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Electrophoretic Analysis of Diversity and Phylogeny of *Pinus brutia* and Closely Related Taxa

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ABSTRACT. Rangewide samples from mature natural stands of *Pinus brutia* Ten. subsp. *brutia*, subsp. *stankewiczii* (Sukaczew) Nahal, subsp. *pithyusa* (Stevenson) Nahal, and subsp. *eldarica* (Medw.) Nahal from throughout the eastern Mediterranean display a continuum of allozyme variation for 30 loci. Ten geographic samples of subsp. *brutia* from Greek islands of the eastern Aegean Sea, and from Turkey, Cyprus, and Iraq have a mean expected heterozygosity of 0.12 (range from 0.10 to 0.14) and similar allelic variation suggesting that they share a common gene pool with minor geographic differentiation. Subspecies *stankewiczii*, a narrow endemic of the Crimea, has expected heterozygosity equal to the mean for subsp. *brutia* populations; subsp. *eldarica* has the lowest expected heterozygosity (0.08) among these closely related pines. Pines of the *P. brutia* group are polymorphic for many loci that are monomorphic and are presumed fixed in a widespread parapatric close relative, *P. halepensis* Mill. Consequently, they have nearly three times more variation than *P. halepensis*. Of all the subspecies sampled, *stankewiczii* has allozyme traits most like those expected in a progenitor of the entire *P. brutia*-*P. halepensis* complex. Its allele frequencies resemble subsp. *brutia* and it has a moderate frequency for an allele that is fixed in *P. halepensis* but which is sparse or lacking in other samples of the *P. brutia* group. Subspecies *pithyusa*'s allele frequencies resemble those of subsp. *stankewiczii* and several subsp. *brutia* populations. Subspecies *eldarica*'s allozyme similarities to the easternmost subsp. *brutia* populations and reduced diversity provide evidence of its derivation from subsp. *brutia*. Allele frequencies of Quetta pine (a provenance from a *P. brutia*-like naturalized race in southwestern Asia) closely match those of subsp. *eldarica* and provide evidence of its derivation from subsp. *eldarica*. Genetic distances between *P. brutia* and *P. halepensis* average 0.35 for Nei's unbiased distance and 0.47 for Cavalli-Sforza and Edwards's chord distance. Allozyme characteristics support the hypothesis that *P. halepensis* was derived from progenitors similar to pines of the extant *P. brutia* group mainly by fixation of alleles that are still polymorphic in pines of the group.

Pinus brutia Ten. subsp. *brutia* is the prominent low- to middle-elevation conifer in Turkey, Crete, Cyprus, and on islands of the eastern Aegean Sea (fig. 1) (Arbez 1974; Critchfield and Little 1966; Kasapligil 1978; Nahal 1962, 1981, 1983; Panetsos 1981). Subspecies *brutia* resembles in appearance and adaptations the widespread Aleppo pine (*P. halepensis* Mill.) of southern Europe, northern Africa, and the eastern Mediterranean. *Pinus brutia* and *P. halepensis* form a distinct subgroup within the Eurasian hard pines and the combined geographic distributions of these two species encircle the Mediterranean.

Narrow endemics closely resembling *P. brutia* subsp. *brutia* are *P. brutia* subsp. *stankewiczii* (Sukaczew) Nahal in the Crimea, *P. brutia* subsp. *pithyusa* (Stevenson) Nahal on the northeastern coast of the Black Sea, and *P. brutia* subsp. *eldarica* (Medw.) Nahal in the central Transcaucasia between the Black and Caspian seas (Nahal 1983) (fig. 1). The subspecies *brutia*, *stankewiczii*, *pithyusa*, and *eldarica* are referred to in this paper as the *P. brutia* group. An exotic pine of unknown genetic origin resembling subsp. *eldarica*, called Quetta pine (also referred to by the common name of Afghanistan pine), grows in southwest Asia in Iran, Afghanistan, the Quetta region of Pakistan, and southern U.S.S.R.

Early taxonomists considered subsp. *brutia* as a variant of *P. halepensis*, citing the seed cone attachment as the major distinguishing feature. But *P. brutia*'s species status was fully recognized when significant differences with *P. halepensis* were found in the composition of gum turpentines (Iconomou et al. 1964; Mirov 1955;

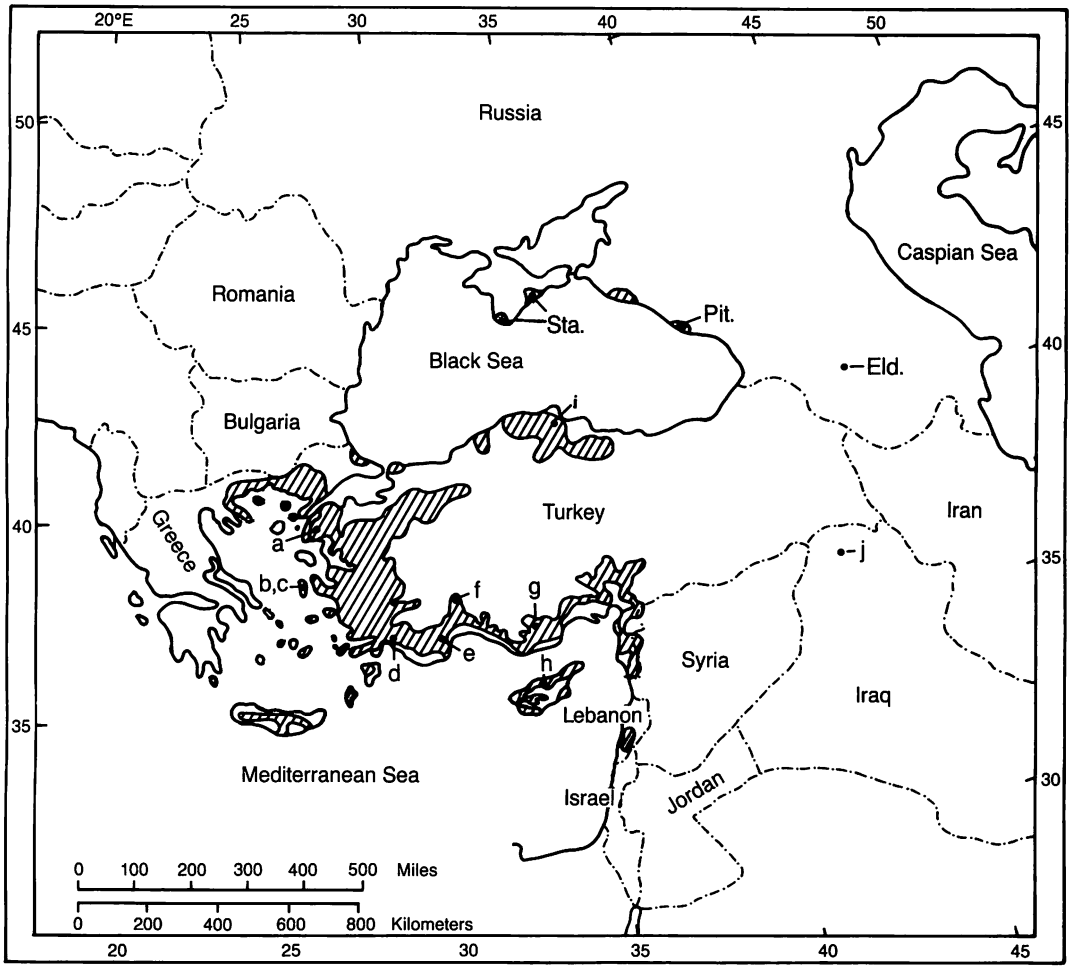

FIG. 1. Natural distribution of taxa in the *Pinus brutia* group (Critchfield and Little 1966) with the locations of 10 population samples (a–j) of subsp. *brutia* and locations of subspp. *stankewiczii* (Sta.), *pithyusa* (Pit.), and *eldarica* (Eld.). A population sample of Quetta pine (Que.) was from an area 2400 km southeast of subsp. *eldarica*.

Mirov and Iloff 1955; Mirov et al. 1966). The morphological traits and turpentine characteristics that distinguish taxa of the *P. brutia–P. halepensis* complex include the following: sessile, projecting cones of the *P. brutia* group are shorter and wider than the pedunculate, strongly reflexed cones of *P. halepensis* (Panetsos 1975; Sefik 1964); subsp. *brutia* seeds are larger in size and about 2.5 times heavier than those of *P. halepensis* (Nahal 1983; Sefik 1964); needles of subsp. *brutia* are longer, thicker, and have fewer marginal teeth than needles of *P. halepensis* (Panetsos 1975); the gum turpentines of subsp. *brutia* consist of α-pinene (about 70 percent), β-pinene, and Δ^3-carene (the latter two about 10 percent each) (Mirov et al. 1966), whereas *P. halepensis* consists largely of α-pinene and myrcene (about 80 and 10 percent, respectively). The turpentines of subsp. *brutia* and *P. halepensis* also differ in their refraction of polarized light; subsp. *brutia* is levorotatory, *P. halepensis* is dextrorotatory (Mirov et al. 1966).

Subspecies *stankewiczii*, *pithyusa*, and *eldarica*, and Quetta pine have cone and seed characteristics resembling those of subsp. *brutia*. Subspecies *eldarica* and Quetta pine have the heaviest seeds of the *P. brutia* group; both also lack or have significantly fewer resin canals per needle than the other subspecies. All four subspecies of *P. brutia* have similar turpentine composition

TABLE 1. Geographic locations of seed samples (mixtures of seed from 25 trees) of *P. brutia*. [a] The information in parentheses (B and numbers) are accession codes, Food and Agriculture Organization of the United Nations, Committee for Mediterranean Forest Research (FAO 1976); letters without numbers are seed lot codes, Institute of Forest Genetics, Placerville, California. [b] Information not available.

Species/population	Country	Location[a]	Latitude (N)	Longitude (E)	Elevation (m)
Subsp. *Brutia*					
a	Turkey	Canakkale-Ayvacik	39°56′	26°25′	250
b	Greece	Chios (AM)	38°36′	26°8′	200
c	Greece	Chios (AN)	38°36′	26°8′	1100
d	Turkey	Marmaris (B 6)	37°0′	28°18′	175
e	Turkey	Düzlercami (B 8)	37°3′	30°25′	175
f	Turkey	Pâmucak (B 9)	37°40′	30°41′	1000
g	Turkey	Silifke (B 12)	36°13′	33°43′	400
h	Cyprus	Cyprus (B 5)	35°8′	33°17′	150
i	Turkey	Camgolü (B 13)	41°50′	35°20′	70
j	Iraq	Iraq (AW)	36°55′	43°15′	900
Subsp. *stankewiczii*					
Sta.	U.S.S.R.	Yalta (D)	44°30′	34°9′	NA[b]
	U.S.S.R.	Sudak (G)	44°45′	35°0′	NA
Subsp. *pithyusa*					
Pit.	U.S.S.R.	Georgia (C)	43°10′	40°30′	NA
Subsp. *eldarica*					
Eld.	U.S.S.R.	Azerbaydzhan (Z)	41°NA	45°NA	400
Quetta pine					
Que.	Pakistan	Quetta (S)	30°NA	70°NA	1700

(Mirov et al. 1966). Subspecies *pityusa* and *eldarica* have levorotatory turpentine like subsp. *brutia*, but the specific rotation of turpentines reveals an unexpected and unexplained similarity between subsp. *stankewiczii* and *P. halepensis*; both are dextrorotatory (Mirov et al. 1966).

Artificial hybrid crosses between *P. brutia* and *P. halepensis* confirmed species-level differentiation by revealing partial breeding barriers (Moulalis et al. 1976). When pollen of subsp. *brutia* was placed on *P. halepensis* ovulate cones, virtually no viable seeds were produced. Viable seeds were produced in reciprocal crosses, but the production was only 25 percent of subsp. *brutia*'s normal yield. The F_1 interspecies hybrids were, however, highly interfertile with either species (60 percent of normal yields) and native stands of *P. halepensis* in Greece contain significant proportions of natural hybrids (Panetsos 1975).

Pines of the *P. brutia* group are recognized for their adaptations to drought and alkaline soils. Breeding and provenance trials are under way in Turkey (Isik 1986), Greece (Panetsos 1981), Australia (Palmberg 1975; Spencer 1985), Israel (Weinstein 1982), and southwestern United States (Fisher and Widmoyer 1978; Weber 1981). Noteworthy results are their resistance to *Matsucoccus josephi* (Mendel 1984), a serious scale insect pest on *P. halepensis* in Israel, as well as highly encouraging early growth of trees in arid regions of the southwestern United States.

Genetic knowledge about the *P. brutia* group is sparse. The objectives of our electrophoretic analyses are to estimate genetic diversity in populations and subspecies and provide information about geographic patterns of variation, systematic relationships, and probable evolutionary histories.

MATERIALS AND METHODS

Seed Materials. Seeds for these analyses (table 1) were from stored reserves at the Institute of Forest Genetics, Placerville, California, USA, and from reserves at the Agricultural Research Organization, Ilanot, Israel. They originated from collections coordinated by the

United Nations Food and Agriculture Organization's Committee for Mediterranean Forest Research (FAO 1976) and from provenance samples supplied by individual collaborators. The individual samples were seed mixtures from 25 or more mature trees at each collection site.

The geographic distribution of subsp. *brutia* was sampled at 10 locations (fig. 1a–j). Samples (a) through (h) covered the center of the subspecies distribution, (i) was from one of the widely scattered stands of northern Turkey, (j) was from a distant, easternmost outlying stand in northern Iraq. We obtained samples from the three subspp., *stankewiczii, pithyusa,* and *eldarica,* that are narrow endemics in the northeastern area of *P. brutia*'s distribution. Quetta pine was sampled from a region more than 2000 km southeast of the main species distribution.

Electrophoretic Analysis. Haploid megagametophytes from individual germinated seeds were assayed using horizontal starch gel electrophoresis when the root tips of embryos had emerged 2 to 5 mm beyond the seed coats (Conkle et al. 1982). The liquid fraction from macerated megagametophytes was analyzed simultaneously in four gel-buffer systems (Conkle et al. 1982; Strauss and Conkle 1986) that we coded by upper case letters: A. Tris citrate gel buffer (pH 8.3) and lithium borate tray buffer (pH 8.3), B. Tris citrate gel buffer (pH 8.8) and sodium borate tray buffer (pH 8.0), D. Morpholine citrate tray and gel buffers (pH 6.1), and E. Morpholine citrate tray and gel buffers (pH 8.1).

Twenty gel stains (Conkle et al. 1982; Millar 1985; Strauss and Conkle 1986) were used to resolve band patterns (fig. 2) for the following enzymes: alanine aminopeptidase [AAP: EC 3.4.11.1: (A) gel and tray buffer], aconitase [ACON: EC 4.2.1.3: (D)], acid phosphatase [ACP: EC 3.1.3.2: (D)], alcohol dehydrogenase [ADH: EC 1.1.1.1: (E)], catalase [CAT: EC 1.11.1.6: (B)], esterase [EST: EC 3.1.1.1: (A)], fructose diphosphatase [FDP: EC 3.1.3.11: (E)], fluorescent esterase [FLEST: EC 3.1.1.1: (A)], glutamate dehydrogenase [GDH: EC 1.4.1.3: (B)], glutamate oxaloacetate transaminase [GOT: EC 2.6.1.1: (B)], glucose-6-phosphate dehydrogenase [G6PD: EC 1.1.1.49: (B)], isocitrate dehydrogenase [IDH: EC 1.1.1.42: (D)], leucine aminopeptidase [LAP: EC 3.4.11.1: (A)], malate dehydrogenase [MDH: EC 1.1.1.37: (D)], menadione reductase [MNR: EC 1.6.99.2: (E)], mannose phosphate isomerase [MPI: EC 5.3.1.8: (B)], 6-phosphogluconic dehydrogenase [6PGD: EC 1.1.1.44: (D)], phosphoglucose isomerase [PGI: EC 5.3.1.9: (E)], shikimate dehydrogenase [SKDH: EC 1.1.1.25: (D)], and superoxide dismutase [SOD: EC 1.15.1.1: (B)]. Evidence from gametic segregation ratios, enzyme band phenotypes, and close similarity to enzyme loci in other pines were the basis for genetic interpretations of 30 loci in populations and subspecies of the *P. brutia* group (Conkle 1981).

The most common allele in *P. halepensis* was named allele 1. The gene and allele notations in this study (fig. 2) are consistent with those in companion papers reporting on variation in *P. halepensis* (Grunwald et al. 1986; Schiller et al. 1986). Allele frequencies for provenances were based on samples of 50 megagametophytes which we treated in computations as equivalent to 25 diploids. Allele frequency differences of 0.2 or larger between samples are statistically significant at the 0.05 probability level.

Allele frequencies for the taxonomic units were analyzed using BIOSYS (Swofford and Selander 1981). Expected Hardy-Weinberg proportions were computed to estimate the amount of genetic variation in populations and subspecies. Genetic distances among and between *P. brutia* populations and subspecies and *P. halepensis* races were estimated by the unbiased genetic distance method (Nei 1978) and by the chord distance method (Cavalli-Sforza and Edwards 1967). Data for all loci were processed using Cavalli-Sforza and Edwards (1967) chord distances and the Wagner distance procedure (Farris 1972) to develop a phylogenetic tree. Arc distances (Cavalli-Sforza and Edwards 1967) and Prevosti distances (Wright 1978) yielded similar results so their values were not included in this report. The complete analysis of *P. halepensis* is available elsewhere (Schiller et al. 1986).

Results

Allele frequencies varied by populations and subspecies (table 2). Seven loci (*Acp, Est, Got1, Got2, Mdh1, Pgi2, Skdh1*) were polymorphic in the *P. brutia* group including Quetta pine but were monomorphic in *P. halepensis. Mpi* had only minor variation in *P. halepensis* and it could be included to bring this total to eight loci. These loci contributed to significantly greater variation in the *P. brutia* group compared with

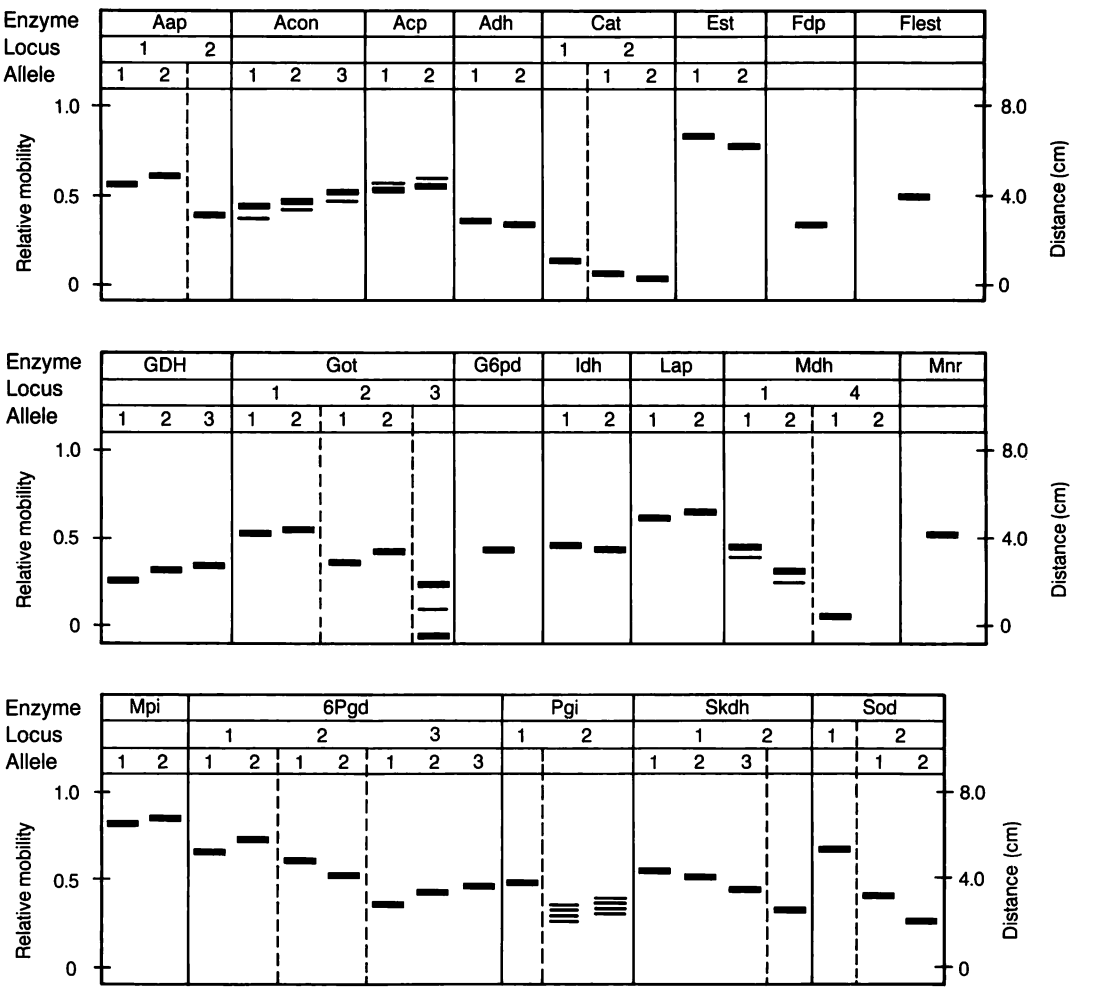

FIG. 2. Enzyme band phenotypes and genetic interpretation for 30 loci in *Pinus brutia* and associated taxa. Enzymes, abbreviations, and Enzyme Commission numbers are: alanine aminopeptidase, *Aap*, 3.4.11.1; Aconitase, *Acon*, 4.2.1.3; acid phosphatase, *Acp*, 3.1.3.2; alcohol dehydrogenase, *Adh*, 1.1.1.1; catalase, *Cat*, 1.11.1.6; esterase, *Est*, 3.1.1.1; fructose diphosphatase, *Fdp*, 3.1.3.11; fluorescent esterase, *Flest*, 3.1.1.1; glutamate dehydrogenase, *Gdh*, 1.4.1.3; glutamate oxaloacetate transaminase, *Got*, 2.6.1.1; glucose-6-phosphate dehydrogenase, *G6pd*, 1.1.1.49; isocitrate dehydrogenase, *Idh*, 1.1.1.42; leucine aminopeptidase, *Lap*, 3.4.11.1; malate dehydrogenase, *Mdh*, 1.1.1.37; menadione reductase, *Mnr*, 1.6.99.2; mannose phosphate isomerase, *Mpi*, 5.3.1.8; 6-phosphogluconic dehydrogenase, *6Pgd*, 1.1.1.44; phosphoglucose isomerase, *Pgi*, 5.3.1.9; shikimate dehydrogenase, *Skdh*, 1.1.1.25; superoxide dismutase, *Sod*, 1.15.1.1.

variation in *P. halepensis* (table 3). The mean number of alleles per locus for subsp. *brutia* populations (a) through (j) was 1.5, mean effective number of alleles was 1.2, 43 percent of loci were polymorphic, and mean expected heterozygosity was 0.12 of the loci. Of the other subspecies, only *stankewiczii* had an expected heterozygosity value as large as the mean for subsp. *brutia* populations; subsp. *eldarica* and Quetta pine had the lowest values of the *P. brutia* group (both 0.08).

Genetic distances between taxa (table 4) were computed using the mean frequencies of 10 populations for subsp. *brutia* and two distance measures. Distances for subsp. *brutia* compared with subspp. *stankewiczii, pithyusa,* and *eldarica* were 0.06, 0.04, and 0.04, respectively (unbiased genetic distance) and 0.19, 0.17, and 0.17, re-

TABLE 2. Allele frequencies for polymorphic loci of species and populations in the *Pinus brutia–P. halepensis* complex. Abbreviations used for *P. brutia* subspecies and the exotic race are Sta. for subsp. *stankewiczii*, Pit. for subsp. *pithyusa*, Eld. for subsp. *eldarica*, and Que. for Quetta pine. [a] Refer to figure 2 for enzyme names, abbreviations for loci, and band phenotypes that were assigned allele numbers. [b] Allele frequencies for introgressed populations are not included.

Locus[a] allele	Taxa/population(s)																
	P. brutia subsp. *brutia*															*P. halepensis*[b]	
	a	b	c	d	e	f	g	h	i	j	Mean	Sta.	Pit.	Eld.	Que.	Eastern	Western
Aap1																	
1	1.0	1.0	0.96	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.45	0.96
2			0.04								+					0.55	0.04
Acon																	
1	0.08	0.17	0.04	0.02	0.16	0.18	0.24	0.13		0.32	0.13	0.24	0.55	0.01	0.06	0.77	0.66
2	0.88	0.83	0.96	0.98	0.84	0.82	0.76	0.85	1.0	0.56	0.85	0.73	0.45	0.99	0.94	0.23	0.34
3	0.04							0.02		0.12	0.02	0.03					
Acp																	
1	0.30	0.25	0.96	0.55	0.36	0.38	0.60	0.62	0.46	0.48	0.50	0.27	0.30	0.85	0.62	1.0	1.0
2	0.70	0.75	0.04	0.45	0.64	0.62	0.40	0.38	0.54	0.52	0.50	0.73	0.70	0.15	0.38		
Adh																	
1	0.02				0.03		0.10		0.04	0.04	0.02	0.18		0.01		1.0	1.0
2	0.98	1.0	1.0	1.0	0.97	1.0	0.90	1.0	0.96	0.96	0.98	0.82	1.0	0.99	1.0		
Cat2																	
1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.83	1.0
2																0.17	
Est																	
1	0.60	0.12	0.13		0.17	0.61	0.22	0.24		0.32	0.24	0.23		0.02	0.06	1.0	1.0
2	0.40	0.88	0.87	1.0	0.83	0.39	0.78	0.76	1.0	0.68	0.76	0.77	1.0	0.98	0.94		
Gdh																	
1																1.0	1.0
2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.96	1.0	0.91	1.0	1.0	1.0		
3										0.04	+	0.09					
Got1																	
1	0.78	0.62	0.96	0.52	0.93	0.85	0.82	0.94	1.0	0.98	0.84	0.74	0.36	0.57	0.62	1.0	1.0
2	0.22	0.38	0.04	0.48	0.07	0.15	0.18	0.06		0.02	0.16	0.26	0.64	0.43	0.38		

TABLE 2. Continued.

Locus[a] allele	Taxa/population(s)																
	P. brutia subsp. *brutia*															*P. halepensis*[b]	
	a	b	c	d	e	f	g	h	i	j	Mean	Sta.	Pit.	Eld.	Que.	Eastern	Western
Got2																	
1	0.78	0.72	0.88	0.76	0.93	0.95	0.96	0.88	1.0	1.0	0.89	0.81	0.92	1.0	1.0	1.0	1.0
2	0.22	0.28	0.12	0.24	0.07	0.05	0.04	0.12			0.11	0.19	0.08				
Idh																	
1																1.0	1.0
2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		
Lap																	
1																1.0	0.99
2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		0.01
Mdh1																	
1	0.46	0.42	0.32	0.09	0.36	0.50	0.24	0.28	0.18	0.44	0.33	0.13	0.20	0.74	0.02	1.0	1.0
2	0.54	0.58	0.68	0.91	0.64	0.50	0.76	0.72	0.82	0.56	0.67	0.87	0.80	0.26	0.98		
Mdh4																	
1																0.62	0.81
2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.38	0.19
Mpi																	
1	0.70	0.93	0.62	0.88	1.0	0.44	0.59	0.82	0.76	0.56	0.73	0.85	1.0	0.98	0.92	1.0	0.99
2	0.30	0.07	0.38	0.12		0.56	0.41	0.18	0.24	0.44	0.27	0.15		0.02	0.08		0.01
6Pgd1																	
1	0.96	1.0	0.78	0.98	0.53	1.0	0.97	0.99	0.89	0.94	0.90	1.0	1.0	0.87	1.0	0.99	0.97
2	0.04		0.22	0.02	0.47		0.03	0.01	0.11	0.06	0.10			0.13		0.01	0.03
6Pgd2																	
1	0.30	0.53	0.62	0.59	1.0	0.72	0.87	0.81	0.63	0.82	0.69	0.88	0.90	0.15	0.06	0.99	0.93
2	0.70	0.47	0.38	0.41		0.28	0.13	0.19	0.37	0.18	0.31	0.12	0.10	0.85	0.94	0.01	0.07
6Pgd3																	
1	0.90	0.97	1.0	0.90	0.97	0.94	1.0	0.93	0.94	0.92	0.95	1.0	0.90	0.99	1.0	0.99	0.97
2								0.07			0.01		0.10	0.01		0.01	0.03
3	0.10	0.03		0.10	0.03	0.06			0.06	0.08	0.04						

TABLE 2. Continued.

Locus[a] allele	Taxa/population(s)																
	P. brutia subsp. *brutia*														*P. halepensis*[b]		
	a	b	c	d	e	f	g	h	i	j	Mean	Sta.	Pit.	Eld.	Que.	Eastern	Western
Pgi2																	
1	0.86	0.58	0.96	0.70	1.0	0.89	0.59	0.94	0.61	0.50	0.76	0.95	0.86	1.0	0.46	1.0	1.0
2	0.14	0.42	0.04	0.30		0.11	0.41	0.06	0.39	0.50	0.24	0.05	0.14		0.54		
Skdh1																	
1	0.22	0.06	0.50	0.04	0.40	0.33	0.55	0.55	0.50	0.80	0.40	0.75	0.70	0.42	0.53	1.0	1.0
2	0.70	0.94	0.50	0.92	0.40	0.67	0.45	0.39	0.50	0.13	0.56	0.25	0.30	0.58	0.47		
3	0.08			0.04	0.20			0.06		0.07	0.04						
Sod2																	
1																1.0	1.0
2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		

spectively (chord distance). Subspecies *stankewiczii* had comparably shorter distances with *P. halepensis* than other subspecies in the *P. brutia* group (0.27 versus 0.35 unbiased genetic distance, 0.42 versus 0.47 chord distance). Quetta pine had relatively short distances with subspp. *eldarica* (0.03 unbiased genetic distance, 0.15 chord distance) and *brutia* (0.04 unbiased genetic distance, 0.16 chord distance), but had the largest of all distances with *P. halepensis* (0.42 unbiased genetic distance, 0.50 chord distance).

Conspecific variation of *P. brutia* and *P. halepensis* represented only a fraction of the variation between the two species. Average genetic distances among the subsp. *brutia* populations (table 4), computed by two methods, were 0.02 for unbiased genetic distance (Nei 1978) and 0.14 for chord distance (Cavalli-Sforza and Edwards 1967). Similar values for *P. halepensis* populations, excluding a geographic area with introgressed populations, were 0.02 and 0.12, respectively (Schiller et al. 1986).

All taxonomic units were used in the construction of a phylogenetic tree (fig. 3). This tree is rooted along the left side at the midpoint of the longest path between taxonomic units. It is helpful to think of this root as representing a hypothetical progenitor with sufficient variation to account for differentiation between extant populations and taxa in this study. The tree, based on genetic distances, represents a possible evolutionary scheme where the populations most like a hypothetical progenitor are closest to the tree root (first subsp. *stankewiczii* and then subsp. *pithyusa*). Taxa with the longest distances from the root are those along the right side of the figure.

The first dichotomy from the tree root separates the *P. brutia* group from *P. halepensis*. The 10 subsp. *brutia* populations are clustered after subspp. *stankewiczii* and *pithyusa* on the upper branch; they have a tendency to associate into three geographic-physiographic groups: low-elevation western (a, b, d), mixed-elevation central (c, e, h), and eastern (g, i, j). Subspecies *eldarica* and Quetta pine are joined on the same branch forming the closest association with populations of the eastern group (first i, then g and j).

DISCUSSION

Electrophoretic variation in allozymes provides enlightening evidence about the evolu-

TABLE 3. Genetic diversity values for *Pinus brutia* subspecies and *P. halepensis* races; averages for 30 loci with standard errors in parentheses. [a] Number of megagametophytes analyzed. An equivalent number of diploid trees equals 0.5 times the number in the table. [b] Effective number of alleles per locus equals 1/sum (allele frequencies squared) (Hiebert and Hamrick 1983).

Subspecies and races	Sample size[a]	Mean alleles per locus	Mean effective alleles per locus[b]	Percent of loci polymorphic	Mean expected heterozygosity
Pinus brutia					
Subsp. *brutia*	480	1.53	1.23	43	0.118
		(0.12)	(0.06)		(0.034)
Subsp. *stankewiczii*	60	1.43	1.17	40	0.118
		(0.10)	(0.05)		(0.029)
Subsp. *pithyusa*	50	1.30	1.16	30	0.097
		(0.09)	(0.05)		(0.031)
Subsp. *eldarica*	50	1.37	1.12	37	0.075
		(0.09)	(0.05)		(0.027)
Quetta pine	50	1.30	1.15	30	0.082
		(0.09)	(0.06)		(0.030)
P. halepensis					
Eastern race	480	1.23	1.10	23	0.055
		(0.07)	(0.05)		(0.026)
Western race	820	1.23	1.06	23	0.035
		(0.08)	(0.03)		(0.017)

tion of taxa in the *P. brutia–P. halepensis* species complex. A simplifying hypothesis is useful for detailing the most probable history of speciation within the complex. We hypothesize that progenitors were the principal source of alleles now present in populations and species of the complex and that mutation and the establishment of new alleles played a minor role in recent speciation. Conifers are capable of maintaining high levels of variation due to outcrossing breeding systems, wind-disseminated pollen and seeds, seed production that often takes place over one-half to three-quarters of their life spans, and cross fertilization of trees from different generations. The differentiation among subspecies and species in this study is therefore assumed to be largely related to a history of maintaining or losing, by fixation, the ancestral variation.

The determination of systematic relationships and current evolutionary status requires interpreting multilocus differentiation. Variation of the loci analyzed in this study (table 2) can be grouped into six classes for comparing subspecies and species:

A. Loci that are monomorphic in all the samples (total of 10 loci; *Aap2, Cat1, Fdp, Flest, Got3, G6pd, Mnr, Pgi1, Skdh2, Sod1*).
B. Loci with polymorphisms characteristic of the eastern Mediterranean race of *P. halepensis* (total 2; *Aap1, Cat2*).
C. Loci that are polymorphic in all or the majority of the *P. brutia* group and *P. halepensis* samples (total 4; *Acon, 6Pgd1, 6Pgd2, 6Pgd3*).
D. Loci polymorphic in populations and subspecies of the *P. brutia* group but monomorphic (or nearly so in the case of *Mpi*) and presumed fixed in *P. halepensis* for one of the alleles present in the *P. brutia* group (total 8; *Acp, Est, Got1, Got2, Mdh1, Mpi, Pgi2, Skdh1*).
E. One locus (*Mdh4*) that was polymorphic in *P. halepensis* and fixed in the *P. brutia* group.
F. Loci fixed or near fixation for alternative alleles in the *P. brutia* group and *P. halepensis* (total 5; *Gdh, Idh, Lap, Sod2, Adh*).

The 10 monomorphic loci in class A are useful for estimating average genetic diversity (table 3). They provide no information about differentiation among the samples.

Class B highlights two loci with variation restricted to eastern Mediterranean *P. halepensis*. That variation was the basis for postulating a primary subdivision in *P. halepensis* (Schiller et al. 1986). The derivation of the allelic variation is uncertain since alleles characteristic of the eastern Mediterranean race of *P. halepensis* are ubiquitous in native stands throughout Israel (Grunwald et al. 1986) and sparse or absent from

TABLE 4. Genetic distance values for *Pinus brutia* subspecies (subsp. *brutia* based on the mean frequencies for 10 populations) and *P. halepensis* races. [a] Cavalli-Sforza and Edwards (1967) chord distances are above the diagonal, Nei's (1978) unbiased genetic distances are below.

Taxa	Bru.	Sta.	Pit.	Eld.	Que.	Hal—e.	Hal—w.
Pinus brutia							
Subsp. *brutia* (Bru.)	0.14[a] 0.02	0.19	0.17	0.17	0.16	0.47	0.46
Subsp. *stankewiczii* (Sta.)	0.06	—	0.18	0.22	0.22	0.43	0.42
Subsp. *pithyusa* (Pit.)	0.04	0.04	—	0.18	0.17	0.48	0.47
Subsp. *eldarica* (Eld.)	0.04	0.09	0.06	—	0.15	0.48	0.46
Quetta pine (Que.)	0.04	0.08	0.05	0.03	—	0.51	0.49
P. halepensis							
Eastern (Hal—e.)	0.36	0.28	0.36	0.36	0.43	—	0.12
Western (Hal—w.)	0.34	0.27	0.35	0.34	0.41	0.02	—

other *P. halepensis* and all *P. brutia*. The alleles may represent variation that evolved after major speciation of *P. brutia* and *P. halepensis*. Another hypothesis is that variation in these two loci once existed in an ancient progenitor and was subsequently eliminated from the *P. brutia* complex and from all but the eastern Mediterranean race of *P. halepensis*.

Class C contains loci that are generally polymorphic throughout taxa of the *P. brutia–P. hal-*

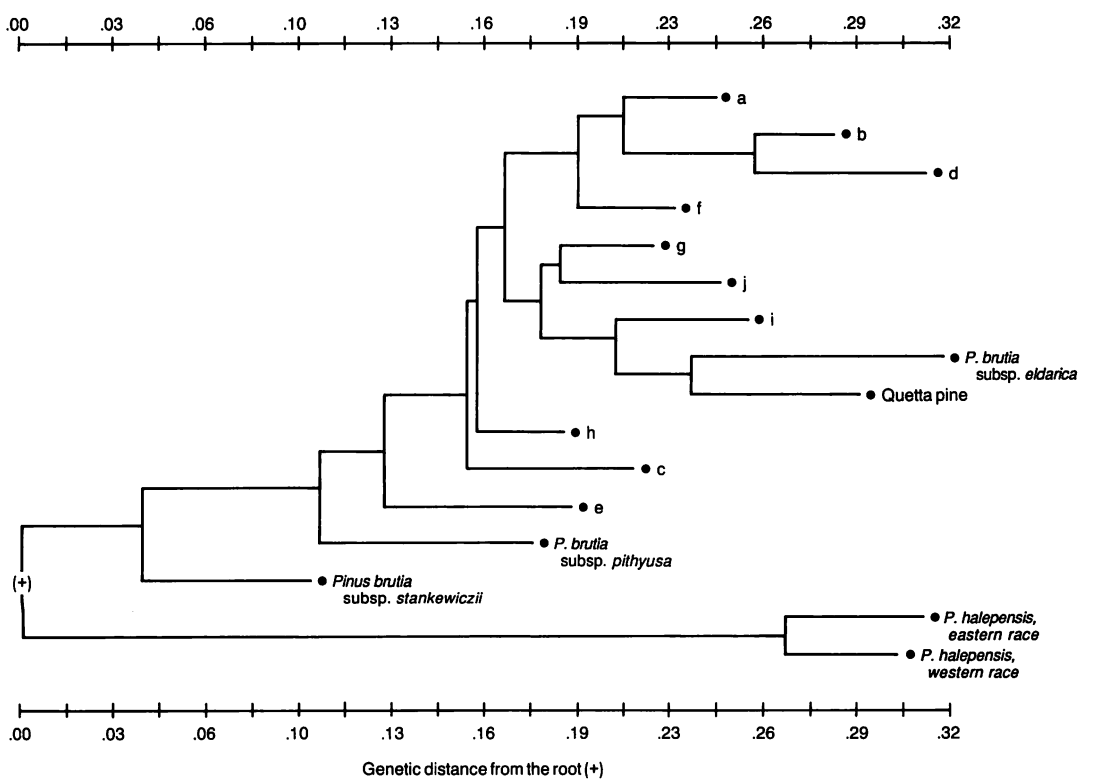

FIG. 3. Phylogenetic tree for Mediterranean pines of the *Pinus brutia–P. halepensis* species complex produced using the Wagner distance procedure and Cavalli-Sforza and Edwards chord distances (Swofford and Selander 1981). Ten populations of *P. brutia* subsp. *brutia* are (a–j). See table 1 and figure 1 for the geographic locations of samples.

epensis complex. These loci, only 13 percent of the total loci sampled, presumably represent conserved variation for they express differentiation by quantitative frequency differences and occasional gene fixation among populations and subspecies. *Acon* is an example locus for this group, with a tendency for the *P. brutia* group to have high frequencies of $Acon^2$ and *P. halepensis* to have high frequencies of $Acon^1$. A third allele ($Acon^3$) reaches a moderate frequency (0.12) in the j-population of subsp. *brutia* and is also present in trace frequencies in two other subsp. *brutia* populations and in subsp. *stankewiczii*. The inclusion of all the *6Pgd* loci in this class signals an unexpected, nonrandom association that may be related to gene duplication and linkage, but no further research was carried out on the *6Pgd* loci.

Class D has 8 loci (27 percent of the total sample and 40 percent of the polymorphic loci) that are variable in *P. brutia* and Quetta pine but are fixed in the eastern and western Mediterranean races of *P. halepensis*. This large group of loci, if the evolutionary hypothesis is correct, provides ample evidence to conclude that *P. halepensis* was derived from *P. brutia*-like progenitors. The alleles that are fixed in *P. halepensis* are in moderate to high frequencies in subsp. *brutia* [range of the mean frequencies in subsp. *brutia* is from 0.24 (Est^1) to 0.89 ($Got2^1$), table 2]. For example, the *Acp* allele (Acp^1) fixed in *P. halepensis* has a mean frequency of 0.5 in subsp. *brutia* (range from 0.25 to 0.96 in populations) and varies from 0.27 to 0.85 in the related subspecies (*stankewiczii* and *eldarica*, respectively).

What evolutionary conditions could account for the fixation of alleles in *P. halepensis* in such a large proportion of loci? If early populations of *P. halepensis* had large effective numbers of parent trees, natural selection would have had to have been intense and prolonged to produce such widespread gene fixation. The loss is better explained by an evolutionary bottleneck (perhaps several), with genetic drift and selection acting together to eliminate alleles. Gene flow among the species had to be eliminated, since even low-level migration of *P. brutia* genes into *P. halepensis* for these loci would have reduced or removed the distinction.

The loci that are polymorphic in subsp. *brutia* and fixed in *P. halepensis* also provide ample evidence that subspp. *stankewiczii*, *pithyusa*, and *eldarica*, and Quetta pine are close relatives of subsp. *brutia*. These relatives are also polymorphic for the majority of loci in the D class.

Class E has one locus (*Mdh4*) which was polymorphic in *P. halepensis* and fixed in the *P. brutia* group. It is the antithesis of class D.

Class F is one of the most interesting, for it includes vital clues to the phylogeny of the entire *P. brutia–P. halepensis* complex. Four loci are fixed for alternative alleles in *P. brutia* and *P. halepensis*. Fixation of alternative alleles represents maximum genetic divergence between taxa. We presume the ancient progenitor was polymorphic for *Gdh*, *Idh*, *Lap*, and *Sod2*; allele 1 became fixed in *P. halepensis*; allele 2 became fixed in the ancestral line leading to the *P. brutia* group.

The clues regarding phylogeny stem from close examination of *Adh* variation (table 2). *Pinus halepensis* was found to be fixed for allele 1. Five of subsp. *brutia*'s ten populations, subsp. *pithyusa*, and Quetta pine were fixed for allele 2. Trace frequencies of the *P. halepensis* allele (Adh^1) were in the a-population of subsp. *brutia* and in subsp. *eldarica*. The frequency of Adh^1 progressively increased in eastern populations of subsp. *brutia* (i, j, and g had 0.04, 0.04, and 0.10 frequencies, respectively). Of all the *P. brutia* subspecies, *stankewiczii* had the highest frequency of Adh^1 (18 percent).

We conclude from *Adh* information that subsp. *stankewiczii* represents the most intermediate subspecies between *P. brutia* and *P. halepensis* of all our samples. Finding moderate frequencies of the *P. halepensis* allele in the eastern populations of subsp. *brutia* further establishes a geographic link with subsp. *stankewiczii*. This evidence supports a conclusion that various stands with progenitor-like variation in regions north and south of the Black Sea, throughout eastern Turkey, and extending into northern Iraq are remnants from the geographic center of origin of extant taxa in the *P. brutia–P. halepensis* complex.

Low-frequency alleles of genes other than *Adh* also provide supporting evidence that these regions are the center of origin. Subspecies *stankewiczii* and the j-population of subsp. *brutia*, alone, had low frequencies (0.09 and 0.04, respectively) of a third *Gdh* allele (Gdh^3). The j-population had a significant proportion (0.12) of a rare *Acon* allele ($Acon^3$); subsp. *stankewiczii* also had a trace frequency of the allele. Five

subsp. *brutia* populations (e, a, j, h, and d) had a third *Skdh1* allele. The j-population of subsp. *brutia* from Iraq is therefore a noteworthy reservoir of rare alleles for *Adh, Gdh, Acon,* and *Skdh1.*

Subspecies *eldarica,* with allozyme frequencies resembling eastern populations of subsp. *brutia* (i, j, and g; table 2 and fig. 3), had the lowest expected heterozygosity (0.08) among the subspecies of *P. brutia* (table 3). Subspecies *eldarica*'s divergence from the other subspecies apparently involved shifting allele frequencies and the loss or near loss of some alleles that are present in other subspecies of the *P. brutia* group.

The origin of pines resembling *P. brutia* that were introduced into southwest Asia is unknown, but close correspondence between allozyme frequencies of subsp. *eldarica* native to central Transcaucasia in Russia and Quetta pine from Pakistan leads us to conclude that Quetta pine was derived directly from subsp. *eldarica.*

This allozyme information supports many conclusions about species relationships that were previously based on phenotypic characteristics. Allozymes indicate a highly significant divergence between *P. brutia* and *P. halepensis.* Subspecies *stankewiczii* has allozyme variation much like that expected in a genetically variable progenitor of the *P. brutia–P. halepensis* complex. Whereas the major components of subsp. *stankewiczii*'s turpentine are the same three that are in subsp. *brutia,* subsp. *stankewiczii*'s rotation of polarized light transmitted through the resin is the same as *P. halepensis,* the rotation for both differs from all other subspecies of the *P. brutia* group. Allozymes indicate Quetta pine was derived from subsp. *eldarica.* Both subsp. *eldarica* and Quetta pine lack resin canals near the abaxial face of needles while subspp. *brutia, pithyusa,* and *P. halepensis* have from 2 to 4 canals (the number in subsp. *stankewiczii* has not been reported).

The evolutionary history of these related pines, reconstructed from allozyme evidence, indicates that the center of origin included the regions bordering the Black Sea, easternmost Anatolia, and eastward extensions into lands between the Black and Caspian seas. Early populations there may once have been widespread, larger in size, and more nearly contiguous. Modern subsp. *brutia* of western Anatolia and adjacent islands in the Aegean and Mediterranean seas is a widespread taxon that maintains significant levels of allozyme variation (0.12 expected heterozygosity) throughout its geographic distribution.

Eastern populations of subsp. *brutia* are now geographically isolated from the main distribution by long distances. Several of the eastern populations resemble subspp. *stankewiczii, pithyusa,* and *eldarica* by possessing rare alleles and by having allele frequencies that distinguish them from the western populations of subsp. *brutia.* Morphological differentiation of subspp. *stankewiczii, pithyusa,* and *eldarica* has been sufficient for some taxonomists to assign species status to them. But enzyme allele frequencies of these subspecies closely resemble the frequencies for subsp. *brutia.*

Evidence from allozymes indicates that geographically widespread *P. halepensis* is a genetically depauperate derivative from subsp. *stankewiczii*-like progenitors. Subspecies *stankewiczii* from the Crimea had genetic distances and distinguishing alleles identifying it as the closest evolutionary link to *P. halepensis.* But *P. halepensis* is monomorphic and presumed fixed for 40 percent of the loci that are variable in the *P. brutia* group. Variation in subspp. *stankewiczii* and *brutia* is 2 to 3 times the variation in *P. halepensis* (expected heterozygosities are 0.06 and 0.04 for eastern and western Mediterranean *P. halepensis,* respectively, and 0.12 for subspp. *brutia* and *stankewiczii*). *Pinus halepensis* samples from Morocco have the lowest expected heterozygosity values (0.02) of all samples from the *P. brutia–P. halepensis* complex (Schiller et al. 1986).

Evolution and differentiation of taxa are related with levels of genetic variation (Gottlieb 1977; Ledig 1986a). Long lived, wind pollinated, geographically widespread plant species characteristically maintain high levels of intrapopulation genetic variation (Brown 1978, 1979; Hamrick et al. 1979) and the assessment of diversity in natural populations is an informative first step in developing gene conservation strategies (Ledig 1986b). The mean expected heterozygosity for pine species is about 0.18 (Ledig 1986a); species values range from a high of 0.33 for *P. longaeva* (Hiebert and Hamrick 1983) to 0.0 for *P. resinosa* (Fowler and Morris 1977; but Allendorf et al. 1982 report trace levels of null alleles) and *P. torreyana* (Ledig and Conkle 1983). Diversity in *P. brutia* is below the mean reported for pine species and is similar to the values reported for rangewide samples of *P. contorta*

[expected heterozygosity 0.12 (Wheeler and Guries 1982)] and *P. banksiana* in Alberta, Canada [expected heterozygosity 0.12 (Dancik and Yeh 1983)].

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