Journal of Apicultural Research

ISSN: 0021-8839 (Print) 2078-6913 (Online) Journal homepage: https://www.tandfonline.com/loi/tjar20

Comparative analysis of mitochondrial genomes of the honey bee subspecies *A. m. caucasica* and *A. m. carpathica* and refinement of their evolutionary lineages

Rustem Ilyasov, Alexei Nikolenko, Varis Tuktarov, Kenji Goto, Jun-Ichi Takahashi & Hyung Wook Kwon

To cite this article: Rustem Ilyasov, Alexei Nikolenko, Varis Tuktarov, Kenji Goto, Jun-Ichi Takahashi & Hyung Wook Kwon (2019) Comparative analysis of mitochondrial genomes of the honey bee subspecies *A. m. caucasica* and *A. m. carpathica* and refinement of their evolutionary lineages, Journal of Apicultural Research, 58:4, 567-579, DOI: 10.1080/00218839.2019.1622320

To link to this article: https://doi.org/10.1080/00218839.2019.1622320

Published online: 10 Jun 2019.

Journal of Apicultural Research, 2019
Vol. 58, No. 4, 567–579, https://doi.org/10.1080/00218839.2019.1622320

ORIGINAL RESEARCH ARTICLE

Comparative analysis of mitochondrial genomes of the honey bee subspecies *A. m. caucasica* and *A. m. carpathica* and refinement of their evolutionary lineages

Rustem Ilyasov[a,b,c]*, Alexei Nikolenko[b], Varis Tuktarov[d], Kenji Goto[d], Jun-Ichi Takahashi[e]* and Hyung Wook Kwon[a,c]*

[a]*Division of Life Sciences, Major of Biological Sciences, Incheon National University, Incheon, Korea;* [b]*Ufa Federal Research Center, Institute of Biochemistry and Genetics, Russian Academy of Sciences, Ufa, Russia;* [c]*Convergence Research Center for Insect Vectors, Incheon National University, Incheon, Republic of Korea;* [d]*Department of Bioecology and Biological Education, Bashkir State Agrarian Universit, Russia;* [e]*Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan*

(Received 28 December 2017; accepted 20 July 2018)

The complete mitochondrial genome sequences of the honey bee subspecies *Apis mellifera caucasica* and *Apis mellifera carpathica* were analyzed for the first time. The length of the mitochondrial DNA (mtDNA) sequences of *A. m. caucasica* and *A. m. carpathica* are 16,341 and 16,336 bps, respectively. Both sequences contain 13 protein-coding genes, 22 transfer RNA genes, 2 ribosomal RNA genes, and 1 AT-rich region. The overall composition of the nucleotides in the mtDNA of *A. m. caucasica*/*A. m. carpathica* is as follows: A (43.2/43.3%), C (9.5/9.6%), G (5.6/5.5%), and T (41.6/41.6%). Most of the mtDNA genes of both subspecies (*ND2, COX1, COX2, ATP8, ATP6, COX3, ND3, ND6, CYTB*, and 14 tRNA genes) are located on the heavy strand, and less (*ND1, ND4, ND4L, ND5, SrRNA, LrRNA*, and 8 tRNA genes) are located on the light strand. Phylogenetic analysis based on the complete mtDNA showed that both subspecies *A. m. caucasica* and *A. m. carpathica* are representatives of the C lineage. Thirty-five unique markers (*ND5*/*Xba*I and 34 SNPs) were found which enable the differentiation of the honey bee subspecies *A. m. caucasica* and *A. m. carpathica*. These genetic markers can contribute to purebred beekeeping and will help to prevent hybridization between these endangered honey bee subspecies in Europe.

Keywords: *Apis mellifera*; honey bee subspecies; *A. m. caucasica*; *A. m. carpathica*; mitochondrial genome; mtDNA; conservation genetics

Introduction

Honey bees are the main insect species maintained by beekeepers for both honey production and pollination activity (Delaplane & Mayer, 2000; Klein et al., 2007; Ollerton, Winfree, & Tarrant, 2011). With human assistance, honey bees now occupy almost all continents and countries in a wide range of climatic conditions and they are the most important pollinators for agriculture, globally. The evolution of honey bees in wide native areas with a broad spectrum of climatic zones has resulted in 30 subspecies in the Old World (Papachristoforou, Rortais, Bouga, Arnold, & Garnery, 2013; Tennant & Chadwick, 2016).

Due to pesticides, intraspecific hybridization and availability of pathogens and pests threatening the honey bees, such as viruses, bacteria, microsporidia, parasitic fungi and mites, most of the honey bee subspecies are endangered and at risk of extinction across the world (Aubert et al., 2007; Coffey, 2007; Cornman et al., 2012; Decourtye, Devillers, Cluzeau, Charreton, & Pham-Delegue, 2004; Genersch et al., 2010; Haddad et al., 2016; Iwasa, Motoyama, Ambrose, & Roe, 2004). The decreasing honey bee populations can lead to a decline of biodiversity and not only of bees but also of plants due to the specificity of flower preference in the pollination activity of different honey bee subspecies (Özdil, Aytekin, Ilhan, & Boztepe, 2012; Franck, Garnery, Celebrano, Solignac, & Cornuet, 2000; Oleksa, Chybicki, Tofilski, & Burczyk, 2011; Palmer, Smith, & Kaftanoglu, 2000; Potts et al., 2010; Uzunov, Kiprijanovska, Andonov, Naumovski, & Gregorc, 2009).

The honey bee subspecies are subdivided into at least four lineages: A (Africa), M (Western Europe), C (Eastern Europe), and O (Middle East). There are great challenges in Middle East and Eastern Europe beekeeping due to hybridization and introgression between honey bee subspecies belonging to the C and O lineages. Most known honey bee subspecies in commercial beekeeping of this region are *Apis mellifera ligustica,* Spinola, 1806, *Apis mellifera carnica,* Pollman, 1879, *Apis mellifera caucasica,* Pollmann, 1889, *Apis mellifera carpathica,* Foti, 1965, *Apis mellifera cecropia,* Kiesenwetter, 1860, *Apis mellifera armeniaca,* Gerstäcker, 1862, *Apis mellifera syriaca,* Skorikov, 1829, *Apis mellifera meda,* Skorikov, 1829, and *Apis mellifera anatoliaca,* Maa, 1953 (Franck et al., 2000; Oleksa et al., 2011; Özdil et al., 2012; Palmer et al., 2000; Uzunov et al., 2009).

The Caucasian bee *A. m. caucasica* and Carpathian bee *A. m. carpathica* are the preferred subspecies and

*Corresponding authors. Emails: apismell@hotmail.com; jit@cc.kyoto-su.ac.jp; hwkwon@inu.ac.kr

they have been intensively used by European beekeepers for more than 100 years (Ruttner, 1988). The honey bee subspecies *A. m. caucasica* and *A. m. carpathica* are very famous and important for the agriculture of countries between the Caspian and Mediterranean Sea. These bee subspecies are well adapted to live in forestry, mountain environmental conditions with hot summers and moderately cold winters. These bee subspecies are components of the Caucasian and Carpathian Mountain ecosystems, which have a key role in biodiversity conservation as well as sustaining unique natural ecosystems (Aizen et al., 2014; Natsopoulou et al., 2017; Ollerton, Erenler, Edwards, & Crockett, 2014; Tandon, Kumar, & Rana, 2016).

The honey bee subspecies *A. m. caucasica* is indigenous to the mountain range and southern valleys of the Caucasus, as well as the eastern end of the Black Sea coast in Anatolia (Ivanova, Bienkowska, & Petrov, 2011). The subspecies *A. m. carpathica* is indigenous to the mountain range and western valleys of the Carpathian's as well as the western part of Romania and Moldova and the Transylvania Plateaus (Bouga et al., 2011). *A. m. carpathica* was not confirmed by Ruttner (1988), who considered it part of *A. m. carnica* in the western part of Romania and *A. m. macedonica* in the southeast (Bouga et al., 2011). The natural range of both subspecies has been artificially extended to Armenia, Austria, Azerbaijan, Belorussia, Bulgaria, Czech Republic, Georgia, Hungary, Poland, Romania, Slovakia, Southern Russia, Turkey, Ukraine, and Uzbekistan (Ivanova et al., 2011). Overlapping habitats along the entire range have led to increasing hybridization and introgression of *A. m. caucasica* and *A. m. carpathica* with each other and with other subspecies (Bouga et al., 2011; Kukrer, Kence, & Kence, 2017; Péntek-Zakar, Oleksa, Borowik, & Kusza, 2015).

At the moment, there is no clear opinion about whether *A. m. caucasica* or *A. m. carpathica* belongs to evolutionary lineage C or O. The phylogenetic conclusions of morphological and mtDNA studies on honey bee subspecies still remain controversial. Morphological measurements (Adl, Gencer, Firatli, & Bahreini, 2007; Kandemir, Ozkan, & Fuchs, 2011; Meixner, Worobik, Wilde, Fuchs, & Koeniger, 2007; Ruttner, 1988) and allozyme studies (Ivanova et al., 2011) assumed that *A. m. caucasica* belongs to the O lineage. However, mtDNA studies (Cornuet & Garnery, 1991; Franck et al., 2000; Garnery, Cornuet, & Solignac, 1992; Garnery et al., 1998; Koulianos, & Crozier, 1997; Mărghitaş, Coroian, Dezmirean, Stan, & Furdui, 2010; Özdil, Yildiz, & Hall, 2009; Palmer et al., 2000; Smith, 2002; Smith, Slaymaker, Palmer, & Kaftanoglu, 1997) assumed that *A. m. caucasica* belongs to the C lineage.

The subspecies *A. m. carpathica* is the least studied of all the honey bee subspecies. Some scientists do not even know about the existence of the honey bee subspecies *A. m. carpathica* and do not mention them in their work (Arias & Sheppard, 1996; Franck et al., 2000; Kandemir et al., 2011; Maa, 1953; Ruttner, 1988; Smith, 2002). Ruttner (1988), based on the analysis of their morphology, considered that *A. m. carpathica* is only an ecotype of the subspecies *A. m. carnica* and *A. m. macedonica* in the western and southern part of Romania, respectively. Morphological measurements (Cauia et al., 2008; Engel, 1999; Foti et al., 1965; Mărghitas et al., 2008; Teleky et al., 2009) and mtDNA studies (Mărghitas et al., 2009; Mărghitas et al., 2010; Bouga et al., 2011; Coroian et al., 2014; Syromyatnikov, Borodachev, Kokina, & Popov, 2018) assumed that *A. m. carpathica* could be a separate subspecies which belonged to the C lineage.

A. m. caucasica and *A. m. carpathica* are not well-studied subspecies, and their taxonomic position and genetic relationship are still controversial. These subspecies reared in the same range have been affected by hybridization with each other. The correct identification of the honey bee subspecies *A. m. caucasica* and *A. m. carpathica* colonies is the only way to preserve their gene pool and intraspecific biodiversity (Bouga et al., 2011; Kukrer et al., 2017).

The phylogenetic relationship and taxonomic position of *A. m. caucasica* and *A. m. carpathica* cannot be clarified based on morphological and partial mtDNA studies. Until now, the complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* have not been sequenced yet. Only a comparative analysis of the complete mitochondrial genomes of these subspecies could answer these questions. In this work, the complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* were sequenced, and *a* comparative analysis with each other and with other subspecies belonging to lineages C and O was done.

Materials and methods

Adult honey bee workers of subspecies *A. m. caucasica* and *A. m. carpathica* were collected from hives in apiaries at the Sochinskii region of the Krasnodarskii krai and at the Maikopskii region of the Republic of Adygea of Russia, respectively. We identified and verified that the subspecies belonged to the honey bee colonies based on morphology using Alpatov's method (Alpatov, 1948). Genomic DNA was extracted from the thoracic muscle tissue with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA samples were stored at –20 °C until further use. Complete mitochondrial DNA was sequenced with Illumina's Next Seq 500 (ILLUMINA, United States) at the Department of Life Sciences of Kyoto Sango University.

The 1,662,186 and 1,541,213 reads were assembled and annotated using the MITOS web server (Universität Leipzig, Germany) (Bernt et al. 2013) and Geneious R9 (BIOMATTERS, New Zealand), the Unipro UGENE 1.28 (UNIPRO, Russia) and CLC Genomics Workbench

Table 1. Nucleotide distribution in complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica*.

	A. m. caucasica (AP018404) 16,341 bp		*A. m. carpathica* (AP018403) 16,336 bp	
Nucleotide	Count	%	Count	%
Adenine (A)	7067	43.2	7066	43.3
Cytosine (C)	1560	9.5	1562	9.6
Guanine (G)	908	5.6	906	5.5
Thymine (T)	6806	41.6	6800	41.6
GC	2468	15.1	2468	15.1
AT	13,873	84.9	13,866	84.9

Table 2. Frequencies of dinucleotides in complete mtDNA sequences of *A. m. caucasica*/*A. m. carpathica*.

1 position\2 position	A	C	G	T
A	0.195/0.194	0.032/0.032	0.022/0.022	0.184/0.185
C	0.044/0.044	0.015/0.015	0.005/0.005	0.032/0.033
G	0.028/0.028	0.006/0.006	0.007/0.007	0.014/0.014
T	0.166/0.166	0.043/0.043	0.022/0.022	0.186/0.185

11 (CLCbio, Denmark) for *A. m. caucasica* and *A. m. carpathica*, respectively. The resultant mtDNA sequences of *A. m. caucasica* comprising 16,341 bps (AP018404) and *A. m. carpathica* comprising 16,336 bps (AP018403) were uploaded on DDBJ database, which is integrated with GenBank. These sequences are available from both DDBJ and GenBank under the same accession numbers.

The phylogenetic analysis was performed using MEGA7 software (Kumar, Stecher, & Tamura, 2016) based on the nucleotide sequences of the complete mtDNA. To explore the phylogeny of *A. m. caucasica* and *A. m. carpathica*, additional complete mtDNA sequences were taken from DDBJ/Genbank database: NC_001566 (*A. m. ligustica*, MD, USA) (Crozier, & Crozier, 1993), KX908209 (*A. m. ligustica*, Gwangju, Korea) (Kim, Kim, & Kim, in press), KP163643 (*A. m. syriaca*, Baqa, Jordan) (Haddad, 2016), and KY926882 (*A. m. syriaca*, China, Yunnan) (Eimanifar et al., 2017). The sequence NC_001566 (*A. m. ligustica)* was used as reference. The sequence GQ162109 (*Apis cerana cerana*, China, Yunnan) (Tan et al., 2011) was used as outgroup.

Pairwise nucleotide sequence differences were estimated based on the complete mtDNA sequences using the Jukes-Cantor models (Jukes & Cantor, 1969), implemented in the MEGA7 software (Kumar et al., 2016). The phylogenetic tree was constructed using the Neighbor-Joining method based on the Jukes-Cantor model with 1000 bootstrap replications (Saitou & Nei, 1987).

Results

The complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* are 16,341 and 16,336 bp, respectively, which are slightly longer than the mtDNA sequence of the fruit fly *Drosophila yakuba* (NC_001322) at 16,019 bp.

We determined the proportion of A, T, G, and C nucleotides and the most important pairs AT and GC. The average contents of the AT and GC nucleotides in both mtDNA sequences were 84.9% and 15.1%, respectively (Table 1). Similarly, the mtDNA of the fruit fly *Drosophila melanogaster* (U37541) and other honey bee subspecies are also highly AT-rich. This could be due to the frequent replacements of 5-methylcytosine and 6-methylguanine in the CG pairs by AT pairs during evolution (Clary & Wolstenholme, 1985; Crozier & Crozier, 1993).

The frequencies of all the dinucleotides were counted in the complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica*. We found that all the dinucleotides have different frequencies. Dinucleotides AA, TA, AT, and TT were the most frequent, and dinucleotides CG, GC, GG, and CC were the rarest. The AA dinucleotide was present at the highest frequency (0.195), and the CG dinucleotide was present at the lowest frequency (0.005) (Table 2).

A comparative analysis of *A. m. caucasica* and *A. m. carpathica* found about a 99% identity in the nucleotide sequence of the complete mtDNA. A high similarity between the complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* can be visualized by the dot plot in which a straight uninterrupted diagonal line confirms the high-level identity between the two mtDNA sequences (Figure 1).

Similar to the reference sequence of *A. m. ligustica* (NC_001566), the mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* contained 13 protein-coding genes, 22 genes for tRNA, 2 genes for rRNA, and a big AT-rich control region (Table 3). No differences were found in the synteny of the mtDNA loci among *A. m. caucasica, A. m. carpathica,* and the reference sequence. Four pairs of genes are slightly overlapped in both subspecies: *ND2* and *tRNA-Cys*, *ATP6* and *ATP8, COX1,* and *tRNA-Leu*, and *COX2* and *tRNA-Asp* (Figure 2).

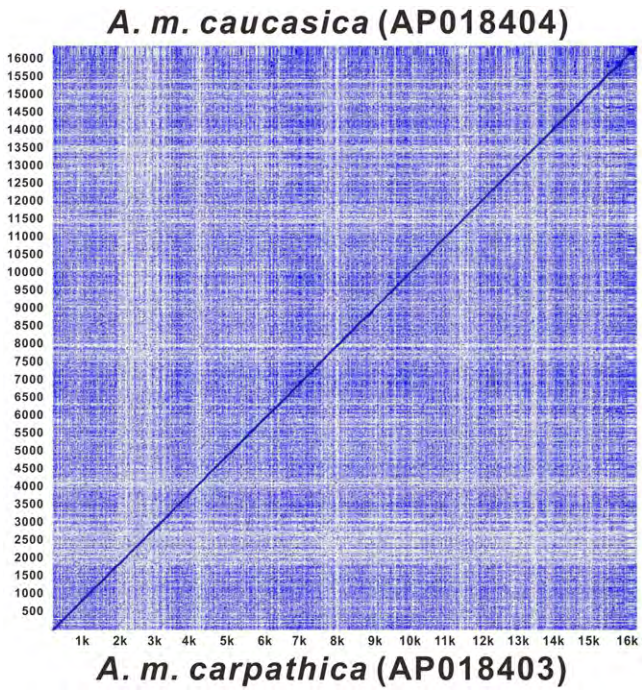

Figure 1. Dot plot comparative analysis of complete mtDNA sequences of honey bee subspecies *A. m. caucasica* and *A. m. carpathica*.

The coding sequence (CDS) of the mtDNA of *A. m. caucasica* and *A. m. carpathica* similar to the reference sequence have one type (TAA) of stop codon and four types of start codons: three genes (*ATP6, COX3,* and *CYTB*) have the ATG type, three genes (*COX1, ND3*, and *ND4*) the ATA type, six genes (*COX2, ATP8, ND1, ND4L, ND5,* and *ND6*) the ATT type, and one gene (*ND2*) the ATC type of start codon.

There are two non-identical genes for transfer RNA for each amino acid serine (Ser) and leucine (Leu) in the mtDNA of *A. m. caucasica, A. m. carpathica,* and the reference sequence. They are the genes for isoacceptor tRNAs. The first *tRNA-Ser*$^{(TCT)}$ recognizes the codon AGN by the anticodon TCT located on the heavy strand at position 138–140, and the second tRNA-Ser$^{(TGA)}$ recognizes the codon UCN by the anticodon TGA located on the heavy strand at position 12230–12232 relative to the reference sequence. The first tRNA-Leu$^{(TAA)}$ recognizes the codon UUR by the anticodon TAA located on the heavy strand at position 3388–3390, and the second tRNA-Leu$^{(TAG)}$ recognizes the codon CUN by the anticodon TAG located on the light strand at position 13267–13269 relatively to the reference sequence. Obviously, the presence of these two isoacceptor tRNA genes in one mtDNA is a result of the adaptive evolution of honey bees, which guarantees the synthesis of important proteins and peptides.

The heavy strand of the mtDNA of *A. m. caucasica* and *A. m. carpathica* encodes 9 protein-coding genes (*ND2, COX1, COX2, ATP8, ATP6, COX3, ND3, ND6*, and *CYTB*) and 14 tRNA genes (*tRNA-Glu, tRNA-Ser, tRNA-Met, tRNA-Gln, tRNA-Ala, tRNA-Ile, tRNA-Trp, tRNA-Leu, tRNA-Asp, tRNA-Lys, tRNA-Gly, tRNA-Asn, tRNA-Thr,* and *tRNA-Ser*), and the light strand of the mtDNA encodes 4 protein-coding genes (*ND1, ND4, ND4L,* and *ND5*), 8 tRNA genes (*tRNA-Cys, tRNA-Tyr, tRNA-Arg, tRNA-Phe, tRNA-His, tRNA-Pro, tRNA-Leu,* and *tRNA-Val*), and 2 rRNA genes (*LrRNA* and *SrRNA*) (Figure 2).

Table 3. Sequentially localization of 37 genes in complete mtDNA sequences of honey bee subspecies *A. m. caucasica* (AP018404) and *A. m. carpathica* (AP018403) in comparison with the reference sequence.

Type	Name	Strand	Region
CDS	*ND2*	heavy	502–1503
	COX1	heavy	1794–3359
	COX2	heavy	3618–4295
	ATP8	heavy	4444–4602
	ATP6	heavy	4584–5264
	COX3	heavy	5285–6064
	ND3	heavy	6185–6538
	ND5	light	6892–8556
	ND4	light	8644–9987
	ND4L	light	9991–10254
	ND6	heavy	10441–10944
	CYTB	heavy	11004–12155
	ND1	light	12302–13219
tRNA	*tRNA-Glu*	heavy	1–66
	tRNA-Ser	heavy	116–178
	tRNA-Met	heavy	221–286
	tRNA-Gln	heavy	296–358
	tRNA-Ala	heavy	360–429
	tRNA-Ile	heavy	433–501
	tRNA-Cys	light	1503–1571
	tRNA-Tyr	light	1592–1659
	tRNA-Trp	heavy	1722–1793
	tRNA-Leu	heavy	3355–3424
	tRNA-Asp	heavy	4294–4362
	tRNA-Lys	heavy	4370–4438
	tRNA-Gly	heavy	6119–6184
	tRNA-Arg	light	6572–6638
	tRNA-Asn	heavy	6734–6802
	tRNA-Phe	light	6810–6878
	tRNA-His	light	8557–8624
	tRNA-Thr	heavy	10267–10344
	tRNA-Pro	light	10365–10432
	tRNA-Ser2	heavy	12201–12267
	tRNA-Leu2	light	13220–13290
	tRNA-Val	light	14662–14731
rRNA	*LrRNA*	light	13291–14661
	SrRNA	light	14732–15517
Misc.	AT-rich region	heavy	15517–16343

Fifteen restriction sites are common for both mtDNA sequences of *A. caucasica* and *A. carpathica*. The unique restriction site of *Xba*I in the gene *ND5* of the mtDNA at position 7825–7830 relatively to the reference sequence was found only in *A. m. caucasica* and not in *A. m. carpathica*. The restriction site of *Xba*I (T↓CTAGA) in the gene *ND5* of the mtDNA has appeared in the *A. m. caucasica* due to the SNP 7830 C > A relatively to the reference sequence, which changed the recognition site from TCTAGC to TCTAGA.

An *in silico* analysis of the complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* identified

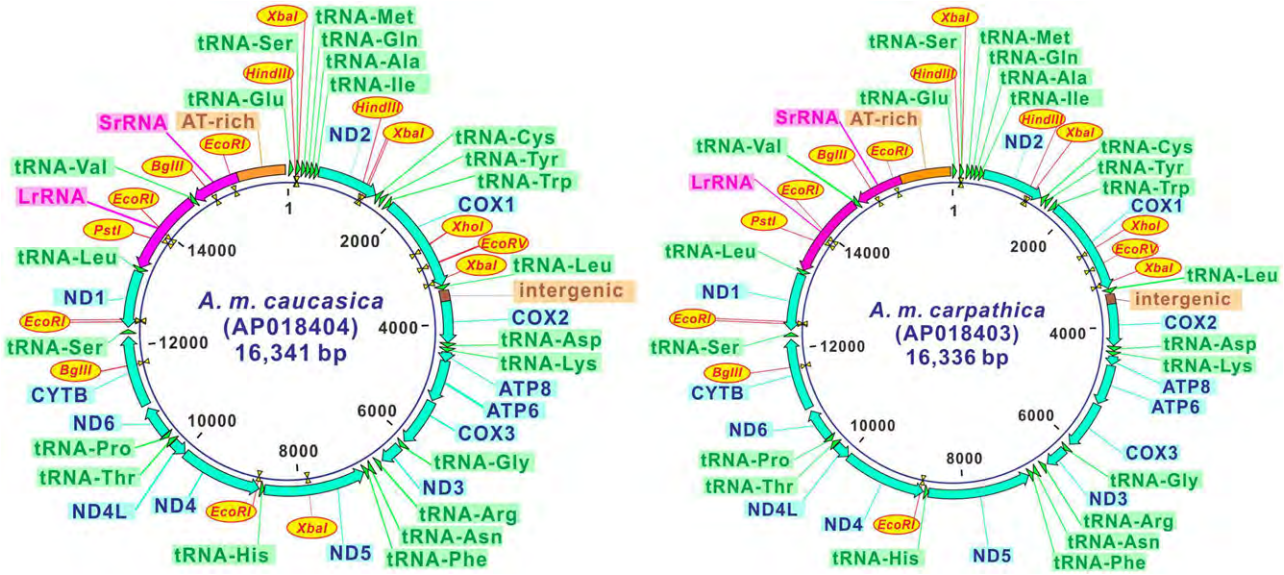

Figure 2. Physical maps of mtDNA of honey bee subspecies *A. m. caucasica* and *A. m. carpathica*. Clockwise arrows indicate heavy strand, counterclockwise indicate light strand. The protein-coding genes labeled by light blue, tRNA genes labeled by green and rRNA genes labeled by pink colors. Restriction enzymes' recognizing sites labeled by yellow highlighted ellipses.

Table 4. ORFs in complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* in comparison with the reference sequence.

No.	Start position	End position	Length in *A. m. caucasica*/ *A. m. carpathica*	Strand	Start codon
1	1788 (*tRNA-Trp*)	3359 (*tRNA-Leu*$^{(TAA)}$)	1572/1572	heavy	ATC
2	2090 (*COX1*)	2434 (*COX1*)	345/345	heavy	ATT
3	3989 (*COX2*)	4291 (*COX2*)	303/303	light	ATT
4	5285 (*COX3*)	6064 (*COX3*)	780/780	heavy	ATG
5	6892 (*ND5*)	8559 (*tRNA-His*)	1668/1668	light	ATC
6	10417 (*tRNA-Pro*)	10944 (*ND6*)	528/528	heavy	ATT
7	12302 (*ND1*)	13225 (*tRNA-Leu*$^{(TAG)}$)	924/924	light	ATA

seven open reading frames (ORFs), which potentially can be translated (Table 4). The lengths of the ORFs are different: the longest size (1668 bp) has an ORF located at position 6892–8559 relatively to the reference sequence, and the shortest size (303 bp) has an ORF located at position 3989–4291 relatively to the reference sequence. Four of the seven ORFs are located on the heavy strand of the mtDNA, and the remaining three are located on the light strand of the mtDNA.

Comparative analysis of the aligned complete mtDNA sequences showed 17 insertion/deletions and 54 SNPs between *A. m. caucasica* and *A. m. carpathica*, of which 36 are transitions, and 18 are transversions. All of the CDS genes of *A. m. caucasica* and *A. m. carpathica* differed from each other by 33 SNPs, of which 27 are transitions (6 of which led to amino acid replacements), and 6 are transversions (5 of which led to amino acid replacements). The MtDNA CDS genes of *A. m. carpathica* contained one deletion site TAA in the *ND4* gene at position 9979–9981 relatively to the reference sequence (Table 5).

A. m. caucasica and *A. m. carpathica* differed from each other in the *COX1* gene by one transition which led to amino acid replacement; in *COX2* by two transitions; in *COX3* by one transition and one transversion which led to an amino acid replacement; in *CYTB* by two transversions; in *ND1* by four transitions and 1 transversion; in *ND2* by three transversions which led to one amino acid replacement; in *ND3* by one transition which led to one amino acid replacement; in *ND4* by seven transitions which led to five amino acid replacements and 1 transversion; in *ND5* by two transitions and two transversions which led to two amino acid replacements, and in *ND6* by four transitions and one transversion.

A. m. caucasica and *A. m. carpathica* differed from each other in the ribosomal RNA genes by 17 insertion/deletions and 1 transition; in the transfer RNA genes by 10 insertion/deletions, 2 transitions and 1 transversion; in the AT-rich region by 14 insertion/deletions, 5 transitions, and 9 transversions, and in the intergenic regions by 13 insertion/deletions, 1 transition and 2 transversions.

Transitions occur more frequently than transversions in the mtDNA of most animals (De Salle, Freedman, Prager, & Wilson, 1987; Wolstenholme & Clary, 1985). The transitions/transversions ratio in the mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* (2.05) is very similar to that of *D. melanogaster* (U37541) (2.06) (Keightley et al., 2009; Seplyarskiy, Kharchenko,

Table 5. Differences between complete mtDNA sequences of *A. m. caucasica* (AP018404) and *A. m. carpathica* (AP018403) in comparison with the reference sequence of *A. m. ligustica* (NC_001566).

Position	*A. m. ligustica* NC_001566 (reference)	*A. m. caucasica* (AP018404)	*A. m. carpathica* (AP018403)	Amino acid replacements	Location
159	A	G	A		*tRNA-Ser* (116–178)
214	A	G	A		Intergenic
311	A	T[a]	A		*tRNA-Gln* (296–358)
345	C	T	C		
492	–	–	Ins TT		*tRNA-Ile* (433–501)
752	C	T	C	Thr > Ile	*ND2* (502–1503)
936	A	G	A	Ser	
1134	C	T	C	Phe	
1933	G	A	G	Ser > Asn	*COX1* (1794–3359)
3370	T	T	Del T		tRNA-Leu (3355–3424)
3567	A	T[a]	A		Intergenic
3632	T	C	T	Phe	*COX2* (3618–4295)
3767	C	T	C	Phe	
4369	A	A	Del A		Intergenic
4427	T	T	Del T		*tRNA-Lys* (4370–4438)
5495	T	A[a]	T	Ser > Thr	*COX3* (5285–6064)
6040	T	C	T	Tyr	
6099	T	A[a]	T		Intergenic
6101	A	Del A	A		
6101	–	–	Ins AT		
6136	–	–	Ins T		*tRNA-Gly* (6119–6184)
6488	A	G	A	Thr > Ala	ND3 (6185–6538)
6540	A	A	Del A		Intergenic
6549	A	A	Del A		
6562	C	C	Del C		
6646	–	Ins T	–		
7408	G	A	G	Ser	*ND5* (6892–8556)
7444	T	T	C	Met	
7587	T	T	A[a]	Met > Leu	
7830	C	A[a]	C	Ala > Ser	
8571	T	T	Del T		*tRNA-His* (8557–8624)
8615	C	C	Del C		
8660	T	A[a]	T	Ala > Ser	*ND4* (8644–9987)
8875	G	A	G	Ser	
9394	C	T	C	Leu	
9772	C	T	C	Leu	
9789	T	T	C	Met > Val	
9906	T	C	T	Met > Val	
9919	T	C	T	Leu	
9956	T	C	T	Asn > Ser	
9979–9981	TAA	TAA	Del TAA	Del Leu	
10264–10265	TA	Del TA	TA		Intergenic
10323	A	A	Del A		*tRNA-Thr* (10267–10344)
10381	A	A	Del A		*tRNA-Pro* (10365–10432)
10539	C	T	C	Ser	*ND6* (10441–10944)
10563	T	T	A[a]	Ser	
10596	C	T	C	Thr	
10825	C	C	T	Leu	
10902	G	A	G	Lis	
10973	A	A	Del A		Intergenic
11001	T	T	Del T		
11832	C	T	C	Leu	*CYTB* (11004–12155)
11999	C	T	C	Asn	
12183	A	A	Del A		Intergenic
12256	T	T	Del T		tRNA-Ser2 (12201–12267)
12283	T	C	T		Intergenic
12505	A	G	A	Leu	*ND1* (12302–13219)
12620	A	G	A	Ile	
12674	C	C	T	Leu	
12971	A	T[a]	A	Val	
13181	T	C	T	Met	
13282	T	T	Del T		*LrRNA* (13291–14661)

(*Continued*)

Table 5. (*Continued*).

14569	A	A	Del A	
14589	A	A	Del A	
14590	T	T	Del T	
14623	A	Del A	A	
14749	C	C	Del C	*SrRNA* (14732–15517)
14778	C	C	Del C	
14793	T	T	Del T	
14868	T	T	Del T	
14926	T	T	Del T	
14955	T	T	Del T	
14996	T	C	T	
15119	A	A	Del A	
15295	T	T	Del T	
15340	G	Del G	Del G	
15444	A	A	Del A	
15474	T	T	Del T	
15503	T	T	Del T	
15522	–	–	Ins T	AT-rich region
15535	T	T	A[a]	
15537	T	T	A[a]	
15539	–	–	Ins T	
15569	–	–	Ins A	
15578	–	–	Ins A	
15644	C	T	C	
15660	A	A	C[a]	
15666	–	–	Ins ATATTATAAATATTT	
15828	T	C	T	
16133	–	Ins TAAA	–	
16146	A	T[a]	A	
16162–16163	TT	Del TT	TT	
16164	T	Del T	A	
16171	T	T	A[a]	
16172	T	A[a]	T	
16173	A	G	A	
16179	C	A[a]	C	
16180	T	A[a]	T	
16198	C	T	C	
16203	T	A[a]	T	
16232	–	Ins T	–	
16262	G	A	G	

Ins: insertions; Del: deletions.
atransversions.

Kondrashov, & Bazykin, 2012). An excess of transitions over transversions have been found in the mtDNA of *D. melanogaster* (U37541), *D. yakuba* (NC_001322), *Drosophila simulans* (AF200854), and *Drosophila mauritiana* (AF200831) (De Salle et al., 1987; Satta et al., 1987; Wolstenholme & Clary, 1985). An excess of transitions over transversions in mtDNA is possible in the third positions of the codons, which do not lead to amino acid replacements.

Most eukaryotes have repetitive motifs in their genomes which can be repeated hundreds of times. The repetitive motifs can be involved in the regulation of gene expression through RNA fragments. Across the complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica*, the two most repetitive 8 nucleotide motifs have been detected: the AATTAATT motif repeated 23 times, and the AATAAATT motif repeated 50 times. These two repetitive motifs differ from each other by only one transversion T > A in the fourth position.

The AT-rich region in the mtDNA of honey bees is flanked by SrRNA and tRNA-Ser and is highly enriched in AT (96%). The AT-rich region of *A. m. caucasica* has 832 bp and that of *A. m. carpathica* has 849 bp, which are slightly larger than the AT-rich region of the reference sequence containing 826 bp. The AT-rich region is well known in honey bee mtDNA as a control region. The reduced GC content is one of the most outstanding features of the control region of the honey bee mtDNA. Due to the presence of the TATA motif, PolyT stretch, and [TA(A)]n-like stretch, the AT-rich region is involved in the initiation of transcription and replication of the mtDNA genes in honey bees (Tan et al., 2011).

Besides the AT-rich region, the mtDNA sequences of *A. caucasica* and *A. carpathica* have 24 intergenic spacers with a common size 813 bp. The longest 192 bp intergenic spacer of *A. caucasica* and *A. carpathica* is located at position 3425–3617 relatively to the reference sequence between the tRNA-Leu$^{(TAA)}$ and COX2

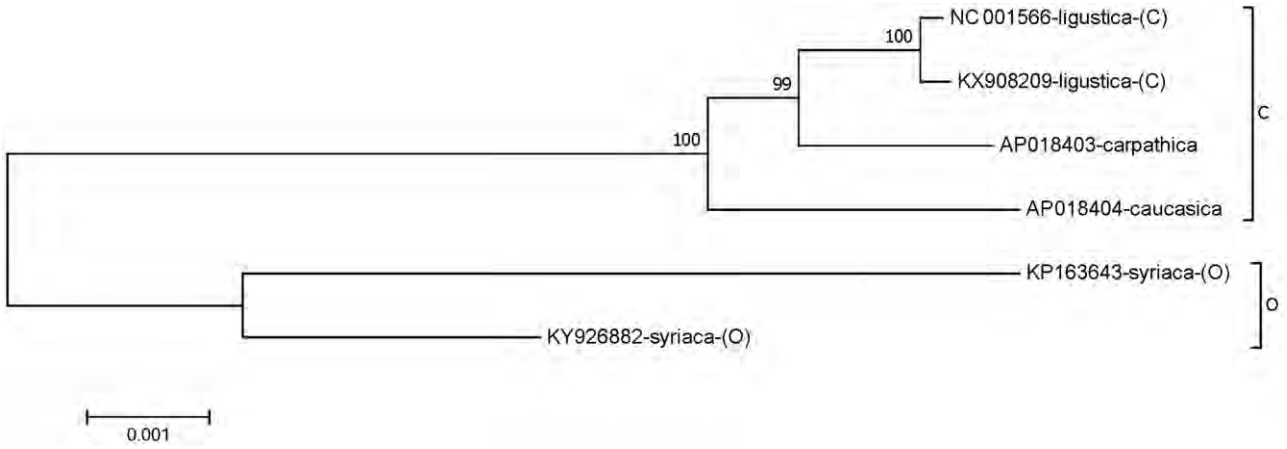

Figure 3. Phylogenetic tree is built on the basis of the complete mtDNA sequences of *A. m. caucasica*, *A. m. carpathica* and samples belonging to the C and O lineages (GenBank). Confidence in the nodes was evaluated by 1000 bootstrap replicates. Sequence of *A. m. ligustica* (NC_001566) used as a reference.

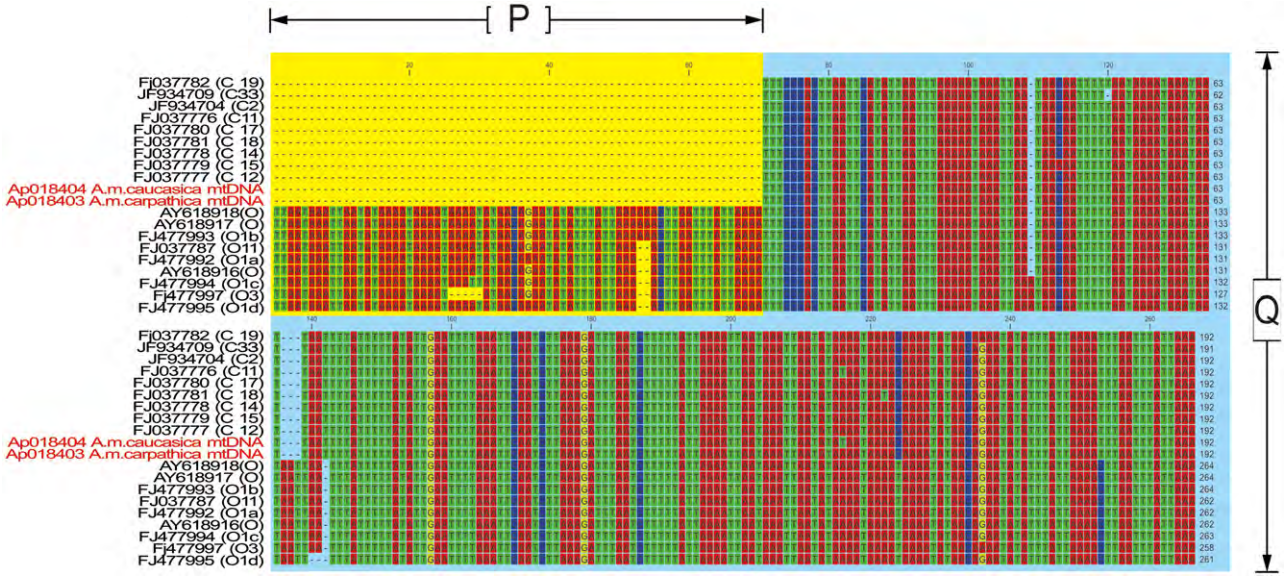

Figure 4. Alignment of tRNA-Leu$^{(TAA)}$ and COX2 intergenic region of *A. m. caucasica* and *A. m. carpathica*, nine samples of C lineage and nine samples of O lineage from GenBank.

genes. This intergenic spacer is very variable between the honey bee *A. mellifera* subspecies from different lineages: subspecies from the C lineage have the smallest size at 191–192 bp, but subspecies from the O lineage have a larger size at 258–264 bp. For comparison, the mtDNA of *A. cerana* has 22 intergenic spacers with a common size 705 bp. The longest 231 bp intergenic spacer of *A. cerana* is located between the tRNA-Met and tRNA-Gln genes (Tan et al., 2011).

The final goal of the DNA comparison is to detect the genetic relationship between samples. The phylogenetic trees enable the visualization of the genetic relationship of honey bee subspecies. Based on the pairwise Jukes-Cantor genetic distances between the complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* and the representatives of the C and O lineages, the phylogenetic tree was constructed (Figure 3).

To further show the evolutionary lineages that belong the samples for comparison, we aligned the sequences of the *tRNA-Leu$^{(TAA)}$–COX2* intergenic region of *A. m. caucasica* and *A. m. carpathica* and 10 representatives from each of the C and O lineages obtained from GenBank (Figure 4).

Discussion

A comparative analysis of mtDNA is the most useful tool for phylogenetic and systematic research on honey bees and other animals (Arias & Sheppard, 1996; Rand & Kann, 1998; Tan et al., 2011). The use of complete mtDNA sequences for phylogenetic studies has become more convenient and reliable now because the complete sequences of mtDNA for many species of the genus *Apis*, e.g., *A. mellifera, A. cerana, A. dorsata, A. florea,*

Table 6. Pairwise divergence, genetic distance (upper) and nucleotide differences (lower) between complete mtDNA sequences of *A. m. caucasica*, *A. m. carpathica,* and two representatives of each C and O lineages.

		Divergence, % (Jukes-Cantor distance)						
		NC 001566 *A. m. ligustica* (C)	KX908209 *A. m. ligustica* (C)	AP018404 *A. m. caucasica*	AP018403 *A. m. carpathica*	KP163643 *A. m. syriaca* (O)	KY926882 *A. m. syriaca* (O)	GQ162109 *A. cerana*
NC 001566 *A. m. ligustica* (C)	Differences, *n*	***	0.7 (0.001)	0.5 (0.004)	0.6 (0.002)	2.7 (0.014)	1.4 (0.012)	19.9 (0.164)
KX908209 *A. m. ligustica* (C)		137	***	1.3 (0.004)	1.3 (0.002)	3.4 (0.015)	2.1 (0.012)	20.6 (0.164)
AP018404 *A. m. caucasica*		89	216	***	0.8 (0.003)	2.8 (0.015)	1.6 (0.012)	19.9 (0.164)
AP018403 *A. m. carpathica*		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 *A. cerana*		3342	3459	3336	3357	3245	3300	***

and *A. koschevnikovi* are available in the GenBank database. The analysis of the complete mtDNA of honey bee subspecies is one of the ways to preserve the natural biodiversity of *Apis mellifera* (Ilyasov, Poskryakov, & Nikolenko, 2016). Comparative analysis of complete mtDNA can help in understanding the phylogenetic relationships of honey bee subspecies with overlapping artificial ranges, e.g., *A. m. caucasica* and *A. m. carpathica*; *A. m. mellifera* and *A. m. carpathica*, and *A. m. mellifera* and *A. m. ligustica*. The results of the comparative analysis of the honey bee subspecies *A. m. caucasica* and *A. m. carpathica* complete mtDNA will enable researchers to stop and prevent their uncontrollable hybridization between themselves.

The phylogenetic relationships of the honey bee subspecies *A. m. caucasica* and *A. m. carpathica* were defined based on the comparative analysis of their complete mtDNA sequences with two samples of *A. m. ligustica* (NC_001566, KX908209) belonging to the C lineage and two samples of *A. m. syriaca* (KP163643, KY926882) belonging to the O lineage, using the mtDNA sequence of *A. c. cerana* (GQ162109) as an outgroup.

The percent of genetic divergence and numbers of nucleotide differences between the complete mtDNA sequences of *A. m. caucasica*, *A. m. carpathica,* and subspecies of the C and O lineages were determined (Table 6). *A. m. caucasica* and *A. m. carpathica* differ from each other by 0.8% due to changes in 127 nucleotides. *A. m. caucasica* and *A. m. carpathica* are most similar (0.5%–0.7% of divergence) to *A. m. ligustica* (NC 001566) and are most different (2.7%–3.4% of divergence) from *A. m. syriaca* (KP163643). *A. m. caucasica* and *A. m. carpathica* significantly differ (19.9%–20%) from the *A. cerana* outgroup sample due to changes in 3336 and 3357 nucleotides, respectively.

Despite the high similarity between the complete mtDNA of *A. m. caucasica* and *A. m. carpathica*, there are 34 SNPs in 11 genes which enabled us to differentiate them: *ND2* (752 T > C, 936 G > A, 1134 T > C), *COX1* (1933 A > G), *COX2* (3632 C > T, 3767 T > C), *COX3* (5495 A > T, 6040 C > T), *ND3* (6488 G > A), *ND5* (7408 A > G, 7444 T > C, 7587 T > A, 7830 A > C), *ND4* (8660 A > T, 8875 A > G, 9394 T > C, 9772 T > C, 9789 T > C, 9906 C > T, 9919 C > T, 9956 C > T), *ND6* (10539 T > C, 10563 T > A, 10596 T > C, 10825 C > T, 10902 A > G), *CYTB* (11832 T > C, 11999 T > C), *ND1* (12505 G > A, 12620 G > A, 12674 C > T, 12971 T > A, 13181 C > T), and *SrRNA* (14996 C > T). The positions of the SNPs were designated relative to the reference sequence. Moreover, the restriction site *Xba*I detected in the gene *ND5* at position 7825–7830 relatively to the reference sequence can differentiate *A. m. caucasica* and *A. m. carpathica*. Use of these *ND5*/*Xba*I and SNP markers in practice will assist in preventing hybridization between *A. m. caucasica* and *A. m. carpathica* and in preserving their purebred gene pool.

The phylogenetic tree based on the complete mtDNA sequences enables differentiation of the C and O lineages as well. The outgroup sample of *A. cerana* located on the tree separately as expected. There are two big clusters that we can observe on the tree. The first cluster united *A. m. syriaca* samples (KP163643, KY926882), belonging to the O lineage. The second cluster united the *A. m. ligustica* samples (NC_001566, KX908209) belonging to the C lineage. Thus, the samples of the C and O lineages can be separated based on the comparative analysis of the complete mtDNA.

The samples for *A. m. caucasica* and *A. m. carpathica* located together with the representatives of the C lineage. This grouping can be explained as the genetic affinity of the subspecies *A. m. caucasica* and *A. m. carpathica* with honey bees belonging to the C lineage. Thus, we can assume that both honey bee subspecies *A. m. caucasica* and *A. m. carpathica* belong to the C lineage. Our complete mitochondrial DNA analysis

confirms the results of other mtDNA studies, which classify *A. m. caucasica* and *A. m. carpathica* as representatives of the C lineage (Bouga et al., 2011; Coroian et al., 2014; Mărghitaş et al., 2010; Özdil et al., 2009; Syromyatnikov et al., 2018).

The *tRNA-Leu$^{(TAA)}$–COX2* intergenic region is composed of two distinct nucleotide sequences, named P (51–69 bp) and Q (194–196 bp), where P can also appear in several variations (P (52–54 bp), P0 (62–69 bp), and P1 (50–51 bp)). Honey bees of each the A, M, and O evolutionary lineages include a variant of the P sequence, combined with a different copy number of the Q sequence, resulting in length polymorphisms of this mtDNA region. Honey bees of the A lineage contain the P0 or P1 element and the 1–4 Q elements; those of the M lineage contain the P element and the 1–4 Q elements; those of the O lineage contain the P0 element and the 1–4 Q elements and those of the C lineage contain only a single copy of the Q element (Garnery et al., 1992). The size of the *tRNA-Leu$^{(TAA)}$–COX2* intergenic region in the honey bees of the O lineage is 256–853 bp, in the honey bees of the A lineage 244–853 bp, in the honey bees of the M lineage 246–838 bp, and in the honey bees of the C lineage 194–196 bp.

The aligned *tRNA-Leu$^{(TAA)}$–COX2* intergenic regions of *A. m. caucasica* and *A. m. carpathica* did not contain the P element and have a size 192 bp, similarly with the 10 representatives of the C lineage. The intergenic regions of the 10 representatives of the O lineage contained the P element and have a larger size at 258–264 bp. According to the comparative analysis of the *tRNA-Leu$^{(TAA)}$–COX2* intergenic region, we can assume that both subspecies *A. m. caucasica* and *A. m. carpathica* are representatives of the C lineage.

Conclusion

The complete mtDNA of *A. m. caucasica* is longer than the complete mtDNA of *A. m. carpathica* by 5 nucleotides. The complete mtDNA of honey bee subspecies *A. m. caucasica* and *A. m. carpathica* differ from each other by about 1%, which is a difference of 127 nucleotides. *A. m. caucasica* and *A. m. carpathica* are more similar with the honey bee subspecies *A. m. ligustica* and less similar with *A. m. syriaca*. Despite the high similarity between *A. m. caucasica* and *A. m. carpathica*, there are 34 unique SNPs in 11 mtDNA genes, which enable their differentiation. The unique restriction marker *ND5/Xba*I at position 7825–7830 relatively to the reference sequence is only present in *A. m. caucasica* and absent in *A. m. carpathica*. The SNPs and restriction markers enable the differentiation of the honey bee subspecies *A. m. caucasica* and *A. m. carpathica*.

The *tRNA-Leu$^{(TAA)}$–COX2* intergenic region of *A. m. caucasica* and *A. m. carpathica* did not contain the P element and only contained a single Q element, similarly with the representatives of the C lineage. Thus, the results of the comparative analysis of the complete mtDNA and the analysis of the structure of the *tRNA-Leu$^{(TAA)}$–COX2* intergenic region equally confirm the affiliation *A. m. caucasica* and *A. m. carpathica* to the C lineage.

Using these genetic markers in practice will enable researchers to save and prevent the hybridization of *A. m. caucasica* and *A. m. carpathica* with each other and with other honey bee subspecies.

Perspectives

Molecular genetics methods are reliable tools for differentiating subspecies of bees. The use of mtDNA markers seems promising in determining the subspecies of bees. Molecular genetic tools enable the recognition of hidden differences in honey bee genomes. Purebred lines of honey bees only can be used successfully for the selection and sustainable management of populations. The published complete mtDNA sequences of *A. m. caucasica* and *A. m. carpathica* will lead to new strategies for the conservation of the subspecies. Most populations of *A. m. caucasica* and *A. m. carpathica* have not been well investigated. The authors are going to extend the research of *A. m. caucasica* and *A. m. carpathica* in the countries where these subspecies exist. It will enable understanding their population structures and biodiversity levels as well as their properties of adaptive evolution.

Acknowledgments

We are grateful to Dr. Hisashi Okuyama for kindly providing data for our analysis. The work of RI was supported by Russian Government Assignments No. AAAA-A16-116020350026-0 and by the Postdoctoral Fellowships in the Incheon National University (2017–2019). The work of NA was supported by the Grant of the Russian Found of Basic Research (No. 17-44-020648 Povolzhye). The work of HWK was supported by Grants of the National Research Foundation of Korea (NRF), of the Korea government (MSIP) (No. 2016R1A2B3011742), and of the Cooperative Research Program for Agriculture Science and Technology Development (RDA) (No. PJ012526).

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Rustem Ilyasov http://orcid.org/0000-0003-2445-4739
Alexei Nikolenko http://orcid.org/0000-0002-9235-680X
Hyung Wook Kwon http://orcid.org/0000-0001-9340-7974

References

Adl, M. B. F., Gencer, H. V., Firatli, C., & Bahreini, R. (2007). Morphometric characterization of Iranian (*Apis mellifera meda*), Central Anatolian (*Apis mellifera anatoliaca*) and Caucasian (*Apis mellifera caucasica*) honey bee populations.

Journal of Apicultural Research, *46*(4), 225–231. doi:10.3896/IBRA.1.46.4.03

Aizen, M. A., Morales, C. L., Vázquez, D. P., Garibaldi, L. A., Sáez, A., & Harder, L. D. (2014). When mutualism goes bad: density-dependent impacts of introduced bees on plant reproduction. *New Phytologist*, *204*(2), 322–328. doi:10.1111/nph.12924

Alpatov, W. W. (1948). The races of honey bees (p. 143). Moscow: Moscow Society of Naturalists (in Russian).

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

Aubert, M., Ball, B., Fries, I., Moritz, R., Milani, N., & Bernardinelli, I. (2007). *Virology and the honey bee* (p. 458). Brussels: Northern Bee Books.

Bernt, M., Donath, A., Jühling, F., Externbrink, F., Florentz, C., Fritzsch, G., … Stadler, P. F. (2013). MITOS: improved de novo Metazoan mitochondrial genome annotation. *Molecular Phylogenetics and Evolution*, *69*(2), 313–319. doi:10.1016/j.ympev.2012.08.023

Bouga, M., Alaux, C., Bienkowska, M., Büchler, R., Carreck, N. L., Cauia, E., … Wilde, J. (2011). A review of methods for discrimination of honey bee populations as applied to European beekeeping. *Journal of Apicultural Research*, *50*(1), 51–84. doi:10.3896/IBRA.1.50.1.06

Cauia, E., Usurelu, D., Magdalena, L. M., Cimponeriu, D., Apostol, P., Siceanu, A., … Gavrila, L. (2008). Preliminary researches regarding the genetic and morphometric characterization of honeybees (*A. mellifera* L.) from Romania. *Lucrări Ştiinţifice Zootehnie Şi Biotehnologii*, *41*(2), 278–286.

Clary, D. O., & Wolstenholme, D. R. (1985). The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *Journal of Molecular Evolution*, *22*(3), 252–271. doi:10.1007/BF02099755

Coffey, M. F. (2007). *Parasites of the honeybee* (p. 81). Oak Park, Carlow, Ireland: Teagasc, Crops Research Centre.

Coroian, C. O., Muñoz, I., Schlüns, E. A., Paniti-Teleky, O. R., Erler, S., Furdui, E. M., … Moritz, R. F. A.. (2014). Climate rather than geography separates two European honeybee subspecies. *Molecular Ecology*, *23*, 2353–2361. doi:10.1111/mec.12731.

Cornman, R. S., Tarpy, D. R., Chen, Y., Jeffreys, L., Dawn, L., Pettis, J. S., … Evans, J. D. (2012). Pathogen webs in collapsing honey bee colonies. *PLoS One*, *7*(8), e43562. doi:10.1371/journal.pone.0043562

Cornuet, J. M., & Garnery, L. (1991). Mitochondrial DNA variability in honeybees and its phylogeographic implications. *Apidologie*, *22*(6), 627–642. doi:10.1051/apido:19910606

Crozier, R. H., & Crozier, Y. C. (1993). The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. *Genetics*, *133*(1), 97–117. doi:10.1111/j.1365-2583.1993.tb00131.x

De Salle, R., Freedman, T., Prager, E. M., & Wilson, A. C. (1987). Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. *Journal of Molecular Evolution*, *26*, 157–164. doi:10.1007/BF02111289

Decourtye, A., Devillers, J., Cluzeau, S., Charreton, M., & Pham-Delegue, M. (2004). Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. *Ecotoxicology and Environmental Safety*, *57*(3), 410–419. doi:10.1016/j.ecoenv.2003.08.001

Delaplane, K. S., & Mayer, D. F. (2000). *Crop pollination by bees* (p. 344). Wallingford, UK: CABI Publishing.

Eimanifar, A., Kimball, R. T., Braun, E. L., Fuchs, S., Grunewald, B., & Ellis, J. D. (2017). The complete mitochondrial genome and phylogenetic placement of Apis nigrocincta Smith (Insecta: Hymenoptera: Apidae), an Asian, cavity-nesting honey bee. *Mitochondrial DNA Part B Resour. 2*, 249–250. doi: 10.1080/23802359.2017.1318683.

Engel, M. S. (1999). The taxonomy of recent and fossil honey bees (Hymenoptera, Apidae, *Apis*.). *Journal of Hymenoptera Research*, *8*(2), 165–196. doi:10.1007/978-1-4614-4960-7_18

Foti, N., Lungu, M., Pelimon, C., Barac, I., Copaitici, M., & Marza, E. (1965). Researches on morphological characteristics and biological features of the bee population in Romania. Proceedings of XXth Jubiliar International Congress of Beekeeping Apimondia. Apimondia. Bucharest, Romania, pp. 171–176.

Franck, P., Garnery, L., Celebrano, G., Solignac, M., & Cornuet, J. M. (2000). Hybrid origins of honeybees from Italy (*Apis mellifera ligustica*) and Sicily (*A. m. sicula*). *Molecular Ecology*, *9*(7), 907–921. doi:10.1046/j.1365-294x.2000.00945.x

Garnery, L., Cornuet, J. M., & Solignac, M. (1992). Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. *Molecular Ecology*, *1*(3), 145–154. doi:10.1111/j.1365-294X.1992.tb00170.x

Garnery, L., Franck, P., Baudry, E., Vautrin, D., Cornuet, J. M., & Solignac, M. (1998). Genetic diversity of the west European honey bee (*Apis mellifera mellifera* and *A. m. iberica*). 1. Mitochondrial DNA. *Genetics Selection Evolution*, *30*(1), 31–47. doi:10.1186/1297-9686-30-S1-S31

Genersch, E., Von Der Ohe, W., Kaatz, H., Schroeder, A., Otten, C., Büchler, R., … Rosenkranz, P. (2010). The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. *Apidologie*, *41*(3), 332–352. doi:10.1051/apido/2010014

Haddad, N. J. (2016). Mitochondrial genome of the Levant Region honeybee, *Apis mellifera syriaca* (Hymenoptera: Apidae). *Mitochondrial DNA Part A*, *27*(6), 4067–4068. doi:10.3109/19401736.2014.1003846

Haddad, N. J., Batainh, A. M., Migdadi, O. S., Saini, D., Krishnamurthy, V., Parameswaran, S., & Alhamuri, Z. (2016). Next generation sequencing of *Apis mellifera syriaca* identifies genes for *Varroa* resistance and beneficial bee keeping traits. *Insect Science*, *23*(4), 579–590. doi:10.1111/1744-7917.12205

Ilyasov, R. A., Poskryakov, A. V., & Nikolenko, A. G. (2016). Seven genes of mitochondrial genome enabling differentiation of honeybee subspecies *Apis mellifera*. *Russian Journal of Genetics*, *52*(10), 1062–1070. doi:10.1134/S1022795416090064

Ivanova, E., Bienkowska, M., & Petrov, P. (2011). Allozyme polymorphism and phylogenetic relationships in *Apis mellifera* subspecies selectively reared in Poland and Bulgaria. *Folia Biologica*, *59*, 3–4. doi:10.3409/fb59_3-4.121-126

Iwasa, T., Motoyama, N., Ambrose, J. T., & Roe, R. M. (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protection*, *23*(5), 371–378. doi:10.1016/j.cropro.2003.08.018

Jukes, T. H., & Cantor, C. R. (1969). Evolution of protein molecules. In H. N. Munro (Ed.), *Mammalian protein metabolism* (pp. 21–132). New York: Academic Press.

Kandemir, I., Ozkan, A., & Fuchs, S. (2011). Reevaluation of honeybee (*Apis mellifera*) microtaxonomy: a geometric morphometric approach. *Apidologie*, *42*(5), 618–627. doi:10.1007/s13592-011-0063-3

Keightley, P. D., Trivedi, U., Thomson, M., Oliver, F., Kumar, S., & Blaxter, M. L. (2009). Analysis of the genome sequences of three *Drosophila melanogaster* spontaneous mutation accumulation lines. *Genome Research*, *19*(7), 1195–1201. doi:10.1101/gr.091231.109

Kim, J. S., Kim, M. J., & Kim, I. (in press). *The complete mitochondrial genome of Italian honey bees Apis mellifera ligustica*

(Hymenoptera: Apidae). KX90820 Submitted to GenBank, 27 September 2016. Unpublished.

Klein, A. M., Vaissiere, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 303–313. doi:10.1098/rspb.2006.3721

Koulianos, S., & Crozier, R. H. (1997). Mitochondrial sequence characterisation of Australian commercial and feral honeybee strains, *Apis mellifera* L. (Hymenoptera: Apidae), in the context of the species worldwide. *Australian Journal of Entomology*, *36*(4), 359–364. doi:10.1111/j.1440-6055.1997.tb01486.x

Kukrer, M., Kence, M., & Kence, A. (2017). Genetic evidences for the impact of anthropogenic factors on honey bee diversity. *BioRxiv*, (1), 1–28. doi:10.1101/154195

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*, 1870–1874. doi: 10.1093/molbev/msw054.

Maa, T. C. (1953). An inquiry into the systematics of the tribus Apidini or honeybees (Himenoptera). *Treubia*, *21*, 525–640.

Mărghitaş, L. A., Coroian, C., Dezmirean, D., Stan, L., & Furdui, E. (2010). Genetic diversity of honeybees from Moldova (Romania) based on mtDNA analysis. *Bulletin of University of Agricultural Sciences and Veterinary Medicine. Animal Science and Biotechnologies*, *67*(1–2), 396–402. doi: 10.15835/buasvmcn-asb:67:1-2:5330

Mărghitas, L. A., Dezmirean, D., Teleky, O. R., Furdui, E., Moise, A., Stan, L., … Coroian, C. (2009). Biodiversity testing of transylvanian honeybee populations using mtDNA markers. *Bulletin of University of Agricultural Sciences and Veterinary Medicine. Animal Science and Biotechnologies*, *66*(1–2), 402–406. doi:10.15835/buasvmcn–asb:66:1-2:3391

Mărghitas, L. A., Teleky, O., Dezmirean, D., Rodica, M., Bojan, C., Coroian, C., … Moise, A. (2008). Morphometric differences between honey bees *Apis mellifera carpatica* populations from transylvanian area. *Lucrări Stiintifice Zootehnie si Biotehnologii*, *41*(2), 309–315.

Meixner, M. D., Worobik, M., Wilde, J., Fuchs, S., & Koeniger, N. (2007). *Apis mellifera mellifera* in Eastern Europe - morphometric variation and determination of its range limits. *Apidologie*, *38*(2), 191–197. doi:10.1051/apido:2006068

Natsopoulou, M. E., McMahon, D. P., Doublet, V., Frey, E., Rosenkranz, P., & Paxton, R. J. (2017). The virulent, emerging genotype B of Deformed wing virus is closely linked to overwinter honeybee worker loss. *Scientific Reports*, *7*, 1–9. doi:10.1038/s41598-017-05596-3

Oleksa, A., Chybicki, I., Tofilski, A., & Burczyk, J. (2011). Nuclear and mitochondrial patterns of introgression into native dark bees (*Apis mellifera mellifera*) in Poland. *Journal of Apicultural Research*, *50*(2), 116–129. doi:10.3896/IBRA.1.50.2.03

Ollerton, J., Erenler, H., Edwards, M., & Crockett, R. (2014). Extinctions of aculeate pollinators in Britain and the role of large-scale agricultural changes. *Science*, *346*(6215), 1360–1362. doi:10.1126/science.1257259

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos*, *120*(3), 321–326. doi:10.1111/j.1600-0706.2010.18644.x

Özdil, F., Aytekin, I., Ilhan, F., & Boztepe, S. (2012). Genetic variation in Turkish honeybees *Apis mellifera anatoliaca*, *A. m. caucasica*, *A. m. meda* (Hymenoptera: Apidae) inferred from RFLP analysis of three mtDNA regions (16S rDNA-COI-ND5). *European Journal of Entomology*, *109*(2), 161–167. doi:10.14411/eje.2012.021

Özdil, F., Yildiz, M. A., & Hall, H. G. (2009). Molecular characterization of Turkish honey bee populations (*Apis mellifera*) inferred from mitochondrial DNA RFLP and sequence results. *Apidologie*, *40*(5), 570–576. doi:10.1051/apido/2009032

Palmer, M. R., Smith, D. R., & Kaftanoglu, O. (2000). Turkish honeybees: genetic variation and evidence of a fourth lineage of *Apis mellifera* mtDNA. *Journal of Heredity*, *91*(1), 42–46. doi:10.1093/jhered/91.1.42

Papachristoforou, A., Rortais, A., Bouga, M., Arnold, G., & Garnery, L. (2013). Genetic characterization of the Cyprian honey bee (*Apis mellifera cypria*) based on microsatellites and mitochondrial DNA polymorphisms. *Journal of Apicultural Science*, *57*(2), 127–134. doi:10.2478/jas-2013-0023

Péntek-Zakar, E., Oleksa, A., Borowik, T., & Kusza, S. (2015). Population structure of honey bees in the Carpathian Basin (Hungary) confirms introgression from surrounding subspecies. *Ecology and Evolution*, *5*(23), 5456–5467. doi:10.1002/ece3.1781

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology and Evolution*, *25*(6), 345–353. doi:10.1016/j.tree.2010.01.007

Rand, D. M., & Kann, L. M. (1998). Mutation and selection at silent and replacement sites in the evolution of animal mitochondrial DNA. *Genetica*, *102/103*, 393–407. doi:10.1023/A:1017006118852

Ruttner, F. (1988). *Biogeography and taxonomy of honeybees* (p. 288). Berlin, Heidelberg: Springer-Verlag. doi:10.1016/0169-5347(89)90176-6

Saitou, N., & Nei, M. (1987). The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406–425.

Satta, Y., Ishiwa, H., & Chigusa, S. I. (1987). Analysis of nucleotide substitutions of mitochondrial DNAs in *Drosophila melanogaster* and its sibling species. *Molecular Biology and Evolution*, *4*, 638–650. doi:10.1093/oxfordjournals.molbev.a040464

Seplyarskiy, V. B., Kharchenko, P., Kondrashov, A. S., & Bazykin, G. A. (2012). Heterogeneity of the transition/transversion ratio in *Drosophila* and Hominidae genomes. *Molecular Biology and Evolution*, *29*(8), 1943–1955. doi: 10.1093/molbev/mss071

Smith, D. R. (2002). Genetic diversity in Turkish honey bees. *Uludağ Arıcılık Dergisi*, *2*(8), 10–17. doi:10.1080/09064700600641681

Smith, D. R., Slaymaker, A., Palmer, M., & Kaftanoğlu, O. (1997). Turkish honey bees belong to the East Mediterranean mitochondrial lineage. *Apidologie*, *28*(5), 269–274. doi:10.1051/apido:19970503

Syromyatnikov, M. Y., Borodachev, A. V., Kokina, A. V., & Popov, V. N. (2018). A molecular method for the identification of honey bee subspecies used by beekeepers in Russia. *Insects*, *9*(1), E10. doi:10.3390/insects9010010

Tan, H. W., Liu, G. H., Dong, X., Lin, R. Q., Song, H. Q., Huang, S. Y., … Zhu, X. Q. (2011). The complete mitochondrial genome of the Asiatic cavity-nesting honeybee *Apis cerana* (Hymenoptera: Apidae). *PLoS One*, *6*(8), e23008. doi:10.1371/journal.pone.0023008

Tandon, V., Kumar, A., & Rana, C. (2016). Pollination-its type, threats and role in environment conservation. *International Journal of Current Research*, *8*(8), 35744–35751.

Teleky, O. R., Bojan, C., Moise, A., Coroian, C., Dezmirean, D., & Mărghitaş, L. A. (2009). Ecotypes differentiation within honeybee (Apis mellifera carpatica) from

Transylvania. *Bulletin of University of Agricultural Sciences and Veterinary Medicine. Animal Science and Biotechnologies*, *64*(1–2), 1–6. doi:10.15835/buasvmcn–asb:64:1-2:2256

Tennant, E., & Chadwick, F. (2016). *The bee book (p. 221)*. London, UK: Dorling Kindersley Limited.

Uzunov, A., Kiprijanovska, H., Andonov, S., Naumovski, M., & Gregorc, A. (2009). Morphological diversity and racial determination of the honey bee (*Apis mellifera* L.) population in the Republic of Macedonia. *Journal of Apicultural Research*, *48*(3), 196–203. doi:10.3896/IBRA.1.48.3.08

Wolstenholme, D. R., & Clary, D. O. (1985). Sequence evolution of *Drosophila* mitochondrial DNA. *Genetics*, *109*(4), 725–744.