Chapter 9

Dark-Colored Forest Bee *Apis mellifera* in Siberia, Russia: Current State and Conservation of Populations

Nadezhda V. Ostroverkhova, Aksana N. Kucher,
Olga L. Konusova, Ekaterina S. Gushchina,
Vadim V. Yartsev and Yury L. Pogorelov

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Abstract

A comprehensive research of two dark-colored forest bee populations in Siberia, identified during a screening study, was conducted using morphometric and molecular genetic methods. The first population is an isolated Yenisei population located in the taiga zone in the Krasnoyarsk Territory, on which bees have not been imported for a long time (50–60 years). The second population is located in the northern areas of the Tomsk region, where beekeeping is more developed. All studied bees had a variant PQQ of the COI–COII mtDNA locus. However, some morphometric parameters of some bee colonies deviated from the *Apis mellifera mellifera* standard, which is probably due to the features of population formation. As a result of the analysis of the variability of 18 microsatellite loci, possible potential DNA markers specific for determining the bee subspecies and/or ecotypes of the dark-colored forest bee have been identified. An algorithm for the search and a comprehensive study of the dark-colored forest bee are proposed.

Keywords: honeybee, dark-colored forest bee, *Apis mellifera mellifera*, genetic diversity, morphometric parameters, COI–COII mtDNA locus, microsatellites, Siberia

1. Introduction

The species *Apis mellifera* L. includes 30 bee subspecies and has a huge natural range covering the whole of Africa, Europe, and the Middle East. On the basis of morphological analysis, all bee subspecies were grouped into four evolutionary branches (A, M, C, and O), which corresponded to the geographic origin of subspecies [1]. However, the data of mtDNA analysis

of honeybee subspecies showed that mtDNA variants do not always correspond to the morphological system: bee subspecies grouped into morphological branches C and O do not significantly differ for the variants of mtDNA. According to mtDNA data, three evolutionary branches are distinguished (A, M, and C), although an additional branch of African origin is identified into branch A [2–4]. For example, bee subspecies of the evolutionary branch C (southern bee subspecies) have the shortest sequence of COI–COII mtDNA locus (variant Q); bee subspecies of branches M and A are characterized by a longer sequence (one of the variants PQ, PQQ, PQQQ, PQQQQ, or PQQQQQ is detected) [5, 6]. Thus, the specificity of bee subspecies for the structure of the COI–COII mtDNA locus makes it possible to determine the origin of the honeybee for the maternal line.

Since honeybees do not have sex chromosomes, as additional information on the origin of bees, data on autosomal loci, for example, on microsatellites, can be used. However, genetic diversity of the autosomal loci in different bee subspecies is still poorly understood. At the same time, molecular genetic studies of 14 subspecies with the use of nuclear markers (SNP) allowed identification of the groups that largely reflect the traditional four morphological branches [7].

There are negative trends in the development of honeybee populations both in Russia and in the world in recent years. The most dangerous processes, having catastrophic consequences, are the mass mortality of bee colonies and uncontrolled hybridization of bees. So far, the reasons for the bee collapse have not yet been fully defined [8]. Mass hybridization between *A. mellifera* subspecies leads to the destruction of the existing evolutionary genetic complexes of individual species, and the emergence of hybrids interbreeds with unwanted phenotypic traits and unpredictable combinations of genetic material. As a result of this process, the level of fitness of bee colonies to environmental factors is reduced, and the loss of pure breed is observed. There is a decrease of economically valuable indicators and bee immunity and the emergence of new diseases [4, 9–11].

The main problem in beekeeping is the preservation of gene pools of native bee populations. One of the unique *A. mellifera* subspecies is the dark-colored forest bee *Apis mellifera mellifera* L. as the most adapted to the harsh climatic conditions of the northern region of Eurasia (a natural range along the northern border of Eurasia, up to about 60°N). In addition, the dark-colored forest bee mastered the forest steppe and forest zones. In contrast to other bee subspecies, *A. m. mellifera* is characterized by a high level of adaptation to adverse environmental factors (e.g., long harsh winter, short period of honey collection) and greater resistance to diseases. Unfortunately, now the dark-colored forest bee *A. m. mellifera* is recognized as an endangered species by the European Society of Beekeepers [12–15]. In connection with this, the issue of conservation of the honeybee populations and the gene pool of *A. m. mellifera* has a biospherical value.

Russia has some unique opportunities to preserve the local populations of the *A. m. mellifera* honeybee. Two major *A. m. mellifera* populations (the Burzyan population in the nature reserve "Shulgan-Tash," Bashkortostan, and the Yenisei population in the Krasnoyarsk Territory, Siberia) in Russia are considered promising for the study of the dark-colored forest bee.

The goal of our work was to search for the dark-colored forest bee populations in Siberia and morphometric and molecular genetic characterization of bee colonies to assess the current state and the possibility of preservation of the *A. m. mellifera* gene pool.

2. Materials and methods

2.1. Region

In Siberia, the honeybee was introduced 230 years ago; it is well adapted to the local climate and plant communities and is an artificial population whose wintering is controlled by people.

Siberia is characterized by unfavorable severe natural and climatic conditions. The most characteristic feature of the climate of Siberia is sharp contrasts of air temperatures in the warm and cold seasons of the year, rapid transitions from summer to winter and from winter to summer, and duration of the off-season (spring and autumn) in some areas does not exceed 1–2 months. In transition periods (spring and autumn), there are sharp temperature fluctuations that, even within 1 day, their amplitude in some places reaches 25–30°C.

For example, the Tomsk region is located in the geographic center of Siberia, in the southeastern part of the West Siberian Plain. Almost the entire territory of the region is within the taiga zone. The climate is temperate continental with considerable daily and annual amplitudes and long winters (5–6 months). The average annual temperature is –0.6°C, while the average temperature in July is +18.1°C and in January is –19.2°C. The frost-free period is 100–120 days. Precipitation is 435 mm.

The Krasnoyarsk Territory is located in the Eastern Siberia. About 70% of the territory is occupied by forests. Due to the long length of the edge in the meridional direction, the climate is very heterogeneous. The climate of the Krasnoyarsk Territory varies from arctic and subarctic to sharply continental and temperate continental. In particular, in the Yenisei district, the average annual temperature is –1.5°C, while the average temperature in July is +18.1°C and in January is –21.6°C. The frost-free period is 100–110 days, and precipitation is 200–350 mm.

2.2. Research algorithm on search for the dark-colored forest bee colonies

At the first stage of the study, we performed the screening of bee colonies inhabited different regions of Siberia (northern and southern territory, isolated apiaries, forest areas, and others) to search for *A. m. mellifera* populations. To search for a dark-colored forest bee *A. m. mellifera*, we investigated honeybee populations in four regions of Siberia: the Tomsk region, the Kemerovo region, the Krasnoyarsk Territory, and the Altai Territory (**Figure 1**).

In the screening study, we use the following algorithm:

(1) mtDNA analysis (variability of the locus COI–COII) to determine the origin of the bee colony in the maternal line. If the variants PQQ and PQQQ of the COI–COII locus are detected in bees of the colony, this bee colony is the *A. m. mellifera* origin in the maternal line (evolutionary branch M) and analyzed by the morphometric method. If the bees of the colony have a variant Q of the COI–COII mtDNA locus, the bee colony maternally originates from the southern breeds (*A. m. carnica*, *A. m. carpatica*, *A. m. caucasica*, *A. m. ligustica*, and others) (evolutionary branches C and O). Analysis of this bee colony has not been conducted (the bee colony is excluded from the research).

(2) Morphometric analysis (parameters of wing, body painting, and others). If the morphometric parameters of bees correspond to the dark-colored forest bee's standard, this bee colony is

considered *A. m. mellifera*. If morphometric parameters are not consistent with the *A. m. mellifera* breed standard, this bee colony is considered a hybrid. Analysis of this bee colony has not been conducted (the bee colony is excluded from the research).

At the first stage of study (the screening study), about 500 bee colonies from various regions of Siberia were examined using morphometric and mtDNA analysis [16–19].

Based on the screening study, the most interesting areas where the dark-colored forest bees live were selected for more detailed investigation: (1) the Tomsk region, Western Siberia; (2) the Krasnoyarsk Territory, Yenisei population, Eastern Siberia (**Figure 1**).

We investigated the Yenisei bee population at the Krasnoyarsk Territory as a unique isolated *A. m. mellifera* population that has existed for more than 60 years in the forest without the importation of new honeybees (**Figure 1**; **Table 1**). Whereas bee colonies from the Krasnoyarsk Krai were obtained from the territory distant from the center and located in sparsely populated

Figure 1. Map of localization of the territories of Siberia where search for *A. m. mellifera* populations was conducted (the screening study): the Tomsk region (A), the Krasnoyarsk Territory (B), the Kemerovo region (C), and the Altai Territory (D). The apiaries selected for the study of the dark-colored forest bee are indicated by dots 1–5: (1) s. Mogochino, (2) s. Teguldet, (3) s. Kolmogorovo, (4) s. Ostyatskoe, and (5) s. Ozernoe. For comparison, the Burzyan dark-colored forest bee population is attracted: E, Bashkortostan, Ural: 6, the reserve "Shulgan-Tash" [24].

Region	Settlement	Latitude	Longitude	Altitude
Tomsk region	Mogochino	57°42′42″	83°34′30″	104
	Teguldet	57°18′00″	88°10′00″	131
Krasnoyarsk Territory (Yenisei population)	Kolmogorovo	59°16′06″	91°19′02″	60
	Ostyatskoe	59°11′12″	91°19′24″	63
	Ozernoe	58°46′56″	92°08′05″	74

Table 1. Geographic location and altitude of apiaries in Siberia, where the dark-colored forest bees were collected for study using microsatellite loci.

areas, in the taiga, the bee colonies from the Tomsk region inhabit the relatively isolated territory, characterized by higher development of beekeeping and constant importation of bees of different origins. Bee colonies from two northern points, as potentially "pure," of the Tomsk region were studied: settlements Mogochino and Teguldet (**Figure 1**; **Table 1**).

The second stage of study of the dark-colored forest bee colonies detected by morphometric and mtDNA methods were studied in detail using microsatellite loci.

2.3. Samples for characterization of the dark-colored forest bee

We defined apiaries and territories, where only the dark forest bee is distributed. For further investigation, two populations (five apiaries) of Siberia (the Tomsk region, the Krasnoyarsk Territory) are selected: s. Mogochino and s. Teguldet in the Tomsk region and s. Kolmogorovo, s. Ostyatskoe, and s. Ozernoe in the Krasnoyarsk Territory (**Table 1**).

Collected honeybees from bee colonies were anesthetized on dry ice and stored in 96% ethanol until use.

Twenty-two dark-colored forest bee colonies from Siberia (5 bee colonies from the Tomsk region and 17 bee colonies from the Krasnoyarsk Territory) were investigated by morphometric (minimum 30 bees from each colony, in total of 673 samples) and molecular genetic methods (mtDNA analysis and microsatellite analysis). In total, 170 bees were examined by mtDNA analysis (5–10 bees from each colony). We analyzed 18 microsatellite loci; the minimum number of individuals analyzed for the locus was 269, and the maximum number of bees was 524 (from 10 to 30 individuals from each bee colony).

2.4. Morphometric method

Morphometric parameters (wing venation), including the cubital index, the hantel index, and the discoidal shift were studied [17, 19].

2.5. Molecular genetic methods

Each bee colony has been studied using the mtDNA analysis (locus COI–COII) and morphometric analysis (morphometric parameters of the wing, including the cubital index, the hantel index, and the discoidal shift, were analyzed) to determine the conformance of the bee colony to the *A. m. mellifera* standard (see details in Refs. [16, 17, 19].

DNA isolation and polymerase chain reaction (PCR) were carried out according to standard techniques with some modifications [20, 21]. To amplify the COI–COII mtDNA locus, the following sequences of primers were used: 5′-CACATTTAGAAATTCCATTA and 5′-ATAAATATGAATCATGTGGA [20]. Amplification products were fractionated in 1.5% agarose gel, and the results were documented with the use of Gel Doc XR+.

We examined variability of 18 microsatellite loci localized on 11 of the 16 chromosomes of the honeybee (**Table 2**). PCR was performed using specific primers and reaction conditions according to Solignac et al. [22]. Amplification products were analyzed with ABI Prism 3730

Locus	Chromosome	Size (pb)	Motive	Annealing temperature (°C)	$MgCl_2$ concentration (mM)	Primer sequence: upper (F) and lower (R)
A008 (rs26723312)	2	160	$(GA)_{15}$… $(GCTCG)_5$	55	1.2	F: CGCGAAGGTAAGGTAAATGGAAC R: GGCGGTTAAAGTTCTGG
Ap049 (rs267233076)	1	142	$(AGG)_7$	58	1.2	F: CCAATAGCGGCGAGTGTG R: GGGCTTCGTACGTCCACC
AC117 (rs267233481)	12	181	$(TTTC)_5$	50	1.5	F: CGGTTCATCTTCCCTTTATTTC R: CCACGGGATTATTATCGTTTATC
Ap066 (rs267233165)	3	100	$(CT)_{11}$	54	1.5	F: TTGCATTCGGTCTCCAGC R: ACTTGCCGCGGTATCTGA
Ap081 (rs267233372)	9	128	$(GT)_8$	60	1.0	F: GGATCGTCGAGGCGTTGA R: GAAAAGTATTCCGCCGAGCA
A088 (rs267233346)	8	150	$(CT)_{10}$.. $(GGA)_7$	55	1.2	F: CGAATTAACCGATTTGTCG R: GATCGCAATTATTGAAGGAG
A113 (rs267233291)	6	220	$(TC)_5TT$ $(TC)_8TT(TC)_5$	60	1.0	F: CTCGAATCGTGGCGTCC R: CCTGTATTTTGCAACCTCGC
Ap243 (rs267233098)	1	260	$(TCC)_9$	50	1.5	F: AATGTCCGCGAGCATCTG R: TGTTTACGAGAATTCGACGGG
A024 (rs267234016)	7	100	$(CT)_{11}$	55	1.2	F: CACAAGTTCCAACAATGC R: CACATTGAGGATGAGCG
A007 (rs267233337)	8	131	$(CT)_{24}$	58	1.2	F: CCCTTCCTCTTTCATCTTCC R: GTTAGTGCCCTCCTCTTGC
A043 (rs267233033)	1	140	$(CT)_{12}$	55	1.5	F: CACCGAAACAAGATGCAAG R: CCGCTCATTAAGATATCCG
A028 (rs267233550)	14	140	$(AG)_6(GAG)_6$	54	1.7	F: GAAGAGCGTTGGTTGCAGG R: GCCGTTCATGGTTACCACG
6339 (rs267233937)	5	146	$(AAT)_9$	55	1.5	F: CGCACACGACATGCATATCC R: ATCTGCTGCAGAGGGTCGAG
H110 (rs267233914)	5	160	$(ATCC)_4$ $(ATCT)_2$	56	1.5	F: CGCTCGCGGTGGATTTCATTT R: GGCAAAAGTGGCGGAGAAAGA
SV185 (rs267233900)	5	272	$(AAC)_{12}$	55	1.5	F: AGCTCACGCAGCACATGC R: GACGTTGTTTCCATCACCACTC
SV220 (rs267233836)	3	185	$(AAT)_{13}$	55	1.5	F: TTTCTCGCGTAGAATGTAGAATAGG R: AAGGATTTGCCTGCTACATGAC
mrjp3	11	350–530	Length polymorphism	55	1.5	F: ATGTAATTTTGAAGAATGAACTTG R: TGTAGATGACTTAATGAGAAACAC

Table 2. Characterization of 18 microsatellite loci, primer sequence, and the amplification conditions.

Genetic Analyzer and GeneMapper Software (Applied Biosystems, Inc., Foster City, CA) in the collective Center for Medical Genomics (Research Institute of Medical Genetics, Tomsk National Research Medical Center, Russian Academy of Sciences). Two microliters of PCR products were mixed with GeneScan 500 ROX size standards (Applied Biosystems, Inc.) and deionized formamide. Samples were run according to the manufacturer's recommendations. These genetic parameters were calculated using the POPGENE 1.31 software [23]: allelic frequencies with standard error, heterozygosity.

For the microsatellite loci specific for evolutionary branch M according to our results, our data on their variability in southern breeds of honeybee (*A. m. carpatica*, *A. m. carnica*) were used (our unpublished data).

For comparison, data on the native Burzyan dark-colored forest bee population (the reserve "Shulgan-Tash," Bashkortostan, Ural) were attracted (**Figure 1**) [24].

3. Results and discussion

In the screening study of the Siberian territories, the dark-colored forest bee populations were identified in the Tomsk region and in the Krasnoyarsk Territory. For bee colonies from these populations, a detailed morphometric and molecular genetic (mtDNA) analysis was carried out. Using of microsatellite loci, research studies of bee colonies were performed (1) to characterize genetic diversity of bees, (2) to find unique or specific DNA markers for the dark-colored forest bee, and (3) to assess the ecological component in the genetic diversity of bees using microsatellite loci studied for which differences in allelic spectrum and allelic frequencies in bees from different dark-colored forest bee populations were identified.

3.1. Morphometric and mtDNA analysis of dark-colored forest bees in Siberia

Using the mtDNA analysis (variability of the COI–COII locus), we performed molecular genetic study of 22 bee colonies (5–10 samples from each bee colony) to exclude the hybridization (mixing) with southern bee subspecies and confirm their origin from the dark-colored forest bee in the maternal line. One variant of the COI–COII mtDNA locus was registered in all studied honeybees of Tomsk and Krasnoyarsk populations: PQQ (typical for the dark-colored forest bee). No variant Q specific for southern races of bee was detected.

Then, bee colonies were investigated by the morphometric analysis to identify the characteristics of both the maternal and paternal lines and to assess the level of hybridization. The results of the morphometric study of honeybees from examined regions of Siberia (the Tomsk region and the Krasnoyarsk Territory) were different. The results of morphometric analysis confirmed the origin of bee colonies of Tomsk population (apiaries of s. Mogochino and s. Teguldet) from the dark-colored forest bee, but some influence of southern races was shown. For example, the parameter "discoidal shift" deviates from the Russian *A. m. mellifera* breed standard: individuals with zero value of discoidal shift were found in bee colony No. 1 from Mogochino (**Table 3**).

Bee colonies obtained from isolated apiaries of the Krasnoyarsk Krai (s. Kolmogorovo, s. Ostyatskoe, and s. Ozernoe) are of considerable interest. The area with these isolated apiaries was not influenced by other subspecies of honeybee for many years, and all studied bees had only variant PQQ of the locus COI–COII mtDNA. However, when comparing the data of the morphometric study of bees from isolated apiaries with Russian and European standards of the *A. m. mellifera*, the decrease of the lower limit values of cubital index was observed in the studied bees, and, as a result, for most bee colonies, the deviation from the mean values of cubital index was shown. In addition, a slight deviation of the other morphometric indices from the *A. m. mellifera* standard in some families of bees is also shown (**Table 3**). There are

Geographic location		Bee colony (№)	Number of studied bees	Sequence composition of the COI–COII mtDNA locus	Cubital index (standard units)		Hantel index (standard units)		Discoidal shift (%)		
Region	**Settlement**				***Lim:min-max***	***M±m***	***Lim:min-max***	***M±m***	**–**	**0**	**+**
Tomsk region	Mogochino	1	30	PQQ	1.26–2.56	1.92±0.05	0.806–1.000	0.879±0.010	70.00	30.00	0.00
		2	43	PQQ	1.36–2.00	1.73±0.02	0.693–0.923	0.821±0.006	100.00	0.00	0.00
	Teguldet	1	30	PQQ	1.44–2.10	1.75±0.03	0.692–1.000	0.854±0.011	100.00	0.00	0.00
		2	30	PQQ	1.28–1.90	1.45±0.05	0.707–0.923	0.823±0.012	93.30	6.70	0.00
		3	30	PQQ	1.26–2.22	1.74±0.04	0.701–0.914	0.825±0.010	100.00	0.00	0.00
Krasnoyarsk Territory	Ostyatskoe	1	30	PQQ	1.24–2.00	1.61±0.04	0.675–0.892	0.795±0.011	100.00	0.00	0.00
		2	30	PQQ	1.39–1.74	1.51±0.02	0.743–0.912	0.849±0.012	83.30	16.70	0.00
		3	30	PQQ	1.23–1.74	1.51±0.03	0.736–0.883	0.837±0.008	83.30	16.70	0.00
		4	30	PQQ	1.20–1.67	1.45±0.02	0.723–0.900	0.837±0.009	97.00	3.00	0.00
		5	30	PQQ	1.24–1.79	1.46±0.03	0.735–0.923	0.842±0.010	87.00	13.00	0.00
	Kolmogorovo	1	30	PQQ	1.32–2.10	1.60±0.05	0.724–0.900	0.820±0.009	97.00	3.00	0.00
		2	30	PQQ	1.12–1.76	1.51±0.03	0.758–0.919	0.845±0.008	93.00	7.00	0.00
		3	30	PQQ	1.28–1.86	1.56±0.04	0.746–0.985	0.810±0.011	97.00	3.00	0.00
		4	30	PQQ	1.07–1.76	1.45±0.04	0.716–0.923	0.830±0.011	97.00	3.00	0.00
		5	30	PQQ	1.13–2.00	1.51±0.05	0.716–0.900	0.841±0.008	96.70	3.30	0.00
	Ozernoe	1	30	PQQ	1.02–2.00	1.62±0.04	0.746–1.000	0.845±0.011	100.00	0.00	0.00
		2	30	PQQ	1.22–2.33	1.59±0.04	0.742–0.967	0.841±0.011	96.70	3.30	0.00
		3	30	PQQ	1.24–2.06	1.61±0.04	0.786–1.000	0.866±0.011	93.30	6.70	0.00
		4	30	PQQ	1.45–1.95	1.65±0.04	0.768–1.000	0.867±0.010	93.30	6.70	0.00
		5	30	PQQ	1.35–2.05	1.65±0.04	0.716–0.951	0.806±0.010	100.00	0.00	0.00
		6	30	PQQ	1.25–2.38	1.55±0.04	0.726–1.000	0.842±0.012	100.00	0.00	0.00
		7	30	PQQ	1.43–2.11	1.71±0.04	0.785–1.000	0.876±0.010	100.00	0.00	0.00

Geographic location		Bee colony (№)	Number of studied bees	Sequence composition of the COI–COII mtDNA locus	Cubital index (standard units)		Hantel index (standard units)		Discoidal shift (%)		
Region	**Settlement**				***Lim:min-max***	***M±m***	***Lim:min-max***	***M±m***	**–**	**0**	**+**
	Standard for *Apis mellifera mellifera*										
I		PQQ, PQQQ, and others			1.30–2.10	1.70	0.600–0.923	No data	No data		
II		PQQ, PQQQ, and others			1.30–1.90	1.5–1.7	0.600–0.923		91–100	5–10	0

Lim, Limits of value of the sing, *M*±*m* average value of the sign, ± the standard error of the mean
I, European breed standard based on values of cubital and hantel indices [25]
II, Russian breed standard

Table 3. Morphometric parameters (wing venation) of honeybee workers from 22 bee colonies from Siberia.

several possible explanations for the results. First, these apiaries are isolated, and there are a limited number of bees. Second, the large scale of variability of the cubital index is the result of adaptation to the environment in a more severe climatic condition. Nevertheless, these isolated apiaries in the Krasnoyarsk Territory may be considered a unique population of the dark-colored forest bee that has existed for a long time without the influence of other bee subspecies.

3.2. Genetic diversity of the dark-colored forest bees in Siberia on the microsatellite loci

Variability of the 18 microsatellite loci in dark-colored forest bees from Siberian populations was studied. For each microsatellite locus, the allelic range, frequency of alleles, and heterozygosity were determined (**Table 4**).

Locus	Alleles (pb)	Allelic frequency		Locus	Alleles (pb)	Allelic frequency	
		Tomsk region	Krasnoyarsk Territory			Tomsk region	Krasnoyarsk Territory
Ap066	90	**0.302±0.029**	0.104±0.013	**A007**	104	0.055±0.013	0.155±0.014
	92	0.008±0.006	0		106	0	0.010±0.004
	94	0	0.004±0.003		108	**0.863±0.020**	**0.807±0.015**
	96	0.175±0.024	**0.375±0.021**		110	0	0.006±0.003
	98	**0.401±0.031**	**0.314±0.020**		112	0.082±0.016	0.015±0.005
	100	0.115±0.020	0.204±0.017		114	0	0.007±0.003
Ho		*0.802±0.036*	*0.620±0.029*	*Ho*		*0.158±0.030*	*0.313±0.025*
He		*0.705±0.014*	*0.709±0.008*	*He*		*0.245±0.032*	*0.324±0.021*
N		*126*	*279*	*N*		*146*	*342*
A024	92	0.287±0.026	**0.666±0.017**	**A008**	151	0.024±0.009	0.017±0.005
	94	**0.351±0.028**	0		157	0	0.019±0.006
	96	0	0.049±0.008		161	0	0.010±0.004
	100	0.044±0.012	0.239±0.016		163	**0.914±0.017**	**0.910±0.012**
	102	0.047±0.012	0.045±0.008		169	0	0.003±0.002
	104	0.007±0.005	0		171	0.055±0.013	0.029±0.007
	106	0.264±0.026	0		173	0.007±0.005	0.012±0.005
Ho		*0.581±0.041*	*0.455±0.026*	*Ho*		*0.131±0.028*	*0.105±0.018*
He		*0.720±0.010*	*0.494±0.017*	*He*		*0.161±0.029*	*0.170±0.021*
N		*148*	*376*	*N*		*145*	*295*
Ap081	116	0.040±0.014	0.004±0.028	**AC117**	169	0.011±0.006	0
	119	0.020±0.010	0		173	0.175±0.023	0.006±0.003
	123	**0.910±0.020**	**0.982±0.006**		177	0.058±0.014	0.137±0.013
	128	0	0.014±0.005		181	**0.456±0.030**	0.195±0.015
	130	0.030±0.012	0		185	0.299±0.028	**0.663±0.018**

Locus	Alleles (pb)	Allelic frequency		Locus	Alleles (pb)	Allelic frequency	
		Tomsk region	Krasnoyarsk Territory			Tomsk region	Krasnoyarsk Territory
Ho		*0.120±0.033*	*0.036±0.012*	*Ho*		*0.453±0.043*	*0.318±0.025*
He		*0.169±0.035*	*0.035±0.011*	*He*		*0.668±0.016*	*0.504±0.018*
N		*100*	*253*	*N*		*137*	*359*
A028	118	0.026±0.013	0	**6339**	146	0.262±0.034	**0.467±0.023**
	120	0.039±0.015	0.003±0.002		149	0.192±0.030	0.122±0.015
	126	**0.795±0.032**	**0.845±0.014**		152	0.128±0.026	0.164±0.017
	132	0.141±0.028	0.015±0.005		155	**0.320±0.036**	0.098±0.014
	134	0	0.135±0.013		159	0.099±0.023	0.144±0.016
	148	0	0.003±0.002		162	0	0.004±0.003
Ho		*0.410±0.056*	*0.281±0.024*	*Ho*		*0.663±0.051*	*0.655±0.031*
He		*0.346±0.044*	*0.268±0.020*	*He*		*0.766±0.012*	*0.710±0.016*
N		*78*	*342*	*N*		*86*	*229*
A043	121	0	0.002±0.002	**SV185**	260	0	0.032±0.007
	128	**0.781±0.024**	**0.981±0.006**		263	0.286±0.030	0.206±0.015
	134	0.021±0.008	0		266	0.103±0.020	**0.346±0.018**
	138	0.017±0.008	0		269	**0.611±0.032**	**0.414±0.019**
	140	0.182±0.023	0.017±0.006		272	0	0.003±0.002
Ho		*0.384±0.040*	*0.022±0.009*	*Ho*		*0.539±0.046*	*0.586±0.026*
He		*0.357±0.031*	*0.037±0.011*	*He*		*0.534±0.025*	*0.666±0.007*
N		*146*	*268*	*N*		*117*	*348*
A088	138	0	0.002±0.002	***mrjp3***	391	0.034±0.014	0.028±0.009
	141	**0.928±0.021**	**0.998±0.002**		437	0.040±0.015	0.163±0.019
	144	0.020±0.011	0		464	0.085±0.021	0.022±0.008
	146	0.053±0.018	0		485	0.006±0.006	0
					501	0	0.028±0.009
					529	**0.835±0.029**	**0.760±0.023**
Ho		*0.118±0.037*	*0.004±0.004*	*Ho*		*0.080±0.029*	*0.309±0.034*
He		*0.136±0.037*	*0.004±0.004*	*He*		*0.292±0.043*	*0.394±0.029*
N		*76*	*236*	*N*		*88*	*181*
Ap243	254	0	0.003±0.003	**Ap049**	117	0.003±0.003	0
	257	**0.468±0.034**	**0.304±0.023**		120	0.201±0.023	0.024±0.006
	260	0.046±0.014	0.003±0.002		127	**0.705±0.026**	**0.759±0.016**
	263	0.266±0.030	**0.554±0.025**		130	0.054±0.013	0.164±0.014

Locus	Alleles (pb)	Allelic frequency		Locus	Alleles (pb)	Allelic frequency	
		Tomsk region	Krasnoyarsk Territory			Tomsk region	Krasnoyarsk Territory
	266	0	0.005±0.004		133	0.003±0.003	0
	269	0.028±0.011	0.096±0.015		136	0.003±0.003	0
	272	0.128±0.023	0.020±0.007		139	0.017±0.007	0.043±0.008
	275	0.064±0.017	0.003±0.002		142	0.013±0.007	0.001±0.001
	284	0	0.015±0.006		152	0	0.008±0.003
Ho		*0.560±0.048*	*0.520±0.035*	*Ho*		*0.517±0.041*	*0.380±0.025*
He		*0.687±0.022*	*0.591±0.018*	*He*		*0.460±0.030*	*0.395±0.020*
N		*109*	*203*	*N*		*149*	*371*
Ap249	207	0	0.020±0.006	**SV220**	170	0	0.093±0.011
	213	0.021±0.012	0.010±0.005		173	0.020±0.011	0.046±0.008
	219	0.111±0.026	0.012±0.005		176	0.065±0.020	0
	221	**0.653±0.040**	**0.958±0.009**		179	0.026±0.013	0.005±0.003
	223	0.125±0.028	0		182	0	**0.394±0.019**
	225	0.090±0.024	0		185	**0.604±0.039**	**0.383±0.019**
					188	0.182±0.031	0.069±0.010
					191	0.104±0.025	0.011±0.004
Ho		*0.500±0.059*	*0.061±0.015*	*Ho*		*0.442±0.057*	*0.475±0.028*
He		*0.537±0.043*	*0.082±0.017*	*He*		*0.586±0.038*	*0.683±0.011*
N		*72*	*248*	*N*		*77*	*324*
A113	212	0.057±0.013	0.042±0.007	**H110**	158	0	0.045±0.008
	214	0	0.001±0.001		160	0	0.037±0.007
	218	**0.631±0.028**	**0.803±0.015**		162	**0.726±0.026**	**0.484±0.018**
	220	**0.299±0.027**	0.151±0.013		164	0	0.016±0.005
	226	0.010±0.006	0		166	0.188±0.023	0.025±0.006
	228	0.003±0.003	0		168	0	0.064±0.009
	232	0	0.003±0.002		170	0.087±0.017	**0.329±0.017**
Ho		*0.409±0.040*	*0.275±0.023*	*Ho*		*0.451±0.042*	*0.412±0.025*
He		*0.509±0.022*	*0.331±0.020*	*He*		*0.431±0.030*	*0.649±0.012*
N		*149*	*367*	*N*		*144*	*376*

N, Number of studied samples; *Ho*, observed heterozygosity; *He*, expected heterozygosity
The predominant alleles in bees in both Siberian populations (allele frequency is ≥ than 20%) are bold.

Table 4. Allele frequency and heterozygosity at 18 loci in the dark-colored forest bee in Siberia.

Microsatellite loci differed in variability: the minimum number of alleles was detected for locus A088 (four alleles), and the maximum number of alleles was registered for locus Ap243 and Ap049 (nine alleles). At the same time, for most loci (A007, A008, Ap081, A028, A043, A088, Ap049, A113, Ap249, and *mrjp3*), one major allele with a frequency of more than 0.63 (from 0.631 for allele "218"of locus A113 to 0.998 for allele "141" of locus A088) was registered.

Some differences were also registered in the frequency of alleles between Tomsk and Krasnoyarsk populations. Thus, at the locus AC117 in bees from the Tomsk population, the allele "181" was most often registered (frequency of allelic registration was 0.46), and allele "185" was registered less often (0.30), whereas in bees from the Krasnoyarsk population, on the contrary, the allele "185" was predominant (frequency of allelic registration was 0.66). Differences in the frequency of registration of predominant alleles were registered for some other loci (Ap066, A024, 6339, and others). At the same time, for most loci A007, A008, Ap081, A028, A043, A088, Ap049, Ap249, A113, H110, and *mrjp3*, the same alleles were predominant in both populations (**Table 4**).

Observed and expected heterozygosity differs among bees of two populations. The lower values of the observed heterozygosity in comparison with the expected heterozygosity are shown for most loci (except, locus A028). Probably, one of the reasons for this situation is the features of the reproductive biology of bees. At the same time, the differences between the bees of the Tomsk and Yenisei populations were revealed for some loci. For example, loci Ap066, A043, Ap049, and H110, the values of the observed heterozygosity were higher values of the expected heterozygosity in bees from Tomsk population in comparison with the bees of the Yenisei population. Possibly, this may be the result of genetic drift, the effect of which may be due to the fact that apiaries of the Krasnoyarsk Territory (Yenisei population) are isolated and there are a limited number of bees. It cannot be ruled out that the loss of the genetic diversity of the bees from the Yenisei population can be the cause of some morphological differences from the *A. m. mellifera* breed standard.

3.3. Comparative analysis of the variability of the microsatellite loci in the *A. m. mellifera* bees from different populations of Russia

It is expected that a vast territory of Eurasia cannot be inhabited by *A. m. mellifera* subspecies with a similar structure of the gene pool in all local populations. Most likely, there are ecological groups (ecotypes), which differ from each other, both for genetic parameters and behavioral, physiological, and morphological characteristics at the level below the subspecies one [17, 18, 24].

In order to identify genetic features (specificity, adaptation to various climatic conditions) of dark-colored forest bees from different populations (different geographic areas) and determine different *A. m. mellifera* ecotypes, the comparative analysis of the variability of nine microsatellite loci was carried out for the bees of *A. m. mellifera* of Siberian and Ural populations using

our own data (the Tomsk region and the Krasnoyarsk Territory) and literature data (the Ural) [24] (**Table 5**).

The complexity of such a comparative analysis is a small study of the bees of different populations of both Russia and Europe. For example, the genetic diversity of bees of the Burzyan population (the Ural, Russia) has been studied only at nine microsatellite loci [24]. Large-scale research of the genetic diversity of the dark-colored forest bee in European populations (Belgium, Sweden, France) dates back to 1998 [26, 27]. At the present time, genetic characteristics of bees in these territories can differ significantly from those described earlier, on the one hand, due to the rapid change of bee generations and, on the other hand, due to mass hybridization processes.

According to our data, Siberian populations (the Tomsk region and the Krasnoyarsk Territory) are the closest in allelic spectrum and allelic frequencies of most studied loci (Ap049, A113,

Parameter		**Allelic frequency**			**Parameter**		**Allelic frequency**		
		Siberia		**Ural**			**Siberia**		**Ural**
		Tomsk region	**Krasnoyarsk Territory (Yenisei population)**	**Bashkortostan (Burzyan population)[1]**			**Tomsk region**	**Krasnoyarsk Territory (Yenisei population)**	**Bashkortostan (Burzyan population)[1]**
Locus Ap049					*Locus A113*				
NB		149	371	326	NB		149	367	326
NA		8	6	3	NA		5	7	4
Min/max		117/142	120/152	129/142	Min/max		212/228	212/232	216/228
Allele* (pb)	127	**0.71**	**0.76**	0	Allele* (pb)	218	**0.63**	**0.80**	0.09
	129	0	0	**0.78**		220	0.30	0.15	**0.85**
Locus Ap243					*Locus H110*				
NB		109	203	326	NB		144	376	326
NA		6	9	3	NA		3	7	3
Min/max		257/275	254/284	254/260	Min/max		162/170	158/170	160/168
Allele* (pb)	254	0	0	**0.62**	Allele* (pb)	160	0	0.04	**0.68**
	257	0.47	0.30	0.32		162	**0.73**	0.48	0
	263	0.27	**0.55**	0		170	0.09	0.33	0
Locus A008					*Locus A088*				
NB		145	295	326	NB		76	236	326
NA		4	7	3	NA		3	2	4
Min/max		151/173	151/173	154/158	Min/max		141/146	138/141	143/155
Allele* (pb)	154	0	0	**0.87**	Allele* (pb)	141	**0.93**	**1.0**	0
	163	**0.91**	**0.91**	0		146	0.05	0	**0.74**

Parameter		Allelic frequency			Parameter		Allelic frequency		
		Siberia		Ural			Siberia		Ural
		Tomsk region	Krasnoyarsk Territory (Yenisei population)	Bashkortostan (Burzyan population)[1]			Tomsk region	Krasnoyarsk Territory (Yenisei population)	Bashkortostan (Burzyan population)[1]
Locus A028					*Locus A043*				
NB		78	342	326	NB		76	236	326
NA		4	5	2	NA		4	3	3
Min/max		118/132	120/148	134/140	Min/max		128/140	121/140	128/140
Allele* (pb)	126	**0.80**	**0.85**	0	Allele* (pb)	128	**0.78**	**0.98**	**0.76**
	134	0	0.13	**0.89**					
Locus A024									
NB		148	376	326	Allele* (pb)	92	0.29	**0.67**	0
NA		6	5	3		94	0.35	0	0
Min/max		92/106	92/102	98/108		98	0	0	**0.63**

NB, Number of studied bees; NA, number of registered alleles; Min/max, minimal/maximal size of alleles (pb).
[1]Data on the Ural (Burzyan population) are taken from Ref. [24].
*Alleles with the frequency more than 30% are indicated. Predominant alleles with the frequency more than 50% are in bold.

Table 5. Parameters of the genetic diversity of nine microsatellite loci in the dark-colored forest bee from different populations of Russia.

Ap243, A024, A008, A088, and A028). The Ural population located to the west of the Siberian region differs from Siberia for some loci: for loci A008, A088, and A028, differences were registered in the spectrum of alleles, for the locus A113—in the frequency of alleles, for the loci Ap243 and A024—in both the spectrum and frequency of alleles. Only for locus A043, a greater similarity in the spectrum and frequency of alleles was detected in the dark-colored forest bee from different populations of Russia.

At the same time, the results of genotyping of some loci deserve special consideration. For example, for loci H110 and Ap049, the differences in the size of alleles in bees from Siberian and Ural populations were found (alleles differ by two nucleotides), which may be due to methodical characteristic. Therefore, the most important task for studying the genetic diversity of bees is the development of a standard allelic ladder for microsatellite loci.

3.4. Characterization of *A. m. mellifera* gene pool and possibilities of its preservation in Siberia

Important conditions for the preservation of the honeybee gene pool, including the dark-colored forest bee, are the precise identification of the species of bees, the development of

diagnostic DNA markers (e.g., microsatellite loci), and the conduction of genetic certification of valuable species.

In order to determine the subspecies status of an individual honeybee, a honeybee colony, or a honeybee population, it is important to compare allelic counts and genotypes across different studies including analysis of populations from different regions, as well as description of the genetic diversity of different bee subspecies. At the present time, comparative genetic-geographic analysis for bees has some problems: (1) no standard reference material, such as a standard allelic ladder, is available for honeybees [4]; (2) a small number of studies are devoted to the analysis of the genetic diversity of bees; and (3) the spectra of analyzed microsatellite markers are often not overlapped, and primary data on the allele spectrum and allele frequencies are not always presented in publications.

At the same time, microsatellite loci as the most informative molecular genetic markers can be useful for the study of the genetic structure of different honeybee populations and bee colonies; evaluation of genetic diversity and introgressive hybridization; differentiation of different subspecies (ecotypes); establishment of evolutionary relationships and adaptive features of four evolutionary branches (A, M, C, and O); search of genetic markers associated with economically significant characteristics, and others [12, 13, 17, 18, 24, 26–40].

We attempted to develop a standard allele ladders for microsatellite loci studied for the dark-colored forest bee of Siberian populations and to search for diagnostic DNA markers of the nuclear genome (microsatellite loci) for differentiation of subspecies *A. m. mellifera* (branch M) and southern breeds of honeybee living in Siberia (*A. m. carpatica*, *A. m. carnica*; branch C).

We conducted a comparative analysis of the spectrum and frequencies of the alleles of some microsatellite loci (A008, A028, A088, *mrjp3*) in the dark-colored forest bee (branch M) of various populations of Russia (Siberia, Ural) and Europe (Belgium, Sweden, France) using our own data and literature data [24, 26, 27]. Our unpublished data on the variability of some microsatellite loci in southern breeds of honeybee (*A. m. carpatica*, *A. m. carnica*; branch C) were also used.

The informativeness of the microsatellite loci studied to describe the subspecies and ecological specificity was different. As possible DNA markers for differentiation of different bee subspecies, microsatellite loci can be divided into three groups.

(1) Loci specific for *A. mellifera* subspecies. For these loci, the predominant alleles of the dark-colored forest bee have been identified, which can be considered specific for evolutionary branch M. In bees of the evolutionary branch C, these alleles are recorded at a low frequency.

For example, for locus A043 the allele "128" is predominant in dark-colored forest bees from different populations of Russia (allelic frequency P_{128}=0.76–0.98) and most European populations (allelic frequency P_{128}=0.68–0.90) (**Table 5**; see detail in Refs. [26, 27]). For bees of the evolutionary branch C, the allele "140" is more characteristic.

For the microsatellite *mrjp3* locus, the differences in the spectrum of alleles and the frequency of allele registration were revealed in honeybees of different evolutionary branches. Allele "529" can be considered specific for *A. m. mellifera*, the evolutionary branch M. This allele is registered with a high frequency (P_{529}=0.76–0.84) in dark-colored forest bees of Siberian populations, and this allele is registered in bees of southern origin (*A. m. carpatica*, *A. m. carnica*) rarely with frequency less than 0.01. On the contrary, alleles "406" and "518" are characteristics of bees of southern origin, the evolutionary branch C, and not registered in *A. m. mellifera* honeybees from Siberian populations.

(2) Locus specific for *A. m. mellifera* ecotypes. For these loci, the spectrum and the frequency of alleles were different for the dark-colored forest bee from different populations of Russia and Europe.

For example, for the locus A008, the differences in the spectrum of alleles and the frequency of allele registration were revealed in dark-colored forest bees of Siberian, Ural, and European populations. For honeybees of the Ural and Europe, shorter alleles of locus A008 were predominant (154 bp and 148 bp, respectively), whereas for bees from Siberia, allele "163" was the most specific. Probably, this locus should be considered a marker related to geographic and environmental conditions (specific adaptation to local conditions) [4, 9, 41, 42].

(3) Nonspecific loci. No specific features in the spectrum and frequency distribution of alleles were found. For example, a close spectrum and frequencies of alleles in bees of different origins (evolutionary branches M and C) are registered for loci AC117, H110, SV185, 6339, and others.

Thus, it is shown that for some loci the specific distribution of allele frequencies was detected in bees, which differ by geographic location and/or origin. These loci can be used to determine the origin of honeybees and/or to identify traces of hybridization.

However, in our opinion, for the determination of bee subspecies (or bee breed), the DNA markers of the nuclear genome should be used with caution, if other signs of bee subspecies, for example, morphometry and/or mtDNA, are not considered. None of the microsatellite loci makes it possible to uniquely determine the origin of the bees (i.e., they are not universal). Further research is needed, and the expansion of genetic-geographic studies of honeybees is relevant.

These studies should be of a complex nature (it is necessary to investigate both morphometric and molecular genetic traits, including mtDNA analysis and nuclear genome markers).

In our studies, we used the following algorithm for the search for *A. m. mellifera* populations and study of dark-colored forest bee colonies (**Figure 2**).

Initially, to determine the origin of the bee colony in the maternal line, each colony should be investigated by the mtDNA analysis (variability of the locus COI–COII). Then, the morphometric analysis should be carried out to determine the origin of the bee colony and its conformance to the bee breed standard and to assess the correspondence of the mtDNA data to the morphometric parameters. As a result of our studies, it has been shown that among the morphometric parameters highly informative and minimally necessary indicators for the determination of

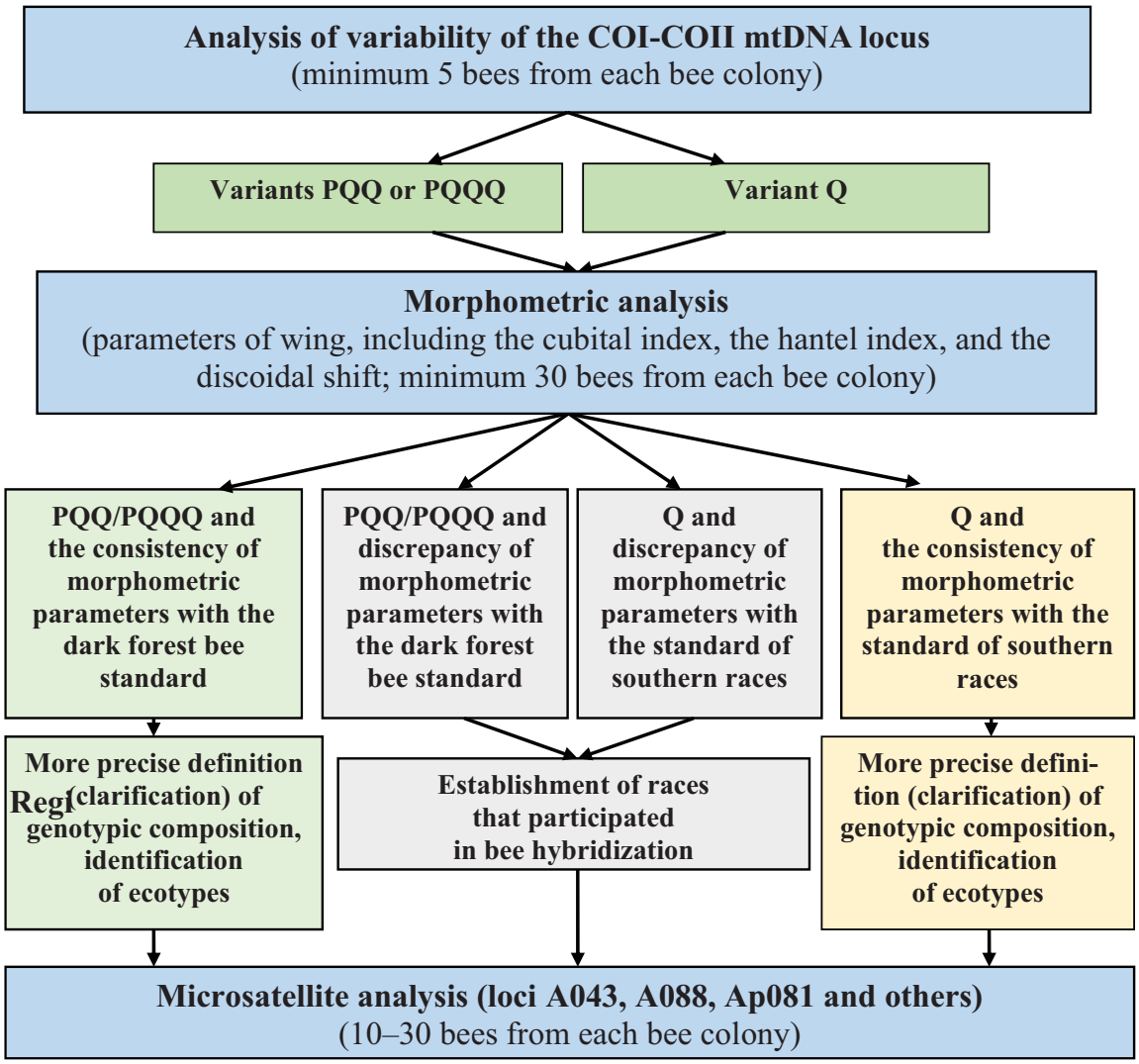

Figure 2. Algorithm of the study of the bee colonies.

A. mellifera subspecies are three parameters of the wing namely the cubital index, the hantel index, and the discoidal shift. These parameters, together with the data on the variability of the COI–COII mtDNA locus, make it possible to differentiate the dark-colored forest bee and bees of southern breeds, as well as hybrids (see details in Refs. [17–19]).

Our data also indicate that only the exterior or just genetic traits may be insufficient to determine the origin of bees and only the simultaneous analysis of morphometric parameters and data on the variability of locus COI–COII of mtDNA allow to evaluate the breed and cases of hybridization objectively.

Finally, a microsatellite analysis should be conducted to study genetic diversity of bee colonies and to clarify their origin (possibly ecotypes) and/or the origin of the hybrids. As the research on the variability of the nuclear DNA markers in different bee subspecies

inhabiting different climatic conditions will increase, the range of informative molecular genetic markers for certain bee subspecies, breeds, and/or ecotypes can be expanded and optimized.

4. Conclusion

A screening study of bee colonies in Siberia made it possible to identify two populations of the dark-colored forest bee in the Krasnoyarsk Territory and the Tomsk Region. These *A. m. mellifera* populations identified in Siberia were described by a complex of morphometric and molecular genetic markers. According to the mtDNA analysis, all studied bee colonies were of the dark-colored forest bee origin in the maternal line (the bees had a variant PQQ of the COI–COII locus). According to the basic morphometric parameters, most bee colonies fully corresponded to the *A. m. mellifera* standard. As possible potential DNA markers, microsatellite loci specific for determining of the bee subspecies (A043, *mrjp3*) and/or ecotypes (A008) of the dark-colored forest bee have been identified from 18 analyzed microsatellites.

Thus, to identify and preserve dark-colored forest bee populations in Siberia, we studied the genetic diversity of local native bees, described the specific polymorphic variants of loci of mtDNA and nuclear genome, and proposed an algorithm for the search and a comprehensive study of the dark-colored forest bee.

As a result of our research, we can draw the following conclusions:

1. It is necessary to establish the exact correspondence of the breed using comprehensive analysis (morphometric and mtDNA methods).
2. Identify and remove hybrid colonies with a discrepancy between morphometric and mtDNA parameters.
3. Given the high variability of microsatellites, it is necessary to cautiously use a small number of individuals and/or microsatellite loci to assess the genetic diversity of bee colonies when microsatellite loci are used to identify bee subspecies.
4. Take into account the genetic-geographic and ecological aspects for the conservation of biodiversity, which is not given much attention.

Development of diagnostic DNA markers is a scientific basis for the evaluation of quality of bee colonies in the dark-colored forest bee farm, created by Tomsk State University. In addition, a complex approach to the analysis of bee colonies (morphometric and molecular genetic analysis) allows obtaining genetic certification of bees, identifying the valuable line (ecotypes) of local bees, and protecting and making rational use of genetic resources of aboriginal bee subspecies.

This is one of the first attempts to introduce molecular genetic markers in the practice of beekeeping in Russia as the real possibility of the definition of bee subspecies (bee breeds). In

the future, a similar comprehensive approach, including analysis of molecular genetic and morphometric markers, will be used for the selection of bee colonies with high economically significant indicators, disease resistance, and other parameters based on genotypic features of honeybees.

Author details

Nadezhda V. Ostroverkhova*, Aksana N. Kucher, Olga L. Konusova, Ekaterina S. Gushchina, Vadim V. Yartsev and Yury L. Pogorelov

*Address all correspondence to: nvostrov@mail.ru

Institute of Biology, National Research Tomsk State University, Tomsk, Russia

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