	0.369	0.316	0.000	0.000	0.000	0.148	0.259	0.607	0.607	0.838	0.630	0.600	0.680
	3	0.023	0.342	0.378	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.277	0.190	0.000	0.000	0.000	0.000	0.000	0.000
	4	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ACO,	**1**	0.558	0.618	0.333	0.583	0.444	0.848	0.840	0.838	0.728	0.794	0.897	0.906	0.662	0.718	0.804	0.714	0.622	1.000	0.567	0.856
	2	0.442	0.382	0.667	0.417	0.556	0.140	0.153	0.162	0.272	0.206	0.103	0.094	0.338	0.282	0.196	0.286	0.378	0.000	0.433	0.144
	3	0.000	0.000	0.000	0.000	0.000	0.012	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ACP,	**1**	1.000	0.947	0.978	1.000	1.000	1.000	1.000	1.000	0.956	1.000	1.000	1.000	0.940	0.995	1.000	1.000	1.000	1.000	1.000	1.000
	2	0.000	0.053	0.022	0.000	0.000	0.000	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.060	0.005	0.000	0.000	0.000	0.000	0.000	0.000
ADH-1,	**1**	1.000	0.757	0.922	1.000	0.972	1.000	0.979	0.985	0.982	1.000	1.000	1.000	0.829	0.778	1.000	1.000	1.000	1.000	1.000	1.000
	2	0.000	0.217	0.078	0.000	0.028	0.000	0.021	0.015	0.018	0.000	0.000	0.000	0.171	0.222	0.000	0.000	0.000	0.000	0.000	0.000
	3	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ADH-2,	**1**	0.977	0.632	0.733	0.944	0.910	0.535	1.000	0.808	0.640	0.971	0.750	0.750	0.519	0.593	0.784	0.852	0.851	0.926	0.767	0.858
	2	0.023	0.125	0.100	0.000	0.090	0.465	0.000	0.146	0.342	0.000	0.103	0.000	0.218	0.171	0.216	0.148	0.108	0.074	0.233	0.132
	3	0.000	0.072	0.078	0.000	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.088	0.060	0.000	0.000	0.000	0.000	0.000	0.000
	4	0.000	0.171	0.089	0.056	0.000	0.000	0.000	0.046	0.018	0.000	0.147	0.250	0.175	0.176	0.000	0.000	0.041	0.000	0.000	0.010
CAT-2,	**1**	1.000	0.993	0.989	1.000	1.000	0.965	0.868	0.846	0.930	1.000	0.956	1.000	0.995	0.981	0.890	0.775	0.946	0.463	0.817	0.854
	2	0.000	0.007	0.011	0.000	0.000	0.035	0.132	0.154	0.070	0.000	0.044	0.000	0.005	0.019	0.110	0.225	0.054	0.537	0.183	0.146
GDH,	**1**	1.000	1.000	0.800	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.991	0.981	1.000	1.000	1.000	1.000	1.000	0.998
	2	0.000	0.000	0.178	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
	3	0.000	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.019	0.000	0.000	0.000	0.000	0.000	0.000
GOT-1,	**1**	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.981	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GOT-2,	**1**	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.986	1.000	1.000	1.000	1.000	1.000	1.000	0.985
	2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.015
GOT-3,	**1**	1.000	1.000	1.000	0.958	1.000	1.000	1.000	1.000	1.000	1.000	0.971	1.000	0.944	1.000	0.579	0.571	0.973	1.000	0.833	0.743
	2	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.056	0.000	0.421	0.429	0.027	0.000	0.167	0.243
	3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015
IDH,	**1**	1.000	0.954	0.989	1.000	1.000	0.988	0.993	0.977	0.965	1.000	1.000	0.969	1.000	1.000	0.887	0.955	1.000	1.000	1.000	1.000
	2	0.000	0.000	0.011	0.000	0.000	0.012	0.007	0.023	0.035	0.000	0.000	0.031	0.000	0.000	0.113	0.045	0.000	0.000	0.000	0.000
	3	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
LAP-1,	**1**	0.988	0.309	0.544	1.000	1.000	0.953	0.986	0.954	0.956	0.971	1.000	1.000	0.458	0.667	0.932	1.000	0.770	0.963	1.000	0.853
	2	0.012	0.664	0.456	0.000	0.000	0.000	0.000	0.000	0.009	0.029	0.000	0.000	0.542	0.333	0.000	0.000	0.000	0.000	0.000	0.000
	3	0.000	0.027	0.000	0.000	0.000	0.047	0.014	0.046	0.035	0.000	0.000	0.000	0.000	0.000	0.068	0.000	0.230	0.037	0.000	0.147
MDH-1,	**1**	1.000	0.993	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	2	0.000	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MDH-2,	**1**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.176	0.021	0.000	0.000	0.037	0.067	0.080
	2	1.000	0.987	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.981	0.824	0.979	1.000	1.000	0.963	0.933	0.920
	3	0.000	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MDH-3,	**1**	1.000	0.921	0.844	0.889	0.986	0.837	0.931	0.862	0.772	0.941	1.000	1.000	0.722	0.750	0.769	1.000	0.838	0.981	0.633	0.722
	2	0.000	0.079	0.156	0.111	0.014	0.163	0.069	0.138	0.228	0.059	0.000	0.000	0.278	0.250	0.231	0.000	0.162	0.019	0.367	0.278
MDH-4,	**1**	0.081	0.283	0.289	0.194	0.125	0.256	0.132	0.146	0.263	0.000	0.015	0.062	0.727	0.769	0.201	0.266	0.122	0.019	0.167	0.185
	2	0.919	0.717	0.711	0.806	0.875	0.744	0.868	0.854	0.737	1.000	0.985	0.938	0.273	0.231	0.799	0.734	0.878	0.981	0.833	0.815
MNR-1,	**1**	0.023	0.053	0.078	0.028	0.201	0.023	0.069	0.077	0.053	0.059	0.118	0.000	0.028	0.000	0.143	0.222	0.284	0.000	0.133	0.308
	2	0.953	0.940	0.900	0.972	0.799	0.930	0.931	0.923	0.947	0.823	0.853	1.000	0.972	1.000	0.394	0.778	0.702	1.000	0.867	0.654
	3	0.024	0.007	0.022	0.000	0.000	0.047	0.000	0.000	0.000	0.118	0.029	0.000	0.000	0.000	0.463	0.000	0.014	0.000	0.000	0.038
MNR-2,	**1**	1.000	1.000	0.956	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.991	1.000	1.000	1.000	1.000	1.000
	2	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000
PGM-1,	**1**	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PGM-2,	**1**	0.125	0.322	0.233	0.000	0.035	0.477	0.104	0.100	0.430	0.206	0.147	0.000	0.630	0.398	0.263	0.089	0.541	0.000	0.350	0.134
	2	0.875	0.678	0.767	1.000	0.965	0.523	0.896	0.900	0.570	0.794	0.853	1.000	0.370	0.602	0.737	0.911	0.459	1.000	0.650	0.866
PGI-1,	**1**	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PGI-2,	**1**	0.965	0.803	0.956	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.971	1.000	1.000	0.986	1.000	1.000	1.000	1.000	1.000	0.952
	2	0.035	0.151	0.044	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.048
	3	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000
6PGD-1,	**1**	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
6PGD-2,	**1**	1.000	0.842	0.922	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.995	0.894	1.000	1.000	1.000	1.000	1.000	0.990
	2	0.000	0.158	0.078	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.106	0.000	0.000	0.000	0.000	0.000	0.010
6PGD-3,	**1**	0.930	0.809	0.778	0.944	0.958	0.930	0.931	0.946	0.912	0.676	0.221	0.313	0.681	0.810	0.997	0.982	1.000	1.000	0.850	0.990
	2	0.070	0.191	0.222	0.056	0.042	0.070	0.069	0.054	0.088	0.324	0.779	0.687	0.319	0.157	0.003	0.018	0.000	0.000	0.150	0.008
	3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.002

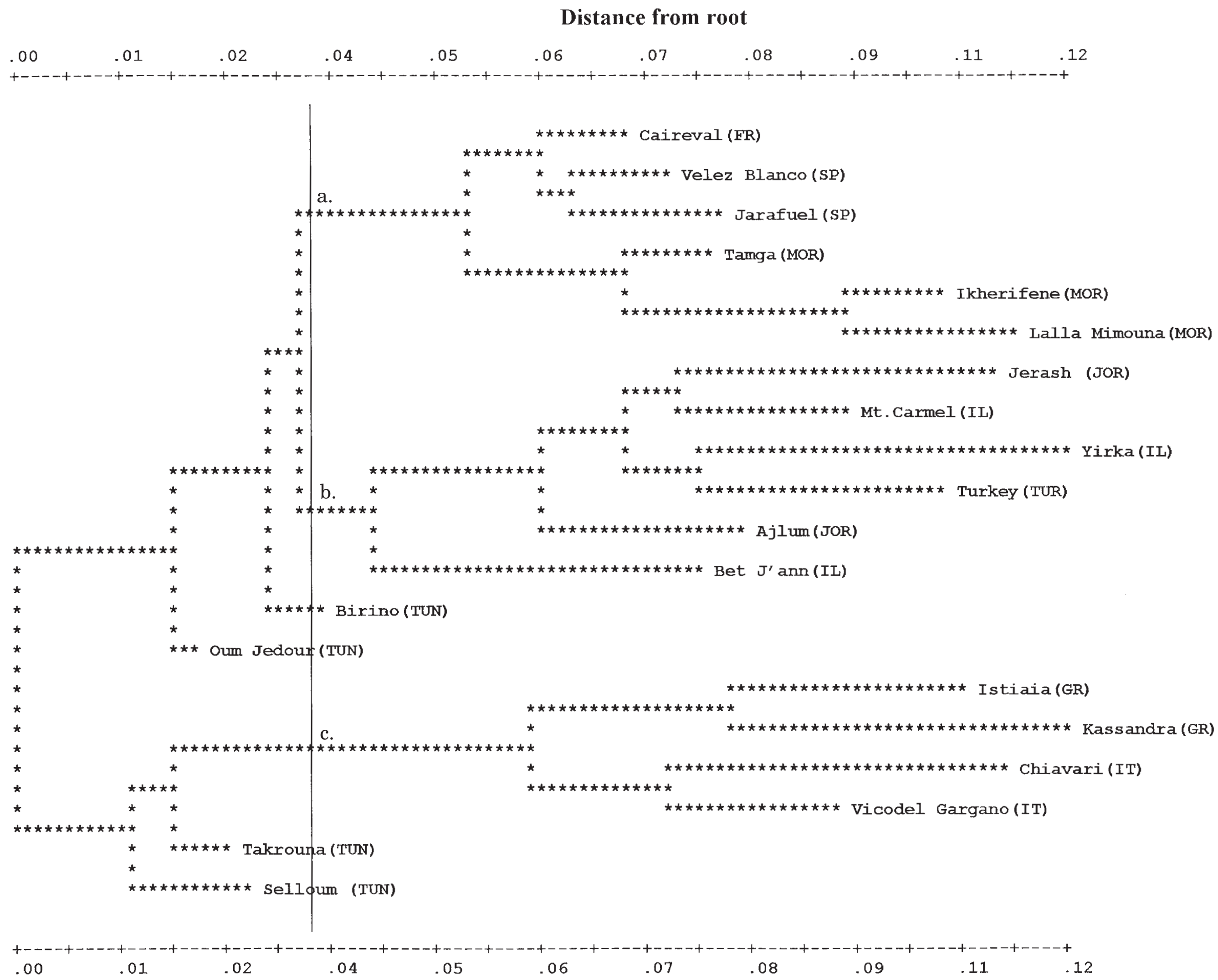

Figure 1. – Wagner tree produced by rooting at midpoint of longest path

populations, are more similar to those appearing in the western Mediterranean group, whereas allele frequencies in the Birino and Oum Dejour populations are more similar to those in the eastern Mediterranean group.

The Aleppo pine populations analyzed were grouped into four groups, according to the mean allele frequencies in each locus. Group one include six populations from France, Spain and Morocco. The second group include four populations from Greece and Italy. The third group include four populations from Tunisia, and the fourth group comprise six populations, from Turkey, Jordan and Israel. The overall means of the allele frequencies in each locus analyzed in the four groups are presented in *Table 4*. Differences among the four groups occurred in most of the loci, with significant differences in the Aap, Aco, Adh_2, Cat_2, Got_3, Lap_1, Mdh_2, Mdh_4, Mnr_1, Pgm_2 and $6Pgd_3$ loci. In comparison with the other groups the eastern Mediterranean group had high allele frequencies in the second loci of the Aap, Cat_2 and Got_3. The Greek-Italian group differed from the other ones mainly in allele frequencies in the Aap, Aco, Adh_1, Adh_2, Gdh, Lap, Mdh_4, Pgi_2, and $6Pgd_2$ loci; in many enzyme systems the third and fourth allele was present only in the Greek-Italian populations.

In the analyzed populations the same four groups, as mentioned above, could also be distinguished on the basis of some genotype frequencies. Populations in the first group showed high levels of homogeneity in several enzyme systems. The locus Aap clearly indicated differences among the groups: genotype 1-1 was the most frequent in the first group, with an average frequency of 0.965, whereas in other groups its mean frequency was not more than 0.450 (*Figure 2*). The *Aap* 1-1 genotype frequencies in the first group also differed significantly ($P<0.001$) from the other three groups. The *Aap* 2-2 genotype was the most frequent one (0.452) in group four, that differed significantly from group one ($P<0.0001$), from group two ($P<0.054$) and from group three ($P<0.161$). Populations from other regions appeared to be less homogeneous. The *Aap* 3-3 genotype was detected only in the second group (the Greek-Italian group), its average frequency was 0.117; this genotype was never detected in trees from other regions. High frequencies of the $Adh_1$1-2 and the *Lap* 2-2 genotypes also characterized the Greek-Italian group. The *Lap* 2-2 and the *Aap* 3-3 genotypes were detected only in the second group. The *Lap* 0-1 genotype was found only in the fourth group, being very frequent in the Israeli populations (*Table 5*). In both, the eastern Mediterranean and the Tunisian populations the Cat_2 1-2 genotype was the most frequent, while it was not detected in the other groups.

Low levels of polymorphism among individuals within the same population were found in some cases. Also in this feature, the four groups could be distinguished according to several parameters. The western Mediterranean group showed the

Table 4. – Avarage allele frequencies in four geographie groups pf *Pinus halepensis* and in *Pinus brutia* from Turkey.

		Pinus halepensis										***Pinus brutia***	
		Group 1		**Group 2**		**Group 3**		**Group 4**		**overall**	**overall**	**overall**	**overall**
Loci	**Allele**	**Mean**	**S.D.**	**Mean**	**S.D**	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
AAP	1	0.975	0.028	0.541	0.033	0.646	0.085	0.340	0.092	0.632	0.264	0.745	0.154
	2	0.011	0.014	0.136	0.090	0.354	0.085	0.660	0.092	0.299	0.280	0.117	0.162
	3	0.004	0.009	0.296	0.083	0.000	0.000	0.000	0.000	0.060	0.125	0.108	0.098
	4	0.011	0.026	0.007	0.013	0.000	0.000	0.000	0.000	0.004	0.015	0.031	0.065
ACO	1	0.697	0.195	0.583	0.171	0.814	0.057	0.761	0.159	0.717	0.168	0.157	0.115
	2	0.303	0.195	0.417	0.171	0.182	0.061	0.240	0.159	0.283	0.169	0.830	0.119
	3	0.000	0.000	0.000	0.000	0.005	0.006	0.000	0.000	0.001	0.003	0.012	0.020
	4											0.002	0.010
ACP	1	1.000	0.000	0.965	0.026	0.989	0.022	1.000	0.000	0.991	0.019	0.413	0.106
	2	0.000	0.000	0.019	0.025	0.011	0.022	0.000	0.000	0.006	0.015	0.534	0.117
	3	0.000	0.000	0.016	0.029	0.000	0.000	0.000	0.000	0.003	0.013	0.001	0.005
	4											0.051	0.041
ADH1	1	0.995	0.011	0.822	0.074	0.987	0.009	1.000	0.000	0.960	0.077		
	2	0.005	0.011	0.172	0.067	0.014	0.009	0.000	0.000	0.039	0.074		
	3	0.000	0.000	0.007	0.013	0.000	0.000	0.000	0.000	0.001	0.006		
ADH2	1	0.884	0.106	0.619	0.089	0.746	0.203	0.840	0.057	0.790	0.147	0.122	0.081
	2	0.036	0.048	0.154	0.052	0.238	0.206	0.152	0.062	0.135	0.119	0.878	0.081
	3	0.005	0.012	0.075	0.012	0.000	0.000	0.000	0.000	0.016	0.031	0.001	0.004
	4	0.076	0.103	0.153	0.043	0.016	0.022	0.009	0.016	0.059	0.080		
CAT	1	0.993	0.018	0.990	0.006	0.902	0.055	0.791	0.171	0.913	0.127	0.997	0.009
	2	0.007	0.018	0.011	0.006	0.098	0.055	0.180	0.183	0.078	0.123	0.003	0.009
GDH	1	1.000	0.000	0.943	0.096	1.000	0.000	1.000	0.001	0.989	0.045	0.154	0.130
	2	0.000	0.000	0.045	0.089	0.000	0.000	0.000	0.001	0.009	0.040	0.773	0.163
	3	0.000	0.000	0.013	0.010	0.000	0.000	0.000	0.000	0.003	0.006	0.060	0.065
	4											0.011	0.041
	5											0.001	0.005
GOT1	1	1.000	0.000	0.995	0.010	1.000	0.000	1.000	0.000	0.999	0.004	0.699	0.207
	2											0.232	0.207
	3											0.008	0.017
	4											0.060	0.099
	5											0.001	0.006
GOT2	1	1.000	0.000	0.997	0.007	1.000	0.000	0.998	0.006	0.999	0.004	0.877	0.095
	2	0.000	0.000	0.004	0.007	0.000	0.000	0.003	0.006	0.001	0.004	0.123	0.095
GOT3	1	0.988	0.019	0.986	0.028	1.000	0.000	0.783	0.186	0.929	0.138		
	2	0.012	0.019	0.014	0.028	0.000	0.000	0.215	0.186	0.071	0.137		
	3	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.006	0.001	0.003		
IDH	1	0.995	0.013	0.986	0.022	0.981	0.012	0.974	0.046	0.984	0.028	0.999	0.005
	2	0.005	0.013	0.003	0.006	0.019	0.012	0.026	0.046	0.014	0.027	0.001	0.004
	3	0.000	0.000	0.012	0.023	0.000	0.000	0.000	0.000	0.002	0.010	0.001	0.003
LAP1	1	0.993	0.012	0.495	0.150	0.962	0.016	0.920	0.091	0.865	0.207	1.000	0.000
	2	0.007	0.012	0.499	0.140	0.002	0.005	0.000	0.000	0.102	0.211		
	3	0.000	0.000	0.007	0.013	0.036	0.015	0.080	0.091	0.033	0.059		
MDH1	1	1.000	0.000	0.998	0.004	1.000	0.000	1.000	0.000	1.000	0.002	0.333	0.084
	2	0.000	0.000	0.002	0.004	0.000	0.000	0.000	0.000	0.000	0.002	0.664	0.086
	3											0.000	0.002
	4											0.001	0.003
	5											0.002	0.006
MDH2	1	0.000	0.000	0.049	0.085	0.000	0.000	0.034	0.034	0.020	0.044		
	2	1.000	0.000	0.948	0.083	1.000	0.000	0.966	0.034	0.979	0.043		
	3	0.000	0.000	0.003	0.007	0.000	0.000	0.000	0.000	0.001	0.003		
MDH3	1	0.969	0.046	0.809	0.091	0.851	0.066	0.824	0.145	0.870	0.113		
	2	0.031	0.046	0.191	0.091	0.150	0.066	0.176	0.145	0.130	0.113		
MDH4	1	0.080	0.072	0.517	0.267	0.199	0.070	0.155	0.083	0.214	0.203		
	2	0.921	0.072	0.483	0.267	0.801	0.070	0.840	0.084	0.785	0.203		
MNR1	1	0.072	0.075	0.040	0.033	0.056	0.024	0.182	0.114	0.095	0.093	0.028	0.071
	2	0.900	0.085	0.953	0.043	0.933	0.010	0.733	0.206	0.867	0.148	0.942	0.075
	3	0.028	0.046	0.007	0.010	0.012	0.024	0.086	0.185	0.038	0.104	0.030	0.043
MNR2	1	1.000	0.000	0.989	0.022	1.000	0.000	0.999	0.004	0.997	0.010	0.860	0.089
	2	0.000	0.000	0.011	0.022	0.000	0.000	0.002	0.004	0.003	0.010	0.139	0.089
	3											0.001	0.006
PGM1	1	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000
PGM2	1	0.086	0.086	0.396	0.170	0.278	0.204	0.230	0.197	0.229	0.190	0.999	0.005
	2	0.915	0.086	0.604	0.170	0.722	0.204	0.771	0.197	0.771	0.190	0.001	0.005
PGI1	1	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000		
	2												
PGI2	1	0.989	0.017	0.936	0.091	1.000	0.000	0.992	0.020	0.982	0.045	0.598	0.113
	2	0.011	0.017	0.049	0.071	0.000	0.000	0.008	0.020	0.015	0.036	0.341	0.107
	3	0.000	0.000	0.015	0.022	0.000	0.000	0.000	0.000	0.003	0.011	0.007	0.028
	4											0.054	0.050
6PGD1	1	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000		
6PGD2	1	1.000	0.000	0.913	0.064	1.000	0.000	0.998	0.004	0.982	0.044	0.470	0.161
	2	0.000	0.000	0.087	0.064	0.000	0.000	0.002	0.004	0.018	0.044	0.489	0.151
	3											0.021	0.052
	4											0.010	0.026
	5											0.002	0.006
	6											0.010	0.022
6PGD3	1	0.674	0.333	0.770	0.061	0.930	0.014	0.970	0.059	0.833	0.218	0.589	0.082
	2	0.327	0.333	0.222	0.070	0.070	0.014	0.030	0.059	0.165	0.218	0.141	0.105
	3	0.000	0.000	0.008	0.016	0.000	0.000	0.000	0.001	0.002	0.007	0.244	0.099
	4											0.022	0.053
	5											0.003	0.011
SKDH1	1											0.706	0.145
	2											0.246	0.138
	3											0.038	0.049
	4											0.010	0.042
	5											0.001	0.003
SKDH2	1											0.702	0.128
	2											0.224	0.129
	3											0.004	0.013
	4											0.071	0.065

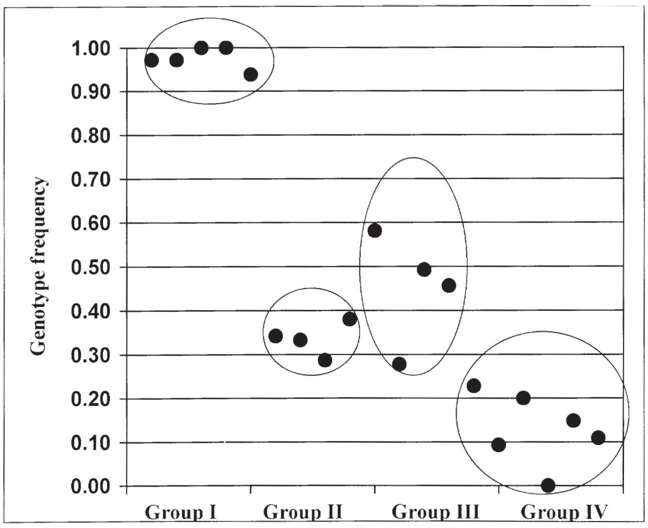

Figure 2. – Aap 1-1 genotype frequencies in the four groups

Table 5. – Lap 0-1 and Lap 2-2 genotype frequencies in the four *P. halepensis* geographic groups.

Group and populations	genotype 2-2	genotype 0-1
Group 1, (France, Spain, Morocco)	0	0
Group 2, (Greece and Italy)	0.35	0
Group 3, (Tunisia)	0	0
Group 4, (Turkey, Jordan, Israel)	0	0.07

lowest degree of diversity within populations (*Table 6*). The mean numbers of alleles per locus ranged between 1.2 and 1.4 in the first group, between 1.8 and 2.0 in the second group, between 1.5 and 1.6 in the third group, and between 1.3 and 1.8 in the fourth group. The percentages of polymorphic loci according to the 95% criterion were between 16 and 20% in the first group, between 44 and 52% in the second group, between 32 and 36% in the third group, and between 12 and 44% in the fourth group. Direct count heterozygosity (observed heterozygosity = Hobs) and expected heterozygosity (HARDY-WEINBERG expectations = Hexp) ranged between 0.025 and 0.051, and between 0.052 and 0.068, respectively, in the first group; between 0.126 and 0.144 and between 0.177 and 0.188, respectively, in the second group; between 0.059 and 0.088, and between 0.076 and 0.129, respectively, in the third group; between 0.049 and 0.124 and between 0.054 and 0.156, respectively, in the fourth group.

There was great interest in the possibility of comparing the overall mean allele frequencies in the 20 circum-Mediterranean populations of *Pinus halepensis* MILL. with these in 32 natural elite seed stands of *Pinus brutia* TEN. subsp. brutia (NAHEL, 1983) of a range-wide distribution in Turkey (*Table 4*). *Table 4* shows that, although the same technique was used, not all the enzyme systems were resolved equally in the two species, therefore, in one or the other species, data for several loci are not available. Significant differences between the two species in the overall average frequencies of alleles in many loci can be observed. The largest differences occurred in the Aco, Acp, Adh_2, Gdh, Got_1, Mdh_1, Pgm_2, Pgi_2, $6Pgd_2$ and $6Pgd_3$ enzyme systems and loci. In these enzyme systems, higher and lower allele frequencies alternated between the first and second loci in the two species.

Discussion

The new seed collections of *Pinus halepensis* MILL. and the *Pinus brutia* TEN. have enabled additional biochemical analysis to be performed to reveal the genetic diversity within and among populations, and between the two species. The results of the present study differ significantly from those of an earlier study by SCHILLER *et al.* (1986) in the number of loci revealed in the enzyme systems analyzed and, hence, in the number of polymorphic enzyme systems. SCHILLER *et al.* (1986) revealed only two alleles in all of the enzyme systems, and found only nine enzyme systems to be polymorphic. In the present study more then two alleles were revealed in many of the loci, and 17 loci were polymorphic according to the 95% criterion. These new findings also changed other genetic parameters such as the overall mean number of alleles per locus, the overall mean percentage of polymorphic loci and the overall mean expected heterozygosity. In the former study (SCHILLER *et al.*, 1986) these data were 1.10, 15.0, and 0.040 respectively; whereas the present study yielded values of 1.53, 32.6 and 0.110, respectively.

In spite of the above-mentioned differences, cluster analysis based on NEI's (1972) genetic distance or similarity procedures, or on distance-Wagner or CAVALLI-SFORZA and Edwards (1967) procedures, revealed geographic aggregations that were very similar to the cluster published in the earlier study by SCHILLER *et al.* (1986); but there were also some differences that resulted from the availability of seeds from Tunisia that were not available before. In the previous study Aleppo pine growing in Israel, i.e., the eastern Mediterranean group, differed from the western Mediterranean group mainly by the frequencies of allele two in the Cat_2 and the Aap loci; the Turkish population available at that time was thought to be an old-time artificial

Table 6. – Genetic variability at 25 loci in 20 circum-Mediterranean populations.

Population	Mean sample size per locus	Mean no. of alleles per locus	Percentage of loci polymorphic*	Mean heterozygosity	
				Direct- count	Hdy-Wbg expected*
Caireval (FR)	43	1.4 ± 0.1	20.0	0.042 ± 0.020	0.054 ± 0.022
Velez Blanco (SP)	36	1.3 ± 0.1	20.0	0.051 ± 0.028	0.055 ± 0.024
Jarafuel (SP)	72	1.4 ± 0.1	16.0	0.045 ± 0.021	0.059 ± 0.024
Tamga (MOR)	17	1.3 ± 0.1	20.0	0.049 ± 0.028	0.067 ± 0.027
Ikherifene (MOR)	34	1.4 ± 0.1	20.0	0.036 ± 0.017	0.068 ± 0.024
Lalla Mimouna (MOR)	16	1.2 ± 0.1	20.0	0.025 ± 0.013	0.052 ± 0.024
Istiaia (GR)	76	2.0 ± 0.2	52.0	0.138 ± 0.036	0.188 ± 0.041
Kassandra (GR)	45	1.9 ± 0.2	48.0	0.126 ± 0.037	0.177 ± 0.038
Chiavari (IT)	108	1.8 ± 0.1	44.0	0.137 ± 0.039	0.186 ± 0.046
Gargano (IT)	108	1.8 ± 0.2	44.0	0.144 ± 0.037	0.180 ± 0.043
Selloum (TU)	43	1.5 ± 0.1	32.0	0.085 ± 0.033	0.111 ± 0.034
Birino (TU)	72	1.5 ± 0.1	32.0	0.059 ± 0.25	0.076 ± 0.025
Oum Jedour (TU)	65	1.5 ± 0.1	36.0	0.059 ± 0.021	0.097 ± 0.027
Takrouna (TU)	57	1.6 ± 0.1	36.0	0.088 ± 0.030	0.129 ± 0.036
Jerash (JOR)	167	1.6 ± 0.1	44.0	0.110 ± 0.039	0.156 ± 0.039
Ajlun (JOR)	54	1.4 ± 0.1	32.0	0.121 ± 0.045	0.123 ± 0.037
Yirka(IL)	37	1.5 ± 0.1	36.0	0.124 ± 0.042	0.118 ± 0.035
Bet J'ann (IL)	27	1.3 ± 0.1	12.0	0.049 ± 0.024	0.054 ± 0.027
Turkey (TUR)	30	1.4 ± 0.1	44.0	0.103 ± 0.033	0.151 ± 0.038
Mt. Carmel (IL)	238	1.8 ± 0.1	44.0	0.116 ± 0.029	0.142 ± 0.033

* A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95

** Unbiased estimate (see NEI, 1978)

one, resembling Moroccan populations; and it was concluded that Aleppo pine migrated to the eastern Mediterranean from North Africa. In the present study the eastern Mediterranean group was enlarged by including the Jordanian and Turkish populations, which implies that all natural Aleppo pine populations in the eastern Mediterranean, i.e., Turkey, Lebanon, Syria, Israel and Jordan belong to the same group. Connections to Tunisian populations were found as the result of similarities mainly in the frequencies of the Aap and Cat_2 allele number two, which strengthens former conclusions about the migration path of the Aleppo pine into the eastern Mediterranean countries. A split among the Tunisian populations was also evident from the results presented in previous studies by Schiller and Grunwald (1987) and Baradat *et al.* (1995) using resin monoterpene composition as a genetic marker. In their study "On geographical variation in the terpene composition of *Pinus halepensis* Mill." Baradat *et al.* (1995) concluded that the Chiavari and Vico del Gargano populations were similar in their terpene compositions and they placed their origin in the Middle East. The results of the present study, too, point to a high similarity in allele frequencies between these two populations. But, contrary to the conclusions of Baradat *et al.* (1995), they are strongly tied to the natural populations of the Balkan Peninsula; this implies that the Italian Chiavari population might be an artificial one. According to Schiller *et al.* (1986) the Albanian, Greek and Italian-Gargano Peninsula Aleppo pine populations were considered as the eastern-European subgroup of the western-Mediterranean group, and that they include introgression from *Pinus brutia*. The results presented in *Table 4* imply that this is not the case, because in the enzyme systems where the third allele is unique to the eastern-European populations, no more than three alleles were detected, whereas in *Pinus brutia* four or five alleles were revealed. In spite of the few similarities between *P. halepensis* and *P. brutia* in allele frequencies (Aap), the idea of Schiller and Mendel (1995) that Aleppo pine in the Balkan peninsula is "a direct descendent of Tertiary Aleppo pine in central Europe, that migrated into the Balkan peninsula due to climate changes, keeping their relative high heterozygosity" might be more plausible. Such an idea is supported by the findings of Morgante *et al.* (1998), who categorically rejected the idea of introgression because of their results gained by chloroplast microsatellites analysis.

Since the beginning of botanical-genetical research within and among Mediterranean pine species, there has been great interest in the relations between the two vicarious species, *P. halepensis* and *P. brutia*. Our present analysis of genetic diversity in these species used large sample sizes (20 populations of *P. halepensis* and 32 populations of *P. brutia*) and the same methods, and therefore enables a partial comparison. Overall mean allele frequencies in the 15 enzyme systems and 18 loci differed significantly (*Table 4*). Among the two pine species, allele frequencies alternated between the first, second and third alleles in 11 of the 18 loci analyzed. In *P. brutia* the second and third alleles had considerably higher frequencies, and the first allele much lower frequencies than in *P. halepensis*. These results are similar to those presented by Conkle *et al.* (1988), who stated that "Allozymes indicate a highly significant divergence between *P. brutia* and *P. halepensis*".

To conclude, the results of allozyme analysis obtained in the present study strengthen conclusions formed previously about the genetic diversity and the relations among circum-Mediterranean *P. halepensis* populations, and between *P. halepensis* and *P. brutia*. These conclusions are that each is a different species not only in morphological characters but also in biochemical and molecular characters.

Citation

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