Journal of Molecular Biology Research; Vol. 8, No. 1; 2018
ISSN 1925-430X E-ISSN 1925-4318
Published by Canadian Center of Science and Education

Variability and Structure of the Repetitive Region of the Major Royal Jelly Protein Gene *mrjp3* in Honeybee *Apis mellifera* of Different Evolutionary Branches

Nadezhda V. Ostroverkhova[1], Aksana N. Kucher[1,2], Nadezhda P. Babushkina[2], Olga L. Konusova[1] & Igor V. Sharakhov[1,3]

[1] Institute of Biology, National Research Tomsk State University, 36 Lenina Avenue, Tomsk 634050, Russia

[2] Research Institute of Medical Genetics, Tomsk National Research Medical Center of the Russian Academy of Sciences, 10 nab. Ushaika, Tomsk 634050, Russia

[3] Department of Entomology, Virginia Polytechnic Institute and State University, 360 West Campus Drive, MC 0346, Blacksburg, Virginia, VA 24061, United States of America

Correspondence: Nadezhda V. Ostroverkhova, Institute of Biology, National Research Tomsk State University, 36 Lenina Avenue, Tomsk 634050, Russia. Email: nvostrov@mail.ru

Received: July 4, 2018 Accepted: July 12, 2018 Online Published: July 31, 2018

doi:10.5539/jmbr.v8n1p122 URL: https://doi.org/10.5539/jmbr.v8n1p122

Abstract

An assessment of the genetic diversity of the microsatellite locus *mrjp3* in honeybee of European *Apis mellifera* subspecies was conducted. Differences in the frequency of alleles for the *mrjp3* locus were found in honeybees of different evolutionary branches. Allele "529" was found to be specific for the *Apis mellifera mellifera*, evolutionary branch M, while alleles "406", "518", and "485" were characteristic of the southern subspecies of bees (*A. m. carnica, A. m. carpatica* (some researchers consider this breed as a derivative of *A. m. carnica)*, and *A. m. caucasica*; evolutionary branches C and O, respectively). A high correspondence of the studied nucleotide sequences (≥99% identity) with the reference sequences (Genbank) was established indicating a high conservation of the repetitive region of the *mrjp3* gene in *A. mellifera* subspecies inhabiting different geographic regions of Europe and Siberia. Locus *mrjp3* is of considerable interest for further study as a candidate marker for differentiation of bee subspecies.

Keywords: microsatellite *mrjp3* locus, *Apis mellifera,* honeybee, royal jelly, subspecies, evolutionary branches

1. Introduction

The major royal jelly protein gene *mrjp3* is considered perspective for the evaluation of bee colonies in royal jelly productivity (Baitala et al., 2010; Parpinelli et al., 2014; Ruvolo-Takasusuki et al., 2016) and/or for differentiation of some honeybee subspecies (Ostroverkhova et al., 2018b). This *mrjp3* gene is a member of the *Mrjp*-subfamily that encodes Major Royal Jelly Proteins (MRJP) in bees of the genus *Apis* and some other Hymenoptera insects (the solitary, parasitoid jewel wasp *Nasonia vitripennis*, the alfalfa leafcutter bee *Megachile rotundata*, several bumblebees, and ants) (Albert et al., 1999a; Albert and Simúth, 2002; Albertová et al., 2005; Su et al., 2005; Kupke et al., 2012; Buttstedt et al., 2013). Proteins encoded by *mrjp* genes show a 20–30% identity at the amino acid level with YELLOW proteins of *Drosophila melanogaster*, indicating their common evolutionary origin. The *yellow* subfamily, a group of genes common throughout arthropods, regulates the processes of sex-specific reproductive maturation and behavior, pigmentation, and together with *mrjp* genes form the *mrjp*/*yellow* family (Drapeau et al., 2006; Ferguson et al., 2011; Buttstedt et al., 2014).

The *mrjp* genes are located in the honeybee chromosome 11 between the *yellow-e3* and *yellow-h* genes; they have a size of about 60 kb and include 9 genes (*mrjp1–mrjp9*) encoding proteins with approximately 60% identity to each other and 1 pseudogen *mrjp-Ψ* (the issue of the *mrjp-Ψ*, recently renamed into *mrjp2*-like, is currently under review) (Schmitzová et al., 1998; Albert et al., 1999a; Drapeau et al., 2006; HGSC, 2006; Helbing et al., 2017). It is believed that the MRJPs (*mrjp* subfamily of genes) most likely evolved precisely in connection with the function of the production of royal jelly (Albert et al., 1999a; Schmitzová et al., 1998).

Royal jelly is a secretion of the hypopharyngeal and mandibular glands of nurse bees and secreted between the 5th and 15th days of worker lifespan (Schmitzová et al., 1998; Imjongjirak et al., 2005). Royal jelly is a special food for larvae of workers and drones up to 3 days old, and for the queen larvae and queens throughout the life (up to 4–5 years). Besides water (60–70%), royal jelly consists of 10–16% sugar, 12–15% crude protein, 3–6% lipids, and traces of salts, free amino acids, and vitamins. Ninety percent (90%) of RJ proteins are MRJPs (Albertová et al., 2005; Schmitzová et al., 1998; Sabatini et al., 2009; Tamura et al., 2009; Ji et al., 2014). MRJPs have important functions for honeybee physiology in general and not just as the nutritional value for developing larvae. It is suggested that MRJPs also have other functions, such as caste and sexual differentiation (Buttstedt et al., 2013, 2014).

A characteristic feature of the *mrjp3* gene structure is the presence at the 3'-end of the repetitive region described as the region of hypervariable numbers of tandem repeats (VNTRs). This fragment is referred to as the microsatellite *mrjp3* locus, which encodes the C-terminal region of the MRJP3 protein represented by a 20-22 fold regularly repeating motif of five amino acid residues (N/K/R)QN(A/G/D)(G/D/N) (Albert et al., 1999b). In the honeybee *Apis melifera* L. from different populations (Europe, Brasilia), from seven to ten alleles of locus *mrjp3* with a size from 380 bp to 610 bp were identified (Albert et al., 1999b; Baitala et al., 2010; Parpinelli et al., 2014). Analysis of the nucleotide sequence of the repetitive region of five alleles showed the presence of two different segments in the structure of the *mrjp3* locus (Albert et al., 1999b).

Previously, we sequenced the most frequently registered alleles of the *mrjp3* microsatellite locus in bees of Siberian populations. A high level of identity between the sequences of the *mrjp3* locus in bees from different Siberian and European populations and in different subspecies of honeybees of the evolutionary branches M and C was noted (Ostroverkhova et al., 2018a). The goal of this work was to study the variability of the *mrjp3* microsatellite locus in honeybees of European *Apis mellifera* subspecies from different geographic regions, and to compare the nucleotide sequences of the *mrjp3* locus in European and Africanized honeybees (evolutionary branches M/C and A).

2. Materials and Methods

The following material is included in the study:

1) samples of a dark-colored forest bee *Apis mellifera mellifera* from Siberia (3 different populations – from the Tomsk Territory, the Krasnoyarsk Territory and the Altai Territory);
2) honeybees from the bee colonies of 3 southern subspecies, obtained from Europe: *Apis mellifera carnica* (from Germany), *Apis mellifera carpatica* (from Ukraine) and *A. m. caucasica* (from the Caucasus, Sochi, Russia).

2.1 Regions of the Honeybee Collections

Worker bees obtained from the dark-colored forest bee colonies living in the apiaries of the Siberian region were investigated: from the Tomsk region, Western Siberia; the Krasnoyarsk Territory, Eastern Siberia (Yenisei population); and the Altai (Figure 1).

In Siberia, the honeybee was introduced about 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. Originally, it was the dark-colored forest bee *Apis mellifera mellifera* L., 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 *Apis mellifera carnica* Poll. and the Carpathian race or *Apis mellifera carpatica* (a derivative of *A. m. carnica*) have been actively imported to Siberia. This importation process had become widespread and nearly uncontrolled by now.

At present, the original *A. m. mellifera* populations have been preserved in some areas of the Tomsk region, the Krasnoyarsk Territory, and the Altai. For example, the Yenisei population of the Krasnoyarsk Territory is a unique isolated population that existed for more than 60 years in a forest that lacks more recently imported honeybees. On the contrary, the Altai is a territory with well-developed beekeeping and closely located apiaries.

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. 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 temperature 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. The climate is sharply continental, where 70% of the territory is occupied by forests. Altai is

located in the southeast of Western Siberia. The intracontinental location and complex relief determines the essentially heterogeneous and contrasting climate of the Altai (from moderate to sharply continental).

Bees of the southern subspecies (*A. m. carnica*, *A. m. carpatica*, evolutionary branch C; *A. m. caucasica*, branch O) were obtained from bee farms of Germany, Ukraine, and the Caucasus (Russia), respectively.

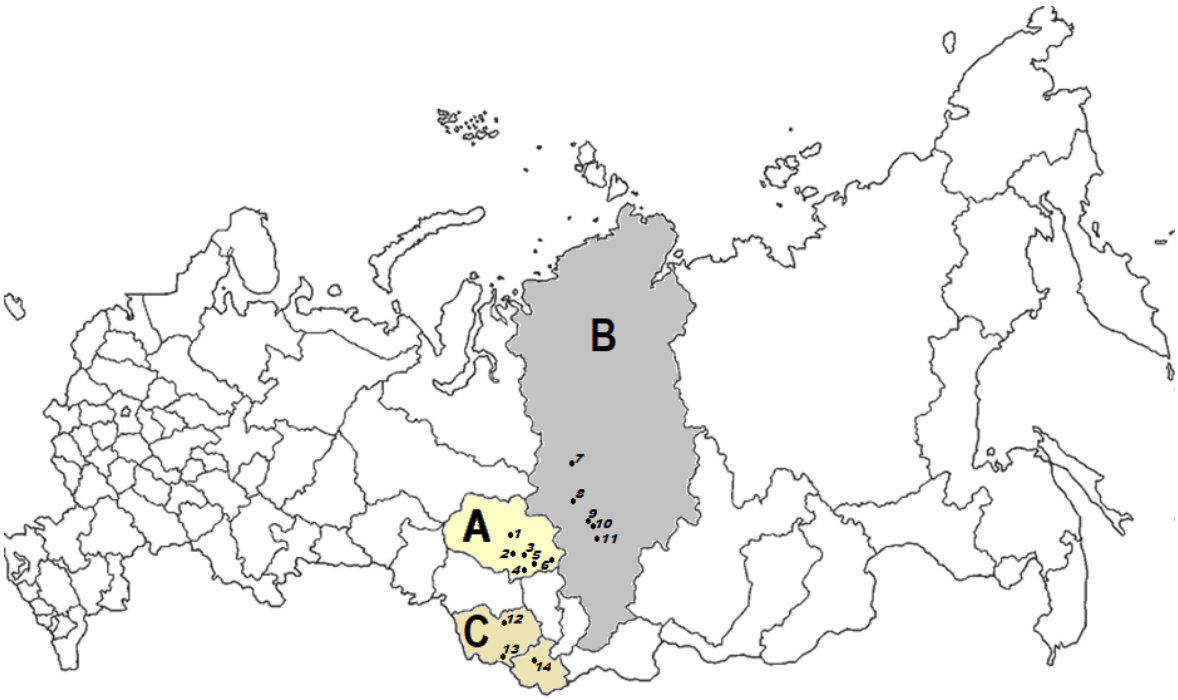

Figure 1. The map of localization of areas (dots A, B, and C) and apiaries (dots 1–14), studied in Siberia: A, the Tomsk region; B, the Krasnoyarsk Krai; C, the Altai. Apiaries where a dark forest bee is identified: *1*, s. Parabel; *2*, s. Podgornoe; *3*, s. Mogochino; *4*, s. Zarechny; *5*, s. Dubrovka; *6*, s. Teguldet; *7*, s. Turuchansk; *8*, s. Yartsevo; *9*, s. Kolmogorovo; *10*, s. Ostyatskoe; *11*, s. Ozernoe; *12*, c. Barnaul; *13*, c. Zmeinogorsk; *14*, s. Ongudai

2.2 Biological Samples

Collected honeybees from bee colonies were anesthetized on dry ice and stored in 96% ethanol until use. The genetic diversity of the *mrjp3* microsatellite locus was investigated in 1058 honeybees from 114 bee colonies of different origin: the dark-colored forest bee *A. m. mellifera*, evolutionary branch M; *A. m. carnica*, evolutionary branch C; *A. m. carpatica* (a derivative of *A. m. carnica*), evolutionary branch C; *A. m. caucasica*, evolutionary branch O. From five to fifteen individuals from each bee colony were examined: 561 bees (*A. m. mellifera*, evolutionary branch M) from Siberia, 129 bees of *A. m. carnica*, branch C, 145 bees of *A. m. carpatica*, branch C, and 166 bees of *A. m. caucasica*, branch O. Each bee colony was analyzed using the variability in the locus COI-COII of mtDNA and morphometric parameters of wing, including the cubital index, the hantel index, and the discoidal shift to determine the origin of the colony and its conformance to the breed standard (Ostroverkhova et al., 2015, 2016). Allelic frequencies and heterozygosity (expected and observed) with standard error were calculated using the POPGENE 1.31 software (Yeh et al., 1999).

We previously conducted the DNA sequencing of 12 samples of bees from different colonies (Ostroverkhova et al., 2018a). Seven samples were from bee colonies of the dark-colored forest bee *A. m. mellifera* from the Tomsk region and the Krasnoyarsk Krai (Yenisei population). Five samples were from bee colonies of the *A. m. carpatica* from Ukraine (Table 1). Bees having alleles of size 406 bp and 518 bp were obtained from bee colonies of the Carpathian breed (*A. m. carpatica*); bees with alleles of size 437 bp and 529 bp were the dark-colored forest bee (*A. m. mellifera*). (The sequences presented in Table 3 have been submitted to GenBank under accession numbers MH673344–MH673347).

Table 1. Geographical coordinates of apiaries in Siberia, where bees were collected for DNA sequencing

Region	Settlement	Geographical coordinates		
		Latitude	Longtitude	Altitude above sea level
Krasnoyarsk Krai	Kolmogorovo	59°16′06″	91°19′02″	60
(Yenisei population)	Ostyatskoe	59°11′12″	91°19′24″	63
Tomsk	Zarechnyi	56°39′03″	85°18′57″	146
region	Tomsk	56°29′51″	84°59′37″	128

Table 2. Parameters of the genetic diversity of the microsatellite locus *mrjp3* in different bee subspecies (evolutionary branches M, C, and O)

Parameter		European subspecies of honeybees*			
		A. m. mellifera	*A. m. carpatica*	*A. m. carnica*	*A. m. caucasica*
		M	C		O
		PQQ or PQQQ**	Q**		
		Siberia, Russia	Ukraine	Germany	Caucasus, Russia
Number of studied bees		561	145	129	166
Number of registered genotypes		20	21	25	17
Number of registered alleles		9	9	8	6
Allele (bp)	391	0.055±0.007	0.110±0.018	0.043±0.013	0
	406	0.009±0.003	**0.486±0.029**	**0.399±0.031**	0.015±0.007
	437	0.084±0.008	0.021±0.008	0	0
	464	0.021±0.004	0.097±0.017	**0.194±0.025**	0.081±0.015
	485	0.016±0.004	0.010±0.006	0.035±0.011	**0.313±0.026**
	495	0.005±0.002	0.003±0.003	0.027±0.010	0.145±0.019
	501	0.010±0.003	0.035±0.011	0.089±0.018	0.072±0.014
	518	0.013±0.003	**0.197±0.023**	**0.194±0.025**	**0.374±0.027**
	529	**0.787±0.012**	0.041±0.018	0.019±0.009	0
Ho		0.278±0.019	0.621±0.040	0.550±0.044	0.729±0.035
He		0.370±0.018	0.700±0.022	0.753±0.017	0.729±0.013

* The subspecies were determined by morphometric analysis and mtDNA analysis (for details see references by Ostroverkhova et al, 2015, 2016). **Variants of COI-COII locus mtDNA are presented. The allele frequency, which is more than 15%, is in bold. Ho – observed heterozygosity; He – expected heterozygosity. In the table the values of allele frequencies and parameters of heterozygosity with a standard error are given.

2.3 Experimental Procedures

DNA isolation and polymerase chain reaction (PCR) was carried out according to standard techniques. To amplify the microsatellite *mrjp3* locus, the following sequences of primers were used: *forward*-5′– ATG TAA TTT TGA AGA ATG AAC TTG; *reverse*-5′– TGT AGA TGA CTT AAT GAG AAA CAC (Albert et al., 1999b). Amplification products were fractionated in 1.5% agarose gel, and the results were documented with the use of Gel-Doc XR+. Amplification products were analyzed with ABI Prism 3730 Genetic Analyzer and GeneMapper Software (Applied Biosystems, Inc., Foster City, CA) in the collective center Medical Genomics (Research Institute of Medical Genetics, Tomsk National Research Medical Center, Russian Academy of Sciences). Two microlitres of PCR products were mixed with GeneScan500-ROX size standards (Applied Biosystems) and deionized formamide. Samples were run according to the manufacturer's recommendations.

A comparative analysis of the nucleotide sequences obtained in this study, and of the sequences presented in the GenBank (NCBI Reference Sequence, GenBank) and published in the articles (Albert et al., 1999; Pozza, 2011; Ruvolo-Takasusuki et al., 2016), was performed.

3. Results

3.1 Genetic Diversity of the mrjp3 Locus in Honeybees from Siberia

We studied variability of the *mrjp3* microsatellite locus in honeybees of different origin (different subspecies of bees). The spectrum and frequency of alleles of *mrjp3* locus were determined (Table 2) and nine alleles of the *mrjp3* locus with a size from 390 bp to 530 bp were identified. These results are consistent with the data of other scientific publications (Albert et al., 1999b).

Table 3. Nucleotide sequences of the repetitive regions of the *mrjp3* gene in bee subspecies of different evolutionary branches

Evolutionary branch M (*Apis m. mellifera*)		**Evolutionary branch C** (*Apis m. carnica, Apis m. carpatica*)	
allele "529", BankIt2134109 529 MH673346 (Siberia); XM_016917723.1	alleles "406", BankIt2134109 406 MH673344 (Siberia), Mrjp3a (Germany); GU434675.1; NM_001011601.1; Z26318.1	allele "518", BankIt2134109 518 MH673345 (Siberia); allele Mrjp3d (Germany); AY663104.1	alleles "437", BankIt2134109 437 MH673347 (Siberia); allele Mrjp3c (Germany)
TCGTTGCGGAAGATATCAC	*TCGTTGCGGAAGATATCAC*	*TCGTTGCGGAAGATATCAC*	*TCGTTGCGGAAGATATCAC*
(AATCAGAATGCTGGC)$_3$	AATCAGAATGCTGGC	(AATCAGAATGCTGGC)$_2$	(AATCAGAATGCTGGC)$_2$
(AATCAGAATG(***C/T***)TGAC)$_2$	(AATCAGAATGCTGAC)$_2$	(AATCAGAATG(***C/T/C***)TGAC)$_3$	(AATCAGAATG(***C/T***)TGAC)$_2$
AATCAGAATGCTAAC	AATCAGAATGCTAAC		AATCAGAATGCTAAC
AATCAGAATGCTGAT	AATCAGAATGCTGAT	AATCAGAATGCTGAT	AATCAGAATGCTGAT
AATCAGAATGCTAAC	AATCAGAATGCTAAC	AATCAGAATGCTAAC	(AATCAGAATGCTAA(***T/C***)) $_2$
AAACAAAATGGTAATAGACAAAATG***G***TA AC	AAACAAAATGGTAATAGACAAAAT GATAAC	AAACAAAATGGTAATAGACAAAATGATA AC	AAACAAAATGGTAATAGACAAAAT GATAAC
(AGACAGAATGATAACAAGCAAAAT(***G/A***) GTAA(***C/T***))$_5$	(AGACAGAATGATAACAAGCAAAAT GGTAAC)$_3$	(AGACAGAATGATAACAAGCAAAATGGT AA(***C/T***))$_5$	(AGACAGAATGATAACAAGCAAAAT GGTAAC)$_4$
AGACAAAATGGTAACAAACAGAATGATA AC	AGACAAAATGGTAACAAACAGAAT GATAAC	AGACAAAATGGTAACAAACAGAATGATA AC	AGACAAAATGGTAACAAACAGAAT GATAAC
(AAGCAAAATGGTAA(***T/C***)AGACA(***A/G***)AA TGATAAC)$_2$	AAGCAAAATGGTAACAGACAGAAT GATAAC	(AAGCAAAATGGTAA(***T/C***)AGACA(***A/G***)AA TGATAAC)$_2$	AAGCAAAATGGTAACAGACAGAAT GATAAC
AAGAGGAATGGTAACAGGCAAAATGAT***C AG***	AAGAGGAATGGTAACAGGCAAAAT GAT***AAT*** CAA	AAGAGGAATGGTAACAGGCAAAATGAT***A AC*** AATCAG	AAGAGGAATGGTAACAGGCAAAAT GAT***CAG***
AATAATCAGAATGATAATAATCGAAATGATAA T	*AATAATCAGAATGATAATAATCGAAATG ATAAT*	*AATAATCAGAATGATAATAATCGAAATGATAA T*	*AATAATCAGAATGATAATAATCGAAATG ATAAT*

Evolutionary branch A (Africanized bees)				
allele "G"	allele "E"	allele "C"	allele "D"	allele "F"
AATCAGAATGCTGGC	AATTCAAGAATTGCTTGGC	ATTATC	GCTGCTTCATTTAAATGTCAGTCTACACATA CATGATTATCTGATATC	
A–TCAGAATGCTGGC	AATCAGAATTGCTGAC	ATTTTGCCTGTTTACCATTC CTCTTGTTATC	ATTTTGCCTGT–ACCATTCCTCTTGT–ATC	CCTGTTACCATTCCTCTTGTT TATC
AATCAGAATGCTGGC	AATCAAAATGCTGAC	ATTCTGTCTGTTACCATTTT GCTTGTTATC	ATTCTGTCTGTTACCATTTTGCTTG–TATC	ATTCTGTCTGTTACCATTTTGC TTGTTATC
(AATCAGAATGCTGAC)$_2$	AATCAGAATTGTTGAC	ATTTTGTCTATTACCATTTTG CTTGTTATC	ATTT–GTCTATTACCATTTTGCTTG–TATC	ATTTTGTCTATTACCATTTTGC TTGGTTATC
AATCAGAATGCTAAC	(AATCAGAATTGCTAAC)$_2$	ATTCTGTTTGTTACCATTTTG TCTGTTACC	ATTCTGTT–GTTACCATTTTGTCTAGTTACC	ATTTCTGGGTTGGTTACCATTT GGTCTATTACC
AATCAGAATGCTGAT	AATCAAAATGCTGAT	(ATTTTGCTTGTTATCATTCT GTCTGTTACC)$_3$	(ATTTTGCTTGTTATCATTCTGTCTGTTACC)$_4$	(ATTTTGCTTGTTATCATTCTG TCTGTTACC)$_4$
AATCAGAATGCTAAC	AATCAGAATGCTAAC	ATTTTGCTTGTTATCATTCTG TCTGTTATC	ATTTTGCTTGTTATCATTCTTGTCTGTTATC	ATTTTGCTTGTTATCATTCTGT CTGTTATC
AAACAAAATGGTAATAGACA AAATGATAAC	CAAAACCAAAAAAATTGGGGTT AATTTAGGAACAAAATTGATAA C	ATTTTGTCTATTACCATTTTG TTTGTTAGC	ATTCTTGACTTTATTACCATTTTGATTGTTAG C	ATTTTGTCTATTACCATTTTGT TTGTTAGC
(A(***G/A***)ACAGAATGATAAC AAGCAAAATGGTAAC)$_2$	AGACATGAATGATAACAAGCAA AATGGTAAC	ATTCTGATTATCAGC	ATTCTGATTATCAGC	ATTCTGATTATCAGC
AGACAGGAATGATAACAAGC AAAATGGTAAC	AGACAGAATGATAACAAGCAAA ATTGGTAAC	ATTCTGATTGTTAGC		
(AGACAGAATGATAACAAGCA AAATGGTAAC)$_2$	(AGACAGAATGATAACAAGCAA AATGGTAA(***C/T***))$_3$	(ATTCTGATTGTCAGC)$_2$	(ATTCTGATTGTCAAGC)$_2$	ATTCTGATTGTCAGC
AGACAAAATGGTAAC AAACAGAATGATAAC	AGACAAAATGGTAAC AAACAGAATGATAAC		ATTCTGATTGTCAAC	(ATTCTGATTGTCA(***A/G***)C)$_2$
(AAGCAAAATG(***A/G***)TAA(***T/C***) AGACA(***A/G***)AATGATAAC)$_2$	(AAGCAAAATGGTAA(***T/C***) AGACA(***A/G***)AATGATAAC)$_2$	(ATTCTGATTGCCAGC)$_2$	ATTCTGATTGCCAGC	(ATTCTGATTGCCAGC)$_3$
AAGAGGAATGGTAACAGGCA AAATGATAAT CAA	AAGAGGAATGGTAACAGGCAAA ATGATAAT CAG	ATTCTGATTG***C***CA***GT***	ATTCTGATTGTCATA	ATTCTGATTGTGATA
AATAATCAGAATGATAAT AATCGAAATGATAAT	*AATAATCAGAATGATAAT AATCGAAATGATAAT*	CTCTTCCGGTTGCCGAGCTG TTCCTGCATT GTGATCACTTCCCGGAAGG GA	ATTCTCGATAAGCCA	TCTTCCGCAACGA

Note. Each line shows identical sequences in different alleles. The nucleotide substitutions in the sequence are in bold type, italics. Nucleotide insertions in the sequence are in bold type in square. Identical sequences at the beginning and at the end of the repeating region of the *mrjp3* gene of several alleles are in italics. A deletion of a nucleotide is dash. The first and second segments are separated by a dash.

To identify the genetic features of honeybees of different origin, the comparative analysis of the variability of the *mrjp3* locus was carried out for *A. m. mellifera* (branch M), *A. m. carpatica* (branch C), *A. m. carnica* (branch C), and *A. m. caucasica* (branch O). Predominant alleles in different subspecies were determined. In honeybees of different evolutionary branches, the allele spectrum of the *mrjp3* locus was similar, but the allele frequencies were different. Allele "529" is considered specific (predominant) for *A. m. mellifera* (frequency of allele registration P_{529}=0.79). This allele is registered in bees of southern origin (*A. m. carpatica*, *A. m. carnica*, and *A. m. caucasica*) with a low frequency (0.00–0.04). On the contrary, alleles "406" (P_{406}≥0.40) and "518" (P_{518}=0.20) are characteristic for bees of the branch C, alleles "485" (P_{485}>0.31) and "518" (P_{518}>0.37) are characteristic for bees of the branch O, but are not registered in the dark-colored forest bees of a unique isolated Yenisei population (Krasnoyarsk Krai) (Ostroverkhova et al., 2018b).

Observed heterozygosity differs from expected heterozygosity in bees of different evolutionary branches. The lower values of the observed heterozygosity in comparison with the expected heterozygosity are shown for most compared bee groups, which is consistent with data on the variability of nine microsatellite loci in honeybees from Siberia (Ostroverkhova et al., 2017); except bees of the Caucasian race, for which the observed heterozygosity is the highest and corresponding to the expected one. Probably, one of the reasons for these differences is the peculiar features of the reproductive biology of bees. However, despite polyandry, the effect of inbreeding can also be observed in connection with a higher probability of participation of drones of the same colony as the queen in the process of fertilization.

3.2 Structure of the mrjp3 Locus in Honeybees of Different Evolutionary Branches

To characterize the structure of the locus *mrjp3* in bees of different origins (evolutionary branches M, C, and A), a comparative analysis of the nucleotide sequences of the *mrjp3* locus was performed between the following bee groups (Table 3):

(1) Evolutionary branch M (the dark-colored forest bee, *A. m. mellifera*): allele "529" (Siberian population);

(2) Evolutionary branch C (*A. m. carnica*, *A. m. carpatica*): reference sequences AY663104.1 (*Apis mellifera carnica* major royal jelly protein 3 (*mrjp3*) gene, complete cds); reference sequences Z26318.1 of *A. m. carnica* (*A. mellifera* mRNA for royal jelly protein (RJP57-1)); alleles *Mrjp3a*, *Mrjp3b*, *Mrjp3c*, *Mrjp3d*, and *Mrjp3e* of *A. m. carnica* (Germany) (Albert et al., 1999b); alleles "406" and "518" of bees of the Carpathian race *A. m. carpatica* from Siberia.

Two reference sequences, GU434675.1 (*Apis mellifera* major royal jelly protein mRNA, complete cds) and NM_001011601.1 (*Apis mellifera* major royal jelly protein 3 (Mrjp3), mRNA) for which the honeybee subspecies is not indicated in Genbank, were attributed to this group conditionally because they showed a 100% identity with the Mrjp3a allele identified in *A. m. carnica.*

(3) Hybrids (BeeWeaver, DH4 strain): reference sequence XM_016917723.1 (PREDICTED: *Apis mellifera* major royal jelly protein 3-like (LOC727045), partial mRNA). The BeeWeaver breed is a hybrid of the Italian and Buckfast breeds (HGSC, 2006). The Buckfast contains heritage from mainly *A. m. ligustica* (North Italy) and *A. m. mellifera* (English, French) and from other subspecies (*A. m. anatoliaca* (Turkish) and *A. m. cecropia* (Greek), two rare and docile African stocks *A. m. sahariensis* and the *A. m. monticola*, but not the "Africanized" *A. m. scutellata*).

(4) Africanized honeybees: alleles C, D, E, F and G (Brazilian population) (Baitala et al., 2010; Pozza, 2011; Parpinelli et al., 2014; Ruvolo-Takasusuki et al., 2016). The Africanized honeybee is a predominantly African polyhybrid resulting from a mixture of European subspecies already present in Brazil (*A. m. mellifera*, *A. m. ligustica*, *A. m. caucasica*, and *A. m. carnica*) and the African bee *A. m. scutellata* (Francoy et al., 2009). Africanized bees were assigned the same phylogenetic branch as the African subspecies (evolutionary branch A) (Arias and Sheppard, 1996).

Initially, the nucleotide sequences of the *mrjp3* locus were compared to European bee subspecies: the dark-colored forest bee *A. m. mellifera* (evolutionary branch M) and bees of southern origin *A. m. carnica* and *A. m. carpatica* (evolutionary branch C), obtained from different populations of Siberia and Europe. Then the nucleotide sequences of the *mrjp3* locus were compared between European subspecies (branches M and C) and Africanized bees (branch A).

3.3 Evolutionary Branches M and C

In the structure of the repetitive region of all compared sequences, two different segments are clearly distinguished. According to the study of the *mrjp3* locus in the honeybee *A. m. carnica* (Albert et al., 1999b), both segments in the repetitive region originated from a common ancestral sequence. The first segment located at the 5′-end of the

gene is more conserved and consists of 6–8 copies of pentadecanucleotide motif AATCAGAATGCT(A/G)A(C/T), where 13 nucleotides are conserved to 100%. The protein, respectively, contains a motif of five amino acid residues – NQNA(D/N/G). In the first segment of the sequence of the locus *mrjp3* of honeybees from Siberia, an additional replacement of the nucleotide in the 11th position (C→T) in all studied alleles, except for allele "406," was shown. This nucleotide substitution results in the replacement of the amino acid alanine with valine (A→V) in the protein sequence (Table 3).

The second segment is less conserved and includes 5 to 20 copies of the 15-nucleotide motif A(A/G)(A/G)CA(A/G)AATG(A/G)TAA(C/T); the protein, respectively, is represented (K/R)QN(D/G)N pentapeptides (Albert et al., 1999b). In bees from Siberia, the second segment includes 14–20 copies of the 15-nucleotide motif, and the single nucleotide substitution G→A was detected in allele "529," leading to a change in the protein sequence (glycine to serine) – (K/R)QN(D/G/S)N (Table 3). The nucleotide sequence of the second segment determines the specificity of the encoded amino acid sequence. Thus, a characteristic feature of the second segment is that the first amino acid of each motif begins with a positively charged lysine or arginine; the last amino acid is asparagine. But if in the first segment the last (fifth) amino acid is variable, then in the second segment, on the contrary, the fifth amino acid is a constant element in the protein. In both segments, the invariant glutamine-asparagine (QN) motif is conserved (Albert et al., 1999b). Thus, the data on the Siberian population are consistent with the results of the sequencing of alleles in the bees of European populations.

It is of interest to consider the second segment not as a 15-nucleotide but as a 30-nucleotide motif; the motifs coincide in all alleles (Table 4). In this case, the sequence of the second segment is as follows: A–$(B)_{3–5}$–C–$(B')_{1–2}$–D. The motifs B and B′, repeated most often, retain the nucleotide sequence of 15-nucleotide structural fragments, but differ from each other in the order of their location: the first 15 nucleotide sequence of motif B corresponds to the 15 nucleotide sequence at the end of motif B' and, conversely, the last 15 nucleotide sequence of motif B corresponds to the 15 nucleotide sequence at the beginning of motif B'. Motifs B and B' are flanked on both sides by different 30-nucleotide sequences (A, C, and D) that are identical in all alleles. The last 30-nucleotide motif (D) differs between alleles with the last three nucleotides (CAG, AAT or AAC). It is supposed that both segments in the repetitive region originated from a common ancestral 15-nucleotide sequence (Albert et al., 1999b). However, it can then be assumed that the second segment could evolve independently by duplicating the 30-nucleotide motif.

The repetitive region ends with a 33-nucleotide sequence that is identical in all alleles. In some cases, the repetitive region and this 33-nucleotide sequence are separated by short fragments of different lengths, but always are represented by complete triplets (for example, AATCAG or CAA). Perhaps this indicates a selection against the shift of the reading frame and the functional significance of the conserved sequence behind the repetitive region of the *mrjp3* locus.

Thus, a high level of identity between the sequences of the *mrjp3* locus in different European subspecies of honeybees (*A. m. mellifera*, branch M; *A. m. carnica* and *A. m. carpatica*, branch C) was detected. This may indicate a high level of conservatism and the functional significance of the *mrjp3* gene.

3.4 Evolutionary Branch A

Another picture is observed when comparing the nucleotide sequences of the repetitive region of the *mrjp3* gene between European bee subspecies (M and C branches) and Africanized bees (branch A): significant differences in the structure of the microsatellite locus *mrjp3* were revealed.

Of the five investigated alleles of Africanized bees (C, D, E, F, and G), alleles E and G had a repetitive region structure similar to the nucleotide sequence of allele "529" specific for *A. m. mellifera* (Siberia) and reference sequence XM_016917723.1 (from BeeWeaver), while three alleles C, D, and F were characterized by another structure (Table 3). For allele G only the deletion of one nucleotide in the first segment and the insertion of the nucleotide in the second segment were detected and that, however, leads to the shift of the reading frame in a small part of the repetitive region of the *mrjp3* gene. Allele E is characterized by high identity of only the second segment, with a large number of single and other insertions of nucleotides, especially in the sequence between the first and second segments.

The alleles C, D, and F (branch A) are characterized, on the one hand, by an inverted repeat structure (first the second segment, then the more conservative first segment is located in these alleles), and on the other hand, by the other nucleotide composition of the repeats of both the first and second segments in comparison with the alleles specific for European bee subspecies (branches M and C) (Table 3).

4. Discussion

The genetic diversity of the microsatellite locus *mrjp3* in bees of different origins and different geographic locations has been analyzed in very few studies (Albert et al., 1999b; Baitala et al., 2010; Parpinelli et al., 2014). For the first time, we studied the variability of the *mrjp3* locus in bees of three evolutionary branches (M, C, and O). We analyzed the bee colonies, which have been living in Siberia for a long time, as well as newly imported bee colonies. Nine alleles of the *mrjp3* locus with a size from 390 bp to 530 bp was identified in honeybees of three evolutionary branches (M, C, and O). Probably, an allele of 529 bp is more characteristic of evolutionary branch M (*A. m. mellifera*), whereas other alleles ("406", "518", and "485") are more specific for southern subspecies of bees (evolutionary branches C and O). For example, alleles "406" and "518" are characteristic of *A. m. carnica* and *A. m. carpatica* (branch C); alleles "485" and "518" are characteristic of *A. m. caucasica* (branch O).

At the same time, similarities in the spectrum and size of alleles are shown for bees of different populations (Russia, Germany, Ukraine). To investigate the specificity of alleles of the *mrjp3* locus in honeybees of different geographic locations, a comparative analysis of the nucleotide sequences of the *mrjp3* repetitive region in honeybees from different populations (Siberia, Europe, Brazil) was carried out.

A high correspondence of nucleotide sequences of the microsatellite locus *mrjp3* in the bees of European and Siberian populations was established, which indicates a high conservation of the repetitive region of the *mrjp3* gene in *A. mellifera* subspecies inhabiting different geographic regions. Only Africanized bees differ from European honeybee subspecies in structure of the *mrjp3* repetitive region.

In connection with the considerable differences in the sequence and structure of the repetitive region of the *mrjp3* gene in European bee subspecies and Africanized bees, it can be assumed that the evolution of the *mrjp3* repetitive region in honeybees was in two independent directions: the evolutionary branches M and C (European bee subspecies) and the African branch A. Furthermore, for European bee subspecies *A. mellifera* (branches M and C) and other *Apis* species (among 20 sequenced alleles), it is noted that despite many nucleotide substitutions leading to amino acid substitutions and deletions, the translational reading frame of *mrjp3* repeat never shifts; no frameshift or precocious STOP codon could be detected. These data support the earlier assumption that there is a selection pressure that eliminates frameshift or termination mutations (Albertová et al., 2005). However, for alleles of Africanized bees, a large number of nucleotide insertions and deletions are shown, and the number of rearrangements is not a multiple of three, the shift of the reading frame occurs.

Surprisingly, the same nucleotide sequence of the repetitive region having the maximum length was detected in bees of different origins and geographic locations: the dark-colored forest bee *A. m. mellifera* (Siberia) and hybrids that have passed a long selection (BeeWeaver and Africanized bees) but have the genetic material of a dark-colored forest bee in their gene pool. These results are consistent with the hypothesis that the repetitive regions in MRJPs contain high amounts of nitrogen-rich amino acids. Their presence significantly increases the nitrogen content of the MRJP proteins. It seems therefore that the repetitive regions are domains storing nitrogen in a biologically processable form (Albert et al., 1999b). In connection with the fact that a correlation between nitrogen content and repeat length was documented in the MRJPs, it can be assumed that the repeat occurred due to selection for an increase in nitrogen storage that results in more efficient nutrition for queens and larvae (Albertová et al., 2005). However, for Africanized bees (if we consider the alleles C, D, and F that are characteristic for evolutionary branch A), amino acids in the protein are not nitrogen-rich.

It can be assumed that nitrogen storage is not the only function of the MRJP3 and other MRJP repeats (at least not for Africanized bees). This supports the assumption that the repetitive regions in MRJPs have other signaling and/or regulatory functions (Albertová et al., 2005). Therefore, further studies of the structure and variability of the repetitive region of the gene *mrjp3* in bees of different origins and different geographic location, as well as analysis of associations of polymorphic variants with economically significant features, may elucidate the biological role of this gene and the MRJP family as a whole.

5. Conclusion

The study of variability, sequence, and structure of the repetitive region of the major royal jelly protein gene *mrjp3* in honeybee *A. mellifera* of different evolutionary branches showed that the European subspecies of the honeybee (evolutionary branches of M and C) are characterized by the conservatism of the locus *mrjp3*. At the same time, the structure of this locus is significantly different in Africanized bees.

Thus, although the *A. mellifera* MRJP family has been extensively studied, little was known about the variability of the *mrjp* family genes in subspecies bees from different geographic location, and the functional significance of

the genes of the *mrjp* family and their role in the formation of individual traits in bees (for example, those that may be economically significant, such as royal jelly production). New knowledge can contribute to the ability to use these molecular markers in population genetic studies and/or as selective markers associated with royal jelly production.

Acknowledgments

We thank Melissa Wade for preliminary editing of the manuscript.

References

Albert, S., Bhattacharya, D., Klaudiny, J., Schmitzová, J., & Simúth, J. (1999a). The family of major royal jelly proteins and its evolution. *Journal of Molecular Evolution*, *49*(2), 290–297.

Albert, S., Klaudiny, J., & Simúth, J. (1999b). Molecular characterization of MRJP3, highly polymorphic protein of honeybee (*Apis mellifera*) royal jelly. *Insect Biochemistry and Molecular Biology*, *29*(5), 427–434.

Albert, S., & Simúth, J. (2002). Characterization of major royal jelly protein-like DNA sequences in *Apis dorsata*. *Journal of Apicultural Research*, *41*(3–4), 75–82. https://doi.org/10.1080/00218839.2002.11101072

Albertová, V., Su, S., Brockmann, A., Gadau, L., & Albert, S. (2005). Organization and potential function of the *mrjp3* locus in four honeybee species. *Journal of Agricultural and Food Chemistry, 53*(20), 8075–8081. https://doi.org/10.1021/jf051417x

Arias, M. C., & Sheppard, W. S. (1996). Molecular phylogenetics of honey bee subspecies (*Apis mellifera* L.) inferred from mitochondrial DNA sequence. *Molecular Phylogenetics and Evolution, 5(*3), 557–566. doi:10.1006/mpev.1996.0050

Baitala, T. V., Faquinello, P., Toledo, V. A. A., Mangolin, C. A., Martins, E. N., & Ruvolo-Takasusuki, M. C. C. (2010). Potential use of major royal jelly proteins (MRJPs) as molecular markers for royal jelly production in Africanized honeybee colonies. *Apidologie, 41*, 160–168. https://doi.org/10.1051/apido/2009069

Buttstedt, A., Moritz, R. F., & Erler, S. (2013). More than royal food – Major royal jelly protein genes in sexuals and workers of the honeybee *Apis mellifera*. *Frontiers in Zoology, 10*, 72. https://doi.org/10.1186/1742-9994-10-72

Buttstedt, A., Moritz, R. F., & Erler, S. (2014). Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gene family. *Biological Reviews, 89*(2), 255–269. https://doi.org/10.1111/brv.12052

Drapeau, M. D., Albert, S., Kucharski, R., Prusko, C., & Maleszka, R. (2006). Evolution of the Yellow/Major royal jelly protein family and the emergence of social behavior in honey bees. *Genome Research, 16*, 1385–1394. https://doi.org/10.1101/gr.5012006

Ferguson, L. C., Green, J., Surridge, A., & Jiggins, C. D. (2011). Evolution of the insect yellow gene family. *Molecular Biology and Evolution, 28*, 257–272. https://doi.org/10.1093/molbev/msq192

Francoy, T. M., Wittmann, D., Steinhage, V., Drauschke, M., Müller, S., Cunha, D. R., … Gonçalves, L. S. (2009). Morphometric and genetic changes in a population of *Apis mellifera* after 34 years of Africanization. *Genetics and Molecular Research, 8*(2), 709–717.

Helbing, S., Lattorff, H. M. G., Moritz, R. F. A., & Buttstedt, A. (2017). Comparative analyses of the major royal jelly protein gene cluster in three *Apis* species with long amplicon sequencing. *DNA Research, 24*(3), 279–287. https://doi.org/10.1093/dnares/dsw064

Honeybee Genome Sequencing Consortium. (2006). Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature, 443*, 931–949. https://doi.org/10.1038/nature05260

Imjongjirak, C., Klinbunga, S., & Sittipraneed, S. (2005). Cloning, expression and genomic organization of genes encoding Major Royal Jelly Protein 1 and 2 of the honey bee *(Apis cerana*). *Journal of Biochemistry and Molecular Biology, 38*(1), 49–57.

Ji, T., Liu, Z., Shen, J., Shen, F., Liang, Q., Wu, L., Chen, G., & Corona, M. (2014). Proteomics analysis reveals protein expression differences for hypopharyngeal gland activity in the honeybee, *Apis mellifera carnica* Pollmann. *BMC Genomics*, *15*, 665. https://doi.org/10.1186/1471-2164-15-665

Kupke, J., Spaethe, J., Mueller, M. J., Rössler, W., & Albert, S. (2012). Molecular and biochemical characterization of the major royal jelly protein in bumblebees suggest a non-nutritive function. *Insect Biochemistry and Molecular Biology*, *42*(9), 647–654. https://doi.org/10.1016/j.ibmb.2012.05.003

Ostroverkhova, N. V., Konusova, O. L., Kucher, A. N., Kireeva, T. N., Vorotov, A. A., & Belikh, E. A. (2015). Genetic diversity of the locus COI-COII of mitochondrial DNA in honeybee populations (*Apis mellifera* L.) from the Tomsk region. *Russian Journal of Genetics*, *51*(1), 80–90. https://doi.org/10.1134/S1022795415 01010X

Ostroverkhova, N. V., Konusova, O. L., Kucher, A. N., & Sharakhov, I. V. (2016). A Comprehensive characterization of the honeybees in Siberia (Russia). In E. Dechechi Chambo (Ed.), *Beekeeping and Bee Conservation – Advances in Research. InTech, Croatia* (pp. 1–37). https://doi.org/10.5772/62395

Ostroverkhova, N. V., Kucher, A. N., Konusova, O. L., Kireeva, T. N., & Sharakhov, I. V. (2017). Genetic diversity of honeybees in different geographical regions of Siberia. *International Journal of Environmental Studies*, *74*(5), 771–781. https://doi.org/10.1080/00207233.2017.1283945

Ostroverkhova, N. V., Kucher, A. N., Babushkina, N. P., & Konusova, O. L. (2018a). Sequence of the *mrjp3* Microsatellite Locus in Honeybees of Different Origin. *Russian Journal of Genetics*, *54*(3), 322–327. https://doi.org/10.1134/S1022795418030109

Ostroverkhova, N. V., Kucher, A. N., Konusova, O. L., & Sharakhov, I. V. (2018b). The *mrjp3* microsatellite marker: determination of honeybee subspecies or/and royal jelly productivity of bee colony. *Far Eastern Entomologist, 353*, 322–327. https://doi.org/10.25221/fee.353.3

Parpinelli, R. S., Ruvolo-Takasusuki, M. C. C., & Toledo, V. A. A. (2014). MRJP microsatellite markers in Africanized *Apis mellifera* colonies selected on the basis of royal jelly production. *Genetics and Molecular Research*, *13*(3), 6724–6733. https://doi.org/10.4238/2014.August.28.16.

Ruvolo-Takasusuki, M. C. C., Pozza, A. P. B. C., Oliveira, A. P. N. Z., Parpinelli, R. S., Costa-Maia, F. M., Faquinello, P., & Toledo, V. A. A. (2016). Improvement and selection of honeybees assisted by molecular markers. In E. Dechechi Chambo (Ed.), *Beekeeping and Bee Conservation – Advances in Research. InTech, Croatia* (pp. 63–75). https://doi.org/10.5772/62426

Pozza, A. P. B. C. (2011) Sequenciamento dos genes *Mrjp3*, *Mrjp5* e *Mrjp8* de abelhas *Apis mellifera* Africanizadas. MARINGÁ, Estado do Paraná Novembro, 61 p.

Sabatini, A., Marcazzan, G., Caboni, M., Bogdanov, S., & Almeida-Muradian, L. (2009). Quality and standardisation of Royal Jelly. *Journal of ApiProduct and ApiMedical Science, 1*(1), 1–6. https://doi.org/10. 3896/IBRA.4.1.01.04

Schmitzová, J., Klaudiny, J., Albert, S., Schröder, W., Schreckengost, W., Hanes, J., Júdová, J., & Simúth, J. (1998). A family of major royal jelly proteins of the honeybee *Apis mellifera* L. *Cellular and Molecular Life Sciences*, *54*(9), 1020–1030. https://doi.org/10.1007/s000180050229

Su, S., Albert, S., Chen, S., & Zhong, B. (2005). Molecular cloning and analysis of four cDNAs from the heads of *Apis cerana cerana* nurse honeybees coding for major royal jelly proteins. *Apidologie*, *36*, 389–401. https://doi.org/10.1051/apido:2005026

Tamura, S., Kono, T., Harada, C., Yamaguchi, K., & Moriyama, T. (2009). Estimation and characterisation of major royal jelly proteins obtained from the honeybee *Apis mellifera*. *Food Chemistry*, *114*, 1491–1497. https://doi.org/10.1016/j.foodchem.2008.11.058

Yeh, F., Yang, R., & Boyle, T. (1999). POPGENE Microsoft Windows-Based Freeware for Population Genetic Analysis Release 1.31. Alberta, Canada University of Alberta.