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Soluble proteins in *Messor structor* (Latreille, 1798) (Hymenoptera: Formicidae) populations from Bulgaria – genetic variability and possible usage as population-genetic markers

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Abstract. The genetic variability of ant species determined as *Messor structor* (Latreille, 1798) or close to it ("*M.* cf. *structor*") from Bulgaria has been studied using polyacrylamide gel electrophoresis analysis of five soluble protein systems (Sp-1, Sp-2, Sp-3, Sp-4 and Sp-5) corresponding to 5 loci. Four of the studied loci were found to be polymorphic. Two alleles were detected at Sp-1 and Sp-2 loci and three – at Sp-3 and Sp-5. The observed and expected heterozygosities (H_o and H_e) ranged from 0.0 (Yambol) to 0.140 (Topolovo) and from 0.170 (Nova Zagora) to 0.401 (Tvarditsa), respectively. The calculated mean value of inbreeding coefficient (F_{IS}) was 0.8263 and demonstrated high level of inbreeding within populations, which correlated with a low level of observed heterozygosity compared to the expected one. The estimated mean fixation index (F_{ST}) value was 0.2746. Allele frequencies of soluble protein loci were used to estimate Nei's (1972) genetic distance and to obtain the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Neighbor-Joining (NJ) dendrograms, where Topolovo and Nova Zagora populations were grouped separately than other populations.

Key words: *Messor structor*, soluble proteins, genetic variability, phylogeny.

Introduction

The harvester ant *Messor structor* (Latreille, 1798) is well adapted to different climatic conditions. Being one of the most widespread steppe species, it occurs throughout the European continent. In Bulgaria this species could be found in medium sized populations along all over the country. Harvester ant occur in plain and mountainous terrains – slopes, outskirts, different woodlands, abandoned fields, pastures, unkept lawns, road right of ways. As haploid social insects, they could be an interesting model for different investigations dedicated to behavior ecology, population genetics and phylogeny. (Brian 1983, Cantagali *et al.* 2010, Höldobler & Wilson 1990).

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Although different DNA and isozyme analysis have been done for the ant genetic characterization, (Cantagali *et al.* 2010, Diel *et al.* 2002, Hagen *et al.* 1988, Krieger *et al.* 1999, Pamilo *et al.* 1975, Ross *et al.* 2003, Schlick-Steiner *et al.* 2005, Tomaszevski *et al.* 1973), *M. structor* populations have not been enough investigated. Schlick-Steiner et al. (2006) have studied genetic diversity of ants determined as *Messor structor*, collected from different countries in Central Europe and have found out two major mtDNA lineages with partial overlapping distribution. According to this study, specimens from both lineages occur in Bulgaria. These results lead to the idea of possible existence of cryptic species within *M. structor* group.

As a simple method, electrophoresis has been applied for studying the protein and isozyme polymorphisms which exist at levels high enough (about 30% of all loci) for the population genetics aims (Kimura 1968, Tomaszevski *et al.* 1973). There was not much information found concerning population-genetic studies of soluble proteins in different eusocial Hymenoptera species – electrophoretic surveys of available polymorphic protein markers have been conducted for three fire ant species and for two forms of *Solenopsis invicta* (Buren) (Ross *et al.* 1987, Shoemaker *et al.*1992). In this aspect and having this lack of information in mind, the aim of the study was to evaluate soluble proteins as suitable genetic markers for characterization of genetic variability among populations of harvester ant *M. cf. structor* in Bulgaria.

Material and Methods

Messor structor samples

Totally 310 *M. srtuctor* workers from ten nests, each from different unicolonial geographically distinct population from Southern part of Bulgaria were tested in this study (Fig. 1). The workers were collected directly from the nest. Three to five of the collected ants from a nest were used for the species identification on the base of classical morphometry. According to key of Agosti & Collingwood (1987) and Atanasov & Dluskyi (1992), workers were determined as belonging to *Messor structor* or close to it ("*Messor* cf. *structor*"). Other individuals were stored at -20ºC until electrophoresis. From 30 to 36 workers per population were analyzed for electrophoretic spectrum of soluble proteins (Table 1).

Electrophoretic analysis

The ants were squashed with quartz sand in 0.8 M tris–phosphate buffer at pH 6.7 and left for extraction for 18 hrs at 4 °C. The total body extracts were centrifuged for 15 min at 5 000 rpm at 4 °C and the individual samples were turned to use for electrophoretic analyses. The electrophoretic separation was done in 7.5% polyacrylamide vertical gel (pH 8.9) at 4.5 mA/cm for 3 hrs, together with 3.3% concentrating gel (pH 6.7) and 0.05 M tris–0.2 M glycine electrode buffer at pH 8.3 (Davis 1964, Maurer 1971, Ivanova *et al* 2000). Soluble proteins were displayed by staining with 5% Commassie Brilliant Blue R250 in 14% trichloroacetic acid.

Fig. 1. Sampling locations.

Table 1. Populations, number of sampled workers tested and allele frequencies (N – number of workers sampled).

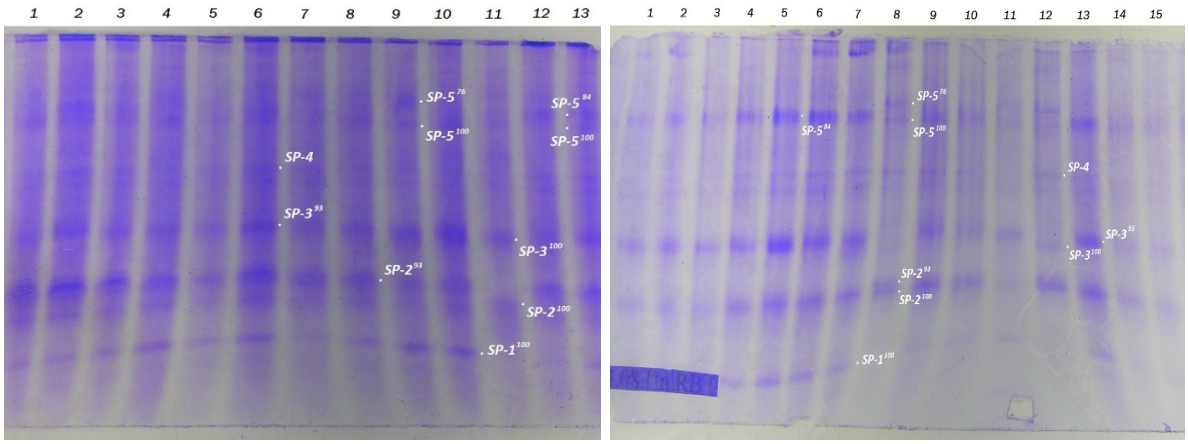
Population	N	Locus										
		Sp-1		Sp-2		Sp-3			Sp-4	Sp-5		
		Sp-1^{100}	Sp-1^{97}	Sp-2^{100}	Sp-2^{96}	Sp-3^{100}	Sp-3^{95}	Sp-3^{87}	Sp-4^{100}	Sp-5^{100}	Sp-5^{89}	Sp-5^{77}
Nova Zagora	30	0.933	0.067	0.050	0.950	0.867	0.133	0	1	0.759	0.190	0.052
Elenovo	36	0.743	0.257	0.181	0.819	0.361	0.639	0	1	0.389	0.542	0.069
Yambol	31	1	0	1	0	0.097	0.774	0.129	1	0.387	0.613	0
Plovdiv	32	1	0	0.786	0.214	0.313	0.406	0.281	1	0.625	0.250	0.125
Pesnopoy	31	0.968	0.032	0.048	0.952	0.194	0.806	0	1	0.625	0.250	0.125
Tsalapitsa	30	1	0	0.517	0.483	0.037	0.630	0.333	1	0.083	0.833	0.083
Topolovo	30	0.233	0.767	0	1	0.283	0.517	0.200	1	0.500	0.067	0.433
Velingrad	30	0.933	0.067	0.283	0.717	0.167	0.733	0.100	1	0.167	0.517	0.317
Tvarditsa	30	0.467	0.533	0.383	0.617	0.067	0.667	0.267	1	0.357	0.589	0.054
Merichleri	30	0.717	0.283	0.383	0.617	0.217	0.783	0	1	0.583	0.167	0.250

Statistical analyses

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, Nei's genetic distance (D), (Nei 1972) and F_{ST} values, (Wright 1965) were calculated using BIOSYS-1 (Swofford 1981) software package. Phylogenetic UPGMA, (Sneath *et al.* 1973) and neighbor-joining, (Saitou & Nei 1987) trees were constructed by usage of Nei's genetic distance, (Nei 1972) and the PHYLIP, (Felsenstein 1993) software package.

Results and Discussion

Samples of protein electrophoretic spectrums could be seen in Fig. 2.



a. **b.**

Fig. 2. Spectrum of soluble proteins of *M. structor* workers (7.5% PAGE): **a.** Elenovo population; **b.** Tsalapitsa population.

The allele frequencies calculated are presented in Table 1. Four of the studied protein loci (marked as Sp-1, Sp-2, Sp-3 and Sp-5) were polymorphic in almost of all of the populations, at the 95% level. Sp-4 locus was fixed in all studied populations. In total, two alleles were detected at Sp-1 (Sp-1^{100} and Sp-1^{97}) and Sp-2 (Sp-2^{100} and Sp-2^{96}) loci and three alleles – at Sp-3 (Sp-3^{100}, Sp-3^{95} and Sp-3^{87}) and Sp-5 (Sp-5^{100}, Sp-5^{89} and Sp-5^{77}) loci.

The most frequent alleles in the most of the populations were Sp-1^{100} (except in Topolovo and Tvarditsa), Sp-2^{96} (except in Yambol, Plovdiv and Tsalapitsa) and Sp-3^{95}(except in Nova Zagora, where Sp-3^{100} was more frequent allele). Sp-5^{100} was detected as more or the most frequent allele in Nova Zagora, Plovdiv, Pesnopoy, Topolovo and Merichleri. Sp-5^{89} was found as more or the most frequent allele in Elenovo, Yambol, Tsalapitsa, Velingrad and Tvatditsa. Differently than Sp-4 locus which was monomorphic in all of the studied populations with fixed Sp-4^{100} allele, Sp-1^{100} allele was fixed in populations of Yambol, Plovdiv and Tsalapitsa, Sp-2^{100} – in Yambol population and Sp-2^{96} – in Topolovo population.

Mean sample size per locus, mean number of alleles per locus, proportions of polymorphism, observed and expected heterozygosity in the populations tested are presented in Table 2.

Table 2. Mean sample size per locus, mean number of alleles, percentage of polymorphic loci, observed and expected heterozygosity in the populations tested (Standard Errors are included).

Population	Mean sample size per locus	Mean number of alleles per locus	Percentage of loci polymorphic	Mean heterozygosity	
				(Ho)	(He)
Nova Zagora	29.8±0.2	2.0±0.3	80.0	0.014±0.008	0.170±0.067
Elenovo	35.8±0.2	2.0±0.3	80.0	0.094±0.065	0.343±0.096
Yambol	31.0 ±0.0	1.6±0.4	40.0	0.000±0.000	0.173±0.107
Plovdiv	31.2±0.8	2.0±0.4	60.0	0.013±0.013	0.310±0.137
Pesnopoy	31.2±0.2	2.0±0.3	40.0	0.083±0.061	0.203±0.100
Tsalapitsa	29.4±0.6	2.0±0.4	60.0	0.028±0.014	0.261±0.113
Topolovo	30.0±0.0	2.0±0.4	60.0	0.140±0.116	0.311±0.134
Velingrad	30.0±0.0	2.2±0.4	80.0	0.013±0.008	0.317±0.111
Tvarditsa	29.6±0.4	2.2±0.4	80.0	0.054±0.017	0.401±0.101
Merichleri	30.0 ±0.0	2.0±0.3	80.0	0.040±0.016	0.364±0.099

According to the results of this study, the mean number of alleles per locus varied from 1.6 (Yambol) to 2.2 (Velingrad and Tvarditsa). The estimated percentage of polymorphic loci was 40% in Yambol and Pesnopoy, 60% in Plovdiv, Tsalapitsa and Topolovo, 80% in all other populations, using the 0.95 criterion (Table 3). The observed and expected heterozygosities (H_o and H_e) ranged from 0.0 (Yambol) to 0.140 (Topolovo) and from 0.170 (Nova Zagora) to 0.401 (Tvarditsa), respectively (Table 3). There are significant deviations of genotype frequencies from Hardy-Weinberg expectations at all of the loci in most populations ($0.005 \geq P$). Chi-Square (df: 1-3) tests showed that the deviations were generally in favour of homozygotes.

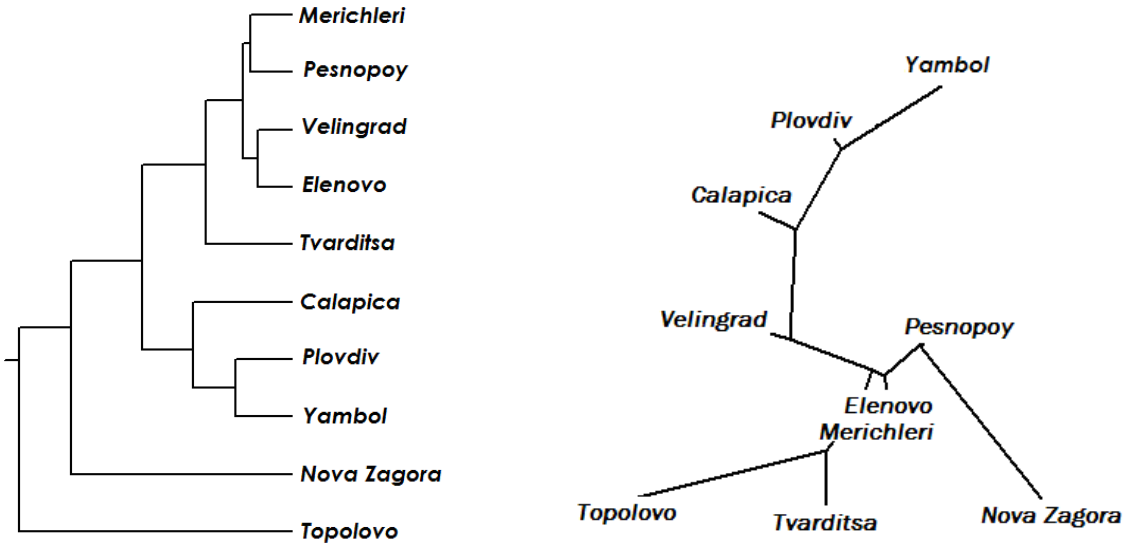
F statistics for the polymorphic loci are presented in Table 3. The estimated mean F_{ST} and F_{IS} values were 0.2746 and 0.8263, respectively. Allele frequencies of soluble protein loci were used to estimate Nei's genetic distance and to obtain the UPGMA and NJ dendrograms, where Topolovo and Nova Zagora populations were grouped separately than other populations (Fig. 3).

Proteins were used fragmentary as electrophoretic markers for genetic characterization of some ants. Shoemaker *et al.* (1992) surveyed enzyme and protein activity in red fire ant *Solenopsis invicta* (Buren) in order to estimate the heterosygosity in populations of these social insects. A total of 110 putative loci were revealed for the fire ants, 15 of which were polymorphic at the 95% level. No polymorphic protein loci were reported in this study (Shoemaker *et al.* 1992). Ross *et al.* (2007) conducted comparative biochemical genetic investigation on three fire ant species in North America with special preference to the two social forms of *S. invicta*. Nineteen enzyme and general protein systems were surveyed yielding the products of 26 presumptive loci. Three of the studied loci were protein – Pro-1, Pro-2 and Pro-3. Rare alleles (frequency less than 0.006) were found at Pro-1 and Pro-3 loci. Pro-3 was defined as polymorphic in *Solenopsis richteri* (Forel) but without useful variability (p_1 – 0.983, q_2 – 0.017) (Ross *et al.* 2007). Differently than in mentioned surveys, in our study, we found most soluble protein loci as polymorphic. Only Sp-4^{100} was fixed in all the studied Bulgarian populations. Sp-$1^{\mathbf{100}}$, Sp-$2^{\mathbf{100}}$ and Sp-$2^{\mathbf{96}}$ alleles were fixed in only some of populations (Table 1) which indicates them as possible genetic markers. It must be also noted that differently that in surveys mentioned above, in all Bulgarian populations, high percentage of polymorphic loci was found (40% - 80%). Data

established by us concerning the polymorphism of protein loci are in agreement with these concerning genetic polymorphism found in the mitochondrial *COI* gene in *Messor* cf. *structor* workers collected from different countries in Central Europe including Bulgaria (Schlick-Steiner *et al.* 2006). Schlick-Steiner *et al.* (2006) founded out two major mtDNA lineages. Polymorphism in *M. structor* populations from the region near Retz (Austria) was described by Arthofer *et al.* (2005), too. On the base of the microsatellite polymorphism found (seven microsatellite loci with 21 alleles), the authors determined *M. structor* as a model organism for studying the habitat fragmentation influence on social and population structure of social insects (Arthofer *et al.* 2005).

Table 3. F-statistics for polymorphic loci studied.

F - statistics for individual alleles		F(IS)	F(ST)	F(IT)
Locus:Sp-1	Sp-1^{100}	0.9175	0.3847	0.9492
	Sp-1^{97}	0.9175	0.3847	0.9492
	Mean	0.9175	0.3847	0.9492
Locus:Sp-2	Sp-2^{100}	0.8401	0.4218	0.9076
	Sp-2^{96}	0.8401	0.4218	0.9076
	Mean	0.8401	0.4218	0.9076
Locus:Sp-3	Sp-3^{100}	0.8197	0.2558	0.8658
	Sp-3^{95}	0.8528	0.1632	0.8769
	Sp-3^{87}	0.9659	0.1388	0.9706
	Mean	0.8665	0.1914	0.8921
Locus:Sp-5	Sp-5^{100}	0.7183	0.1611	0.7637
	Sp-5^{89}	0.8777	0.2276	0.9055
	Sp-5^{77}	0.5875	0.1325	0.6422
	Mean	0.7485	0.1812	0.7941
Summary of F - statistcs at all loci		F(IS)	F(ST)	F(IT)
Locus: Sp-1		0.9175	0.3847	0.9492
Locus: Sp-2		0.8401	0.4218	0.9076
Locus: Sp-3		0.8665	0.1914	0.8921
Locus: Sp-5		0.7485	0.1812	0.7941
Mean		0.8263	0.2746	0.874



a. **b.**

Fig. 3. a. UPGMA dendrogram (Sneath *et al.* 1973); **b.** Neighbour-joining dendrogram (Saitou & Nei 1987).

The expected heterozygosity (H_e) by polymorphic protein loci in Bulgarian populations was higher than the observed one (H_o) in all tested populations and ranged from 0 to 0.170. Calculated mean F_{IS} value over all loci was very high (0.8263), demonstrating high level of inbreeding within populations, (Conner & Hartl 2004) which was in correlation with a low level of observed heterozygosity and disequilibrium. Arthofer *et al.* (2005) noted that deviation from Hardy-Weinberg equilibrium might be partially due to habitat fragmentation.

F_{ST}, which shows the correlation between genes of different individuals within population, (Nei 1977, Weir *et al.* 1984) indicates extend of genetic differentiation among the populations. The mean F_{ST} value of 0.2746 from our study indicates that 27.46% of the overall genetic diversity observed was among populations, which shows that more than 72% of the observed genetic variability resides within populations analysed in our study. This value indicates relatively high level of genetic differentiation among the populations for polymorphic loci studied (Table 3).

The topology of both UPGMA and NJ dendrograms (Fig. 3) confirm the level of genetic diversity within *Messor* cf. *structor* populations studied and the genetic differentiation among them. In the dendrograms constructed two populations (Topolovo and Nova Zagora) are clearly separated than others which are grouped in two different sub-clusters.

Conclusions

In conclusion, this study provides new information on the genetic variability in populations of harvester ant *M.* cf. *structor* in Bulgaria on the base of protein electrophoretic analysis. The indicated protein electrophoretic markers could be used appropriately together with other genetic markers for comparisons, discrimination and characterization of *M. structor* populations. Further complex investigations, including different methods and additional genetic markers have to be done, in order to analyze in details genetic structure of *M. structor* populations in Bulgarian and European.

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