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Genetic variation of *Marchalina hellenica* (Hemiptera: Margarodidae) sampled from different hosts and localities in Greece

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Abstract

Random amplified polymorphic DNA (RAPD) analysis was applied to 120 individuals of *Marchalina hellenica* (Gennadius) representing six populations collected in northern, central and southern mainland Greece. One population was sampled on one species of fir tree and the others on two species of pine trees. Four random decamer primers were used to evaluate genetic variation among the populations examined. The results revealed intra- and interpopulation polymorphism both related to host type and region of origin. Phylogenetic analysis based on genetic distances estimated by the RAPD frequencies revealed an important genetic differentiation in samples collected on fir trees in southern Greece and to a lesser extent in samples from pine trees in central and northern Greece. Furthermore, considerable subdivision and restricted gene flow among the populations examined were observed. The results are discussed in relation to the biology and geographical distribution of *M. hellenica* in Greece.

Introduction

Marchalina hellenica (Gennadius) (Hemiptera: Margarodidae) is an important insect in honey production. The honeydew produced by *M. hellenica* constitutes an important source of food for foraging honeybees, *Apis mellifera* Linnaeus (Hymenoptera: Apidae). Approximately 65% of the annual production of honey in Greece is derived from honeydew (Santas, 1983; Thrasyvoulou & Manikis, 1995; Bacandritsos, 1998; 2002). *Marchalina hellenica* is a sap-sucking insect, which lives in the cracks and under the scales of the bark of pine trees, concealed under large quantities of a white cotton-like wax that it secretes. It has one generation per year. The adult female does not feed, lives for a short time and lay its eggs in the folds of pine-tree bark and underneath the mass of the cottonish white secretions during March to May (Nikolopoulos, 1964, 1965; Fimiani & Sollino, 1994; Erlinghagen, 2001). According to Nikolopoulos (1965) and Erlinghagen (2001) the females of *M. hellenica* have two nymphal instars and the species overwinters in the second instar which lasts from October to March or April.

It is considered that *M. hellenica* reproduces parthenogenetically since males are rare (Sureyya & Hovasse, 1930; Kailidis, 1991; Erlinghagen, 2001). In Greece, Nikolopoulos (1964) has found and described the male larvae and the winged adult male. Recently, Erlinghagen (2001) has also found male larvae in southern Greece. The species is found in the eastern Mediterranean and particularly in mainland Greece, in Aegean and eastern Mediterranean islands, in Turkey (Kailidis, 1965; Nikolopoulos, 1965; Santas, 1983; Tremblay, 1995), as well as on the island of Ischia in Italy (Fimiani & Sollino, 1994; Pollini, 1998). In Greece, the species lives on various pine species, mainly *Pinus brutia* Tenore and

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Pinus halepensis Miller and to a smaller extent *Pinus sylvestris* L. and *Pinus nigra* Arnold (all Pinaceae), which grow in almost all areas and altitudes ranging from sea level to about 1000 m. An interesting finding is that *M. hellenica* is able to capture new hosts. This has been illustrated by artificial infestation of fir-trees, *Abies cephalonica* Loudon (Pinaceae). Fir trees on the Menalo (altitude 1200 m) and Helmos (altitude 2200 m) mountains in Peloponnese, southern Greece were successfully infested with samples of *M. hellenica* that originated from pines during the years 1995–1997. The insects were established successfully on fir trees and relatively high populations were recorded each year (Bacandritsos, 2002).

Given that *M. hellenica* is an insect of primary economic importance in honey production, many studies on its biology have been conducted during the past decades. However, the genetic affinities of the species are still unknown. The present study was aimed at examining the genetic variation among populations of the insect. Particularly, the random amplified polymorphic DNA–polymerase chain reaction (RAPD–PCR) method was applied to samples of *M. hellenica* originating from pine and fir trees from various regions of Greece.

Materials and methods

A total of 120 adult females representing six populations were analysed using the RAPD–PCR method. Five populations were sampled on pine trees (*P. halepensis* and *P. brutia*) and one on fir trees (*A. cephalonica*) from various localities in Greece during April to May 2001 (fig. 1). Each sample was put in a self-sealing plastic bag, which slightly inflated and contained a piece of paper towel to reduce condensation. Bags were placed in plastic containers containing ice packs and transferred to the laboratory. Each sample was stored in Eppendorf tubes at –23°C until analysis. DNA was extracted according to the following method. In an Eppendorf tube one individual was crushed in 300 µl of extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS). An aliquot (150 µl) of 3 M sodium acetate, pH 5.2 was added and the tube was placed at –23°C for 20 min. Then the tubes were centrifuged at 13,800 *g* for 10 min and the supernatant was transferred to another tube. DNA was purified by successive extractions with phenol, phenol: chloroform: isoamyl alcohol (25: 24: 1; v: v: v) and chloroform: isoamyl alcohol (24: 1; v: v) and precipitated with ice-cold absolute ethanol overnight at –23°C. The precipitated DNA was pelleted by centrifugation at 13,800 *g* for 20 min, and after washing with 70% ethanol, was vacuum dried and resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

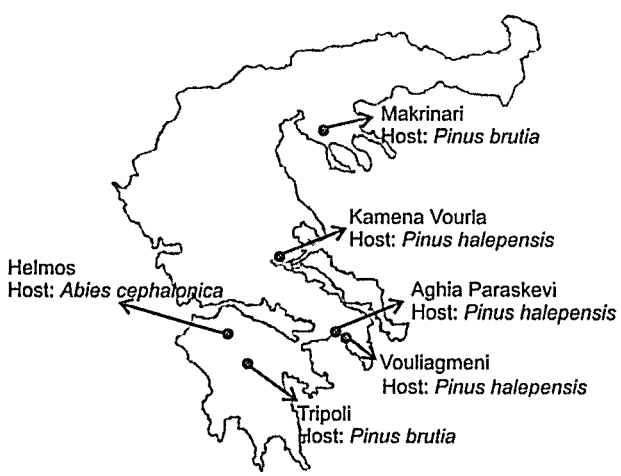

Fig. 1. Host-trees and sampling sites in mainland Greece of the six *Marchalina hellenica* populations used in the study.

Nine decamer primers were used in the study, eight of them (OPA-02, OPA-03, OPA-06, OPA-07, OPA-13, OPA-17, OPA-18, and OPA-20) were purchased from Operon Technologies (Alameda, California, USA) and one (BAM: 5'-ATGGATCCGC-3' by designed by DuTeau (Black *et al.*, 1992)) was synthesized by Minotech (Heraklion, Greece). However, only the primers OPA-03, OPA-13, OPA-20 and BAM gave well-resolved and reproducible RAPD bands with most of the samples examined. The other primers either failed to produce any band or did not give satisfactory products with most of the insects examined. Thus, only data obtained by the above mentioned primers were used in the analysis. Polymerase chain reactions were performed using the same thermocycler (Techne Cyclogene Thermalcycler) in 25 µl reaction mixture containing 100 ng of template DNA, 1 unit of Taq polymerase (Minotech, Heraklion, Greece), 0.2 mM dNTPs, 20 ng of each primer, 2.5 mM $MgCl_2$, and 1X reaction buffer (50 mM KCl, 10 mM Tris HCl, pH 8.5, and 0.1% Triton X-100) supplied by the enzyme manufacturer. Amplification conditions involved one preliminary denaturation at 94.5°C for 4 min, a total of 35 cycles of a strand denaturation at 94°C for 40 s, annealing at 39°C for 1 min, and primer extension at 72°C for 1 min, followed by final extension period of 7 min at 72°C. Then, the amplified products were screened in a 2% agarose gel containing 0.5 µl $^{-1}$ ethidium bromide in TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.0). DNA bands were visualized under UV light and were scored for their presence or absence using a binomial code (1/0). The molecular weight of each band was estimated by comparison to a co-migrating 100-bp ladder. To avoid problems related to variation in the number and intensity of amplification products (Hadrys *et al.*, 1992; Black, 1993; Williams *et al.*, 1993), band mobility comparisons were made only within gels and based on the same PCR reaction. Each PCR reaction from the same DNA extraction was replicated twice to check for reproducibility of the RAPD patterns produced. Any weak DNA pattern generated due to unsatisfactory amplification was excluded from the analysis.

The statistical analysis of RAPD data was performed according to the method proposed by Lynch & Milligan (1994). Allele frequency within each population was estimated under the assumption that variation in banding patterns represents allelic segregation at independent loci. Each locus was treated as a two allelic system corresponding to the presence or absence of the amplified band, where the 'null' allele may fail to amplify due to the loss of a primer site or because an insertion has caused the distance between primer sites to exceed the capacity of the PCR (Lynch & Milligan, 1994). Under these assumptions an asymptotically unbiased estimator of the null allele frequency is

$$\hat{q} = \hat{x}^{1/2}\left(1 - \frac{Var(\hat{x})}{8\hat{x}^2}\right)^{-1}$$

where $\hat{x}$ is the proportion of N sampled individuals that do not exhibit the marker and $Var(\hat{x}) = \hat{x}(1-\hat{x})/N$. Nei's pairwise

genetic distances (Nei, 1972) between populations, estimated heterozygosity and *Fst* values were calculated according to the method proposed by Lynch & Milligan (1994). The estimated heterozygosity were calculated taking into consideration only polymorphic bands whose frequency was less than (*1–3/N*), where *N* is the number of individuals analysed per population. The number of migrants per population and generation was estimated according to the equation: $N_em = [(1/F_{ST})-1]/4$ (Waples, 1987). Genetic distances calculated for data from all primers were used to estimate genetic relationships between the populations by means of the unweighted pair group method of analysis (UPGMA) (Sneath & Sokal, 1973). *Fst* was also estimated following the Bayesian approach proposed by Holsinger *et al.* (2002) and using the software Hickory v0.8. The estimation of *Fst* was performed using the 'f free model' which gave the lower value of deviance information criterion (Spiegelhater *et al.*, 2002).

Furthermore, possible separations among populations were examined using multivariate approaches. Thus, data of RAPD scores for the individuals that were analysed by all primers were submitted to both principal component (PCA) and multidimensional scaling analyses (MDS). Both analyses were performed using the SPSS statistical package. Principal component analysis transforms a set of correlated variables to a new set of uncorrelated variables called principal components from which the first two usually account for the most variation of the original data. Plotting the scores of the first two components for each individual is a useful way to identify clusters in the data. Multidimensional scaling analysis is an algebraic reconstruction method for finding the co-ordinates of the points, preferably in a short number of dimensions, whose interpoint distances are in agreement with the given dissimilarities (similarities) between the points (individuals). The data can easily be plotted and possible clusters could be detected. A detailed description of the nature and application of these methods can be found in Chatfield & Collins (1980).

Insect specimens of the six populations examined have also been kept in the Laboratory of Entomology and Agricultural Zoology of the University of Thessaly for further study with different molecular methods.

Results

Each random primer produced a unique banding pattern of amplified DNA. However, all the primers did not produce any diagnostic bands able to discriminate the six populations of *M. hellenica* examined. A total of 35 reproducible and well-resolved bands, ascribed to 'loci', were scored for the six populations with all primers used. The number of bands produced ranged from 6 to 11 per primer and their size from 350 to 1400 bp. The estimated allele frequencies are given in table 1. The number of

Table 1. The frequency of the null allele of the 35 random amplified polymorphic DNA (RAPD) markers scored in six *Marchalina hellenica* populations from Greece estimated by the method of Lynch & Milligan (1994).

RAPD markers*	Aghia Paraskevi	Kamena Bourla	Helmos	Makrinari	Bouliagmeni	Tripoli
$OPA03_{1300}$	0.000	0.632	0.671	0.548	0.000	0.000
$OPA03_{1150}$	0.837	0.837	0.447	0.922	0.922	1.000
$OPA03_{1100}$	0.000	0.548	0.894	0.671	0.000	0.000
$OPA03_{950}$	0.000	0.000	0.000	0.000	0.000	0.000
$OPA03_{850}$	0.000	0.000	0.224	0.316	0.000	0.000
$OPA03_{750}$	0.000	0.224	0.447	0.387	0.000	0.000
$OPA03_{650}$	0.000	0.000	0.000	0.224	0.000	0.000
$OPA03_{600}$	0.000	0.000	0.000	0.000	0.000	0.000
$OPA13_{1200}$	0.000	0.000	0.316	0.000	0.392	0.000
$OPA13_{1100}$	0.000	0.500	0.000	0.000	0.000	0.000
$OPA13_{980}$	0.000	0.000	0.316	0.343	0.392	0.000
$OPA13_{900}$	0.000	0.000	0.000	0.243	0.000	0.000
$OPA13_{800}$	0.000	0.000	0.000	0.243	0.000	0.000
$OPA13_{700}$	0.000	0.000	0.775	0.000	0.000	0.000
$OPA13_{570}$	0.000	0.408	0.000	0.343	0.000	0.000
$OPA13_{550}$	0.000	0.408	0.707	0.000	0.000	0.000
$OPA13_{490}$	0.000	0.000	0.000	0.000	0.000	0.000
$OPA13_{350}$	0.000	0.000	0.000	0.000	0.000	0.000
$OPA13_{300}$	0.000	0.000	0.000	0.000	0.000	0.000
BAM_{1400}	0.000	0.922	0.707	0.949	0.975	0.975
BAM_{1300}	0.000	0.922	0.775	0.806	0.000	0.000
BAM_{900}	0.000	0.000	0.000	0.000	0.000	0.000
BAM_{700}	0.000	0.000	0.000	0.000	0.000	0.000
BAM_{650}	0.387	0.224	0.000	0.000	0.224	0.500
BAM_{500}	0.000	0.000	0.000	0.000	0.000	0.000
BAM_{450}	0.000	0.000	0.000	0.000	0.000	0.224
BAM_{400}	0.000	0.000	0.000	0.000	0.000	0.000
BAM_{380}	0.894	0.922	1.000	0.975	0.894	0.949
BAM_{350}	0.447	0.316	0.000	0.224	0.447	0.224
$OPA20_{1200}$	0.365	0.000	0.542	0.277	0.000	0.000
$OPA20_{850}$	0.000	0.000	0.767	0.000	0.000	0.000
$OPA20_{800}$	1.000	1.000	0.594	1.000	1.000	1.000
$OPA20_{700}$	0.000	0.000	0.728	0.000	0.000	0.316
$OPA20_{650}$	0.000	0.000	0.000	0.000	0.000	0.000
$OPA20_{350}$	0.000	0.000	0.000	0.000	0.000	0.000

*Each marker is assigned by the name of the random primer and its size in base pairs.

Table 2. Number of individuals (N), random amplified polymorphic DNA bands (n) analysed per *Marchalina hellenica* population, number (m), average proportion (P) of monomorphic bands found per primer in each population and estimated heterozygosity (H).

		Aghia Paraskevi		Kamena Vourla		Helmos		Makrinari		Vouliagmeni		Tripoli	
Primer	n	N	m	N	m	N	m	N	M	N	m	N	m
OPA-3	8	20	7	20	4	20	3	20	2	20	7	20	8
OPA-13	11	18	11	12	8	20	7	17	7	13	9	12	11
OPA-20	6	15	5	9	6	17	2	13	5	14	6	10	5
BAM	10	20	7	20	5	20	8	20	6	20	6	20	5
P			0.857		0.657		0.571		0.571		0.800		0.829
H			0.049		0.109		0.169		0.140		0.051		0.046

individuals that were analysed per primer varied from 9 to 20 because any inadequate banding pattern generated due to unsatisfactory amplification was excluded from the analysis (table 2). Bands that were reproduced after at least two replications, regardless of their intensity, were considered reliable. An example of RAPD profiles produced by the OPA 3 and BAM primers is illustrated in fig. 2.

Monomorphic bands constantly present in all population varied between primers from 25 to 100%. Intrapopulational polymorphism varied considerably among populations and primers. However, the highest levels of intrapopulational variation were observed in samples from Makrinari and Helmos, where the proportions of monomorphic bands for combined data from all primers attained 57.1%, followed by samples from Kamena Vourla with a corresponding value of 65.7%. The highest proportion of monomorphic loci was observed in the population from Aghia Paraskevi (85.7%) (table 2). Table 3 shows the number of distinct genotypes that were observed among the six populations. The RAPD genotype consists of a sequence of 35 numbers (0 or 1) corresponding to the presence or absence of the bands scored. Because the number of individuals per region examined by each primer was not equal, only individuals that were analysed by all primers were used in the analysis. Thus, a total of 75 individuals complied with the former criterion and 34 distinct genotypes were identified. The populations on fir trees in southern Greece and on pines in central and northern Greece exhibited a higher number of genotypes (8–12) than those observed on pines in the three regions of southern Greece, i.e. a total of 11 different genotypes, four from each region.

Table 3. Number of genotypes of populations of *Marchalina hellenica* collected in various regions of Greece.

Population	Host-tree	Number of individuals examined	Number of distinct genotypes
Aghia Paraskevi, SG*	Pine	15	4
Vouliagmeni, SG	Pine	12	4
Tripoli, SG	Pine	9	4
Kamena Vourla, CG	Pine	9	8
Makrinari, NG	Pine	14	9
Helmos, SG	Fir	16	12

*SG, southern Greece; CG, central Greece; and NG, northern Greece.

Nei's (1972) pair wise genetic distances (*D*) calculated for populations originating from different regions ranged from $D = 0.041$ (Aghia Paraskevi-Tripoli) to $D = 0.202$ (Helmos-Aghia Paraskevi) with an average value of $D = 0.096$ (table 4). The

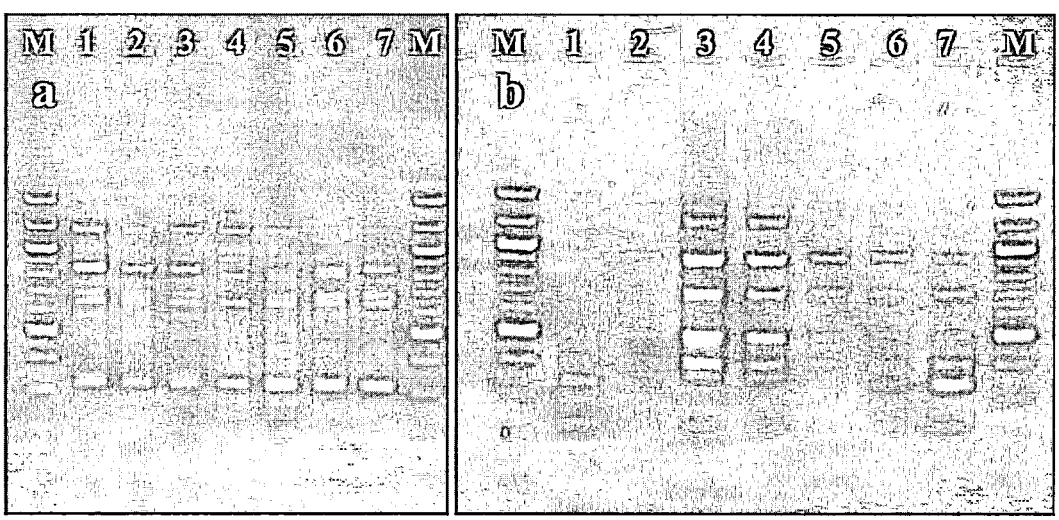

Fig. 2. Banding pattern obtained by the random primers (a) OPA-20 (lane 1, Aghia Paraskevi; lane 2, Vouliagmeni; lane 3, Tripoli; lanes 4 and 5, Helmos; lane 6, Makrinari; lane 7, Helmos) and (b) BAM (lane 1, Aghia Paraskevi; lane 2, Tripoli; lanes 3 and 4, Helmos; lane 5, Makrinari; lane 6, Kamena Vourla; lane 7, Vouliagmeni). M, DNA marker (New England Biolabs Inc.) sizes 100 to 1500 base pairs.

Table 4. Matrix of Nei's genetic distances for six *Marchalina hellenica* populations.

	Region	Host	1	2	3	4	5	6
1	Aghia Paraskevi, SG*	Pine	0.000					
2	Kamena Vourla, CG	Pine	0.105	0.000				
3	Helmos, SG	Fir	0.202	0.124				
4	Makrinari, NG	Pine	0.105	0.034	0.117			
5	Vouliagmeni, SG	Pine	0.044	0.082	0.188	0.074		
6	Tripoli, SG	Pine	0.041	0.079	0.186	0.082	0.018	0.000

*SG, southern Greece; CG, central Greece; and NG, northern Greece.

UPGMA dendrogram revealed a genetic divergence among the populations examined (fig. 3). The population sampled on fir trees in Helmos was clearly separated from those collected on the two pine species. Furthermore, a geographical separation in populations sampled from pines was observed since they were located in two distinct clusters. The first cluster consisted of populations from southern Greece (Aghia Paraskevi, Tripoli and Vouliagmeni) and the second one from samples collected in central (Kamena Vourla) and northern Greece (Makrinari).

The scattergrams resulting from the PCA and MDS are presented in figs 4 and 5, respectively. In general, there is a concordance between the analysis based on Nei's genetic distance and the multivariate approach, although some differences were also revealed. In both PCA and MDS analyses the samples collected on firs in Helmos were separated from those on pines. Regarding the populations from pines, most of the samples from Makrinari, northern Greece formed a distinct cluster. However, the samples from Kamena Vourla, central Greece were mixed with those collected in regions of southern Greece.

The values of estimated heterozygosity calculated according to the method of Lynch & Milligan (1994) differed among the populations examined. The highest values were observed in the populations sampled on firs in Helmos, south Greece (0.169) and on pines in Makrinari, northern Greece (0.140) (table 2). The F_{ST} values calculated (0.469 for all samples and 0.426 for those collected on pines) using the estimated heterozygosity values for the population examined suggested the presence of high genetic subdivision among populations. Relatively high *Fst* values (0.384 for all samples and 0.328 for those collected on pines) were also obtained following the Bayesian approach proposed by Holsinger *et al.* (2002).

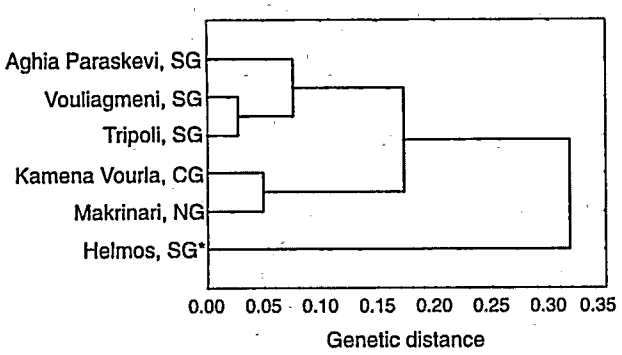

Fig. 3. Unweighted pair group method of analysis dendrogram based on Nei's genetic distances between populations of *Marchalina hellenica* sampled from various regions in Greece. SG, southern Greece; CG, central Greece; and NG, northern Greece. *Population collected on firs, the others on pines.

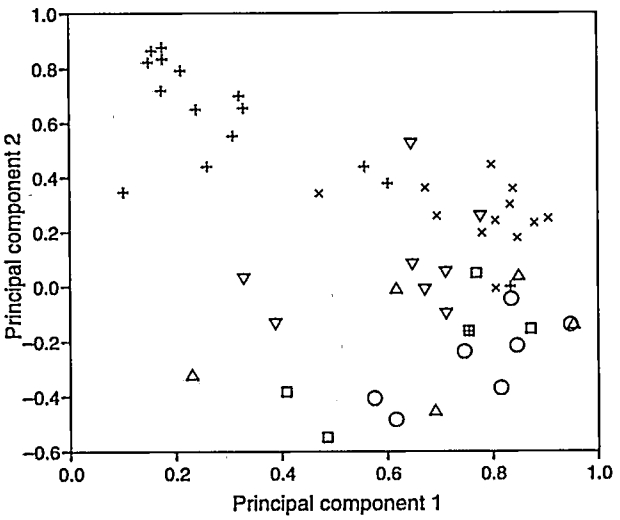

Fig. 4. Principal components analysis of the scores of random amplified polymorphic DNA bands in *Marchalina hellenica* populations sampled from various regions of Greece. ○, Tripoli; △, Vouliagmeni; ×, Makrinari; ▽, Kamena Vourla; +, Helmos; □, Aghia Paraskevi. Principal components 1 and 2 explain the 49.7 and 14.0% of the total variation of the data, respectively. Samples from Helmos were collected on fir, the others on pines.

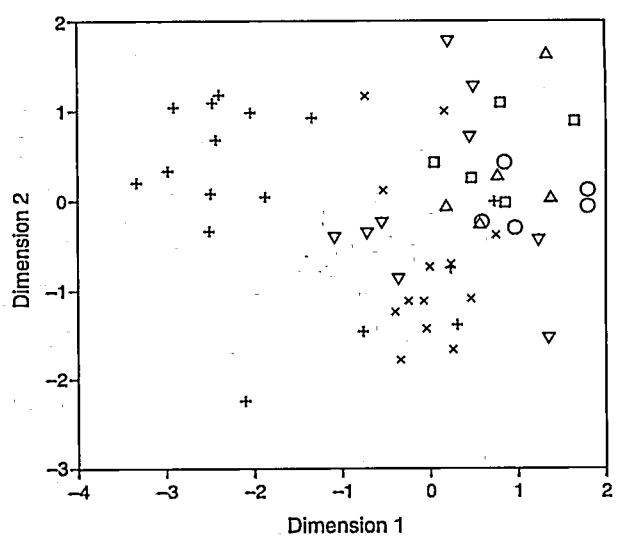

Fig. 5. Multidimensional scaling analysis of the scores of random amplified polymorphic DNA bands in *Marchalina hellenica* populations sampled from various regions of Greece. ○, Tripoli; △, Vouliagmeni; ×, Makrinari; ▽, Kamena Vourla; +, Helmos; □, Aghia Paraskevi. Samples from Helmos were collected on fir, the others on pines.

Discussion

Marchalina hellenica is a sap-sucking insect which is widely distributed in pine forests throughout Greece (Kailidis, 1965; Nikolopoulos, 1965; Santas, 1983). Its way of life has two important characteristics that should be taken into consideration in population genetics studies. First, the species reproduces mainly parthenogenetically, since males are rare (Sureyya & Hovasse, 1930; Erlinghagen, 2001; Kailidis, 1991) and second, its migratory ability is low. Adult females are apterous and consequently they are not able to disperse over long distances. The question that arises is whether geographically distant populations of the species are genetically divergent. The results of the present study failed to reveal a specific marker enabling discrimination between the six populations examined. However, data analysis for genetic polymorphism showed a degree of both intra- and interpopulation genetic variation. The intrapopulation variation observed was associated with host type and region of origin. Among the samples collected on pines, lower proportions of monomorphic loci for the combined data of all primers were found at Makrinari in northern Greece and Kamena Vourla, central Greece (57.1 and 65.5%, respectively) than that observed in southern Greece (80.0–85.7%), although the only sample from fir trees at Helmos in southern Greece also showed a low ratio of monomorphic bands (57.1%). The number of distinct genotypes identified corroborates with the observed pattern of intrapopulation variation in that the lowest number of genotypes was found on pines in southern Greece.

The Nei's (1972) pairwise genetic distances, which provide an estimate of the mean number of mutations separating the genes of two populations, revealed genetic variation among the populations examined. The most striking result was the association between region of origin, host type and genetic divergence. UPGMA cluster analysis based on Nei's genetic distances clearly separated the only population of *M. hellenica* sampled on fir from those on pines. It seems, therefore, that the capture of a new host and the presumable new environmental pressure are probably responsible for the genetic differentiation of the population from fir. Furthermore, the populations on pines were separated into two main groups. The first group consisted of the samples from southern Greece and the second one from those collected further north. It is worth mentioning, however, no genetic differentiation related to the species of host pine was observed. Similar results were obtained when data of RAPD scores were analysed using multivariate analyses, although both PCA and MDS failed to separate the samples from pines in central Greece from those collected on pines further south. *Marchalina hellenica* is widely distributed in pine forests in many regions of Greece. However, its low migratory ability could lead to genetic differentiation of geographically distant populations. The phylogenetic reconstruction confirms the former hypothesis by revealing genetic divergence among populations from northern and southern regions of Greece.

The different genetic structure of *M. hellenica* populations is further supported by the estimated heterozygosity levels which suggest a relatively low genetic diversity in samples originated from pines in southern Greece. In addition, the high *Fst* values which were estimated according to the methods of Lynch & Milligan (1994) (0.469 for all samples, 0.426 for samples from pines) and Holsinger *et al.* (2002) (0.384 for all samples, 0.328 for samples from pines) confirm the presence of genetic subdivision among those populations. The estimated effective number of migrants per generation (N_em) calculated for all samples and those collected on pines according to the method of Lynch & Milligan (1994) were both quite low (0.28 and 0.34, respectively). The corresponding values calculated using the Bayesian method of Holsinger *et al.* (2002) were 0.4 and 0.5. Theses values denote a restricted gene flow between sampling sites which presumably reflects the low migratory ability of the species.

Marchalina hellenica is a useful insect in honey production and consequently its population performance should be taken into consideration for maximizing honey yield. According to Jones (1980) and Wallner (1987) the level of heterogeneity in natural populations affects their success in relation to biotic or abiotic environmental factors. For example, reduced genetic diversity can lead to a reduction of fitness and increase the probability of extinction (Frankham, 1995a,b). The present study has revealed low heterogeneity levels in the insect populations on pines in southern Greece, however, data regarding biological or population parameters of *M. hellenica* and honeydew production at the collection sites are not available for a direct comparison. Nevertheless, it is worthwhile that information regarding the genetic affinities of *M. hellenica* populations is considered in management programmes and especially in cases where artificial introductions of the species into uninfested forests or into localities where local populations pre-exist are contemplated.

Acknowledgements

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