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ANIMAL GENETICS

Genetic Differentiation of Local Populations of the Dark European Bee *Apis mellifera mellifera* L. in the Urals

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Abstract—For the last two centuries, beekeepers in Russia and Europe have been introducing bees from the southern regions to the northern ones, subjecting the genetic pool of the dark European bee *Apis mellifera mellifera* L. subspecies to extensive hybridization. In order to reconfirm on the genetic level the previously published morphological data on the native bee population in the Urals, the Bashkortostan Republic, and the Perm Krai, we analyzed the polymorphism of the mitochondrial (mtDNA COI–COII intergenic locus) and nuclear (two microsatellite loci, ap243 and 4a110) DNA markers. Four local populations of the dark European bee *A. m. mellifera* surviving in the Urals have been identified, and their principal genetic characteristics have been determined. Data on the genetic structure and geographical localization of the areals of the dark European bee local populations in the Urals may be of use in restoring the damaged genetic pool of *A. m. mellifera* in Russia and other northern countries.

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INTRODUCTION

The natural habitation areal of the honey bee *Apis mellifera* Linnaeus 1758 [1] extends to Europe, Africa, and western Asia [2, 3]. The species is subdivided into 29 subspecies clustered into four evolutionary branches [4–6]: the African branch (A), the Middle-East branch (O), and two European branches (C and M) [3, 7–10]. Mitochondrial DNA and microsatellite loci studies supported the morphometric data, indicating that the honey bee subspecies group into four evolutionary branches [5, 11, 12].

Among the 29 bee subspecies, only *Apis mellifera mellifera* Linnaeus 1785 [1], commonly known as the dark European bee and referred to as the dark hylile bee or the Central Russian bee in Russia, is characterized by a huge habitation areal that occupies all of northern Eurasia and is covered by vegetation typical for forests and steppe-forests. The *A. m. mellifera* subspecies of the honey bee is uniquely adapted for the extremely cold and long winters and diseases associated with long wintering periods (e.g., nosema disease), as well as for the collection of the yearly harvest of honey during the short period of the rapid blossoming of the linden under the strongly continental climate of the Eurasia [13].

Over the last two centuries, the habitation areal of *A. m. mellifera* has grown substantially smaller as a consequence of intensive deforestation, the introduction of southern subspecies into the northern territories, and the emergence of new diseases caused by the microsporidian *Nosema ceranae* [14], varroosis, and ascospherosis, such as type C nosema disease. Numerous experimental crossings of different honey bee subspecies within a single apiary resulted in the uncontrollable subspecies hybridization across the entire habitation areal [13, 15, 16]. Commercial beekeeping in Europe and in Russia is now based primarily on the southern subspecies such as *A. m. ligustica* [17], *A. m carnica* [18], *A. m. caucasica* [19], *A. m. carpatica* [20], and *A. m. armeniaca* that were introduced into the North Eurasia [21].

Due to hybridization and unrestricted gene flows between natural and commercial bee populations [12, 22], the genetic pool of the native dark European bee *A. m. mellifera* is considered lost in many European countries [15]. For example, wide-scale introduction of the southern bees in Germany led to a complete replacement of the *A. m. mellifera* subspecies by the *A. m. carnica* subspecies [23, 24]. In Russia, the *A. m. mellifera* subspecies was displaced almost everywhere by the *A. m. caucasica* and *A. m. carpatica* subspecies [13, 16]. The majority of beekeepers in the Nordic countries and on the British Islands more readily raise *A. m. ligustica*, *A. m. carnica*, or the artificially bred buckfast race [15]. In such a way, the natural habitation areal of *A. m. mellifera* has substantially narrowed in all European countries.

However, the previously published results of morphological studies of bees on the territories of the Perm krai and the Bashkortostan Republic suggested that the gene pool of the dark European bee *A. m. mellifera* may not be absolutely lost in Russia [13, 16, 25–27]. According to the morphometric data, beekeepers in

Fig. 1. Distribution of the honey bee *A. mellifera* local populations analyzed in this work.

Russia have in their possession sufficient resources to restore the *A. m. mellifera* gene pool preserved on the territories of the Bashkortostan Republic, the Tatarstan Republic, and the Udmurtia Republic, as well as Altai krai, Perm krai, and Kirov oblast [16, 26, 27].

Each year, beekeeping in Russia encounters the problem of decreased productivity of bee families and their mass mortality following wintering, which appears to be the consequence of their decreased adaptation to the environmental conditions due to hybridization with the southern subspecies [13, 26, 27]. The Burzyanskaya population of the dark European bee *A. m. mellifera* is maintained by the efforts of the Shulgan-Tash Reserve staff and survives because of its geographical isolation by the Urals forest and mountain groups [13, 16, 28]. No legal means for preserving the gene pools of the native bee populations have been developed in Russia so far. Each extant population of the dark European bee in Russia is exposed to a constant threat of extinction as a result of hybridization with the introduced bee subspecies [27–29].

To restore the aboriginal gene pool of the *A. m. mellifera* honey bee in the Urals, precise identification of the subspecies is required. Until recently, only morphometric approaches were utilized towards this end in Russia [13, 26]. But, while morphometric features are important for bee classification, they are not quite convenient to be used for subspecies identification, since they are affected by environmental factors and natural selection [6]. Genetic markers, such as the intergenic locus COI–COII, which is unique for the *Apis* genus, are the most informative for bee studies [30]. The variability of the nucleotide sequence length for this locus is used to differentiate the subspecies belonging to the four evolutionary branches and to identify the dark European bee *A. m. mellifera* [4, 31, 32].

The technique of differentiation between the *A. m. mellifera* subspecies and the *A. m. caucasica* and *A. m. carnica* belonging to the evolutionary branch C makes it possible to investigate the extant genetic pool of the dark European bee on the territory of the Bashkortostan Republic and Perm krai. This technique is based on well-defined differences in the typical COI–COII mtDNA locus variants between representatives of the M evolution branch and the C, PQ, PQQ, and PQQQ evolution branch variants, which are present only in the *A. m. mellifera* subspecies (evolutionary branch M), and the Q variant, which is characteristic of the subspecies introduced from the southern regions (evolutionary branch C) [14]. This technique was modified by researchers from the Institute of Biochemistry and Genetics of the Ufa Scientific Center of the Russian Academy of Sciences and allows the amplification of bee DNA fragments that are 200 bp shorter than those in the works of the European scientists, which makes the analysis less difficult and time-consuming [13, 26].

Microsatellite loci are unique markers for studying the population and genetic structure, as well as the hybridization rate of bee subspecies [30, 33, 34]. Our work is primarily devoted to the investigation of the *A. m. mellifera* populations in the Bashkortostan Republic and Perm krai. A number of local bee populations in the Urals are exposed to hybridization with *A. m. caucasica* and *A. m. carpatica*, the subspecies belonging to the evolutionary branch C. The purpose of our work was to obtain data on the habitation areal and the population structure of the dark European bee *A. m. melifera* population, which is subdivided into several local populations in the Urals, on the basis of studying the variability of the mitochondrial (COI–COII) and two nuclear (microsatellites ap243 and 4a110) loci.

MATERIALS AND METHODS

To carry out the present work, worker bee samples were collected in 11 apiaries in three regions of the Bashkortostan Republic and in 11 apiaries in seven regions of Perm krai, totaling 550 bee colonies (Fig. 1). Bee samples were preserved in 96% ethanol.

Total DNA was isolated from the thoracic muscles by an extraction technique with guanidinium thiocyanate–phenol–chloroform mixture [35]. PCR amplification of the mtDNA COI–COII locus was performed in accordance with a previously published

method [13, 26]. Statistical analysis was performed with FSTAT 2.9.3.2 and Genepop 4.2.2 software. The dendrogram was built with STATISTICA 8.0 software.

The intergenic mtDNA COI–COII locus, which is localized between the 3'-end of the COI gene and the 5'-end of the COII gene, was amplified with the locus-specific oligonucleotide primers F-Nik CACATTTAGAAATTCCATTA and R-Nik ATAAATATAAATCATGTGGA under the modified conditions described by Nikonorov et al. [26]. These allow the amplification of shorter fragments, namely 200 bp shorter, than the technique previously developed by the European researchers [26].

PCR amplification of the corresponding locus in the honey bee subspecies *A. m. mellifera* of the evolutionary branch M resulted in a 400-bp PQ fragment, 600-bp PQQ fragment, and 800-bp PQQQ fragment, while amplification reveals only a 300-bp Q fragment in the southern subspecies of the evolutionary branch C (Fig. 2) [13, 26]. This unique polymorphism of the mtDNA COI–COII intergenic locus length serves as a marker for the precise differentiation between the local and introduced bee subspecies in Russia.

For the study of the genetic structure of the dark European bee subspecies *A. m. mellifera* in the Urals, two microsatellite loci previously described for *A. mellifera*, 4a110 and ap243 [36, 37], were used. PCR was carried out in 15 μL of a mixture containing 50–200 nM of each primer, 100 μM of each dNTP, 1.2–1.5 mM $MgCl_2$, 1× buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 0.5 U of the *Taq* polymerase (Sintol), and 2 μL of the DNA extract. The PCR conditions were as follows: initial denaturation 3 min at 94°C; 30 cycles consisting of denaturation 30 s at 94°C, primer annealing 30 s at 55°C, and elongation 30 s at 72°C each; final extension 5 min at 72°C. The fragment analysis of the obtained PCR products was performed in an Applied Biosystems Sequencer automatic sequencing device. In the analyzed dark European bee *A. m. mellifera* in local populations in the Urals, we have detected three alleles for the ap 243 microsatellite locus (254, 257, and 260 bp) and three alleles for the 4a110 microsatellite locus (160, 163, and 168 bp).

RESULTS

To search for extant local populations of the dark European bee *A. m. mellifera* in the Bashkortostan Republic and Perm krai, we took advantage of the mtDNA COI–COII locus amplified fragment length polymorphism: the longer PQQ fragment is typical of the local Ural *A. m. mellifera* bees and the shorter Q fragment is characteristic of the introduced southern bee subspecies belonging to the evolutionary branch C.

The frequencies of the PQQ and Q variants varied within a range of 0.57–1.00 (Table 1) in the local populations of the dark European bee *A. m. mellifera* in the Bashkortostan Republic and Perm krai. The Visherskaya, Tatyshlinskaya, and Burzyanskaya populations were characterized by an extremely high frequency of the PQQ variant (0.99), while in the Yuzhno-Prikamskaya population its frequency was a little lower (0.90). The observed high frequency of the PQQ variant (≥0.90) makes it possible to conclude that these populations belong to the *A. m. mellifera* subspecies by their maternal lineage. The Iglinskaya population was characterized by the lowest frequency of the PQQ variant (0.57), which suggests that it has undergone hybridization with the introduced southern subspecies belonging to the evolutionary branch C.

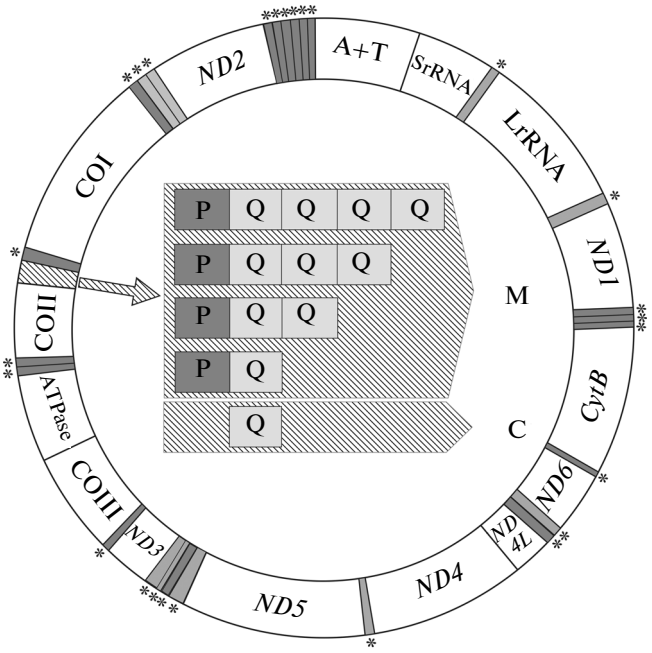

Fig. 2. Localization of the COI–COII intergenic locus on the circular mitochondrial DNA of the honey bee *A. mellifera* and the distinctive features of its intraspecific polymorphism. tRNA genes are indicated with asterisk.

The frequencies of the alleles for two microsatellite loci, ap243 and 4a110, were unevenly distributed in the populations of the dark European bee *A. m. mellifera* (Table 1). The most frequent were the 254 and 257 bp alleles of the locus ap243 and the 160 and 168 bp alleles of the locus 4a110.

The genetic distances (*D*) [38] between the local populations of the dark European bee *A. m. mellifera* in the Urals were calculated using FSTAT software on the basis of the allele frequencies for the ap243 and 4a110 microsatellite loci and ranged from 0.01 to 0.12 (Table 2). The genetic distances between the Visherskaya, Yuzhno-Prikamskaya, Tatyshlinskaya, and Burzyanskaya local populations varied from 0.01 to 0.03. At the same time, the distances between these populations and the hybrid Iglinskaya populations were in the range from 0.05 to 0.12. The high genetic distances between the local dark bee *A. m. mellifera* populations in the Urals indicate that

Table 1. Frequencies of the PQQ (branch M) and Q (branch C) variants of the mtDNA COI–COII locus and the ap243 and 4a110 microsatellite loci alleles in local populations of the honey bee *Apis mellifera* in the Urals

Loci	Population				
	Burzyanskaya	Tatyshlinskaya	Visherskaya	Yuzhno-Prikamskaya	Iglinskaya
	$N = 66$	$N = 111$	$N = 33$	$N = 111$	$N = 229$
COI–COII variants					
PQQ	0.99	0.99	1.00	0.90	0.57
Q	0.01	0.01	0.00	0.10	0.43
ap243 alleles					
254 bp	0.45	0.37	0.36	0.38	0.77
257 bp	0.32	0.54	0.43	0.45	0.16
260 bp	0.23	0.09	0.21	0.17	0.07
4a110 alleles					
160 bp	0.58	0.48	0.57	0.48	0.71
163 bp	0.00	0.00	0.00	0.01	0.01
168 bp	0.42	0.52	0.43	0.51	0.28

Table 2. Genetic distances (D) between the local populations of the honey bee *A. mellifera* in the Urals obtained on the basis of the allele frequencies for the ap243 and 4a110 microsatellite loci ($P < 0.05$)

Population	Burzyanskaya	Tatyshlinskaya	Visherskaya	Yuzhno-Prikamskaya	Iglinskaya
Burzyanskaya	–	0.03	0.01	0.02	0.05
Tatyshlinskaya		–	0.02	0.01	0.12
Visherskaya			–	0.01	0.09
Yuzhno-Prikamskaya				–	0.10
Iglinskaya					–

they are genetically divergent, which may be the result of hybridization with unrelated subspecies.

DISCUSSION

The allele frequencies for the ap243 and 4a110 microsatellite loci obtained for the *A. m. mellifera* population in the Urals were used to calculate the genetic parameters and *F*-statistics coefficients [39] (Table 3). In the population genetics of the honey bee, *F*-statistics allows statistical assessment of the observed and the expected according to Hardy–Weinberg equilibrium levels of heterozygosity in the population. *F*-statistics may also be considered a measure of correlation between genes at different levels of the subdivided population, which is influenced by such evolutionary processes as mutations, migration, natural selection, inbreeding, and the Wahlund effect.

Positive values for the F_{IS} and F_{IT} inbreeding coefficients indicate that the crossing of closely related individuals prevails in the bee population in the Urals, both on the level of subpopulations and on the whole population level. The F_{ST} coefficient, which has a value close to zero, shows the low degree of genetic structure in the population, indicating that the local *A. m. mellifera* populations in the Urals are genetically close to each other. In the bee population in the Urals, the allele frequency and genotype distribution do not follow the Hardy–Weinberg distribution, which is caused by active genetic processes and the negative effects of the environment. The local populations of the dark European bee in the Urals are characterized by a deficit of heterozygotes, both on the level of subpopulations and the whole population. It is well known that heterozygote deficit is typical of many *A. mellifera* populations in which no introduction or migration takes place and is

Table 3. *F*-statistics and heterozygosity in the extant dark European bee *A. m. mellifera* population in the Urals obtained on the basis of the ap243 and 4a110 microsatellite loci allele frequencies in the Burzyanskaya, Tatyshlinskaya, Visherskaya, and Yuzhno-Prikamskaya local populations

F_{ST}	F_{IS}	F_{IT}	H_o	H_s	H_T
0.01	0.24	0.25	0.35	0.47	0.48

F_{ST}—coefficient of population subdivision; F_{IS}—inbreeding coefficient for local populations; F_{IT}—inbreeding coefficient for the whole population; H_o—observed heterozygosity level for the whole population; H_T—expected heterozygosity level for the whole population; H_s—expected heterozygosity level in local populations.

caused by such features of bee biology and development as the interchange of haploid and diploid generations in a large number of bee families under dramatically limited space conditions.

A dendrogram depicting the genetic relations between the local *A. m. mellifera* populations in the Urals was built with the STATISTICA 8.0. software on the basis of genetic distances *D* [38] by the Neighbor-Joining method [40] (Fig. 3).

It can be seen on the dendrogram that the Iglinskaya population is separated from all of the other populations, which group together. The position of the Iglinskaya population indicates its significant genetic divergence from other local dark European bee populations, which may be the result of the hybridization of local bees with the introduced southern subspecies belonging to the evolutionary branch C. The other four local populations of the dark European bee grouping together suggest that they are genetically related by the nuclear loci. In such a way, the dendrogram clearly defines the four extant local populations of the dark European bee *A. m. mellifera*: the Visherskaya, Yuzhno-Prikamskaya, Tatyshlinskaya, and Burzyanskaya populations.

To summarize, the genetic studies of the *A. m. mellifera* populations in the Urals, Bashkortostan Republic (the Southern Urals), and Perm krai (the Middle Urals) revealed four extant local populations of the dark European bee (namely, the Visherskaya, Yuzhno-Prikamskaya, Tatyshlinskaya, and Burzyanskaya populations) based on an analysis of the mitochondrial DNA COI–COII locus and two nuclear microsatellite loci (ap243 and 4a110),. We believe that the obtained data will assist in the further search for new localizations of the surviving population of the dark European bee *A. m. mellifera* in Russia and other countries. In the future, we will broaden the range of the analyzed loci and expand the territories under study.

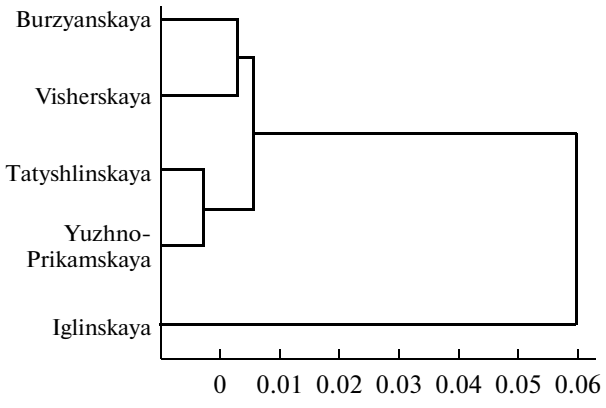

Fig. 3. Dendrogram depicting genetic relationships between the local honey bee *A. mellifera* populations in the Urals obtained on the basis of the analysis of the ap243 and 4a110 microsatellite loci polymorphism.

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