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ANIMAL GENETICS

New SNP Markers of the Honeybee Vitellogenin Gene (*Vg*) Used for Diagnostics of Subspecies *Apis mellifera mellifera* L. in Russia

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Abstract—Preservation of the gene pool of honeybee subspecies *Apis mellifera mellifera* is of vital importance for successful beekeeping development in the northern regions of Eurasia. An effective method of genotyping honeybee colonies used in modern science is the mapping of sites of single nucleotide polymorphism (SNP). The honeybee vitellogenin gene (*Vg*) encodes a protein that affects reproductive function, behavior, immunity, longevity, and social organization in the honeybee *Apis mellifera* and is therefore a topical research subject. The results of comparative analysis of honeybee *Vg* sequences show that there are 26 SNP sites that differentiate M and C evolutionary branches and can be used as markers in selective breeding, DNA-barcoding, and the creation of genetic passports for *A.m.mellifera* colonies.

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INTRODUCTION

In a long-term evolutionary process, the species *Apis mellifera* was divided into 30 subspecies geographically isolated within their natural habitat [1]. This divergence was not attended by genetic isolation; i.e., hybridization occurs between subspecies populations along the edges of habitats. Beekeeping contributed to this process via the transportation of certain subspecies to the habitats of other subspecies. Hybrid honeybees are unsuitable for selective breeding for biological and economic traits, since controlling the crossbreeding is a difficult task: each queen bee can mate on the wing with more than 12 drones. Artificial insemination cannot settle this problem, since worker bees usually do not accept an inseminated queen bee and replace it by a proper newly bred one. On the other hand, artificial insemination can negatively affect a queen's health and egg quality: some eggs remain unfertilized, and this can lead to an increase in the number of drones raised from those eggs.

Beekeeping is thought to be successful only in the case of breeding one subspecies within each region. Western and northern Eurasia is the native habitat of *Apis mellifera mellifera*. This subspecies, derived from the evolutionary branch M, is extremely important for beekeeping in northern regions because of its high level of adaptation to a strong continental climate with long cold winters [2].

At present, as a result of human economic activities, populations of this northern subspecies remain in small numbers in Russia, Switzerland, Denmark, Sweden, Norway, France, and Spain [3, 4]. Genetic monitoring of exported and imported bee colonies and gene pool purity preservation are therefore indispensable for successful selective breeding and reproduction of *A.m.mellifera*. Under conditions of intense hybridization, subspecies diagnostics methods based on characteristics of chitinous body parts, microsatellite polymorphism, and intergenic COI-COII mtDNA locus structure [5] are not usable. In modern techniques of selective breeding and systematics in beekeeping, the use of SNP markers has proved to be effective [6]. There were found 1136 [6], or, according to other authors [7], 1183, SNP sites scattered over the honeybee genome. They are used for subspecies identification, estimation of the introgression rate, and bee breeding all over the world.

To detect SNPs that differentiate *A.m.mellifera* from branch C, the authors chose the *Vg* gene, which encodes vitellogenin, the major precursor of honeybee yolk protein, a high-density monomeric phospholipoglycoprotein with Mw of 180 kDa [8–10]. It was found previously that *Vg* has a pleiotropic effect, leading to the appearance of various phenotypic traits in queens and worker honeybees [11]. The blood concentration of vitellogenin in queens was proved to correlate positively with the egg production rate [12]. Vitellogenin also plays an important role in the caste differentiation process [13–14]. In honeybees, vitellogenin is produced by fat bodies, released into hemolymph, and cumulates in oocytes as a nutritional supply for the embryo [10].

The honeybee genome contains only one copy of *Vg*, while some insect species have several copies [15].

Table 1. PCR primers of the *Vg* gene exons in the honeybee *Apis mellifera* [15]

Exon	Primer
2	F 5'-tcttgttcgttccaggttcc-3'
	R 5'-gacagtttcagccgacttcc-3'
3	F 5'-cctttcgatccattccttga-3'
	R 5'-gtcaaaacggattggtgctt-3'
4	F 5'-tcgaaggggaagaatttcaa-3'
	R 5'-acgagcaattcctcaacacc-3'
5	F 5'-gtcggacaatttcacgtcct-3'
	R 5'-gttcgagcatcgacacttca-3'
6	F 5'-agagccagggatacgtcaaa-3'
	R 5'-gagtcatctcgaggctcacc-3'
7	F 5'-ttctggctgaggtcaggatt-3'
	R 5'-aatttcgaccacgactcgac-3'

The honeybee *Vg* gene consists of seven exones; the sequences of six of them are published in GenBank database [16].

The purpose of the work at hand, based on comparative analysis of the honeybee *Vg* gene, was to find new, 1
previously unknown SNPs tht can be used in beekeeping as genetic markers for differentiating evolutionary branches M and C.

MATERIALS AND METHODS

Twelve worker bees were selected from apiaries located in the natural habitat of *A.m.mellifera*: the settlements of Kagarmanovo, Kaga, and Sermenevo of Beloretskii district, the settlements of Galiakberovo, Yaumbaevo, and Irgizly of Burzyanskii district, the settlements of Kustarevka, Sabanchi, and Uyadybash of Tatyshlinskii district, Bashkortostan; the settlement of Nytva of Nytvinskii district; and two apiaries in the settlement of Porshakova of Krasnovisherskii region and Perm krai. The intergenic COI-COII mtDNA locus structure and allele spectra of nine microsatellite loc—Ap243, 4a110, A24, A8, A43, A113, A88, Ap049, and A28 [2, 3, 5]—were investigated in order to prove the assignment of samples to the studied subspecies.

DNA was extracted from the tissue of honeybee pectoral flight muscles using a DNA-EXTRAN-2 extraction kit (Syntol) [17].

Polymerase chain reaction (PCR) was performed using Amplifier Terzik MC2 in a 15 μl volume mixture containing 1 U Taq-DNA polymerase, 2–4 mM $MgSO_4$, 200 μM of each dNTP, 0.5 μM of each primer, and 20–100 ng DNA. For PCR and sequencing of six *Vg* exons (from exon 2 to exon 7), oligonucleotide primers were used (Table 1). The first *Vg* exon was not analyzed, since there are no data on this exon in GenBank. Purification of the amplificates and sequencing were performed in the APPLIED BYOSYSTEMS automatic sequencer (Syntol).

The obtained sequences of the *Vg* gene were analyzed by the program MEGA 4.1. Sequences were aligned with reference to the *Vg* gene sequences of *A.m.mellifera* published in GenBank (LG4; NC_007073.3 (4020743-4026919); AADG 06005159.1 (56573-62749)); the first nucleotide of the start codon of the reference sequence corresponded to position 4020743 of the fourth chromosome LG4 of the honeybee [18].

RESULTS

The sequences of six *Vg* gene exons obtained for honeybees of the Ural region were submitted to the GenBank database under the following accession numbers: exon 2 (KJ572309 – KJ572320), exon 3 (KJ645883 – 645894), exon 4 (KJ572297 – KJ572308), exon 5 (KJ572285 – KJ572296), exon 6 (KJ532136 – KJ532147), exon 7 (KJ532124 – KJ532135). All worker bees in the study were homozygous for the *Vg* gene. The total number of submitted sequences of 6 *Vg* gene exons is 72.

The GenBank currently contains data on *Vg* gene exons 2–7 for 19 African samples of honeybees (branch A), ten samples from eastern Europe (branch C), 12 samples from western Europe [15], and 12 samples from the Ural region studied in the work at hand (branch M).

Comparative analysis of the obtained and reference *Vg* sequences showed the following SNPs: transition (substitution of a purine for another purine, or of a pyrimidine for another pyrimidine) and transversion 2
(substitution of a purine for a pyrimidine or vice versa). In exon 2 two transitions were found; in exon 3, three transitions were found; in exon 4, two transitions were found; in exon 5, five transitions and two transversions were found; in exon 6, one transition (the only nonsynonymous one) was found; in exon 7, six transitions (Table 2) were found.

The total number of SNPs found in the compared sequences is 20, of which 18 substitutions were transitions (90%) and 2 were transversions (10%). Both of transversions of *Vg* exon 5 in positions 4528 and 4533 were nonsynonymous and resulted in amino acid substitutions of Ley for Ile and of Arg for Ser, respectively. One of 18 transitions located in exon 6 position 5229 was nonsynonymous (6%) and resulted in amino acid substitution of Ala for Thr.

In the *Vg* gene exon 2 of a DNA sample obtained from a bee in the settlement of Uyadybash of Tatyschlinskii region, a deletion of 9 nucleotides was found

Table 2. Base substitution sites of the *Vg* gene in honeybees of the Ural region, compared with the reference sequences submitted to GenBank

Samples	Exon 2		Exon 3			Exon 4		Exon 5		
	substitution sites									
	526	574	1373	1418	1793	2443	2458	3978	4528	4533
Reference sequence Vg GenBank	A	A	T	A	T	T	T	C	C	G
Bashkortostan										
Beloretskii district, Kagarmanovo	G	G	C	A	T	C	C	C	C	G
Beloretskii district, Kaga	G	G	C	A	T	T	T	C	C	G
Beloretskii district, Sermenevo	G	G	C	A	T	C	T	C	C	G
Burzyanskii dictrict, Galiakberovo	G	G	C	G	T	T	T	T	A*	G
Burzyanskii dictrict, Yaumbaevo	G	G	C	A	C	C	C	C	A*	C**
Burzyanskii dictrict, Irgizly	G	G	C	A	T	T	T	C	C	G
Tatyshlinskii district, Kustarevka	A	A	C	A	T	T	T	C	C	G
Tatyshlinskii district, Sabanchi	G	G	C	A	T	T	T	C	C	G
Tatyshlinskii district, Uyadybash	G	G	C	A	T	T	T	C	C	G
Perm krai										
Krasnovisherskii district, Porshakova	A	G	C	A	T	T	T	C	C	G
Nytvinskii district, Nytva	G	G	C	A	T	T	T	C	A*	G
Krasnovisherskii district, Porshakova	G	G	C	A	T	C	C	C	A*	C**

Samples	Exon 5				Exon 6	Exon 7					
	substitution sites										
	4554	4555	4800	4812	5229	5608	5677	5680	5692	5878	5935
Reference sequence Vg GenBank	A	T	G	A	G	T	T	C	C	T	T
Bashkortostan											
Beloretskii district, Kagarmanovo	A	T	A	A	G	T	T	T	T	T	T
Beloretskii district, Kaga	A	T	G	A	A***	–	C	T	T	T	C
Beloretskii district, Sermenevo	A	T	G	G	A***	C	C	T	T	T	C
Burzyanskii dictrict, Galiakberovo	A	T	A	A	A***	C	C	T	T	C	T
Burzyanskii dictrict, Yaumbaevo	G	C	G	A	A***	C	C	T	T	C	T
Burzyanskii dictrict, Irgizly	A	T	A	A	G	C	C	T	T	T	C
Tatyshlinskii district, Kustarevka	A	T	G	A	A***	C	C	T	T	C	T
Tatyshlinskii district, Sabanchi	A	T	G	G	A***	C	T	C	C	T	C
Tatyshlinskii district, Uyadybash	A	T	A	G	A***	C	C	T	T	T	C
Perm krai											
Krasnovisherskii district, Porshakova	A	T	G	A	A***	C	C	T	T	T	T
Nytvinskii district, Nytva	A	T	G	A	A***	C	C	T	T	C	T
Krasnovisherskii district, Porshakova	G	C	G	A	A***	C	C	T	T	C	T

* Substitution of Ley for Ile; ** substitution of Arg for Ser; *** substitution of Ala for Thr. The symbol "–" indicates that there is no data on this site because of the inadequate length of the *Vg* sequence obtained.

in the position 794—802; it did not lead to a reading frame shift and amino acid substitution but shortened the sequence by three amino acids. The same deletion in *Vg* exon 2 was observed previously for samples of isolates from Egypt, which were submitted to the GenBank database under the following accession numbers: JN557265 (isolate L2371), JN557266 (isolate L2372), JN557273 (isolate L2411), JN557274 (isolate L2412), JN557275 (isolate L2421), JN557276 (isolate 2422).

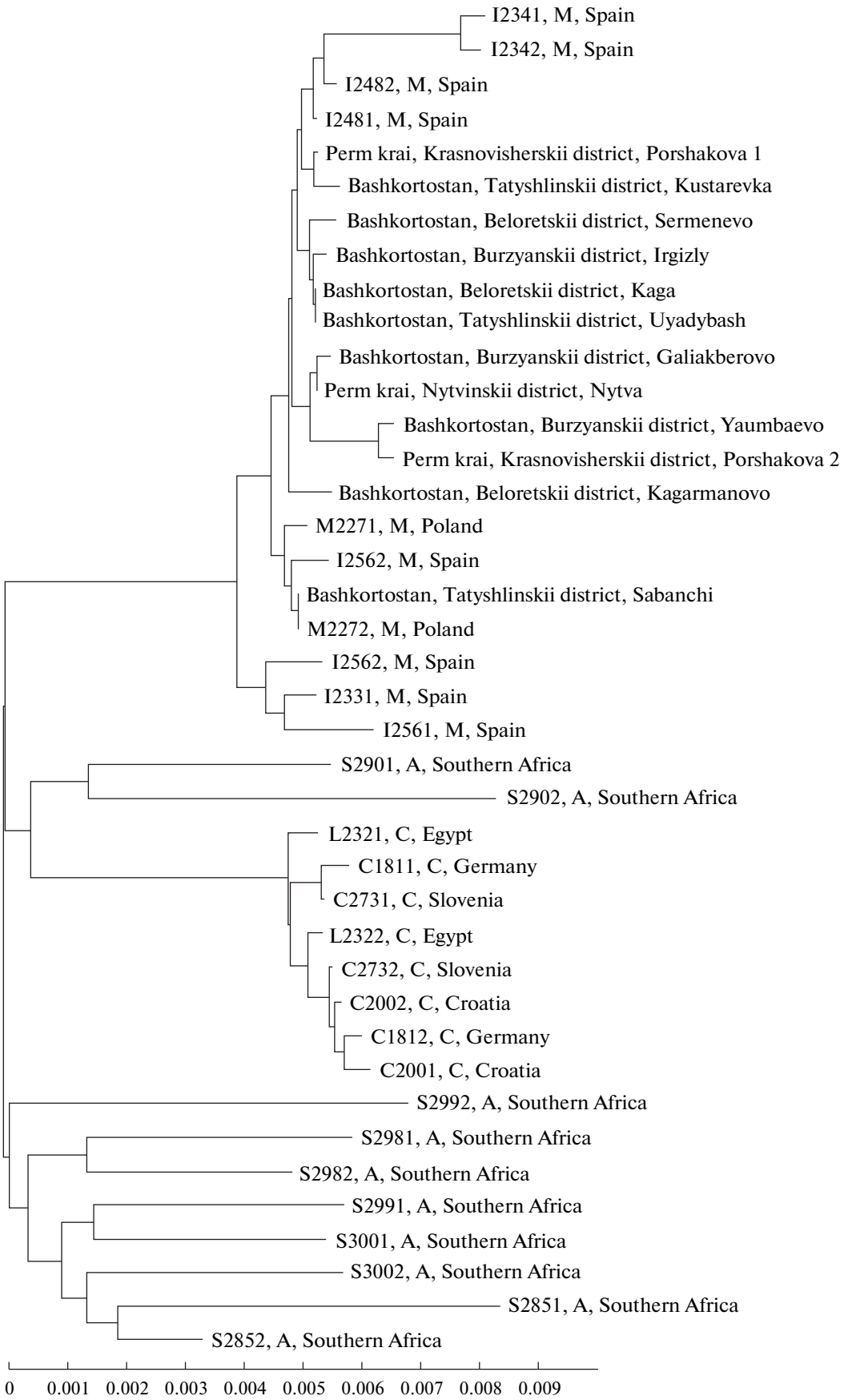
I2341, M, Spain
I2342, M, Spain
I2482, M, Spain
I2481, M, Spain
Perm krai, Krasnovisherskii district, Porshakova 1
Bashkortostan, Tatyshlinskii district, Kustarevka
Bashkortostan, Beloretskii district, Sermenevo
Bashkortostan, Burzyanskii district, Irgizly
Bashkortostan, Beloretskii district, Kaga
Bashkortostan, Tatyshlinskii district, Uyadybash
Bashkortostan, Burzyanskii district, Galiakberovo
Perm krai, Nytvinskii district, Nytva
Bashkortostan, Burzyanskii district, Yaumbaevo
Perm krai, Krasnovisherskii district, Porshakova 2
Bashkortostan, Beloretskii district, Kagarmanovo
M2271, M, Poland
I2562, M, Spain
Bashkortostan, Tatyshlinskii district, Sabanchi
M2272, M, Poland
I2562, M, Spain
I2331, M, Spain
I2561, M, Spain
S2901, A, Southern Africa
S2902, A, Southern Africa
L2321, C, Egypt
C1811, C, Germany
C2731, C, Slovenia
L2322, C, Egypt
C2732, C, Slovenia
C2002, C, Croatia
C1812, C, Germany
C2001, C, Croatia
S2992, A, Southern Africa
S2981, A, Southern Africa
S2982, A, Southern Africa
S2991, A, Southern Africa
S3001, A, Southern Africa
S3002, A, Southern Africa
S2851, A, Southern Africa
S2852, A, Southern Africa
0 0.001 0.002 0.003 0.004 0.005 0.006 0.007 0.008 0.009

Table 3. Base substitution sites of the *Vg* gene differentiating evolutionary branches M and C

Honeybee subspecies	Exon 2			Exon 3				Exon 4				
	substitution sites											
	964	997	1039	1415	1460	1901	1970	1976	2788	2887	2888	2920	2938
Evolutionary branch M	T	C	C	T	C	C	G	C	T	A	A	C	T
Evolutionary branch C	C	T	T	C	T	T	A	T	C	T	C	T	C

Honeybee subspecies	Exon 5							Exon 6					
	substitution sites												
	3981	4242	4288	4316	4500	4508	4509	5114	5210	5225	5306	5321	5328
Evolutionary branch M	T	T	C	A	G	G	G	C	C	C	G	T	A
Evolutionary branch C	C	A	T	G	A	A	A	T	T	T	A	A	G

DISCUSSION

According to comparative analysis of *Vg* gene sequences obtained for honeybees from the Ural region and reference sequences submitted to the GenBank database for the bees of branch C, 26 SNPs that sharply differentiate evolutionary branches M and C were found (Table 3). These SNPs can be used as genetic markers to search for the remaining *A.m.mellifera* isolates that formed in Russia as a result of hybridization with honeybee colonies derived from the Caucasus, Central Asia, and Eastern Europe.

Comparative analysis of *Vg* genes in branches M and C showed that there are three differentiating positions in exon 2; in exon 3, there are five positions; in exon 4, there are five positions; in exon 5, there are seven positions; in exon 6, there are six positions; in exon 7, there are no differentiating positions. Thus, in the analysis of branches M and C, exons 5 and 6 were the most informative, exons 2, 3 and 4 were moderately informative, and exon 7 was not informative.

The cluster analysis was performed by program MEGA 4.1 and used the sequences obtained for branch M honeybees and sequences for relative species contained in the GenBank database, including branch M (isolates I2331, I2332, I2341, I2342, I2481, I2482, I2561, I2562, Spain; isolates M2271, M2272, Poland), branch A (isolates S2851, S2852, S2901, S2902, S2981, S2982, S2991, S2992, S3001, S3002, Southern Africa), and branch C (isolates C1811, C1812, Germany; isolates C2001, C2002, Croatia; isolates C2731, C2732, Slovenia; isolates L2321, L2322, Egypt). The genetic relations of the honeybees of the studied branches are depicted in a dendrogram (see Figure).

All of the analyzed sequences of the *Vg* gene clustered in three groups corresponding to three evolutionary branches of honeybees: A (Africa), M (Ural region and western Europe), C (central Asia and eastern Europe). Bees derived from the Ural region and western European isolates of branch M formed one cluster; this confirms their genetic proximity. Bees from two African isolates showed proximity with the bees of the branch C, which probably results from mistaken categorization of these isolates as belonging to the branch A [15].

Thus, comparative analysis of *Vg* gene sequences can be useful for phylogenetic modeling in *Apis mellifera*, and 26 SNP sites can be used as markers to differentiate M and C evolutionary branches in selective breeding, DNA-barcoding, and creating genetic passports for *A.m.mellifera* colonies.

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Neighbor-joining dendrogram of genetic relations of honeybees from evolutionary branches M and C based on comparative analysis of *Vg* gene sequences.

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SPELL: 1. tht, 2. transversion