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Allozyme Polymorphism and Phylogenetic Relationships in *Apis mellifera* Subspecies Selectively Reared in Poland and Bulgaria

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The genetic variability of honey bee populations of three subspecies selectively reared in Poland (*A. m. carnica* and *A. m. caucasica*) and Bulgaria (*A. m. macedonica* – type *rodopica*) was studied using isoenzyme analysis of six enzyme systems (MDH-1, ME, EST-3, ALP, PGM and HK) corresponding to 6 loci. All loci were found to be polymorphic in the studied populations. Three alleles were detected at each locus: MHD-1 (MDH^{65}, MDH^{80} and MDH^{100}), Me (ME^{90}, ME^{100} and ME^{106}), EST-3 (EST^{94}, EST^{100} and EST^{118}), ALP (ALP^{80}, ALP^{90} and ALP^{100}), PGM (PGM80, PGM^{100} and PGM^{114}) and HK (HK^{87}, HK^{100} and HK^{110}). The observed and expected heterozygosities (H_o and H_e) ranged from 0.196 (*A. m. macedonica* SM) to 0.265 (*A. m. carnica* MV) and from 0.224 (*A. m. macedonica* SM) to 0.273 (*A. m. carnica* GR), respectively. Allele frequencies of all loci were used to estimate Nei's (1972) genetic distance, which was found to range from 0.003 (between *A. m. macedonica* TR and SM and between *A. m. carnica* GR and MV populations) to 0.057 (between *A. m. macedonica* SM and *A. m. caucasica* populations). The estimated mean F_{ST} value from allozyme data was 0.0364. A UPGMA dendrogram was obtained by genetic distance matrix methods; *A. m. macedonica* (type *rodopica*), *A. m. carnica* and *A. m. caucasica* populations represented different clades.

Key words: Honey bee, *Apis mellifera*, allozymes, genetic variability, phylogeny.

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Apis mellifera L. is native to Europe, Africa, and Asia and across this vast natural range honeybee populations show considerable differences in many biological characters as a result of historical patterns of isolation and adaptation to particular habitats. Based on morphological, behavioral and molecular evidence, about 26 subspecies and numerous ''ecotypes'' of *A. mellifera* have been described (MEIXNER *et al*. 2009). These subspecies are classified into four main lineages: C (Carnica group); M (north and western European group); A (African group); and the O group (Oriental group) (RUTTNER 1992). M and C branches predominate in Europe, composed of west Mediterranean and north European 'M-subspecies' (*A. m. iberiensis* and *A. m. mellifera*) and central and southeast European 'C-subspecies' such as *A. m. ligustica*, *A. m. cecropia*, *A. m. macedonica* and *A. m. carnica* (DE LA RUA *et al*. 2009; SOLORZANO *et al*. 2009). *A. m. ligustica* and *A. m. carnica* have attracted the attention of many beekeepers worldwide. Originally, *A. m. ligustica* occured in the Italian Peninsula but it has been commercially transported throughout the world. These bees have hybridized extensively with *A. m. mellifera* and *A. m. carnica* in the north. Already in the 18th century the Carniolan bee *A. m. carnica* was well known throughout Europe (RUTTNER 1988). The original range of this subspecies extends across central and eastern Europe. Moreover, the small Carinthian hives, traditionally employed by beekeepers along the Austrian-Slovenian border, facilitated the transport of *A. m. carnica* bees outside their natural range. The intense dissemination of *A. m. ligustica* and *A. m. carnica* throughout the European continent has resulted in the almost complete replace-

ment of *A. m. mellifera* by *A. m. carnica* in central European countries (JENSEN *et al*. 2005; DE La RUA *et al*. 2009). The Grey Caucasian honey bee *A. m.caucasica* (which belongs to the O group) is also among the preferred subspecies. It has been intensively used by beekeepers for more than 100 years (RUTTNER 1988). The natural range of *A. m. caucasica* has been artificially extended from the Caucasus to western Turkey and Bulgaria (IVANOVA *et al*. 2007). A significant number of hives have been introduced into Russia, Ukraine, Germany, Poland and France (GROMISZ 1978, 1997; RUTTNER 1988). *A. m. macedonica* extends across eastern Europe from the Ukraine and Bulgaria, to the northern part of Greece. Eastern Europe has also been subject to the introduction of foreign honeybee subspecies and in Bulgaria *A. m. macedonica* has been hybridized with *A. m. ligustica*, *A. m. carnica* and *A. m. caucasica* for more than three decades (IVANOVA *et al*. 2007; IVANOVA 2010; IVANOVA *et al*. 2010a).

Various genetic tools, such as DNA sequence analysis and allozyme electrophoresis, have recently been applied to the study of honey bee genetic diversity. Allozymes have been used successfully for studying the discrimination between subspecies (NUNAMAKER *et al*. 1984; SYLVESTER 1982, 1986; DALY 1991; BOUGA *et al*. 2005; IVANOVA *et al*. 2010b) and for revealing the existence of hybrid zones between them (SHEPPARD & MCPHERON 1986). They were also used for analyzing the phylogeny of *A. mellifera* on the basis of genetic distance matrices (SHEPPARD & HUETTEL 1988) and for the detection of genetic differences between commercial and natural honey bee populations (SCHIFF & SHEPPARD 1995).

In this study honey bee populations of *A. m. carnica*, *A. m. caucasica* and *A. m. macedonica*, which are under selective control in Poland and Bulgaria, were studied using polyacrylamide gel electrophoresis on six different gene-enzyme systems. The purpose of the research was to investigate and characterize genetic variability in populations and phylogenetic relationships between subspecies studied using allozyme analysis.

Material and Methods

Honey bee samples

Samples were collected from selection bases in Poland and Bulgaria. Worker honey bees from colonies with instrumentally inseminated queens were used for this study. Two lines of *A. m. carnica* (marked as GR and MV) and *A. m. caucasica*, both subspecies reared in Poland and two local Bulgarian lines *A. m. macedonica* – type "*rodopica"* (PETROV 1995) honey bees (marked here as TR and SM) were used for this research. In total ive colonies per population, 8 to 10 individuals per colony) were tested. Collected worker bees were transported to the laboratory alive and stored at -20^{o}C until use.

Allozyme analysis

Thorax homogenization and electrophoresis in polyacrylamide gels were done according to IVANOVA (1996). Six enzyme systems were studied: MDH (malate dehydrogenase, EC 1.1.1.37); ME (malic enzyme, EC 1.1.1.40); EST (esterase, EC 3.1.1), ALP (alkaline phosphatase, EC 3.1.3.1); PGM (Phosphoglucomutase, EC 5.4.2.2) and HK (Hexokinase, EC 2.7.1.1). Buffers and electrophoretic conditions for each enzyme system were as in BOYER (1961), GAHNE (1967), SHAW and PRASAD (1970) and IVANOVA (1996). Enzyme activities were visualized by histochemical staining () and allozymes were numbered according to their relative anodal mobility.

Statistical Analyses

Allele frequencies, mean number of alleles per locus, proportion of polymorphic loci at the 95% level, observed (H_o) and expected (H_e) heterozygosities, deviation from Hardy-Weinberg equilibrium and Nei's genetic distances (D) (NEI 1972), were calculated using BIOSYS-1 (SWOFFORD & SELANDER 1981). Phylogenetic trees were constructed using NEI's (1972) genetic distance, by the UPGMA (SNEATH & SOKAL 1973) method using the PHYLIP (FELSENSTEIN 1993) software package.

Results

The enzyme systems studied (MDH-1, ME, EST-3, ALP, PGM and HK) were polymorphic in all of the populations, at the 95% level, having two to three different alleles in populations (Tables 1 and 2). In total, three alleles were detected at Mdh-1 (MDH^{65}, MDH^{80} and MDH^{100}), Me (ME^{90}, ME^{100} and ME^{106}), Est-3 (EST^{94}, EST^{100} and EST^{118}) Alp (ALP^{80}, ALP^{90} and ALP^{100}), Pgm (PGM^{80}, PGM^{100} and PGM^{114}) and Hk (HK^{87}, HK^{100} and HK^{110}).

The mean number of alleles per locus varied from 2.0 (*A. m. macedonica* SM) to 2.7 (*A. m. caucasica*). The estimated percentage of polymorphic loci was 66.7% – in most of the populations, 83.3% in *A. m. macedonica* SM population and, 100% in *A.m.caucasica* population, P = 0.95%.

Table 1

Allele frequencies in the populations studied

Locus and alleles	*A. m. carnica G*R	*A. m. carnica* MV	*A. m. caucasica*	*A. m. macedonica* TR	*A. m. macedonica* SM
MDH-1					
65	0.4	0.48	0.235	0.36	0.405
100	0.52	0.5	0.551	0.64	0.595
80	0.08	0.02	0.214	0	0
ME					
100	0.891	0.9	0.826	0.879	0.935
106	0.109	0.1	0.174	0.086	0.065
90	0	0	0	0.034	0
EST-3					
100	0.974	0.972	0.944	0.976	0.946
118	0	0.028	0.028	0.008	0.054
94	0.026	0	0.028	0.016	0
ALP					
80	0.389	0.324	0.24	0.535	0.575
100	0.407	0.5	0.38	0.465	0.425
90	0.204	0.176	0.38	0	0
PGM					
100	0.935	0.913	0.923	0.935	0.957
114	0.065	0.087	0.058	0.065	0.043
80	0	0	0.019	0	0
HK					
87	0.017	0.016	0	0.063	0
100	0.983	0.984	0.929	0.896	0.978
110	0	0	0.071	0.042	0.022

Table 2

Percent of polymorphic loci, observed (Ho) and expected (He) heterozygosity values in the populations studied

Population	Mean no. of alleles per locus (± S.E.)	Percent Polymorphic loci (P=0.95)	H_o	H_e
A. m. carnica GR	2.3±0.2	66.7	0.232±0.095	0.273±0.111
A. m. carnica MV	2.3±0.2	66.7	0.265±0.106	0.266±0.103
A. m. caucasica	2.7±0.2	100	0.234±0.109	0.326±0.101
A. m. macedonica TR	2.5±0.2	83.3	0.23±0.115	0.258±0.075
A. m. macedonica SM	2±0	66.7	0.196±0.083	0.224±0.086

Table 3

Nei's genetic distances

Population	*A. m. carnica* GR	*A. m. carnica* MV	*A. m. caucasica*	*A. m. macedonica* TR	*A. m. macedonica* SM
A. m. carnica GR	***	0.003	0.013	0.012	0.01
A. m. carnica MV		***	0.021	0.014	0.013
A. m. caucasica			***	0.038	0.041
A. m. macedonica TR				***	0.003
A. m. macedonica SM					***

The observed and expected heterozygosities (H_o and H_e) ranged from 0.196 (*A. m. macedonica* SM) to 0.265 (*A. m. carnica* MV) and from 0.224 (*A. m. macedonica* SM) to 0.273 (*A. m. carnica* GR), respectively (Table 2).

We detected significant deviations of genotype frequencies from Hardy-Weinberg expectations at most of the loci in most populations ($P \geq 0.001$). Chi-Square (df: 1-3) tests showed that the deviations were generally in favor of homozygotes.

The estimated mean F_{ST} value was 0.0364 which shows that 3.64% of the overall genetic diversity observed was among populations, as opposed to 96.36% within populations.

Genetic distance values (NEI 1972) were calculated using the allele frequencies (Table 1) and ranged from 0.003 (between *A. m. macedonica* TR and SM and between *A. m. carnica* GR and MV populations) to 0.057 (between *A. m. macedonica* SM and *A. m. caucasica* populations) – Table 3.

In the UPGMA phylogenetic tree, *A. m. caucasica* clustered separately from *A. m. carnica* and *A. m. macedonica.* The populations studied formed three clades (Fig. 1).

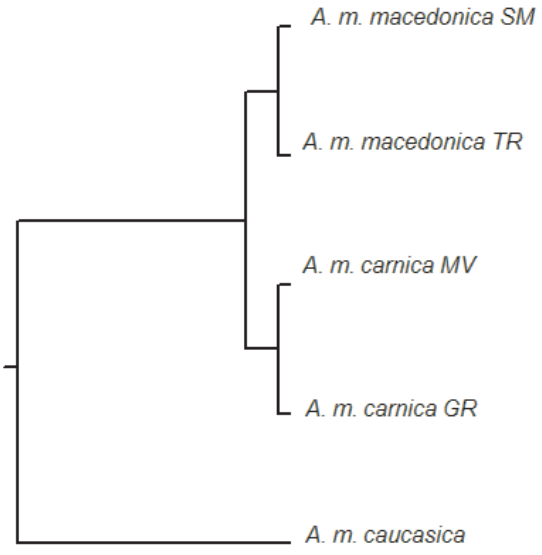

Fig. 1. Relationships between populations as shown in UPGMA dendrogram.

Discussion

All studied enzyme loci were found to be polymorphic. In similar investigations, in total five alleles at the MDH-1 locus were detected (GARDSIDE 1980; NUNAMAKER *et al*. 1984; BADINO *et al*. 1983, 1985, 1988; SHEPPARD 1988; SHEPPARD & BERLOCHER 1984, 1985; SHEPPARD & MCPHERON 1986; LOBO *et al.* 1989; MEIXNER *et al*. 1994; KANDEMIR & KENCE 1995; KANDEMIR *et al*. 2000; BOUGA *et al*. 2005) in different populations from Europe, Brazil and the USA. In the present research we observed three alleles at this locus in Polish populations of *A. m. carnica* and *A. m. caucasica* and two alleles in Bulgarian populations of *A. m. macedonica*, where MDH^{80} was absent. MDH^{100} was the most common allele in all populations. MDH^{80} was found to reach highest frequency in *A. m. caucasica* (0.214) population, while its frequency was lower in both *A. m. carnica* lines – 0.02 and 0.08 for MV and GR, respectively. DEDEJ *et al*. (1996) reported two MDH-1 alleles (MDH^{100} and MDH^{65}) for *A. m. macedonica* in Greece but according to BOUGA *et al.* (2005) this locus has three alleles (MDH^{100}, MDH^{80} and MDH^{65}) in Greece and the most frequent of them is MDH^{80}. The frequency of MDH^{100}, according to BADINO *et al*. (1988), decreases from southern to northern Greece, but remains high in the eastern region near the border with Turkey. One more allele – MDH^{125} was found in *A. m. carnica* from Serbia (IVANOVA 2010) where in a population from Alexinatz four alleles at the MDH-1 locus were observed.

Three alleles were found at the ME locus (ME^{70}, ME^{100} and ME^{106}) in *A. mellifera* populations in Norway (SHEPPARD & BERLOCHER 1984), Italy (SHEPPARD & BERLOCHER 1985) and western Czechoslovakia (SHEPPARD & MCPHERON 1986). This locus was nearly fixed in Kenya where, in one colony, a previously unknown allele, ME^{117} was found (MEIXNER *et al*. 1994). The ME locus was found to be invariant in honeybee populations from Turkey (KANDEMIR *et al*. 2000, 2005). DEDEJ *et al*. (1996) reported no polymorphism in the ME locus, but according to BOUGA *et al*. (2005) this locus is polymorphic with two alleles – ME^{100} and ME^{79} in *A. m. macedonica* populations from Greece. ME^{100} was fixed in *A. m. carnica* populations from Serbia (IVANOVA *et al*. 2010b). In our study ME^{100} and ME^{106} alleles were detected and the frequency of ME^{100} was higher in all populations studied. The ME^{106} allele achieved its lowest frequency in "macedonica" bees (0.065-0.086) and highest frequency in "caucasica" bees (0.174). The frequencies of this allele in Polish "carnica" bees was 0.109 and 0.1 (for GR and MV lines, respectively) - Table 1. One more allele – ME^{90} was observed in an *A. m. macedonica* TR population where its frequency was calculated as 0.034.

The EST-3 locus was polymorphic and exhibited three alleles, EST^{70}, EST^{100} and EST^{130} in Czechoslovakian (SHEPPARD & MCPHERON 1986) and in central Anatolian honey bees (KANDEMIR & KENCE 1995). Three alleles were also detected

in *A. m. macedonica* from Greece (BOUGA *et al*. 2005). IVANOVA *et al*. (2004) reported that EST^{100} was fixed in Rhodopes mountainous regions of Bulgaria and its frequency is rather high in Thrace regions of Bulgaria and Turkey (0.96). In the present investigation (Table 1) the EST-3 locus had three alleles in *A. m. caucasica* and *A. m. macedonica* TR populations and two (EST^{94} and EST^{100} or EST^{100} and EST^{118}) – in others. EST^{100} was the most common allele in all populations studied. In previous studies (IVANOVA 2010; IVANOVA *et al*. 2010) three other alleles at the EST-3 locus (EST^{80}, EST^{88} and EST^{105}) were described in populations of *A. m. carnica* and *A. m. macedonica* from Serbia, Montenegro, Bulgaria and Greece.

The ALP locus was polymorphic with two alleles, ALP^{100} and ALP^{80}. ALP^{80} was the more frequent allele in Greece (BOUGA *et al*. 2005) and in Bulgaria (IVANOVA *et al*. 2010). In the present research the ALP locus had three alleles in "carnica" and "caucasica" and two in "macedonica" honey bees. ALP^{80} was the more frequent allele in Bulgarian populations (*A. m. macedonica*) while ALP^{100} was the most common allele in Polish *A. m. carnica* populations. In Polish *A. m. caucasica* ALP^{90} and ALP^{100} exhibited equal frequencies (0.38) – Table 1.

The PGM locus was studied by many researchers (MESTRINER & CONTEL 1972; BRUECKNER 1974; NUNAMAKER & WILSON 1980; BADINO *et al*. 1983; SHEPPARD & BERLOCHER 1985) but DEL LAMA *et al*. (1985) first reported the presence of three alleles at this locus in Africanized bee populations and two alleles in *A. m. carnica* originating from Germany. MEIXNER *et al*. (1994) found three alleles of which PGM^{120} was previously unreported. The PGM locus was found to be polymorphic with two alleles (PGM^{100} and PGM^{114}) in populations from Serbia, Montenegro, Bulgaria and Greece (IVANOVA *et al*. 2010a, b), where PGM^{100} was the more common (in most of the populations) or fixed (in a Serbian population) allele. In the present study a third allele (PGM^{80}) was found in an *A. m. caucasica* population from Poland. Its frequency was calculated as 0.019.

The HK locus was monomorphic in Norwegian, Italian (SHEPPARD & BERLOCHER 1985), Czechoslovakian (SHEPPARD & MCPHERON 1986), Greek (BADINO *et al*. 1988) and German (DEL LAMA *et al*. 1990) honeybee populations. It is polymorphic with two alleles (HK^{87} and HK^{100}) in Africanized bee populations from Brazil and Central America (DEL LAMA *et al*. 1988, 1990). Later studies determined four alleles at this locus (KANDEMIR & KENCE 1995). KANDEMIR *et al*. (2000) detected one more allele – HK^{77} in honey bee populations from Turkey. In our study three alleles were found at the HK locus (HK^{87}, HK^{100} and HK^{110}). HK^{100} was the more common variant in all populations studied. In the Bulgarian *A. m. macedonica* TR population all three alleles were present, while in other populations two were found – HK^{87} and HK^{100} – in "carnica" bees from Poland, HK^{100} and HK^{110} – in *A. m. caucasica* and *A. m. macedonica* SM populations. In a previous study one more allele – HK^{121} was found in Serbian *A. m. carnica* populations (IVANOVA *et al*. 2010b).

A high percentage of polymorphic loci (66.7%-100%) was found in all populations. The F_{ST} value of 0.0364 indicates a low level of genetic differentiation among populations.

The UPMGA phylogenetic tree clustered the population of *A. m. caucasica* reared in Poland in a separate branch and *A. m. carnica* and *A. m. macedonica* populations – in two other closely related clusters.

The results of this research provide new information concerning the genetic variability in *A. m. carnica, A. m. caucasica*, and *A. m. macedonica* honeybee populations from Poland and Bulgaria. Data on allozyme variability in "carnica" and "caucasica" populations from Poland and their comparison with Bulgarian *A. m. macedonica* based on allozyme analysis are reported and discussed here for the first time.

Further investigation, based on a complex approach including different methods is necessary in order to analyze in detail the genetic structure of honey bee populations from different *A. mellifera* subspecies in European countries.

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