MONOTERPENOID DIVERSITY IN RELATION TO MORPHOLOGY OF *PINUS BRUTIA* AND *PINUS HALEPENSIS* IN AN EAST MEDITERRANEAN AREA (ATTIKI, GREECE): IMPLICATIONS FOR PINE EVOLUTION

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Pinus halepensis and *P. brutia* hybridize extensively. The analysis of monoterpenoid and morphological variability of 122 pine trees at five sites showed that the within-site heterogeneity is high while the entire provenance diversity is comparable with that of a circum-Mediterranean scale. Four morphotypes and four chemotypes were recognized. The congruence between morphological and monoterpenoid classification of pines was high (coefficient of contingency 89%) while thirteen family groups of genetically closely related trees were found by means of a specifically designed sampling scheme of continuous classification – identification and re-sampling. Putative hybrids contributed substantially to the observed diversity and there is considerable heterogeneity between sites. Low-altitude sites, presumably susceptible to sea-level fluctuations, alluvial deposition and human influence, are in general more diverse at both levels of genetic complexity than inland sites. The hybrid phenotypes were not merely intermediate forms of parental taxa, and in a multivariate sense they are located on a parabola in the space of morphological properties. The arrangement of hybrids in the monoterpenoid space did not reveal any clear-cut pattern except that three major composite axes can summarize 98.9% of the entire monoterpenoid variation. The observed pattern of variation was interpreted as a result of waves of introgressive influence of eastern *P. brutia* population on *P. halepensis,* which was caused by marine transgressions in Pliocene and eustatic sea-level fluctuations in Pleistocene. The human influence on this introgression accelerated the process, and amplified the resulted pattern, although it blurred in many instances the biogeographic routes of germplasm mixing. Its bearing on the evolution of pines is discussed, and it was found that the 'Eocene refugia hypothesis' of C.I. Millar holds by analogy also in central Aegean pines.

Keywords. Diversity, east Mediterranean, introgressive hybridization, monoterpenes, morphometry, *Pinus brutia*, *Pinus halepensis*, Pleistocene, Pliocene.

INTRODUCTION

Although the origin of pines can be safely stated as being in the Mesozoic of northern hemisphere, phylogenetic analyses within the genus showed that many subgeneric

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patterns observed in extant taxa can be traced back in the Eocene. Millar's (1993) hypothesis states that Eocene refugia, together with the concomitant extinction of many taxa of the boreotropical flora as a result of the late Eocene cooling episode, resulted in extensive range expansion of pine species. This expansion triggered a multitude of processes that can be summarized under the term 'introgressive hybridization'; a term coined by Anderson (1953) to describe all phenomena associated with the genomic mixing of two geographically separated species. In these outlined scenarios, formerly isolated pine taxa came in contact and extensively hybridized. These contact phenomena either resulted in speciation, with the aid of temperature fluctuations during the Oligocene and Miocene, which caused periodic contractions and expansions of low- and mid-latitude ranges, or created situations where two pine species co-occurred syntopically together with their hybrids. Introgressions in pines have been reported from North American provenances on the basis of needle essential oil data (e.g. von Rudloff, 1975).

The global pattern of re-invasions was affected by local geomorphological and tectonic processes, which created contact opportunities and range disjunctions. The situation peaked in Pliocene marine transgressions and Pleistocene glaciations (Pielou, 1979, 1991; Fady *et al.*, 1992) with differences both in the duration of various phases of the re-invasions and in the nature of the raised barriers, i.e. sea corridors, adverse climatic conditions or biotic factors. In the Pliocene-Quaternary period, the eastern Mediterranean became the theatre of large-scale episodes, such as the Messinian salinity crisis, the Pliocene revolution, and the basinal subsidence (Fabricius *et al.*, 1984; Batin, 1992), while in the Pleistocene at least three major eustatic sea regressions, corresponding to the Mindel, Riss and Wurm glaciations, caused a dramatic change in the geography of the eastern Mediterranean. These changes can be observed easily in the Greek territory (mainland, Ionian and Aegean Sea), where the isobath lines delimitating various island complexes such as Cyclades or northern Sporades in the Aegean, and Cephallonia, Ithaca and Leukas in the Ionian, are well inside the sea-level fluctuations in the Pleistocene (c.160m after Kurten, 1972; see also Creutzburg, 1966).

Plio-Pleistocene geographic reconstructions broadly correspond to biogeographic divisions and associated palaeogeographic scenarios assembled by various authors (Rechinger, 1951, Runemark, 1971, and Greuter, 1975 for Aegean vascular plants; Beug, 1975 for human-influenced plant succession; Bottema, 1975 for Quaternary vegetation; Snogerup, 1967a,b for the cruciferous genus *Erysimum*; Reille *et al.*, 1980, Reille, 1992, and Pons, 1984 for *Pinus halepenesis*; Trihas & Legakis, 1987 for Coleoptera; Petrakis, 1991 for heteropterous insects and their host and food plants; Batin, 1992 for Odonata: Zygoptera: *Platycnemis pennipes*; Ikeya & Poulianos, 1979 for Acheulian – 1 Myr BP – hunter-gatherers in Greece; Hoffman, 1980 for palaeolithic – 40 Kyr BP – architectural industries in river Nile delta).

If Millar's hypothesis that Eocene temperature fluctuations triggered the refugia recolonization processes, which became the major evolutionary theatre of pines, is correct, then Plio-Pleistocene eustatic events would have similarly caused, qualitatively, the same evolutionary processes. Given the restricted time-scale, the extended life-span of pine trees and the comparatively slower chromosomal evolutionary rate of conifers with respect to other angiosperms (Prager *et al.*, 1976), these evolutionary events have not resulted in complete speciation, as of yet. However, these events must be manifested by the extensive hybridization pattern of the participating species, mainly *P. brutia* Miller and *P. halepensis* Tenore. Schiller and his colleagues (Schiller *et al.*, 1986) have already found evidence of such an introgression of *P. brutia* to *P. halepensis* in the Balkan peninsula on the basis of isoenzyme variation, and they went further to hypothesize that the eastern European race of Aleppo pine originated from this process. That the entire Miocene-Quaternary period could offer such opportunities for pines is not only confirmed by palaeobotanic investigations (Klaus, 1989) but is also the result of extensive observations, of several authors about the Pleistocene glaciations and interglacial range expansions (Lepart & Debussche, 1991). It is believed that they have played an important role in the genotypic diversification of the genus *Pinus* (see for example Forrest, 1987 for *P. contorta* Douglas ex Loudon and the closely related *P. banksiana* Lambert; Pielou, 1991: 232 for several Nearctic pines).

Because in *Pinus* the widely used enzyme variation has been proved to be a poor predictor of intraspecific and intervarietal variability at higher levels of complexity (see for example Linhart *et al.*, 1989 for *P. ponderosa*; Schiller *et al.*, 1986 for *P. halepensis*) the potential covariation of morphological and chemical (monoterpene) traits could offer a better insight into the evolutionary processes maintaining such an extensive hybridization pattern (see Zavarin *et al.*, 1989 for an example of typified morphological characteristics and monoterpenoid differentiation in *P. edulis* Engelm.). A relationship between two consecutive levels of genetic complexity involving several (monoterpenoid profile) and many (morphology, anatomy) genes respectively is very likely to be manifested. This was the case of the *P. halepensis* × *P. brutia* hybrid detection method of Korol *et al.* (1995) on the basis of allozyme and morphological traits. The purpose of this study is to investigate the hybridization status between *P. halepensis* and *P. brutia* through the simultaneous analysis of morphological variation and the needle monoterpene variability, as well as the implications it may have for the evolution of genus *Pinus* in Mediterranean type climates. On the basis of anatomical, morphological and distribution data, these taxa are considered to be two distinct species (Nahal, 1962; Panetsos, 1981) although only the first is consider indigenous to the Mediterranean Basin (Naveh & Vernet, 1991: 21), probably because of its post-fire germination ability, which characterizes the annual species – therophytes – of Mediterranean type vegetation (Trabaud, 1991).

MATERIALS AND METHODS

Selection of the study area

Attiki was selected (Fig. 1) as the focus area of this study for several reasons. (i) It is located well within the geographical area where these pine species exhibit

FIG. 1. Map of the central and southern Aegean (scale 1 : 100,000). Triangles: sites sampled for the purpose of this study (1, Schinias; 2, Mt Parnis; 3, 'Demokritos' wood; 4, Kaesariani; 5, Elefsis). Dashed line: Quaternary phytogeographic divisions proposed by Rechinger (1951). Thin line: contemporary coastline. Thick line: the last glacial maximum coastline (after van Andel & Shackleton, 1982). Dot and dash line: the early Pleistocene coastline (Creutzburg, 1966). Lakes are not shown. Double arrows: Riss glacial connections.

germplasm mixing (Naveh & Vernet, 1991). Rechinger (1951) never attempted to separate these two species in his proposed phytogeographical maps of the Aegean. It is premature to say that an extensive hybrid zone exists, but several intermediate forms between typical *P. brutia* and *P. halepensis* (Gaussen *et al.*, 1964) can be found within this area. (ii) It provides several habitat types, ranging from coastal pinewoods of steep sea-facing slopes with minimal human impact, i.e. no historical record of reafforestation, only restricted wood-cutting and range-management practice by burning, which can be safely considered as remnants of ancient extensive pine forests (Petrakis, 1991), to mountainous, up to 1200m a.s.l., areas anthropogenically influenced either through reforestation activities or through burning for creation of pastures for sheep and goat. (iii) Palaeogeographical reconstructions (Creutzburg, 1966) suggest that the area remained intact, from subsidence, since the late Pliocene with various connections at different times with the Asia Minor land-mass and the associated offshore islands, as a result of marine regressions (Figs 1 and 2). These close contacts of Attiki with the area of origin of *P. brutia* created potentially at least two waves of introgressive influences of this species into the native *P. halepensis*. The first can be located in the late Pliocene corridor from Samos island to the Cycladean land-mass (offshore of Mykonos island), and the second is a Riss connection between Ikaria island and Cycladean land-mass to the north and a second Riss glacial connection between northern Dodecanese and Kinaros island (east of

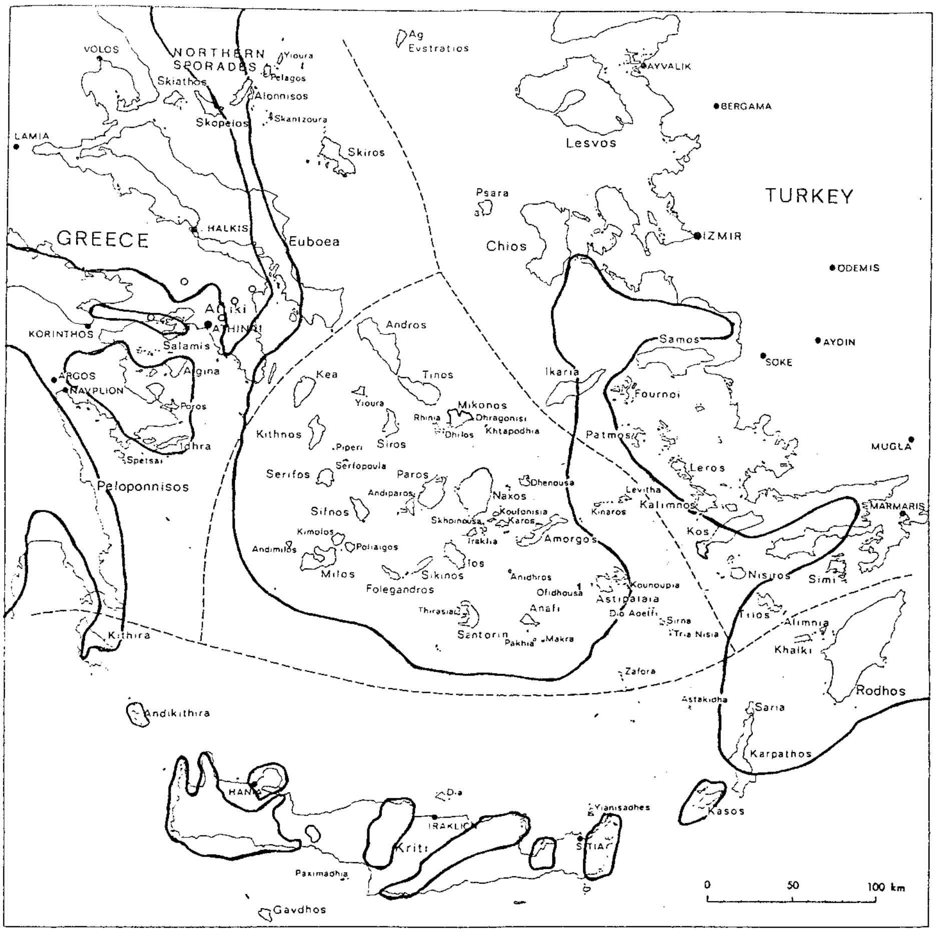

FIG. 2. Pliocene map of the central and southern Aegean (scale 1:100,000). Dashed line: the Quaternary phytogeographic divisions proposed by Rechinger (1951). Thin line: contemporary coastline. Thick line: Pliocene coastline (Creutzburg, 1966).

Amorgos island) to the south (see Fig. 1 for the last glacial maximum, 18 Kyr BP). All these connections have been roughly traced both in influences received by local floras (Runemark, 1971; Greuter, 1975) and in geographical variability discussions of plant species complexes (e.g. Strid, 1970 for *Nigella arvensis* complex), and animal species and species assemblages (e.g. Heller, 1976 for enid landsnails; Batin, 1992 for *Platycnemis pennipes* Pallas, zygopterous insects). Within this area, five sites were selected to cover the entire habitat and topoclimatic variability (Table 1).

Sample processing and analysis

Branches were sampled from 122 tagged pine trees, which according to taxonomic criteria (Gaussen *et al.*, 1964; Vidakovic, 1991) were either roughly categorized as *P. halepensis* or *P. brutia* by various degrees of conformation to identification keys. The sampling effort in each site was roughly proportional to the existing diversity of forms and monoterpenoid profiles. Three trees were initially sampled from each site in a random design from various parts of their crowns (twigs undamaged by herbivores) to cope with plant plasticity effects (Bradshaw, 1965, 1973) and branch autonomy phenomena (Sprugel *et al.*, 1991). Morphological and monoterpene composition data were submitted in a minimum variance agglomerative centroid clustering algorithm (Ward method in Orloci & Kenkel, 1984). As a distance measure, Euclidean distances were used for monoterpene data, and the unit complement of the Goodman-Kruskal gamma coefficient for morphological data (Goodman & Kruskal, 1954), because these data are a mixture of ordinal and category variables. Cluster analysis produced four recognizable groups corresponding to four morphotypes. After this initial clustering attempt, additional samples were allocated to existing groups by means of the generalized Mahalanobis distance criterion (Orloci & Kenkel, 1984). The assignment was made on the basis of the minimization of the misclassification probability of a particular sample to the already devised groups. If the misclassification probability of a new sample was high ($P > 0.05$) for all existing groups (chemotypes and morphotypes), the cluster analysis was repeated by incorporating the new sample in the initial data set of three trees from each site. When two consecutive samples from one site were successfully assigned to the devised groups, we stopped sampling new trees from this particular site. There is no statistical method to estimate the best size of sample for trees in this case of a hierarchically structured data body. Instead we used an approximation of the modified Zippin population-size estimation method (Southwood, 1966; Petrakis, 1991), substantially affected by logistic resources.

This procedure of objective assignment of new samples was prevented several times by matrix singularity problems (Orloci & Kenkel, 1984). These problems are usually the result of strong covariation among morphological characters and monoterpene quantities, especially in cases where many variables are used to describe the individuals to be analysed. Because of the slow chromosomal evolutionary rate of pines, this singularity problem is a reflection of the slow morphological evolution (Prager *et al.*, 1976). To cope with this problem we applied a variable (i.e. a morphological character or a monoterpene constituent) weighting algorithm. We retained in the analysis only those variables that accounted for 99% or more of the observed variation in this data set (Jancey, 1979; see Tables 2 and 3).

Samples collected from the field were transferred to the laboratory, within 2h, for morphological and chemical analyses. Only samples taken in August were used in this

TABLE 1. Parameters, morphological and chemotypical composition of the sites in which the trees were collected

Site	Elefsis	Kaissa-riani	Demokritos wood	Mt Parnis	Schinias coast
Distance from the sea (km)	0.1	12	30	40	0.1
Stand area (ha)	2	60	50	4000	250
Years from last fire	>50	25	>40	>40	>45
Mean intertree distance (m)	4	5	20	12	10
m a.s.l.	20	350	150	600	5
Coexisting *Pinus* spp.	None	None	*P. canariensis*	*P. nigra*	*P. pinea*
Understorey dominant plants	#1	#2	#3	#4	#5
Anthropogenic impact [0–9], where 0: no signs of human impact, 9: scattered in agricultural land	9	7	4	2	1
	Number of trees sampled from each site				
Chemotypes #6					
CA		9			
CB1	7			13	
CB2			8		
CC					15
CD1	21				
CD2	16	11			
CD3	14		8		
Total number of trees	58	20	16	13	15
Morphotypes #7					
MA1	21				
MA2	15				
MB1			8		
MB2	7	11			
MC1	15				
MC2		9			
MD1			8	13	
MD2					15

#1, none; #2, *Quercus coccifera*, *Thymus capitatus*, *Brachypodium pinnatum*; #3, *Quercus coccifera*, *Cistus monspeliensis*, *C. creticus*; #4, sparse herbaceous *Fabaceae* and *Poaceae*; #5, *Quercus coccifera*, *Pistacia lentiscus*, *Asparagus aphyllus*, *Brachypodium distahyon*; #6, significant interaction is manifested between sites and chemotypes (contingency coefficient = 0.81, $P < 0.005$); #7, contingency coefficient = 0.75, $P < 0.005$.

TABLE 2. Morphological characters examined in the morphometric analysis *of P. halepensis, P. brutia* and their hybrids. Morphotype (MA, MB, MC, MD) averages are given together with their rank in weighting them by a sum of squares criterion. Measurements are specific to this data set and may differ markedly from those reported from other areas of species range. Terms such as 'typical *P. brutia*' – for morphotype MA – are referred to the ease of the identification of the tree according to conventional identification keys (Gaussen *et al.*, 1964; Vidakovic, 1991)

n	Morphological character specification	MA[#1]	MB	MC	MD	Rank
	Leaf characters					
1	No. stomatal rows (adaxial surface)	8.02	8.08	8.00	6.00	14
2	No. stomata per 5µm (adaxial surface)	11.07	9.05	9.79	8.62	12
3	No. resin canals (cross section)	5.95	6.49	5.52	3.67	10*
4	Position of resin canals (µm, adaxial surface)	37.75	45.00	38.33	60.00	8*
5	Degree of ornamentation [0–4]	1.025	1.30	2.20	2.00	4*
6	Length of longest leaf (of branch) (mm)	177.75	121.50	112.33	117.00	1*
7	Width of longest leaf (mm)	1.20	1.09	0.99	0.93	2*
8	Colour [0–4] from light greenish–yellow to dark green)	2.25	2.00	0.67	0.50	12
9	No. layers of hypodermal cells	2.35	1.74	2.13	2.02	12
	Cone characters					
10	Length (mm)	63.74	68.56	64.94	78.15	3*
11	Maximum width of recently opened cones (mm)	58.51	53.56	50.79	63.72	6*
12	Degree of asymmetry [0–4]	0.25	1.00	1.67	2.00	5*
13	Apophysis of ovuliferous scale height (mm)	10.23	13.85	11.22	14.24	12
14	Apophysis of ovuliferous scales width (mm)	15.99	14.78	15.93	13.15	7*
15	Apophysis lower margin spinesence [0–4]	0.88	1.00	0.33	1.00	12
16	Umbo height (mm)	2.99	3.10	3.48	3.79	13
17	Umbo width (mm)	5.46	5.47	6.08	6.19	13
18	Umbo prickle/spine [0,1,2]	0.25	0.25	0.00	0.00	13
19	Peduncle length (mm)	0.27	8.41	6.73	17.47	13
20	Peduncle curvature 0/1 (presence/absence)	0	0.50	0.33	1.00	12
	Twig characters					
21	Colour [0–4]	3	2.12	2.50	2.25	11
22	Smoothness 0/1 (presence/absence)	0.75	1.00	0.67	1.00	12
	Seed characters					
23	Length (mm)	8.38	6.98	6.60	6.73	13
24	Width (mm)	4.78	3.87	3.60	3.86	15
25	Wing length (mm)	16.38	15.61	18.17	18.03	12
26	Wing width (mm)	7.98	7.18	7.87	7.59	9*
27	Seed wing degree of development around the seed [1–4]	2.75	2.44	2.00	1.00	13

*, characters used in cluster analysis of pine trees as they retained almost all the variation in the data set; [#1], because several characters from each tree were examined to cope for plant plasticity, these values are averages and for this they may not be integers; [ ], numbers included in square brackets denote ranges of arbitrary category integer units.

TABLE 3. Monoterpenes detected and incorporated in this study. Chemotype (CA, CB, CC, CD) average composition and the rank of the respective monoterpenes according to the sum of squares weighting criterion is also given. For highly ranked monoterpenes, the actual sum of squares is also given in parentheses

n	Monoterpene	CA	CB	CC	CD	Rank (SS)
1	Thujene		0.07		0.08	14
2	α-pinene	72.54	35.25	14.60	21.90	2 (285.48)
3	Camphene	2.67	1.58	0.24	1.17	14
4	Sabinene	0.03	0.51		0.26	13
5	β-pinene	7.91	3.45	10.12	57.32	1 (783.13)
6	Myrcene	1.93	4.14	43.38	2.73	4 (87.86)
7	α-phellandrene	0.10	0.16	0.00	0.08	16
8	3-carene	4.50	17.93	3.63	0.50	5 (4.89)
9	α-terpinene	0.18	0.44	1.18	0.11	12
10	Limonene	2.71	20.33	2.33	3.54	7 (3.16)
10	Ocimene	1.92	5.85	1.02	2.24	8 (2.01)
11	γ-terpinene	0.21	0.47	2.07	0.28	16
13	Terpinolene	0.97	3.89	14.11	1.37	11
14	Linalool	1.44	2.45	0.30	0.46	13
15	Fenchol	0.08	0.20	0.00	0.18	15
16	Borneol	0.02	0.06	0.00	0.13	15
17	4-terpineol	0.10	0.41	4.52	0.39	6 (3.21)
18	α-terpineol	1.55	1.29	0.42	4.37	3 (146.28)
19	linalyl acetate	0.00	0.00	0.00	0.31	15
20	methyl thymyl ether	0.00	0.00	0.82	0.00	15
21	fenchyl acetate	0.31	0.59	0.18	0.52	15
22	terpenyl acetate	0.00	0.06	0.00	1.86	9 (1.54)
23	neryl acetate	0.31	0.96	0.66	0.06	15
24	geranyl acetate	0.47	0.39	0.30	0.47	10 (0.76)

study, because these pine species are dormant by the end of the summer and dormant trees provide leaves with reproducible monoterpene profiles (von Rudloff, 1975). The morphological characters used are shown in Table 2. For chemical analysis each sample was sorted according to the phenological scheme proposed elsewhere (Petrakis, 1991). In this way needles were sorted into three age classes (f: newly emerged needles, g: mature needles, h: senescent needles). Needles were cut into 3–5mm pieces and they were steam-distilled, without their brachyblast sheaths, for about 2h. The length of the piece of the needle to be steam-distilled was a compromise between the facilitation of the removal of the terpenes from the resin canals of the needle (von Rudloff, 1975) and the avoidance of losing the more volatile constituents. The essential oils obtained were subjected to Gas Chromatography and Mass Spectrometry analyses on a Varian and a Hewlett-Packard 8590-VG Trio 1000 systems. GC analyses were performed on DB-1 and DB-5 capillary columns, and the quantification of the constituents was based on the peak areas integration (Roussis *et al.*, 1995). The secondary metabolites were identified by comparison of their retention times and mass spectra with those of authentic data and the NIST/NBS and Wiley libraries. Although several sesquiterpenes and diterpenes were identified in this way (48 compounds in all) only the 24 monoterpenes are used for the purpose of this analysis. Several attempts to use the various phenological phases of the needles as operational taxonomic units, showed that the differences in monoterpene composition were well below the variability observed between trees. For this, in subsequent comparisons all needle ages are pooled.

RESULTS AND DISCUSSION

Morphological variability

The pattern depicted in Fig. 3(a) through the application of a variance-minimizing polythetic centroid algorithm shows three levels of recognized groups. The higher level includes four main morphotypes indicated as MA (corresponds to typical *P. brutia*), MB (hybrid phenotype morphologically related to *P. brutia*), MC (hybrid phenotype morphologically closer to *P. halepensis*), MD (corresponds to typical *P. halepensis*). At the intermediate level, two subgroups within each morphotype were recognized as subtypes (Fig. 3a,b) because there are detectable and significant differences between them, according to Tukey's Honestly Significant Difference test (Zar, 1994). The recognition of other groups below the subgroup level stopped at thirteen basic types that cannot be split further on the basis of the morphological and monoterpene properties selected in this study. They consist of trees with very similar morphological and monoterpene constitution. These basic types, denoted by capital letters, are very basic units of morphological and chemical variation and in all dendrograms they form compact top branches (amalgamation distances less than 0.01 in the morphological space and less than 0.1 in the monoterpene space). The complete congruence between the morphological and monoterpene basic types indicates the existence of genetically very similar trees corresponding to what Linhart *et al.* (1989) have named 'family groups'.

Morphotypes MA and MD match the identification key presented in *Flora Europaea* (Gaussen *et al.*, 1964) corresponding to *P. brutia* and *P. halepensis*, although their subtypes MA2 and MD1 fit very poorly the range of variability of various taxonomic characters such as the length of the needle, the length and curvature of the peduncle, and the number of hypodermal cells. The other two hybrid morphotypes (MB and MC) diverge from this identification scheme on the basis of several isolated characters or, more importantly, combinations of them. The morphological variability is higher in the *brutia*-morphotype MA that geographically is restricted to Elefsis. The *halepensis*-morphotype is widespread except Elefsis and Kaesariani. Two sites, on Mt Parnis and at Schinias coast, are exclusively dominated by this particular morphotype while 'Demokritos' wood (north-eastern slope of Mt Hymettus) is occupied by this morphotype and the codominant morphotype MB.

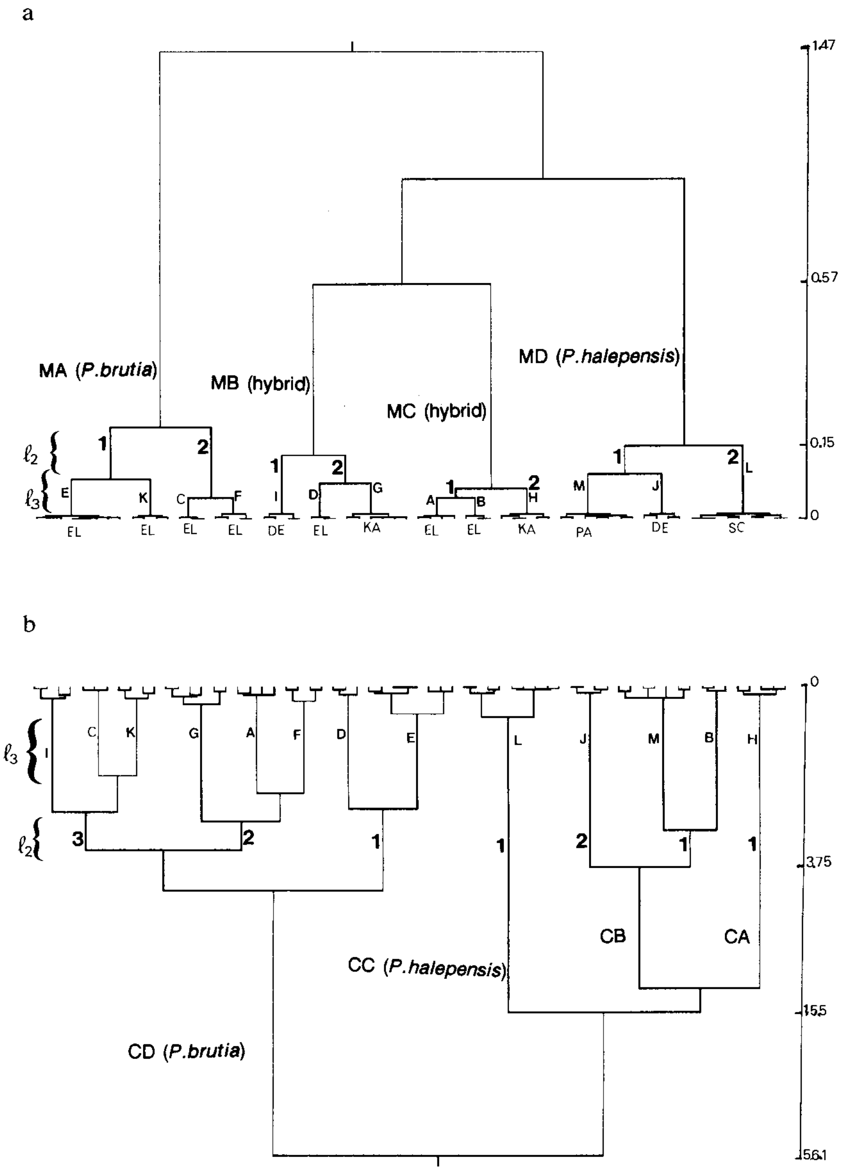

FIG. 3. Dendrograms showing the hierarchical classification of the thirteen basic types indicated by single capital letters at the hierarchical level l_3, (a) in a morphological variable space, and (b) in a monoterpene variable space. Sites are represented by the first two letters EL, Elefsis; KA, Kaesariani; DE, Demokritos wood; PA, Mt Parnis; SC, Schinias. The numbers indicate the subtypes at the hierarchical level l_2. MA, MB, MC and MD indicate morphotypes and CA, CB, CC and CD indicate chemotypes; their taxonomic affiliation, if any, is given in parentheses.

The site at Kaesariani (western slope of Mt Hymettus) is covered by hybrids belonging either to MB or MC morphotype.

Not all morphotypes are equally homogenous. The *brutia*-type MA is presented as the morphologically most diverse, followed by the *halepensis*-type MD. The intermediate types MB and MC seem to be more homogenous than those morphologically closer to putative parental typified taxa. This is in contrast to the findings of other studies of hybridization, where the variability in hybrid populations is higher than in the population of parental taxa. For example Rushton (1978, 1979), by using principal component analysis to study the relative position of hybrids and their putative parental oak species *Quercus petraea* and *Q. robur*, found that two principal axes accounted for 63.4% of the total morphological variation. In his scatter-plot diagram the range of variation of hybrids, measured as the product of the ranges of the points on both principal axes weighted by the variation accounted for by each axis, exceeds the amount of variation of the typified parental taxa. It is obvious that such comparisons are strongly affected by the typification of the parental taxa, which is highly subjective. However, whatever the range of variability in the original description of the taxon, the classification presented in Fig. 3(a) shows a continuum of variation between *P. brutia* and *P. halepensis*. Because cluster analysis is not suitable in representing continuous variation, we analysed the same data set by applying multidimensional scaling (Kruskal, 1964a,b) of the unit complement of gamma coefficients to obtain a reduced dimensionality presentation of the thirteen basic types of trees.

The scatter-plot in Fig. 4(a) represents the relationship between ‘basic types’ but the points correspond to individual trees. Only ‘basic types’ are labelled and many overlapping trees are removed to facilitate inspection. This three-dimensional configuration retains the entire amount of variation ($R^2 = 0.990$) in the original variable space (27 morphological variables). The salient feature of the morphological continuum is the parabolic nature of the hybridization axis. Hybrids depart from the straight line connecting the edge morphotypes MA and MD. The parabolic hybridization axis has no prominent direction to any of the three major axes of morphological variation. This indicates that hybrid types are not merely intermediate phenotypes but they are differentiated from their parental phenotypes on many characters. Not all locations on the parabola were found to be occupied by phenotypes and the arrangement is prominently discontinuous. Moreover, the ‘basic types’ are arranged at the top of the dendrogram in Fig. 3(a) in the same way as they are spread over the hybridization parabola.

The implications of the structure revealed in Fig. 4(a) are many. Firstly, it seems that despite the considerable variability around parental taxa the pattern of the morphological variation is anisotropic, i.e. hybrid phenotypes are not equally diverse in morphology. This may be well the result of intense natural selection on hybrids that eliminates intermediate phenotypes. Indeed, some morphological correlates were found in oviposition preference of the pine processionary moth (*Thaumetopoea pityocampa* Den. & Schiff), such as the number of resin canals in the needle and the

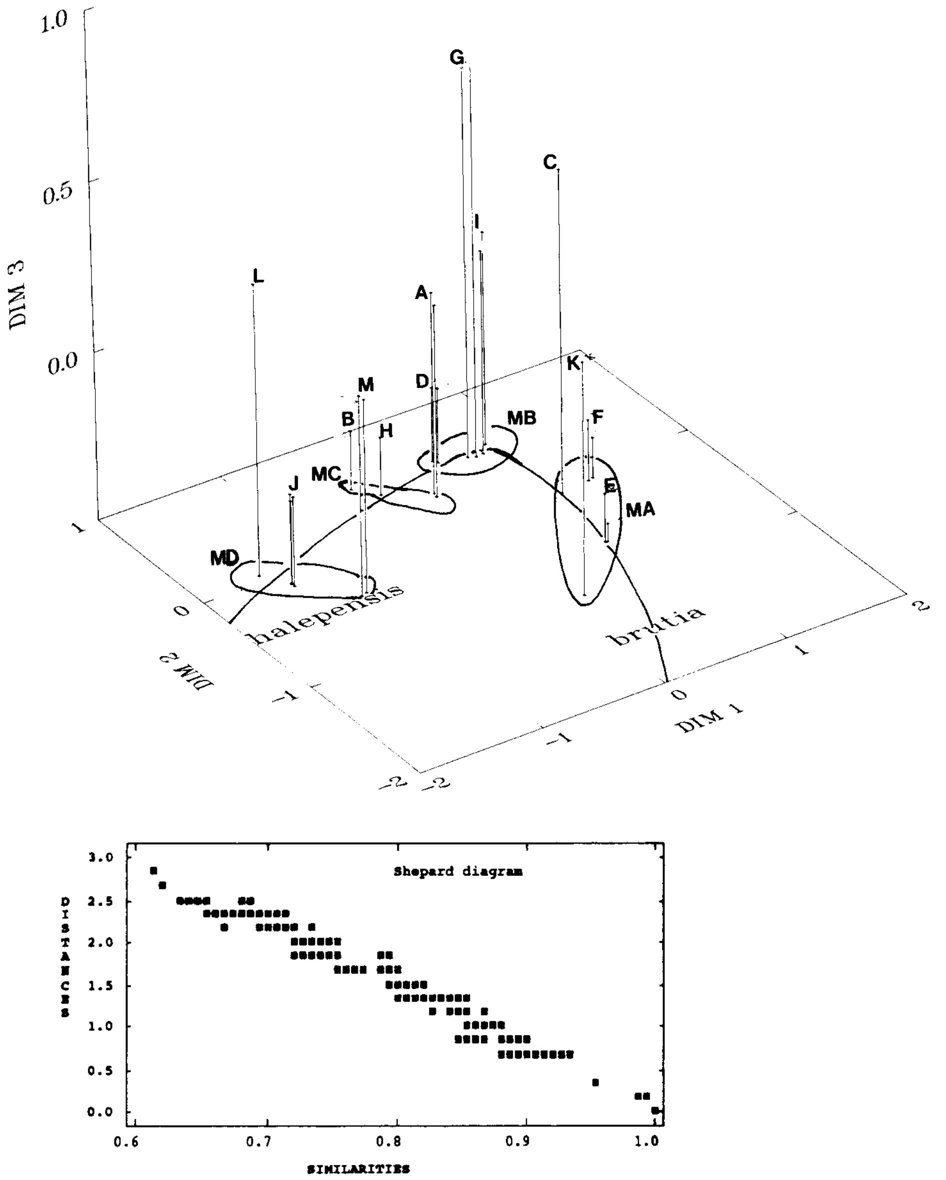

FIG. 4. (a) Multidimensional scaling three-dimensional presentation of pine trees in a morphological space. Trees are indicated by their basic type capital symbol (as in Fig. 3). Several trees are tied in one point while others are removed to facilitate inspection. Free-hand drawn group lines are morphotypes and the parabola connects morphotype centroids in reduced space. The Shepard diagram indicates the relationship between distances of trees in the original space (27 dimensions) and their Spearman similarity coefficient in the reduced space (3 dimensions). The linear form of the Shepard diagram indicates that three axes are enough to represent the variability exhibited in 27 axes ($R^2 = 0.990$).

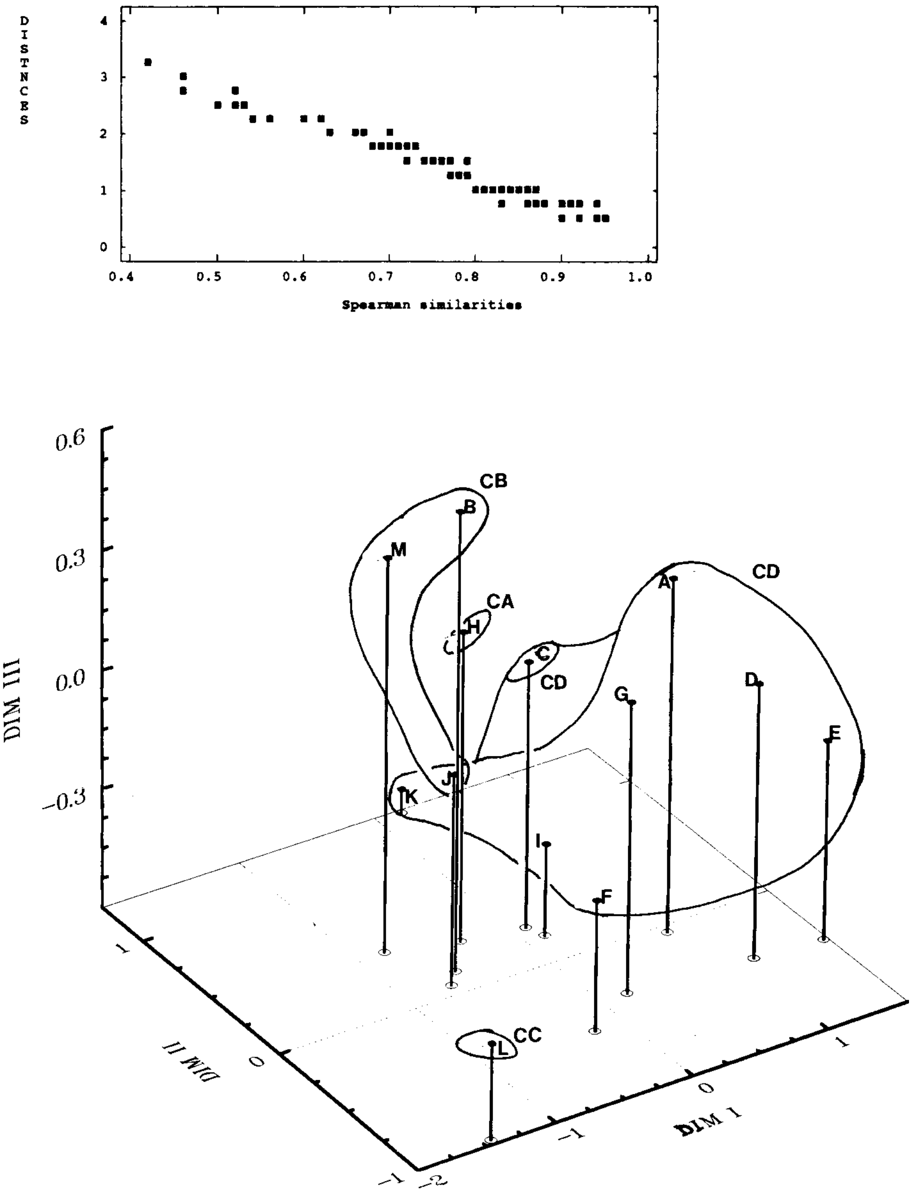

FIG. 4. (b) Multidimensional scaling three-dimensional presentation of pine basic types in a monoterpene space. The Shepard diagram is the same as in Fig. 4(a), and here $R^2 = 0.989$. Free-hand group lines are chemotypes and associated subtypes.

needle width. Other characters or character combinations were found also to be associated with herbivory by *T. pityocampa* larvae and *Grypotes staurus* (*Hemiptera*: *Cicadellidae*), and small mammals feeding on cones (*Apodemus sylvaticus* and *Rattus norvegicus*) tend to prefer either *P. brutia* or the syntopic *P. halepensis* in different locations (unpublished observation). If we generalize the concept of the 'adaptive landscape' (Barton, 1988) from one allele frequency to the notion of the morphotype, which can serve as the polygenic analogue of the single allele frequency, it seems that the detected morphotypes are adaptive peaks corresponding to specific advantageous character combinations possessed by hybrid types. Although our approach does not directly permit an assessment of the degree of reproductive isolation between morphotypes, their geographic distribution shows that it is not zero.

Second, it seems that the situation of the two pines in Attiki is the outcome of a series of introgressive contacts of *P. brutia* with *P. halepensis*, because the geographic corridors (Figs 1 and 2) through which these contacts could be done, had already existed. The hybridization pattern evidenced in this study, with well-defined hybrid morphotypes (MB and MC) in the current interglacial, implies that the introgression of the germplasm of one pine species to another related but previously isolated one, creates new genotypes, some of which are particularly successful. In other words, there are some points on the hybridization axis where the hybrid phenotypes seem to be better adapted to biotic or abiotic factors. In a subsequent geographic isolation of these hybrid types, the geographic pattern can be evidenced as a dichotomous – or possibly polytomous – speciation event. Millar's (1993) Eocene refugia hypothesis describes such speciation events seen through the coarse scale of several (c.64) million years. Pliocene-Quaternary in Attiki exemplifies a hybridization situation between two pine species, where the refugia are not created in glacial maxima but in interglacial periods because of the uprising of the sea level.

Third, these central Aegean pines seem to diverge from Runemark's (1971) view that the evolution in the Aegean is almost at a standstill and the floristically rich mid-Tertiary central Aegean land-mass is now represented by small islands with relict endemics outnumbering newly evolved species. Indeed, *Pinus* is not a member of the present Cycladean flora (Rechinger, 1951). The reasons may not be entirely anthropogenic, but the fragmentation of the mid-Tertiary central Aegean land-mass created disjunctions in the ranges of several organisms that now are seen to have diverged at subspecific levels (Pieper, 1971). Pines seem to belong to this group of organisms, which were severely affected by the fragmentation that followed the sea uprising (in the previous interglacial the sea level was 90m higher than the present one – Pirazzoli, 1996), and they became extinct from Cyclades. The contribution of humans to such extinctions is not easily estimated, and the conventional anthropogenic plant indicators are also members of the natural vegetation (Jahns, 1993). Various authors (Beug, 1975; Bottema, 1975; Pignatti, 1983; Allen, 1990, Quezel *et al.*, 1990, Reille, 1992; Jahns, 1993) have correlated the emergence of *Pinus* stage in the ecological succession to human influence, although the role of other processes, such as the species-area effect, cannot be excluded (see for example Greuter, 1975). Volcanism also may have contributed to the extinctions. Volcanic eruptions have been invoked as a causal agent of many extinctions, especially of herbaceous plants occupying cliff terraces (see in Strid, 1970). However, their importance in explaining pine extinctions is hampered by anthropogenically caused depauperation of local floras. It can be speculated that the empty parts of the hybridization parabola (Fig. 4a) correspond to phenotypes dominating extinct pine populations such as those of the Cyclades island complex. Morphological data collected from herbarium specimens of *P. brutia* from the east Aegean islands (P. V. Petrakis, unpublished) showed that in remote areas on some islands (e.g. Ikaria, Lesbos, Samos and Crete) there are morphotypes that fall at the far end of the right tail (i.e. the *brutia* arm MA) of the hybridization parabola in Fig. 4(a). The lack of this morphotype from Attiki and Cyclades denotes that local extinctions may be responsible for the gaps in the hybridization morphological continuum.

Monoterpenoid variability

In Fig. 3(b) the hierarchical arrangement of the thirteen ‘basic types’ is shown on the basis of their composition in 24 monoterpenes. Table 3 shows the mean composition of the recognized chemotypes (CA, CB, CC, CD). Monoterpene and morphological subtypes (hierarchical level l_2 in Fig. 3a,b) are considered only as an intermediate level of clustering that facilitates the search of the degree of congruence between the two classifications (Table 4a,b). As in morphometric classification the same thirteen ‘basic types’ (hierarchical level l_3 in Fig. 3a,b) were recognized that include trees very similar in monoterpene composition. The distance between individual trees in the same ‘basic type’ is less than 5% of the amalgamation distance of subtypes. This level of similarity renders ‘basic types’ suitable operational taxonomic (in the mathematical sense) units. Individual trees of ‘basic types’ are coming with no exception from the same site, although a site may contain several such ‘basic types’. Among the study sites only those at Mt Parnis and Schinias coast are monotypic in terms of ‘basic types’.

Chemotypes do not perfectly match morphological categories. Chemotype CD includes all *P. brutia* trees of morphotype MA and the hybrid *brutia*-like morphotype MB together with one ‘basic type’ from the *halepensis*-like morphotype MC (Fig. 3a,b). Chemotypes CC and CA comprise only one ‘basic type’. The first is included in the *P. halepensis* morphotype and the latter in the hybrid *halepensis*-like morphotype MC. Chemotype CB includes typical ‘basic types’ of *P. halepensis* as well as hybrid ones.

Chemotype CA is exclusively found in Kaesariani, and in the sample it comprises nine trees. Morphologically these trees fall in the hybrid category MB and their monoterpene composition is characterized by the highest proportion of α-pinene (Table 3), twice as much as that of chemotype CB. Unfortunately the usual presentation of results in the literature, as percentage composition of various monoterpenes, does not allow for direct comparisons, because percentages are affected not only by the relative amounts of various compounds but also by their number. Nevertheless, CA is closer to *P. halepensis* chemotypes as presented by Schiller & Grunwald (1987a) with respect to α-pinene, but deviates from all other chemotypes by them and Mirov *et al.* (1966), Iconomou *et al.* (1964) and Zafra & Garcia-Peregrin (1976) with

TABLE 4. Two-way frequency table, showing the relationship between morphotypes and chemotypes (a), and their subtypes (b). Cells are percentage proportions of trees with respect to the total number of trees examined, chemotypes are rearranged to indicate the diagonal structure of the table

(a)

	Morphotypes			
Chemotypes	MA (*brutia*)	MB	MC	MD (*halepensis*)
CD	29.51	21.31	6.56	
CA			7.38	
CB			5.74	17.21
CC				12.30

(b)

	Morphological subtypes							
Chemotaxonomic subtypes	MA1	MA2	MB2	MB1	MC2	MC1	MD1	MD2
CD1	11.48		5.74					
CD2		6.56	9.02			6.56		
CD3	5.74	5.74		6.56				
CA					7.38			
CB1						5.74	10.66	
CB2							6.56	
CC								12.30

respect to β-pinene, myrcene and 3-carene. Within our data set the monoterpenes having the highest weight in discriminating phytochemically similar groups are β-pinene, α-pinene, α-terpineol and myrcene (Table 3), followed by 3-carene, 4-terpineol, limonene, ocimene and terpenyl acetate. The discrepancies in the amounts of monoterpenes can be attributed to the plant part that is used as source of the essential oil and the sample-collection procedures. The work of Zafra & Garcia-Peregrin (1976) and Dmytriyev *et al.* (1989), who used needles and twigs as an essential oil source, being exempted, all the other workers (Iconomou *et al.*, 1964; Mirov *et al.,* 1966; Schiller & Grunwald, 1987a,b) have used cortex or xylem resin. This makes our study not directly comparable with several other studies.

According to Croteau's (1987) unifying proposal, not more than 50 enzymes, mainly cyclases, participate into the cyclization biosynthetic routes of monoterpenes (see also Waterman & Mole, 1989), while secondary transformations of the basic monoterpenes to hydroxylated and oxygenated forms involve usually one or two enzymes. No localization of these enzymes has been reported in specific plant parts. The basic tissue responsible for their production in conifers is the needle resin canal epithelium (Bell & Woodcock, 1975). For this, it is expected that the basic monoterpenoid profile of an individual tree can be obtained by using eventually any resin-rich tissue source. In conifers, no surface localization has been determined as in other spermatophytes bearing trichomes or vacuolated terpene-rich upper epidermis cells (Harborne, 1988) and we coped with phenomena related to branch autonomy by randomizing samples over crowns. According to the 'source-sink transportation' hypothesis (Kramer & Kozlowski, 1979), photosynthates and metabolites are transferred from the source to the stronger sinks irrespective their proximity to the source tissue. In the proposed scheme, leaves and twigs are higher in the hierarchy of sink strengths than wood, roots, cambium and storage tissues. Our preliminary data showed that average needle monoterpenes, although varying between leaf ages and given that no severe attack by herbivores occurred, retain the very basic relative proportion in the needle essential oil. We found variations well inside the range of 5% and usually below 2%, which is compatible to the findings of Zafra & Garcia-Peregrin (1976) for Spanish *P. halepensis* populations. These variations were also well inside the limits set by von Rudloff (1975) for reproducible results of chemosystematic value.

The chemotype CD corresponds broadly to *P. brutia* chemotypes (Schiller & Grunwald, 1987b), widespread in circum-Mediterranean areas, except for the low myrcene content. Because myrcene is believed to be among the differentially occurring monoterpenes in the *brutia–halepensis* species group together with 3-carene (Schiller & Grunwald, 1987a; Dmytriyev *et al.*, 1989), this chemotype expectedly includes *P. brutia* and *P. brutia* × *halepensis* hybrids. In Table 4(a) it can be seen that CD consists of MA and MB morphotypes. Hybrids morphologically closer to *P. halepensis* (i.e. MC) are equidominated by the CD, CA and CB chemotypes. Geographically it predominates in the southern and western sites. In all these sites it is accompanied by one of the other chemotypes, except CC, which is exclusively found in Schinias where it is the sole occupant (Table 1). South-western sites presumably received directly the effects of sea uprising and were recolonized several times by plants. It was found (van Andel & Shackleton, 1982) that in the last glacial maximum, these lowlands were part of a lake (Fig. 2).

The chemotype CC, which corresponds to the morphologically typical *P. halepensis*, seems to be quite common in Crimea (Dmytriyev *et al.*, 1989), Turkey and Israel (Schiller & Grunwald, 1987b). The existence of many different chemotypes in the same province implies differential introgressive influence of various sites from the eastwards-originated *P. brutia*.

The CB chemotype grows in sites that have received the least influence in the Plio-Pleistocene period as a result of the higher altitude and the distance from the sea. It is known that the most susceptible vegetation types to the Plio-Pleistocene tectonic and eustatic events are the littoral and the phryganic (i.e. low xerophytic bushes of lowlands) ones (Runemark, 1969). Typically it comprises *P. halepensis* and the morphologically similar hybrids. The monoterpenoid profile of this chemotype is quite close to that reported for Granada (Spain) *P. halepensis* chemotypes (Zafra & Garcia-Peregrin, 1976). This proximity is intensified by the remarkable similarity in the amount of sabinene found in some trees of 'Demokritos' site and in pines from Granada. It seems that ancient populations of *P. halepensis* contained higher proportions of sabinene but they have diverged on a west–east Mediterranean gradient of decreasing proportions of these monoterpenes. The waves of introgressive influence of *P. brutia* may have substantially contributed to this differentiation. Unfortunately, other data sets with range-wide coverage of *P. halepensis* have not recognized sabinene as a constituent of the cortex or xylem oleoresin (e.g. Schiller & Grunwald, 1987a).

At this point, the importance of human introductions of plant genetic material has to be considered. Human-related introduction of Moroccan populations to Adana, Turkey, although established and naturalized, did not introgress into syntopic local *P. brutia* populations. Schiller *et al.* (1986) did not find specific marker genes characterizing the east Mediterranean group of *P. halepensis*.

In contrast to the hypothesis of Nahal (1962), supported by Bucci *et al.* (1998) on the basis of paternally inherited simple-sequence repeat markers, that *P. halepensis* expanded in the last 10 Kyr through establishment by migration of a small number of individuals, human-induced introduction did not result in genomic mixing with local races or other species within the halepensis complex. A proximate cause is the time scale, given the low evolution rate of conifers. Literature data (Beug, 1975; Bottema, 1975; Reille, 1992) suggest that pines, wherever they emerge in a Pleistocene successional phase, replace deciduous oak and steppe in a cyclic pattern alternating between wet and dry periods. Pines emerge only recently – the last 2 Kyr – in pollen diagrams, such as the case of pinewoods on Corsica (Reille, 1992).

On the other hand, detailed reviews and archaeo-ecological investigations in the study area – Attica and Boeotia (Hughes, 1983; Rackham, 1983; Sallares, 1991), revealed that pine forests were not genetically disturbed or severely destroyed by early inhabitants of the area, i.e. in neolithic and classical times. Pine trees, as a rule, were not planted for afforestation purposes. Pine stands merely provided materials for temporary constructions (weapons and shelters) in Pleistocene mobile economies (Coles & Higgs, 1975). In addition, wood-cutting was restricted in gentle mountain slopes leaving undisturbed the rocky steep slopes from which natural afforestation resumed. Shipbuilding was based on wood stock cut from special places such as lowland wind-protected forests (Hughes, 1983). For the archaic and ancient inhabitants the forests were sacred places protected by gods where only 'temene' and 'templa' are allowed. The common warfare practice of forest-burning is related to this religious feeling rather than the deprivation of the enemy of valuable materials for weapon construction. However, these activities were never as severe as other catastrophic events, such as natural fires (Hughes, 1983; Sallares, 1991; Trabaud, 1991). Human-induced afforestation is only very recently practised in Greece (in the last 50 years) while treelets are always grown from the local seed stock (Dr N. Eustathioides, Directorate of Natural Parks and Game Reserves, personal communication). In conclusion, human influence on pinewood genetic composition seems to be unimportant. Deforestation is the prevailing human impact and local pinewoods in prehistorical and historical times, as a rule, regenerate from local seed banks.

There are a number of monoterpenes, reported for first time from the needles *P. brutia* and *P. halepensis*, in this study. Among them α-terpineol is proved to be the third in rank influential monoterpene on the phenogram shown in Fig. 3(b). It is proportionally highest in CD1 and CD2 subtypes that comprise pines found in south-western sites. The possibility of this to be a steam-distillation artefact (von Rudloff, 1975) is negligible because it was found in foliage volatile blend solvent extracted from Tenax TA-70 trap. Possibly it has been derived from the confamilial γ-terpinene, by hydroxylation (Croteau, 1987), with which it is negatively correlated. In this way it represents a novel character that appears in Attiki. Both, γ-terpinene and α-terpineol are reciprocally replaced in hybrid and 'parental' chemotypes (Table 3). The same holds for 4-terpineol, which attains the highest percentage in the CC chemotype and ocimene, which is highest in the hybrid chemotype CB grown on Mt Parnis, 'Demokritos' wood and in the '*halepensis*' morphotype (i.e. MD) in Elefsis. These monoterpenes seem to be immobile within needles because no sink exists for them in other plant organs. Their role, if any, is localized in needles. This may be the reason why these terpenoids have not been found in the monoterpenoid profile of other plant parts. Indeed, in steam-distillates of gum terpentine of the same trees, we found α-terpineol, γ-terpinene, ocimene and 4-terpineol in very low quantities, much lower than those of the foliage.

On the other hand, myrcene is negatively correlated to the confamilial compound ocimene. Myrcene is the major monoterpene constituent of the CC chemotype in the Schinias coast and its percentage is only comparable with – possibly introduced – Crimean *P. halepensis* (Dmytriyev *et al.*, 1989). Myrcene seems to be an ancestral character of *P. halepensis*, which is replaced but does not disappear from hybrid chemotypes. It is a salient feature of the reduced dimensionality presentation (Fig. 4b) of the 'basic-types' that hybrid chemotypes are not intermediate combinations of parental chemotypes in terms of their overall monoterpene constitution, although in terms of individual monoterpenes this may not hold.

The high congruence between morphological and monoterpene classification is caused by the entire monoterpenoid profile. The coefficient of contingency (Wilkinson, 1990) between morphological and monoterpene types was high (0.74, $P<0.005$). In addition, the high number of monoterpenes does not disturb the reduced dimensionality presentation of the basic types (Fig. 4b). Almost the entire variation in the data set (98.9%) can be accommodated by three dimensions. This implies that three major trends exist in the monoterpenoid variability in this data set. However, these trends are associated with many monoterpenes and no simple correlational structure seems to exist. The composite nature of major trends in monoterpenoid variability supports the importance of monoterpenes in pine chemotaxonomy although they cannot be routinely treated – at present – as characters because of the difficulty in discriminating character states. Monoterpenes are able to indicate thin partitioning of natural groups in complex data sets such as *P. brutia*, *P. halepensis* and their hybrids in Attiki. A two-way table analysis of the congruence between morphological and monoterpene subtypes (Table 4) produced a better coefficient of contingency (0.89, $P<0.005$). The fact that the coefficient of contingency increases with increasing resolution in both phenograms indicates that they are equally important in pine classification and biogeography. Interestingly, the interaction of subtypes with sites is the same as that of major types (0.87 and 0.86, $P<0.001$). Evidently, in this group of pines, introgressive hybridization was not accompanied by elimination of genes responsible for morphological differences. Gene elimination has been previously invoked, for the explanation of the observed hybridization patterns in some Nearctic pines (Zavarin *et al.*, 1989).

Evolution of pines in the Aegean – conclusion

Our data demonstrated that the genetic heterogeneity of pine populations, interpreted either as morphological or as monoterpenoid variability, can be high even in a very small geographic scale. Besides this heterogeneity, family groups of genetically related individuals (sensu Linhart *et al.*, 1981) are found at two consecutive scales of genetic complexity, namely the morphology and the monoterpene constitution. In this study family groups were treated as 'basic types', which were possibly produced from one or a few individuals having a common ancestry, by short-range seed dispersal (Lepart & Debussche, 1991). This is in striking agreement with the hypothesis of Raven (1973) on the mode of evolution in Mediterranean type areas (Petrakis & Roussis, 1997). Raven stated that the exploitation of the mosaic nature of the Mediterranean environment by woody plants, which are modally out-crossing, demands for high variability, while they have to maintain their genetic contact to the population. The various morphotypes and chemotypes of pines that occur in sympatry is a manifestation of the coexistence of high variability with the close genetic affinity.

The types of pines in the *brutia–halepensis* complex, which were found in Attiki, are related to other, within this complex, pines from several Aegean populations. The Plio-Pleistocene physiography of the Aegean Sea (Fig. 2), together with the Pleistocene sea transgressions and tectonic events (Fig. 1), permitted numerous waves of contact between these two pine species. In Attiki, areas more susceptible to sea-level uprising were found to comprise chemotypes and associated morphotypes more similar to east Mediterranean ones. The Cycladean pine gap, together with the present sea uprising, is evidence of a typical interglacial isolation of mainland Greek and eastern populations. This isolation is believed to have shaped the present littoral and lowland Aegean flora and vegetation (Runemark, 1969). In this isolation the introgressive influence of the oriental *P. brutia* into *P. halepensis* formed a taxonomic complex of which only extreme types are recognized in conventional identification keys (Gaussen *et al.*, 1964), while the rest are not in general intermediate – in a linear sense – forms located on a linear hybridization axis. The majority of putative hybrids are arranged on a paraboloid hybridization axis. The departure of hybrids from a linear arrangement between the putative parental species may be partly a result of the lack of gene elimination from introgressive influences from other extinct (in the Pliocene) pine species such as *P. canariensis* Smith (Klaus, 1989). In a phenetic similarity context, by using monoterpene-based characters, we found that syntopic populations of *P. brutia*, *P. halepensis* and *P. canariensis* showed greater similarity between the last two species while *P. brutia* was clearly separated (Roussis *et al.*, 1995). At the same time, other pine species, such as *P. pinea* L., are now occurring on sandy, well-drained soils in isolated declining populations. They are certainly affected by anthropogenic factors but they are also replaced by the more opportunistic and pioneer forms of the *brutia–halepensis* group; *P. halepensis*, and to a lesser degree *P. brutia*, are well-known post-disturbance and bare-ground invaders (Lepart & Debussche, 1991) and this ability is probably retained in their hybrids. Schinias coastal wood exemplifies this phenomenon and this site appears to be monotypic in terms of chemotypes probably occupied by the competitively superior hybrids. Importantly, we observed no attack of common pine pests on this chemotype dominating Schinias pinewood.

In interglacial warm stages, when island populations of pines are separated, because of the rising of the sea level, it seems that ecological processes such as competitive replacement and selective herbivory (Petrakis *et al.*, 1994) act synergistically towards the local extinction of susceptible phenotypes (Petrakis *et al.*, 1999). Extinction is more a probability in fragmented ranges, because of stochastic factors such as reproductive drift (Runemark, 1969, 1971), and the Aegean islands are too small geographic fragments to support viable tree populations (Bennett *et al.*, 1991). Only a subset of the phenotypes, which has been created in a glacial contact, is able to persist in the following sea uprising and the isolation, which is accompanied by additional range contraction as many lowland ranges are flooded. What phenotypes are more likely to survive the interglacials depends on the prevailing selective forces in the generated ecological scenery. The data of this study are not suitable to test this last argument because our samples came from intact trees to avoid plant-induced responses to herbivore damage. Substantially augmented data sets are needed from several places in the Aegean from damaged and intact pine trees, which constitutes our future line of research (Roussis *et al.*, 1995).

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