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ANIMAL GENETICS

Phylogenetic Relationships among Honey Bee Subspecies *Apis mellifera caucasia* and *Apis mellifera carpathica* Based on the Sequences of the Mitochondrial Genome

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Abstract—The sequences of the complete mitochondrial genome of the honey bee *Apis mellifera* L. subspecies *Apis mellifera caucasia* Pollmann, 1889 (AP018404, 16341 bp) and *Apis mellifera carpathica* Foti et al., 1965 (AP018403, 16336 bp) are sequenced for the first time. Mitochondrial DNA (mtDNA) of both subspecies contains 13 protein-coding genes, 22 tRNA genes, two rRNA genes, and one AT-rich regulatory region. The ratio of transitions to transversions (tr/tv) in complete mtDNA between *A. m. caucasia* and *A. m. carpathica* was 2.05, which reflects the formation of adaptations to changing environmental conditions. Genes with the highest GC content—*COX1* (24%), *COX2* (19.6%), *CYTB* (19.1%), *COX3* (17.2%), and *ND1* (17.2%)—can be highly polymorphic and can be used in phylogenetic and population studies of bees. The majority of mtDNA genes of both subspecies are located on the heavy strand (9 protein-coding genes and 14 tRNA genes), while other genes (4 protein-coding genes, 2 rRNA genes, and 8 tRNA genes) are located on the light strand. Cluster analysis of the complete mtDNA sequence and assessment of the structure of the *tRNA-Leu*(UUR)–*COX2* intergenic region with a single Q element 192 bp in length showed that both subspecies *A. m. caucasia* and *A. m. carpathica* are representatives of the line C with haplotypes C2 and C2j, respectively. Honey bee subspecies *A. m. caucasia* and *A. m. carpathica* can be differentiated from each other by 34 unique SNPs in 11 mtDNA genes and the *Xba*I restriction marker in the *ND5* gene. These genetic markers can contribute to the preservation of purebred gene pools of honey bee subspecies *A. m. caucasia* and *A. m. carpathica* within their natural range.

Keywords: *Apis mellifera*, honey bee subspecies, *A. m. caucasia*, *A. m. carpathica*, mitochondrial genome, mtDNA, haplotypes, conservation genetics

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INTRODUCTION

The honey bee is used by humans for the production of resources specific to beekeeping and for the pollination of agricultural plants [1–3]. As a result of evolution, approximately 30 subspecies of the honey bee have been formed, distributed over a wide range of climatic conditions in the Old World [4, 5]. Good adaptability of the bees allowed humans to distribute them to almost all countries of the world [6–8].

Despite the wide ecological plasticity, large population size, and wide distribution, the number of bee populations is decreasing all over the world every year [9, 10]. The decline of the bee population is occurring for various reasons: use of pesticides and insecticides in the agriculture, uncontrolled mass transport of bees, intraspecific hybridization, spread of new diseases, and global climate changes [11–17]. It was shown that a decrease of honey bee populations would lead to decreased genetic diversity and adaptability of the population, as well as to decrease in the biodiversity of ecosystems [18–23].

The honey bee subspecies are subdivided into at least five evolutionary lines: line A over the entirety of Africa, line M in Western Europe, line C in Eastern Europe, line O in West Asia, and line Y in Northeast Africa and Southwest Asia. The subspecies of bees from different lines differ more significantly than subspecies from the same line. Hybridization of bee sub-

species from different lines can have such consequences as a decrease in the population size, fitness, and adaptability, as well as loss of economically useful traits [9, 10, 24].

The geographical ranges of the subspecies from different evolutionary lines have adjacent boundaries and often overlap, which has led to the formation of hybrid zones at the boundaries of the ranges. Human activity has intensified the formation of hybrid populations of bees [9, 10, 24]. The most demanded bees in the global commercial beekeeping belong to subspecies of the evolutionary line C: *A. m. ligustica* Spinola, 1806 [25], *A. m. carnica* Pollmann, 1889 [26], *A. m. caucasia* Pollmann, 1889 [26], and *A. m. carpathica* Foti et al., 1965 [27]. The widespread use of bees of these subspecies within the ranges of the natural distribution of local subspecies led to the destruction of the native gene pools of many subspecies from Europe and West Asia. In Russia, the Caucasian honey bee *A. m. caucasia* and the Carpathian honey bee *A. m. carpathica* are the most common subspecies in beekeeping after the European dark bee *A. m. mellifera* [18–24].

The natural geographic range of the Caucasian honey bee *A. m. caucasia* covers the mountain ranges and valleys of the Caucasus Mountains and Eastern Anatolia [28]. The natural geographic range of the Carpathian honey bee *A. m. carpathica* covers the ranges and valleys of the Carpathian Mountains and Western Transylvania [29]. These bees are ideally adapted to hot summers and mild winters and are irreplaceable components of the natural ecosystems of the Caucasus and Carpathian Mountains [30–33]. As a result of large-scale transport, these subspecies were distributed over the territories of Armenia, Austria, Azerbaijan, Belarus, Bulgaria, the Czech Republic, Georgia, Hungary, Poland, Romania, Slovakia, southern Russia, Turkey, Ukraine, and Uzbekistan [28]. Such wide artificial distribution of the subspecies *A. m. caucasia* and *A. m. carpathica* have led to their mass hybridization and introgression with local subspecies native to each area, as well as with each other [29, 34, 35].

Caucasian honey bee *A. m. caucasia* and Carpathian honey bee *A. m. carpathica* are the least scientifically studied subspecies, despite the demand and active use in beekeeping. Often subspecies *A. m. carpathica* and *A. m. caucasia* are omitted from the lists of subspecies [18, 36–39].

The subspecies *A. m. carpathica* has long been considered the ecotype of subspecies *A. m. carnica* in Western Romania or *A. m. macedonica* in Eastern Romania [29, 37]. Other studies, on the basis of morphometry [27, 40–43] and mtDNA [24, 29, 44–47], recognize the taxonomic independence of the subspecies *A. m. carpathica*. Identification of the evolutionary line of subspecies *A. m. caucasia* is debatable: on the basis of morphometry [39, 48–50] and allozymes [28], the subspecies is assigned to the line O, and on the basis of mtDNA [18, 19, 23, 38, 45, 51–54], it is assigned to the line C.

Identification of the subspecies and assessment of the introgression level are used as the basis for the preservation of the gene pool of bee populations [29, 35]. We have identified the complete mtDNA nucleotide sequences of the subspecies *A. m. caucasia* and *A. m. carpathica* in order to clarify their taxonomic status and reconstruct their phylogenetic relationships. On the basis of the mtDNA analysis, we showed that honey bee subspecies *A. m. caucasia* and *A. m. carpathica* belong to the evolutionary line C and interact with each other as two independent subspecies.

MATERIALS AND METHODS

Adult worker bees of *A. m. caucasia* were collected in an apiary in the Sochi district of Krasnodar krai, Russia (43°45′ N, 39°95′ E); adult specimens of *A. m. carpathica* were collected in an apiary in the Maykopsky district of the Republic of Adygea, Russia (44°61′ N, 40°07′ E). Identification of the bee colonies as representatives of subspecies *A. m. caucasia* and *A. m. carpathica* was additionally confirmed using the morphometric method [55]. Total DNA was extracted from the thoracic muscle tissue using the Wizard Genomic DNA Purification Kit (PROMEGA, Madison, WI, United States) according to the manufacturer's recommendations. DNA samples were stored at −20° C until further use.

MtDNA was sequenced using NextSeq 500/550 High Output Kit v. 2 (75 cycles) (ILLUMINA, United States) on the basis of the paired-end reads (2 × 150 bp) on an Illumina Next Seq 500 sequencer (ILLUMINA, United States) at Kyoto Sangyo University (Kyoto, Japan), following the manufacturer's instructions. Genomic libraries were prepared using the Nextera DNA library preparation kit (ILLUMINA, United States) according to the manufacturer's instructions. The genomes of *A. m. caucasia* and *A. m. carpathica* were assembled using 1662186 and 1541213 reads, respectively, with an average coverage of 75 using Geneious R9 (BIOMATTERS, New Zealand). Genome annotation was performed using MITOS (Universität Leipzig, Germany) [56], Geneious R9 (BIOMATTERS, New Zealand), Unipro UGENE 1.28 (UNIPRO, Russia), CLC Genomics Workbench 11 (CLCbio, Denmark), and tRNAscan-SE (CA, United States) [57].

The complete mtDNA nucleotide sequences were deposited in the GenBank/DDBJ databases under the accession numbers AP018404 for *A. m. caucasia* (16341 bp) and AP018403 for *A. m. carpathica* (16336 bp). Comparative analysis of the total mtDNA was performed in MEGA7 [58] using the following sequences from GenBank: NC_001566 (*A. m. ligustica*, Maryland, United States) [59], KX908209 (*A. m. ligustica*, Gwangju, Korea) [60], KP163643 (*A. m. syriaca* Sko-

rikov, 1929 [61], Baqa, Jordan) [62], KY926882 (*A. m. syriaca*, Yunnan, China) [63], *A. m. ligustica* NC_001566 (16324 bp) (Bethesda, United States) (reference sequence), and *A. c. cerana* F. GQ162109 (Yunnan, China) (15895 bp) [64] (outgroup).

Sequences of the *tRNA-Leu*(UUR)–COX2 intergenic region of *A. m. caucasia* and *A. m. carpathica* were aligned with the following nucleotide sequences from GenBank: *A. m. carnica* (FJ037782) haplotype C19, *A. m. ligustica* (JF934709) haplotype C33, *A. m. carnica* (JF934704) haplotype C2, *A. m. carnica* (FJ037776) haplotype C11, *A. m. ligustica* (FJ037780) haplotype C17, *A. m. carnica* (FJ037781) haplotype C18, *A. m. ligustica* (FJ037778) haplotype C14, *A. m. carnica* (GQ433623) haplotype C2j, *A. m. ligustica* (FJ037777) haplotype C12, *A. m. syriaca* (AY618918) haplotype O, *A. m. syriaca* (AY618917) haplotype O, *A. m. syriaca* (FJ477993) haplotype O1b, *A. m. syriaca* (FJ037787) haplotype O11, *A. m. syriaca* (FJ477992) haplotype O1a, *A. m. syriaca* (AY618916) haplotype O, *A. m. lamarckii* Cockerell, 1906 (FJ477994) [65] haplotype O1c, and *A. m. syriaca* (FJ477997) haplotype O3.

The divergence of nucleotide sequences and the Jukes–Cantor genetic distances [66] were calculated using UNIPRO UGENE 1.28 (Russia) and CLC Genomics Workbench 11 (CLCbio, Denmark). Phylogenetic analysis based on DNA sequences was performed using MEGA7 [58] and Statistica 8.0 (StatSoft, Inc., Tulsa, OK, United States) and JMP14 (SAS Institute Inc., North Carolina, United States). Phylogenetic trees were reconstructed using the neighbor-joining method [67] based on the Jukes–Cantor distances with 1000 bootstrap replications. A physical map of the complete mitochondrial genome was reconstructed using CLC Genomics Workbench 11 (CLCbio, Denmark) and Artemis 17.0.1 (The Sanger Institute, Hinxton, Cambridge, UK).

RESULTS

The complete mtDNA sequences of *A. m. caucasia* (AP018404), 16341 bp, and *A. m. carpathica* (AP018403), 16336 bp, were slightly longer than the mtDNA sequence of *Drosophila yakuba* (NC_001322), 16019 bp (Fig. 1). We calculated the ratios of A, T, G, and C nucleotides and the most important AT and GC pairs in the complete mtDNA of *A. m. caucasia* and *A. m. carpathica* (Table 1). The average content of AT nucleotides, 84.9%, and GC nucleotides, 15.1%, is similar to the content in *Drosophila melanogaster* (U37541) and bee subspecies *A. m. ligustica* and *A. m. syriaca*. This may be the result of frequent CG to AT substitutions in the evolutionary process [59].

Similar to the reference sequence of *A. m. ligustica* (NC_001566), mtDNA sequences of *A. m. caucasia* and *A. m. carpathica* contained 13 protein-coding genes (*ND2*, *COX1*, *COX2*, *ATP8*, *ATP6*, *COX3*, *ND3*, *ND5*, *ND4*, *ND4L*, *ND6*, *CYTB*, and *ND1*), 22 tRNA genes (*tRNA-Glu*, *tRNA-Ser*(AGN), *tRNA-Met*, *tRNA-Gln*, *tRNA-Ala*, *tRNA-Ile*, *tRNA-Cys*, *tRNA-Tyr*, *tRNA-Trp*, *tRNA-Leu*(UUR), *tRNA-Asp*, *tRNA-Lys*, *tRNA-Gly*, *tRNA-Arg*, *tRNA-Asn*, *tRNA-Phe*, *tRNA-His*, *tRNA-Thr*, *tRNA-Pro*, *tRNA-Ser*(UCN), *tRNA-Leu*(CUN), and *tRNA-Val*), two rRNA genes (*16S rRNA* and *12S rRNA*), and an AT-rich regulatory region (Table 2).

Table 1. Nucleotide content of complete mtDNA of *A. m. caucasia* and *A. m. carpathica*

Nucleotides	*A. m. caucasia*/*A. m. carpathica*	
	number of nucleotides	content, %
Adenine (A)	7067/7066	43.2/43.3
Cytosine (C)	1560/1562	9.5/9.6
Guanine (G)	908/906	5.6/5.5
Thymine (T)	6806/6800	41.6/41.6
GC	2468/2468	15.1/15.1
AT	13873/13866	84.9/84.9

The heavy mtDNA strand in *A. m. caucasia* and *A. m. carpathica* contains nine protein-coding genes (*ND2*, *COX1*, *COX2*, *ATP8*, *ATP6*, *COX3*, *ND3*, *ND6*, and *CYTB*) and 14 tRNA genes (*tRNA-Glu*, *tRNA-Ser*(AGN), *tRNA-Met*, *tRNA-Gln*, *tRNA-Ala*, *tRNA-Ile*, *tRNA-Trp*, *tRNA-Leu*(UUR), *tRNA-Asp*, *tRNA-Lys*, *tRNA-Gly*, *tRNA-Asn*, *tRNA-Thr*, and *tRNA-Ser*(UCN)); the light mtDNA strand contains four protein-coding genes (*ND1*, *ND4*, *ND4L*, and *ND5*), eight tRNA genes (*tRNA-Cys*, *tRNA-Tyr*, *tRNA-Arg*, *tRNA-Phe*, *tRNA-His*, *tRNA-Pro*, *tRNA-Leu*(CUN), and *tRNA-Val*), and two rRNA genes (*16S rRNA* and *12S rRNA*) (Fig. 2).

It is known that the values of genetic diversity and variability depend on the GC content: the higher the GC content, the higher the gene variability [68]. We assessed the GC content for all the studied mtDNA genes. The possible most variable protein-coding genes are *COX1*, *COX2*, *CYTB*, *COX3*, and *ND1*; the least variable protein-coding genes are *ND4*, *ND3*, *ND2*, *ND6*, and *ATP8*. Since the GC content in the mtDNA is less than 40%, which is considered low [69], it is likely that most mtDNA genes are highly conserved (Table 2).

We reconstructed a physical map of the complete mtDNA of honey bees *A. m. caucasia* and *A. m. carpathica*. No differences were found in the synteny of the complete mtDNA of *A. m. caucasia* and *A. m. carpathica* with the reference *A. m. ligustica* sequence. Four gene pairs *ND2* and *tRNA-Cys*, *ATP6* and *ATP8*, *COX1* and *tRNA-Leu*(UUR), and *COX2* and *tRNA-*

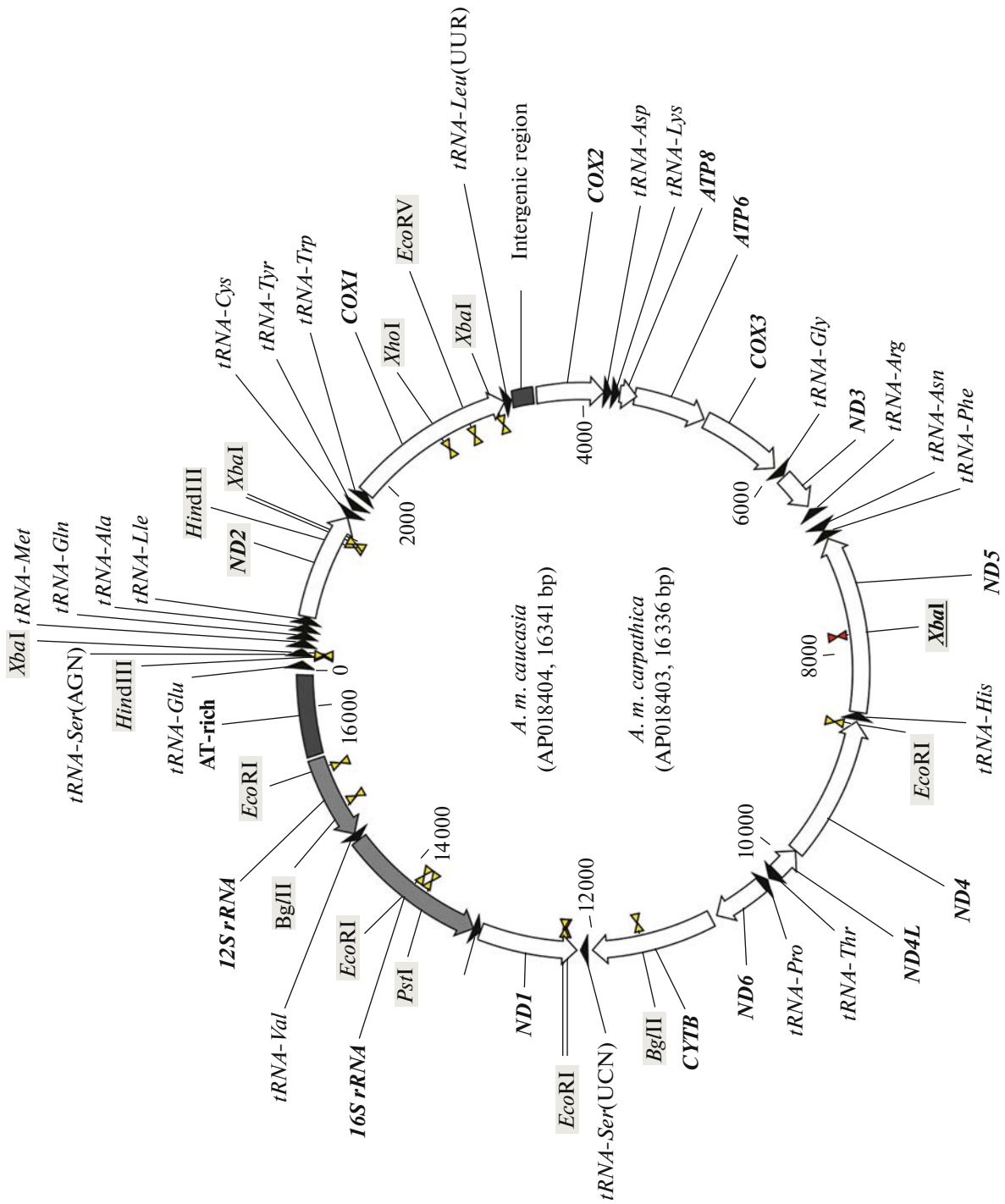

Fig. 1. Circular physical map of complete mtDNA of *A. m. caucasia* and *A. m. carpathica*. The recognition sites of restriction enzymes are highlighted in the background, and the restriction site unique for *A. m. caucasia* is underlined.

Asp had small overlapping regions in both bee subspecies (Fig. 1).

Protein-coding mtDNA genes of *A. m. caucasia* and *A. m. carpathica*, in much the same way as the reference sequence of *A. m. ligustica*, have a single type of stop codon (TAA) and four types of start codons: ATG codon in genes *ATP6*, *COX3*, and *CYTB*; ATA codon in genes *COX1*, *ND3*, and *ND4*; ATT codon in genes *COX2*, *ATP8*, *ND1*, *ND4L*, *ND5*, and *ND6*; ATC codon in gene *ND2*.

In the mtDNA of *A. m. caucasia* and *A. m. carpathica*, as well as in the reference *A. m. ligustica* sequence, there are two isoacceptor tRNA genes for the amino acids serine (Ser) and leucine (Leu). The first *tRNA-Ser*(AGN) gene recognizes the AGN codon by the TCT anticodon at position 138–140 on the heavy strand, and the second *tRNA-Ser*(UCN) gene recognizes the UCN codon by the TGA anticodon on the heavy strand at position 12230–12232 relative to the reference *A. m. ligustica* sequence. The first *tRNA-Leu*(UUR) gene recognizes the UUR codon by the TAA anticodon in position 3388–3390 on the heavy strand, and the second *tRNA-Leu*(CUN) gene recognizes the CUN codon by the TAG anticodon located on the light strand at position 13267–13269 relative to the reference *A. m. ligustica* sequence. Obviously, the presence of these two isoacceptor tRNA genes in single mtDNA is the result of the adaptive evolution of honey bees, which ensures the uninterrupted translation of the most important proteins and peptides.

A total of 15 restriction sites are common for the complete mtDNA of *A. m. caucasia* and *A. m. carpathica*; however, the *Xba*I (T↓CTAGA) restriction site in the *ND5* mtDNA gene, in position 7825–7830 relative to the reference *A. m. ligustica* sequence, was typical only of *A. m. caucasia,* but not of *A. m. carpathica*. This mtDNA restriction site appeared in *A. m. caucasia* owing to SNP 7830C>A, which changed the TCTAGC sequence to TCTAGA, the *Xba*I recognition site.

In the mtDNA of the honey bee subspecies *A. caucasia* and *A. carpathica*, there are 24 intergenic spacers with a total size of 813 bp. The largest intergenic spacer, 192 bp, is located between the *tRNA-Leu*(UUR) and *COX2* genes. The size of this intergenic spacer varies among the subspecies of honey bee *A. mellifera* from different lines: subspecies of line C have the shortest spacer of 191–192 bp, and subspecies of line O have a longer spacer of 258–264 bp (Fig. 2). For comparison, the mtDNA of *A. cerana* has 22 intergenic spacers with a total size of 705 bp, where the longest intergenic spacer of 231 bp is located between the *tRNA-Met* and *tRNA-Gln* genes [64].

Comparative analysis of the aligned sequences of complete mtDNA of *A. m. caucasia* and *A. m. carpathica* showed 17 indels and 54 SNPs; the latter included 36 transitions and 18 transversions. Protein-coding genes of the complete mtDNA of *A. m. caucasia* and

Table 2. Genes of complete mtDNA of honey bees *A. m. caucasia* and *A. m. carpathica*

Gene type	Gene	Size, bp	GC content, %
Protein-coding	*ND2*	1002	13.4
	COX1	1566	24
	COX2	678	19.6
	ATP8	159	11.3
	ATP6	681	15.3
	COX3	780	17.2
	ND3	354	13.6
	*ND5**	1665	14.2
	*ND4**	1311	13.7
	*ND4L**	264	14
	ND6	504	12.5
	CYTB	1152	19.1
	*ND1**	918	17.2
tRNA	*tRNA-Glu*	66	4.5
	tRNA-Ser(AGN)	61	19.7
	tRNA-Met	66	21.2
	tRNA-Gln	55	12.7
	tRNA-Ala	70	10.0
	tRNA-Ile	69	13.0
	*tRNA-Cys**	69	13.0
	*tRNA-Tyr**	68	11.8
	tRNA-Trp	72	8.3
	tRNA-Leu(UUR)	70	18.6
	tRNA-Asp	69	10.1
	tRNA-Lys	69	20.3
	tRNA-Gly	66	7.6
	*tRNA-Arg**	67	13.4
	tRNA-Asn	69	14.5
	*tRNA-Phe**	69	11.6
	*tRNA-His**	68	14.7
	tRNA-Thr	59	8.5
	*tRNA-Pro**	69	14.5
	tRNA-Ser(UCN)	67	13.4
	tRNA-Leu(CUN)*	71	12.7
	*tRNA-Val**	70	11.4
rRNA	*16S rRNA**	1362	15.6
	*12S rRNA**	818	3.4

* Genes of the light mtDNA strand.

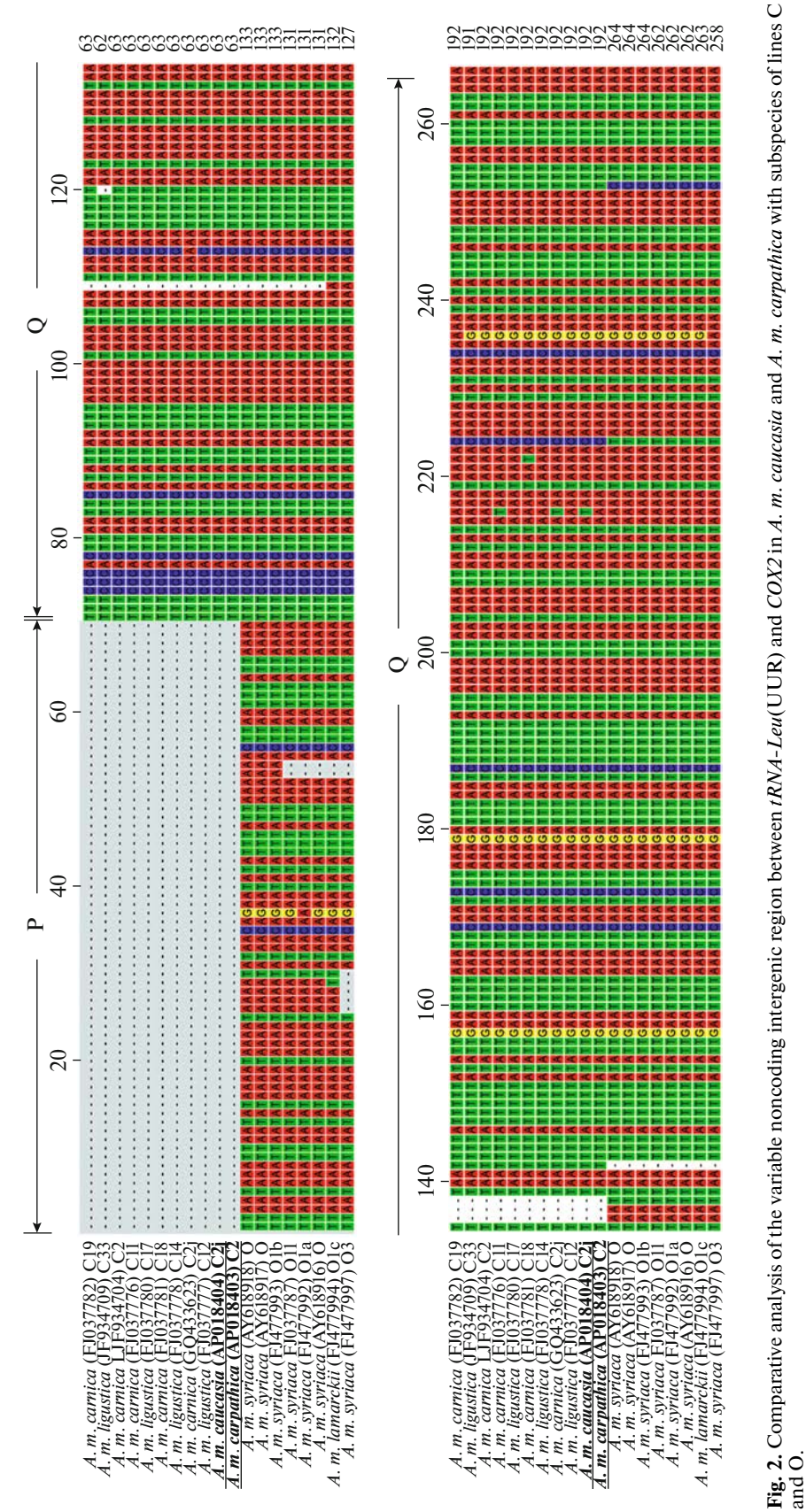

Fig. 2. Comparative analysis of the variable noncoding intergenic region between *tRNA-Leu*(UUR) and *COX2* in *A. m. caucasia* and *A. m. carpathica* with subspecies of lines C and O.

Table 3. Genetic differences and distances (above the diagonal) and numbers of nucleotide substitutions (below the diagonal) between complete mtDNA sequences of samples of *A. m. caucasia*, *A. m. carpathica*, *A. m. ligustica*, and *A. m. syriaca*, as well as *A. cerana* as outgroup

Samples		NC001566 *A. m. ligustica* (C)	KX908209 *A. m. ligustica* (C)	AP018404 *A. m. caucasia* (C)	AP018403 *A. m. carpathica* (C)	KP163643 *A. m. syriaca* (O)	KY926882 *A. m. syriaca* (O)	GQ162109 *Apis cerana*
		genetic differences, % (Jukes–Cantor genetic differences)						
NC 001566 *A. m. ligustica* (C)	Number of SNP, *N*	***	0.7 (0.001)	0.8 (0.005)	0.8 (0.005)	2.7 (0.014)	1.4 (0.012)	19.9 (0.164)
KX908209 *A. m. ligustica* (C)		137	***	1.3 (0.005)	1.3 (0.005)	3.4 (0.015)	2.1 (0.012)	20.6 (0.164)
AP018404 *A. m. caucasia* (C)		89	216	***	0.8 (0.005)	2.8 (0.015)	1.6 (0.012)	19.9 (0.164)
AP018403 *A. m. carpathica* (C)		104	221	127	***	3.0 (0.014)	1.8 (0.012)	20 (0.164)
KP163643 *A. m. syriaca* (O)		454	565	477	503	***	2.1 (0.006)	19.3 (0.162)
KY926882 *A. m. syriaca* (O)		218	331	247	279	322	***	19.8 (0.160)
GQ162109 *Apis cerana*		3342	3459	3336	3357	3245	3300	***

A. m. carpathica differed from each other in one indel and 33 SNPs, of which there were 27 transitions (six nonsynonymous substitutions) and six transversions (five nonsynonymous substitutions). We identified one transversion in the *COX1* gene, two transversions in the *COX2* gene, one transversion in the *COX3* gene, two transversions in the *CYTB* gene, four transitions and one transversion in the *ND1* gene, three transversions in the *ND2* gene, one transversion in the *ND3* gene, seven transitions and one transversion in the *ND4* gene, two transitions and two transversions in the *ND5* gene, and four transitions and one transversion in the *ND6* gene. The rRNA genes of *A. m. caucasia* and *A. m. carpathica* differed in 17 indels and one transition. The tRNA genes of *A. m. caucasia* and *A. m. carpathica* differed in ten indels, two transitions, and one transversion. All intergenic noncoding regions of *A. m. caucasia* and *A. m. carpathica* differed in 27 indels, six transitions, and 11 transversions.

To compare *A. m. caucasia* and *A. m. carpathica* with representatives of *A. m. ligustica* (line C) and *A. m. syriaca* (line O), we calculated the percentage (%) of genetic differences, the Jukes–Cantor genetic distances, and the number of single nucleotide substitutions (SNPs) for the complete mtDNA sequence (Table 3). The greatest differences with *A. m. caucasia* and *A. m. carpathica* were observed for representatives of the outgroup, *A. cerana* (20.3% genetic differences and 3346 SNP), and of the line O, *A. m. syriaca* (2.3% genetic differences and 376 SNPs). The smallest differences with *A. m. caucasia* and *A. m. carpathica* were observed for the representatives of line C, *A. m. ligustica* (1.1% genetic differences and 157 SNPs). *A. m. caucasia* and *A. m. carpathica* honey bees differed in 127 SNPs and had 0.8% genetic differences.

On the basis of pairwise Jukes–Cantor genetic distances between the complete mtDNA sequences, we reconstructed a dendrogram of phylogenetic relationships between the representatives of *A. m. caucasia*, *A. m. carpathica*, *A. m. ligustica* (line C), *A. m. syriaca* (line O), and the outgroup *A. cerana* (Fig. 3). On the dendrogram, representatives of the subspecies from lines C and O were clearly grouped separately, and *A. cerana* was located in the outgroup.

DISCUSSION

The gene pool of a population comprises a set of genomes of all its individuals and is assessed using nuclear and mitochondrial DNA markers [29, 35]. Nucleotide sequences of the complete mtDNA can be used for subspecies identification and phylogenetic reconstructions [18, 19, 37, 54, 59, 70]. Complete mtDNA sequences are currently available in GenBank for five bee species of genus *Apis*: *A. mellifera*, *A. cerana*, *A. dorsata*, *A. florea*, and *A. koschevnikovi* [24, 37,

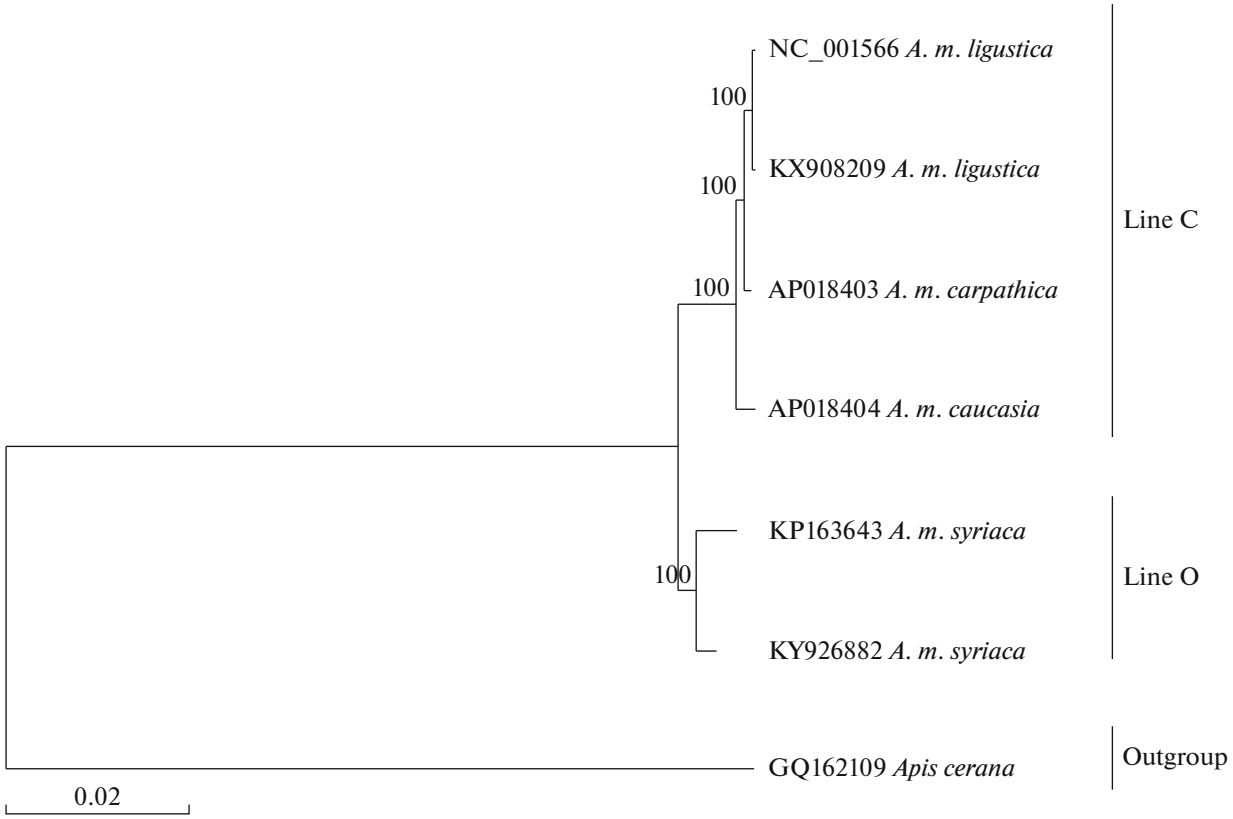

Fig. 3. Phylogenetic relationships between representatives of *A. m. caucasia*, *A. m. carpathica*, *A. m. ligustica*, *A. m. syriaca*, and outgroup *A. cerana* based on cluster analysis of total mtDNA using the neighbor-joining method and Jukes–Cantor genetic distances. Numbers indicate the bootstrap values.

64, 71, 72]. Comparative analysis of complete mtDNA has already become an effective method of taxonomic identification and can be used to preserve the gene pool of local subspecies of bees [10, 24, 72].

The mitochondrial genomes of *A. m. caucasia* and *A. m. carpathica*, similar to mtDNA of other Hymenopterans, have nucleotide content of 43% A, 41% T, 6% G, and 10% C; have AT content of 85%; and contain the highest frequencies of dinucleotides AA (19%), AT (18%), TT (18%), and TA (16%) and the lowest frequencies of dinucleotides GG (1%), GC (1%), CG (1%), and CC (2%) [24, 59, 64, 72, 73]. The average GC content in *A. m. caucasia* and *A. m. carpathica* mtDNA is 15%. The values of genetic diversity and variability are in direct proportion to the GC content: the higher the GC content, the higher the genetic diversity and gene variability. A GC content in mtDNA of less than 40% is considered low [69]. The mtDNA of *A. m. caucasia* and *A. m. carpathica* contains no protein-coding genes with GC content higher than 40%. Genes with the highest GC content—*COX1* (24%), *COX2* (19.6%), *CYTB* (19.1%), *COX3* (17.2%), and *ND1* (17.2%)—can become informative markers in phylogenetic and population studies of honey bees (Table 1).

The tr/tv ratio of transitions to transversions is the most important characteristic of the mutation process. In mtDNA of most animals, transitions occur more frequently than transversions [68, 74]. For most of the known eukaryotes, the normal ratio is tr/tv > 1, while tr/tv < 1 indicates a high frequency of single nucleotide mutations and indels or a low efficiency of the DNA repair process. The variability of the tr/tv ratio in a genome may indicate a local change in the mutational mechanism in the process of adaptation to changing environmental conditions [75–77].

The tr/tv ratio of the total mtDNA was 2.05 between *A. m. caucasia* and *A. m. carpathica*, which is similar to the 2.06 tr/tv ratio for the mtDNA of *Drosophila melanogaster* and *D. yakuba* [78, 79]. Therefore, the mtDNA of honey bee subspecies *A. m. caucasia* and *A. m. carpathica* reflects the process of continuous adaptation to changing environmental conditions.

The AT-rich (AT content of 96%) noncoding region between the *12S rRNA* and *tRNA-Ser* mtDNA genes in *A. m. caucasia* and *A. m. carpathica* is 832 and 849 bp, respectively, which is slightly larger than the corresponding region in the reference *A. m. ligustica* sequence, 826 bp. Owing to the presence of TATA, Poly-T, and $[TA(A)]_n$-like motifs, the AT-rich non-

coding region performs a regulatory function and is involved in the initiation of transcription and replication of mtDNA genes in the honey bee [64].

Most eukaryotes have repeating motifs in their genomes, which can be repeated hundreds of times and involved in the transcriptional and post-transcriptional regulation of gene expression through microRNA. In the complete mtDNA of *A. m. caucasia* and *A. m. carpathica*, we found two repeating 8-nucleotide motifs: the AATTAATT motif, repeated 23 times, and the AATAAATT motif, repeated 50 times; the latter can perform the function of transcriptional and post-transcriptional regulation of gene expression. These two repeating motifs differ from each other only in a single T > A transversion in the fourth position.

Another large noncoding region, located between the *tRNA-Leu*(UUR) and *COX2* genes, consists of two types of nucleotide sequences, which are called P (51–69 bp) and Q (194–196 bp) elements, where P can occur in several variants: P (52–54 bp), P0 (62–69 bp), and P1 (50–51 bp) (Fig. 2). The *tRNA-Leu*(UUR)–*COX2* intergenic region in the bees of evolutionary lines A, M, and O includes a P element in combination with a varying number of copies of the Q element, which leads to polymorphism in the length of this mtDNA region. Bee subspecies of line A contain the P0 or P1 variants and 1–4 Q elements (244–853 bp); bee subspecies of line M contain the P variant and 1–4 Q elements (246–838 bp); bee subspecies of line O contain the P variant and 1–4 Q elements (256–853 bp); bee subspecies of line C do not contain a P element and have a single copy of the Q element (194–196 bp) [52]. Sequences of the *tRNA-Leu*(UUR)–*COX2* intergenic region of *A. m. caucasia* and *A. m. carpathica* did not contain a P element and were 192 bp, similar to nine representatives of the subspecies *A. m. carnica* and *A. m. ligustica* from line C. The *tRNA-Leu*(UUR)–*COX2* intergenic regions of eight representatives of bee subspecies *A. m. syriaca* and *A. m. lamarckii* from line O contained a P element and had a larger size, 258–264 bp (Fig. 2).

Alignment of the sequences of the *tRNA-Leu*(UUR)–*COX2* intergenic region of *A. m. caucasia* and *A. m. carpathica* with accessions from GenBank showed that *A. m. carpathica* is similar to the *A. m. carnica* accession (GQ433623), haplotype C2j, and *A. m. caucasica* is similar to the *A. m. carnica* accession (JF934704), haplotype C2. Thus, the bee subspecies *A. m. caucasia* and *A. m. carpathica* are representatives of the C line, haplotypes C2 and C2j, respectively (Fig. 2).

Despite the great similarity of the complete mtDNA of *A. m. caucasia* and *A. m. carpathica*, they differed in 34 SNPs in 11 genes: *ND2* (752T>C, 936G>A, 1134T>C), *COX1* (1933A>G), *COX2* (3632C>T, 3767T>C), *COX3* (5495A>T, 6040C>T), *ND3* (6488G>A), *ND5* (7408A>G, 7444T>C, 7587T>A, 7830A>C), *ND4* (8660A>T, 8875A>G, 9394T>C, 9772T>C, 9789T>C, 9906C>T, 9919C>T, 9956C>T), *ND6* (10539T>C, 10563T>A, 10596T>C, 10825C>T, 10902A>G), *CYTB* (11832T>C, 11999T>C), *ND1* (12505G>A, 12620G>A, 12674C>T, 12971T>A, 13181C>T), and *12S rRNA* (14996C>T). SNP positions were numbered relative to the reference sequence of *A. m. ligustica*. Moreover, the *Xba*I restriction site, found in the *ND5* gene at position 7825–7830 relative to the reference sequence, was present only in the bee subspecies *A. m. caucasia* and was absent in the subspecies *A. m. carpathica*. The aforementioned mtDNA markers can be very useful for distinguishing between the two subspecies of honey bee *A. m. caucasia* and *A. m. carpathica*.

Bee subspecies *A. m. caucasia*, *A. m. carpathica*, *A. m. ligustica*, and *A. m. syriaca* have 0.80% genetic differences and the Jukes–Cantor genetic distance of 0.005 (Table 3). It was shown that the range of genetic differences between insect subspecies is 0.80–8.00%, and the Jukes–Cantor genetic distances are 0.005–0.100 [24, 63, 64, 72, 80]. Genetic distances and differences between the compared bee samples are consistent with the differences between insect subspecies. Therefore, *A. m. caucasia*, *A. m. carpathica*, *A. m. ligustica*, and *A. m. syriaca* are, indeed, separate subspecies of bees and not ecotypes.

We used the Jukes–Cantor genetic distance matrix for the complete mtDNA sequence in the cluster analysis to reconstruct a dendrogram of phylogenetic relationships (Table 3). The representative of the outgroup of bees *A. cerana* is located separately on the dendrogram, as expected. The dendrogram shows two large clusters. The first cluster combines representatives of *A. m. syriaca* (KP163643 and KY926882), which belong to the line O. The second cluster comprises representatives of *A. m. ligustica* (NC_001566 and KX908209), which belong to the line C. Thus, the dendrogram based on the complete mtDNA can be used to clearly differentiate between the bee subspecies belonging to lines C and O. The grouping of *A. m. caucasia* and *A. m. carpathica* bees together with representatives of *A. m. ligustica* proves that they belong to line C, as previously suggested by some researchers [29, 45–47, 70] (Fig. 3).

Thus, we have identified the nucleotide sequences of the complete mtDNA of bees from subspecies *A. m. caucasia* and *A. m. carpathica* with lengths of 16341 and 16336 bp, respectively. The mtDNA of both subspecies contains 13 protein-coding genes, 22 tRNA genes, two rRNA genes, and an AT-rich regulatory region. The nucleotide content of the mitochondrial genomes of *A. m. caucasia* and *A. m. carpathica* is 43% A, 41% T, 6% G, and 10% C, with 85% AT content. The tr/tv ratio of total mtDNA was 2.05 between *A. m. caucasia* and *A. m. carpathica*, which reflects the process of continuous adaptation to changing environmental conditions.

The majority of the mtDNA genes (*ND2*, *COX1*, *COX2*, *ATP8*, *ATP6*, *COX3*, *ND3*, *ND6*, *CYTB*, and 14 tRNA genes) are located on the heavy strand, and a smaller number of genes (*ND1*, *ND4*, *ND4L*, *ND5*, *SrRNA*, *LrRNA*, and eight tRNA genes) are located on the light strand. The genes with the highest GC content—*COX1* (24%), *COX2* (19.6%), *CYTB* (19.1%), *COX3* (17.2%), and *ND1* (17.2%)—are probably the most polymorphic and have been successfully used in phylogenetic and population studies of bees.

On the basis of the structure of the *tRNA-Leu*(UUR)–*COX2* intergenic region (absence of a P element), bee subspecies *A. m. caucasia* and *A. m. carpathica* can be identified as belonging to the evolutionary line C with haplotypes C2 and C2j, respectively. Bee subspecies *A. m. caucasia* and *A. m. carpathica* mtDNA can be differentiated from each other by 34 unique SNPs in 11 mtDNA genes and the *Xba*I restriction marker in the *ND5* gene.

The *A. m. caucasia* and *A. m. carpathica* bees differ from each other, as well as from the *A. m. ligustica* and *A. m. syriaca* bees, in 0.8% of total mtDNA and have the Jukes–Cantor genetic distance of 0.005. These values of the Jukes–Cantor genetic distances are within the range of intraspecific differences between insect subspecies. Therefore, *A. m. caucasia* and *A. m. carpathica* are, indeed, separate subspecies of bees and not ecotypes.

Despite the studies conducted, populations of *A. m. caucasia* and *A. m. carpathica* remain insufficiently studied both in Russia and in the European countries. We hope to continue the research on bees of subspecies *A. m. caucasia* and *A. m. carpathica* within their natural geographic range in order to assess the structure and genetic diversity of the populations, to prevent intraspecific hybridization, and to preserve the unique local gene pools.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals have been followed.

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