

Genetic Variability of Species of the Genus *Eupelmus* Dalman, 1820 (Hymenoptera: Eupelmidae) Based on Allozyme Markers

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Abstract: Four enzyme systems (MDH, ME, PGM and HK) of three parasitoid species of the genus *Eupelmus*, i.e. *E. vesicularis* (Retzius), *E. urozonus* Dalman and *E. microzonus* Förster were studied. A total of five populations were examined, three of them belonging to *E. urozonus*. Polymorphism of four alleles of MDH-1, PGM, HK-1 and HK-2 loci and three alleles of ME locus was observed. Taxonomic markers for the tested *Eupelmus* spp. were found. The degree of polymorphism and heterozygosity of each species were calculated. UPGMA cluster analysis confirmed that *E. vesicularis*, which belongs to the subgenus *Macroneura*, is discriminated from *E. urozonus* and *E. microzonus*, which belong to the subgenus *Eupelmus*. One of the populations of *E. urozonus* from Osogovo Mt. was more similar to a population from Vitosha Mt. than to another population from Osogovo Mt.

Key words: Hymenoptera, *Eupelmus*, genetic variability, allozymes

Introduction

Among the members of the superfamily Chalcidoidea (Hymenoptera), Eupelmidae is a relatively small family (NOYES 2016) composed of three subfamilies: Eupelminae Walker, Calosotinae Bouček and Neanastatinae Kalina (GIBSON *et al.* 1997). The genus *Eupelmus* Dalman, 1820 is the most diversified within the Eupelminae and includes 109 extant species in the Palearctic Region (NOYES 2016). GIBSON (1995) recognised three subgenera within the genus *Eupelmus*: *Eupelmus*, *Episolidelia* Girault, 1914 and *Macroneura* Walker, 1837. Species of *Eupelmus* are primary or secondary ectoparasitoids of immature stages of various holometabolous insects that are concealed or protected in plant tissue (GIBSON 2011).

Eupelmus (Eupelmus) urozonus Dalman, 1820 is a cosmopolitan polyphagous species that develops as a primary and secondary parasitoid of a wide range

of insect hosts (BOUČEK & ASKEW 1968, THOMPSON 1955). It is a part of a species group that includes morphologically similar species (BOUČEK 1988).

Eupelmus (Eupelmus) microzonus Förster, 1860 is a well known polyphagous parasitoid, with hosts belonging to twelve families of four insect orders; its plant associations comprise Asteraceae, Fabaceae, Fagaceae, Lamiaceae, Papaveraceae, Poaceae, Rosaceae and Zygophyllaceae (NOYES 2016).

Eupelmus (Macroneura) vesicularis (Retzius, 1783) is a widespread species characterised by unusually broad trophic links. Its hosts include various insect orders such as Coleoptera, Diptera, Hymenoptera, Lepidoptera, Orthoptera and Hemiptera (GIBSON 1990).

Electrophoretic techniques offer a great capability to identify species using a variety of genetic

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Table 1. *Eupelmus* populations examined in the present study

Population	Locality	GPS coordinates	Host-plants	Date of collection
<i>E. vesicularis</i>	Ihtimanska Sredna Gora Mts., Verinsko Village	N 42°29'20.6" E 23°45'52.5"	<i>Rosa</i> sp.	22.02.2016
<i>E. microzonus</i>	VelikoTarnovo, Arbanasi Village	N 43°05'50.1" E 25°40'38.3"	<i>Dianthus giganteus</i>	01.08.2015
<i>E. urozonus</i>	Osogovo Mts., Granitsa Village	N 42°15'30.0" E 22°44'26.2"	<i>Rosa</i> sp.	04.03.2016
	Osogovo Mts., Eremitiya Village	N 42°13'09.6" E 22°50'06.0"	<i>Rosa</i> sp.	05.03.2016
	Vitosha Mts., Marchaevvo Village	N 42°36'58.0" E 23°10'53.6"	<i>Rosa</i> sp.	20.03.2016

markers for diagnosing of insect parasitoids. They have been used to measure genetic variability in natural populations. Allozyme electrophoresis is a suitable tool that can be applied successfully in agriculture entomology to resolve many problems (MENKEN & ULENBERG 1987) including identification of pests and natural enemies, phylogenetic relationships, patterns of gene flow among populations, as well as for many other purposes (MENKEN 1990).

Isoenzyme and allozyme genetic variability in the *Eupelmus* parasitoids is poorly studied. Due to this fact, the aim of the present study was to characterise the genetic variability and differentiation among five *Eupelmus* populations from various locations in Bulgaria belonging to three *Eupelmus* species based on isoenzyme analysis.

Materials and Methods

Eupelmus samples

More than 180 specimens of three different species from the genus *Eupelmus* were used for polyacrylamide gel electrophoresis (PAGE). The insects were reared from seeds and galls in laboratory conditions. Identification of the species was based on the keys by ASKEW & NIEVES-ALDREY (2000), GIBSON & FUSU (2016) and KALINA (1981). The specimens were aspirated with a pooter and anaesthetised with ethyl acetate and stored at -20°C prior to electrophoretic analysis. The examined populations of *Eupelmus* spp. and their collection dates, host-plants and localities are presented in Table 1.

Enzyme electrophoresis

The whole body of each specimen was squashed using quartz sand in 0.8 M tris-phosphate buffer at pH=6.7, and laid for extraction for 18 hours at 4°C. Then the homogenates were centrifuged for 15 minutes at 5000 rpm and 10µl of the supernatant were syringed into wells in the gel. The electrophoretic

separation was carried out according to the technique described by DAVIS (1964) and MAURER (1971) using 7.5% polyacrylamide vertical gel at 4.5 mA/cm for 3 hours, together with concentrating gels (3.3%) at pH=6.7, and 0.05 M tris-0.2 M glycine electrode buffer at pH=8.3. The following enzymes were manifested: malate dehydrogenase (MDH –EC 1.1.1.37), malic enzyme (ME –EC 1.1.1.40), phosphoglucomutase (PGM – EC 5.4.2.2) and hexokinase (HK – EC2.7.1.1). Buffers and histochemical staining for each enzyme were as in SHAW & PRASAD (1970) and SPENCER *et al.* (1964).

Statistical analysis

After the detection of the isoenzyme activity regions, the phenotypes of the discovered loci were recorded. Allele frequencies, mean number of alleles per locus, proportion of polymorphic loci, observed (H_o) and expected (H_e) heterozygosity, deviation from the Hardy-Weinberg equilibrium and Nei's genetic distance (D) (NEI 1972) were calculated using BIOSYS-1 (SWOFFORD & SELANDER 1981). A phylogenetic tree was constructed using NEI's (1972) genetic distance by UPGMA (SNEATH & SOKAL 1973) method using the PHYLIP (FELSENSTEIN 1993) software package.

Results

Four enzymes (malate dehydrogenase, malic enzyme, phosphoglucomutase and hexokinase) corresponding to five loci (MDH-1, ME, PGM, HK-1 and HK-2) produced clear bands which were used for a detailed study. Most of the studied loci were polymorphic in most of the populations included in this investigation (Table 2).

Polymorphism with total of four alleles of MDH-1 locus was detected in the studied species (Table 2). One species, *E. vesicularis*, showed a unique fixed allele of MDH-1 locus.

MDH-1¹⁰⁰, MDH-1⁸⁴ and MDH-1⁷⁸ alleles were present in both of *E. urozonus* and *E. microzonus*. In *E. urozonus* population from Eremyia Village the MDH-1¹⁰⁰ allele was recorded with the highest frequency (0.194). The MDH-1⁸⁴ allele was detected with the highest frequency in *E. microzonus* (0.658). The MDH-1⁷⁸ allele was the most common in the all *E. urozonus* populations. This allele demonstrated the highest frequency in the population from Granitsa Village (0.836). The Mdh-1⁵⁹ allele only occurred in *E. vesicularis* (1.000).

Polymorphism with three alleles was observed in the ME locus. The ME¹³⁶ allele occurred in *E. microzonus* (0.059) and *E. urozonus* populations from Eremyia Village (0.125) only (Table 2). Me¹⁰⁰ was the most common allele in all tested populations of the studied species. It demonstrated the highest frequency in *E. microzonus* (0.941). Among the populations of *E. urozonus*, this allele was with the highest frequency in population from Granitsa Village (0.923) and with the lowest in population from Eremyia Village (0.594). The ME⁷⁷ allele was

detected in the *E. urozonus* and *E. vesicularis* populations but absent in *E. microzonus* one. With the highest frequency this allele occurred in *E. vesicularis* (0.313).

Polymorphism with four alleles was observed in the PGM locus (Table 2). The PGM¹²³ allele was specific for *E. vesicularis* (1.000). The other three alleles were typical for *E. urozonus* and *E. microzonus*. The PGM¹⁰⁰ allele was the rarest in the gene pool of all populations where it existed. The PGM⁸⁵ allele demonstrated the highest frequency in *E. microzonus* population (0.636). The PGM⁷⁵ allele was the most common in all populations of *E. urozonus*, with the highest frequency in population from Marchaev Village (0.847).

Polymorphism with four alleles was observed in both of HK-1 and HK-2 loci (Table 2). The HK-1¹²⁷ allele was specific for *E. microzonus* (1.000). HK-1¹¹⁴ occurred only in the populations of *E. urozonus* from Eremyia and Marchaev Village (0.444 and 0.028). The HK-1¹⁰⁸ allele was specific for *E. vesicularis* (1.000). HK-1¹⁰⁰ was specific for *E. uro-*

Table 2. Allele frequencies at enzyme loci in *Eupelmus* populations tested

	<i>Eupelmus urozonus</i> Eremyia	<i>Eupelmus urozonus</i> Marchaev	<i>Eupelmus urozonus</i> Granitsa	<i>Eupelmus vesicularis</i> Verinsko	<i>Eupelmus microzonus</i> Arbanasi
Locus/allele	Frequencies				
MDH-1					
MDH-1 ¹⁰⁰	0.194	0.024	0.014	0.000	0.079
MDH-1 ⁸⁴	0.056	0.427	0.151	0.000	0.658
MDH-1 ⁷⁸	0.750	0.548	0.836	0.000	0.263
MDH-1 ⁵⁹	0.000	0.000	0.000	1.000	0.000
ME					
ME ¹³⁶	0.125	0.000	0.000	0.000	0.059
ME ¹⁰⁰	0.594	0.909	0.923	0.688	0.941
ME ⁷⁷	0.281	0.091	0.077	0.313	0.000
PGM					
PGM ¹²³	0.000	0.000	0.000	1.000	0.000
PGM ¹⁰⁰	0.067	0.010	0.024	0.000	0.091
PGM ⁸⁵	0.267	0.143	0.175	0.000	0.636
PGM ⁷⁵	0.667	0.847	0.802	0.000	0.273
HK-1					
HK-1 ¹²⁷	0.000	0.000	0.000	0.000	1.000
HK-1 ¹¹⁴	0.444	0.028	0.000	0.000	0.000
HK-1 ¹⁰⁸	0.000	0.000	0.000	1.000	0.000
HK-1 ¹⁰⁰	0.556	0.972	1.000	0.000	0.000
HK-2					
HK-2 ¹³⁷	0.000	0.000	0.000	0.000	1.000
HK-2 ¹²⁵	0.364	0.028	0.000	0.000	0.000
HK-2 ¹¹⁶	0.000	0.000	0.000	1.000	0.000
HK-2 ¹⁰⁰	0.636	0.972	1.000	0.000	0.000

Table 3. Mean number of alleles per locus, proportion of polymorphic loci, observed (H_o) and expected heterozygosity (H_e)

Population of <i>Eupelmus</i>	Mean no. of alleles per locus	Percent polymorphic loci (P = 0.99)	H_o	H_e
<i>E. urozonus</i> Ereмиya	2.6±0.2	100.0	0.046±0.033	0.497±0.027
<i>E. urozonus</i> Marchaevo	2.4±0.2	60.0	0.018±0.008	0.212±0.086
<i>E. urozonus</i> Granitsa	2.0±0.4	60.0	0.021±0.011	0.151±0.069
<i>E. vesicularis</i> Verinsko	1.2±0.2	20.0	0.025±0.025	0.092±0.092
<i>E. microzonus</i> Arbanasi	2.0±0.4	60.0	0.055±0.034	0.231±0.120

Table 4. Nei's (1972) genetic distance between the examined populations of *Eupelmus* based on allozymes

Population	<i>Eupelmus microzonus</i>	<i>Eupelmus vesicularis</i>	<i>E. urozonus</i> Granitsa	<i>E. urozonus</i> Marchaevo	<i>E. urozonus</i> Ereмиya
<i>Eupelmus microzonus</i>	*****				
<i>Eupelmus vesicularis</i>	1.874	*****			
<i>E. urozonus</i> Granitsa	0.983	1.901	*****		
<i>E. urozonus</i> Marchaevo	0.892	1.872	0.020	*****	
<i>E. urozonus</i> Ereмиya	1.006	1.941	0.112	0.141	*****

zonus. This allele was fixed in the gene pool of population from Granitsa Village (1.000).

The HK-2¹³⁷ allele was specific for *E. microzonus* (1.000). HK-2¹²⁵ was presented only in the gene pool of *E. urozonus* populations from Ereмиya and Marchaevo Village (0.364 and 0.028). HK-2¹¹⁶ was unique for *E. vesicularis* (1.000). HK-2¹⁰⁰ occurred in the gene pool of all *E. urozonus* populations, with the highest frequency in population from Granitsa Village where it was fixed (1.000) (Table 2).

The mean number of alleles per locus varied from 1.2 (in *E. vesicularis*) to 2.6 (in *E. urozonus* from Ereмиya Village) (Table 3). The percent of polymorphic loci was as follows: 20% (for *E. vesicularis*); 60% (for *E. microzonus*, *E. urozonus* from Granitsa and Marchaevo Village) and 100% (for *E. urozonus* from Ereмиya Village) according to 0.99 criterion. The observed heterozygosity (H_o) ranged from 0.018 (*E. urozonus* from Marchaevo Village) to 0.055 (*E. microzonus*) and the expected heterozygosity (H_e) ranged from 0.092 (*E. vesicularis*) to 0.497 (*E. urozonus* from Ereмиya Village) (Table 3). There were significant deviations of genotype frequencies from Hardy-Weinberg expectations in almost all loci of the tested populations, except the Me locus in *E. microzonus* and *E. vesicularis*. Chi-Square (df = 3) test showed that the deviations were in result of excess of homozygotes and deficiency of heterozygotes.

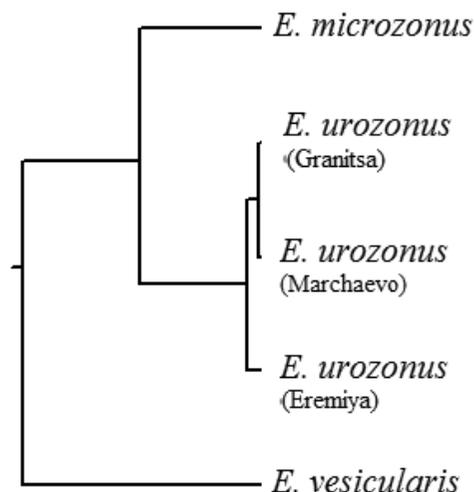


Fig. 1. UPGMA dendrogram of the studied populations of *Eupelmus* based on NEI's (1972) genetic distance

The values of the genetic distance (NEI 1972) were calculated using the allele frequencies and ranged from 0.020 (between *E. urozonus* from Marchaevo and *E. urozonus* from Granitsa Village) to 1.941 (between *E. urozonus* from Ereмиya and *E. vesicularis*) (Table 4).

The resulting UPGMA dendrogram (SNEATH & SOKAL 1973) separated the *Eupelmus* species into 2 distinct clusters. *E. urozonus* and *E. microzonus* formed one cluster with two separate subclus-

ters and *E. vesicularis* – formed the second cluster. *E. urozonus* population from Granitsa Village was more similar with *E. urozonus* population from Marchaev Village than the same of Eremiya Village (Figure 1).

Discussion

The results obtained in this study indicate that MDH, ME, PGM and HK isozyme markers could be applied to explore the genetic variability and to provide reliable biochemical characters for discriminating among *Eupelmus* species. PINTUREAU (1993) examined three enzyme systems (EST, MDH and SOD) by electrophoresis to determine their ability to discriminate several species of *Trichogramma* (Chalcidoidea: Trichogrammatidae). He ascertained that MDH and SOD are not appropriate markers for species diagnosis unlike EST. Our results about MDH pointed that this enzyme system is appropriate for studying *Eupelmus* genetic diversity. FUSU (2010) observed distinct polymorphism of ME locus with four alleles as well as polymorphism of PGM locus, with three alleles and monomorphism of MDH-1 and MDH-2 loci in *E. vesicularis* by means of cellulose acetate electrophoresis. In our study, MDH-1 locus showed clear polymorphism among the studied species from Bulgaria with diagnostic allele MDH-1⁵⁹ for *E. vesicularis*. ME, PGM, HK-1 and HK-2 loci were also found to be polymorphic. ME and PGM loci in our investigation correspond to ME and PGM loci described by FUSU (2010). PGM¹²³, HK-1¹⁰⁸ and HK-2¹¹⁶ alleles found in this study were specific for *E. vesicularis*. HK-1¹²⁷ and HK-2¹³⁷ alleles were specific for *E. microzonus*. The allele frequencies for all loci showed considerable differences among the populations from three species.

The expected heterozygosity was higher than the observed one as well as higher than the values calculated by other authors for populations of other wasp species (METCALF *et al.* 1975, PAMILO *et al.* 1978a, LESTER & SELANDER 1979, MENKEN 1982, UNRUH *et al.* 1986, ROUX & ROQUES 1996). Relatively high value of expected heterozygosity can be explained by the solitary lifestyle of *Eupelmus* spp. and the development of their larvae in various habitats (stems, galls, seeds) in contrast to some social insects, whose larvae develop in environmentally buffered nests.

The observed heterozygosity for all populations in our study is less than the value of observed heterozygosity in Neotropical sawfly *Digelasinus diversipes* (0.094± 0.025) (Kirby, 1882) (Hymenoptera: Symphyta: Argidae) (BORASCHI & DEL LAMA 2004). According to MENKEN (1991), Hymenoptera har-

bour low levels of genetic variability and show approximately a third of the level of heterozygosity of diploid-diploid insects (BERKELHAMER 1983, GRAUR 1985, UNRUH *et al.* 1986) except sawflies (SHEPPARD & HEYDON 1986, WOODS & GUTTMAN 1987, BOATO & BATTISTI 1996, BORASCHI & DEL LAMA 2004, ROSENMEIER & PACKER 1993).

The low levels of genetic variability in Hymenoptera are not a surprising phenomenon. A number of authors have reported results showing lower values of mean heterozygosity in the Hymenoptera compared to other insect orders (SNYDER 1974, METCALF *et al.* 1975, PAMILO *et al.* 1978a, LESTER & SELANDER 1979, MENKEN 1982, BERKELHAMER 1983, GRAUR 1985, ROUX & ROQUES 1996).

Various explanations for low levels of genetic variation in Hymenoptera have been proposed by many authors (SUOMALAINEN 1962, CROZIER 1970, HARTL 1971, 1972, SNYDER 1974, METCALF *et al.* 1975, PAMILO *et al.* 1978a, 1978b, LESTER & SELANDER 1979, PAMILO & CROZIER 1981, BERKELHAMER 1983, WAGNER & BRISCOE 1983, MENKEN 1991).

The lower value of observed heterozygosity in all *Eupelmus* populations tested could be explained by the haplodiploidy, genetic bottlenecks (MENKEN 1991), effective population size (PAMILO *et al.* 1978a) and the likelihood of inbreeding (HAMILTON 1967).

All Hymenoptera have a haplodiploid mechanism of reproduction that involves two types of parthenogenesis according to the sex of the offspring. Arrhenotoky is the dominant mode of reproduction in which males develop from unfertilized eggs and are haploid and females develop from fertilized eggs and are diploid. In contrast to arrhenotoky, thelytokous parthenogenesis occurs less frequently and represents a mode of asexual reproduction in which diploid females are produced from unfertilized eggs and males are absent. Thelytoky in Hymenoptera can be induced by nuclear genes or by infection with symbiotic microorganisms and includes two genetically based forms (automictic and apomictic thelytoky) and endosymbiont-induced form (HEIMPEL & DE BOER 2008, LEACH *et al.* 2009).

The presence of male specimens in both *E. urozonus* and *E. microzonus* show that they reproduce by arrhenotoky. *E. vesicularis* males are unknown from North America (GIBSON 1990) and in Europe even if males exist they are rarely collected and the species reproduces by thelytokous parthenogenesis (FUSU 2010). According to RABELING & KRONAUER (2013), arrhenotokous females have meiotic oogenesis that enables the production of offspring with combinations of traits differing from those of either parent. In contrast, thelytokous females reproduce

by automixis leading to an irreversible increase of homozygosity in populations.

An important factor limiting the amount of genetic variation is the genetic bottleneck (MENKEN 1991) whose effects last for long periods in many generations (NEI *et al.* 1975). LESTER & SELANDER (1979) gave a typical example with the parasitic wasp *Diachasmimorpha juglandis* (Muesebeck, 1961), that may survive periodic bottlenecks. The occurrence of such events could lead to a reduction of the population size resulting into the arise of favourable conditions for inbreeding. One of the reasons explaining the observed monomorphism in two species of stingless bees (WAGNER & BRISCOE 1983) is inbreeding.

PAMILO *et al.* (1978a) noted the significant role of effective population size as an important factor affecting the amount of genetic variation. According to LESTER & SELANDER (1979), a smaller effective population size and a shorter time for fixation of new mutants have haplodiploid populations than diploid populations.

Conclusions

This paper presents information concerning genetic diversity of *Eupelmus*. Species of the subgenera *Eupelmus* and *Macroneura* were clearly distinguishable at the MDH-1, ME, PGM, HK-1 and HK-2 loci. The MDH-1⁵⁹, PGM¹²³, HK-1¹⁰⁸ and HK-2¹¹⁶ alleles

were absent from the gene pool of the species of the subgenus *Eupelmus* (*E. urozonus* and *E. microzonus*) and could be used as taxonomic markers for determination of *E. vesicularis*. HK-1¹²⁷ and HK-2¹³⁷ were key alleles for discrimination and characterization of *E. microzonus*. The values of the genetic distance (NEI 1972) and the branches of the dendrogram clearly confirmed the genetic differentiation among the studied populations of the studied species of *Eupelmus*.

Further studies including more species and an increased number of analysed loci will be helpful to clarify some questions at both inter- and intraspecific levels of genetic heterogeneity for *Eupelmus* species.

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