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Novel haplotypes of the COI-COII mtDNA region in the dark forest bee, *Apis mellifera mellifera* L., 1758

Новые гаплотипы локуса COI-COII мтДНК у темной лесной пчелы *Apis mellifera mellifera* L., 1758

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КЛЮЧЕВЫЕ СЛОВА: *Apis mellifera mellifera*, локус *COI-COII* мтДНК, разнообразие, адаптивная эволюция, Сибирь.

ABSTRACT: An assessment of the genetic diversity of the *COI-COII* mtDNA locus in dark forest bee *Apis mellifera mellifera* L., 1758 in Siberia (Russia) was conducted. Two variants of the *COI-COII* mtDNA locus were registered: PQQ and PQQQ (allele frequencies 0.83 and 0.17, respectively). According to the *Dra I COI-COII* test, the haplotypes of PQQ and PQQQ variants identified in Siberian bees correspond to the most frequent European haplotypes M4 and M4′, but their nucleotide sequences are novel in *A. m. mellifera*. At the same time, a high similarity of the studied nucleotide sequences of the intergenic region *COI-COII* with the reference sequences (Genbank) described in dark forest bees living in Northern Europe (the Netherlands, United Kingdom, and Ireland) (99% identity) was established, which may indicate the significance of these mtDNA variants for adaptation to cold climatic conditions.

РЕЗЮМЕ: Проведено исследование генетического разнообразия локуса *COI-COII* мтДНК у темной лесной пчелы *Apis mellifera mellifera* L., 1758 на территории Сибири. Зарегистрировано два варианта локуса *COI-COII* мтДНК — PQQ и PQQQ с частотой встречаемости 0,83 и 0,17, соответственно. Согласно *Dra I*-тесту, гаплотипы вариантов PQQ и PQQQ, выявленные у пчел сибирских популяций, соответствуют наиболее часто встречающимся европейским гаплотипам M4 и M4′, но их нуклеотидные последовательности представляют собой новые варианты у *A. m. mellifera*. Вместе с тем, значительное сходство (99% идентичность) исследованных нуклеотидных последовательностей межгенного района локуса *COI-COII* мтДНК показано с референс-последовательностями, представленными в Генбанке (Genbank) и описанными у темной лесной пчелы, обитающей в Северной Европе (Нидерланды, Великобритания и Ирландия), что может свидетельствовать о значимости этих вариантов мтДНК для адаптации к суровым условиям обитания.

Introduction

In Europe, ten subspecies of the honeybee, *Apis mellifera* Linnaeus, 1758, among the 30 currently recognized worldwide are described [Sheppard, Meixner, 2003; De la Rúa *et al*., 2009; Meixner *et al*., 2013]. These European subspecies belong to different evolutionary branches, and most subspecies (*Apis mellifera ligustica* Spinola, 1806; *Apis mellifera carnica* Pollmann, 1879; *Apis mellifera macedonica* Ruttner, 1988; and others) are assigned to the evolutionary lineage C. The distribution area of the C lineage subspecies includes the Apennine and Balkan peninsulas and reaches the Alps in the north and Sicily and the Aegean islands in the south [Ruttner, 1988]. Only two subspecies, *Apis mellifera mellifera* Linnaeus, 1758 (or the dark forest bee) and *Apis mellifera iberiensis* Engel, 1999 are attributed to the lineage M, and the *A. m. mellifera* subspecies occupied a vast territory ranging from northern Iberian Peninsula in the south to Scandinavia in the north, and from the British Isles in the west to the Ural Mountains in the east [Ruttner, 1988; Pinto *et al*., 2014]. In addition, the populations of the *A. m. mellifera* subspecies are found

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further east from the Ural Mountains in Siberia (eastern part of Russia) [Ostroverkhova *et al*., 2018].

In the recent years, the natural range of the *A. m. mellifera* subspecies, which is considered endangered in Europe, has been significantly reduced due to the mass bee hybridization [Jensen, Pedersen, 2005; Soland-Reckeweg *et al*., 2009; Munoz *et al*., 2015]. For example, in Western Europe, where the dark forest bee lived, there is a rapid spread of commercial subspecies such as the Italian honeybee *A. m. ligustica* and the Carniolan honeybee *A. m. carnica* [Bouga *et al*., 2011; Pinto *et al*., 2014]. As a result, in Europe, a change in the representation of the honeybee subspecies and their genotypic composition is registered.

In Northern Europe, protected areas are being created to conserve a unique *A. m. mellifera* gene pool, and a search for the preserved populations of the dark forest bee is being conducted [Pinto *et al*., 2014]. A significant pure population of the dark forest bee was therefore found in Ireland [Hassett *et al*., 2018]. In Russia, there are two large reserves of dark forest bees, known as the Burzyan (Bashkortostan, the Urals) and the Yenisei (Krasnoyarsk Krai, Siberia) populations. If the Burzyan population has been actively studied for a long time in different directions, including the biological, genetic, ecological aspects [Nikonorov *et al*., 1998; Ilyasov *et al*., 2007, 2011, 2016], the data for the Yenisei population are rare [Ostroverkhova *et al*., 2018]. However, without knowledge of the biological, genetic and ecological characteristics of native subspecies (ecotypes, races), as well as without an assessment of the level of gene introgression, the *A. m. mellifera* gene pool conservation programs and the use of local populations as a source of genetic material for sustainable beekeeping will not be successful [De la Rúa *et al*., 2009; Soland-Reckeweg *et al*., 2009; Meixner *et al*., 2010; Oleksa *et al*., 2011; Pinto *et al*., 2014].

The mitochondrial genome, namely, length and sequence variations of mtDNA (for example, 16S rDNA, ND5, *COI-COII* intergenic region), has been actively used for bee population genetic studies, as well as in differentiating evolutionary lineages and subspecies and determining the genetic structure of the honeybee [Franck *et al*., 1998; De la Rúa *et al*., 2001, 2002; Bodur *et al*., 2007; Muñoz *et al*., 2009, 2012; Stevanovic *et al*., 2010].

The genetic diversity and structure of the *COI-COII* locus of the intergenic spacer region of mtDNA (tRNAleu-cox2 intergenic region includes a non-coding sequence formed by two elements (P and Q) and part of cox2 region) in the different European dark bee populations have been analyzed by *Dra I*-RFLPs (Restriction Fragment Length Polymorphisms) and sequencing of the amplified intergenic *COI-COII* sequence [Rortais *et al*., 2011; Pinto *et al*., 2014; Hassett *et al*., 2018]. A total about 7,000 bee colonies belonging to the M lineage were investigated, and more than 100 haplotypes were found in dark forest bees.

The most common haplotypes in *A. m. mellifera* bees are M4b, M4d and M4a' (59% studied colonies), but frequencies of the haplotypes differ between various populations. For example, only one haplotype (M4a') was registered in bees from Scotland, the haplotype M4d prevailed in bees from the Netherlands (67% colonies), two haplotypes M4b and M4d predominated in bees from Norway (70% colonies), and the most common haplotype in the total Irish population was M4e (41% colonies). It is important to emphasize that the populations of Northern Europe (Norway, Scotland, the Netherlands, and Ireland) are the most homogenous and the "purest" populations with average membership proportions in the *A. m. mellifera* cluster higher than 0.97 [Pinto *et al*., 2014; Hassett *et al*., 2018].

To date, little information is available on diversity and genetic structure of *A. m. mellifera* populations in Siberia. Only a few studies have been conducted to assess the genetic diversity and hybridization status of Siberian dark forest bee by morphometrics, mtDNA and microsatellite loci analysis [Ostroverkhova *et al*., 2015, 2018, 2019]. Initially, using morphometry and analysis of mtDNA polymorphism (*COI-COII* locus), we searched for *A. m. mellifera* populations in Siberia [Ostroverkhova *et al*., 2015, 2018]. The "purest" *A. m. mellifera* populations were found in the taiga of the Krasnoyarsk Krai (Yenisei population) and the north of the Tomsk Region (Ob population) [Ostroverkhova *et al*., 2018, 2019].

The objective of this work was to study the variability and the nucleotide sequence of the *COI-COII* locus of the intergenic spacer region of mtDNA in *A. m. mellifera* bees in Siberia. This is the first study reporting on maternal diversity patterns using *Dra I* test and sequence data for Siberian *A. m. mellifera* populations.

Materials and methods

Regions of the honeybee collections

Worker bees of the dark forest bee colonies living in Siberia were investigated: from the Tomsk Region, the Krasnoyarsk Krai (Yenisei population), and the Altai Krai (Fig. 1). In Siberia, the honeybee *A. mellifera* was introduced 230 years ago; bee colonies are artificial populations whose wintering is controlled by humans. It was originally a dark forest bee *A. m. mellifera* that was cultivated in Siberia as the most adapted to the harsh climatic conditions of the region. At the end of the last century, honeybees of southern subspecies, for example, *A. m. carnica*, had been actively imported into Siberia, which led to mass bee hybridization. At the same time, *A. m. mellifera* populations have been preserved in some areas of Siberia. For example, the Yenisei population of the Krasnoyarsk Krai is a unique, long-time isolated population that existed for more than 60 years in the deep taiga kept by the Old Believers [Ostroverkhova *et al*., 2018].

Siberia is a large region of the northeastern part of Eurasia and stretched from the Urals to the Baikal ridge. The territory of Siberia has a wide variety of natural and climatic conditions (Table 1). The climate of Siberia varies from typical continental (the Tomsk Region) to sharply continental (the Krasnoyarsk Krai) with considerable daily and annual temperature amplitudes and long winters (5–6 months). The average annual temperatures are from –10.5 °C in the north to 1–2 °C in the south of Siberia. The warmest month is July; the average July temperature varies from 3.6 to 21–22 °C in different areas of the region. The winter is cold; the average January temperature varies from –18 °C in the south to –30 °C in the north of Siberia. In winter, the territory is covered with snow; snow covers the land for 260 days in the north and 170–180 days in the south of the region. Precipitation is, on average, 300–600 mm. Most of the territory of Siberia is within the taiga zone, where forests cover about 60% of the area (for details, see [Ostroverkhova *et al*., 2019]).

Biological samples

Between 2015 and 2018, a sampling was carried out in three regions of Siberia: the Tomsk Region (11 apiaries), the Krasnoyarsk Krai (Yenisei population, 6 apiaries), and the Altai Krai (3 apiaries) (Fig. 1). In total, we examined 20 apiaries in Siberia, where the dark forest bee was found. In each apiary, from 2 to 29 bee colonies were examined; a total of 182 colonies. Workers were collected from the inner part of hives, placed in absolute ethanol, and stored at –20 ºC until DNA extraction.

To determine conformance bee colonies to the *A. m. mellifera* breed standard, each bee colony was analyzed using the variability in the locus *COI-COII* of mtDNA and morphometric parameters of wing, including the cubital index, the hantel index, and the discoidal shift. To exclude mixed colonies, three individuals from each colony were examined; a total of 546 workers [Ostroverkhova *et al*., 2015, 2018].

To study the structure of the locus *COI-COII* of mtDNA, we initially examined the variability of this locus using the *Dra I COI-COII* test [Rortais *et al*., 2011]; 182 individual bee samples in total. Then we conducted the DNA sequencing of the *COI-COII* intergenic region of *A. m. mellifera* samples from different geographic locations. For sequencing of the *COI-COII* locus, one bee sample was taken from each apiary. Only for three apiaries (Asino, Dubrovka, and Zmeinogorsk), where different *COI-COII* locus variants were identified in bees, two bee samples were examined. Thirteen bee samples were from the apiaries of the Tomsk Region, six samples were from the Krasnoyarsk Krai, and four samples were from the Altai Krai. A total of 23 bee DNA samples from the *A. m. mellifera* colonies (18 DNA samples with the variant PQQ and 5 DNA samples with the variant PQQQ) were selected (Table 1).

Experimental procedures

DNA isolation and polymerase chain reaction (PCR) was carried out according to standard techniques. Total DNA was extracted from the thorax of worker bees using a phenol-chloroform-isoamyl alcohol (25:24:1) protocol [Sambrook *et al*., 1989]. To amplify the *COI-COII* mtDNA locus, the following sequences of primers were used: F-5'-CACATTTAGAAATTCCATTA, R-5'- ATAAATATGAATCATGTGGA [Nikonorov *et al*., 1998]. PCR included an initial denaturation of 5 mins at 95 °C, followed by 35 cycles of 95 °C for 1 min, 57 °C for 2 mins and 72 °C for 2 mins with a final extension of 7 min at 72 °C [Nikonorov *et al*., 1998]. The size of the amplified DNA fragments was determined using a 1.5% agarose gel, and the fragment patterns (i.e., haplotypes) were determined on a 10% polyacrylamide gel after *Dra I* digestion of PCR products. The results were documented with the use of Gel-Doc XR+.

For sequencing of the mtDNA *COI-COII* locus, 18 DNA samples of bees with the PQQ variant (6 apiaries of the Krasnoyarsk Krai; 9 apiaries of the Tomsk Region; and 3 apiaries of the Altai Krai) and 5 DNA bee samples with the variant PQQQ (4 apiaries of the Tomsk Region, and an apiary of the Altai Krai) were selected; a total 23 bee DNA samples from the *A. m. mellifera* colonies (Table 1).

Sequencing of the samples was carried out in the Collective Center of Medical Genomics (Research Institute of Medical Genetics, Tomsk National Research Medical Center, Russian Academy of Sciences) using an Applied Biosystems 3730 automatic analyzer with the BigDye®Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Data analysis was performed using Sequencing Analysis v. 5.4 software. The resulting nucleotide sequences were aligned with the BioEdit program [Hall, 1999]. The resulting sequences were compared with the published mitochondrial DNA sequences available in Genbank (NCBI Reference Sequence Database, https://www.ncbi.nlm.nih.gov). Haplotype sequences obtained during the present study were deposited in 2019 in GenBank as accession numbers MN331472 and MN331473.

Results

Genetic diversity of the *COI-COII* mtDNA locus in *A. m. mellifera* in Siberia

We studied variability of the *COI-COII* mtDNA locus in the *A. m. mellifera* honeybees from Siberia (Fig. 1). Two variants of the *COI-COII* mtDNA locus that belong to the M lineage were registered: PQQ and PQQQ. The variant PQQ was the most frequent; the frequency of PQQ was 0.83. For example, all studied bees from a long-time isolated Yenisei population (the Krasnoyarsk Krai) had the PQQ variant of the *COI-COII* mtDNA locus. The PQQQ variant was registered with a frequency of 0.17 and was identified only in bees from the Tomsk Region (Zarechny, Teguldet, Dubrovka villages and the vicinity of the town of Asino) and the Altai Krai (the vicinity of the city of Zmeinogorsk).

Structure of the *COI-COII* mtDNA locus in *A. m. mellifera* in Siberia

We studied the nucleotide sequences of different variants (PQQ and PQQQ) of the *COI-COII* intergenic region in the *A. m. mellifera* bees from the Siberian populations. According to the *Dra I COI-COII* test, all haplotypes identified in the *A. m. mellifera* bees (182 samples) are the most common haplotypes in the European bees — M4 (PQQ) and M4' (PQQQ).

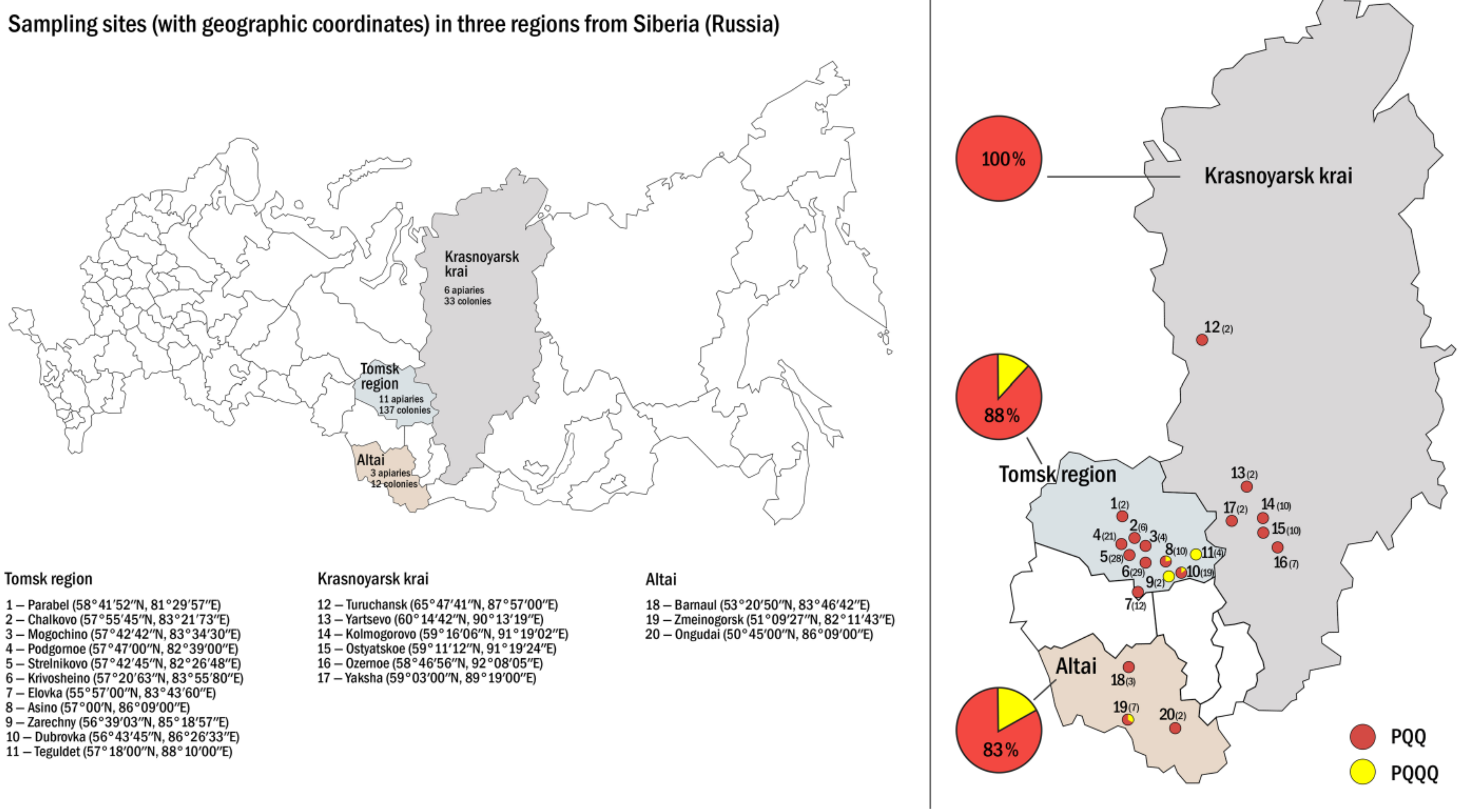
For sequencing of the *COI-COII* mtDNA locus, 23 bee DNA samples from the *A. m. mellifera* Siberian colonies were studied: 18 DNA samples of bees with the PQQ variant and 5 DNA samples of bees with the PQQQ variant. The results showed that the second and third repetitive segments Q2 and Q3 of variants PQQ and PQQQ of the *COI-COII* mtDNA locus were identical to each other and had a size of 195 bp, but differ from the first repetitive segment Q1, the size of which was 197 bp. The Q2/Q3 repeats had two deletions (nucleotide T at position 34 and nucleotide A at position 149) and a transition T→C at position 4 (Fig. 2).

A comparative analysis of the nucleotide sequences of the *COI-COII* locus of Siberian bees with reference sequences in the *A. m. mellifera* subspecies presented in the Genbank did not show full nucleotide correspondence with any of the described haplotypes, identified by the presence of 1–2 nucleotide indels (M4a–M4n

Table 1. Geo-coordinates, climatic conditions, and landscape type of the sampling sites of bee colonies used for sequencing.

Таблица 1. Географические координаты, климатические условия и ландшафт точек сбора пчелиных семей, использованных для секвенирования.

Geographical region	Climate type	Sampling localities (Map code)	Number of bee colonies per sampling location	Latitude, N	Longitude, E	Altitude	Mean annual temperature (ºC)	Mean relative humidity (%)	Mean annual precipitation (mm)	Biome type
Tomsk Region	Typical continental	Parabel	1	58°41′52″	81°29′57″	71	–1,4	70–88	500	Taiga
		Mogochino	1	57°42′42″	83°34′30″	104	–0,9	76	450–500	
		Podgornoe	1	57°47′00″	82°39′00″	97	–0,8	76	450–500	
		Chalkovo	1	57°55′45″	83°21′73″	81	–1,0	76	480–500	
		Strelnikovo	1	57°42′45″	82°26′48″	67	–0,9	75	470–500	
		Krivosheino	1	57°20′63″	83°55′80″	96	–0,8	76	400–450	
		Dubrovka	2	56°43′45″	86°26′33″	119	0,5	76	523	
		Asino	2	57°00′	86°09′	122	0,4	76	537	
		Teguldet	1	57°18′00″	88°10′00″	142	–0,5	76	250–482	
		Zarechny	1	56°39′03″	85°18′57″	117	–0,6	76	400–550	
		Elovka	1	55°57′00″	83°43′60″	128	0,9	74	390–415	
Krasnoyarsk Krai	Sharply continental	Turuchansk	1	65°47′41″	87°57′00″	35	–4,3	60–80	450	
		Kolmogorovo	1	59°16′06″	91°19′02″	60	–1,5	75	460	
		Ostyatskoe	1	59°11′12″	91°19′24″	63	–1,5	75	460	
		Ozernoe	1	58°46′56″	92°08′05″	74	–1,5	75	460	
		Yaksha	1	59°03′00″	89°19′00″	60	–1,5	75	200–350	
		Yartsevo	1	60°14′42″	90°13′19″	60	–1,5	75	460	
Altai Krai		Ongudai	1	50°45′00″	86°09′00″	831	–1,1	59–75	380–450	Mountain taiga
	Conti-nental	Barnaul	1	53°20′50″	83°46′42″	191	2,6	65–75	433	Forest-steppe
		Zmeinogorsk	2	51°09'27"	82°11'43"	383	2,4	70–80	690	



Tomsk region

1 – Parabel (58°41′52″N, 81°29′57″E)
2 – Chalkovo (57°55′45″N, 83°21′73″E)
3 – Mogochino (57°42′42″N, 83°34′30″E)
4 – Podgornoe (57°47′00″N, 82°39′00″E)
5 – Strelnikovo (57°42′45″N, 82°26′48″E)
6 – Krivosheino (57°20′63″N, 83°55′80″E)
7 – Elovka (55°57′00″N, 83°43′60″E)
8 – Asino (57°00′N, 86°09′00″E)
9 – Zarechny (56°39′03″N, 85°18′57″E)
10 – Dubrovka (56°43′45″N, 86°26′33″E)
11 – Teguldet (57°18′00″N, 88°10′00″E)

Krasnoyarsk krai

12 – Turuchansk (65°47′41″N, 87°57′00″E)
13 – Yartsevo (60°14′42″N, 90°13′19″E)
14 – Kolmogorovo (59°16′06″N, 91°19′02″E)
15 – Ostyatskoe (59°11′12″N, 91°19′24″E)
16 – Ozernoe (58°46′56″N, 92°08′05″E)
17 – Yaksha (59°03′00″N, 89°19′00″E)

Altai

18 – Barnaul (53°20′50″N, 83°46′42″E)
19 – Zmeinogorsk (51°09′27″N, 82°11′43″E)
20 – Ongudai (50°45′00″N, 86°09′00″E)

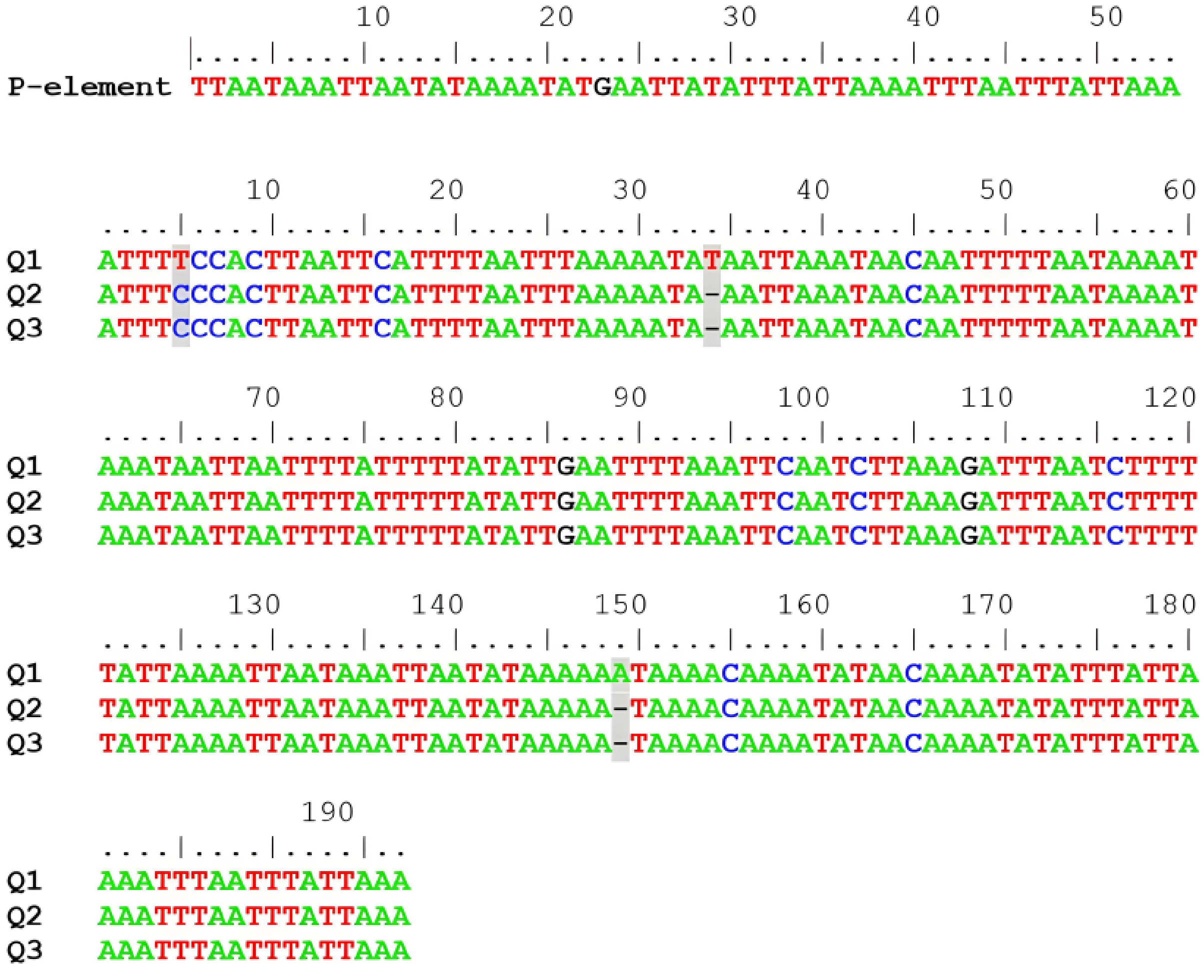
Fig. 1. The map of localization of areas in Siberia (the Tomsk Region, the Krasnoyarsk Krai, the Altai Krai) and apiaries (dots 1–20), where a dark forest bee is identified. The distribution of *COI-COII* mtDNA locus variants (PQQ and PQQQ) in *Apis mellifera mellifera* bees from Siberian apiaries and their frequency in three regions of Siberia are presented on the right side of the figure.

Рис. 1. Карта регионов Сибири (Томская область, Красноярский край, Алтайский край) и локализации пасек (точки 1–20) на территории Сибири, где выявлена темная лесная пчела. Распределение вариантов локуса *COI-COII* мтДНК (PQQ и PQQQ) у темных лесных пчел на пасеках и их частота в трех регионах Сибири представлены в правой части рисунка.

or M4'a) [Rortais *et al*., 2011; Pinto *et al*., 2014; Hassett *et al*., 2018]. The haplotype M4 of PQQ variant detected in the Siberian bees showed a 99% identity to the sequences presented in the GenBank under the numbers KF274629 (*Apis mellifera mellifera* haplotype M4f *COI-COII* intergenic spacer, partial sequence; and cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial) and KF274628 (*Apis mellifera mellifera* haplotype M4e *COI-COII* intergenic spacer, partial sequence; and cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial), described in the *A. m. mellifera* (GenBank). The haplotypes M4f and M4e presented in the Genbank differ among themselves by a single T/C substitution at position 653.

The haplotype M4' of PQQQ variant registered in honeybees of the Siberian populations showed a 99% identity to the sequence presented in the Genbank under the number KF274638 (*Apis mellifera mellifera* haplotype M4a' *COI-COII* intergenic spacer, partial sequence; and cytochrome oxidase subunit II (COII) gene, partial cds; mitochondrial). The revealed differences between the haplotype M4 of PQQ variant in Siberian bees and haplotypes M4f and M4e, and between the haplotype M4' of PQQQ variant in Siberian bees and haplotype M4a' are associated precisely with deletions of nucleotides A and T in Q2 and Q3 repeats in Siberian bees.

Interestingly, the nucleotide sequence *COI-COII* of the mtDNA in *A. m. mellifera* bees from Siberian populations showed 100% identity to the mtDNA sequence (Genbank Sequence ID: MN733955) of the recently described subspecies of honey bee — *Apis mellifera sinisxinyuan* Chen et al., 2016 [Chen *et al*., 2016]. The *A. m. sinisxinyuan* subspecies is distributed in the Yili River Valley in western China and assigned to the M evolutionary lineage [Chen *et al*., 2016; Yang *et al*., 2020]



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                     10        20        30        40        50
                     |....|....|....|....|....|....|....|....|....|....|....
P-element            TTAATAAATTAATATAAAATATGAATTATATTTATTAAAATTTAATTTATTAAA

                10        20        30        40        50        60
           ....|....|....|....|....|....|....|....|....|....|....|....|
Q1         ATTTTCCACTTAATTCATTTTAATTTAAAAATATAATTAAATAACAATTTTTAATAAAAT
Q2         ATTTCCCACTTAATTCATTTTAATTTAAAAATA-AATTAAATAACAATTTTTAATAAAAT
Q3         ATTTCCCACTTAATTCATTTTAATTTAAAAATA-AATTAAATAACAATTTTTAATAAAAT

                70        80        90       100       110       120
           ....|....|....|....|....|....|....|....|....|....|....|....|
Q1         AAATAATTAATTTTATTTTTATATTGAATTTTAAATTCAATCTTAAAGATTTAATCTTTT
Q2         AAATAATTAATTTTATTTTTATATTGAATTTTAAATTCAATCTTAAAGATTTAATCTTTT
Q3         AAATAATTAATTTTATTTTTATATTGAATTTTAAATTCAATCTTAAAGATTTAATCTTTT

               130       140       150       160       170       180
           ....|....|....|....|....|....|....|....|....|....|....|....|
Q1         TATTAAAATTAATAAATTAATATAAAAAATAAAACAAAATATAACAAAATATATTTATTA
Q2         TATTAAAATTAATAAATTAATATAAAAA-TAAAACAAAATATAACAAAATATATTTATTA
Q3         TATTAAAATTAATAAATTAATATAAAAA-TAAAACAAAATATAACAAAATATATTTATTA

                    190
           ....|....|....|..
Q1         AAATTTAATTTATTAAA
Q2         AAATTTAATTTATTAAA
Q3         AAATTTAATTTATTAAA
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Fig. 2. The pattern of the P element sequence (54 bp) and the Q element (Q1, Q2, and Q3) sequences (197, 195, 195 bp, respectively) of the *COI-COII* intergenic region of haplotypes M4 and M4' in *Apis mellifera mellifera* bees from Siberia. Nucleotide substitutions are highlighted, deletions are indicated by a dash and highlighted.

Рис. 2. Структура Р-элемента (54 п.н.) и Q-элементов (Q1, Q2 и Q3) (197, 195, 195 п.н., соответственно) межгенной области *COI-COII* мтДНК (гаплотипы М4 и М4') у пчел *Apis mellifera mellifera* сибирских популяций. Нуклеотидные замены выделены цветом, делеции обозначены тире и выделены цветом.

Discussion

Since 2010, we have analyzed more than 2,000 bee colonies of Siberian populations using polymorphism analysis of the *COI-COII* mtDNA locus (without the *Dra I* test and sequencing) [Ostroverkhova *et al*., 2015, 2019]. According to these results, local *A. m. mellifera* populations have been identified in Siberia [Ostroverkhova *et al*., 2018, 2019].

In the present study, the genetic diversity and structure of the *COI-COII* mtDNA locus in the dark forest bee *A. m. mellifera* of Siberian populations were examined using *Dra I* test and sequencing of the *COI-COII* mtDNA locus. Samples from 182 colonies were selected from the Tomsk Region, the Krasnoyarsk Krai, and the Altai Krai. The results showed two variants of the *COI-COII* mtDNA locus: PQQ (83.0% of colonies) and PQQQ (17.0% of colonies). A total of two haplotypes of the *COI-COII* mtDNA locus were identified (M4 of the PQQ variant and M4' of the PQQQ variant), and their nucleotide sequences are novel in *A. m. mellifera*.

A global assessment of the diversity found in the M evolutionary lineage (i.e., description of haplotype frequencies, fragment length and size, nucleotide sequences of the *COI-COII* mtDNA) was made with 6,628 bee colonies of different European populations (Belgium, Denmark, United Kingdom, France, Ireland, Italy, Norway, Spain, and Sweden) [Rortais *et al*., 2011]. Later, a total of 90 *A. m. mellifera* colonies from European populations including England, France, Belgium, Denmark, the Netherlands, Switzerland, Scotland, and Norway, and 255 *A. m. mellifera* colonies from Ireland were investigated [Pinto *et al*., 2014; Hassett *et al*., 2018]. Among the 100 haplotypes identified in the dark forest bee, the most common variants (59% studied bee colonies) are M4 and M4'. It is these haplotypes of the mtDNA *COI-COII* locus that were identified in the dark forest bees of Siberian populations.

The following observations should be noted. First, the PQQ and PQQQ sequences, specific to Siberian bees including the long-time isolated population of the Krasnoyarsk Krai, belong to the M4 and M4' haplogroups. Second, the sequences M4f, M4e, and M4a', with which the greatest similarity is shown in Siberian bees, were found in bees living in regions of the Northern Europe [Pinto *et al*., 2014; Hassett *et al*., 2018]. The haplotype M4f was registered in bees of only 2 colonies from the Netherlands and in bees of 9 colonies from Ireland. The haplotype M4e was found in bees of 3 colonies from the Netherlands and of 3 colonies from England, but was the main mtDNA variant in bees from Ireland (it is registered in bees of 150 colonies; 41% bee colonies). The haplotype M4a' was detected in all studied bees from Scotland (in 10 bee colonies) and in a single colony from Ireland. Third, the nucleotide sequences *COI-COII* of the mtDNA in *A. m. mellifera* bees from Siberian populations and the subspecies *A. m. sinisxinyuan* discovered in China showed 100% identity [Chen *et al*., 2016].

Thus, the PQQ and PQQQ variants identified in Siberian bees are represented by novel *A. m. mellifera* haplotypes that differ from the previously described variants by 1–2 bp indels. However, the high similarity of the mtDNA nucleotide sequences (99% identity) found for bees living in different regions such as Siberia and Northern Europe (the Netherlands, Scotland, England, and Ireland) may indicate the significance of these mtDNA variants for adaptation of bees to harsh climatic conditions. This assumption is supported by data on another subspecies of the evolutionary M-lineage — *A. m. sinisxinyuan*, which diverged from *A. m. mellifera* about 132,000 years ago [Chen *et al*., 2016]. The *A. m. sinisxinyuan* subspecies living in Xinyuan prefecture (Xinjiang Uygur Autonomous Region, China) is winter-tolerant and adapted to temperate climates. The natural environment in Xinyuan is characterized by long, cold winters due to a combination of high latitude and altitude, with a frost season of 185–205 days [Chen *et al*., 2016]. Genetic variation in the genome including mitochondrial DNA of temperate populations of *A. m. sinisxinyuan* and *A. m. mellifera* can be characterized by adaptations to the extended cold winter [Chen *et al*., 2016; Yang *et al*., 2020].

Finally, Siberian populations of *A. m. mellifera*, especially in the Krasnoyarsk Krai and the Tomsk Region, are homogeneous and genetically similar in mtDNA sequences. It is known that honeybee colonies were imported into Siberia several times in different years, that is, we should expect a greater genetic diversity of the bees. However, only two haplotypes of the mtDNA locus in the dark forest bee of Siberian populations have been identified, which may indicate the adaptive value of these variants. Consequently, selection may have played a role in shaping honeybee regional mtDNA variation and that cold climate was one of the selective pressures. As the predominant mtDNA sequences were found in bees from different populations of Europe, China, and Siberia, their frequency and distribution support the hypothesis of a bottleneck event in which a few bee colonies contributed a limited number of mtDNAs and then spread (the founder effect).

In recent years, it has been shown that mtDNA variations might impact various phenotypic traits and might have a role in bioenergetics pathways, metabolic rates and energy consumption depending on environment as mitochondrial metabolism is important for maintaining body temperature [Lavrov, Pett, 2016; Ladoukakis, Zouros, 2017; Klucnika, Ma, 2019; Sun *et al*., 2019; Sanno *et al*., 2021]. Some factors such as climate, food availability, and environmental pressures that produce high levels of stress can exert a strong influence on mitochondrial genomes and promote the persistence of certain genotypes in order to compensate for the metabolic requirements of the local environment [Noll *et al*., 2022]. Therefore, selection on mtDNA sequences may lead to haplotypes adapted to different environments [Blier, Lemieux, 2001; Fontanillas *et al*., 2005; Bandyopadhyay *et al*., 2008; Ballard, Melvin, 2010; Pichaud *et al*., 2012].

Mitochondrial polymorphism has been shown to be associated with different parameters, such as resistance to insecticides in arthropod pests [Van Leeuwen *et al*., 2008], fitness traits and temperature sensitivity in fruit flies [James, Ballard, 2003; Pichaud *et al*., 2013], muscle composition in pigs [Fernández *et al*., 2008], environmental adaptations in the greater white-toothed shrew *Crocidura russula* (Hermann, 1780), Arctic charr, and other animals [Blier, Lemieux, 2001; Fontanillas *et al*., 2005]. For example, high altitude populations of mammals, birds, fish, and invertebrates in Tibet show adaptive mtDNA haplotypes compared with low altitude populations [Li *et al*., 2018]. Using grasshoppers as a model, the evolutionary molecular response of insect mitochondrial genes to the reduction of atmospheric oxygen content and high-altitude environments has been investigated for the first time. It has been shown that positive selection drives adaptive evolution in mitochondrial genomes, both with respect to flight and to survival in challenging environments [Li *et al*., 2018]. In addition, a study of the mitochondrial genomes of two closely related but ecologically distinct species of *Myodes* Pallas, 1811 voles showed that the *rutilus* haplotype individuals inhabits cooler and drier habitats than the *glareolus* haplotype individuals. This difference may result from local adaptation or from the geographic context of introgression, and mtDNA may have played an important role in the adaptive evolution of these species [Boratyński *et al*., 2014].

Thus, in some species, the mitochondrial genome is associated with adaptive evolution to different climatic conditions and may be highly sensitive to the interaction with environment. In the dark forest bee, we identified two haplotypes of the mtDNA *COI-COII* locus, which are structurally similar to the haplotypes described in *A. m. mellifera* bees living in Northern Europe, which may indicate the significance of habitat conditions. It is important to understand if the identified structural features of the mtDNA *COI-COII* locus are universal or specific characteristics of the *A. m. mellifera*. Sequencing of the mitogenome of different subspecies of honeybees will be useful for such studies [Henriques *et al*., 2019]. As noted by Yang *et al*. [2020], sequencing the complete mitochondrial genome of *A. m. sinisxinyuan* (GenBank: MN733955) would be helpful to better elucidate the evolution and adaptation of the honey bee. Until now, climatic adaptation of mitochondrial genomes of honeybees remains an unsolved problem.

Conclusion

This study allowed us for the first time to investigate the variability and nucleotide sequence of the *COI-COII* mtDNA locus and identify novel mtDNA haplotypes in the *A. m. mellifera* honeybees living in Siberia for a long time, including isolated territories in the deep taiga of the Krasnoyarsk Krai. Our study contributes to the understanding of the genetic basis of *A. mellifera* adaptation to temperate climates at the genomic level.

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Conflict of interest. The authors declare that they have no potential conflict of interest in relation to the present study.

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