Research Article

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Genetic variability in Populations of *Messor barbarus* (L., 1767) (Hymenoptera: Formicidae) from Bulgaria Based on Isoenzyme Analysis

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Abstract: Genetic heterogeneity in 13 populations of *Messor barbarus* (Linnaeus, 1767) (Formicidae) from Bulgaria has been studied using analysis of seven enzyme and protein systems, which have been found to be appropriate genetic markers for characterization of genetic variability within and between populations. Totally, 49 allelic variants were found for the studied loci. A comparative analysis of gene pool and genotypic structure of the tested populations have been carried out. Moderate to high percentage of polymorphism (59.1–86.4) and low levels of heterozygosity have been calculated (0.030–0.066). Deviations from Hardy-Weinberg equilibrium in almost all analyzed loci, in favor of homozygotes, have been recorded. The mean value of inbreeding coefficient (F_{IS}) has been found to be high (0.8212), demonstrating a high level of inbreeding in the studied populations. The obtained data of the genetic variability provide new information concerning polymorphism and phylogenetic relations between the studied populations.

Key words: *Messor barbarus*, isoenzymes, genetic variability, phylogenetics

Introduction

Ants are social insects, which play an important role in all terrestrial ecosystems. Various reproductive mechanisms including one or more fertile females, single or multiple fertilization, are observed in their colonies. This determines differences in the genotypic structure of their populations and makes them an interesting model for genetic research. Various genetic markers (isoenzymes, microsatellite and mitochondrial DNA) were used for the study of genetic variations of intrapopulation and interpopulation level of various representatives of the fanily Formicidae (Tomaszewski *et al.* 1973, Pamilo *et al.* 1975, Hagen *et al.* 1988, Krieger *et al.* 1999, Diehl *et al.* 2002, Ross *et al*. 2003, Pamilo *et al.* 2005, Schlick-Steiner *et al.* 2005, Schlick-Steiner *et al.* 2006, Ross *et al.* 2007, Cantagalli *et al*. 2010).

Messor barbarus (Linnaeus, 1767) is species inhabiting dry and sandy soils. It is characterised by polymorphism expressed in the presence of transitional forms with the ants workers. This diversity is due to the specialization of the various size categories of the ants workers collecting different-sized plant seeds. Similarly to other representatives of the genus *Messor* Forel, 1890, they build underground colonies with complex designed granaries, which store seeds in dry conditions, preventing germination (Hölldobler & Wilson 1990). They influence the formation of the physical, chemical and hydrological properties of the soil (Cammeraat *et al.* 2002) and affect vegetation, helping to spread seeds (Pacini 1990, MacMahol *et al.* 2000).

Studies based on isoenzyme analysis provide valuable information on the genetic relationship of specimens in the colonies and the amount of genetic

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variation within populations and among populations (Bader 1998). Studies on genetic diversity and interspecies differentiation of ants of the genus *Messor* (Hasegawa & Yamaguchi 1995, Arthofer *et al.* 2005, Schlick-Steiner *et al.* 2006, Steiner *et al.* 2011) are scanty. Detailed information on using the isoenzyme polymorphism in analyzing the degree of genetic heterogeneity in their populations is missing. This study is dedicated to the genetic characterization of populations of *Messor barbarus* in southern Bulgaria on the basis of isoenzyme polymorphism and analyzing the phylogenetic relationships between them.

Materials and Methods

Totally, 447 ants workers of *Messor barbarus* were studied. They were collected from 13 locations in Bulgaria (Table 1). The analyzed populations are designated according to the name of the nearest located settlement (Fig. 1). The material is collected and processed during July and August in the period 2011-2014. From each locality, 5 to 10 specimens were separated and stored in 75% alcohol until their taxonomic determination through classical morphometry.

We used a total extract of each individual. We homogenised the material in Eppendorf tubes using quartz sand. As an extracting solution we used trisphosphate buffer (pH 6.7) diluted with distilled water (ratio 1:7). We extracted the material overnight at a temperature of 4°C. We centrifuged at 5000 rpm for 15 min at 4°C and used the supernatant in the native form.

We examined the following enzyme and protein systems: soluble proteins (SP), nonspecific esterases (EST, EC 3.1.1.1), acid phosphatases (ACP, EC 3.1.3.2), malate dehydrogenases (MDH, EC 1.1.1.37), malate enzymes (ME, EC 1.1.1.40), superoxide dismutases (SOD, EC 1.15.1.1) and aspartate aminotransferases (AST, EC 2.6.1.1), by means of a native gel electrophoresis in polyacrylamide gel. We have implemented the visualization of isoenzymes through specific histochemical staining by the method of Shaw & Prasad (1970), Schmidtke & Engel (1972) and Korochkin *et al.* (1977).

For the purposes of the statistical analysis, we used the software product BIOSYS-1 (Swofford & Selander 1981). We calculated allele frequencies, average number of alleles per locus, degree of polymorphism, observed and expected heterozygosity (Ho and He), deviation from Hardy-Weinberg equilibrium, genetic distance by the method of Nei (D) (Nei 1972) and the fixation index of Wright (F_{ST}) (Wright 1965). We constructed UPGMA (Sneath & Sokal 1973) and Neighbour-Joining (NJ) (Saitou & Nei 1987) dendrograms using the software packages PHYLIP (Felsenstein 1993) and PAST (Hammer *et al.* 2001).

Results and Discussion

The intensity and distribution of the fractions in the individual spectra of the studied specimens demonstrated the genetic control of the enzyme and protein systems analyzed. We identified 19 variable loci and recorded the presence of a total of 49 alleles. We calculated the allele frequencies and identified the specificity in the composition of the gene pool. The comparative analysis of genotypic structure of the studied populations revealed high levels of homozygosity.

Polymorphism and heterozygosity

The average number of alleles per locus ranged from 1.8 to 2.2 (Table 1). The degree of polymorphism was highest in Ahtopol population (86.4%) and lowest at Dolnoslav and Lyubimets (59.1%). Heterozygosity, viewed as an indicator for the genetic diversity, is a convenient parameter to study the genetic variation (Ting & Cohong 2011). The recorded heterozygosity (Ho) ranged from 0.030 (with Novo Selo) to 0.066 (with Byaga and Harmanli). In all analysed populations of *Messor barbarus*, the expected heterozygosity (He) was higher than the experimentally detected. We found significant differences between the observed and expected genotypic frequencies, according to the Hardy-Weinberg equilibrium, in favour of homozygotes. The lower degree of observed heterozygosity and the higher frequency of homozygous specimens are associated with the ef-

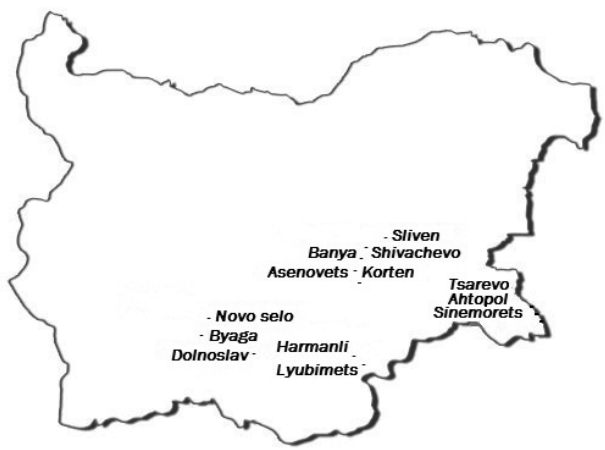

Fig. 1. Studied populations of *Messor barbarus* sampled for examination of isoenzyme analysis

Table 1. Mean sample size per locus, mean number of alleles per locus, percentage of polymorphic loci, observed and expected heterozygosity in the populations tested (Standard errors are included)

Population	Mean saple size per Locus	Mean no. of alleles per locus	Percentage of loci polymorphic	Mean heterozygosity	
				Mean heterozygosity – Direct- Count (Ho)	Mean heterozygosity – HdyWbg Expected (He)
Asenovets	28.4 ±1.2	2.0 ±0.2	72.7	0.055 ±0.017	0.341 ±0.048
Ahtopol	35.1 ±4.0	2.2 ±0.2	86.4	0.055 ±0.017	0.351 ±0.041
Banya	49.4 ±3.0	2.2 ±0.2	81.8	0.062 ±0.023	0.388 ±0.048
Byaga	21.0 ±2.4	1.8 ±0.1	63.6	0.066 ±0.026	0.252 ±0.048
Korten	44.5 ±2.0	2.0 ±0.1	77.3	0.056 ±0.017	0.357 ±0.046
Novo selo	27.2 ±2.1	2.0 ±0.2	68.2	0.030 ±0.008	0.291 ±0.050
Sinemorets	27.9 ±2.7	1.9 ±0.2	63.6	0.042 ±0.023	0.292 ±0.052
Sliven	27.0 ±2.1	1.9 ±0.2	63.6	0.063 ±0.024	0.296 ±0.054
Shivachevo	42.4 ±2.3	2.0 ±0.2	63.6	0.045 ±0.026	0.279 ±0.051
Lyubimets	24.7 ±2.9	2.0 ±0.2	59.1	0.062 ±0.030	0.287 ±0.055
Harmanli	24.7 ±2.9	2.0 ±0.2	68.2	0.066 ±0.029	0.297 ±0.051
Dolnoslav	35.9 ±2.6	1.9 ±0.2	59.1	0.052 ±0.018	0.295 ±0.055
Tsarevo	27.0 ±2.0	2.0 ±0.2	77.3	0.062 ±0.022	0.321 ±0.048

Table 2. F statistics for polymorphic loci studied: F_{ST} – fixation index; F_{IS} and F_{IT} – inbreeding coefficients

Summary of F - statistics at all loci	F(IS)	F(ST)	F(IT)
Locus: Sp-1	0.8792	0.1376	0.8958
Locus: Sp-2	0.4486	0.3043	0.6164
Locus: Sp-3	0.7641	0.2516	0.8235
Locus: Sp-5	0.7929	0.0895	0.8114
Locus: Sod-1	0.9498	0.2757	0.9636
Locus: Sod-2	0.9056	0.2334	0.9276
Locus: Me-1	0.9954	0.1124	0.9959
Locus: Ast-1	0.9415	0.1145	0.9482
Locus: Acp-1	0.6565	0.3587	0.7797
Locus: Mdh-1	0.9002	0.2092	0.9210
Locus: Mdh-2	0.5360	0.3572	0.7018
Locus: Est-1	0.8441	0.3291	0.8954
Locus: Est-2	0.9010	0.2021	0.9210
Locus: Est-3	0.6079	0.1958	0.6846
Locus: Est-5	0.5373	0.2078	0.6334
Locus: Est-6	0.8044	0.3126	0.8655
Locus: Est-7	0.9706	0.1246	0.9743
Locus: Est-8	0.9792	0.0842	0.9809
Locus: Est-9	0.8974	0.1430	0.9120
Mean	**0.8212**	0.2082	0.8584

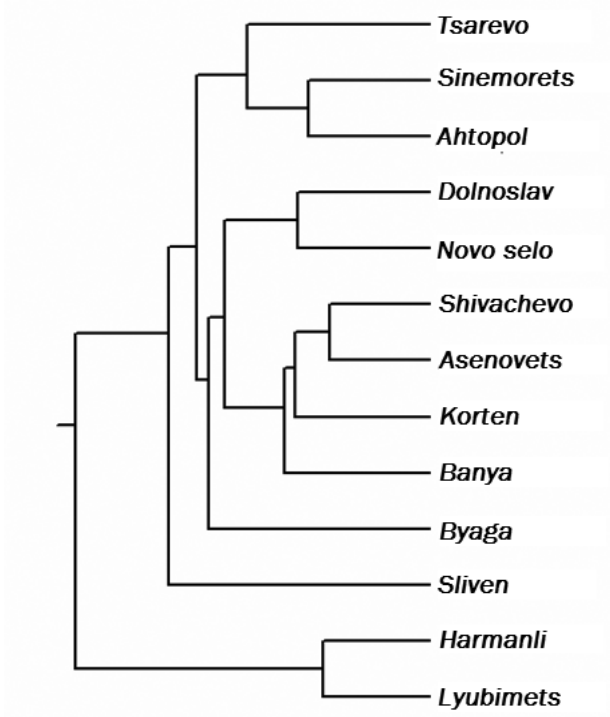

Fig. 2. UPGMA dendrogram (Sneath & Sokal 1973) representing relationships of populations of *Messor barbarus*

fect of inbreeding. In most of the populations studied, we established deviations from Hardy-Weinberg equilibrium in almost all analyzed loci.

Fixation index

Fixation index (F_{ST}) is used to determine the genetic differentiation in the population on the basis of allele frequencies. The average value of F_{ST} (0.2082) (Table 2), calculated on the basis of the recorded isoenzyme polymorphism, shows that 20.82% of the genetic variation was observed between the separate populations of *Messor barbarus*, which is associated with the level of interpopulation differentiation and 79.18% of genetic variation is intrapopulation. We recorded the highest degree of interpopulation variability in Acp-1 (0.3587) and Mdh-2 (0.3572)

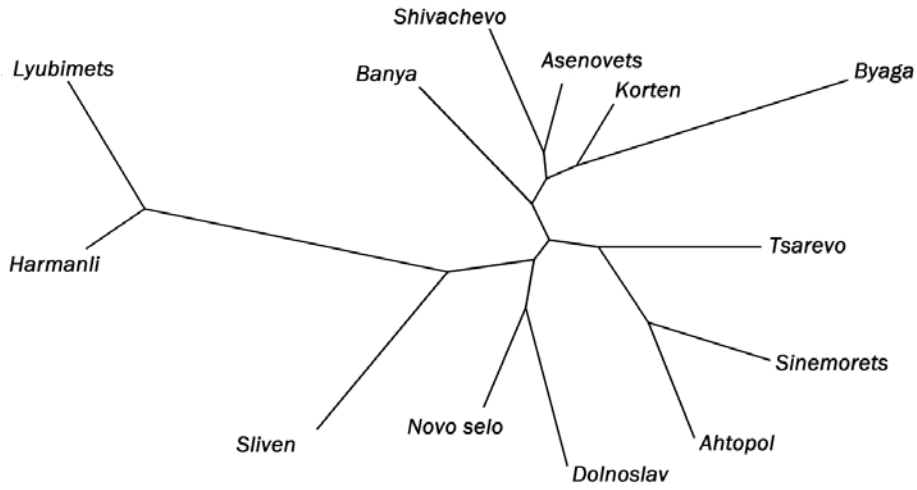

Fig. 3. Neighbor-joining dendrogram (Saitou & Nei 1987) presenting relationships of populations of *Messor barbarus*

loci, and the lowest degree in Est-8 (0.0842) and Sp-5 (0.0895) loci. The parameters, designated as F_{IS} and F_{IT}, are inbreeding-coefficients. F_{IS} is used to determine the genetic variation within the population and F_{IT} – among all the populations studied. The summarized results show that the average value of F_{IS} for all loci of *Messor barbarus* species is high – 0.8212 (Table 2). This demonstrates a high level of inbreeding in the populations studied (Conner & Hartl 2004) and correlates with the high average value of F_{IT} (0.8584) showing a deficit of heterozygotes in these populations and corresponding to the level of 85.84% of the expectations according to Hardy-Weinberg equilibrium.

Genetic distance

The obtained data on genetic variation in populations of *Messor barbarus* provide an opportunity to identify and analyze the phylogenetic relationships between the studied populations. Based on the allele frequencies, we calculated the genetic distance according to Nei (Nei 1972). In this basis, by applying the two methods of graphic analysis (UPGMA и NJ), we have built UPGMA and NJ dendrograms (Fig. 2, 3). The juxtaposed populations of *Messor barbarus* are grouped in two clusters. The first cluster includes Lyubimets and Harmanli populations, which is divergent from the remaining populations grouped in the second cluster. The Tsarevo, Sinemorets and Ahtopol populations are distinctly separated in a distinct subcluster. The detected specificity in the composition of the gene pools determines the genetic differentiation of the populations of *Messor barbarus*.

Conclusion

Through the present electrophoretic analysis, the genetic heterogeneity of populations of *Messor barbarus* was determined. Polymorphism was characterised and, on this basis, the composition of the gene pool and genotypic structure of the studied populations was analysed. Specificity in allelic composition of the tested populations and differences in allele frequencies were identified. A comparative analysis of genotypic structure of the studied populations has been carried out and intraspecific phylogenetic relationships have been studied. The comparative analysis of allele frequencies in the tested populations of *Messor barbarus* revealed differences in their values, thus demonstrating dependence on the territorial location of populations. This can be explained with colonisation of close geographical locations and the founder effect.

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