Local differentiation among Mediterranean populations of Aleppo pine in their isoenzymes*)

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Summary

Genetic variation among 19 circum- Mediterranean populations of Aleppo pine *(Pinus halepensis* MILL.) was investigated using starch gel electrophoresis to estimate allele frequencies for 30 loci. There are two major subdivisions: a western Mediterranean group from Spain to Greece and from Morocco to Libya; and an eastern Mediterranean group which includes a population from central Italy. Populations of the eastern group have loci with significant alleles frequencies that are absent from western populations. Geographic trends in allele frequencies and genetic distances among populations indicate that the western group consists of four geographic races: (i) western European populations from Spain to Italy; (ii) eastern European populations from the Balkan area, eastern Italy and Libya, with alleles from *P. brutia* TEN.; (iii) Moroccan, with populations homozygous for most loci; and (iv) a North African race- Algeria and probably Tunisia.

When the alleles from *P. brutia* are disregarded, *P. halepensis* has low amounts of variation, only six out of 30 loci being polymorphic. Heterozygosity of samples from the western group averages .03, ranging from .02 in Morocco to .04 in Algeria; average heterozygosity of the eastern group is .05. The average genetic distance among western and eastern group is .03, reflecting an overall genetic uniformity within the species.

Introgression of alleles from *P. brutia* in *P. halepensis* growing in the Balkan peninsula has raised the number of polymorphic loci and the heterozygosity, and given rise to the eastern European race.

Key words: Pinus halepensis, Pinus brutia, Matsucoccus josephi, Introgression.

Zusammenfassung

Die genetische Variation von 19 mediterranen *Pinus halepensis* MILL. Populationen wurde mittels Stärkegel-Elektrophorese untersucht, um die Allelhäufigkeit für 30 Loci zu bestimmen. Diese Analysen zeigten, daß es zwei größere Unterabteilungen gibt: (i) Eine west-mediterrane Gruppe, die sich von Spanien bis Griechenland und von Marokko bis Libyen erstreckt; und (ii) eine ost-mediterrane Gruppe, welche auch eine Population von Zentral-Italien enthält. Die Populationen der ost-mediterranen Gruppe haben Loci mit auffälligen Allelhäufigkeiten, die bei west-mediterranen Populationen nicht vorhanden sind. Die geographische Tendenz der Allelhäufigkeit, sowie der Abstand zwischen den Populationen zeigen, daß die west-mediterrane Gruppe aus 4 geographischen Rassen besteht: (i) Die west-europäische Rasse, welche die Populationen von Spanien bis West-Italien enthält. (ii) Die ost-europäische Rasse, welche die Populationen des Balkangebietes, Ost-Italiens und Libyens enthält. Sie unterscheidet sich durch den Besitz von *P. brutia* TEN. Allelen. (iii) Die marokkanische Rasse, mit Populationen, die für die meisten Loci homozygot sind. (iv) Die nordafrikanische Rasse, mit Populationen aus Algerien und wahrscheinlich Tunesien. Bei Nichtbeachtung der *P. brutia* Allele hat *P. halepensis* nur eine geringfügige Variation: nur 6 von 30 Loci sind polymorph. Der durchschnittliche Heterozygotiegrad der Populationsproben der west-mediterranen Gruppe beträgt .03 und reicht von .02 in Marokko bis .04 in Algerien. Der durschnittliche Heterozygotiegrad der ostmediterranen Gruppe ist .05. Der durchschnittliche genetische Abstand zwischen der west- und ost-mediterranen Gruppe beträgt .03, was eine überwiegende genetische Identität der Art zum Ausdruck bringt. Die Introgression von *P. brutia*-Allelen bei *P. halepensis* der Balkanhalbinsel erhöht die Anzahl der polymorphen Loci und den Heterozygotiegrad. Sie begründet die ost-europäische *P. halepensis* Rasse.

Introduction

Aleppo pine *(Pinus halepensis* MILL.) is the only species of the *P. halepensis* - *P. brutia* TEN. complex with circum-Mediterranean distribution (MIROV, 1967; CRITCHFIELD and LITTLE, 1966). Its present range is the result of geomorphological and climatic changes in the Tertiary and Quaternary; the species may have been moved about to some extent by human activity in the last three millenniums, so that some populations that appear to be native may have distant geographic origins (NAHAL, 1962; PANETSOS, 1981). It extende from 9° long. W in Morocco to 36° long. E in Jordan, and from 45° lat. N in the upper Rhone Valley in France to 31° 30' lat. N in Israel. Its main area of distribution is southern Europe and North Africa west of 25° long. E; at about this longitude in Greece it comes into contact with *P. brutia* and forms natural hybrids (PAPAJOANNOU, 1936; PANETSOS, 1975). Occurrences of very limited extent in Asia Minor and the Near East form distinct outliers and their existence poses intriguing questions regarding past distribution patterns and migration pathways. *Pinus halepensis* grows on different forest sites throughout the Mediterranean. It is extremely drought-tolerant (OPPENHEIMER, 1967) and adapted to dry Mediterranean climates (NAHAL, 1981). It occupies low-altitude sites, and reaching elevations of 2100 m (PANETSOS, 1981). Aleppo pine populations are often separated by non-forested areas giving its range the appearance of long chains of genetic islands. We know of no comprehensive studies of range-wide morphological or other variation. Provenance trials (ECCHER *et al.*, 1982; PALMBERG, 1975; PANETSOS, 1981; WEINSTEIN, 1982) are in early growth stages, and the amount of data available is of mainly local significance; so far, there is but limited information about the genetic variation in the species (see below).

Owing to the importance of *P. halepensis* for reforrestation in dry areas, there is considerable interest in knowledge on geographic patterns of genetic variation because of the disjunct area of distribution, prolonged isolation preventing gene exchange, hybridization and introgression with *P. brutia* (the eastern vicariad of *P. halepensis),* and wide range of evironmental conditions prevailing throughout its area. However, so far, no quantitative information on the gene pool of Aleppo pine is available. In this paper data are given on variation throughout its range to fill, at least partly, gaps in our knowledge. Subsequent papers

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will describe variation in *P. brutia* and closely related species, examine introgression between *P. halepensis* and *P. brutia* in detail, and analyze variation among native and planted stands of *P. halepensis* in Israel.

Materials and Methods

Samples of *P. halepensis* consisted of two groups: (i) 18 individual seedlots, each of an unknown number of trees (except for the collection from Libya of only six to ten trees) supplied by the F.A.O. and IUFRO, and by the Institute of Forest Genetics, Placerville, California; and (ii) 10 seedlots from 11 to 35 seed trees each collected in Israel by the Israeli authors, *(Table 1, Fig. 1.).*

Analyses were performed using megagametophytes (the haploid endosperm surrounding the embryo) taken from germinating seeds when the radicles emerged 3—5 mm from the seedcoats. About 75 seeds were analyzed from each seedlot. Enzymes in liquid extracts from individual seeds were separated simultaneously in four gel electrode buffer systems followed by the incubation of gel slices in 22 stain solutions (Conkle *et al.*, 1982). The genetic interpretation of bands on gels was based on experience with other pine species (Conkle, 1982). Enzymes are designated in the text by capital letters; loci are italicized and contain small letters. Multiple loci are numberd sequentlly from the edge of the gel toward the cathode. The most common allele of a locus was designated allele "1".

Statistical analyses were performed using BIOSYS computer program (Swofford and Selander, 1981). To evaluate genetic relationships among samples, two quantitative measures of genetic distance were computed: unbiased genetic distance (Nei, 1975; algebraic average of allelic differences) and chord distance (Cavallis-Sforza and Edwards, 1967; algebraic sum of all allelic differences). Nei's unbiased genetic distances were reported to permit comparison of our data with those from other species. Chord distances were used in conjunction with the distance Wagner procedure to estimate phylogenetic relationships. Midpoint and outgroup rooting, using *P. brutia* as the outgroup produced the same phylogenetic tree.

Results

Only nine loci in *P. halepensis* are polymorphic: Aap_1, *Aco*, Adh_2, Cat_2, Lap_1, Mdh_3, Mdh_4, *Mpi*, and $6Pgd_2$, each with two alleles (for codes of loci see *Table 2).* Variation in *Aco*, Mdh_3, Mdh_4, *and* $6Pgd_2$ occurs throughout the geographic range; heterozygosity for these loci, averaged over all populations, are .28, .30, .24 and .13, respectively. The five other loci *(*Aap_1, Adh_2, Cat_2, Lap_1, and *Mpi)* are associated with introgression from *P. brutia* or with variation between the western and eastern groups *(Table 2).*

To interpret variation among populations of *P. halepensis,* we first considered samples with alleles from *P. brutia.* Significant frequencies of Adh_2 and Lap_1 alleles that are fixed in *P. brutia* (M. T. Conkle, G. Schiller and Clara Grunwald, in preparation) are present in five samples: Italy (g), Albania (h), Greece (i,j) and Libya (q). Elsewhere throughout the range of *P. halepensis,* Adh_2 allele 2 and Lap_1 allele 2 (both characteristic of *P. brutia),* are presented in only a few scattered populations at frequencies of .05 or less *(Table 2).* The five populations have intermediate (.22 for Lap_1 in population g) or high frequencies of *P. brutia* alleles (> .85 for Lap_1 in populations h, i, j, q). There is no evidence of introgression in *P. halepensis* populations from Adana, Turkey (r) and Israel (s).

Two loci provide evidence for a major genetic subdivision among populations of *P. halepensis* that remain after excluding the five provenances with *P. brutia* alleles. Two

Table 1. — Geographic origin of *Pinus halepensis* seed samples.

Item	Country	Provenance[1]	Longitude (° ′)	Latitude (° ′N)	Elevation (m)
a	Spain	Jaen	03 47 W	37 46	500
b	Spain	Murcia	02 00 E	37 45	400
c	France	Gemenos (24)	05 04 E	43 25	150
d	Italy	Imperia (25)	08 03 E	43 54	200
e	Italy	Otricoli (26)	12 38 E	42 24	400
g	Italy	Vico del Gargano (27)	16 00 E	41 54	225
f	Italy	Patemisco (28)	17 20 E	40 39	5
h	Albania	Albania (1)	19 25 E	40 37	2
i	Greece	Elea (2)	21 32 E	37 46	200
j	Greece	Euboea (3)	23 18 E	38 58	200
k	Morocco	Jebel Afra (AS)	07 55 W	30 44	1500
l	Morocco	Jebel Masker (AO)	05 09 W	32 18	2000
m	Morocco	Irherifene (AP)	04 37 W	35 08	900
n	Algeria	Telagh (31)	NA	NA	NA
o	Algeria	Quarsensis (32)	05 04 E	35 05	NA
p	Algeria	Senalba (30)	NA	NA	NA
q	Libya	Wadi Latrun	22 30 E	32 30	250
r	Turkey	Adana (AI)	35 10 E	36 40	250
s	Israel	8 populations	34 55 E	31 02	80
			-35 23 E	-32 58	-900

[1] The numbers in parentheses are accession codes of the FAO, Committee for Mediterranean Forest Research. Letters are seedlot codes, Institute of Forest Genetics, Placerville, California.

NA = information was not available.

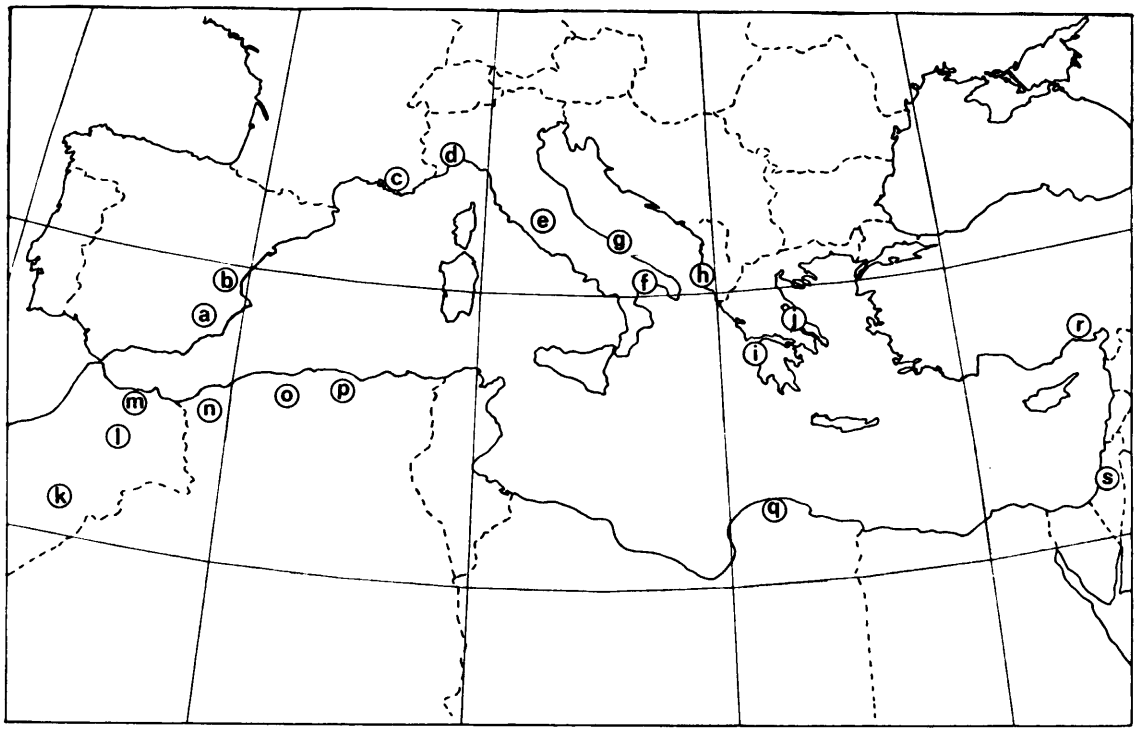

Figure 1. — Locations of population samples of *Pinus halepensis* MILL.

loci in the ten- stand composite sample from Israel (s) have significant proportions of alleles that are absent, or at low frequencies, in most other populations; allele-2 frequencies are .13 for Cat_2 and .49 for Aap_1 *(Table 2).* The diagnostic value of alleles of the loci are adequate to identify the eastern samples with ease. Population (e) from central Italy has high allele frequencies for the same loci, allele-2 frequencies being .40 for Cat_2 and .72 for Aap_1.

Western Mediterranean samples are not affected by introgression from *P. brutia* and lack alleles characteristic of the Israeli material. Populations from Spain (a, b), France (c) and Italy (d, f) have higher frequencies of *Aco* allele 2 (average .57 ± .07, range .33 to .72) than populations from Morocco (k, l, m) and Algeria (n, o, p) (average .15 ± .06, range .01 to .40). Mdh_3 allele-2 frequencies are higher for these six African populations (average .18 ± .03) than for the five western European populations *(Fig. 2).* Mdh_3 allele 2 is at low frequency (.06) in the westernmost Spanish sample (a) and is absent from the other Spanish (b), French (c) and Italian (d, f) populations *(Table 2).*

Island-like populations of *P. halepensis* scatterd across the Atlas Mountains of North Africa differ from the Moroccan samples. Allele-2 frequencies for *Aco* and Mdh_4 are low in Morocco (.09 and .01, respectively) and intermediate in Algeria (.22 and .37). Two Algerian samples have low frequencies of Aap_1 allele characteristic of populations from Israel, but the allele is not found in samples from Morocco *(Table 2).*

The northernmost population in Morocco (m) has a very high frequency of $6Pgd_2$ allele-2 (.93) in comparison with all other *P. halepensis* samples. Other allele frequencies for this sample are similar to Morocco populations (k) and (l).

Allozyme loci are useful for estimating the average amount of genetic variation in *P. halepensis* seed sources *(Table 4).* Populations from France (c), Morocco (k, m), Libya (q) and Turkey (r) have the lowest proportions of polymorphic loci (10%); European populations with alleles from *P. brutia* (g, i, j) and populations from Algeria (o, p have the highest values (20% or more). Average values for heterozygosity per individual, estimated from population allele frequencies using HARDY-WEINBERG predictions, are lowest for populations from Morocco and Turkey (about .02) and highest for the introgressed populations. Heterozygosities average .03 for the western European populations, .04 for the North African, and .06 for the eastern group. Heterozygosities for Moroccan populations are half of those for North African populations (.02 and .04, respectively).

The phylogenetic tree estimated from chord distances provides a graphic representation of relationships among *P. halepensis* populations *(Fig. 3).* It incorporates into a single diagram many of the associations already noted for individual loci. The phylogenetic tree is rooted on the left margin at the algebraic location of a hypothetical progenitor population with variation intermediate between the most divergent samples. Nineteen provenances of *P. halepensis* occupy the upper portion of the tree, with a *P. brutia* composite sample positioned on the lowest line *(Fig. 3).* The seven uppermost populations include European provenances (but not those with *P. brutia* alleles), the Israeli composite (s), and the sample from central Italy (e). Below those populations, a branch links the three Moroccan samples and the Turkish sample (r) displaying close affinity with Morocco (l). The next three samples are from Algeria. Beginning at Italy (g) and continuing to Libya (q) are populations containing alleles characteristic of *P. brutia.*

Unbiased genetic distances and chord distances are summarized in *Table 3* for populations of the western group, the eastern group (the composite from Israel, s, and the related sample from Italy, e), and for the *P. brutia* composite. From isoenzyme results, the samples of the western group can be assigned to four races: (1) western Europe, consisting of samples from Spain (a, b), France (c), and Italy (d, f); (2) eastern Europe, consisting of the introgressed samples from Italy (g), Albania (h), Greece (i, j), and

Table 2. — Allele frequencies for 9 polymorphic isozyme loci in 19 populations of *Pinus halepensis*.

Locus Allele 1/	Populations																		
	-European-										-North African-							-Eastern-	
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s
Aap1																			
1	1.0	.98	.83	.95	.28	1.0	.90	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.90	.93	1.0	1.0	.51
2		.02	.17	.05	.72		.10								.10	.07			.49
Aco																			
1	.28	.50	.67	.38	.60	.33	.80	.63	.54	.69	.99	.80	.96	.60	.83	.90	1.0	.81	.79
2	.72	.50	.33	.62	.40	.67	.20	.37	.46	.31	.01	.20	.04	.40	.17	.10		.19	.21
Adh2																			
1	1.0	1.0	1.0	.98	1.0	.98	.72	.78	.78	.64	1.0	1.0	1.0	1.0	.99	.98	.89	.99	1.0
2				.02		.02	.28	.22	.22	.36					.01	.02	.11	.01	
Cat2																			
1	1.0	1.0	1.0	1.0	.60	1.0	1.0	1.0	.97	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.87
2					.40				.03										.13
Lap1																			
1	1.0	1.0	1.0	1.0	.98	.96	.78	.14	.04	.14	1.0	1.0	1.0	.95	1.0	1.0		1.0	1.0
2					.02	.04	.22	.86	.96	.86				.05			1.0		
Mdh3																			
1	.94	1.0	1.0	1.0	1.0	1.0	.62	1.0	1.0	1.0	.81	.96	.75	.80	.82	.75	.80	1.0	1.0
2	.06						.38				.19	.04	.25	.20	.18	.25	.20		
Mdh4																			
1	.64	.68	.97	.97	.25	.67	.17	.30	.78	.44	1.0	.97	1.0	.64	.86	.39	.88	.92	.68
2	.36	.32	.03	.03	.75	.33	.83	.70	.22	.56		.03		.36	.14	.61	.12	.08	.32
Mpi																			
1	1.0	1.0	1.0	.97	1.0	1.0	1.0	1.0	1.0	.94	1.0	1.0	1.0	1.0	1.0	.95	1.0	1.0	1.0
2				.03						.06						.05			
6Pgd2																			
1	.70	.90	1.0	1.0	1.0	1.0	.90	.84	.78	.89	.91	.95	.07	1.0	.93	.92	1.0	1.0	.99
2	.30	.10					.10	.16	.22	.11	.09	.05	.93		.07	.08			.01

1/ Abbreviations are as follows: Aap, alanine aminopeptidase; Aco, aconitase; Adh, alcoholdehydrogenase; Cat, catalase; Lap, leucine aminopeptidase; Mdh, malic dehydrogenase; Mpi, mannose-6-phosphate isomerase; 6Pgd, 6-phosphogluconate dehydrogenase. Twenty-one additional loci were found monomorphic: acid phosphatase (1 locus); aldolase (1 locus); esterase (1 locus); fructose 1-6-diphosphatase (1 locus); fluorescent esterase (1 locus); glutamate dehydrogenase (1 locus); superoxide dismutase (2 loci); glutamate-oxaloacetate transaminase (3 loci); glucose-6-phosphate dehydrogenase (1 locus); isocitric dehydrogenase (1 locus); malic dehydrogenase (1 locus); menadione reductase (2 loci); phosphglucose isomerase (2 loci); shikimate dehydrogenase (1 locus); 6-phosphogluconate dehydrogenase (2 loci).

Libya (q); (3) Morocco (k, l, m) and the similar Turkish sample (r); and (4) Algeria (n, o, p).

Unbiased genetic distances and chord distances among *P. halepensis* populations (excluding the introgressed populations) averages .02 and .12 respectively. Averages for both measures of genetic distance *(Table 3,* values on the diagonal) are lowest among samples from western Europe (.003 and .065) and from Algeria (.005 and .072). Both distance measures also have low values between samples from western Europe and Algeria (.009 and .091). All *P. halepensis* groups have large average distances with *P. brutia* (.34 for genetic and .50 for chord distances) indicating substantial differences between the two species. The introgressed *P. halepensis* populations of eastern Europe have intermediate distances with other western Mediterranean samples (.04 and .18, respectively) and large distance values with *P. brutia* (.26 and .44).

Discussion and Conclusions

Allozyme analyses of seed sources provide new information about geographic patterns and relative amounts of genetic variation in *P. halepensis.* Previous research on variation involves provenance trials (Eccher *et al.,* 1982; Palmberg, 1975; Panetsos, 1981; Weinstein, 1982) and analyses of morphological, physiological and resin characters (Ancilloti and Giannini, 1975; Calamassi *et al.,* 1980, Calamassi, 1982; Giannini and Scarascia, 1981; Grunwald *et al.,* 1983; Iconomou *et al.,* 1964; Melzack *et al.,* 1981, 1982; Mirov *et al.,* 1966; Pelizzo and Tocci, 1978).

Of particular interest in the context of our study are proposals to divide the species into discrete geographical races. Nahal (1962) recognized the following groups on the basis of pollen morphology: West Europe, North Africa and the Near East, Yet, only limited significance should be attributed to his classification owing to the small number of seed sources at his disposal. Panetsos (1981), summarizing data on growth rates in provenance trials and morphological characters, distinguished four geographical races: Moroccan, North African, West European und East European; however, this classification does not take into account the Near Eastern occurrences of Aleppo pine extending from Israel and Jordan to Turkey, and the boundaries between provenances groups are not defined.

The data from our analyses provide evidence for the subdivision of *P. halepensis* into two groups, a West Mediterranean group and an East Mediterranean group.

The eastern group, represented by the Israeli composite sample (s) and probably including the native Jordanian and Lebanese-Syrian provenances, has distinct marker alleleles at intermediate to high frequencies for the two loci Aap_1 and Cat_2. The same marker alleles are at high frequencies also in a sample from central Italy, about 50 km NE of Rome (e) *(Table 2).* It appears that the similarities between populations from Israel and central Italy were

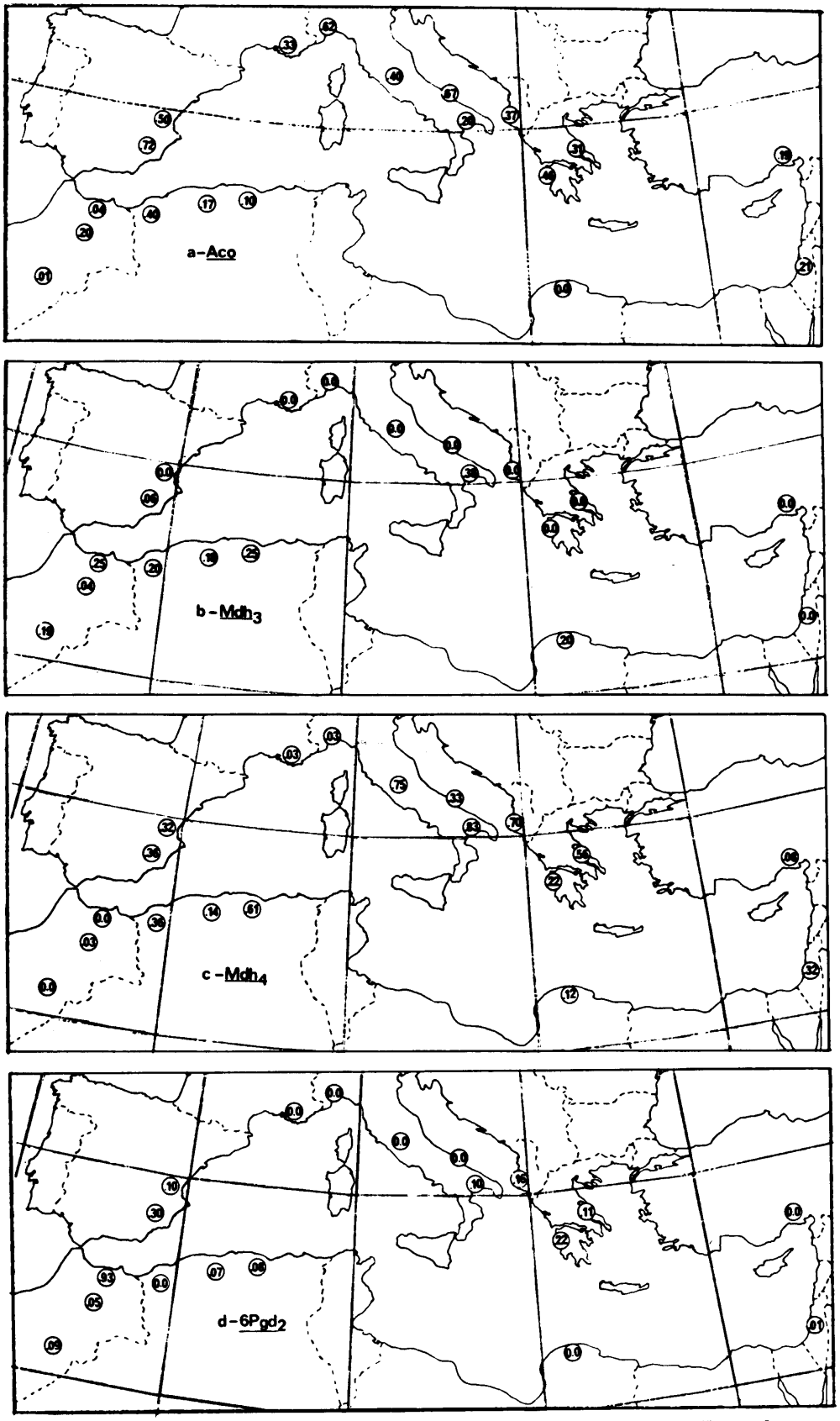

Figure 2. — Allele-2 frequencies for four loci of *Pinus halepensis;* Aco, Mdh_3, Mdh_4, $6Pgd_2$.

brought about by independent events like convergent evolution and natural selection, although the possibility of seed transfer by man in the distant past may not be excluded beforehand.

The allozyme data support the subdivision of the western Mediterranean group of *P. halepensis,* which extends from Spain and Morocco to Greece and Libya (25° Long. E limits the eastern extension of the group). As metioned above,

Table 4. — Diversity statistics for populations of *Pinus halepensis*.

Groups, Populations		Mean Sample Size per Locus[1]	Mean No. of Alleles per Locus	Percentage of Loci Polymorphic	Mean Heterozygosity, Hardy-Weinberg Expected Value
Western Mediterranean					
Western European					
Spain	a	69	1.1	13	.035
Spain	b	79	1.1	13	.040
France	c	69	1.1	10	.027
Italy	d	71	1.2	17	.025
Italy	f	67	1.1	13	.035
--Mean			1.1	13	.032
Eastern European					
Italy	g	65	1.2	23	.075
Albania	h	71	1.2	17	.059
Greece	i	80	1.2	20	.056
Greece	j	87	1.2	20	.065
Libya	q	68	1.1	10	.025
--Mean			1.2	18	.056
Moroccan					
Morocco	k	84	1.1	10	.017
Morocco	l	81	1.1	13	.019
Morocco	m	85	1.1	10	.020
Turkey	r	70	1.1	10	.017
--Mean			1.1	11	.018
Algerian					
Algeria	n	71	1.1	13	.046
Algeria	o	74	1.2	20	.038
Algeria	p	72	1.2	23	.049
--Mean			1.2	19	.044
--Group Mean			1.1	15	.038
Eastern Mediterranean					
Italy	e	66	1.2	17	.062
Israel	s	416	1.2	13	.045
--Group Mean			1.2	15	.054
--Overall Mean (Standard Error of the Mean)			1.1 (.01)	15 (1.0)	.040 (.004)

[1] Number of megagametophytes analyzed per population.
[2] Standard errors for the individual population heterozygosities ranged from .017 for the smallest values to .029 for the largest.

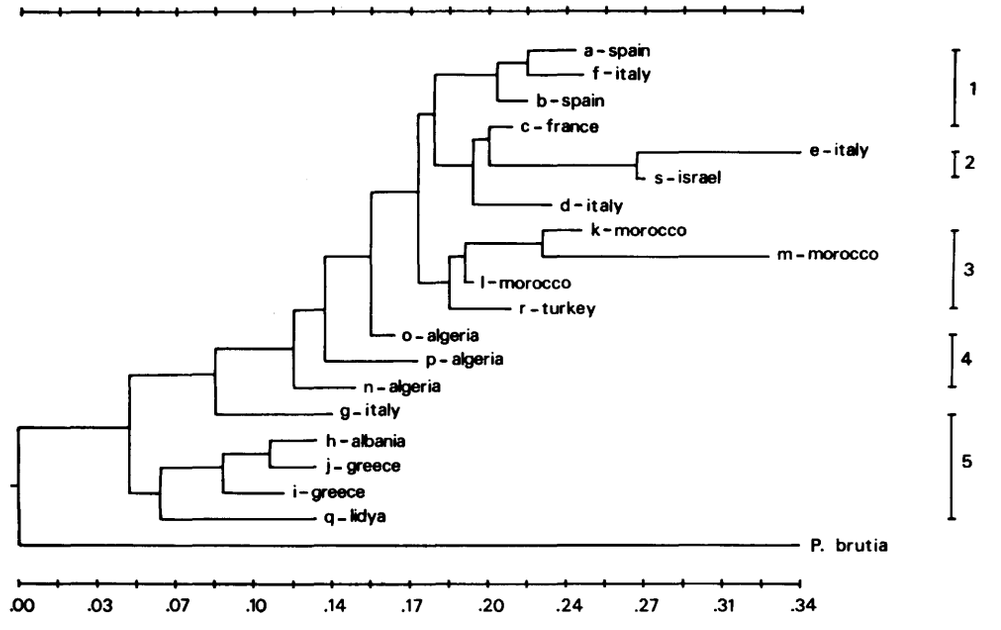

Figure 3. — Phylogenetic tree constructed using Cavallis-Sforza and Edwards (1967) chord distances and distance Wagner procedure (Farris, 1972) of populations of *Pinus halepensis*. 1- West European race (a, b, c, d, f); 2- East Mediterranean group (e, s); 3- Moroccan race + Turkish population (k, l, m, r); 4- Algerian race (n, o, p); 5- East European race, introgressed populations (g, h, i, j, q).

Table 3. — Average genetic distance above the diagonal (NEI, 1978) and average chord distances below the diagonal (CAVALLIS-SFORZA and EDWARDS, 1967) among and within five geographic subdivisions of *Pinus halepensis* and a composite for *P. brutia* obtained by averaging the allele frequencies from ten populations.

Group[1]							
---Halepensis--- Western Mediterranean		(1)	(2)	(3)	(4)	(5)	(6)
Western Europe	(1)	.065/.033	.038	.018	.009	.023	.319
Eastern Europe	(2)	.177	.114/.018	.045	.033	.049	.260
Morocco	(3)	.112	.194	.100/.015	.014	.037	.359
Algeria	(4)	.091	.163	.105	.072/.005	.020	.328
Eastern Mediterranean	(5)	.129	.199	.172	.130	.075/.011	.340
---Brutia---	(6)	.490	.422	.513	.489	.499	NA[2]

[1] Population samples were grouped as follows: (1) a, b, c, d, f; (2) g, h, i, j, q; (3) k, l, m, r; (4) n, o, p; (5) e, s; (6) a composite of *P. brutia samples.*
[2] NA = estimate not available, one sample value.

four geographic races are recognized according to their allele frequencies: (1) a western European race in Spain, France and part of Italy; (2) an eastern European race in Greece, Albania, and probably in Yugoslavia, Italy and possibly Libya; (3) a Moroccan race; and (4) a North African race in Algeria with probable inclusion of Tunisia.

The natural geographic subdivision of European *P. halepensis* from the African ones is supported by differences in allele frequencies for two loci (*Aco* and Mdh_3); allele frequencies differ among the regions and are relatively consistent for populations within the regions. *Aco* allele 2 is at higher frequency in western European than in Africa; Mdh_3 allele 2 is virtually absent from western Europe, but is present at intermediate frequencies throughout North Africa with a average frequency of .18. These data support the conclusion by PANETSOS (1981) that *P. halepensis* from western Europe differs from that growing in North Africa. In agreement with PANETSOS (1981) our data suggest that the geographic subdivision of African populations is between Morocco and Algeria. Mdh_4 allele 2 is almost absent in Morocco and abundant in most other *P. halepensis* populations. Moroccan populations have the lowest heterozygosities (.02) of all geographic subdivisions; West European seed sources average .03 and Algerian sources - .04.

The northernmost sample from Morocco (m) has an unusual gene frequency, apparently caused by random drift; $6Pgd_2$ allele 2 is near fixation but is at low frequency elsewhere. The sample is from a region with small isolated populations.

The provenance from the environs of Adana, Turkey (r), is one of three very small populations within the geographic range of, and in close proximity to, native stands of *P. brutia* (KAYACIK, 1954; 1963; 1973), but lacks alleles indicating hybridization or introgression by *P. brutia* and also lacks marker genes for Aap_1 and Cat_2 that characterize the East Mediterranean group. The allele frequencies in this population match those of Moroccan population (l); its heterozygosity, along with population (k) from Morocco, is the lowest of Aleppo pine populations, while that of the nearest Greek and Israeli samples is among the highest. Because of its small extent, this population could have lost variation by random drift. The absence of *P. brutia* alleles in this population could be because they were lost or, because of its artificial origin, possibly from Moroccan seeds. In this connection it is worth noting that *P. halepensis* population on Rhodes is according to PANETSOS (1981) of North African origin because of its smooth bark. Finding trees with smooth bark in the Turkish population would support the hypothesis of their western origin.

The West Mediterranean group of *P. halepensis* includes an East European race, which consist of populations from eastern Italy, the Balkan peninsula and, probably, Libya. The occurrence of *P. brutia* alleles in those populations (g, h, i, j, q) increases the heterozygosity in comparison with that in pure *P. halepensis.* Relatively high heterozygosity (.18) for *P. halepensis* near Athens, Greece was noted by LOUKAS *et al.* (1983); although the bases for estimating heterozygosity differed (LOUKAS and co-workers studied 17 pollen loci, which only about seven were common to the 30 loci from megagametophytes in our study), further research will probably find that variation in the Athens samples is associated with alleles from *P. brutia.*

Introgression shows up not only in the gene pool (as evidenced by electrophoresis), but also in the growth rate (PANETSOS, 1981) and shape of the trees and their reaction to lateral light. In unpublished trials by SCHILLER involving measurements of phototropism of seedlings of various provenances as an indicator of stem straightness (SCHMIDT, 1943; SCHRÖCK, 1958), exposure to lateral light produced the smallest curvature in seedlings from Balkan peninsula provenances. Further presumed evidence of the effect of introgression is the high resistance, in experimental plantations in Israel, of Greek provenances to the Israeli pine bast scale *Matsucoccus josephi* (MENDEL, 1984) (*P. brutia* is known to be unaffected by the scale).

The sample from Libya (q) also contains alleles typical of *P. brutia* and we speculate that it too is introgressed. It was, however, from a small number of trees and we feel that our classification of the Libyan population with the eastern European introgressed population is tentative.

Major differentiation among *P. halepensis* and *P. brutia* may have occurred as early as the Tertiary, with *P. halepensis* becoming widespread throughout the western Mediterranean. NAHAL (1962) and PANETSOS (1981) hypothesized that the center of origin of *P. halepensis* is in central and southern Europe. Our French seed source (c) is closest to their postulated center of origin and to areas where fossils similar to *P. halepensis* were found (NAHAL, 1962). Assuming a rate of mutation of 10^{-7} and calculating the divergence time from the French seed source (NEI, 1975), the time span obtained is 10,000—75,000 years for the Algerian seed

source, and 80,000 years for the Israeli composite sample; these dates correspond to the late Pleistocene.

The sequence of events leading to the divergence of western and eastern groups of *P. halepensis* is obscure. Major significance is attached to the existence of distinct alleles for Cat_2 and Aap_1 in populations throughout Israel and the population (e) in central Italy. These alleles could have been derived from independent mutation events which may have occurred subsequent to the geographic isolation of eastern and western populations. There is chance that the loci are linked on the same chromosome (Conkle, 1982).

The phylogenetic tree constructed from genetic distances implies that the eastern group is related to the western European race of the western group. The absence of Mdh_3 allel 2 (the allele characteristic of African populations) from the Israeli populations suggests an evolutionary link between *P. halepensis* in Europe and Israel. Low frequencies of Lap_1 allele 2 that mark the eastern group, are found also in West European and Algerian populations, suggesting a link between the eastern group of *P. halepensis* and the West European race *via* North Africa. Desertification during interpluvials, probably in the Riss-Wuerm interglacial (Said, 1982), may have broken any physical connections between populations of Aleppo pine in North Africa and the Near East.

The natural geographic subdivision of European from African Aleppo pine is supported by differences in allele frequencies for two loci (*Aco* and Mdh_3). The similarity of variation in Mdh_3 among African populations is important for inferring the derivation of *P. halepensis* of Algeria and probably Tunisia and Libya, from Moroccan populations. In the Tertiary Morocco was connected to Spain (Melville, 1967), and Aleppo pine spread over North Africa after the disappearance of the Tethyan Sea. Populations in Algeria and Libya have Mdh_3 allele frequencies identical to two Moroccan populations (k, m), with equal allele frequencies of about .20; elsewhere in Europe and in eastern Mediterranean (with only two exceptions a, g), allele 2 is absent. We reason that this similarity establishes that populations from Algeria, Libya and Tunisia are more closely related to Moroccan populations than to European ones. Reduced variation in *Aco* and Mdh_4, and the accompanying low estimates for heterozygosity are probably due to loss of low-frequency alleles, since the present Moroccan populations are relict populations isolated in mountainous area (Panetsos, 1981).

Introgression of populations of the western group by genes from *P. brutia* may be a recent evolutionary event. *Aco* and and Mdh_3 are unaffected by introgression. Both loci have allelic differences between European and African races, indicating that the races were established prior to introgression. Contact in the Balkan area between Aleppo pine and *P. brutia* resulted in gene flow into *P. halepensis*, and *vice versa*. Changes in climate have brought about the disappearance of *P. brutia* from this area. Introgression found today is therefore the remnant of phantom hybrids of *P. halepensis* × *P. brutia*, thereby recalling the phantom hybrid of *Eucalyptus cypellocarpa* L. Johnson × *E. globulus* Labill. in an area where only *E. cypellocarpa* and its hybrid occure nowadays (Kirkpatrick *et al.*, 1973).

Pinus halepensis has low amounts of variation in comparison with other pines. This low polymorphism was unexpected, since the species has a very extensive distribution and grows under widely different environments (Nahal, 1962). From observation, we class Aleppo pine with the early successional species with short life span, early cone production, massive seed production, and high capacity for regeneration after fire; characteristics of tree species with average heterozygosity in the range between .13 and .16 (Hamrick, Mitton *et al.*, 1982; Hamrick, Linhart *et al.*, 1979). Thus, *P. halepensis* has evolved into a species with good adaptability or plasticity, but lacking adaptive changes permitting growth of particular populations in given environments.

This interpretation of enzyme variation in *P. halepensis* has important implications for future improvement of the species. The geographic differentiation within the species implies that provenance tests will be valuable for identifying seed sources with desirable characters. Since Aleppo pine has low variation, phenotypic selection or selection based on progeny tests will result in discouragingly slow improvement. Introgression found in the Greek provenances, resulting in higher heterozygosity and better growth and stem form, leads to expect that improvement of Aleppo pine could be achieved using *P. brutia* as a source of new genetic variation.

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Reliable Plantlet Formation from Seedling Explants of Populus tremuloides (Michx.)

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Abstract

Seedlings have been ignored in tissue culture of *Populus* species due to the relative ease of obtaining or regenerating suitable explants from older individuals. Cotyledon and hypocotyl cultures of *Populus tremuloides* exhibit remarkable morphogenic capabilities that can be manipulated to form shoots, roots, or callus. Plantlets are obtained by production of multiple adventitious buds under a high cytokinin-to-auxin ratio, followed by shoot elongation and root formation. To date, over 600 propagules have been hardened into soil, making this a reliable procedure. Investigation of four different quaking aspen crosses reveals substantial variation in organogenic features both within and between crosses. This can be exploited to establish shoot cultures of individual seedlings, yielding a continuous harvest of rootable shoots at monthly intervals for up to five subculture periods. Such a system might find applications in isolation and proliferation of unique individuals from conventional and nonconventional breeding practices.

Key words: Aspen (*Populus*), Micropropagation, Seedling explant culture, Plantlet.

Zusammenfassung

Weil bei Pappelarten relativ leicht von älteren Individuen durch Gewebekultur gute Explantate erzielt werden können, wurden bisher Pappel-Sämlinge als Ausgangsmaterial vernachlässigt. Hypokotyl- und Kotyledonenkulturen von *Populus tremuloides* zeigen bemerkenswerte Fähigkeiten zur Manipulation, um Wurzeln, Sprosse oder Kallus zu bilden. Plantlets sind durch Herstellung multipler Adventivknospen bei einem hohen Cytokinin: Auxinverhältnis zu erhalten, wobei Wurzelbildung und Triebverlängerung folgen. Da bisher über 600 Abkömmlinge im Boden eingewöhnt werden konnten, dürfte dies ein zuverlässiges Verfahren sein. Die Untersuchungen von vier verschiedenen Aspen-Kreuzungen zeigen wesentliche Unterschiede in organogenetischen Merkmalen, sowohl innerhalb der Kreuzungen als auch zwischen diesen. Dies kann ausgenutzt werden, um Sproßkulturen einzelner Sämlinge zu erhalten und ständig neue bewurzelungsfähige Sprosse in monatlichen Intervallen bis zu fünf Einzelkulturperioden zu erzielen. Ein solches System könnte bei der Isolierung und Vermehrung besonders guter Individuen aus konventionellen und unkonventionellen Züchtungspraktiken Anwendung finden.

Introduction

Of all the commercially important forest species that have been propagated *in vitro*, none have enjoyed the success of members of the genus *Populus*. Indeed, the first woody species produced from callus using tissue culture techniques was *P. tremuloides* (Winton, 1970). Since that time, there have been reports of poplars regenerated from sporophytic (Venverloo, 1973; Winton, 1971; Wolter, 1968) and gametophytic (anther) callus (Sato, 1974; Wang *et al.*,