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Microsatellite Markers Reveal Genetic Diversity among Honey Bee Populations from Some Balkan Peninsula Regions and Distinctive Characteristics of the Local for Bulgaria Apis mellifera rodopica

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Abstract. The genetic polymorphism in honey bee populations from some Balkan peninsula countries was investigated using microsatellite DNA analysis. The local for Bulgaria *A. m. rodopica,* Petrov, 1991 population was compared with *A. m. macedonica* and *A. m. carnica* populations, originating from Greece and Serbia, respectively. In total, 88, 64 and 58 alleles were found for the nine microsatellite loci in the gene pool of the studied Bulgarian, Greek and Serbian populations. Genetic parameters and relationships between the investigated honey bee populations from some Balkan peninsula regions were analyzed. Twenty private alleles were detected for the population of the local Bulgarian *A. m. rodopica*, eight – for the Greek population of *A. m. macedonica* and 11 – for the *A. m. carnica* population from Serbia. Clear diagnostic markers, appropriate for distinguishing the local Bulgarian honey bee were found and described. It was concluded that together with the other molecular, biochemical, morphological and ethological indicators, described earlier, they could be taken into consideration when conduct activities for the conservation of the local Bulgarian honey bee *A. m. rodopica*.

Key words: *A. m. rodopica,* microsatellites, polymorphism, genetic distinction.

Introduction

The conservation of genetic polymorphism and the gene pool of local populations is a priority goal of many biological studies. In recent years, different activities have been focused on the relationship between genetic variability (Meixner et al., 2013; Rahimi et al., 2019) and the vitality of honey bees (Francis et al., 2014) and are related to proving the hypothesis that the health of the bee colonies depends on their genotypes and adaptation to the conditions of the particular environment, which includes not only climate and vegetation, but also prevalent diseases,

pesticide pollution and breeding activities (Meixner et al., 2014).

In this aspect, for the period 2008 – 2014, different scientific teams developed and published standardized methods for genetic and breeding analysis (Costa et al., 2012; Meixner et al., 2014), investigated and analyzed comparatively populations with various European origins of *Apis mellifera*, studied their genetic characteristics and selectively significant productive and behavioral traits (Büchler et al., 2014; Hatjina et al., 2014a, b; Uzunov et al., 2014a).

The local Bulgarian honey bee show high queen fertility, high honey productivity in local conditions, good wintering ability, low defensive behavior, good hygienic behavior (Ivanova, 2018). These valuable qualities are confirmed by comparative studies of European populations of *A. mellifera*. The results obtained show that the local Bulgarian honey bee has the highest comparative survival rates (Büchler et al., 2014; Hatjina et al., 2014b; Meixner et al., 2014b), low swarming tendency, high level of gentleness and highest level of hygiene behavior (Büchler et al., 2014; Uzunov et al., 2014a). Previous relative molecular-genetic studies show that the local Bulgarian honey bee belongs to the *A. m. macedonica* subspecies, but can be clearly distinguished from the populations of this subspecies that inhabit the territories of the Republics of Northern Macedonia and Greece (Francis et al., 2014; Uzunov et al., 2014b).

At present, the National program for breeding of *A. m. rodopica* is applied in Bulgaria and a law on beekeeping is in force, which prohibits the import and breeding of honey bees with foreign origin (Petrov & Ganev, 2013). Despite this fact, many beekeepers, for commercial reasons, disrupt the law and carry out uncontrolled imports of honey bee queens with foreign origin (mainly *A. m. carnica*, but also some hybrids), which affects the composition of the gene pool of the local populations. All bases of the National Bee Breeding Association, as well as the established gene bank in Bulgaria, store, protect and distribute the local honey bee *A. m. rodopica*.

Having in mind the valuable characteristics of the local Bulgaria honey bee, a system of genetic markers (enzymatic, DNA microsatellite and mitochondrial) was presented by Ivanova (2018). These markers, in concert with morphometric and ethological indicators, could be used for distinguishing the local Bulgarian honey bee *A. m. rodopica*, Petrov, 1991 from the other origins of *A. m. macedonica*, as well as from the populations of other *A. mellifera* subspecies distributed in Europe.

The present study aims to update the genetic marker system for distinguishing the local Bulgarian honey bee *A. m. rodopica*, Petrov, 1991 in accordance with its valuable biological and productive characteristics.

Materials and Methods

Worker bees (N=330) from *A. m. rodopica*, *A. m. macedonica* and *A. m. carnica* populations were sampled for this study. The honey bees from the Agricultural University – Plovdiv and the gene bank of National Bee Breeding Association in Sliven belong to the local *A. m. rodopica*, used in Bulgaria as a basis for a National bee breeding program (Petrov & Ganev, 2013). Populations of *A. m. macedonica,* originating from Greece and A. *m. carnica*, originating from Serbia were also included in the study and compared to the local Bulgarian honey bee.

The collected honey bees were frozen in containers at -20°C and then moved into tubes with absolute alcohol until ready for DNA extraction process. DNA extraction, PCR protocol and microsatellite DNA analysis were done as it was described by Nikolova (2011).

Total DNA was isolated from a single worker bee with prior rinsing in insect buffer for one hour followed by mechanical disruption of the tissue, using NEW Omni TH_Q. DNA yields of all extractions were estimated by DNA spectrophotometry according to the manufacturer's instructions.

PCR yields were estimated by comparison of band intensity to a DNA Mass Ladder on a 2% agarose gel stained with ethidium bromide by blinded reviewers who had not participated in DNA extraction.

PCR amplifications were carried out in 10μL of a mixture containing 5-10 ng of DNA template, 400 nM of each primer, 1.2–1.5 mm MgCl2, 1 X QIAGEN Multiplex PCR reaction buffer 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-400(ROX) Size Standard and genotyped on the ABI 3130 Genetic Analyzer using GeneScan™ Analysis Software.

All honeybee samples were analyzed for nine microsatellite loci: Ac011; A024; A043; A088; Ap226; Ap238; Ap243; Ap249 and Ap256.

Population-genetic analyzes have been computed using GenAlEx v.6.42 (Peakall & Smouse, 2006). GenAlEx assignment test was applied to determine the logarithmic probability and the degree of affiliation of a given genotype to a population studied.

Results

Based on the microsatellite DNA analysis performed, allelic diversity in the composition of the gene pool of the studied populations was characterized. The highest number of alleles (23) was diagnosed at the Ap256 locus and the smallest (8) – at the Ap024 locus.

Table 1 presents information concerning the number of alleles per each microsatellite locus in the studied populations of *A. m. rodopica*, *A. m. macedonica* and *A. m. carnica*.

The results demonstrate that the total numbers of alleles for the studied 9 microsatellite loci in the investigated populations varies between 58 (for *A. m. carnica* from Serbia) and 88 (for the local Bulgarian *A. m. rodopica*), which reveals the presence of significantly greater allelic diversity in the gene pool of the Bulgarian honey bee population compared to that of the other investigated populations from Greece and Serbia (Table 1).

Table 2 contains data concerning the identified private alleles in the studied populations.

A total of 39 private alleles have been identified in the comparative analysis of the studied Balkan peninsula populations as follows (Table 2): 20 – for the local Bulgarian bee *A. m. rodopica* (Petrov, 1991), 8 – for *A. m. macedonica* from Greece, and 11 – for *A. m. carnica* from Serbia).

The comparative analyzes indicate that four alleles of the Ac011 locus are diagnostic: 117 – for *A. m. rodopica*; 118 and 112 – for *A. m. macedonica* from Greece; 109 – for *A. m. carnica* from Serbia. Four alleles of the A024 locus are also diagnostic: 102 and 104 – for *A. m. rodopica*; 97 – for *A. m. macedonica* from Greece, 83 – for *A. m. carnica* from Serbia. The A043 locus is represented by a total of three diagnostic alleles: 123 and 126 for *A. m. rodopica*; 129 – for *A. m. carnica* from Serbia. Three alleles of the A088 locus can also be used as diagnostic: 128 – for *A. m. carnica* from Serbia; 130 – for *A. m. macedonica* from Greece; 142 – for *A. m. rodopica*. With respect to the A226 locus, the allele 240 also appears to be diagnostic for *A. m. macedonica* from Greece.

Locus A238 is represented by a total of five diagnostic alleles: 210, 252, and 260, occurring in populations of *A. m. rodopica*; 244 – found in populations of *A. m. macedonica* from Greece and 250 – in *A. m. carnica* population from Serbia. Concerning A243 locus, seven alleles are diagnostic, 6 of which characterize the local Bulgarian honey bee – these are the alleles 200, 210, 237, 240, 253 and 280. The allele 231 is found to be diagnostic for *A. m. carnica* originating from Serbia.

Table 1. Number of alleles per a locus in the studied populations.

Population	Ac 011	A 024	A 043	A 088	Ap 226	Ap 238	Ap 243	Ap 249	Ap 256	Total
A.m. rodopica, Bulgaria	7	7	7	14	9	10	11	10	13	88
A.m. macedonica, Greece	7	6	4	7	9	8	6	7	10	64
A. m. carnica, Serbia	10	5	5	4	5	9	5	3	12	58

Table 2. Private alleles and their frequencies in the populations studied.

Population	Locus	Allele	Allele frequency
A. m. rodopica, Bulgaria	Ac011	117	0.483
	A024	102	0.077
	A024	104	0.077
	A043	123	0.077
	A043	126	0.063
	A088	142	0.063
	Ap238	210	0.063
	Ap238	252	0.129
	Ap238	260	0.092
	Ap243	200	0.056
	Ap243	210	0.056
	Ap243	237	0.068
	Ap243	240	0.156
	Ap243	253	0.202
	Ap243	280	0.058
	Ap249	227	0.061
	Ap249	260	0.109
	Ap249	290	0.065
	Ap249	300	0.060
	Ap256	245	0.060
A. m. macedonica, Greece	Ac011	118	0.500
	Ac011	112	0.500
	A024	97	0.150
	A088	130	0.500
	Ap226	240	0.430
	A238	244	0.250
	Ap256	168	0.150
	Ap256	208	0.132
A. m. carnica, Serbia	Ac011	109	0.125
	A024	83	0.225
	A043	129	0.100
	A088	128	0.455
	Ap238	250	0.250
	Ap243	231	0.300
	Ap249	225	0.370
	Ap256	195	0.079
	Ap256	202	0.312
	Ap256	212	0.063
	Ap256	217	0.125

The A249 locus is represented by five diagnostic alleles: 227, 260, 290 and 300 – for the local Bulgarian honey bee; 225 – for *A. m. carnica* from Serbia. Locus A256 is presented by seven diagnostic alleles. The alleles 168 and 208 are found in populations of *A. m. macedonica* from Greece. Alleles 195, 202, 212 and 217 are diagnostic for *A. m. carnica* from Serbia and 245 – for the local Bulgarian honey bee.

The average calculated value for the gene flow (*Nm*) is 1.723 (varying between 0.402 for Ap243 to 3.282 for A043) and for the fixation index (*Fst*) is 0.152 (varying between 0.071 for A043 to 0.249 for Ap226).

The differentiation between the studied honey bee populations from Bulgaria, Greece and Serbia is presented in Figure 1 by grouping together and in pairs with the local Bulgarian *A. m. rodopica* based on the GenAlEx assignment test.

Discussion

The local Bulgarian honey bee is most adapted to the specific conditions of the country and also characterized by a high level of survival, high fertility and productivity, with a strong hygienic behavior, with a low tendency to swarming and other significant biological features (Büchler et al., 2014; Hatjina et al., 2014a, b; Meixner et al., 2014; Uzunov et al., 2014a). Its gene pool must be preserved through science-based selection and conservation activities in Bulgaria. In this aspect, the microsatellite DNA analysis carried out in this study provides new information on additional diagnostic genetic markers for its discriminating.

A high level of genetic similarity among the studied populations from all over the country based on isoenzyme and microsatellite analyses was found (Georgieva et al., 2016; Ivanova, 2018), which has also demonstrated high consolidation among the selectively controlled and uncontrolled honey bee populations in Bulgaria. This is a serious circumstance giving a reason to assume that a significant part of the bee populations on the Bulgarian territory has the characteristics of the local honey bee *A. m. rodopica*.

The presence of private alleles in populations is an indicator for their specific population-genetic characteristics or for clear differences between them. The search for diagnostic markers among the more widespread alleles in the population is considered as successful if these alleles are detected in one population while absent in another one, with a frequency above 5%. Based on the results of our study, it becomes clear, that all the 39 identified private alleles in the studied populations from Bulgaria, Greece and Serbia could be used as successful diagnostic markers for discriminating the studied origins as their frequency is above the proposed one (5%) – Table 2.

In this study, the average calculated value for gene flow (*Nm*) is 1.723. *Nm* value varies from 0.402 (Ap243) to 3.282 (A043) for the studied loci, which indicates moderate to high levels of genetic differentiation between the studied populations from Bulgaria, Greece and Serbia. In accordance with the *Nm* data, the results obtained for *Fst* values calculated in pairs indicate also moderate to high levels of genetic differentiation for the studied populations.

Comparative analyses of the studied honey bee populations from some Balkan peninsula regions show distinct characteristics that could be successfully used for their discrimination. Data from the assignment tests done, demonstrate that the populations of the local Bulgarian honey bee *A. m. rodopica* are grouped separately from *A. m. macedonica* originating from Greece, as well as from *A. m. carnica* originating from Serbia (Figure 1).

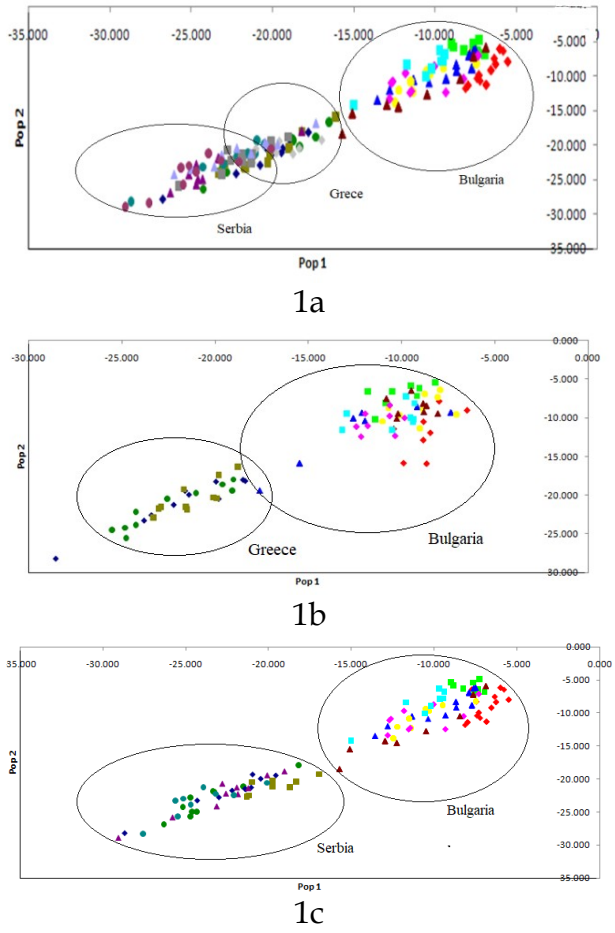

Fig. 1. Distribution of individuals in the honey bee populations studied: a) grouping the populations from Bulgaria (local *A. m. rodopica*), Greece (*A. m. macedonica*) and Serbia (*A. m. carnica*) together; b) grouping of the local Bulgarian honey bee *A. m. rodopica* (to the right) and *A. m. macedonica* originating from Greece; c) grouping of the local Bulgarian honey bee *A. m. rodopica* (to the right) and *A. m. carnica* originating from Serbia.

These results could be taken as further proof that the local Bulgarian honey bee could be distinguish by the other European *Apis mellifera* populations, as well as by the Balkan peninsula honey bee populations, based on different genetic approaches, which is in agreement with previously reported data (Ivanova et al., 2012; Francis et al., 2014). Simultaneously with the findings of other authors (Nedić et al., 2014; Uzunov et al., 2014b), this study provides data on the genetic diversity of *Apis melifera* populations in the Balkan Peninsula, as well as possible approaches for their distinction.

Conclusions

Together with the previously described valuable biological and productive characteristics, morphometric, ethological, isoenzymatic and other DNA markers, the characterized here genetic markers update the system of criteria and activities for the protection and conservation of the gene pool of the local Bulgarian honey bee *A. m. rodopica*, Petrov, 1991.

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