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Biochemical systematics and patterns of genetic divergence between the *Troglophilus* species of Crete and Rhodos (Orthoptera, Rhaphidophoridae)

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ABSTRACT. Four populations of *Troglophilus spinulosus* from Crete, one population of *T. lagoi* from Rhodos and one population of *Dolichopoda paraskevi* from Crete have been studied for genetic variation at 19 enzymatic loci by electrophoresis. Three different levels of genetic divergence emerged: mean Nei's genetic distance was 0.025 among *T. spinulosus* populations, 0.347 between *T. spinulosus* and *T. lagoi* and 0.837-0.918 between *D. paraskevi*-*T. spinulosus* and *D. paraskevi*-*T. lagoi* respectively. Relationships among *T. spinulosus* populations were studied by Principal Component Analysis in order to clarify their debated taxonomic position; relationships among all study populations were assessed by two different methods of tree reconstruction, Maximum Likelihood and Neighbor-Joining. The low degree of genetic differentiation among *T. spinulosus* populations and the different data analyses all indicate that this is the only *Troglophilus* species of Crete.

KEY WORDS: *Troglophilus*, electrophoresis, genetic distance, biochemical systematics.

INTRODUCTION

Rhaphidophoridae is an Orthopteran family including, in the Mediterranean area, only two genera, *Troglophilus* and *Dolichopoda*, both colonising cave environments. In this paper we report data on the levels of genetic differentiation and genetic variability in five populations of the cave crickets belonging to the genus *Troglophilus*. In particular, we have studied two species, *T. spinulosus* Chopard, 1921 and *T. lagoi* Menozzi, 1934 endemic of Crete and Rhodos islands respectively. The cavernicolous Orthopteran fauna of Greece, in spite of its biogeographic interest, is relatively unknown, mainly because species identification has been often based on the study of few individuals and in some cases subadults (WILLEMSE, 1985; KOLLAROS et al., 1991).

Particularly debated have been the systematics of the *Troglophilus* of Crete: *T. spinulosus* was described by Chopard (1921). Throughout the years two other species have been recognised *T. roeweri,* Werner, 1927 and *T. petrochilosi,* Boudou-Saltet, 1978. The validity of these species has been often questioned (KOLLAROS et al., 1987); finally KOLLAROS et al. (1991) studying 30 morphological characters were not able to obtain a pattern of discrimination that could account for the presence of three species in Crete, leading them to conclude that *T. spinulosus* is the only species present on this island.

Allozyme markers have proved to be a powerful tool in analysing levels of genetic affinities; we used this approach to inquire into the taxonomic status of the *Troglophilus* of Crete. Moreover we studied a population of *T. lagoi* for evaluating the degree of genetic divergence between morphologically distinct species within the genus and a population of *Dolichopoda paraskevi* Boudou-Saltet, 1973 from Crete to assess the degree of genetic divergence within the family.

MATERIAL AND METHODS

Sampling

For each collecting site we report the cave name and location, the collecting period, the number (N) of study samples and the population symbol. The *Troglophilus* populations of Crete were sampled in the following four caves: Katholiko cave, Khania peninsula, Western Crete, VII-1995, N=14 (SPR); Doxa cave, Iraklion, Central Crete, VII-1995, N=5 (SPS); Milatos cave, Aghios Nikolaos,

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Eastern Crete, IV-1994, N=3 (SPP1); Mikro Katafygi cave, Sitia, Eastern Crete, VII-1995, N=12 (SPP2). The population of *T. lagoi* of Rhodos was from the Tolomeo cave, Rodino, VII-1996, N=19 (LAG); the population of *D. paraskevi* of Crete was from the Atzigano cave, Aghios Nikolaos, Eastern Crete, VII-95, N=3 (PAR). The samples were transported alive to the laboratory and frozen at -80°C. Sampling localities are shown in Fig. 1.

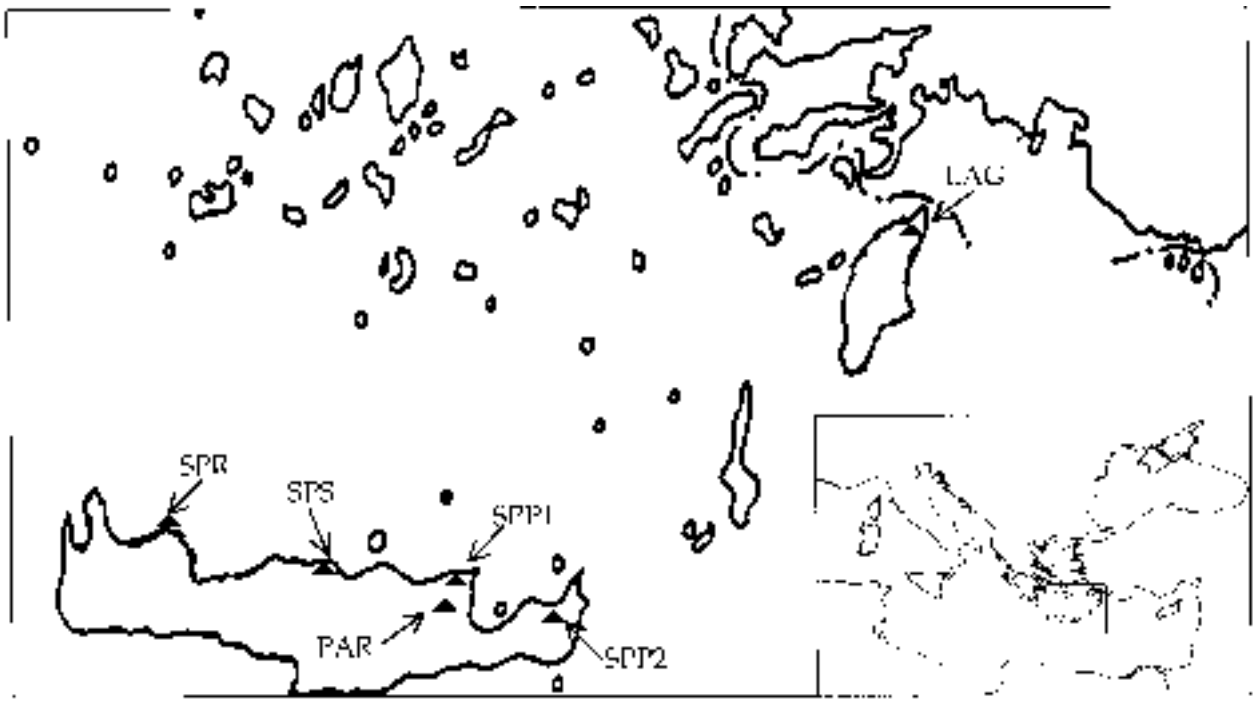

Fig.1. – Sampling locations of study population, for symbols see Materials and Methods.

Electrophoresis

Horizontal electrophoresis was performed on 12% starch gel by using crude homogenates of caudal femor muscle. Fourteen enzymatic proteins were investigated, encoded by 19 loci, namely: Acid phosphatase (EC 3.1.3.2; *Acph*); a- Amylase (EC 3.2.1.1; *a- Amy*); Aldeyde oxidase (EC 1.2.3.1; *Ao-1, Ao-2*); Carbonic anhydrase (EC 4.2.1.1; *Ca-1, Ca-2, Ca-3*); Creatine kinase (EC 2.7.3.2; *Ck*); Esterase (EC 3.1.1.1; *Est-1, Est-2*); Exochinase (EC 2.7.1.1; *Hk*); Isocitrate dehydrogenase (EC 1.1.1.42; *Idh*); Lactate dehydrogenase (EC 1.1.1.27; *Ldh*); Mannose phosphate isomerase (EC 5.3.1.8; *Mpi*); Peptidase (EC 3.4.11; *Pep-1, Pep-2*); Phosphoglucomutase (EC 2.7.5.1; *Pgm*); Phosphohexose isomerase (EC 5.3.1.9; *Phi*); Tetrazolium oxidase (EC 1.15.1.1; *To*). Details for buffer systems and staining procedures are reported in CHRISTENSEN (1977), DE MATTHAEIS et al. (1998) and KETMAIER et al. (1998).

Statistical analysis

From electrophoretic data, allele frequencies, variability parameters (H_e= expected mean heterozygosity under Hardy-Weinberg equilibrium; H_o= observed mean heterozygosity; P= proportion of polymorphic loci according to the 5% criterion; A= mean number of alleles per locus) and genetic distances (D; Nei, 1978) were calculated.

Principal Component Analysis (PCA) was performed on multilocus genotype profiles to investigate relationships among *Troglophilus* populations from Crete. Genetic heterogeneity among these populations was investigated by means of F-statistics: we calculated θ values (θ corresponds to F_{ST} in Wright's notation) for pairs of populations, according to WEIR & COCKERHAM (1984). From θ values indirect estimates of gene flow were obtained, by using the relation $Nm = 1/4\ [(1/F_{ST})-1]$ (WRIGHT, 1965). Relationships among all study populations were assessed by two different methods of tree reconstructions: Maximum Likelihood (FELSENSTEIN, 1993; ML) and Neighbor-Joining (SAITOU & NEI, 1987; NJ); trees were rooted by using PAR as outgroup. Robustness of the obtained trees was tested by the bootstrap method (FELSENSTEIN, 1985) with 1000 replications.

The programs BIOSYS-1 (SWOFFORD & SELANDER, 1981), PHYLIP 3.5 (FELSENSTEIN, 1993), FSTAT (GOUDET, 1995) and STATISTICA for Windows were employed for data analyses.

RESULTS

Six loci were polymorphic in one population at least (*Ao-1*; *Ao-2*; *Idh*; *Mpi*; *Pep-2*; *Pgm*), six loci were monomorphic and fixed for the same allele in all study populations (*a- Amy*; *Est-1*; *Est-2*; *Ldh*; *Phi*; *To*), while the remaining seven loci showed fixed alternative alleles

between groups of populations (*Acph*; *Ca-1*; *Ca-2*; *Ca-3*; *Ck*; *Hk*; *Pep-1*); no fixed alternative alleles were detected within the Crete populations. Allele frequencies are reported in Table 1, which also shows variability estimates for all study populations. The most polymorphic population was LAG (H_o = 0.055); on the contrary SPS and PAR showed no genetic variation. D (Nei, 1978) values are reported in Table 2. Three levels of genetic differentiation were highlighted. D ranged from 0.805 to 0.918 comparing different genera (*Troglophilus* vs *Dolichopoda*), from 0.346 to 0.399 comparing two *Troglophilus* species (*lagoi* vs *spinulosus*), finally D varied from 0.006 to 0.041 among Crete populations. Within these populations SPP1 was the most differentiated in terms of D values (D_{mean}= 0.038). PCA produced a somewhat similar result, emphasising also the separation of SPP2 (Fig. 2); the cumulative proportion of total variance for Factor 1 and Factor 2 was 0.704. Crete populations proved to be genetically structured, Nm not exceeding 0.825 (Table 2), indicating a general reduction of gene flow. ML and NJ analyses produced trees with identical topologies and very robust in terms of bootstrap values (Fig. 3).

TABLE 1

Allele frequencies and variability estimates in 5 *Troglophilus* and 1 *Dolichopoda* populations at 19 loci.

Loci/Pop.	SPS	SPP1	SPP2	SPR	LAG	PAR
Acph						
A	0.000	0.000	0.000	0.000	1.000	1.000
B	1.000	1.000	1.000	1.000	0.000	0.000
a-Amy						
A	1.000	1.000	1.000	1.000	1.000	1.000
Ao-1						
A	0.000	0.000	0.000	0.167	0.447	1.000
B	1.000	1.000	1.000	0.833	0.553	0.000
Ao-2						
A	0.000	0.000	0.000	0.000	0.000	1.000
B	0.000	0.000	0.000	0.417	0.000	0.000
C	1.000	1.000	1.000	0.583	1.000	0.000
Ca-1						
A	0.000	0.000	0.000	0.000	0.000	1.000
B	1.000	1.000	1.000	1.000	1.000	0.000
Ca-2						
A	1.000	1.000	1.000	1.000	0.000	1.000
B	0.000	0.000	0.000	0.000	1.000	0.000
Ca-3						
A	1.000	1.000	1.000	1.000	1.000	0.000
B	0.000	0.000	0.000	0.000	0.000	1.000
Ck						
A	0.000	0.000	0.000	0.000	0.000	1.000
B	0.000	0.000	0.000	0.000	1.000	0.000
C	1.000	1.000	1.000	1.000	0.000	0.000
Est-1						
A	1.000	1.000	1.000	1.000	1.000	1.000
Est-2						
A	1.000	1.000	1.000	1.000	1.000	1.000
Hk						
A	0.000	0.000	0.000	0.000	1.000	0.000
B	1.000	1.000	1.000	1.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.000	1.000
Idh						
A	0.000	0.667	0.000	0.250	0.000	0.000
B	1.000	0.333	1.000	0.750	1.000	0.000
C	0.000	0.000	0.000	0.000	0.000	1.000
Ldh						
A	1.000	1.000	1.000	1.000	1.000	1.000
Mpi						
A	0.000	0.667	0.278	0.000	0.214	0.000
B	1.000	0.333	0.722	1.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.786	0.000
D	0.000	0.000	0.000	0.000	0.000	1.000
Loci/Pop.	SPS	SPP1	SPP2	SPR	LAG	PAR
Pep-1						
A	0.000	0.000	0.000	0.000	0.000	1.000
B	1.000	1.000	1.000	1.000	1.000	0.000
Pep-2						
A	1.000	1.000	1.000	1.000	0.947	1.000
B	0.000	0.000	0.000	0.000	0.053	0.000
Pgm						
A	0.000	0.000	0.250	0.125	0.000	0.000
B	1.000	1.000	0.750	0.875	1.000	0.000
C	0.000	0.000	0.000	0.000	0.000	1.000
Phi						
A	1.000	1.000	1.000	1.000	1.000	1.000
To						
A	1.000	1.000	1.000	1.000	1.000	1.000
Variability estimates						
H_e*	0.000	0.056	0.043	0.075	0.050	0.000
H_o	0.000	0.035	0.010	0.022	0.055	0.000
P**	0.000	10.5	10.5	21.1	15.8	0.000
A	1.000	1.1	1.1	1.2	1.2	1.000

* *unbiased estimate (see Nei, 1978)*

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

TABLE 2

Above diagonal, genetic distance D (Nei, 1978) for all study populations; below diagonal θ (upper) and Nm (lower) values for *T. spinulosus* populations.

Pop.	SPS	SPP1	SPP2	SPR	LAG	PAR
SPS	*****	0.044	0.006	0.013	0.399	0.865
SPP1	0.586	*****	0.031	0.041	0.397	0.836
	0.176					
SPP2	0.543	0.237	*****	0.017	0.356	0.805
	0.210	0.804				
SPR	0.232	0.264	0.326	*****	0.346	0.843
	0.825	0.697	0.516			
LAG	-	-	-	-	*****	0.918
	-	-	-	-		
PAR	-	-	-	-	-	*****
	-	-	-	-	-	

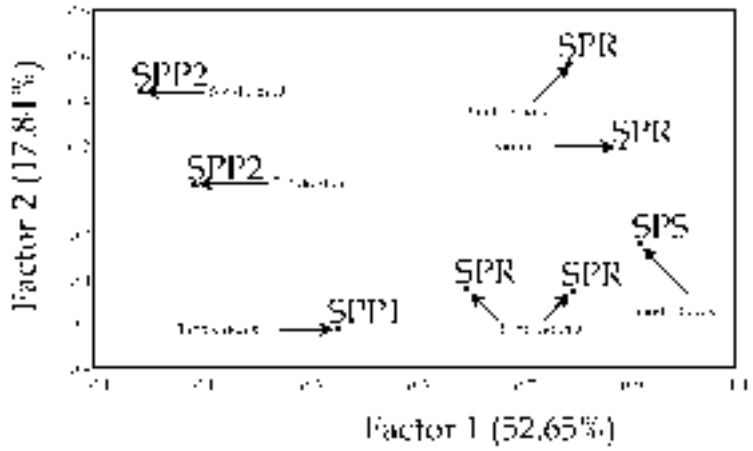

Fig. 2. – Two dimensional plot of multilocus genotype profiles based on Principal Component Analysis. Percentages refer to portion of the overall variance explained by each Factors.

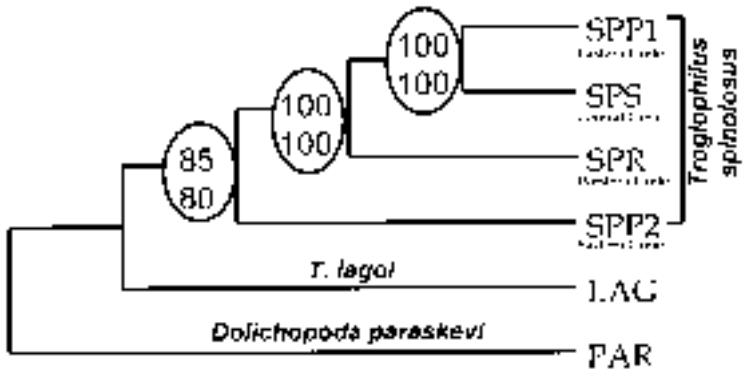

Fig. 3. – Majority rule consensus tree obtained by ML and NJ bootstrap analyses. Circled nodes include bootstrap percentages of 1000 replications for ML and NJ (first and second value, respectively). Bootstrap values are shown only for nodes for which the two phylogenetic methods had a bootstrap support of 70% or greater.

DISCUSSION

The allozyme data on *Troglophilus* populations from Crete indicate a low degree of genetic differentiation among them, the highest D value being 0.044 between SPS and SPP1. D values of this order of magnitude are frequently reported for comparisons among local populations belonging to the same species, either considering surface or cave organisms (AYALA, 1983; SBORDONI, 1982). Moreover, in the only other genetic study on *Troglophilus*, comparable D values are reported between conspecific populations of *T. cavicola* and *T. andreinii* respectively (SBORDONI et al., 1981). The patterns of relationships among the four Crete populations here studied, as assessed by PCA, ML and NJ analyses, always indicate a close affinity among SPS/ SPP1/ SPR, while SPP2 was relatively more isolated. It is interesting to note that SPP1 and SPP2 do not group together, SPS is linked to SPP1 while SPR and SPP2 are subsequently nested to them. Thus, it means that phyletic relationships among these populations do not match a geographic trend; this evidence is in disagreement with the pattern of species distribution proposed by BOUDOU-SALTET (1978). She stressed that, supporting the existence of three *Troglophilus* species in Crete, *T. roeweri*, *T. spinulosus* and *T. petrochilosi* should be distributed in Western, Central and Eastern Crete respectively. On the contrary, genetic data here presented strongly support the hypothesis of KOLLAROS et al. (1991) that *T. spinulosus* is the only species of *Troglophilus* in Crete. F- statistics analyses revealed a considerable degree of genetic structuring among *T. spinulosus* populations, θ values ranging from 0.232 (SPS vs SPR) to 0.586 (SPS vs SPP1), to which correspond Nm values of 0.825 and 0.176. Also analysing the levels of gene flow, it is not possible to recognise a clear geographic pattern, since Nm is higher between populations from Eastern and Western Crete than between populations from Central and Eastern Crete (Table 2). Anyhow, these data are just a rough indication of the degree of genetic structuring of *T. spinulosus*, due to the scattered distribution of study populations. Further research on a higher number of *T. spinulosus* populations is needed to understand the importance of the availability of routes for dispersal (in particular bioclimatic conditions outside the caves), hopefully considering both very close populations and more distant ones in order to calibrate rates of dispersal and to discriminate between present and historical gene flow.

The mean genetic distance between *T. spinulosus* and *T. lagoi* is 0.374, and the same value was found by SBORDONI et al. (1981) between *T. cavicola* and *T. andreinii*. If we use the equation proposed by NEI (1975) to date the beginning of independent evolution of *T. lagoi* and *T. spinulosus* we obtain an estimate of T= 1.870 Myr. This dating does not agree with the geological time estimates regarding the separation of Crete and Rhodos; according to MEULENKAMP et al. (1972) Crete became isolated at the end of Messinian (about 5 Mya) and has remained isolated ever since. We propose the following explanations to account for these results: a) *T. lagoi* and *T. spinulosus* may not be sister species: only studying levels of genetic affinity among several other *Troglophilus* species such as the Anatolian ones could we resolve this point; b) if *T. lagoi* and *T. spinulosus* are actually sister species and their mutual separation is related to the breakdown of the connection between Crete and Rhodos, this probably means that the Nei's formula underestimates evolutionary times in this group of cave crickets and a calibration *ad hoc* is needed.

We are presently studying the degree of genetic differentiation among several *Troglophilus* species from

Anatolia, Greece and Italy in order to provide a more accurate evolutionary scenario for this genus.

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