Allozyme variation in populations of the karyotypically polymorphic vole *Microtus* (*Terricola*) *thomasi* (Mammalia, Rodentia) from Greece

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ABSTRACT. Five distinct karyotypic forms of the vole *Microtus (Terricola) thomasi* are known in Greece so far : the earlier described forms *"thomasi"* (2n=44, FN=44) and *"atticus"* (2n=44, FN=46), and the more recently discovered ones with a)2n=41,42, FN=42,43,44, b)2n=40, FN=42, and c)2n=38, FN=40. The present study gives information on genetic relationships between vole populations belonging to the different karyotypic forms, based on allozyme variation. Eighteen loci were typed on cellulose acetate plates in 102 voles collected from six localities of mainland Greece, and the allelic data obtained were analysed using the biostatistical program BIOSYS-1. Populations studied show high intra- and interpopulation electrophoretic variability. A UPGMA dendrogram revealed clear separation of the *"atticus"* karyotype population from all others. Among the latter, two main groups exist : one including the population with the typical *"thomasi"* karyotype and another comprising all populations with polymorphic karyotypes. The clustering of the six populations studied is in agreement with karyological data given in recent literature.

KEY WORDS : Allozymes, voles, *Microtus (Terricola) thomasi*, Greece, karyotype variation

INTRODUCTION

Voles of the genus *Microtus* (subgenus *Terricola*) of the Arvicolidae family are represented in Greece by three species; *Microtus* (*Terricola*) *subterraneus*, *M. (T.) felteni*, and *M.(T.) thomasi* (MITCELL-JONES et al., 1999). *Microtus* (*Terricola*) *thomasi* Barrett-Hamilton, 1903, is endemic to the SW Balkan peninsula; in Greece it is distributed in the mainland (except Thrace and Eastern and Central Macedonia) and on the Evvoia island. It can be found from sea level up to an altitude of 1700 m.

Extensive karyological investigations (GIAGIA & ONDRIAS 1973; GIAGIA 1985; GIAGIA-ATHANASOPOULOU et al., 1995; GIAGIA-ATHANASOPOULOU & STAMATOPOULOS, 1997) have shown that *M.* (*T.*) *thomasi* occurs as several different karyotypic forms in Greece. Table 1 shows these forms, their geographical distribution and the way each has evolved from another. Discovery of this karyotypic polymorphism triggered interest in the study of its possible role in the speciation process (KING 1993; SEARLE 1993; FRAGUEDAKIS-TSOLIS et al. 1997).

Some morphological and ethological studies indicate that the *"thomasi"* and *"atticus"* karyotypic forms can be regarded as two different species (MILLER 1910; MILLER 1912; KRATOCHVIL 1971; PETROV & ZIVCOVIĆ 1972; STAMATOPOULOS & ONDRIAS 1986), whereas other morphological, behavioural, immunological and biochemical studies suggest these two forms are conspecifics (NIETHAMMER 1974; CORBET 1978; PETROV & ZIVCOVIĆ 1979; NIETHAMMER & KRAPP 1982; NIKOLETOPOULOS et al. 1992; PETROV 1992; TSEKOURA et. al. 2002). In support of the latter opinion, no reproductive isolation was observed between these two forms under laboratory conditions (GIAGIA-ATHANASOPOULOU & STAMATOPOULOS unpublished data).

The present study aims to clarify the genetic relationships between some of the Greek *M.*(*T.*) *thomasi* populations representing all known karyotypic forms.

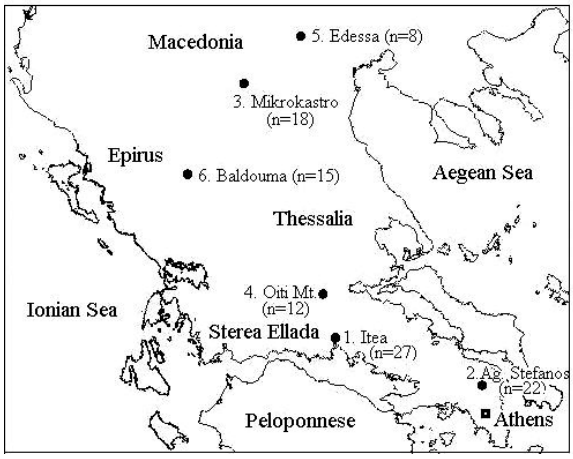

Fig. 1. – Map of Greece showing sampling localities of the present study and numbers of individuals collected from each locality.

MATERIAL AND METHODS

102 individuals of *M.(T.) thomasi* were collected from six localities of mainland Greece taking care that each of the populations belonged to one of the six known karyotypic forms (Table 1, Fig. 1). All individuals were live-trapped between September 1995 and May 2001, and each was karyotypically analysed by GIAGIA-ATHANASOPOULOU (personal communication).

TABLE 1

The six known karyotypic forms of *Microtus (Terricola) thomasi*, their distribution in Greece and the populations that represent these forms in the present study (2n=diploid chromosome number, FN=fundamental number, n=number of individuals) (karyotypic data were obtained from GIAGIA & ONDRIAS 1973; GIAGIA 1985; GIAGIA-ATHANASOPOULOU et al. 1995; GIAGIA-ATHANASOPOULOU & STAMATOPOULOS 1997)

Karyotypic form	Karyotype	Distribution	Populations
"thomasi"	2n=44, FN=44	Parts of Central Greece and the central part of South Peloponnese	Itea (Fokida pref., Sterea Ellada) (n=27)
Pericentric inversion of the X chromosome			
"atticus"	2n=44, FN=46	Parts of Central Greece and the largest part of Peloponnese	Ag. Stefanos (Attiki pref., Sterea Ellada) (n=22)
Tandem fusion between the X chromosome and a small acrocentric autosome			
"subalpine"	2n=42, FN=42	Parts of Pindos mountain range, as well as areas of eastern Epirus and Western Macedonia	Mikrokastro (Kozani pref., Macedonia) (n=18)
Robertsonian centric fusion of an autosome			
"Rb-subalpine"	2n=40, FN=42	Epirus and mountainous Central Greece	Oiti Mt. (Fthiotida pref., Sterea Ellada) (n=12)
Unnamed	2n=41,42, FN=42,43,44	A small area around Baldouma village, pref. of Ioannina (Epirus)	Baldouma (Ioannina pref., Epirus) (n=15)
Unnamed	2n=38, FN=40	A zone along the Greek-FYROM borders (Central-West Macedonia)	Edessa (Pella pref., Macedonia) (n=8)

TABLE 2

The enzymes and loci analyzed, their code numbers, the tissues they were extracted from and the buffers used (Buffers : A = 25mM Tris - 190mM glycine - pH 8.5, B = 40mM Tris - 10mM citrate - pH 7.6, C = 40mM phosphate - pH 6.3).

Enzyme	Tissue	Locus	E.C. number	Buffer
Aconitase	Heart	Aco-1	4.2.1.3.	B
Adenosine deaminase	Spleen	Ada-1	3.5.4.4.	A
Adenylate kinase	Heart	Ak-1	2.7.4.3.	B
Creatine kinase	Heart	Ck-1	2.7.3.2.	B
Glutamate oxaloacetate transaminase	Kidney	Got-1,2	2.6.1.1.	C
Glucose dehydrogenase	Kidney	Gpd-1	1.1.1.47.	A
Glucose phosphate isomerase	Kidney	Gpi-1	5.3.1.9.	A
Isocitrate dehydrogenase	Kidney	Idh-1,2	1.1.1.42.	B
Lactate dehydrogenase	Kidney	Ldh-1,2	1.1.1.27.	A
Malic enzyme	Liver	Mod-1	1.1.1.40.	B
Malate dehydrogenase	Kidney	Mor-1,2	1.1.1.37.	B
Mannose phosphate isomerase	Kidney	Mpi-1	5.3.1.8.	A
Nucleoside phosphorylase	Kidney	Np-1	2.4.2.1.	A
Phosphoglucomutase	Kidney	Pgm-1	2.7.5.1.	A

After the animals were humanely killed, their heart, liver, spleen and kidneys were removed and placed in a deep-freezer (-75° C). Skulls and skins of the specimens are deposited in the collections of the Zoological Museum of Patra University.

The tissues were homogenized and used for the electrophoretic analysis of 14 enzymes coded by 18 loci (Table 2) on prepared cellulose acetate plates (Helena Laboratories). The electrophoretic procedure was carried out according to TSEKOURA et al. (2002), following methods developed for other small mammals (SEARLE 1985; FRAGUEDAKIS-TSOLIS et al. 1997; HAUFFE et al. 2002).

The allelic data obtained were analysed using BIOSYS-1 (SWOFFORD & SELANDER 1981).

RESULTS

Of the 18 loci examined, 13 (72.2%) were found polymorphic. Three of these 13 loci (*Gpi-1*, *Mpi-1, Idh-1*) were polymorphic in only one of the six populations (Ag. Stefanos) while another locus (*Np-1*) was polymorphic in all six populations. Each monomorphic locus was fixed for the same allele in all populations (Table 3).

The mean values of the expected (He) and observed (Ho) heterozygosity, the percentages of polymorphic loci (P%), and the mean number of alleles per locus (A) for each of the examined populations are also shown in Table 3. Itea is the population with the most polymorphic loci (50.00%) and also shows the highest mean number of alleles per locus (1.56), whereas Edessa and Baldouma are the least polymorphic populations both with 22.22% of loci polymorphic and 1.22 alleles per locus. Low values of heterozygosity characterize all six poplulations.

This excess of homozygotes is also revealed by the values of the F_{IS}, F_{IT} and F_{ST} indices (WRIGHT 1951 ; NEI 1977) (Table 4). Indeed, although the genetic loci *Gpi-1*, *Idh-1* and *Ldh-1* present negative values of the fixation index F_{IS} and the locus *Idh-1* also presents a negative value of F_{IT}, these values are very small in contrast with the high positive values obtained for all the other cases ; this strong general tendency of the *F*-statistics for positive values is theoretically known to indicate a deficiency of heterozygotes. The mean value of the fixation index F_{ST} of all polymorphic loci reveals that the genetic differentiation among the six populations is responsible for 51.6% of the total genetic variability, whereas intrapopulation polymorphism is the cause of the remaining 48.4% of this variability (WRIGHT 1951).

The values of Rogers' genetic similarity varied from 0.614 (Ag. Stefanos and Edessa) to 0.964 (Oiti Mt. and Edessa), while those of Nei's genetic distance varied from

TABLE 3

The allelic frequencies for all polymorphic loci, the mean values of expected (He) and observed (Ho) heterozygosity, the percentage of polymorphic genetic loci (P%) and the mean number of alleles per locus (A) of each studied population (n = number of individuals collected) (Populations: 1.Itea, 2.Ag. Stefanos, 3.Mikrokastro, 4.Oiti Mt., 5.Edessa, 6.Baldouma).

Genetic loci		Populations					
		1	2	3	4	5	6
Ada-1	a	0.685	0.000	0.806	0.708	1.000	1.000
	b	0.315	1.000	0.194	0.292	0.000	0.000
Got-1	a	0.889	0.500	0.000	0.000	0.000	0.000
	b	0.111	0.500	1.000	1.000	1.000	1.000
Got-2	a	0.648	0.500	0.000	0.000	0.000	0.000
	b	0.352	0.500	1.000	1.000	1.000	1.000
Gpd-1	a	0.333	0.568	1.000	1.000	1.000	1.000
	b	0.667	0.432	0.000	0.000	0.000	0.000
Gpi-1	b	0.000	0.886	0.000	0.000	0.000	0.000
	c	1.000	0.114	1.000	1.000	1.000	1.000
Idh-1	a	1.000	0.909	1.000	1.000	1.000	1.000
	b	0.000	0.000	0.000	0.000	0.000	0.000
	c	0.000	0.091	0.000	0.000	0.000	0.000
Ldh-1	a	1.000	1.000	0.472	0.083	0.125	0.933
	b	0.000	0.000	0.528	0.917	0.875	0.067
Ldh-2	a	0.241	1.000	0.722	0.167	0.250	0.967
	b	0.759	0.000	0.278	0.833	0.750	0.033
Mod-1	a	0.500	0.000	0.194	0.375	0.571	1.000
	b	0.500	1.000	0.806	0.625	0.429	0.000
Mor-1	a	0.926	0.955	1.000	1.000	1.000	0.700
	b	0.076	0.045	0.000	0.000	0.000	0.300
Mor-2	a	0.130	0.000	0.056	0.000	0.000	0.000
	b	0.870	1.000	0.944	1.000	1.000	1.000
Mpi-1	a	0.000	0.409	0.000	0.000	0.000	0.000
	b	1.000	0.591	1.000	1.000	1.000	1.000
Np-1	a	0.481	0.955	0.222	0.042	0.063	0.133
	b	0.481	0.045	0.750	0.917	0.938	0.867
	c	0.037	0.000	0.028	0.000	0.000	0.000
	d	0.000	0.000	0.000	0.042	0.000	0.000
n		27	22	18	12	8	15
He		0.187	0.143	0.115	0.085	0.071	0.048
Ho		0.053	0.035	0.080	0.051	0.071	0.048
P%		50.00	44.44	33.33	27.78	22.22	22.22
A		1.56	1.44	1.39	1.33	1.22	1.22

0.004 (Oiti Mt. and Edessa) to 0.374 (Ag. Stefanos and Edessa) (Table 5).

The UPGMA dendrogram of the genetic relationships between the six populations, resulting from the Nei's distance values, is shown in Fig. 2. It demonstrates a clear separation of the Ag. Stefanos population, with the rest of the examined populations being divided into two groups: one including the Itea population and another one comprising all the others. The population of Baldouma branches next, while the other populations form two branches : one of them involves the Mikrokastro population, and the other the populations of Oiti Mt. and Edessa.

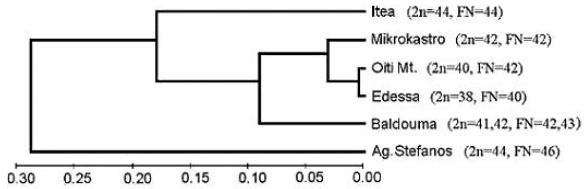

Fig. 2. – UPGMA dendrogram of genetic relationships among the six populations, based on Nei's unbiased genetic distances.

TABLE 4

The values of F_{IS} ,F_{IT} and F_{ST} fixation indices of all polymorphic loci

Locus	F_{IS}	F_{IT}	F_{ST}
Ada-1	0.576	0.805	0.541
Got-1	1.000	1.000	0.673
Got-2	0.961	0.980	0.485
Gpd-1	0.775	0.883	0.479
Gpi-1	-0.128	0.850	0.867
Idh-1	-0.100	-0.015	0.077
Ldh-1	-0.056	0.635	0.654
Ldh-2	0.830	0.915	0.499
Mod-1	0.321	0.593	0.401
Mor-1	0.689	0.744	0.175
Mor-2	0.328	0.381	0.079
Mpi-1	1.000	1.000	0.366
Np-1	0.286	0.605	0.447
Mean	0.545	0.780	0.516

DISCUSSION

The percentage of polymorphic loci (72.2%) of the populations studied is quite high compared to those calculated by GRAF (1982), which varied from 27.3% to 42.1%, for a number of arvicolid species not including *M.* (*T.*) *thomasi*. That author also calculated a mean value of the percentage of heterozygosity per locus for the Arvicolidae family (4.3% ± 2.5%), which he considered relatively high compared to other mammals. Our values vary from 3.0% (Baldouma) to 8.0% (Mikrokastro) and are similar to those of Graf. Therefore, we could conclude that the populations examined in the present study are characterized by a pronounced genetic variability.

The *F*-statistics reveal a general excess of homozygotes in the populations examined (F_{IS},F_{IT} >0), evincing the tendency of most polymorphic loci to stabilize some of the alleles, perhaps resulting from a population subdivision into smaller units (tribes, families), a fact that has also been confirmed for other species of this genus (NYGREN & RASMUSON 1980). The presence of loci that exhibit negative values of F_{IS} and F_{IT} (*Gpi-1*, *Idh-1*, *Ldh-1*), could be due to positive selection of heterozygotes.

TABLE 5

Values of ROGERS' genetic similarity (below diagonal) and NEI'S unbiased genetic distance (above diagonal) between the six populations studied.

Population	Itea (2n=44, FN=44)	Ag. Stefanos (2n=44, FN=46)	Mikrokastro (2n=42, FN=42)	Oiti Mt. (2n=40, FN=42)	Edessa (2n=38, FN=40)	Baldouma (2n=41,42, FN=42,43)
Itea (2n=44, FN=44)	-	0.193	0.162	0.186	0.184	0.182
Ag. Stefanos (2n=44, FN=46)	0.738	-	0.225	0.337	0.374	0.309
Mikrokastro (2n=42, FN=42)	0.775	0.698	-	0.030	0.030	0.063
Oiti Mt. (2n=40, FN=42)	0.779	0.634	0.919	-	0.004	0.119
Edessa (2n=38, FN=40)	0.771	0.614	0.910	0.964	-	0.086
Baldouma (2n=41,42, FN=42,43)	0.748	0.667	0.880	0.836	0.871	-

The mean value of the fixation index F_{ST} (0.516) reveals that the populations studied present about equal inter- and intrapopulation genetic variability.

Moreover, GRAF (1982) calculated mean Nei's genetic distances for different taxonomic levels within the family Arvicolidae. However, a recent study on Greek populations of *M.(T.) thomasi* showed that their genetic distances do not agree with those given by Graf for the population level, and appear to be much greater (TSEKOURA et al. 2002). This conclusion is also verified by our results, suggesting that *M.(T.) thomasi* exhibits an intraspecific variability much higher compared to other European *Microtus* species. Therefore, it appears that GRAF's conclusions should be revised to include the case of *M. (T.) thomasi*, which does not seem to follow GRAF's pattern. Nonetheless, it is worth mentioning that, as has also been indicated in TSEKOURA et al. (2002), our results show that the population of Ag. Stefanos presents the highest genetic distances from all the other populations.

The UPGMA dendrogram (Fig. 2) indicates a clear separation of the *"atticus"* karyotype population (Ag. Stefanos) from all others. Among the latter ones, consisting of two main groups, the population with the original *"thomasi"* karyotype (Itea) separates from all populations with polymorphic karyotypes. With the exception of the Ag. Stefanos population, this clustering is in agreement with the pattern of karyotype evolution within *Microtus thomasi* in the area of Greece, as indicated in Table 1. The genetic differentiation of the population of Ag. Stefanos which has a derived karyotype ("*atticus*", 2n=44, FN=46), is possibly due to particular environmental conditions that prevailed in this sampling area alone (intensive urbanization and other human activities). This area was selected because it is the closest available to the *terra typica* of the originally described vole taxon *Pitymys atticus* Miller, 1910, from which later the "*atticus"* karyotype was firstly described (GIAGIA & ONDRIAS 1973 ; GIAGIA 1985).

Within the cluster of the polymorphic karyotypes, the population of Baldouma (2n=41,42, FN=42,43) separates from the others, which form two groups : one composed of the population of Mikrokastro (2n=42, FN=42-*"subalpine"*) and another composed of the populations of Oiti Mt. (2n=40, FN=42-*"Rb-subalpine"*) and Edessa (2n=38, FN=40). The Edessa population karyotype, as already mentioned, is closely related to the *"Rb-subalpine"* one (GIAGIA-ATHANASOPOULOU & STAMATOPOULOS 1997), which, in turn, has emerged from the *"subalpine"* type (GIAGIA-ATHANASOPOULOU et al. 1995). The Baldouma population can be regarded as a hybrid one, because it consists of individuals with the *"subalpine"* karyotype (2n=42, FN=42) and hybrids with 2n=41, FN=43, resulting from crosses of *"Rb-subalpine"* (2n=40, FN=42) individuals with 2n=42, FN=44 ones, the latter not being included in our sample (Table 1) (GIAGIA-ATHANASOPOULOU & STAMATOPOULOS 1997). Therefore, this population is expected to have intermediate karyotypic characteristics between the populations of Mikrokastro and Oiti Mt.. Our results confirm this point, since the population of Baldouma exhibits similar values of genetic distance from both these two populations (Table 5, Fig. 2).

The results of the electrophoretic analysis and the clustering of the populations examined agree to a large extent with the karyological data. This correspondence encourages us to make further attempts to clarify genetic relationships among the Greek populations of this species. Our main approach will concern more electrophoretic and other molecular studies of populations belonging to the already known karyotypic forms and any as yet undiscovered ones.

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