Chapter 1

A Comprehensive Characterization of the Honeybees in Siberia (Russia)

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Additional information is available at the end of the chapter

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Abstract

A comprehensive study of some populations of honeybee (332 colonies) in Siberia (Tomsk region, Krasnoyarsk Krai (Yenisei population), Altai) using morphometric and molecular genetic methods was conducted. Infestation of bees (132 colonies) by *Nosema* has also been studied. Three variants of the COI-COII mtDNA locus were registered: PQQ, PQQQ (typical for *Apis m. mellifera*), and Q (specific for southern races). It was established that 64% of bee colonies from the Tomsk region and all colonies studied from the Krasnoyarsk and the Altai territories originate from *Apis m. mellifera* on the maternal line. According to the morphometric study, the majority of bee colonies of the Tomsk region are hybrids; in some colonies the mismatch of morphometric and mtDNA data was observed. Moreover, the majority of bee colonies infected by *Nosema* were hybrids. Yenisei population may be considered as a unique *Apis m. mellifera* population. Microsatellite analysis (loci A008, Ap049, AC117, AC216, Ap243, H110, A024, A113) showed the specific distribution of genotypes and alleles for some loci in the bees, which differ by geographical location. Loci A024 and Ap049 are of considerable interest for further study as candidate markers for differentiation of subspecies; locus A008 can be considered informative for determining of different ecotypes of *Apis m. mellifera*.

Keywords: honeybee, COI-COII locus, microsatellites, *Nosema*, Siberia

1. Introduction

In Siberia, the honeybee was introduced about 230 years ago. It was the dark-colored forest bee *Apis mellifera mellifera* L., or the Middle Russian race (a term adopted in Russia), that was cultivated

in Siberia as the most adapted to the harsh climatic conditions of the region. At the end of the last century, bees of southern races, such as the Carpathian race or *Apis mellifera carpatica* (a derivative of *A. m. carnica*) and the Caucasian gray mountain race (*Apis mellifera caucasica* Gorb.), have been actively imported to Siberia. This process had become widespread and almost uncontrollable, which leads to a high level of crossbreeding of bees.

At present, one of the beekeeping problems in different countries is a massive bee hybridization, which leads to the reduction of the range of native subspecies, the formation of hybrids, and "deterioration" of the genotypic composition of honeybees. Hybrid populations are less adapted to environmental conditions that rapidly change during the year and are characterized by the higher morbidity and low immunity [1–3].

Introgressive hybridization modifies the genetic pool of local honeybee populations leading to the loss of their genetic identity [4]. The process of hybridization of different subspecies of honeybee can cause the destruction of the established gene complexes, leading to decrease in adaptive properties of organisms and populations and the change in biological and economically significant indicators of bees. The observed widespread hybridization of honeybees and the formation of hybrid bees can certainly contribute to the spread of disease. The extent of hybridization, characteristics of hybrid bees, the study of genetic processes that occur during hybridization, and evaluation of the effects of hybridization are of considerable interest.

The goal of this study is the morphometric and molecular genetic (mtDNA and microsatellite analysis) characterization of honeybees in Siberia and the assessment of the infestation of bee colonies by *Nosema*.

2. Materials and methods

2.1. Region

Bees and bee colonies were investigated in three regions of Siberia: the Tomsk region, the Krasnoyarsk Krai, and the Altai Krai (**Figure 1**).

The Tomsk region is located in the geographic center of Siberia, in the southeastern part of the West Siberian Plain. The distance between the northern and southern boundaries of the meridian is about 600 kilometers; therefore, the climate of the southern and northern regions is markedly different. A climatic characteristic of the northern region is a more severe and prolonged winter season. Almost the entire territory of the region is within the taiga zone, where forests cover about 60% of the territory. 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 100–105 days. Precipitation is 435 mm.

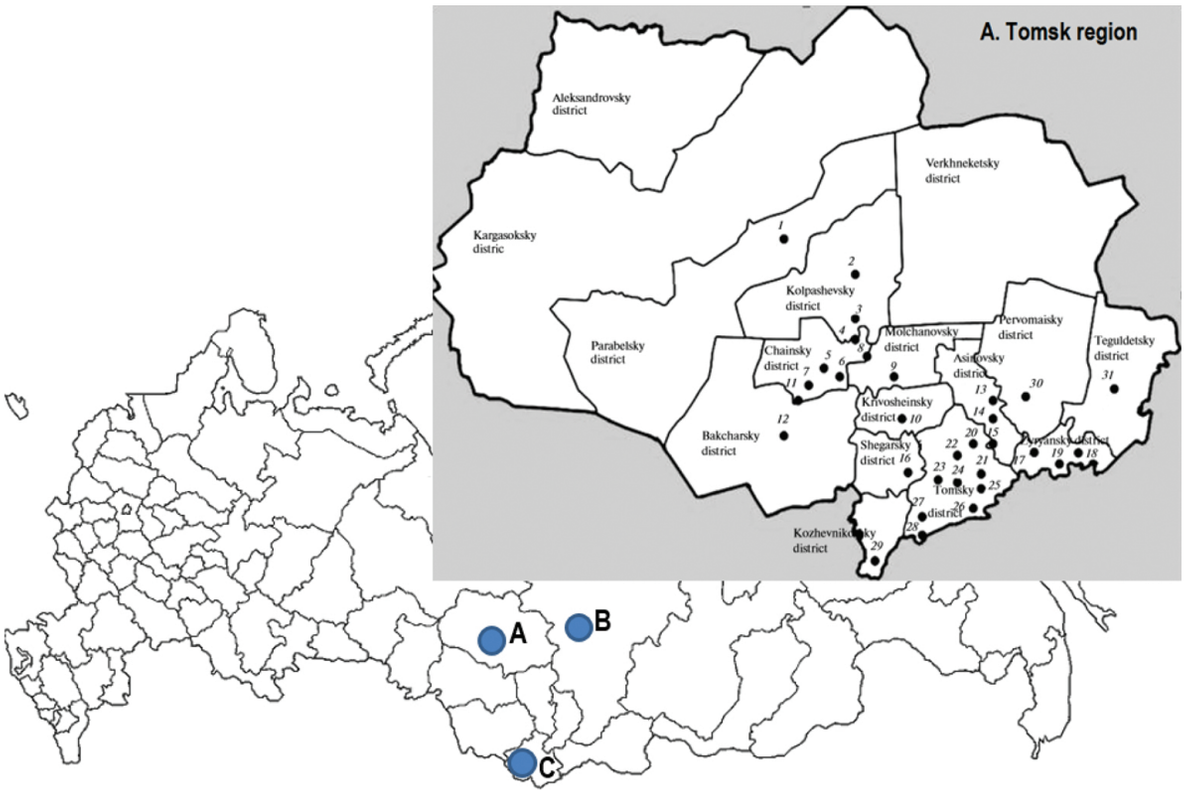

Figure 1. Map of localization of studied areas of Siberia (dots A–C) and apiaries of the Tomsk region (dots 1–31): A, the Tomsk region; B, the Krasnoyarsk Krai; C, the Altai Krai. *1*, s. Parabel; *2*, vicinity of g. Kolpashevo; *3*, d. Novoabramkino; *4*, s. Leboter; *5*, s. Podgornoe; *6*, d. Strelnikovo; *7*, s. Gorelovka; *8*, d. Sarafanovka; *9*, s. Sokolovka, s. Mogochino; *10*, s. Krivosheino; *11*, s. Vysoky Yar, d. Krylovka; *12*, s. Bakchar, s. Parbig; *13*, d. Tihomirovka; *14*, ur. Kuzherbak; *15*, s. Novikovka; *16*, s. Kargala; *17*, s. Dubrovka; *18*, s. Okuneevo; *19*, s. Zyryanskoe; *20*, d. Kuskovo; *21*, p. Zarechnyi (Mezheninovskoe rural settlement); *22*, d. Bodazhkovo, s. Semiluzhki, p. Zarechnyi (Malinovskoe rural settlement); *23*, d. Nizhne-Sechenovo, d. Berezkino, s. Zorkaltsevo, s. Rybalovo, d. Kudrinsky uchastok, d. Gubino; *24*, p. Sinii Utes, d. Magadaevo, d. Prosekino, s. Kolarovo, vicinity of Tomsk; *25*, d. Bolshoe Protopopovo; *26*, s. Mezheninovka; *27*, d. Kandinka, s. Kurlek; *28*, s.Yar; *29*, d. Elovka; *30*, d. Krutolozhnoe; *31*, s. Teguldet. Apiaries located at a distance less than 15 km from each other are marked as a single point.

The Krasnoyarsk Krai is located in the Eastern Siberia. The climate is sharply continental, where 70% of the territory is occupied by forests.

The Altai Krai is located in the south-east of Western Siberia. The region contains almost all natural zones of Russia—the steppe and forest steppe, taiga, and mountains. The climate of the Altai Territory is highly heterogeneous because of various geographical conditions. Foothills have a temperate climate, the transition to continental.

2.2. Samples

The samples are obtained from different geographic parts (ecologically and climatically different districts) of the Tomsk region, including districts with a high beekeeping activity (the southern districts) or districts with a low apicultural activity (the northern districts), according to the local knowledge of specialists from the Society of Beekeepers. Honeybees from the apiaries of the Krasnoyarsk Krai and the Altai Krai were also investigated for comparison.

A total of 332 bee colonies (60 apiaries) from Siberia were investigated by morphometric (3043 honey bee workers) and molecular genetic methods (2073 bees by mtDNA analysis and from 252 to 515 bees by microsatellite analysis): 318 bee colonies from the Tomsk region; 10 colonies from the Krasnoyarsk Krai, and 5 colonies from the Altai Krai (**Figure 1**).

Bee colonies from the Krasnoyarsk Krai were collected from the unique isolated Old Believers population, which existed for more than 60 years in forest without the importation of new honeybees.

Bee colonies from the Altai Krai have been collected in the apiary, located in the foothills.

Infestation of bee colonies by *Nosema* infections were studied in 1983 samples obtained from 132 bee colonies from 68 apiaries of Siberia during 2012–2015.

2.3. Morphometric method

Morphometric parameters (wing venation), including the cubital index, the hantel index, and the discoidal shift, were studied (**Figure 2**).

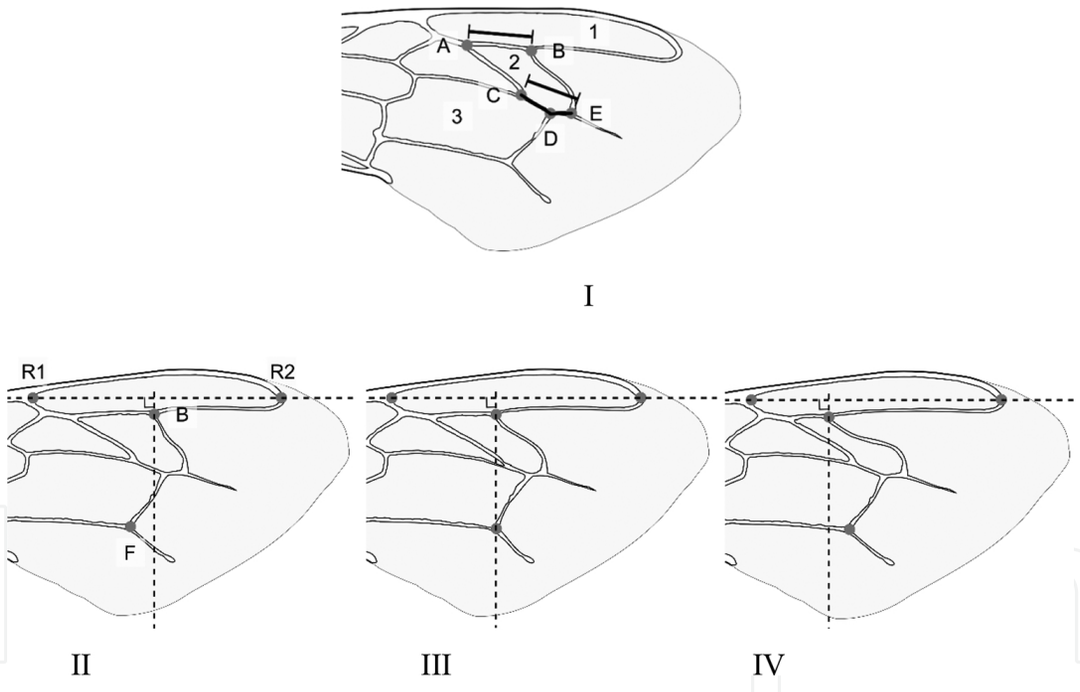

Figure 2. Scheme of the front wing venation of honeybee (I) and discoidal shift (II, III, and IV), showing the position of the horizontal and vertical lines (dashed lines). A, B, C, D, and E—the key points and segments that are used in determining the wing index (cubital index: CD/DE; hantel index: CE/AB). Options of discoidal shift: II—negative (point *F* is located to the left of the perpendicular line); III—zero (point *F* located on a perpendicular line); IV—positive (point *F* is located to the right of the perpendicular line). Designation of sells: 1, radial; 2, cubital; 3, discoidal.

2.4. mtDNA analysis

DNA isolation and polymerase chain reaction (PCR) was carried out according to standard techniques with some modifications [5,6]. To amplify the COI–COII mtDNA locus, the

following sequences of primers were used: 3′-CACATTTAGAAATTCCATTA, 5′-ATAAA-TATGAATCATGTGGA [5]. Amplification products were fractionated in 1.5% agarose gel, and the results were documented with the use of Gel-Doc XR+.

2.5. Microsatellite analysis

Variability of eight microsatellite loci was studied: A008 (=A8), Ap049, AC117, AC216, Ap243, H110, A024, and A113. PCR was performed using specific primers and reaction conditions according to Solignac et al. [7]. Amplification products were analyzed with ABI Prism 3730 Genetic Analyser (Applied Biosystems, Inc., Foster City, CA) and GeneMapper Software (Applied Biosystems, Inc.). Two microliters of PCR products were mixed with GeneScan500-ROX size standards (Applied Biosystems, Inc.) and deionized formamide. Samples were run according to the manufacturer's recommendations. These genetic parameters were calculated: allelic frequencies and standard error.

2.6. Infestation of honeybees by *Nosema*

From 10 to 70 bees were randomly selected from each bee colony and were examined for the presence of *Nosema*. Bee samples were stored in 70% (v/v) ethanol at room temperature prior to testing. The analysis was performed separately for each bee. The midgut of each sample was isolated, and one part of the midgut was used for the detection of *Nosema* spores under a light microscope, while the other part was used for DNA extraction. The midgut was suspended in 200 μL of distilled water and examined by dark-field microscopy for the presence of *Nosema* spores [8]. DNA was extracted from the midgut using a DNA purification kit, PureLink™ Mini (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

After extraction, the samples were submitted to duplex-PCR [9,10]. The primer sequences utilized to amplify the 218-bp fragment corresponding to the 16S ribosomal gene of *N. ceranae* were 218MITOC–FOR 5′–CGGCGACGATGTGATATGAAAATATTAA–3′ and 218MITOC–REV 5′–CCCGGTCATTCTCAAACAAAAAACCG–3′[9]. The primer sequences used to amplify the 321 bp fragment corresponding to the 16S ribosomal gene of *N. apis* were 321APIS–FOR 5′–GGGGGCATGTCTTTGACGTACTATGTA–3′ and 321APIS–REV 5′–GGGGGGCGTTTAAAATGTGAAACAACTATG–3′[9]. PCR was performed using specific primers and reaction conditions according to Hamiduzzaman et al. [10]. PCR products were analyzed on 1.5 % (m/v) agarose gels and visualized using UV illumination (Gel Doc XR+, BioRad, Foster City, CA, USA). All analyses were carried out in duplicate, positive and negative controls were used, and identical results were obtained.

In addition to the use of specific primers and fragment size to identify the species present, a selection of fragments (both *N. ceranae* and *N. apis)* was verified by DNA sequencing. Sequencing was done in both directions using forward or reverse primer (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA). DNA sequencing was performed using ABI Genetic Analyzer 3730 (Applied Biosystems) according to the manufacturer's protocol.

3. Results and discussion

Using the mtDNA analysis (locus COI-COII), we performed molecular genetic analysis of bee colonies (5–6 samples from each bee colony) to determine the origin of bee colony on the maternal line.

3.1. Genetic diversity of COI-COII mtDNA locus

An assessment of the genetic diversity of the COI-COII mtDNA locus in honeybee populations from the Tomsk region was conducted (see details in reference [11]). Three variants of the COI-COII mtDNA locus were registered: PQQ, PQQQ (typical for Middle Russian race), and Q

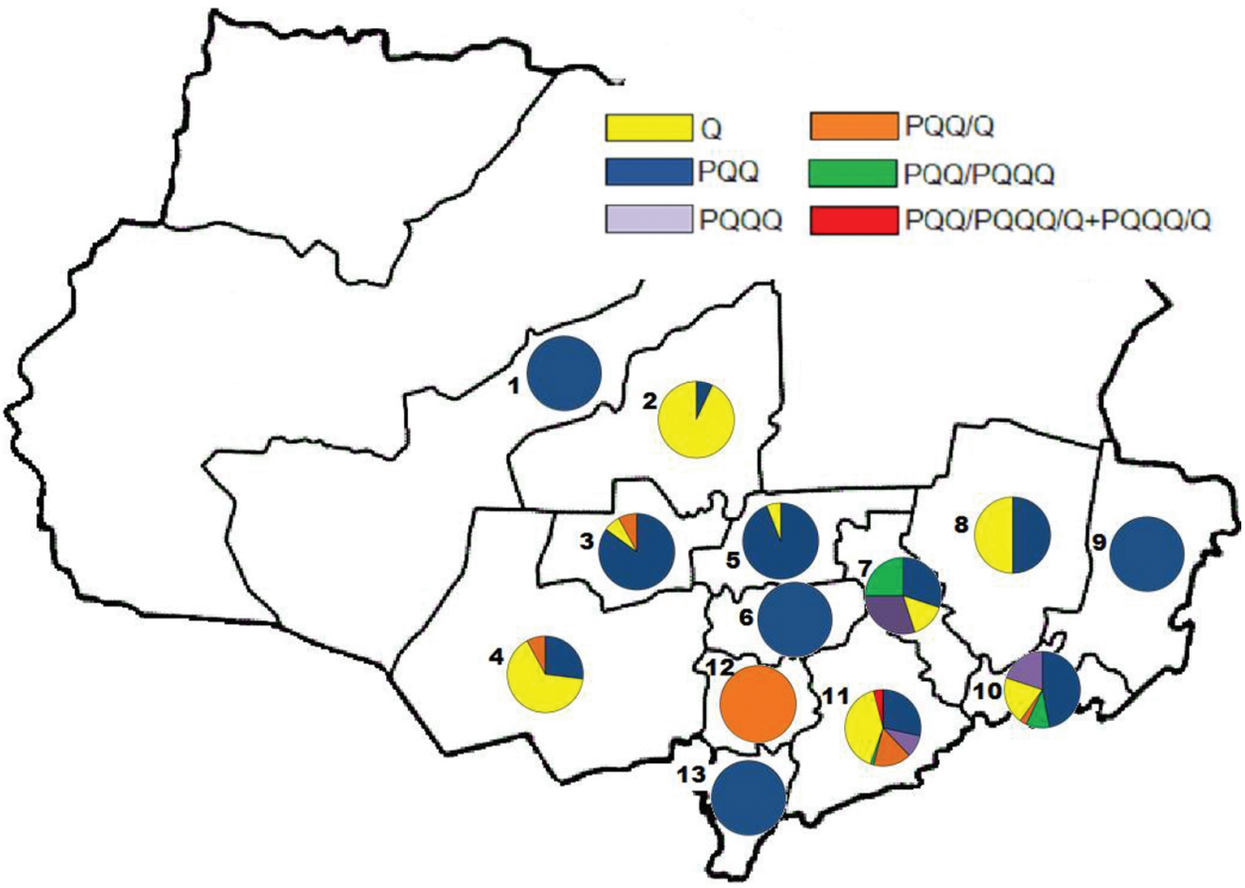

Figure 3. Distribution of COI-COII mtDNA locus variants for the districts (numbers 1–13) of the Tomsk region. Northern districts: 1, Parabelsky; 2, Kolpashevsky; 3, Chainsky; 4, Bakcharsky; 5, Molchanovsky; 6, Krivosheinsky; and southern districts: 7, Asinovsky; 8, Pervomaisky; 9, Teguldetsky; 10, Zyryansky; 11, Tomsky; 12, Shegarsky; 13, Kozhevnikovsky. Variants PQQ/PQQQ/Q (1%) and PQQQ/Q (3%), which are found only in the Tomsk district, are combined.

(typical for southern races). We established that 64% of bee colonies on the maternal line originate from the Middle Russian race, 28% of colonies originate from southern subspecies, and 8% are mixed bee colonies. The southern parts of the Tomsk region (with a high beekeeping activity) show a higher genetic diversity of honeybees as compared with the northern regions, which are dominated by bee colonies (96%) and apiaries (73%) that are homogeneous for the genetic variant of locus COI-COII. The bee colonies derived from the Middle Russian breed were genetically heterogeneous for the COI-COII locus: the PQQ variant was registered in 86.1% of the total number of bee colonies of the Middle Russian race, PQQQ was registered in 9.4%, and another 4.5% of bee colonies showed the presence of individuals with both allele PQQ and allele PQQQ.

Based on the analysis of mtDNA (locus COI-COII), assessment of the genetic diversity of the honeybee in apiaries of the Tomsk region has shown that the genetic structure of bee populations in the Tomsk region is complex and mosaic, especially in the southern parts of the region (**Figure 3**). No large areas with an array of bees having a homogeneous genetic (race) composition and maternally originating from the Middle Russian race have been found; a few apiaries were revealed, in which all bees originated from the Middle Russian breed.

In the study of variability of the COI-COII mtDNA locus in honeybees from apiaries of the Krasnoyarsk Krai and the Altai Krai, two variants of the COI-COII locus specific for Middle Russian race were identified: only variant PQQ was registered in honeybees of Krasnoyarsk Krai (Yenisei population) and two variants (PQQ and PQQQ) were found in honeybees from the Altai Krai. No a variant Q specific for southern races of bee was detected.

Due to the fact that mtDNA analysis allows assessing only the maternal component in the genome of the honeybee, 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.

3.2. Morphometric study of honeybees

The results of the morphometric study of honeybees from examined regions of Siberia (the Tomsk region, the Krasnoyarsk Krai, and the Altai Krai) were different.

According to the morphometric study, the majority of the studied bee colonies of the Tomsk region are hybrids between the Middle Russian race of bees and bees of southern origin (predominantly Carpathian race). Data on the distribution of subspecies and hybrids in the apiaries of the Tomsk region on the basis of cubital index are shown in **Figure 4**. Some of the apiaries, which cultivate the Middle Russian bees, were found in the northern and southern parts of the Tomsk region.

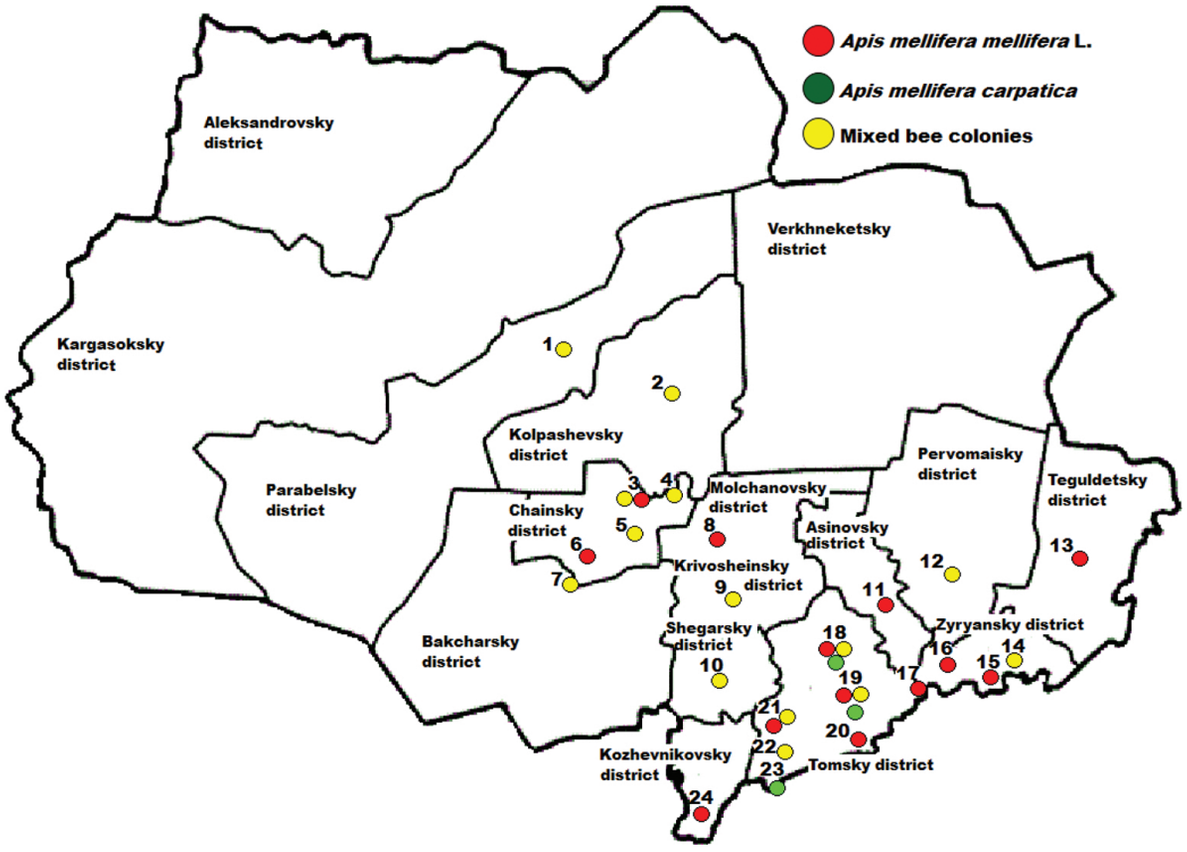

Figure 4. Distribution of subspecies and hybrids in the apiaries of the Tomsk region on the basis of cubital index of bee workers. Studied settlement are indicated by numbers: 1, s. Parabel; 2, vicinity of g. Kolpashevo; 3, Podgornoe; 4, s. Leboter; 5, d. Strelnikovo; 6, s. Gorelovka; 7, s. Vysoky Yar, d. Krylovka; 8, s. Mogochino; 9, s. Krivosheino, Sokolovka; 10, s. Kargala; 11, ur. Kuzherbak; 12, d. Krutolozhnoe; 13, s. Teguldet; 14, s. Okuneevo; 15, s. Zyryanskoe; 16, s. Dubrovka; 17, s. Novorozhdestvenskoe; 18, s. Kornilovo, s. Semiluzhki, p. Zarechnyi (Malinovskoe rural settlement); 19, p. Sinii Utes, d. Magadaevo, d.6 Prosekino, s. Kolarovo, vicinity of Tomsk; p. Zarechnyi (Mezheninovskoe rural settlement); 20, s. Mezheninovka; d. Arkashovo; 21, s. Zorkaltsevo, s. Rybalovo, d. Kudrinsky uchastok, d. Gubino; 22, s. Kurlek; 23, s.Yar; 24, d. Elovka. Apiaries located at a distance less than 15 km from each other are marked as a single point.

Bee colonies obtained from isolated apiaries of the Krasnoyarsk Krai 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 the Russian and European standards of the *Apis. 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 (**Table 1**). There are several possible explanations for the results. First, this may be the result of genetic drift, the effect of which may be because of the fact that 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 more severe climatic conditions. Nevertheless, these isolated apiaries in the Krasnoyarsk Krai may be considered as a unique population of the Middle Russian bee that exists for a long time without affecting other subspecies of honeybee.

Geographical location: settlement	Bee colony, №	Cubital index, standard units		Hantel index, standard units		Discoidal shift, %		
		Lim: min max	*M ± m*	*Lim: min max*	*M ± m*	–	0	+
Ostyatskoe	1	1.24 2.00	1.61±0.04	0.675 0.892	0.795±0.011	100.0	0	0
	2	1.39 1.74	1.51±0.02	0.743 0.912	0.849±0.012	83.3	16.7	
	3	1.23 1.74	1.51±0.03	0.736 0.883	0.837±0.008	83.3	16.7	0
	4	1.20 1.67	1.45±0.02	0.723 0.900	0.837±0.009	97.0	3.0	0
	5	1.24 1.79	1.46±0.03	0.735 0.923	0.842±0.010	87.0	13.0	0
Kolmogorovo	1	1.32 2.10	1.60±0.05	0.724 0.900	0.820±0.009	97.0	3.0	0
	2	1.12 1.76	1.51±0.03	0.758 0.919	0.845±0.008	93.0	7.0	0
	3	1.28 1.86	1.56±0.04	0.746 0.985	0.810±0.011	97.0	3.0	0
	4	1.07 1.76	1.45±0.04	0.716 0.945	0.830±0.011	97.0	3.0	0
Yaksha	1	1.31 1.85	1.59±0.02	0.711 0.846	0.775±0.008	100.0	0	0
Standard for *Apis mellifera mellifera*								
I		1.30 2.10	1.70	0.600 0.923	No data	No data		
II		1.30 1.90	1.5 to 1.7	0.600 0.923		91–100	5–10	0.00

Minimum 30 samples from each bee colony were studied.
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 indexes [12]; II, Russian breed standard.

Table 1. Morphometric parameters (wing venation) of honeybee workers from 10 bee colonies of the Krasnoyarsk Krai (Yenisei population).

The results of morphometric analysis confirmed the origin of bee colonies of Altai population from the Middle Russian race, but some influence of the southern races have been shown. For example, the parameter "Discoidal shift" deviates from the Russian breed standard: individuals with a positive value and zero of discoidal shift were found in bee colony No. 7 (**Table 2**).

If bee colonies from the Krasnoyarsk Krai were obtained from the territory distant from the center and located in sparsely populated areas, in the taiga, the bee colonies from the Altai Krai inhabit the territory, characterized by high development of beekeeping and a constant active importation of bees of different origins.

3.3. The accordance of morphometric parameters and data of mtDNA analysis in honeybees in Siberia

The results of the outward morphological characters-based diagnostics of honeybees (the cubital index, the hantel index, and the discoidal shift) received from 11 bee colonies differing in the variants of the COI-COII mtDNA locus are presented (**Table 2**). Only for 4 of the 11 bee colonies, a full compliance with the criteria of the breed according to the morphometric and mtDNA analysis (the three *Apis mellifera mellifera* colonies and one family of *Apis mellifera carpatica*) was shown. The remaining seven colonies are hybrid, and for three colonies a significant imbalance between genetic and morphometric parameters was shown. Hence, in order to determine the breeds in the conditions of mass bee hybridization, it is important to consider not only the features of mtDNA, but morphometric parameters as well, among which the discoidal shift is probably the most important.

These data are consistent with the results of the research of hybrid apiary, where for many years (over 30) the Middle Russian bee was bred, but the last 10 years, the southern races have been actively imported [6]. More than 50% of individuals refer to the southern races according to mtDNA analysis (variant Q of the locus COI-COII; "southern" mitotype). But none of these individuals corresponded to the southern race according to morphometric analysis (**Table 3**). In 33% of cases, individuals with "southern" mitotype had two morphometric features characteristic to the Middle Russian race.

For bees, originating from the Middle Russian race (variant PQQ of the locus COI-COII), full compliance between mitotype and morphometric parameters was found in approximately 6% of the individuals. 18% of bees had mitotype and two morphometric parameters which specific to the Middle Russian bees.

This indicates a process of cross-breeding of Middle Russian and southern races on this apiary. However, the process of "ousting of genes" is derived differently for bees of different origin: for bees of Middle Russian race the process of "ousting of genes" is smaller in scale, as among individuals with variant PQQ a smaller percentage of bees with "southern" morphometric characters was registered in comparison with the same data shown for bees with "southern" mitotypes.

Geographical location			Bee colony, №	Number of studied bees	Sequence composition of the COI-COII mtDNA locus	Cubital index, standard units			Hantel index, standard units		
region	**District**	**Settlement**				***Lim:*** ***min*** ***max***	***M***	***sd***	***Lim:*** ***min*** ***max***	***M***	***sd***
					Apis mellifera mellifera[*]						
Tomsk region	Tomsky	p. Zarechnyi	1	30	PQQQ	1.39 2.23	1.66	0.216	0.712 0.932	0.826	0.052
		s. Kurlek	2	28	PQQQ	1.74 3.29	2.14	0.376	0.857 1.053	0.937	0.055
	Zyryansky	s. Dubrovka	3	30	PQQ	1.43 2.47	1.69	0.232	0.672 0.933	0.849	0.060
	Molchanovsky	s. Mogochino	4	30	PQQ	1.26 2.56	1.92	0.290	0.806 1.000	0.879	0.055
			5	43	PQQ	1.36 2.00	1.73	0.181	0.693 0.926	0.821	0.038
Altai Krai	Zmeinogorsky	Vicinity of c. Zmeinogorsk	6	29	PQQ	1.19 2.00	1.55	0.232	0.758 0.967	0.858	0.062
			7	30	PQQQ	1.50 2.50	1.80	0.245	0.722 0.984	0.845	0.059
Krasno-yarsk Krai	Yeniseisky	p. Yaksha	8	30	PQQ	1.31 1.85	1.59	0.132	0.711 0.846	0.775	0.044
					Southern breeds[*]						
Tomsk region	Tomsky	s. Semiluzhki	9	50	Q	1.68 3.64	2.51	0.374	0.867 1.210	1.050	0.047
		s. Kurlek	10	29	Q	1.30 2.29	1.66	0.220	0.735 0.965	0.878	0.060
		p. Sinii Utes	11	30	Q	1.83 2.87	2.37	0.334	0.815 1.053	0.931	0.065
Standart of breeds	*A. m. mellifera*[**]		PQQ, PQQQ and other			1.30 2.10	1.70	–	0.600 0.923	–	–
	A. m. mellifera[***]					1.30 1.90	1.6	–	0.600 0.923	–	–
	A. m. carnica[**]		Q			2.40 3.00	2.7	–	≥ 0.925	–	–
	A. m. caucasica[**]		Q			1.70 2.30	2.0	–	No data	–	–

Lim, limits of values; *M,* arithmetic mean; *sd,* standard deviation.
[*]Breed indicated according to the data of mtDNA analysis.
[**]European breed standard based on values of cubital and hantel indexes [12].
[***]Russian breed standard. Discoidal shift are given according to Russian standards.

Table 2. Morphometric parameters (wing venation) of honeybee workers of 11 bee colonies from apiaries of Siberia.

Geographical location			Bee colony, №	Number of studied bees	Sequence composition of the COI-COII mtDNA locus	Discoidal shift, %		
region	**District**	**Settlement**				**–**	**0**	**+**
			Apis mellifera mellifera[*]					
Tomsk region	Tomsky	p. Zarechnyi	1	30	PQQQ	73.30	26.70	0.00
		s. Kurlek	2	28	PQQQ	32.10	53.60	10.70
	Zyryansky	s. Dubrovka	3	30	PQQ	73.33	26.67	0.00
	Molchanovsky	s. Mogochino	4	30	PQQ	70.00	30.00	0.00
			5	43	PQQ	100.0	0.00	0.00
Altai Krai	Zmeinogorsky	Vicinity of c. Zmeinogorsk	6	29	PQQ	94.00	6.00	0.00
			7	30	PQQQ	46.70	46.70	6.60
Krasnoyarsk Krai	Yeniseisky	p. Yaksha	8	30	PQQ	100.0	0.00	0.00
			Southern breeds[*]					
Tomsk region	Tomsky	s. Semiluzhki	9	50	Q	4.00	20.00	76.00
		s. Kurlek	10	29	Q	72.40	27.60	0.00
		p. Sinii Utes	11	30	Q	6.70	76.70	16.70
Standart of breeds	*A. m. mellifera*[**]		PQQ, PQQQ and other			–	–	–
	A. m. mellifera[***]					91–100	5–10	0.00
	A. m. carnica[**]		Q			0–5	0–20	80–100
	A. m. caucasica[**]		Q			60–70	20–30	3–5

Lim, limits of values; *M,* arithmetic mean; *sd,* standard deviation.
[*]Breed indicated according to the data of mtDNA analysis.
[**]European breed standard based on values of cubital and hantel indexes [12].
[***]Russian breed standard. Discoidal shift are given according to Russian standards.

Table 2. Continued.

mtDNA		Variant PQQ		Variant Q	
Number of studied bees, %		44.44		55.56	
Race		*Apis mellifera mellifera*	Southern race	*Apis mellifera mellifera*	Southern race
The combination of features characteristic for different races	3 parameters $x^1 + x^2 + x^3$	5.6	7.4	7.4	0.0
	2 parameters, total, including	18.5	13.0	33.3	14.8
		1.9	1.9	1.9	11.1

mtDNA		Variant PQQ		Variant Q	
	$x^1 + x^2$	3.7	11.1	0	3.7
	$x^1 + x^3$	13.0	0	31.5	0
	$x^2 + x^3$				
	1 parameter, total	13.0	18.5	14.8	33.3

x^1, x^2, x^3 are parameters of cubital index, hantel index, and discoidal shift, respectively.

Table 3. The accordance of morphometric parameters in individuals with different genetic variants of the COI-COII mtDNA locus (see details in reference [6]).

Thus, the result of study of hybrid apiaries and bee colonies indicate, on the one hand, the importance and the necessity of a comprehensive approach to the exact characterization of honeybee races. On the other hand, the results are of scientific interest for the study of genetic processes during hybridization of different subspecies of honeybee and for analyzing the process of "ousting of genes" of one race by genes of other race. For example, hybridization between the Middle Russian bee and Carpathian bee is of interest because the races belong to different evolutionary branches.

For such studies, microsatellite loci are the most informative molecular genetic markers. Microsatellite markers can be useful for the study of genetic structure of different honeybee populations and bee colonies, evaluation of genetic diversity and introgressive hybridization, differentiation of different subspecies (ecotypes), the establishment of evolutionary relationships and adaptive features of four evolutionary branches (A, M, C, and O), mapping quantitative trait loci (QTL), and search of genetic markers associated with economically significant characteristics [3,7,13–46].

Characterization of the allele spectrum of microsatellite loci and analysis of their variability in subspecies, colonies, and individuals in the honeybee populations is the initial stage of any of the above research.

3.4. Microsatellite analysis

Variability of eight microsatellite loci (A008 (=A8), Ap049, AC117, AC216, Ap243, H110, A024, and A113) in honeybee from Siberian region was studied. Seven loci were polymorphic and only for AC216 locus one homozygous genotype was registered in all the studied bees (allele 91 bp). For each locus, the range and frequency of genotypes and alleles were determined (**Table 4**).

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
A008	152–152	0.006	P_{152}=0.0311±0.0054
	152–162	0.049	P_{162}=0.8049±0.0123

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
	152–170	0.002	P_{166}=0.0010±0.0031
	162–162	0.736	P_{168}=0.0010±0.0031
	162–168	0.002	P_{170}=0.0213±0.0045
	162–170	0.016	P_{172}=0.0243±0.0048
	162–172	0.039	P_{174}=0.0825±0.0086
	162–174	0.033	P_{176}=0.0029±0.0017
	166–172	0.002	P_{178}=0.0262±0.0050
	170–170	0.006	P_{180}=0.0039±0.0019
	170–174	0.016	
	172–172	0.004	
	174–174	0.037	
	174–176	0.004	
	174–178	0.031	
	174–180	0.008	
	176–178	0.002	
	178–178	0.010	
	n=515		
Ap049	118–127	0.002	P_{118}=0.0010±0.0001
	121–127	0.002	P_{121}=0.0069±0.0025
	121–130	0.006	P_{127}=0.6581±0.0149
	121–139	0.006	P_{130}=0.1759±0.0120
	127–127	0.529	P_{139}=0.1403±0.0109
	127–130	0.187	P_{142}=0.0010±0.0001
	127–139	0.053	P_{152}=0.0168±0.0040
	127–152	0.019	
	130–130	0.055	
	130–139	0.045	
	130–152	0.002	
	139–139	0.081	
	139–152	0.013	
	142–152	0.002	
	152–152	0.002	
	n=506		

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
AC117	175–175	0.008	P_{175}=0.0910±0.0092
	175–179	0.020	P_{179}=0.0879±0.0090
	175–183	0.145	P_{183}=0.8211±0.0123
	179–179	0.012	
	179–183	0.131	
	183–183	0.683	
	n=489		
H110	162–162	0.567	P_{162}=0.7522±0.0167
	162-166	0.116	P_{166}=0.0627±0.0093
	162–170	0.254	P_{170}=0.1851±0.0150
	166–166	0.003	
	166–170	0.003	
	170–170	0.057	
	n=335		

n, number of studied samples is indicated in bold.

Table 4. Characterization of variability of seven microsatellite loci in honeybees from Siberia.

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
Ap243	255–255	0.401	P_{255}=0.5278±0.0222
	255–263	0.167	P_{263}=0.3175±0.0207
	255–269	0.056	P_{269}=0.0833±0.0123
	255–272	0.028	P_{272}=0.0635±0.0109
	255–275	0.004	P_{275}=0.0079±0.0039
	263–263	0.175	
	263–269	0.075	
	263–272	0.040	
	263–275	0.004	
	269–269	0.004	
	269–272	0.028	
	272–272	0.012	
	272–275	0.008	

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
	n=252		
A024	94–94	0.344	P_{94}=0.4736±0.0186
	94–98	0.036	P_{96}=0.1014±0.0112
	94–100	0.033	P_{98}=0.0375±0.0070
	94–102	0.175	P_{100}=0.0194±0.0051
	94–104	0.014	P_{102}=0.2097±0.0152
	96–96	0.067	P_{104}=0.1528±0.0134
	96–104	0.058	P_{106}=0.0056±0.0028
	96–106	0.011	
	98–98	0.019	
	100–100	0.003	
	102–102	0.089	
	102–104	0.067	
	104–104	0.083	
	n=360		
A113	208–212	0.003	P_{208}=0.0013±0.0013
	210–210	0.003	P_{210}=0.0144±0.0043
	210–218	0.021	P_{212}=0.2350±0.0153
	210–220	0.003	P_{214}=0.0026±0.0018
	212–212	0.177	P_{218}=0.5953±0.0177
	212–214	0.005	P_{220}=0.1084±0.0112
	212–218	0.078	P_{222}=0.0013±0.0013
	212–220	0.013	P_{224}=0.0013±0.0013
	212–222	0.003	P_{226}=0.0183±0.0048
	212–226	0.005	P_{228}=0.0196±0.0050
	212–228	0.003	P_{232}=0.0026±0.0018
	212–232	0.005	
	218–218	0.475	
	218–220	0.117	
	218–226	0.021	
	218–228	0.003	
	220–220	0.018	
	220–224	0.003	

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
	220–226	0.010	
	220–228	0.034	
	n=383		

Table 4. Continued.

Microsatellite loci differed in variability: the minimum number of alleles was detected for loci AC117 and H110 (3 alleles) and the maximum number of alleles was registered for loci A008 (10 alleles) and A113 (11 alleles). At the same time, for six of the seven polymorphic loci (except locus A024), one major allele with a frequency of more than 0.5 (from 0.5278 for allele "255"of locus Ap243 to 0.8211 for allele "183" of locus AC117) was registered regardless of the number of detected alleles.

To identify the features of honeybee from different geographical areas, the comparative analysis of the variability of the studied loci was carried out for the bees of *Apis mellifera mellifera* (= dark-colored forest bee, Middle Russian race) of four populations (Siberia, the Urals, and Europe) using our own data (Tomsk region and Krasnoyarsk Krai) and literature data [15,16,47] (**Tables 5 and 6**). The Ural population (Bashkir population) located in the nature reserve is a unique population of the dark-colored forest bee (Burzyan bee).

Locus	Alleles (pb)	Allelic frequency					
		Russia			Europe**		
		Krasnoyarsk Krai	Tomsk region	Ural* (Bashkortostan)	Belgium (Chimay)	Sweden (Umea)	France (eight geographic areas)
A008	148				0.783	0.727	0.267–0.969
	152		0.006				
	154			0.897			0–0.083
	155						0–0.033
	156			0.053	0.133	0.227	0.017–0.300
	157						0–0.050
	158			0.053		0.023	0–0.117
	159						0–0.017
	160				0.050		0–0.100
	162	1.000	0.912		0.033		0–0.034
	164					0.023	0–0.020
	166		0.003				0–0.017
	170		0.003				

Locus	Alleles (pb)	Allelic frequency					
		Russia			Europe**		
		Krasnoyarsk Krai	Tomsk region	Ural* (Bashkortostan)	Belgium (Chimay)	Sweden (Umea)	France (eight geographic areas)
	172		0.032				
	174		0.044				
N		***120***	***170***	***48***	***60***	***44***	***634***
A024	94	0.216	0.741		No data	No data	
	96	0.358					
	98	0.132		0.896			0.804
	100	0.034	0.020				
	102	0.025	0.227				
	104	0.216	0.012				
	106	0.020		0.104			0.130
	108						0.065
N		***102***	***172***	***48***			***46***
A113	202				0.083	0.024	0.017–0.267
	204						
	208						0–0.017
	210	0.021	0.009				
	212		0.174				0–0.030
	214		0.006		0.033		0.010–0.500
	216			0.063			0–0.017
	218	0.898	0.540	0.865			0–0.020
	220	0.081	0.183	0.042	0.833	0.857	0.433–0.810
	222		0.003	0.032		0.024	0–0.041
	224		0.003		0.017	0.048	0–0.060
	226		0.040			0.048	0–0.034
	228		0.043		0.017		0.017–0.071
	230						0–0.052
	232						0–0.017
	234				0.017		0–0.017
	236						0–0.020
	238						0–0.017

Locus	Alleles (pb)	Allelic frequency					
		Russia			Europe**		
		Krasnoyarsk Krai	Tomsk region	Ural* (Bashkortostan)	Belgium (Chimay)	Sweden (Umea)	France (eight geographic areas)
	240						0–0.010
N		***118***	***175***	***48***	***60***	***44***	***634***

N, number of studied samples.
*data from reference [47].
**data from references [15,16].
The minimum and maximum values of allelic frequencies represented for loci A008 and A113 in honeybees of France populations; allelic frequencies for locus A024 are given for bees of only Northern France population.

Table 5. Allele frequency at three loci in honeybees from different geographic areas of Russia and Europe.

Locus	Alleles (pb)	Allelic frequency		
		Siberia		Ural
		Krasnoyarsk Krai	Tomsk region	Bashkortostan
Ap049	118	0.005		
	121	0.005	0.003	
	123			0.917
	127	0.810	0.711	
	130	0.138	0.249	0.063
	138			0.021
	139	0.014	0.037	
	152	0.029		
Number of studied samples		***105***	***175***	***48***
Ap243	254			0.646
	255	0.280	0.524	
	257			0.354
	263	0.542	0.254	
	269	0.140	0.056	
	272	0.037	0.143	
	275		0.024	
Number of studied samples		***107***	***63***	***48***
H110	160			0.615
	162	0.624	0.837	
	163			0.302

Locus	Alleles (pb)	Allelic frequency		
		Siberia		Ural
		Krasnoyarsk Krai	Tomsk region	Bashkortostan
	166	0.376	0.056	
	168			0.083
	170		0.107	
Number of studied samples		***117***	***135***	***48***

N, number of studied samples.

Table 6. Allele frequency at three loci in honeybees from different populations of Russia.

Siberian populations (Tomsk region and Krasnoyarsk Krai) are closest in spectrum and allele frequencies of most studied loci (A008, Ap049, A113, Ap243, H110). The Ural population located to the west of Siberian region differs from Siberia for some loci: for locus A008 differences were registered in the spectrum of alleles, for the locus A024—in the frequency of alleles, for the loci Ap049 and Ap243—in both the spectrum and frequency of alleles. It is remarkable that the Ural population has a greater similarity in the spectrum of alleles of loci A024 and A008 to European populations.

The differences in the spectrum of alleles and the frequency of allele registration for locus A008 were revealed in honeybees of Siberia, the 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 "162" was the most specific. Probably this locus should be considered as a marker related to geographic and environmental conditions (specific adaptation to local conditions) [1,3,48,49] because the different populations of dark-colored forest bee (European, Ural, and Siberian populations) were compared in this study.

For some loci, for example A113, allelic spectrum overlaps, but the frequency of the alleles was different in honeybees of different populations. Different factors of population dynamics (such as founder effect, genetic drift, natural selection) can be causes of this phenomenon.

Thus, it is shown that for some loci the specific distribution of genotypes and alleles were detected in the bees, which differ by geographical location. Further research is needed and the expansion of gene-geographic studies of honeybee is relevant.

To assess the informativeness of studied loci for the differentiation of different subspecies of honeybee, the comparison of the spectrum of predominant alleles in bees of different evolutional branches (M and C) and from different geographical localization was conducted (**Table 7**). Comparison of the data on the variability of microsatellite loci studied in bees of different origin and different geographical location allows making some conclusions and adjustments with respect to informativeness of these loci as markers for differentiation of subspecies of honeybee.

For locus A008, the differences in the spectrum of the most common alleles are registered between the *Apis m. mellifera* living in different geographical regions (as shown above), and between the two southern races (*Apis m. caucasica* and *Apis m. carpatica*).

For locus A113 clear differences in length of the most frequently detected allele were not detected both among bees of a common origin and between bees belonging to different races. Probably this locus cannot be considered informative for determining of the subspecies.

Loci A024 and Ap049 are of considerable interest for further study as candidate markers for inclusion in the diagnostic panel, differentiating subspecies. So, in general, for the locus A024 the majority of bees and bee colonies *Apis m. mellifera*, regardless of their habitat, are characterized by shorter length of alleles. Perhaps, for locus Ap049 the differences exist in the allelic spectrum between bees belonging to different races.

	Geographical location	**Sequence composition of the COI-COII mtDNA locus (breed)**	**Predominant allele**	**Allelic frequency**
Locus A008	Tomsk region	PQQ/PQQQ	162	0.71–1.00
	Krasnoyarsky Krai	PQQ	162	1.00
	Ural (Bashkir population)[1]	PQQ	154	0.63–1.00
	Tomsk region[2]	Q	174	0.58–0.61
	Sochi area[3]	Q	158	0.88–1.00
	Europe[4]	*A.m.mellifera*	148	0.27–0.97
Locus A113	Tomsk region	PQQ/PQQQ	218	0.67–0.82
			212	0.61
			220	0.50
	Krasnoyarsky Krai	PQQ	218	0.85–0.95
	Ural (Bashkir population)[1]	PQQ	218	0.50–1.00
			220	0.50
	Tomsk region[2]	Q	212	0.94–1.00
	Sochi area[3]	Q	222	0.50
	Europe[4]	*A.m.mellifera*	220	0.433–0.857
Locus A024	Tomsk region	PQQ/PQQQ	94	0.60–0.90
			102	0.54
	Krasnoyarsky Krai	PQQ	98	0.50
			96	0.50–0.71
	Ural (Bashkir population)[1]	PQQ	98	0.50–1.00
			106	0.50
	Tomsk region[2]	Q	104	0.65

	Geographical location	Sequence composition of the COI-COII mtDNA locus (breed)	Predominant allele	Allelic frequency
	Sochi area[3]	Q	106	0.88–1.00
	Europe[4]	*A.m.mellifera*	98	>0.80
Locus AP049	Tomsk region	PQQ/PQQQ	127	0.62–0.92
			130	0.77
	Krasnoyarsky Krai	PQQ	127	0.50–0.96
	Ural (Bashkir population)[1]	PQQ	129	0.50–1.00
			130	1.00
	Tomsk region[2]	Q	139	0.66–1.00
	Sochi area[3]	Q	139	1.00

*Data on allelic frequencies, the frequency of which = or > 0.5 are shown.
[1]Data on the Ural (Bashkir population) are taken from reference [47].
[2]Our own data for the Carpathian breed (*Apis m. carpatica*) imported into the territory of the Tomsk region from Carpathian breed nursery (d. Mukachevo, Ukraine).
[3]Data on the Caucasian honeybee (*Apis m. caucasica*) from the Sochi area are taken from reference [47].
[4]Data on the European population are taken from references [15,16].

Table 7. Comparative analysis of the frequency of the most common alleles* of microsatellite loci in honeybees of different maternal origins and geographic localization.

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. However, no standard reference material, such as a standard allelic ladder, is available for honeybees [3]. In addition, the spectrums of analyzed microsatellite markers often do not overlap and primary data on the allele spectrum and allele frequencies are not always presented in publications. In general, the present stage of the study of variability of microsatellite loci in *Apis mellifera* can be considered as a period of accumulation of information. At this stage of the study of honeybee it should be with caution relate to the use autosomal loci to determine the subspecies of honeybee.

3.5. Infestation of honeybees by *Nosema* in Siberia

Importation of races of southern origin to the territory of Siberia, where the Middle Russian breed for a long time lived, on the one hand, led to a massive hybridization of bees, a loss of purebred, decreased immunity, and increased incidence of bees. On the other hand, the import of bee families from other areas (the European part of Russia, Uzbekistan), disadvantageous in the epidemiological situation, led to the spread of diseases that have not previously registered in the territory of Siberia.

This situation was evaluated for nosemosis: the distribution *Nosema* infection throughout Siberia was studied, the species of microsporidia were determined, and the origin of bee colonies infected with *Nosema* was investigated.

Nosemosis is a parasitic disease of adult honeybees (*Apis mellifera* L.) caused by two described species of microsporidia, *Nosema apis* [50] and *Nosema ceranae* [51]. The disease occurs throughout the world, causes significant detriment to honey production, and results in economic losses. The original assumption was that *N. apis* specifically infects the European honeybee *A. mellifera*, causing nosemosis, and that *N. ceranae* is a specific pathogen of the Asian honeybee, *A. cerana*. Recently, it became evident that *N. ceranae* is also widespread in the *A. mellifera* population throughout the world and is already found in North and South America, across Europe and Asia [52–58]. It has been subsequently detected across Canada and the United States [59,60] and has been confirmed in Central America [61], Australia [62], and North Africa [63].

The geographical distribution of *Nosema* in Russia is not well known [64,65]. In addition, information on the prevalence of *N. ceranae* in Russia, including Siberia, is not complete [66]. Previously, nosemosis in honeybees in Siberia was attributed exclusively to *N. apis*. The problem of the distribution of *Nosema* and the consequences of infection for honeybees has not yet been resolved. The effects of the *Nosema* infection on survival and productivity of honeybees are not well studied.

For the period of 2012–2015, a screening study of 132 bee colonies from 68 apiaries of Siberia for the presence of *Nosema* spores was carried out [65]. For an objective evaluation, the different methods were used: microscopy and PCR. We found that honeybees of 33 colonies from 132 studied (25.0%) and 21 apiaries from 68 studied (30.9%) had spores detectable by light microscopy. As it is difficult to distinguish *N. ceranae* and *N. apis* morphologically, a PCR assay based on 16S ribosomal RNA has been used to differentiate *N. apis* and *N. ceranae*. To characterize further the identity of which species of *Nosema* was present, we performed PCR using primers specific for either *N. apis* or *N. ceranae. Nosema* positive samples (determined from light microscopy of spores) of adult worker bees from 33 bee colonies (21 apiaries) were tested to determine *Nosema* species using PCR primers of the 16S rRNA gene specific for *N. ceranae* or *N. apis*.

The samples of 28 bee colonies from 33 infected colonies (84.8%) from 19 apiaries were positive by PCR using *N. apis* specific primers, and the samples from three colonies (3/33, 9.1%) were positive for *N. ceranae* (only two of apiaries). Samples co-infected with both *N. ceranae* and *N. apis* were registered in two bee colonies (2/33, 6.0%) from two apiaries. To confirm the PCR findings, the DNA fragments were sequenced. Sequence analysis revealed a complete sequence identity for *N. apis* (GenBank Accession No U97150) and *N. ceranae* (GenBank Accession No DQ486027).

Nosema-infected bees were found in samples collected from five districts and mainly in the southern climatic areas (temperate continental parts of Siberia) (**Figure 5**). In the northern district (**Figure 5**, C – Chainsky) bees infected by *Nosema apis* are imported from Uzbekistan. It was established that *Nosema ceranae* revealed in bees from the southern districts of the Tomsk region (**Figure 5**, Shegarsky and Tomsky districts) was introduced with infected bees from southern regions of Russia.

The studied bees from apiaries of Krasnoyarsk Krai and Altai Krai were not infected with *Nosema*.

Reports on the impact of *N. ceranae* infections on honeybee health and colony survival are contradictory, and various symptoms of the disease have been described [4,48,52,54–56,59,60,67–76]. Adult bees become infected by ingesting *Nosema* spores, which germinate in the midgut and infect cells of the midgut epithelium. *Nosema* infection caused by *N. apis* is characterized mainly by dysentery, whereas *N. ceranae* is described as causing death of individuals and colonies not preceded by any visible symptoms [9,68]. *Nosema apis* infection is restricted to the midgut epithelium [77], whereas *N. ceranae* has also been detected by molecular methods in other bee tissues such as malpighian tubules and hypopharyngeal glands [78].

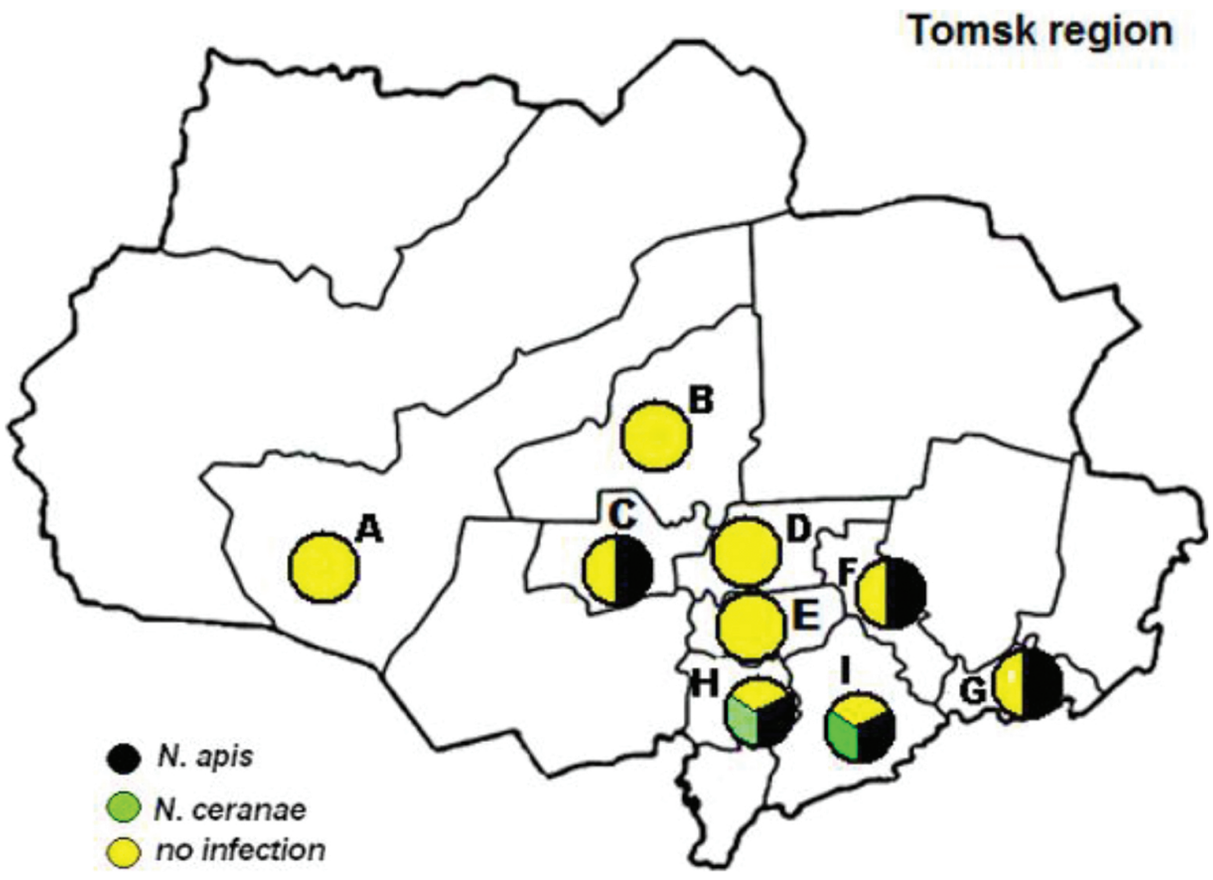

Figure 5. Distribution of *Nosema* in the honeybee colonies (*Apis mellifera* L.) throughout the Tomsk region (Western Siberia) (dots *A*–*I*). Bee colonies not infected by *Nosema* are indicated in yellow; bee colonies corresponding to infection by *N. apis* or *N. ceranae* are indicated in black and green, respectively. Sectors in circles indicate representation cases (existence/absence) of an infection without frequency. Literas (A–I) indicate the districts of the Tomsk region: A, Parabelsky; B, Kolpashevsky; C, Chainsky; D, Molchanovsky; E, Krivosheinsky; F, Asinovsky; G, Zyryansky; H, Shegarsky; I, Tomsky.

Perhaps, *N. ceranae* is the most aggressive of the two *Nosema* species in relation to the host and appears to be replacing *N. apis* in some populations of honeybees.

Currently, several reasons for the widespread presence of the parasite *N. ceranae* in the world and its displacement of *N. apis* are discussed in the literature. On the one hand, nosemosis produced by *N. ceranae* is considered a global problem because this parasite has wide prevalence in multiple hosts [79]. *Nosema ceranae* is a more aggressive parasite compared with *N. apis,* and consequently, it is more widespread than *N. apis*. On the other hand, the killing of honeybee colonies by *N. ceranae* could be a regional problem rather than a global phenomenon [80], and the virulence of *N. ceranae* could be influenced by climatic conditions [81–85] or might actually depend on honeybee race and honeybee genetic diversity [4,48,74,75,86–88].

It is assumed that the level of infestation in honeybees can be associated with the race and the origin (local or non-local) of the bees. Some differences in the resistance to *Nosema* have been shown in Russian bee breeds [86]. Levels of *N. ceranae* infestation differed significantly between lineages and colonies for both Russian and Italian workers [87]. Unlike genetically homogeneous Italian lines [87], Russian bee lineages have a high genetic diversity and are characterized by high resistance to disease. Differences in infection levels were significant between local and introduced bee colonies [4,74]. The use of local honeybees provides a higher chance of colony survival because of their adaptation to regional environmental factors such as climate and vegetation [48,75].

To determine if the infection incidence of bees by *Nosema* is associated with the races of bees, we analyzed breeds of bee derived from *Nosema*-positive colonies using morphometric (wing venation) and molecular-genetic (mtDNA) analyses (**Table 8**). The results of molecular genetic analysis (COI-COII locus) of honeybees have been published in reference [11]. According to the mtDNA analysis, PQQ and PQQQ variants of the locus COI-COII (*A. m. mellifera*, evolutionary branch M) were detected in two colonies (families No. 2 and 4), and Q variant (evolutionary branch C) was registered in four colonies (families No. 1, 3, 5, and 7). Family No. 6 had bees with different variants of the COI-COII locus (PQQ and Q) and apparently was formed by mixing two colonies having different origins. As a result, morphometric studies have shown that colonies No. 1, 3, 4, and 6 can be considered as subspecies of *A. m. mellifera* and that colonies No. 2, 5, and 7 are hybrids. However, according to the combined morphometric and mtDNA analysis, only family No. 4 can be considered as *A. m. mellifera*, whereas six *Nosema*-infected bee colonies did not correspond to any of the standards but were honeybee hybrids (**Table 8**). Furthermore, some colonies that were observed not only differed in morphometric parameters compared with the standards but in a mismatch of morphometric data and results of the mtDNA analysis for the two honeybee colonies. Honeybees infected with *N. apis* (colony No. 3) and bees infected with *N. ceranae* (colony No. 1) correspond to the *A. m. mellifera* race (branch M) according to the morphometric analysis, whereas the results of the mtDNA analysis confirmed the origin of these bees from branch C. Thus, our results indicate that examined honeybees infected with *Nosema* could be of hybrids of the two races (*Apis m. mellifera* and *Apis m. carpatica*).

№ colonies	*Nosema* species	Sequence composition of the COI-COII mtDNA locus	Morphometric parameters			
			Cubital index, standard units		Hantel index, standard units	
			Lim: min max	M ± m	Lim: min max	M ± m
1	*N. ceranae*	Q	1.30 2.29	1.66 ± 0.04	0.735 0.965	0.878 ± 0.011
2	*N. ceranae*	PQQQ	1.74 3.29	2.14 ± 0.07	0.857 1.053	0.937 ± 0.010
3	*N. apis*	Q	1.35 2.11	1.70 ± 0.03	0.667 0.917	0.804 ± 0.011
4	*N. apis*	PQQ	1.45 2.80	1.78 ± 0.06	0.754 1.0	0.846 ± 0.013
5	*N. apis*	Q	1.41 2.82	1.90 ± 0.06	0.656 1.176	0.880 ± 0.018
6	*N. apis*	PQQ/Q	1.28 2.80	1.73 ± 0.06	0.707 1.0	0.834 ± 0.015
7	*N. apis*	Q	1.43 2.35	1.86 ± 0.04	0.733 1.057	0.885 ± 0.011
Standard breeds (subspecies)**						
A. m. mellifera		PQQ, PQQQ and other	1.3 2.1	1.7	0.600 0.923	No data
A. m. carpatica		Q	2.3 3.0	2.65	≥0.925	No data

M ± *m*, average value of the sign ± the standard error of the mean.
*Thirty samples of bees were examined in each family.
**Definition of subspecies was carried out based on European standard honeybee [12].

Table 8. Characterization honeybee colonies infested by *Nosema*[*].

For comparison, the assessment of the origin of the bee colonies not infected with *Nosema* (24 families from 38 analyzed) was carried out using morphometric and mtDNA analysis. Among the 24 bee colonies not infected with *Nosema,* 18 bee colonies were identified as *A. m. mellifera* (75.0 %), 3 colonies were identified as *A. m. carpatica* (12.5 %), while 3 colonies were identified as hybrids (12.5 %).

At present, the cold climate is considered as one of the limiting factors of *N. ceranae* distribution. It appears that the spread of *N. ceranae* across the globe is reduced in colder climates [81,82], as *N. ceranae* spores are capable of surviving high temperatures (60 °C) and desiccation, but they are intolerant of cold (4 °C) [81,82,89]. The marked decrease in *N. ceranae* spore germina-

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tion was observed after even a short exposure to low temperatures (4 °C) [82]. In warmer climates, *N. ceranae* is more competitive than *N. apis* [48,82], but the spores of *N. ceranae* appear to be much more vulnerable than the spores of *N. apis*, in particular, to freezing, and the apparent replacement of *N. apis* for *N. ceranae* remains enigmatic [83].

The different prevalence of *N. ceranae* may simply reflect its time of arrival, by natural spread or by the importation of infected honeybees, and mobility of bees within a country. Reduced or inhibited *N. ceranae* spore germination at low temperatures should hamper the infectivity and spread of this pathogen in climatic regions characterized by a rather cold winter season [82]. The presence of *N. ceranae* in the Tomsk region (Western Siberia, Russian) was reported previously by us [65,66] confirms the fact of a widespread *N. ceranae* infection in honeybee population throughout the world. However, we found *N. ceranae*-infected bee colonies in cold climate with long winters and humid summers, and this parasite is not associated with colony depopulation or honeybee collapse. We established that these previously infected colonies had been imported from other areas of Russia. The fact that *N. ceranae* is registered in the territory of Siberia with its severe climatic conditions does not agree with data on a weak survival of spores at low temperatures. At the same time, the colonies infected with *Nosema* (*N. apis* or *N. ceranae*) are found predominantly in the southern areas of the Tomsk region, which is characterized by more developed beekeeping and active delivery of breeds of southern origin (*A. m. caucasica* and *A. m. carpatica*) that leads to massive honeybee hybridization. Introgressive hybridization modifies the genetic pool of local honeybee populations, leading to the loss of their genetic identity [4]. The process of hybridization of different subspecies of honeybees can cause a destruction of evolutionarily developed gene complexes, leading to a decrease in the adaptive properties of organisms and populations and to a change in biological and economically significant characteristics of honeybees. The observed widespread hybridization of honeybees and the formation of hybrid bees will certainly contribute to the spread of disease.

In our research, the majority of bee colonies infected by *Nosema* were hybrids. This finding is consistent with the view that hybrid forms are poorly adapted to changing environmental conditions and less resistant to the disease. Therefore, our results on the *Nosema* infestation of bee colonies are not surprising. At the same time, it is impossible to make a conclusion about the pathogenicity of a parasite based on our data. Perhaps, hybrids are characterized by other developmental conditions of the parasite in comparison with pure breeds that do not realize the pathogenicity of *N. ceranae* in the host. Also, there is an open question about the distribution of a *Nosema* in the northern part of the Tomsk region (influence of a cold climate, insignificant number of hybrids, etc.) where the colonies infected with *Nosema* were not detected except Chainsky district (*N. apis*-infected bees were imported from Uzbekistan). Siberia can be an ideal location to study how the spread of this disease correlates with climatic conditions and how the disease moves to particularly remote areas. This is an especially intriguing thought since changes in disease prevalence and pathogen virulence because of climatic change are widely discussed [80]. Obviously, more research is needed to elucidate the full effect of *N. ceranae* infection in *A. mellifera* colonies in different geographical areas and to understand if individual virulence levels and colony virulence levels differ between the two parasites.

4. Conclusion

This study of honeybees in Siberia shows the need for a comprehensive approach to the study of various aspects of the honeybee, such as differentiation of subspecies, the role of environmental (geographical) factors in the formation of the genetic diversity of bees, and the incidence of bees.

The primary task of the study of the genetic diversity of honeybees is to determine their subspecies composition. When performing gene-geographical research, it is important to consider the assessment of adaptive and selective significance of genetic markers. This is also important for the planning and conducting of works having applied nature.

Along with exterior characters used for a long time to identify the breed of honeybees, molecular genetic techniques are actively applied. However, in connection with the high level of hybridization of bees, when about one-third of bee colonies show an imbalance between genetic and morphometric parameters, and in some cases, their complete mismatch occurs, a comprehensive analysis of the bees is necessary.

The presence of hybrid forms in an area where the genetic diversity is studied, on the one hand, creates unfavorable background for conservation of gene pools of unique subspecies (for example, dark-colored forest bee), on the other hand, makes it difficult to search for adaptively significant and economically valuable traits (possible distortion of results and their interpretation). Therefore, it should be taken into account in conducting such studies. The above 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.

In the conditions of widespread crossbreeding of bees, genetic methods to control the purity of bee colonies must also be improved. Research in this direction is carried out by international and Russian researchers [43,47,90]. Therefore, on the basis of extensive research carried out on the territory of Eastern Europe (search of informative markers was conducted among more than 1,000 SNP using five different analytical methods), five panels, consisting of 48, 96, 144, 192, and 284 markers informative for determining the ancestral origin of species have been developed. The authors propose to use the results of this study to identify and evaluate the impurity of C-lines (in particular, *Apis m. ligustica* and *Apis m. carnica*) to the M-line (*Apis m. mellifera*) [43]. Russian researchers have only begun such studies, but the results obtained at this stage suggest that populations of honeybees living on the territory of Russia are characterized by wide genetic variability, and it is unlikely to develop a uniform panel of markers for the entire territory of the Russia for differentiation of the various breeds of bees. It is necessary to integrate the scientific achievements and results of the various laboratories and scientific groups of all over the world to establish general regularities of the genetic variability of the bees and to assess the adaptive and selective potential of honeybees.

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