ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., 64 (1), 2012: 199-204

Genetic Variability in a Local Bulgarian Honey Bee Population

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Abstract: Local Bulgarian honey bee *Apis mellifera rodopica* from Selective Center Plovdiv has been studied for genetic variability using analysis of six enzymic systems (MDH, ME, EST, ALP, PGM and HK) corresponding to six loci (Mdh 1, Me, Est 3, Alp, Pgm and Hk) and DNA analysis of 9 microsatellite loci (Ac011; A024; A043; A088; Ap226; Ap238; Ap243; Ap249 and Ap256). All of the studied loci were found to be polymorphic. Two to five alleles were detected with allozymic analysis: two alleles – at Mdh-1 and Pgm loci; three alleles – at Me, Alp and Hk loci. Est-3 locus was polymorphic with five alleles. It was calculated that the average number of alleles per locus is 3; the percent of polymorphic loci (P=0.95) is 83.3 and the observed and expected heterozygosity – 0.24 and 0.259, respectively. Three to seven alleles were detected using microsatelite DNA analysis: three alleles – at Ap243 and Ap249 loci; four alleles – at Ac011, A043, A088, Ap226 and Ap238; six alleles – at A024 and seven alleles – at Ap256. It was found that the observed heterozygosity varies between 0.444 and 0.567 and the expected heterozygosity – between 0.435 and 0.548. This research provides new information regarding the genetic variability in selected local Bulgarian honey bees and will be useful for selection and conservation purposes.

Key words: *Apis mellifera*, microsatellites, allozymes, polymorphism, Bulgaria

Introduction

Since 1930 honey bees have been studied in Bulgaria for the purpose of selection (Lazarov 1935, 1936). Detailed researches about the morphological features of the local bee were carried out in the period of 1967-1975 and the obtained results were used as a basis for organization of the selection work with bees in Bulgaria for the period 1971-1990 (Velichkov 1970). The local Bulgarian bee was threatened by many activities, including queen breeding and importation of foreign bees which have had an impact on the genetic variability of the honey bees throughout the country.

Allozymes were used as genetic markers in different studies on the honey bee genetic diversity, as well as appropriate tools for understanding subspecies discrimination and for characterization of their race status. In addition, they were used to analyze the phylogeny of *A. mellifera* on genetic basis and to detect significant genetic differences between commercial and feral honey bee populations (Bouga *et al.* 2005).

The allozyme variability of honey bee populations from Bulgaria was studied by Ivanova *et al.* (2007, 2010b, 2011). Different methods of DNA analyses (mtDNA, RAPD) have been fragmentarily applied in this direction (Ivanova, Bouga 2009; Ivanova *et al.* 2010a). Until now the microsatellite DNA analysis, which is an important part of the complex of methods used for studying the discrimination of honey bee populations in Europe was not applied for Bulgarian honey bees. In the current study local honey bees from Selective Center in Plovdiv, Bulgaria were genotyped by usage of six different gene-enzyme systems and 9 microsatellite loci.

The tested population represents *A. m. macedonica* (Ruttner 1988) local type '*rodopica*' (Petrov 1990).

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The objective of this study was to investigate and characterize genetic variability in selectively reared local honey bees *A. m. rodopica* from Selective Center Plovdiv in Bulgaria and to give information about allele frequencies, number of alleles per locus, levels of polymorphism, observed and expected heterozygosity.

Materials and Methods

Totally about 350 worker bees were tested for both analyses.

Allozyme analysis

The thorax homogenization, electrophoresis in 7.5% polyacrylamide gel, buffers and electrophoretic conditions for each enzymic system and histochemical staining were done according to Ivanova (1996) and Ivanova *et al.* 2010a. Six enzymic 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).

Microsatellite DNA analysis

For microsatellite DNA analysis samples were stored in absolute ethanol until extracting. Total DNA was isolated from a single bee worker with prior rinsing in insect buffer for one hour (Garnery *et al.* 1998), followed by mechanical disruption of the tissue, using NEW Omni TH_Q. DNA isolation was performed using Puregene protocol and reagents (QIAGEN formerly Systems, formerly Gentra, Sacramento, CA) with slight modifications. The protocol was modified by incubation of Proteinase K step overnight, cooling the sample at 4 °C for 5 min prior to protein precipitation, and cooling the sample to -20 °C for several hours during the isopropanol and ethanol wash steps. All samples were eluted in 50 µL of proprietary elution buffer and stored at 4 °C prior to DNA quantification and PCR use.

DNA yields of all extractions were estimated by DNA spectrophotometry (SpectraMax M2, Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. PCR yields were estimated by comparison of band intensity to a DNA Mass Ladder (Roche Molecular Biochemicals, IN, USA) on 2% agarose gel stained with ethidium bromide by blinded reviewers who had not participated in DNA extraction.

All honeybee samples were analyzed for nine microsatellite loci: Ac011; A024; A043; A088; Ap226; Ap238; Ap243; Ap249 and Ap256 (Estoup *et al.* 1995; Solignac *et al.* 2003; Rowe *et al.* 1997; Haberl, Tautz 1999; Evans 2000; Green *et al.* 2001). PCR amplifications were carried out in 10 µL of a mixture containing 5-10 ng of DNA template, 400 nM of each primer, 1.2-1.5 mM $MgCl_2$, 1 X QIAGEN Multiplex PCR reaction buffer (ready mixture of dGTP, 2'-deoxycytidine 5"triphosphate (dCTP), 2'-deoxythymidine 5"triphosphate (dTTP), and of *Taq* polymerase) and 1 X Q-Solution buffer. After denaturating step of 15 min at 95 °C, samples were processed through 30 cycles consisting of 30 sec at 94 °C, 90 sec at an optimal annealing temperature and 60 sec at 72 °C. The last elongation step was lengthened to 30 min at 72 °C. Aliquots of fluorescently labeled amplified DNA were mixed with formamide solution and GENESCAN-500(ROX) Size Standard (PE Applied Biosystems, FosterCity, CA) and genotyped on the ABI 3130 Genetic Analyzer using GeneScan™ Analysis Software.

Statistical Analyses

The results of isoenzymic analysis were statistically performed using BIOSYS-1 (Swofford, Selander 1981). Population genetic statistics based on microsatellite DNA analysis were computed using GenAlEx package software version 6.3 (Peakall, Smouse 2006). Genetic diversity within populations was evaluated by computing allele frequencies and observed and unbiased expected heterozygosity. Unbiased estimates and standard deviations of heterozygosity were calculated according to Nei (1987).

Results and Discussion

Data about allozyme polymorphism detected and allele frequencies are presented in Table 1. The average number of alleles per locus was calculated as 3±0.4. The estimated percentage of polymorphic loci was 83.3. The observed and expected heterozygosities (H_o and H_e) were 0.24±0.111 and 0.259±0.08, respectively. There were not significant deviations of genotype frequencies from Hardy-Weinberg expectations at most of the loci in population (0.99 > P > 0.1).

In total, for the local honey bee population, two alleles were detected at Mdh-1 (MDH^{65}, and

Table 1. Number of alleles, alleles and allele frequencies for each allozyme locus and mean number of alleles per locus for the population.

Locus	Number of alleles	Alleles	Allele frequency
Mdh 1	2	65 100	0.4 0.6
Me	3	90 100 106	0.013 0.901 0.086
EST 3	5	80 94 100 105 118	0.008 0.006 0.958 0.008 0.02
Alp		80 90 100	0.507 0.033 0.46
Pgm		100 114	0.924 0.076
Hk		87 100 110	0.06 0.927 0.013
Mean number of alleles per locus	3±0.4		

Table 2. Number of alleles, alleles and allele frequencies for each microsatellite locus and mean number (Na) of alleles per locus for the population.

Locus	Number of alleles	Alleles	Allele frequency
Ac 011	4	110 115 117 119	0.352 0.102 0.48 0.063
A024	6	90 92 96 98 100 102	0.114 0.063 0.239 0.359 0.148 0.077
A043	4	128 130 134 36	0.239 0.416 0.108 0.237
A088	4	136 140 145 147	0.455 0.045 0.136 0.364
Ap226	4	220 235 238 260	0.112 0.393 0.432 0.063
Ap238	4	210 236 238 246	0.063 0.159 0.369 0.409
Ap243	3	238 252 253	0.102 0.852 0.046
Ap249	3	210 218 220	0.500 0.261 0.239
Ap256	7	165 171 173 175 177 180 187	0.091 0.045 0.088 0.364 0.148 0.045 0.219
Na	4.333		

MDH^{100}), three – at Me locus (ME^{90}, ME^{100} and ME^{106}), five – at Est-3 locus (EST^{80}, EST^{94}, EST^{100}, EST^{105} and EST^{118}), three – at Alp (ALP^{80}, ALP^{90} and ALP^{100}), two – at Pgm (PGM^{100} and PGM^{114}) and three – at Hk (HK^{87}, HK^{100} and HK^{110}) locus.

In similar studies, five alleles were detected at Mdh-1 locus (Meixner *et al*. 1994; Kandemir *et al*. 2000; Ivanova *et al.* 2010b) and two or three of them were found in *A. m. macedonica* honey bee populations from Greece (Dedej *et al.* 1996; Bouga *et al*. 2005).

Concerning Me locus, three alleles (ME^{90}, ME^{100} and ME^{106}) were found in *A. mellifera* populations in Norway, Italy and western Czechoslovakia (Sheppard, Berlocher 1984, 1985; Sheppard, Mcpheron 1986). 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 in *A. m. macedonica* populations from Greece.

Est-3 locus 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 detected in *A. m. macedonica* from Greece (Bouga *et al*. 2005), but in our research we detected five alleles.

Concerning the Alp, two alleles (ALP^{100} and ALP^{80}) were detected in Greece (Bouga *et al*. 2005)

Table 3. Number of tested specimens (N), observed heterozygosity (Ho) and expected heterozygosity (He).

Parameters	Microsatellite loci								
	Ac 011	A024	A043	A088	Ap226	Ap238	Ap243	Ap249	Ap256
N	44	44	44	44	44	44	44	44	44
Ho	0.444	0.567	0.444	0.556	0.567	0.557	0.565	0.478	0.444
He	0.471	0.465	0.548	0.435	0.466	0.546	0.523	0.442	0.479

and in Bulgaria (Ivanova *et al*. 2010a). In the present research, a third allele ALP^{90} was found at this locus.

Del Lama *et al*. (1985) first reported the presence of three alleles at Pgm 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. PGM locus was found to be polymorphic with two alleles (PGM^{100} and PGM^{114}) in populations from Bulgaria (Ivanova *et al*. 2007, 2010a,b) and the results of present study confirm this.

HK locus was found as monomorphic in Norwegian, Italian, Czechoslovakian, Greek and German (Sheppard, Berlocher 1985; Sheppard, Mcpheron 1986; Badino *et al.* 1988; Del Lama *et al.* 1990) honey bee populations. It was detected to be 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 the present study three alleles were found in Bulgarian population studied.

All nine microsatellite loci were polymorphic in the studied population of local Bulgarian honey bees. Number of alleles observed for Ac011, A024, A043, A088, Ap226, Ap238, Ap243, Ap249 and Ap256 loci, were, 6, 4, 4, 4, 4, 3, 3 and 7 respectively (Table 2).

The average number of alleles per locus was 4.333. The observed heterozygosity values were between 0.444 and 0.567, and the expected heterogzygosity values – between 0.435 and 0.548 (Table 3).

All 9 microsatellite loci studied in this investigation were polymorphic. Gene heterozygosity is a suitable parameter for investigating genetic variation and according to Ott (2001) a polymorphic locus must have a heterozygosity of at least 0.10. Having that information in mind, results presented in our study show a high degree of genetic diversity and relative high selection potential.

Microsatellite studies on honey bee populations have been generally carried out for European and African honey bee subspecies (Frank *et al.* 1998, 2001), whereas, recent studies were published for island populations and Mediterranean honey bee populations (Dall'olio *et al.* 2007; Franck *et al.* 2001; Bodur *et al.* 2007). According to these studies, the expected heterozygosity levels were highest among African honeybee populations which ranged between 0.76 and 0.90 (Franck *et al*. 2001) and were lowest among Western Mediterranean honey bees reported as 0.26–0.68 (Garnery *et al.* 1998; Franck *et al*. 2001). In our study the values of expected heterogzygosity were between 0.435 and 0.536 which corresponds to the reported data concerning Mediterranean honeybee populations.

Concerning the different studied loci in the Bulgarian populations, our results showed that the most frequent allozyme alleles were MDH^{100}, ME^{100}, EST^{100}, PGM^{100}, HK^{100} and ALP^{80} and the most frequent microsatellite alleles were Ac 011 – 110, A024 – 98, A043 – 130, A088 – 136, Ap226 – 238, Ap238 – 246, Ap243 – 252, Ap249 – 210, Ap256 – 175. All they could be used as appropriate genetic markers for Bulgarian honey bees. Future investigation based on comparisons with other honey bee subspecies will dive additional information about private alleles which are also important for discrimination of Bulgarian populations.

The results of this research provide new information concerning the genetic variability of local Bulgarian honey bees and would be useful for selection and conservation purposes. Data about microsatellite DNA polymorphism in local Bulgarian honey bees is reported here for the first time.

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Received: 11.10.2011
Accepted: 16.12.2011