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GENETICS

Morphogenetic Diversity of the Honeybee *Apis mellifera* L. from the Mountain-Forest Zone of Crimea

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Abstract—By using morphometric and molecular-genetic methods, the population of honeybees of apiaries located in the mountain-forest zone of the Crimean Peninsula under conditions of prolonged isolation from other bee farms of the peninsula was studied. The hypothesis that the bees of this apiary may show signs of lost Crimean bees was analyzed. The complex of morphological traits makes the bees of the examined apiaries closer to Italian, Krajina, and Ukrainian steppe bees and, to a lesser extent, to Carpathian bees. The results of molecular genetic analysis of the initial part of the mtDNA *COI* gene from GenBank for the races under consideration revealed the evolutionary proximity of the bees studied to representatives of Italian bees. The bees of the isolated apiary are united into a single haplotype. The sequences of the mtDNA *COI* locus were obtained from Carpathian bees from Moscow oblast and Tajikistan. In terms of differences between the Italian bee haplotype and haplotypes of bees from other regions related to Carpathian bees, it is suggested that the haplotypes of the apiary studied are more similar to Carpathian bees. The investigations do not reject the hypothesis that bees of the isolated apiary may have traits typical of Crimean bees.

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INTRODUCTION

The natural habitat of the honeybee *Apis mellifera* L. covers Africa, Europe, and the Middle East. This species is characterized by intraspecific differentiation, making it possible to distinguish geographical races (subspecies) adapted to local conditions (Ruttner, 1988). The foundations for studying the external traits of honeybee races were laid by Kozhevnikov (1900). Morphometric analysis of the discoid bias, cubital index, length of the proboscis, length and width of the right front wing, the number of hooks on the rear wing, and other traits was suggested by Alpatov (1948). In terms of a complex of such traits, some researchers identify local races of bees (Konusova et al., 2010). According to the modern classification, there are from 25 (Ruttner, 1992) to 28 (Engel, 1999) races of honeybees. Some races are widespread (for example, *A. m. mellifera* L.); others are narrow regional fauna (for example, *A. m. cypria* Pollmann). It is known that in a relatively isolated area on the Crimean Peninsula there was a special race of bees (Alpatov, 1938). Based on morphometric studies of several traits, the author suggested distinguishing the bees of the southern coast of Crimea into a separate subspecies (race), *A. m. taurica* Alpatov (Alpatov, 1938). Comparative analysis carried out by Alpatov (1938) showed that the relative length of legs in the Crimean bee is greater than that of the *A. m. mellifera* and Ukrainian bees, and similar to that of the bees from the Caucasus. He marked the proximity of the Crimean and Caucasian races of bees for the tarsal and cubital indexes. In terms of body color, Alpatov also associated the Crimean bee with the *A. m. caucasica*, thus suggesting that this variety of the Crimean bees can be attributed to Caucasian bees.

It is known that morphometric traits often depend on environmental factors such as seasons or the impact of the anthropogenic factor. This creates some uncertainty in the allocation of a particular race of bees and requires comparison with other results, including those obtained by genetic methods of research. The prospect of using such techniques is associated in particular with nucleotide sequence analysis of mtDNA. The currently known sequences of the complete mitochondrial genome include those of the *A. m. mellifera* (KJ396189.1) and Italian *A. m. ligustica* (L06178.1) (Crozier, R.H. and Crozier, Y.C., 1993), as well as individual *COI* sections of *A. m. carnica* (AY114464.1), *A. m. caucasica* (AY114482.1), and others. The prospect of such methods is shown, for example, in comparative analysis of the mtDNA *COI* site, which makes it possible to identify differences between *A. m. ligustica* and *A. m. mellifera* from Læsø Island in Denmark (Itenov and Petrsen, 1991). The *COI* gene sequence for bees *A. m. carpatica* has not yet been investigated.

Table 1. Morphometric parameters of bees from three apiaries in Crimea

Race, apiary	Cubital index, %	Positive discoid bias, %	Proboscis length, mm	Wing, mm	
				length	Width
Unknown, R	43.4 ± 1.1	100	6.42 ± 0.02	8.00 ± 0.02	2.14 ± 0.01
Carpathian,					
F	42.3 ± 0.9	100	6.30 ± 0.06	8.10 ± 0.05	2.17 ± 0.03
U	42.6 ± 0.9	100	6.42 ± 0.04	8.05 ± 0.07	2.14 ± 0.01

R, "Red Caves"; F, Feodosia; U, village of Ukromnoe; for Tables 1 and 2.

Currently, the Crimean honeybee race is lost. At the same time, one cannot exclude the possibility of recovering it, since, according to some studies with uncontrolled reproduction of other races of bees imported into Crimea, those bees acquired features characteristic of the Crimean race of bees. It was suggested that the genotype of bees of the apiary located in the mountain-forest zone of the Crimean Peninsula, in conditions of prolonged isolation, may contain genes of the lost Crimean race.

This paper aimed at studying the features of the gene pool of bee colonies of the apiary located in the mountain-forest zone of the Crimean Peninsula in conditions of prolonged isolation from other bee farms in comparison with bees of various races from other regions.

MATERIALS AND METHODS

Morphometric analysis. The material for analysis (~500 specimens of working bees) was collected from private apiaries in Feodosia (F), the village of Ukromnoe (U) of Simferopol district, and the apiary located in the mountain-wood zone of Crimea near "the Red Caves" (R). It is expected that according to beekeepers (oral reports) the honeybee race in apiaries F and U is Carpathian. Initially the bee colonies were brought to apiary R from Central Asia and for 30 years were bred in the mountain-forest zone of Crimea without importation of queens of known races.

We studied the length of the proboscis, cubital index, discoid bias, and the width and the length of the right underwing. The measurement method was described in detail in Alpatov (1948). Measurements were carried out using MBS (LOMO, Russia) and VHX-1000E (Keyence, Japan) microscopes; statistical analysis was carried out in Statistica 6.0 and the Keyence application.

Molecular genetic analysis. The material for analysis was taken from the same Crimean apiaries and also from the apiaries of the village of Khistevarza (Kh) (Bobojan, Gafurovskii district, Sogd oblast, Tajikistan), the city of Gzhel (G) (Moscow region), and Scryabin Moscow State Academy of Veterinary Medicine and Biotechnology (Moscow) (M). The alleged races for apiary G are Carpathian and Italian; for apiary M, it is Carpathian. Total DNA was isolated from the legs of adult bees using reagents Diatom-200 (IsoGen, Moscow). Isolation of DNA, PCR, and sequencing were carried out in the laboratory of molecular diagnostics, Institute of Ecology and Evolution. The methods were described in detail previously (Triselyova et al., 2014). Primers LCO1490 and HCO2198 were used. The analyzed fragment contained 680 bp. Data processing was carried out using the software packages MEGA 5 (Tamura et al., 2011), Network ver. 4.6.1.1 (Bandelt et al., 1999), and ARLEQUIN ver. 3.5 (Excoffier and Lischer, 2011). Further comparative analysis was carried out using GenBank sequences of a similar site of the Russian (KJ396188.1) and Italian (LO6178.1) races. As an outgroup we used nucleotide sequences of two species of wild bees, *Apis cerana* (GQ162109.1) and *Apis florea* (NC021401.1).

RESULTS

Morphometric analysis. The main results of the morphometric measurements of honeybee specimens of various origins are shown in Table 1.

According to the cubital index, the bees of apiary R have greater differences from the bees of apiaries F and U than the latter two have between each other. Analysis of the proboscis of the working bees found that in terms of this criterion the bees of apiary R do not differ from the bees of apiary U, while in the bees of apiary F its length is the smallest. The situation with the width of the wing is similar. At the same time, the length of the underwing increases in the series: bees of apiary R, bees of apiary U related to them, and bees of apiary F. Thus, in terms of the morphological traits studied, the most related bees are those from apiaries R and U. The most common body color in the studied three apiaries was gray.

Molecular genetic analysis. Alignment of the obtained sequences of the initial section of mtDNA *COI* (680 bp) of the honeybee specimens (n = 41) revealed four variable sites (all sites are parsimonious-informative). The total number of substitutions is three transitions and one transversion. The nucleotide composition of this fragment is as follows: 11.6% cytosine, 40.7% thymine, 32.8% adenine, and 14.9% guanine. The nucleotide variability in the entire sample is

0.2% (S.E. = 0.001). The described site contained six haplotypes, two of which are unique and four haplotypes of which are found in several samples at a frequency from 5 to 19.

Phylogenetic relationships between the haplotypes are illustrated in the median network (Fig. 1a). The figure shows that all honeybee individuals of the apiaries of the Crimean Peninsula, as well as those of Moscow, Moscow region, and Tajikistan, are combined into a single haplogroup. Two haplotypes are represented by individuals from apiaries F, U, G, and M. Individuals from apiary R, bees from Tajikistan, and one individual for each of apiaries F and M have separate haplotypes. Inclusion into the median network of a portion of the similar mtDNA *COI* gene from GenBank of *A. m. ligustica* and *A. m. mellifera* showed that in terms of this site all the studied honeybee individuals are closer to the Italian race.

Analysis of the sequence of the initial section of the mtDNA *COI* gene of 680 np in bees from apiaries of different geographical regions showed a certain molecular diversity in bees from apiaries of Feodosia, the village of Ukromnoe, and the Veterinary Academy (Table 2). Bees from the isolated apiary of the mountain-wood zone of Crimea, apiaries in Moscow region, and those in Tajikistan are represented by singular haplotypes.

Taking into account the similarity of the morphometric parameters of bees of the unknown race in “the Red Caves” apiary compared to the bees of the Italian, Krajina, and to a lesser extent Carpathian races, as well as the known similarity of the Crimean (Alpatov, 1938) and Caucasian bee races, we additionally used GenBank nucleotide sequences of the Caucasian (AY114472.1) and Krajina (AY114464.1) races, which partly coincide with the analyzed sequences of the honeybee individuals that we studied. The compared site contained 434 bp, starting from 247 bp. Alignment of all sequences of mtDNA *COI* of honeybee specimens, including GenBank specimens of the Italian, Caucasian, and Krajina races ($n = 44$), revealed three variable sites. All sites are parsimonious-informative. The nucleotide composition of this fragment included 15.7% cytosine, 41.9% thymine, 32.7% adenine, and 9.7% guanine. The described site contained four haplotypes; one haplotype was unique, and the remaining three haplotypes were found in several specimens at a frequency from 2 to 27.

In terms of this short site, the bees from apiaries F, U, M, and G, as well as the Italian, Krajina, and Caucasian races, constitute a single haplotype (Fig. 1b). The bees from apiaries R and Kh are represented by singular haplotypes, as well as one haplotype combining an individual from apiary F and an individual from the apiary of the Veterinary Academy.

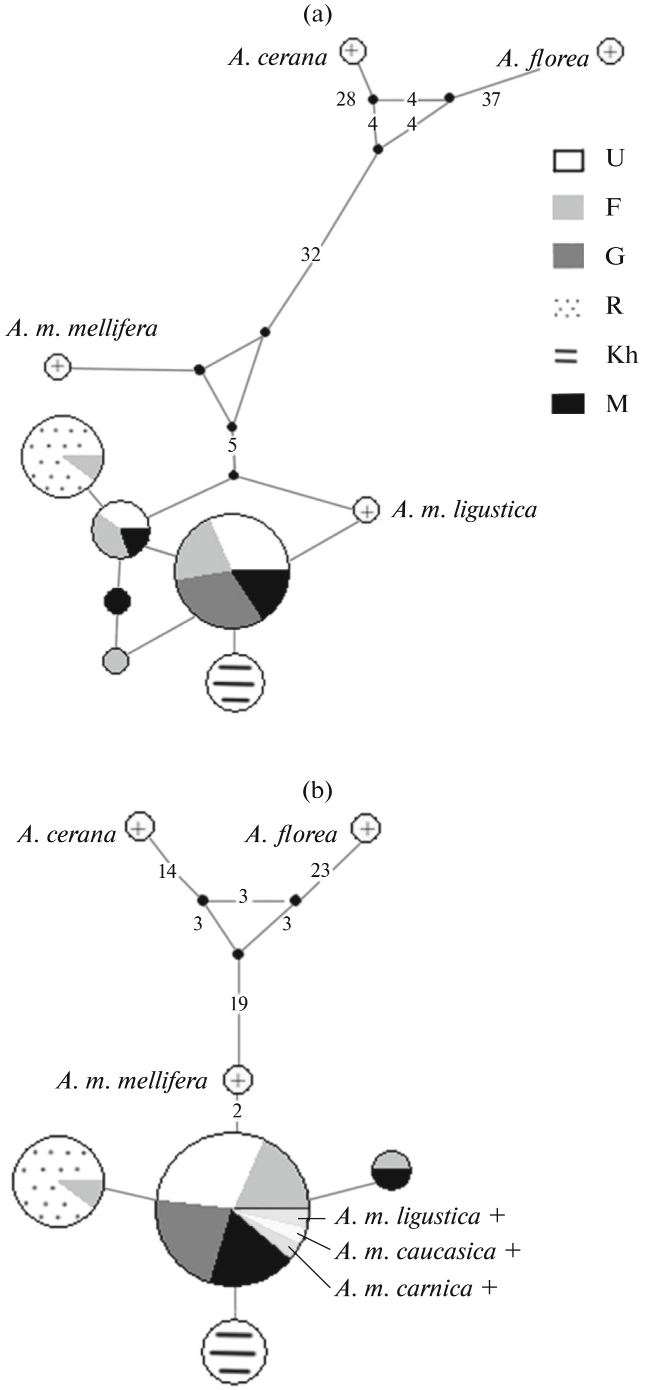

Fig. 1. Median net of haplotypes of the honeybee *Apis mellifera* L. of different races and different collection sites, built based on the mtDNA *COI* gene fragment of 680 (a) and 434 (b) base pairs. Numbers on branches indicate numbers of nucleotide substitutions, lack of numbers between haplotypes is one substitution; for clarity, the length of branches does not correspond to the number of mutations between haplotypes. Circles indicate singular haplotypes; the circle diameter corresponds to the number of specimens; + indicates that the analyzed nucleotide sequence was derived from GenBank. Black dots show hypothetical ancestors. G, Gzhel; M, Moscow; R, “the Red Caves” (mountain-forest zone of Crimea); U, Ukromnoe village; F, Feodosia; Kh, Khistevarz. The external group includes species *A. cerana* F. and *A. florae* F.

Table 2. Molecular diversity of honeybees from different apiaries

Apiary	*n*	Number of haplotypes/unique haplotypes	Pi	*H*	π	Number of variable sites, transitions
F	7	4/1	1.0476 ± 0.7848	0.7143 ± 0.1809	0.0015 ± 0.0013	3
U	8	2/0	0.4286 ± 0.4286	0.4286 ± 0.1687	0.0006 ± 0.0007	1
M	6	3/1	0.9333 ± 0.7379	0.7333 ± 0.1552	0.0014 ± 0.0013	2

M, Moscow; *n*, the number of bees; Pi, average number of pairwise differences among haplotypes; *H*, haplotype diversity; π, nucleotide variability.

Table 3. Morphometric characteristics of bees of different races

Subspecies of bees, color, region	Cubital index, %	Proboscis length, mm	Wing, mm		Reference
			length	width	
Crimean, gray, Crimean Peninsula	57.98 ± 0.77	6.44 ± 0.16	–	–	Alpatov, 1938
Caucasian gray mountain, gray					
Abkhazia	58.33 ± 0.48	6.653 ± 0.01	–	–	The same
Central Caucasus range	55.14 ± 0.67	6.856 ± 0.01	–	–	″
Krasnodar	56.81 ± 4	6.24 ± 0.28	7.04 ± 0.16	2.03 ± 0.09	Morev et al., 2013
Carpathian, gray					
Transcarpathia	33–43	6.6–7	–	–	Gaidar et al., 2004
Krasnodar	45.05 ± 2.8	6.73 ± 0.31	6.94 ± 0.26	2.03 ± 0.13	Morev et al., 2013
Italian, yellow	35–45	5.8–6.4	–	–	Lebedev and Bilash, 1991
Central Russian, dark gray	60–65	6.188 ± 0.042	6.704 ± 0.005	1.885 ± 0.002	Krivtsov et al., 1999
Ukrainian steppe, gray	30–55	6.3–6.7	–	–	Krivtsov and Sokol'skii, 2010
Krajina, dark gray	40–45	6.3–6.8	–	–	The same

"–" indicates no data.

DISCUSSION

Analysis of morphological parameters is commonly used to identify both subspecies and geographic honeybee races (Bilash and Kravtsov, 1985; Il'yasov, 2006). For example, morphometric analysis made it possible to identify three bee populations in Bulgaria, including one narrowly regional population in the highlands (Petrov, 1995). At the same time, the example of a few lines of purebred Carpathian bees showed the impossibility of attributing bees to a particular line in terms of isolated external features (Tormosina, 1985).

Comparative analysis of the cubital index brings the bees from Crimean apiaries together with the Carpathian (the highest value of the trait), Italian, Krajina, and Ukrainian steppe races (Tables 1, 3). The trait of the proboscis length in bees of the unknown race also points to its proximity to the Italian, Krajina, or Ukrainian steppe races. In addition, the length of the proboscis in bees from the studied apiary (R) also coincides with that known for the Crimean race (Tables 1, 3) (Alpatov, 1938). However, it is known that the length of the proboscis, such an important morphometric parameter for the life of bees, is subject to clinal variability and varies by the region of habitat of bees (Alpatov, 1929). Thus, this trait makes it possible to identify differences between apiaries with non-purebred bees of the same race, but it is not a criterion for attributing the bees from apiary R into a separate race. The length and width of the wing also do not make it possible to attribute conclusively bees of the analyzed apiary to the known races. The complex of morphometric parameters brings the bees from apiary R together with bees of the Italian, Krajina, and Ukrainian steppe races, and to a lesser extent with bees of the Carpathian race. However, taking into account the statement of the beekeepers that bees from apiaries U and F belong to the Carpathian race, one can also assume the proximity of the bees from apiary R to this race. This is confirmed by the same color of Carpathian bees and those of the race investigated. Some deviation from the known morphometric parameters of the Carpathian race may be associated with the fact that the bees from apiaries U and F are not purebred. It is known that standard values for the Carpathian race may differ from those for purebred bees (Gaidar et al., 2004). This assumption is also confirmed by certain molecular diversity of the bees from apiaries U, F, and M (Table 2).

Analysis of the mtDNA *COI* gene sequences of 680 and 434 bp draws attention to the fact that in both cases the bees from apiaries R and Kh form individual haplotypes (Fig. 1). The literature discusses the status of bees of the Carpathian race. One hypothesis suggests that the Carpathian race is a separate bee race (Tormosin, 1985; Morev et al., 2013). According to another hypothesis, Carpathian bees are close to Krajina bees, but are very different from the Italian bee race (Gubin, 1972). However, according to the results of studying the *COI–COII* intergenic region, the races of *A. m. caucasica*, *A. m. carnica*, and *A. m. ligustica* do not differ in terms of variability of its length (Brandorf et al., 2012). This corresponds to the notion that all these races are included in one evolutionary branch—C (Ruttner, 1988).

The obtained results of molecular genetic analysis of the initial segment of the mtDNA *COI* gene (Fig. 1a) reveal the evolutionary proximity of the bees studied to representatives of the Italian race. At the same time, we note the fact that the haplotypes studied are different from the haplotype characteristic of the Italian race (Fig. 1). This makes it possible, taking into account the complex of morphological traits, to suggest that the analyzed haplotypes belong to the Carpathian race. Then, for this *COI* site, the Italian and Carpathian races must be recognized as evolutionarily related. The proximity of the locus may be associated with the possible crossing of these races of bees, suggesting the presence of nonpurebred populations in all of the apiaries analyzed. In the populations of bees from apiaries R and Kh, represented by singular haplotypes, unlike the bees from apiaries U and F, one can assume long-term inbreeding or hybridization with a local race.

As indicated above, bees from the studied apiaries are related to the bees of the evolutionary branch C, and what is more, Ruttner attributed the Caucasian bees, along with several others, to a separate evolutionary branch O (Ruttner, 1988). Thus, as a whole, in terms of the complex of the traits studied, bees of the unknown race from apiary R cannot be attributed to representatives of the Crimean race of bees. At the same time, experiments on crossing the Krajina and Dark European bees revealed that it is the maternal heredity that dominates the hybrids (Ruttner, 1967). Taking this view, when analyzing the trait of the proboscis length and some differences in the mtDNA *COI* locus, one cannot completely reject the hypothesis that bees of this isolated apiary could have not interbred with the bees that live in mountainous areas of Crimea and carry traits that are typical of the Crimean race of bees.

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