Original research

PHYLOGENETIC RELATIONSHIPS OF RUSSIAN FAR-EAST *APIS CERANA* WITH OTHER NORTH ASIAN POPULATIONS

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Abstract

Apis cerana* Fabricius, 1793 is the eastern honeybee species distributed throughout Asia from the tropical climate in the southern part to the temperate climate in the northern part. We sequenced and annotated the complete mitochondrial DNA (mtDNA) of *A. cerana* from Vladivostok, Primorsky Krai of the Russian Far East and uploaded it to the database GenBank (AP018450). MtDNA sequence has 15,919 bp length, AT-content 84% and GC-content 16% and contains 22 tRNA genes, 13 protein-coding genes, two ribosomal RNA genes, one AT-rich region and four non-coding intergenic regions (NC1-4). All protein-coding genes start with ATT and ATG codons, except for ATC, the start codon of the ATP8 gene, which and stop with the common stop codons TAA and TAG. A comparative analysis of complete mtDNA of *A. cerana* from China, Indonesia, Korea, Malaysia, Russia, Taiwan, Thailand, Vietnam, and Japan found that the Russian Far East *Apis cerana* differed from others on the subspecies level. Based on the comparative analysis of complete mtDNA (~16,000 bp), nuclear DNA (nDNA) gene Vitellogenin (VG) (~4,100 bp) and morphological measurements (six parameters), we assumed that the Russian Far-East *A. cerana* can be a distinct northern Asia population and can be described as a separate unique subspecies of *A. c. ussuriensis* subsp. nov. *A. c. koreana* subsp. nov. is also validated and described as a new subspecies.

Keywords: *Apis cerana, Apis cerana ussuriensis*, mitochondrial genome, new subspecies, Russian Far East, vitellogenin

INTRODUCTION

The *Apis cerana* Fabricius, 1793 and *Apis mellifera* Linnaeus, 1758, the most important honeybee species in the world, have separately inhabited for eight million years Asia and Europe with Africa, respectively (Ruttner, 1988). *Apis cerana* occurs across southern and southeastern Asia up to Russia in northern Asia and extends from Japan in the east and Afghanistan in the west. It occupies a large range of climatic conditions, from cool regions in higher latitudes and altitudes, dry semi-desert environments and tropical climates. *A. cerana* has a high

genetic and phenotypic variations occurring across a range of spatial scales and adaptations to various climatic conditions, which led to the division into several biology ecotypes in the geographic cline of the north to south (Ruttner, 1988, Radloff et al., 2010). The most northern Russian Far East population of *A. cerana* is feral and occurs in the Russian territories of Primorsky Krai and Khabarovsky Krai to the 47° 54′ N (Proshchalykin et al., 2014).

The complete mtDNA can be a more effective and useful tool to study population genetics and phylogeographics of *A. cerana* (Ilyasov et al., 2018b). In this article, we have analyzed the complete sequence of the mtDNA of *A. cerana* from the Russian Far East in comparison with other *A. cerana* populations to denominate the unique strain and uncover its phylogenetic relationships with other subspecies. In addition, the nuclear DNA (nDNA) gene *Vitellogenin* (*VG*) and morphometry studies were used as supporting data. The phylogenetic uniqueness of *A. cerana* from the Russian Far East was proved based on three types of tests of the mitochondrial DNA (mtDNA), nuclear DNA (nDNA) and morphology.

MATERIAL AND METHODS

The sampling of *Apis cerana* bees

Three types of tests based on the mtDNA, nDNA, and morphology were performed on *A. cerana* bees. In order to sequence the mtDNA and measure the morphology, the adult honeybee workers of the feral Russian Far East *A. cerana* were collected in the forests of the Primorsky Krai near the city of Vladivostok in 2018 (N43°13′ E132°03′). In order to sequence the nDNA, the adult *A. cerana* workers were collected from apiaries in South Korea (*A. c. koreana* subsp. nov. from the Sangju, Gyeo-

Fig. 1. Geographical distribution of *A. cerana* samples used in the comparative analysis of complete mtDNA sequences and two possible ways of their migration in northern Asia.

ngsangbukdo and Sancheong, Gyeongsangnam-do), Japan (*A. c. japonica* Radoszkowski, 1887 from the Kitahiroshima, Hokkaido) and Taiwan (Taichung, Taiwan) in 2015 (Fig. 1).

Extraction of DNA

The DNA was extracted from the thoracic muscle tissue using the Wizard Genomic DNA Purification Kit (PROMEGA, Madison, WI, USA) according to the manufacturer's recommendations. DNA samples were stored at -20°C until further use. Genomic libraries were prepared with the Nextera DNA Library Preparation Kit (ILLUMINA, USA) according to the instructions of the manufacturer. Sequencing was prepared with the NextSeq 500/550 High Output Kit v2 (75 cycles) (ILLUMINA, USA) following the instructions of the kit.

Analysis of mitochondrial DNA

The complete mitochondrial DNA was sequenced with paired-end read runs (2 x 150 bp) using the Illumina Next Seq 500 (ILLUMINA, United States) following the instructions of the reagents at the Department of Life Sciences of Kyoto Sangyo University. A complete mtDNA was obtained as a result of assembling of 1,662,000 reads with average coverage level seventy-five by Geneious R9 (BIOMATTERS, New Zealand) and annotation by MITOS web servers (Germany) (Bernt et al., 2013) and tRNAscan-SE (CA, USA) (Lowe & Eddy, 1997). The resultant mtDNA consisting of 15,919 bp was uploaded into the DDBJ/GenBank database (AP018450). The phylogenetic analysis was performed using the MEGA7 software (Kumar, Stecher, & Tamura, 2016) based on the nucleotide sequences of complete mtDNA.

Analysis of nuclear DNA

The polymerase chain reaction (PCR) of the *Vitellogenin* gene (*VG*) exons (exons 2-7) of *A. cerana* samples was performed on the Applied Biosystems Veriti HID 96-Well Thermal Cycler, 0.2mL based on the already developed primers (Kent et al., 2011) and the TaKaRa PCR Amplification Kit (100µl PCR x 100 reactions) (TAKARA BIO INC., Shiga, Japan) according to the manufacturer's instructions. All PCR products were purified by the QIAquick PCR Purification Kit (250) (QIAGEN, Hilden, Germany) following the instructions of the manufacturer.

The nucleotide sequences of the *VG* exons of *A. cerana* samples were determined through the sequencing of the PCR products using the Sanger dideoxy method (Sanger, Nicklen, & Coulson, 1977) on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's instructions at the Division of Life Sciences of the Incheon National University. All products were sequenced from both strands.

The nucleotide sequences of the *VG* exons 1-7 of *A. cerana* were uploaded into the DDBJ/GenBank database. The accession numbers for all *VG* exons for the first sample of *A. cerana* from Vladivostok, Primorsky Krai, Russia are MH755745, MH755780, MH755815, MH755850, MH755885 and MH755920 (4125 bp). The numbers for the second sample of *A. cerana* from Vladivostok, Primorsky Krai, Russia are MH755746, MH755781, MH755816, MH755851, MH755886 and MH755921 (4125 bp). For the *A. c. koreana* from Sangju, Gyeongsangbukdo, South Korea, they are MH755726, MH755761, MH755796, MH755831, MH755866 and MH755901 (4125 bp). For the *A. c. koreana* from Sancheong, Gyeongsangnam-do, South Korea, they are MH755735, MH755770, MH755805, MH755840, MH755875, and MH755910. For the first sample of *A. c. japonica* from Kitahiroshima, Hokkaido, Japan, they are MH755741, MH755776, MH755811, MH755846, MH755881, and MH755916 (4125 bp). For the second sample of *A. c. japonica* from Kitahiroshima, Hokkaido, Japan, they are MH755742, MH755777, MH755812, MH755847, MH755882, and MH755917 (4125 bp). For the first sample of *A. cerana* from Taichung, Taiwan, they are MH755747, MH755782, MH755817, MH755852, MH755887, and MH755922 (4128 bp). For the second sample of *A. cerana* from Taichung, Taiwan, they are MH755748, MH755783, MH755818, MH755853, MH755888, and MH755923 (4128 bp).

The comparative analysis of *A. cerana* based on the complete mtDNA was performed with the use of the following sequences from the GenBank: AP017314 (*A. c. japonica* from Kyoto, Japan, 15917 bp), AP017941 (*A. c. japonica* from Amami Japan, 15778), AP017983 (*A. c. cerana* from Jiangsu, China, 15460 bp), KM244704 (*A. c. cerana* from Yunnan, China, 15712 bp), AP017984 (*A. cerana* from Taipei, Taiwan, 15376), AP018149 *A. cerana* from Sabah, Malaysia, Borneo, 15884), AP018431 (*A. c. koreana* from Jeollanam-do, South Korea, 15925), KX908206 (*A. c. koreana* from Chungcheongbukdo, South Korea, 15904) and NC 001566 (*A. m. ligustica*, USA, out-group, 16324 bp).

The comparative analysis of *A. cerana* based on *VG* was performed with the use of the following sequences from the GenBank: KT725235 (*A. c. cerana* from Yunnan, China, 4125 bp), KZ288206 (781683-788069), *Apis*CC1.0 (*A. c. cerana* from Yunnan, China, 4125 bp), and exons JN557295, JN557387, JN557201, JN557573, JN557481, JN557109 (*A. m. mellifera* from Poland, isolate M2261, out-group, 4074 bp).

Analysis of morphology

The comparative analysis of *A. cerana* based on the morphology was performed using the Statistica 8.0 statistical package (StatSoft Power Solutions, Inc., USA) and JMP14 statistical discovery software (SAS Institute Inc., North Carolina, USA) based on the factor analysis (principal component analysis, PCA) and cluster analysis (Neighbor-Joining (NJ) clustering method based on the Euclidean distances) from previously published morphological data (forewing length and width, cubital index, hind leg length, metatarsal index, and length of tergite 3+4th) of samples from Korea (*A. c. koreana* from Wanju, Jeollabukdo, Korea N=10 and from Yangyang, Gangwondo, Korea N=10), China (*A. c. cerana* from Yunnan, China, N=120 and from Beijing, China, N=4), Thailand (*A. cerana* from Thailand, N=8), Vietnam (*A. cerana* from Vietnam, N=17), Japan (*A. c. japonica* from Kyoto, Japan, N=8), Russia (*A. cerana* from Vladivostok, Primorsky Krai, Russia, N=11) and the out-group (*A. mellifera* from Korea, N=10) (Lee & Choi, 1986; Ruttner, 1988; Tan et al., 2003) which were measured according to methods described by Ruttner (1988).

Methods of statistics

Pairwise nucleotide sequence divergences were estimated using Unipro UGENE 1.28 (UNIPRO, Russia) and CLC Genomics Workbench 11 (CLCbio, Denmark) on the basis of complete mtDNA sequences with Jukes-Cantor (Jukes & Cantor, 1969), Tamura-Nei (Tamura & Nei, 1993) and p-distance models (Nei & Kumar, 2000). Pairwise Euclidean distances between *A. cerana* populations based on morphology data were calculated using Statistica 8.0. Based on pairwise alignments, amino acid identity (%) was calculated for homologous genes. Phylogenetic trees were constructed using the Reltime method (Tamura et al., 2012) and branch lengths estimated inferred using the Neighbor-Joining method (Saitou & Nei, 1987) based on Jukes-Cantor model with 1000 bootstrap replications (Saitou & Nei, 1987). The circular physical map of the complete mtDNA of the Russian Far East *A. cerana* was constructed using CLC Genomics Workbench 11 and Artemis 17.0.1 (The Sanger Institute, Hinxton, Cambridge, UK). The Student's t-test was used for testing a hypothesis based on a difference between means of morphology data.

RESULTS

Variation in the complete mtDNA

The complete mitochondrial genome sequence of *A. cerana* from the Russian Far East (15,919 bp in length) contained a total of thirteen protein-coding genes, twenty-two *tRNA* genes, two *rRNA* genes (*16S rRNA* and 12S *rRNA*) and four non-coding control regions (*NC1- NC4*). The mtDNA of the Russian Far East *A. cerana* was slightly shorter than that of *A. mellifera* mtDNA (16,343 bp) and *Drosophila yakuba* (16,019 bp). All non-coding intergenic sequences in Korean *A. cerana* mtDNA (1,252 bp) were slightly shorter than that for *D. yakuba* (1,262 bp) and *A. mellifera* (1,639 bp) (Crozier & Crozier, 1993) (Tab. 1).

Table 1.

Properties of the complete mtDNA of the Russian Far East *A. cerana*

Nucleotide	Count (Frequency)
GC-content	2,540 (0.16)
AT-content	13,373 (0.84)
Adenine A	6,729 (0.42)
Cytosine C	1,542 (0.10)
Guanine G	998 (0.06)
Thymine T	6,644 (0.42)
Dinucleotide AA	2,973 (0.19)
Dinucleotide AC	475 (0.03)
Dinucleotide AG	358 (0.02)
Dinucleotide AT	2,921 (0.18)
Dinucleotide CA	675 (0.04)
Dinucleotide CC	239 (0.02)
Dinucleotide CG	70 (0.01)
Dinucleotide CT	558 (0.04)
Dinucleotide GA	471 (0.03)
Dinucleotide GC	116 (0.01)
Dinucleotide GG	136 (0.01)
Dinucleotide GT	275 (0.02)
Dinucleotide TA	2,609 (0.16)
Dinucleotide TC	712 (0.05)
Dinucleotide TG	432 (0.03)
Dinucleotide TT	2,886 (0.18)
Nucleotide A 1 / 2 / 3 positions	240 (0.47) / 128 (0.25) / 249 (0.48)
Nucleotide C 1 / 2 / 3 positions	37 (0.07) / 70 (0.14) / 22 (0.04)
Nucleotide G 1 / 2 / 3 positions	50 (0.1) / 19 (0.04) / 11 (0.02)
Nucleotide T 1 / 2 / 3 positions	188 (0.37) / 298 (0.58) / 233 (0.45)
Length, bp	15,919
Weight (single-stranded), kDa	4,904.98

The overall nucleotide composition of the Russian Far East *A. cerana* was A (42%), T (42%), G (6%), C (10%) and a significant AT bias (84%). These mtDNA properties are very similar to *A. c. japonica* Radoszkowski, 1887 (AP017941, AP017985, AP017314), *A. c. cerana* Fabricius, 1793 (AP017984, AP017983, KM244704, GQ162109) (Tan et al., 2011; Okuyama, Tingek, & Takahashi, 2017), *A. m. ligustica* Spinola, 1806 (NC_001566) (Crozier & Crozier, 1993), *A. m. caucasica* Pollmann, 1889 (AP018404), *A. m. carpathica* Barac, 1977 (AP018403), *A. m. meda* Skorikov, 1929 (KY464957) and *A. m. syriaca* Skorikov, 1929 (KY926882). Russian Far East *A. cerana* mtDNA was characterized by the highest frequencies of AA (19%), AT (18%), TT (18%) and TA (16%) dinucleotides and the lowest frequencies of GG (1%), GC (1%), CG (1%) and CC (2%) dinucleotides. This could be a consequence of the AT-richness of most honeybee mtDNA.

Similar to other *A. cerana* samples (Lee et al., 2016), the mtDNA sequence of the Russian Far East *A. cerana* has four relatively big intergenic regions. The non-coding control region *NC1* (228 bp) is located between genes *tRNA-MET*

and *tRNA-GLN, NC2* (89 bp) between genes *tRNA-LEU*$^{(TAA)}$ and *COX2, NC3* (68 bp) between genes *COX3* and *tRNA-GLY and NC4* (51 bp) located between genes *tRNA-PRO* and *ND6*. All non-coding intergenic and AT-rich regions in the complete mtDNA constitute about 8% of the entire mtDNA size.

We found that Russian Far East *A. cerana* belonged to haplotype Japan01 of *the NC2* region and identical with sequences KP064995 and AP018431. The *NC1* region of the Russian Far East *A. cerana* was found to be similar to the *A. cerana ACNC101* haplotype and differed from the Korean *A. c. koreana* samples KP064870 and KP064972 by only one insertion 31insT, relatively to the *NC1* sequence of Russian Far East *A. cerana*. Ten *A. cerana* haplotypes of the *NC1* region (*ACNC101 - ACNC110*) were published earlier (Lee et al., 2016; Ilyasov et al., 2018a). This haplotype with 31insT insertion is new and is named *ACNC111* (Fig. 2).

The motif TACTTA in the AT-rich intergenic non-coding regions may correspond to the binding site for the mitochondrial transcription terminator (mtTERM), a transcription attenuation factor (Tan et al., 2001). The motif TACTTA was presented in the complete mtDNA Russian Far East *A. cerana* eight times including reverse direction. Across the complete mtDNA sequences of Russian Far East *A. cerana*, the two most repetitive eight-nucleotide motifs were detected: the AATTAATT motif repeated forty-eight times including reverse direction and the AATAAATT motif seventy-four times including reverse direction. These two repetitive motifs differ from each other by only one transversion T>A in the fourth position.

The gene composition and arrangement of the Russian Far East *A. cerana* were similar to the typical structure of complete mtDNA of some honeybee species of the genus *Apis* (Crozier & Crozier, 1993; Tan et al., 2011). Most of the mtDNA genes (*CYTB, COX1, COX2, COX3, ND2, ND3, ND6, ATP6,* and *ATP8*) were located on the light strand except for four subunit genes *(ND1, ND4, ND4L,* and *ND5*), two *rRNA* genes (12S and *16S rRNA*) and eight *tRNA* genes, which were located on the heavy strand. The mtDNA genes overlapped a total of thirty bp in five locations ranging from one to nineteen bp between genes *tRNA-GLN* and *tRNA-ALA* (4 bp), *ND2* and *tRNA-CYS* (1 bp), *COX1* and *tRNA-LEU*$^{(UUR)}$ (5 bp), *COX2* and *tRNA-ASP* (1 bp), *ATP8* and *ATP6* (19 bp). Obviously, the overlapping of mtDNA genes originates from the prokaryotic genome with the polycistronic type of transcription (Tab. 2).

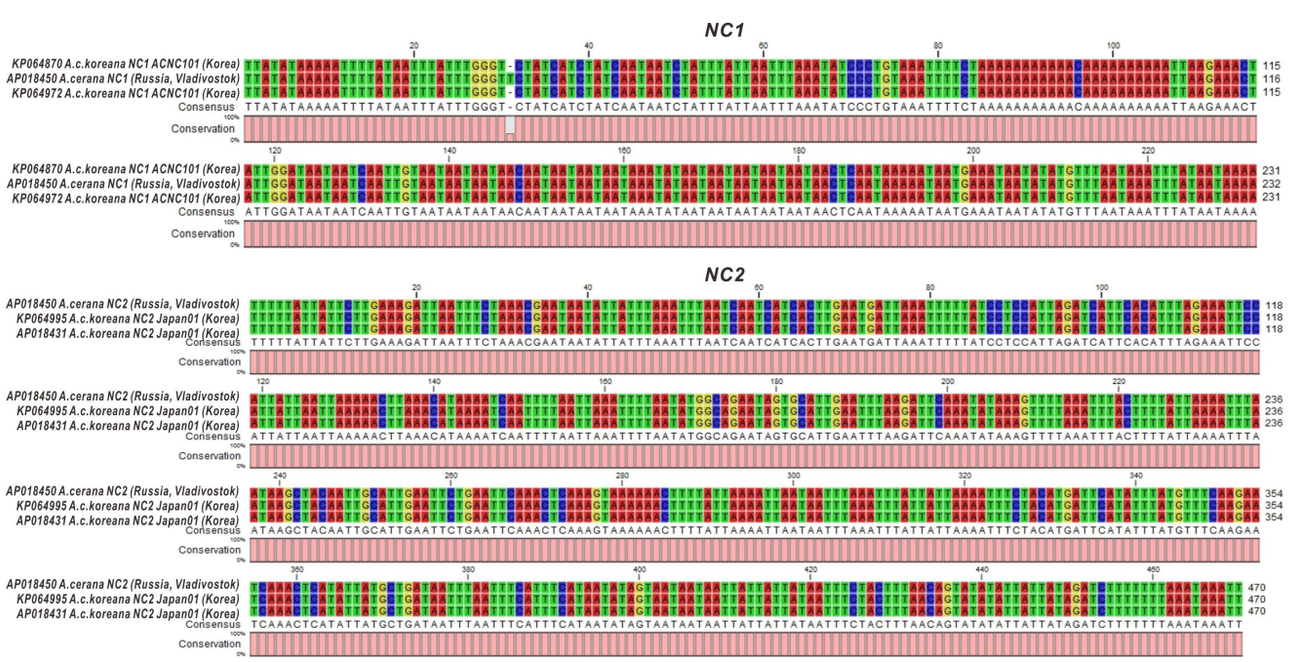

Fig. 2. Alignment of NC1 and NC2 non-coding intergenic region sequences of the Russian Far East A. cerana with close relative haplotypes *ACNC101* and *Japan01*, respectively.

Table 2.

Short annotation of the complete mtDNA of the Russian Far East *A. cerana*

No.	Genes	Start position	End position	Size, bp
1	*tRNA-Ser(AGN)*	1	60	60
2	*tRNA-Glu*	64	129	66
3	*tRNA-Met*	164	229	66
4	*tRNA-Gln*	462	527	66
5	*tRNA-Ala*	524	589	66
6	*tRNA-Ile*	608	673	66
7	*ND2*	674	1669	996
8	**tRNA-Cys*	1669	1734	66
9	**tRNA-Tyr*	1740	1808	69
10	*tRNA-Trp*	1825	1893	69
11	*COX1*	1894	3459	1566
12	*tRNA-Leu(UUR)*	3455	3524	70
13	*COX2*	3614	4294	681
14	*tRNA-Asp*	4294	4361	68
15	*tRNA-Lys*	4368	4439	72
16	*ATP8*	4446	4607	162
17	*ATP6*	4589	5266	678
18	*COX3*	5284	6063	780
19	*tRNA-Gly*	6136	6202	67
20	*ND3*	6203	6556	354
21	**tRNA-Arg*	6577	6645	69
22	*tRNA-Asn*	6665	6732	68
23	**tRNA-Phe*	6751	6821	71
24	**ND5*	6828	8495	1668
25	**tRNA-His*	8496	8561	66
26	**ND4*	8579	9910	1332
27	**ND4L*	9913	10176	264
28	*tRNA-Thr*	10200	10266	67
29	**tRNA-Pro*	10282	10359	78
30	*ND6*	10411	10923	513
31	*CYTB*	10936	12084	1149
32	*tRNA-Ser(UCN)*	12108	12174	67
33	**ND1*	12187	13101	915
34	**tRNA-Leu(CUN)*	13102	13170	69
35	**16S rRNA*	13171	14499	1329
36	**tRNA-Val*	14500	14566	67
37	**12S rRNA*	14567	15353	787
	Total mtDNA	1	15919	15919

* - the genes which are transcribed from the heavy strand of mtDNA.

All thirteen protein-coding genes (11,058 bp in length) of the Russian Far East *A. cerana* mitochondrial genome were annotated through comparison with the published sequences of other honeybees. In all, 3698 amino acids were encoded, of which the genes *COX3, ATP6*, and *CYTB* were regarded ATG as the start codon, while the gene *ND4* started with ATA, the gene *ATP8* with ATC and the genes *ND3*, *ND4L*, *ND5*, *COX1*, *ND6*, *COX2*, *ND1*, *ND2* with ATT. All thirteen protein-coding genes were terminated with the TAA stop codon. The twenty-two *tRNA* genes ranged in size from 60 bp at the *tRNA-SER*$^{(AGN)}$ to 78 bp at *tRNA-PRO*.

The *rRNA* genes consist of 2116 bp (12S *rRNA* of 787 bp and *16S rRNA* of 1329 bp). The 12S *rRNA* is located between *tRNA-VAL* and the AT-rich region and the *16S rRNA* between *tRNA-LEU*$^{(CUN)}$, and *tRNA-VAL*. The two 12S *rRNA* and *16S rRNA* genes share similar structures and functions in organisms ranging from bacteria to mammals, even though the sequences exhibit numerous inter- and intraspecific nucleotide variations. Thus, they provide a reliable method for the taxonomic classification of animals.

The complete mtDNA of the Russian Far East *A. cerana* was visualized on the circular physical map which allows the observing of flanking regions and order of loci (colocalization or synteny) (Fig. 3). Fourteen genes *12S rRNA, 16S rRNA, ND1, ND4, ND4L, ND5, tRNA-ARG, tRNA-CYS, tRNA-HIS, tRNA-LEU*$^{(CUN)}$*, tRNA-PHE, tRNA-PRO, tRNA-TYR,* and *tRNA-VAL* are transcribed from the heavy strand, while twenty-

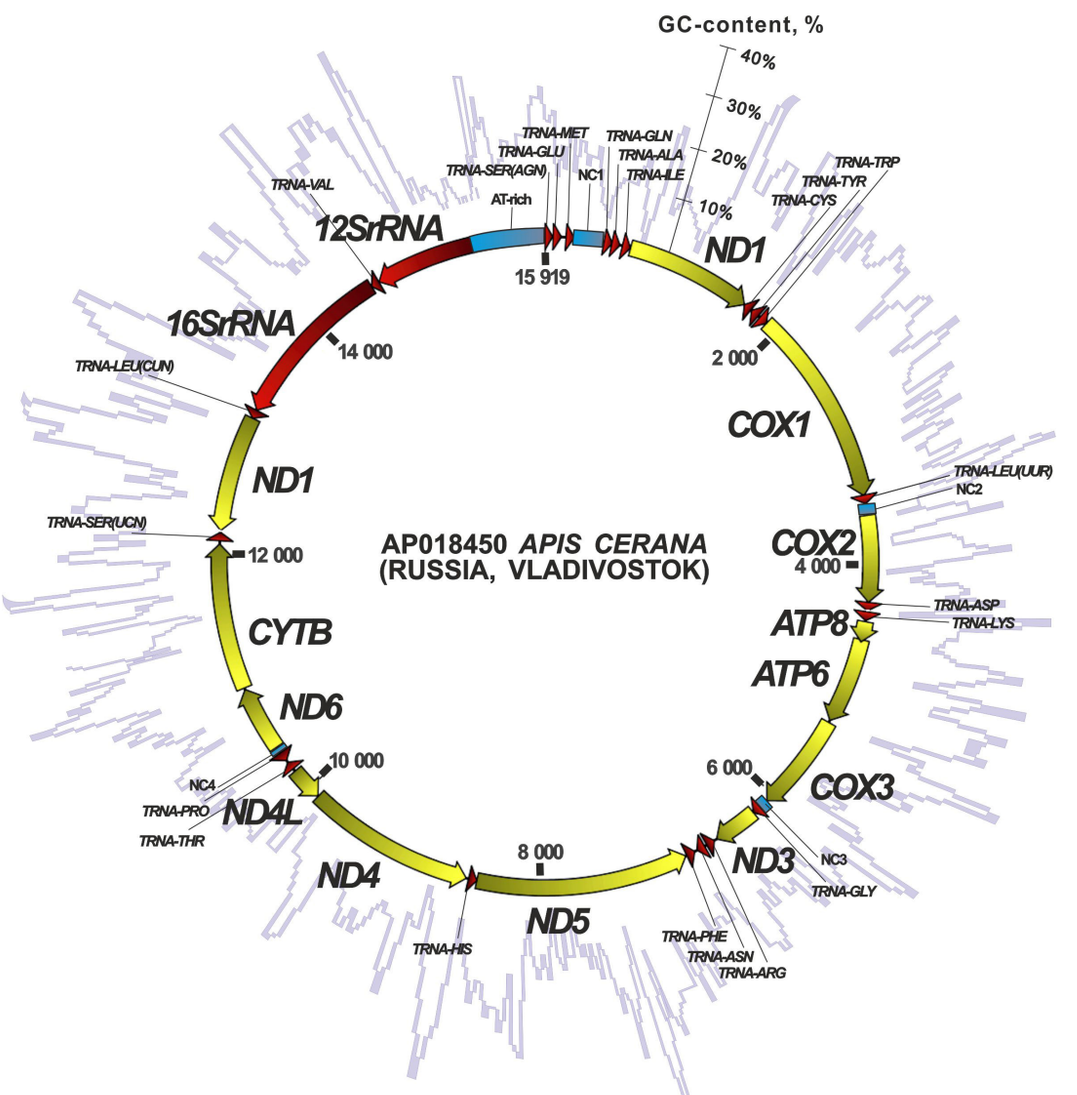

Fig. 3. Circular physical map of the complete mtDNA of the Russian Far East *A. cerana ussuriensis* subsp. nov.

three genes *ATP6, ATP8, COX1, COX2, COX3, CYTB, ND2, ND3, ND6, tRNA-ALA, tRNA-ASN, tRNA-ASP, tRNA-GLN, tRNA-GLU, tRNA-GLY, tRNA-ILE, tRNA-LEU(UUR), tRNA-LYS, tRNA-MET, tRNA-SER$^{(AGN)}$, tRNA-SER(UCN), tRNA-THR,* and *tRNA-TRP* are transcribed from the light strand of mtDNA. Three overlap points were found in the light strand between genes *ATP8* and *ATP6*, *COX1* and *tRNA-LEU$^{(TAA)}$* while *COX2* and *tRNA-ASP*, and one overlap between gene *ND2* of light strand and gene *tRNA-CYS* of the heavy strand. This overlapping of mtDNA genes proves their origin from the prokaryotic genome with the polycistronic type of transcription (Tab. 2).

The average GC-content in the Russian Far East *A. cerana* mtDNA is 16% while the maximal level is less than 40%. A less than 40% GC-content is considered as low frequency. The GC-content was depicted on the map as a light grey histogram outside of the DNA circle (Fig. 3), and the poor regions showed a lower level of genetic diversity and divergence than the rich regions (Kent et al., 2012).

A comparative analysis of the non-coding regions of mtDNA showed that *NC1* region of the Russian Far East *A. cerana* differed from the published in GenBank haplotype *ACNC101* (KP064870) in one deletion (31delT), whereas *NC2* region of the Russian Far East *A. cerana* was identical with the published in GenBank haplotype *Japan01* (KP064995).

The Russian Far East *A. cerana* population has not been well studied. Only thirteen nucleotide sequences of three mitochondrial genes are presented in GenBank: *COX1* - eleven sequences (Ilyasov et al., 2014), *COX2* - one sequence and

Table 3.

Comparison of complete mtDNA of the Russian Far East *A. cerana* with published in GenBank gene sequences of other Russian Far East *A. cerana* samples

Genes	GenBank accession number	Replacement positions relative to the GenBank sequences	Nucleotide (amino acid) replacements
16S rRNA	AF153093	326insT	1 (0)
COX1	KM242593	6CT	1 (0)
	KM242594	6CT	1 (0)
	KM242601	6CT	1 (0)
	AF153107	796AG(Ile-Val)	1 (1)
	KM242596	6CT, 239AG(Lys-Ser)	2 (1)
	KM242599	6CT, 239AG(Lys-Ser)	2 (1)
	KM242600	6CT, 239AG(Lys-Ser)	2 (1)
	KM242602	6CT, 239AG(Lys-Ser)	2 (1)
	KM242597	6CT, 239AG(Lys-Ser), 258CT	3 (1)
	KM242598	6CT, 239AG(Lys-Ser), 258CT, 541TC	4 (1)
	KM242595	6CT, 36GA, 249AG, 258CT, 541TC	5 (0)
COX2	AF153121	6CT, 15TC, 24AG, 39TC, 42GA, 63CT, 105AT, 120CT, 127-128TC-AT(Met-Ser), 157TC, 162CT, 166TC, 177TC, 201TA, 289-291ATT-GTA (Ile-Val), 291TA, 303TC, 321TC, 330TC, 333AT, 342TC, 360TA, 377GA(Ser-Lys), 400TC, 417AT, 426CT, 438TC, 448TC, 462TC, 489TC, 495TA, 496TC, 498AT, 528TA, 567TC, 570CT, 597TA, 621TC, 625-627 ATT-GTA (Ile-Val), 633AT, 651CT, 652TC, 654AT, 663TA, 666TC, 678CT	48 (4)

16S rRNA - one sequence (Tab. 3). A comparative analysis of the GenBank complete mtDNA gene sequences from the Russian Far East *A. cerana* found fifty-three nucleotide replacements, which distributed from one to forty-eight between samples. Most of the nucleotide differences do not lead to amino acid replacements due to the location on the first and the second position of the triplets (Tab. 3).

The tr/tv ratio in the complete mtDNA are 1.93 and 2.46 between the Russian Far East and Chinese *A. cerana* samples, 1.93 and 2.14 between Russian Far East and Japanese *A. cerana* samples, 0.95 and 2.11 between Russian Far East and Korean *A. cerana* samples, 1.10 and 2.39 between Russian Far East and southeastern Asia *A. cerana* samples and 0.47 between all *A. cerana* and *A. mellifera* samples. The tr/tv ratio among *A. cerana* samples is similar to that between *Drosophila melanogaster* lines and mtDNA of four *Drosophila* species (*D. melanogaster*, *D. simulans, D. yakuba,* and *D. erecta*) which is 2.06 (Seplyarskiy et al., 2012) (Tab. 4).

The percent of nucleotide differences and Genetic distances (Jukes-Cantor / Tamura-Nei / p-distance) was calculated based on the complete mtDNA sequences of all *A. cerana* samples. A comparative analysis of the complete mtDNA

Table 4.

The transitions and transversions in the complete mtDNA and in coding sequences between all *A. cerana* samples

		AP018450, *A. cerana*, Russia, Vladivostok	KM244704, *A. c. cerana*, China, Yunnan	AP017983, *A. c. cerana*, China, Jiangsu	AP017314, *A. c. japonica*, Japan, Kyoto	AP017941, *A. c. japonica*, Japan, Amami	KX908206, *A. c. koreana*, Korea, Chungcheongbukdo	AP018431, *A. c. koreana*, Korea, Jeollanamdo	AP017984, *A. cerana*, Taiwan, Taipei	AP018149, *A. c. cerana*, Malaysia, Sabah, Borneo	NC 001566, *A. m. ligustica*, USA, outgroup
		Transversions in complete mtDNA / transversions in CDS									
AP018450, *A. cerana*, Russia, Vladivostok			29 /12	26 /15	15 /5	14 /5	18 /8	42 /10	99 /65	308 /138	1506 /957
KM244704, *A. c. cerana*, China, Yunnan		56 /41		9 /3	17 /9	17 /9	21 /12	53 /18	88 /59	274 /130	1438 /950
AP017983, *A. c. cerana*, China, Jiangsu		64 /52	26 /19		14 /12	14 /12	18 /15	50 /21	85 /58	225 /131	1367 /951
AP017314, *A. c. japonica*, Japan, Kyoto		29 /21	60 /49	70 /60		2 /1	9 /5	43 /13	89 /62	311 /135	1492 /953
AP017941, *A. c. japonica*, Japan, Amami		30 /23	61 /51	71 /62	9 /8		6 /5	45 /13	89 /62	285 /135	1452 /952
KX908206, *A. c. koreana*, Korea, Chungcheongbukdo		38 /25	73 /54	80 /63	30 /18	31 /20		48 /16	92 /65	316 /136	1498 /955
AP018431, *A. c. koreana*, Korea, Jeollanamdo		40 /24	63 /48	73 /57	45 /33	45 /35	39 /23		122 /71	317 /142	1510 /959
AP017984, *A. cerana*, Taiwan, Taipei		237 /197	251 /209	253 /214	244 /206	242 /204	253 /207	245 /204		228 /139	1320 /947
AP018149, *A. c. cerana*, Malaysia, Sabah, Borneo		339 /272	337 /273	334 /277	338 /278	342 /280	355 /281	337 /274	345 /294		1492 /931
NC 001566, *A. m. ligustica*, USA, outgroup		715 /550	713 /554	704 /556	718 /557	710 /554	725 /558	709 /546	691 /543	694 /539	

Transitions in complete mtDNA / transitions in CDS

Table 5.
The pairwise genetic distances (upper triangle), a percentage of genetic divergences, numbers of SNPs and amino acid replacements (lower triangle) based on complete mtDNA sequences of all *A. cerana* samples

	AP018450, *A. cerana*, Russia, Vladivostok	KM244704, *A. c. cerana*, China, Yunnan	AP017983, *A. c. cerana*, China, Jiangsu	AP017314, *A. c. japonica*, Japan, Kyoto	AP017941, *A. c. japonica*, Japan, Amami	KX908206, *A. c. koreana*, Korea, Chungcheong-bukdo	AP018431, *A. c. koreana*, Korea, Jeollanamdo	AP017984, *A. cerana*, Taiwan, Taipei	AP018149, *A. cerana*, Malaysia, Sabah, Borneo	NC 001566, *A. m. ligustica*, USA
Genetic distances Jukes-Cantor / Tamura-Nei / p-distance										
AP018450, *A. cerana*, Russia, Vladivostok		0.006/ 0.002/ 0.002	0.006/ 0.002/ 0.002	0.005/ 0.001/ 0.001	0.005/ 0.001/ 0.001	0.005/ 0.001/ 0.001	0.005/ 0.002/ 0.002	0.022/ 0.006/ 0.006	0.042/ 0.013/ 0.013	0.159/ 0.097/ 0.088
KM244704, *A. c. cerana*, China, Yunnan	1.8 /85 /13		0.002/ 0.001/ 0.001	0.005/ 0.001/ 0.001	0.005/ 0.001/ 0.001	0.006/ 0.001/ 0.001	0.007/ 0.003/ 0.003	0.022/ 0.005/ 0.005	0.040/ 0.013/ 0.013	0.155/ 0.096/ 0.087
AP017983, *A. c. cerana*, China, Jiangsu	3.3 /90 /16	1.5 /35 /5		0.005/ 0.001/ 0.001	0.006/ 0.001/ 0.001	0.006/ 0.001/ 0.001	0.008/ 0.003/ 0.003	0.022/ 0.005/ 0.005	0.037/ 0.013/ 0.013	0.152/ 0.096/ 0.087
AP017314, *A. c. japonica*, Japan, Kyoto	0.9 /44 /7	2.8 /77 /13	3.5 /84 /16		0.002/ 0.001/ 0.001	0.003/ 0.001/ 0.001	0.003/ 0.002/ 0.002	0.022/ 0.005/ 0.005	0.042/ 0.014/ 0.014	0.158/ 0.096/ 0.087
AP017941, *A. c. japonica*, Japan, Amami	1.2 /44 /8	2.2 /78 /14	2.9 /85 /17	0.7 /11 /3		0.005/ 0.001/ 0.001	0.006/ 0.002/ 0.002	0.022/ 0.005/ 0.005	0.041/ 0.014/ 0.014	0.156/ 0.096/ 0.087
KX908206, *A. c. koreana*, Korea, Chungcheong-bukdo	1.1 /56 /13	1.9 /94 /19	3.4 /98 /22	0.9 /39 /12	1.2 /37 /13		0.003/ 0.003/ 0.003	0.023/ 0.006/ 0.006	0.044/ 0.014/ 0.014	0.159/ 0.096/ 0.087
AP018431, *A. c. koreana*, Korea, Jeollanamdo	1.5 /82 /12	2.4 /116 /19	3.9 /123 /22	1.3 /88 /15	1.9 /90 /16	1.1 /87 /17		0.024/ 0.007/ 0.008	0.043/ 0.014/ 0.014	0.159/ 0.097/ 0.088
AP017984, *A. cerana*, Taiwan, Taipei	5.5 /336 /46	4.5 /339 /48	3.1 /338 /47	5.7 /333 /45	5.12 /331 /44	5.6 /345 /51	6.1 /367 /52		0.038/ 0.014/ 0.014	0.148/ 0.095/ 0.087
AP018149, *A. cerana*, Malaysia, Sabah, Borneo	4.8 /647 /89	5.5 /611 /88	6.7 /559 /85	4.9 /649 /87	5.3 /627 /90	4.9 /671 /93	5.2 /654 /95	6.9 /573 /93		0.156/ 0.095/ 0.087
NC 001566, *A. m. ligustica*, USA	20.6 /2221 /585	21.2 /2151 /585	22.2 /2071 /583	20.5 /2210 /581	20.9 /2162 /580	20.5 /2223 /588	20.6 /2219 /587	22.2 /2011 /584	20.5 /2186 /590	

Divergence, % / number of SNPs / Amino acid replacements

sequences showed that the Russian Far East *A. cerana* differed from Chinese *A. c. cerana* by 2.55% (88 SNPs, 15 amino acid replacements), from Japanese samples by 1.10% (44 SNPs, 8 amino acid replacements), from Korean *A. c. koreana* by 1.30% (69 SNPs, 13 amino acid replacements), from southeastern Asia *A. cerana* by 5.15% (492 SNPs, 68 amino acid replacements)

and from *A. m. ligustica* by 20.60% (2221 SNPs, 585 amino acid replacements). In comparison, Japanese *A. c. japonica* differed from Chinese *A. c. cerana* in complete mtDNA sequence by 2.85% (81 SNPs, 15 amino acid replacements), Japanese *A. c. japonica* differed from Korean *A. c. koreana* by 1.33% (64 SNPs, 14 amino acid replacements) and Korean *A. c. koreana* differed from Chinese *A. c. cerana* by 2.90% (108 SNPs, 21 amino acid replacements). Southeastern Asia *A. cerana* differed from northern Asia *A. cerana* by 5.21% (486 SNPs, 69 amino acid replacements). The out-group honeybee species *A. mellifera* differed from *A. cerana* in the complete mtDNA sequence by 21.0% (2161 SNPs, 585 amino acid replacements) (Tab. 5).

Based on the comparative analysis of complete mtDNA sequences of all *A. cerana* samples, the phylogenetic tree was constructed (Fig. 4), on which those from the northern Asian countries of China, Korea, Japan and Russia and the southeastern Asian countries of Taiwan and Borneo of are separate. In the northern group, *A. cerana* samples from China, Russia, and Japan were subdivided into three separate groups according to their geographical habitat. The Korean *A. cerana* samples were subdivided into two groups, which were distantly related to the Russian and Japanese samples, respectively. Such a divergence in complete mtDNA could reflect the migration and geographical isolation of *A. cerana* during its evolution.

A median network reflecting genetic divergences of the complete mtDNA of *A. cerana* samples was constructed according to transversions in all protein-coding mtDNA genes in comparison with the Russian Far East *A. cerana* (Fig. 5). All transversions in the complete mtDNA were placed on connection lines between samples in the median network. The length of the lines in the median network depended on the number of transversions. Amino acid replacements were present on the lines as well (Fig. 5).

Two Korean *A. cerana* samples differed from each other by fourteen SNPs in the complete mtDNA, and on the median network, one was located closer to the Japanese samples while the other was closer to the Russian Far East *A. cerana* samples. Korean *A. c. koreana* samples differed from the Japanese *A. c. japonica* and the Russian Far East *A. cerana* samples on average by nine SNPs in the complete mtDNA.

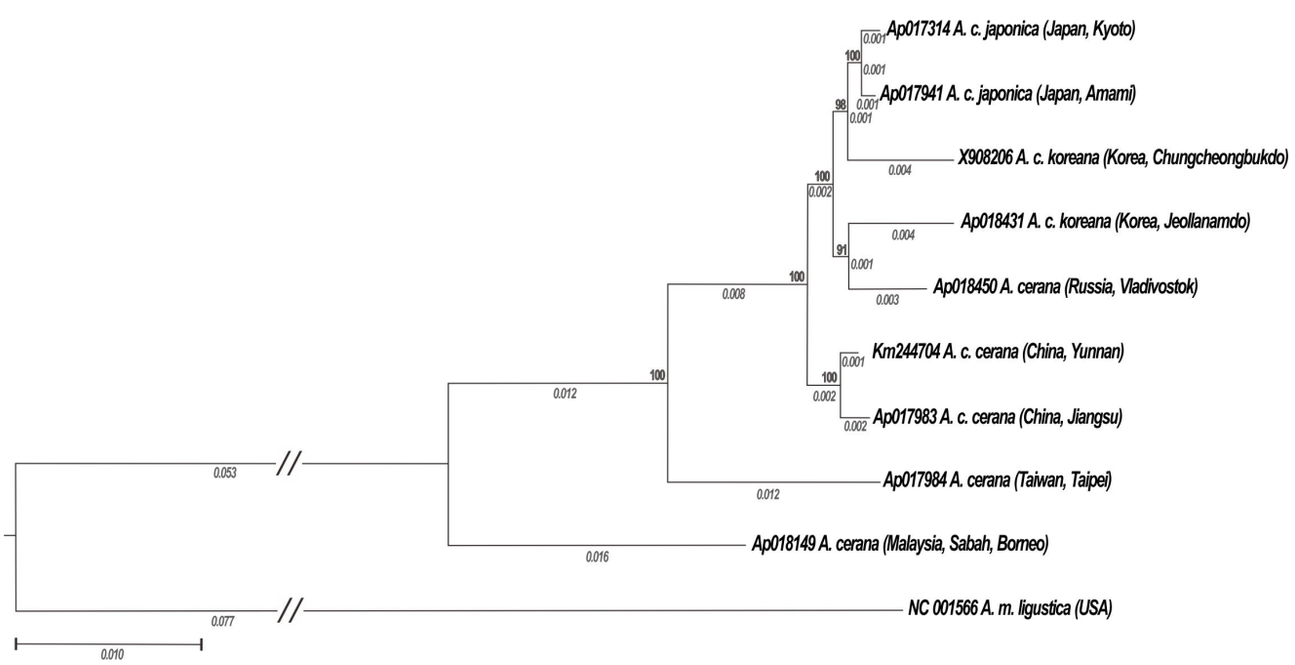

Fig. 4. Phylogenetic tree constructed using a Neighbor-joining algorithm with 1000 bootstrap replications and Jukes-Cantor genetic distances counted based on sequences of complete mtDNA of *A. cerana* samples and rooted with *A. m. ligustica* out-group sample.

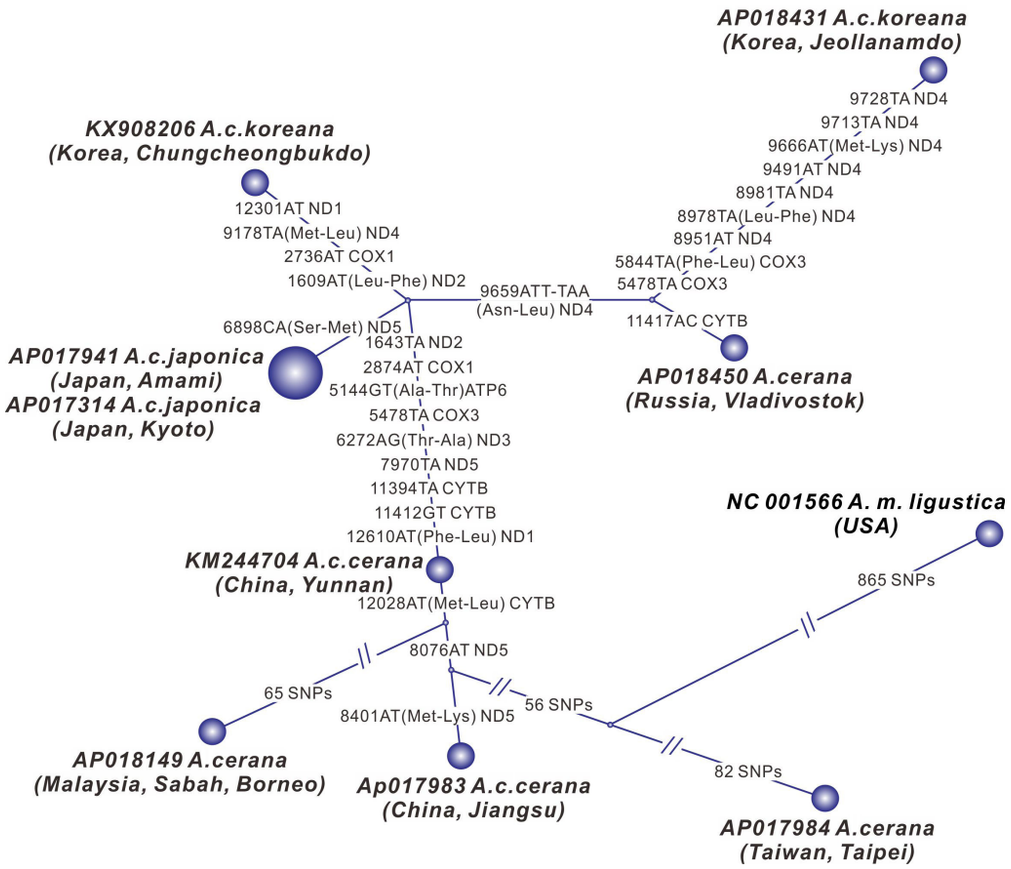

Fig. 5. Phylogenetic relationships of *A. cerana* samples visualized in the median network constructed using transversions in protein-coding mtDNA genes with SNP positions relative to beginning of the complete mtDNA sequence of Russian Far East *A. cerana* sample.

Two southeastern Asia *A. cerana* samples from Borneo and Taiwan differed from all mainland *A. cerana* samples on average by 112 SNPs in the complete mtDNA. The out-group *A. m. ligustica* sample differed from all *A. cerana* samples on average by 951 SNPs in the complete mtDNA.

Variation in gene Vitellogenin of nDNA

Due to mtDNA possesses maternal inheritance, the gene *VG* of nDNA possessing both maternal and paternal inheritance was analyzed. The percent of nucleotide differences and Genetic distances(Jukes-Cantor/Tamura-Nei/p-distance) were calculated based on the sequences of the gene *VG* of nDNA of all *A. cerana* samples. Comparative analysis of the *A. cerana* nDNA gene *VG* sequences showed that the Russian Far East *A. cerana* differs from the Chinese samples by 1.25% (51 SNPs, 24 amino acid replacements), from the Japanese samples by 0.93% (38 SNPs, 21 amino acid replacements), from the Korean samples - by 0.88% (37 SNPs, 22 amino acid replacements), from the southeastern Asia samples by 1.4% (55 SNPs, 24 amino acid replacements) and from *A. m. mellifera* by 9.05% (312 SNPs, 158 amino acid replacements). The Japanese *A. cerana* samples differ from the Chinese samples in nDNA sequence gene *VG* by 0.80% (28 SNPs, 7 amino acid replacements) and from the Korean samples by 0.80% (26 SNPs, 8 amino acid replacements). The Korean samples differ from the Chinese samples by 0.80% (28 SNPs, 8 amino acid replacements). Southeastern Asia *A. cerana* differed from the northern Asia samples by 1.00% (39 SNPs, 12 amino acid replacements). The sister honeybee species *A. mellifera* differs from *A. cerana* in the gene *VG* nDNA sequence by 8.78% (299 SNPs, 148 amino acid replacements) (Tab. 6).

The phylogenetic tree was constructed based on the comparative gene *VG* analysis of nDNA sequences of all *A. cerana* samples (Fig. 6). Similar to the complete mtDNA data, northern (China, Korea, Japan, and Russia) and southeast-

Table 6.

The pairwise genetic distances (upper triangle), a percentage of genetic divergences, numbers of SNPs and amino acid replacements (lower triangle) based on sequences of the gene *VG* of nDNA of all *A. cerana* samples

	A. cerana, Russia, Vladivostok 01	*A. cerana*, Russia, Vladivostok 02	*A. c. cerana*, China, Jiangxi	*A. c. cerana*, China, Yunnan	A. c. japonica, Japan, Kitahiroshima 01	A. c. japonica, Japan, Kitahiroshima 02	*A. c. koreana*, Korea, Gyeongsangbukdo	*A. c. koreana*, Korea, Gyeongsangnamdo	*A. cerana*, Taiwan, Taichung 01	*A. cerana*, Taiwan, Taichung 02	*A. m. mellifera*, Poland
	Genetic distances Jukes-Cantor / Tamura-Nei / p-distance										
A. cerana, Russia, Vladivostok 01		0.002/ 0.003/ 0.001	0.013/ 0.013/ 0.005	0.013/ 0.013/ 0.005	0.008/ 0.008/ 0.004	0.009/ 0.009/ 0.004	0.010/ 0.010/ 0.005	0.010/ 0.011/ 0.005	0.014/ 0.014/ 0.005	0.014/ 0.014/ 0.005	0.081/ 0.082/ 0.026
A. cerana, Russia, Vladivostok 02	0.3/ 12/ 6		0.012/ 0.012/ 0.004	0.012/ 0.012/ 0.005	0.010/ 0.010/ 0.004	0.010/ 0.010/ 0.005	0.008/ 0.008/ 0.004	0.008/ 0.008/ 0.004	0.013/ 0.012/ 0.004	0.013/ 0.013/ 0.004	0.081/ 0.082/ 0.026
A. c. cerana, China, Jiangxi	1.3/ 52/ 22	1.2/ 48/ 22		0.007/ 0.007/ 0.001	0.007/ 0.007/ 0.001	0.006/ 0.006/ 0.001	0.007/ 0.007/ 0.001	0.007/ 0.007/ 0.001	0.009/ 0.009/ 0.001	0.009/ 0.010/ 0.001	0.077/ 0.078/ 0.022
A. c. cerana, China, Yunnan	1.3/ 53/ 26	1.2/ 49/ 24	0.8/ 27/ 6		0.008/ 0.008/ 0.002	0.006/ 0.006/ 0.001	0.007/ 0.006/ 0.001	0.007/ 0.007/ 0.001	0.008/ 0.008/ 0.001	0.008/ 0.008/ 0.001	0.077/ 0.078/ 0.022
A. c. japonica, Japan, Kitahiroshima 01	0.8/ 32/ 20	1.0/ 40/ 22	0.8/ 30/ 6	0.8/ 31/ 10		0.002/ 0.002/ 0.001	0.007/ 0.007/ 0.002	0.007/ 0.007/ 0.002	0.009/ 0.009/ 0.002	0.008/ 0.008/ 0.002	0.076/ 0.077/ 0.023
A. c. japonica, Japan, Kitahiroshima 02	0.9/ 37/ 20	1.0/ 41/ 22	0.7/ 25/ 4	0.7/ 26/ 8	0.2/ 7/ 2		0.006/ 0.006/ 0.001	0.005/ 0.005/ 0.001	0.008/ 0.008/ 0.001	0.007/ 0.008/ 0.001	0.075/ 0.076/ 0.022
A. c. koreana, Korea, Gyeongsangbukdo	1.0/ 42/ 24	0.8/ 32/ 18	0.8/ 30/ 6	0.8/ 27/ 8	0.8/ 28/ 8	0.7/ 25/ 6		0.002/ 0.002/ 0.001	0.008/ 0.008/ 0.001	0.008/ 0.008/ 0.001	0.077/ 0.078/ 0.022
A. c. koreana, Korea, Gyeongsangnamdo	1.0/ 43/ 25	0.8/ 31/ 19	0.8/ 27/ 7	0.8/ 28/ 9	0.8/ 27/ 9	0.7/ 24/ 7	0.2/ 7/ 1		0.008/ 0.008/ 0.001	0.009/ 0.009/ 0.001	0.078/ 0.079/ 0.022
A. cerana, Taiwan, Taichung 01	1.4/ 56/ 24	1.4/ 54/ 24	0.9/ 36/ 8	0.8/ 31/ 10	0.9/ 36/ 8	0.8/ 31/ 6	0.8/ 32/ 8	0.9/ 33/ 9		0.001/ 0.001/ 0.001	0.077/ 0.078/ 0.022
A. cerana, Taiwan, Taichung 02	1.4/ 56/ 23	1.4/ 54/ 23	1.0/ 38/ 9	0.8/ 31/ 11	0.9/ 34/ 9	0.8/ 29/ 7	0.9/ 34/ 9	0.9/ 35/ 10	0.1/ 4/ 1		0.076/ 0.077/ 0.022
A. m. mellifera, Poland	9.0/ 311/ 157	9.1/ 312/ 158	8.7/ 296/ 143	8.7/ 297/ 145	8.7/ 295/ 146	8.6/ 292/ 144	8.7/ 298/ 147	8.8/ 300/ 148	8.8/ 297/ 145	8.7/ 295/ 146	

Divergence, % / number of SNPs / Amino acid replacements

ern Asia (Taiwan and Borneo) *A. cerana* samples were located separately on the gene *VG* of the nDNA-based phylogenetic tree. In the northern group, *A. cerana* samples were subdivided into four separate groups according to their geographical habitat: China, Russia, Japan, and Korea. The Chinese *A. c. cerana* samples were located separately from all others.

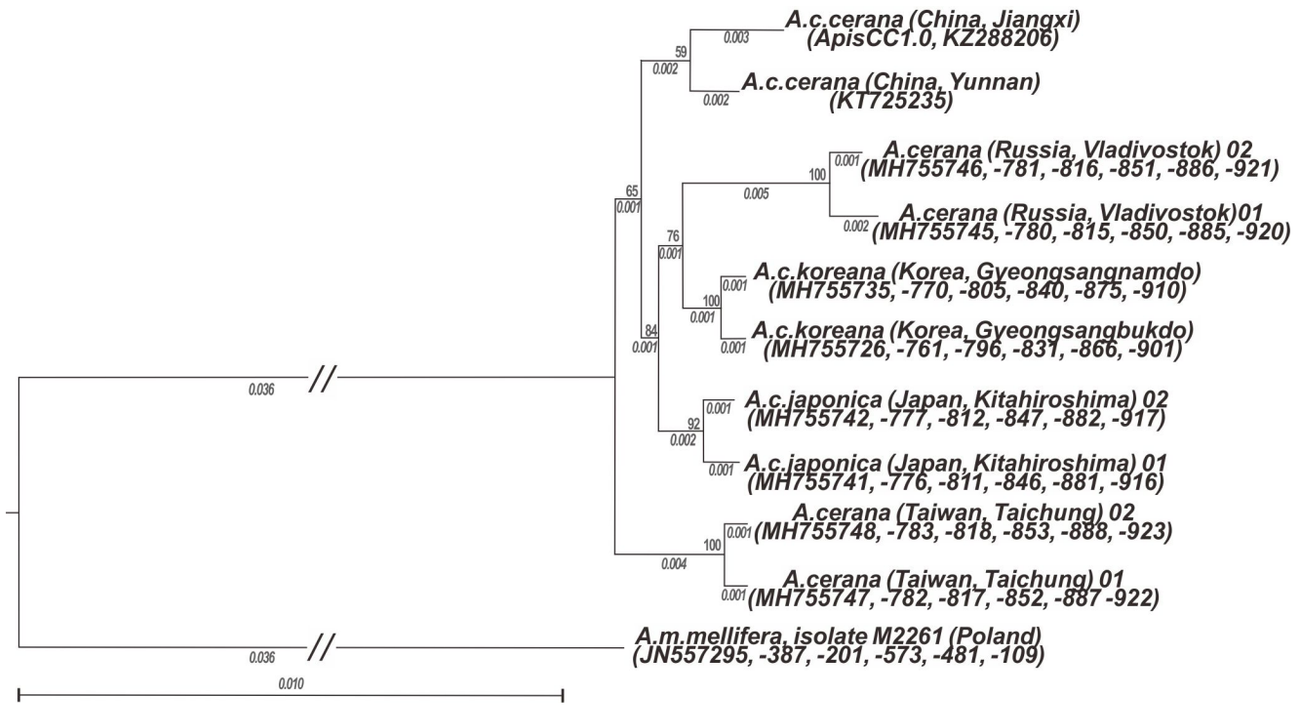

Fig. 6. Phylogenetic relationships of *A. cerana* samples visualized by the tree constructed using a Neighbor-joining algorithm with 1000 bootstrap replications and Jukes-Cantor genetic distances counted based on sequences of nDNA gene VG exons and rooted with *A. m. mellifera* outgroup sample.

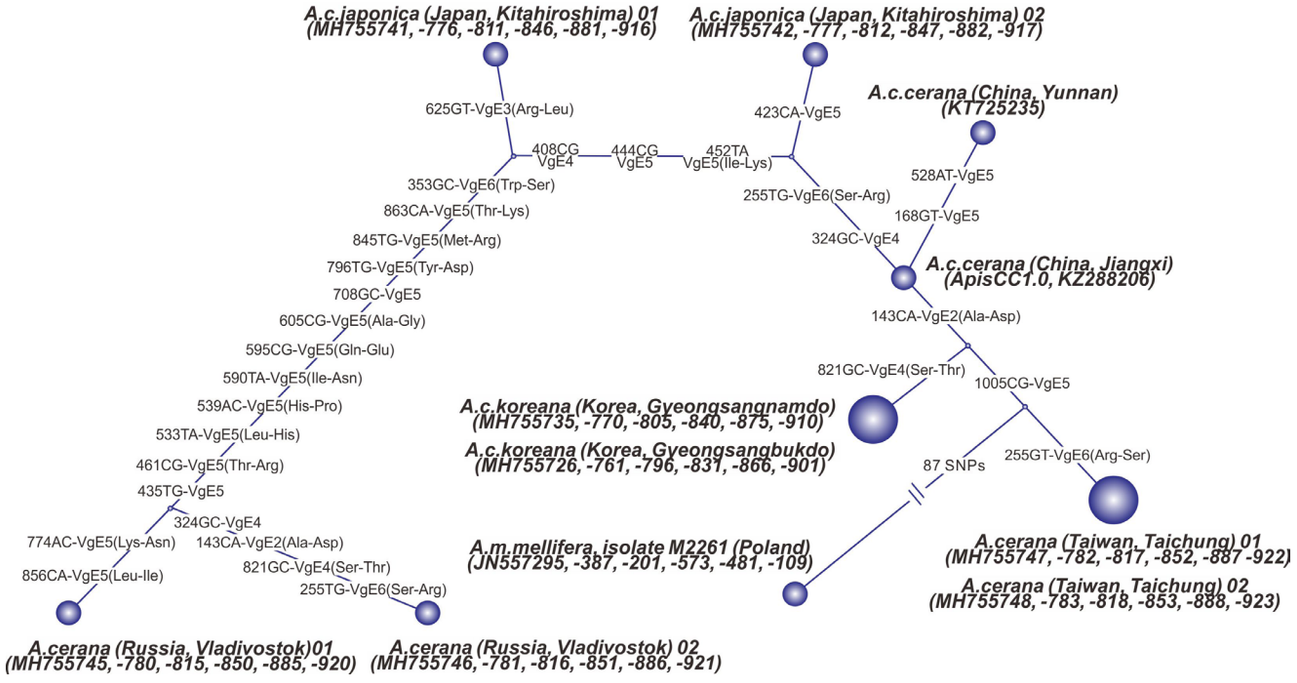

Fig. 7. Phylogenetic relationships of *A. cerana* samples visualized by the median network constructed using transversions in the gene VG of nDNA with SNP positions relative to the beginning of each exon of the first Russian Far East *A. cerana* sample.

A level of genetic divergences based on the gene *VG* of nDNA of *A. cerana* samples can be demonstrated by the median network. The median network was constructed based on transversions in the nDNA gene *VG* in comparison with the Russian Far East *A. cerana* samples (Fig. 7). All transversions in the nDNA gene *VG* were placed on connection lines between samples on the median network. The length of the lines on the median network depended on the number of transversions. Amino acid replacements were presented on the lines of the median network as well (Fig. 7).

On the median network based on the gene, *VG* of nDNA *A. cerana* samples were subdivided according to their geographical position. Two

Table 7.

The pairwise Euclidean distances (upper triangle), a percentage of differences calculated based on morphology data of all *A. cerana* and *A. mellifera* outgroup samples

		A. cerana, Vladivostok, Russia (N=11)	*A. c. cerana*, Yunnan, China (N=120)	*A. c. cerana*, Beijing, China (N=4)	*A. c. japonica*, Kyoto, Japan (N=8)	*A. c. koreana*, Jeollabukdo, Korea (N=10)	*A. c. koreana*, Gangwondo, Korea (N=10)	*A. cerana*, Thailand (N=8)	*A. cerana*, Vietnam (N=17)	*A. mellifera*, Korea (N=10)
		Euclidean distances								
A. cerana, Vladivostok, Russia (N=11)			4.13	3.84	2.25	2.47	2.95	4.08	4.09	6.76
A. c. cerana, Yunnan, China (N=120)		2.32		1.77	4.31	2.71	2.22	2.20	1.37	3.80
A. c. cerana, Beijing, China (N=4)		2.67	1.34		3.55	1.72	1.34	1.44	1.05	4.55
A. c. japonica, Kyoto, Japan (N=8)		2.14	1.80	2.45		2.75	3.15	3.84	4.10	7.24
A. c. koreana, Jeollabukdo, Korea (N=10)	% of differences*	1.79	1.53	1.84	2.28		0.68	2.13	1.76	5.01
A. c. koreana, Gangwondo, Korea (N=10)		2.54	1.22	1.11	1.55	1.75		1.65	1.22	4.87
A. cerana, Thailand (N=8)		5.88	4.50	3.12	2.66	5.06	4.28		0.97	5.30
A. cerana, Vietnam (N=17)		3.28	2.93	2.59	2.13	2.47	2.71	2.46		4.59
A. mellifera, Korea (N=10)		6.38	7.60	8.82	9.23	7.11	7.80	11.58	9.35	

* Statistical significance, Student's t-test, $p<0.05$

Russian Far East *A. cerana* samples differed between each other by six SNPs and were located in one common group separate from other *A. cerana* populations. In contrast to mtDNA data, the nDNA data did not subdivide two Korean *A. cerana* samples. Two Japanese *A. cerana* samples differed from each other by five SNPs based on nDNA data. Korean, Chinese and Japanese *A. cerana* samples differed less between one another (7 SNPs on average) than from the Russian Far East *A. cerana* samples (20 SNPs on average). The out-group sample of *A. m. mellifera* differed from all *A. cerana* samples on average by 156 SNPs in all exons of gene *VG* nDNA (Fig. 7).

Variation in the morphology

A comparative analysis of the morphology data of *A. cerana* samples showed that the Russian Far East *A. cerana* differed from Chinese samples by 2.00%, Japanese samples by 3.14%, Korean samples by 1.66%, southeastern Asia samples by 4.58% and *A. mellifera* by 6.38%. The Japanese *A. cerana* samples differed from the Chinese samples in the morphology analysis by 2.12%, the Japanese samples from the Korean samples by 1.92%, and the Korean samples from the Chinese samples by 1.42%. The southeastern Asia *A. cerana* samples differed from the northern Asia samples by 3.47%. The *A. mellifera* differed from *A. cerana* in the morphology analysis by 8.35%. The morphological differences between populations are statistically significant based on Student's t-test, $p<0.05$ (Tab. 7).

The phylogenetic tree was constructed based on the comparative analysis of the morphology data of all *A. cerana* samples, (Fig. 8). The samples were subdivided into three groups. The first group consisted of Korean *A. c. koreana* samples, the second of Japanese *A. c. japonica* and Russian Far East *A. cerana* samples, and the third of two Chinese *A. c. cerana* and two southeastern Asia *A. cerana* samples from Vietnam and Thailand. The samples from Vietnam and Thailand were closer to each other than with the mainland Chinese *A. c. cerana*. The sister honeybee species *A. mellifera* was located separately from all *A. cerana* samples based on the morphology data analysis.

A genetic divergence level based on morphology data of *A. cerana* samples was demonstrated by the two and three-dimensional PCA plots. The PCA plots were constructed based on the morphology data of *A. cerana* samples in

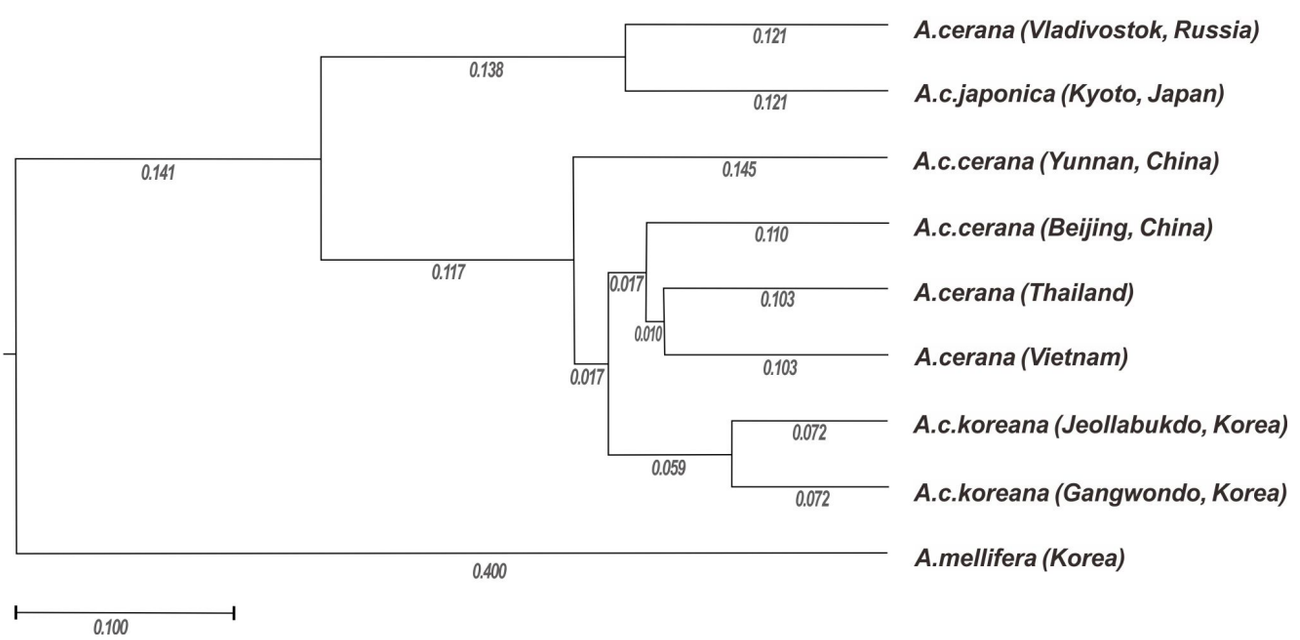

Fig. 8. Phylogenetic tree constructed using a Neighbor-joining algorithm and Euclidian distances based on morphology data of *A. cerana* samples and rooted with *A. mellifera* out-group sample.

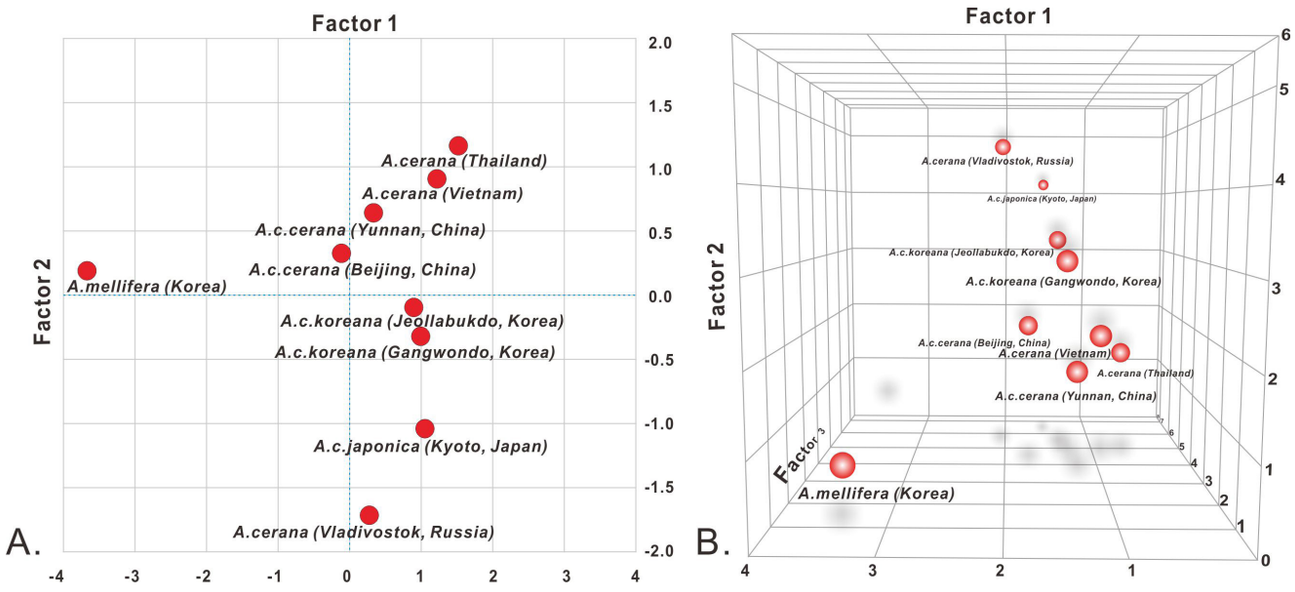

Fig. 9. Phylogenetic relationships of *A. cerana* samples and outgroup *A. mellifera* visualized by Factor PCA analysis based on morphology data. A. Two-dimensional plot. B. Three-dimensional plot.

comparison with out-group *A. mellifera* (Fig. 9).

As far as the morphology possessed the dependence from the environment *A. mellifera* samples were selected from the Asian habitat in order to perform the greatest match with climatic conditions of *A. cerana*. The three-dimensional plot in contrast to the two-dimensional, allowed additional features of phylogenetic honeybee relationships to be seen and thus provided more information.

On both PCA plots, the *A. mellifera* out-group sample was located separately from all *A. cerana* samples. The southern Chinese *A. c. cerana* samples were lying more closely to the south-eastern Asia *A. cerana* samples whereas the northern Chinese samples more closely to northern Asia *A. cerana* samples (Russian, Japanese, and Korean). The western Korean *A. c. koreana* samples were lying more closely to the northern Chinese *A. c. cerana* samples whereas Eastern Korean *A. cerana* samples more closely to the Japanese *A. c. japonica* samples. The Russian Far East *A. cerana* samples were located distantly from the other *A. cerana* samples. Similar to the phylogenetic tree, the southeastern Asia *A. cerana* samples from Vietnam and Thailand were located near each other and the Chinese *A. c. cerana* samples on two- and three-dimensional plots. The Korean *A. c. koreana* samples were located between the Chinese *A. c. cerana* and Japanese *A. c. japonica* samples. This distribution of samples on both plots matched to their geographical distribution on the physical map and partially reflected the migration patterns of honeybees throughout Asia.

DISCUSSION

AT and GC content in mtDNA of Russian Far East *A. cerana*

The AT-content in the mtDNA of Russian Far East *A. cerana* was 84%, similar to AT-content in many such insect species as *A. c. cerana* 84%, *A. c. koreana* 84.1%, *A. mellifera* 84.9%, *Bombus hypocrita* 85.3%, *B. ignitus* 86.8%, *Cephus cinctus* 82%, *Enicospilus* sp. 85.2%, *Melipona bicolor* 86.7%, *Polistes humilis* - 84.7% and *Spathius agrili* - 84% (Tan et al., 2011; Ilyasov et al., 2018a).

Non-coding regions in mtDNA of the Russian Far East *A. cerana*

A phylogenetic analysis based on non-coding region *NC1* found ten haplotypes of *A. cerana* (*ACNC101, ACNC102, ACNC103, ACNC104,*

ACNC105, ACNC106, ACNC107, ACNC108, ACNC109) subdivided into Group A and Group B (Lee et al., 2016). Ilyasov et al. (2018a) found one more new haplotype *ACNC110* in *A. c. koreana*. In the current study, we found new haplotype *ACNC111* of Russian Far East *A. cerana,* which was very similar to *A. c. koreana* but differed from it by one insertion 31insT, with number of position relatively the sequence KP064870. The comparative analysis of non-coding region *NC1* showed that Russian Far East *A. cerana* belongs to the Mainland Asian group, which differed from the populations of *A. c. koreana* (Korea) and *A. c. japonica* (Japan). Such a difference is presumed to be the result of natural selection and the adaptive evolution of *A. cerana* in northern Asian environment.

A phylogenetic analysis based on non-coding region *NC2* identified six haplotypes of *A. cerana* (*Japan1, Nepal1, ThaiS1, BurmaN1, BurmaN2, and BurmaN3*) belonging to two *A. cerana* lineages: Mainland Asian and Sundaland (Smith, 2011). We found that Russian Far East *A. cerana* belongs to the Japan1 haplotype. A predominance of Japan1 haplotype in the non-coding region *NC2* throughout Asia suggests a common origin of the whole *A. cerana* population and distribution across all of Asia.

Phylogenetic pattern of *Apis cerana* populations

All trees based on the three tests are characterized by a similarity of clustering into the two big groups of southeastern and northern Asia. The southeastern Asia *A. cerana* samples are grouped with one Chinese *A. c. cerana* from Beijing only on the morphology-based tree. This is because *A. cerana* lives in neighboring countries where all honeybee movements are unrestricted. The out-group samples of *A. mellifera* are located separately on all trees. On the mtDNA based tree, two Korean *A. c. koreana* samples in one large shared group subdivided into one part distantly combined with Japanese *A. c. japonica* and another with Russian Far East *A. cerana*, while Chinese *A. c. cerana* was located separately from all *A. cerana* groups. On the nDNA-based tree, all *A. cerana* samples from the different populations were located separately in one big shared group, which distantly consisted of Russian Far East *A. cerana*, Japanese *A. c. japonica* and Korean *A. c. koreana*, whereas Chinese *A. c. cerana* samples were located separately from all *A. cerana* groups. On the morphology tree, two Chinese *A. c. cerana* samples in one big shared group subdivided into one part distantly combined with southeastern Asia *A. cerana* samples and another located separately from all *A. cerana* samples, whereas the group of Korean *A. c. koreana* and combined group of Russian Far East *A. cerana* and Japanese *A. c.* japonica was located separately from all *A. cerana* groups.

Median networks and two- and three-dimensional plots constructed based on mtDNA, nDNA, and morphology data supported the topology of phylogenetic trees - South and North Asian *A. cerana* samples were grouped separately. The out-group samples of *A. mellifera* were located separately on plots and networks. The Chinese *A. c. cerana* samples were predominantly located between the southeastern and northern Asia *A. cerana* samples. Similar to the tree, on the mtDNA-based network the Korean *A. c. koreana* samples subdivided into two groups, one combined with the Russian Far East *A. cerana* samples and the other with Japanese *A. c. japonica* samples.

On the median networks, we can mark the most important transversions, which have a part in adaptation to local environments. On the mtDNA-based median network, the Russian Far East *A. cerana* sample differed from one Korean *A. c. koreana* from Jeollanam-do by 10 SNPs in genes *ND4*, *COX3*, *CYTB* (3 amino acid replacements), another Korean *A. c. koreana* from Chungcheongbukdo by 8 SNPs in genes *ND2*, *ND4*, *COX1*, *CYTB* (3 amino acid replacements), both Japanese *A. c. japonica* from Amami and Kyoto by 5 SNPs in genes *ND4*, *ND5*, *CYTB* (3 amino acid replacements), one Chinese *A. c. cerana* from Yunnan by 13 SNPs in genes *ND1*, *ND2*, *ND3*, *ND4*, *ND5*, *COX3*, *CYTB*, *ATP6* (4 amino acid replacements) and another Chinese *A. c. cerana* from Jiangsu by 16 SNPs in genes *ND1*, *ND2*, *ND3*, *ND4*, *ND5*, *COX3*, *CYTB*, *ATP6* (6 amino acid replacements) (Fig. 5). On the nDNA-based median

network, Russian Far East *A. cerana* samples differed from two Japanese *A. c. japonica* from Kitahiroshima on average by 17 SNPs in *VG* gene Exons 2, 3, 4, 5, 6 (on average, 14 amino acid replacements), two Korean *A. c. koreana* from Gyeongsangnam-do on average by 22 SNPs in *VG* gene Exons 2, 4, 5, 6 (on average, 18 amino acid replacements) and two Chinese *A. c. cerana* from Yunnan and Jiangxi on average by 22 SNPs in *VG* gene Exons 2, 4, 5, 6 (on average, 15 amino acid replacements). These mtDNA and nDNA SNPs can be used for the identification and differentiation of Russian Far East *A. cerana* samples from other *A. cerana* populations.

Based on the analysis of trees and networks we assume that the Korean *A. c. koreana* samples subdivided into two separate groups because of historical migration patterns. *A. cerana* came from China to the northern territories, isolated in the Korean peninsula, and further migrated to the Russian Far East and the Japanese archipelago. The population of *A. c. cerana* on the China mainland was not entirely isolated and interpopulation gene flow still occurs along major environmental gradients. Thus, our data support the earlier statement that subspecies *A. c. japonica* originally came to Japan from the Korean peninsula (Takahashi & Yoshida, 2003). The three major northern Asia *A. cerana* populations in Russia, Korea, and Japan have probably been isolated for a relatively short evolutionary time and the speciation process is ongoing.

Genetic distances between *Apis cerana* populations

Three genetic distances between samples (Jukes-Cantor, Tamura-Nei, p-distance) were calculated. All three genetic distances mostly matched one another, so we used the Jukes-Cantor distance for discussion. A comparative analysis of all north Asian *A. cerana* population shows that in the study of mtDNA the Russian Far East *A. cerana* population is most similar to the Japanese *A. c. japonica* population (genetic distance 0.005 and divergence 1.10%) and most differs from the Chinese *A. c. cerana* population (genetic distance 0.006, divergence 2.55%). In the study of nDNA, it is most similar to the Korean *A. c. koreana* population (genetic distance 0.010, divergence 0.88%) and most differs from the Chinese *A. c. cerana* population (genetic distance 0.013, divergence 1.25%). In the morphology study, it is most similar to the Japanese *A. c. japonica* population (Euclidian distance 2.250, divergence 2.14%) and most differs from the Chinese *A. c. cerana* population (Euclidian distance 3.985, divergence 2.50%).

A comparison showed that in the study of mtDNA the Korean *A. c. koreana* population differs more from the Chinese *A. c. cerana* population (genetic distance 0.007, divergence 2.90%) than from the Japanese *A. c. japonica* population (genetic distance 0.006, divergence 1.33%). In the study of nDNA, it differed more from the Chinese *A. c. cerana* population (genetic distance 0.007, divergence 0.80%) than from Japanese *A. c. japonica* population (genetic distance 0.007, divergence 0.75%). In the study of morphology, it differed more from the Japanese *A. c. japonica* population (Euclidian distance 2.950, divergence 1.92%) than from the Chinese *A. c. cerana* population (Euclidian distance 1.998, divergence 1.43%).

In the study of mtDNA The Japanese *A. c. japonica* population differed more from the Korean *A. c. koreana* population (genetic distance 0.006 and divergence 1.33%) than from the Chinese *A. c. cerana* population (genetic distance 0.005 and divergence 2.85%) In the study of nDNA, it differs equally from the Chinese *A. c. cerana* and Korean *A. c. koreana* populations (genetic distance 0.007 and divergence 0.75%). In the study of morphology, it differs more from the Chinese *A. c. cerana* population (Euclidian distance 3.930, divergence 2.21%) than from the Korean *A. c. koreana* populations (Euclidian distance 2.950, divergence 1.92%).

The ranges of inter- and intraspecific genetic distance and genetic divergence in different insect taxa were calculated by Tan et al. (2011), Han et al. (2016) and Eimanifar et al. (2017). We assumed that the level of genetic divergence between individuals within the insect subspecies varied from 0.00% to 0.80% and the genetic distance from 0.000 to 0.005. The level of genetic divergence between subspecies within

insect species varied from 0.80% to 8.00%, and the genetic distance varied from 0.005 to 0.100. The level of genetic divergence between species within the genera varied from 8.00% to 17.00%, and the genetic distance from 0.100 to 0.200. Thus, the genetic divergence range of 0.80% - 8.00% and genetic distance of 0.005 - 0.100 matched the range of intraspecific levels of differences in insects (Tan et al., 2011; Han et al., 2016; Eimanifar et al., 2017). The percent of divergence and genetic distance among the Russian Far East, Korean, Japanese and Chinese *A. cerana* populations matched the intraspecific-level range of differences in other insects. Based on differences in the complete mtDNA analysis we suggested that the Russian Far East, Korean, Japanese and Chinese honeybee samples could be representatives of the distinct subspecies of *A. cerana.*

Two samples of Chinese *A. c. cerana* and two samples of Korean *A. c. koreana* greatly differ from each other with a divergence of 1.5% and 1.2% and Jukes-Cantor genetic distances 0.002 and 0.003, respectively. We assumed it to the result of introgressive hybridization with other introduced *A. cerana* subspecies from neighboring countries. As well, two ecotypes of subspecies could be in different parts of the countries, geographically and anthropogenically isolated from each other for a relatively long time. We suppose that different climatic zones in China are inhabited by different *A. cerana* subspecies (Tan et al., 2003; Tan, Warrit, & Smith, 2007; Lee et al., 2016).

The mtDNA appears to evolve at a similar rate in a wide array of insects, about 2.3% per one million years (Myr). Since the molecular clock idea had been available, the provisional dating of evolutionary events was possible (De Salle et al., 1987; Johns & Avis, 1998). Based on the rate of mtDNA evolution, we could estimate that the Russian Far East *A. cerana* population diverged from the Chinese *A. c. cerana* population about 1.1 Myr ago, from the Japanese *A. c. japonica* 0.5 Myr ago, from the Korean *A. c. koreana* 0.6 Myr ago and from the southeastern Asia *A. cerana* population 2.2 Myr ago. The Korean *A. c. koreana* population separated from the Chinese *A. c. cerana* population about 1.3 Myr ago and from the Japanese *A. c. japonica* 0.6 Myr ago. The Japanese *A. c. japonica* population separated from the Chinese *A. c. cerana* population about 1.2 Myr ago. The northern Asia *A. cerana* population separated from southeastern Asia about 2.3 Myr ago. The *A. cerana* population diverged from the *A. mellifera* population about 9.1 Myr ago. Thus, we assumed that the *A. cerana* subspecies divergence occurred about 1 - 0.5 Myr ago similarly to *A. mellifera.*

Due to the uniqueness of the Russian Far East *A. cerana* population, we designated the Russian Far East *A. cerana* population as a unique subspecies of the *Apis cerana ussuriensis* subsp. nov. The *Apis cerana koreana* subsp. nov. is validated and described as a new subspecies, but further in-depth studies have to prove this statement. The Russian Far East *A. c. ussuriensis* subsp. nov. had such unique properties as the ability to withstand the cold climate and winter in northern Asia and is similar to *A. m. mellifera*. Because of the gene pool and population structure of the Russian Far East *A. c. ussuriensis* are endangered (Pesenko et al., 1989), this subspecies should be included in the international biodiversity conservation program.

Description of new subspecies

***Apis cerana koreana* Ilyasov, Park, Takahashi et Kwon, subsp. nov.**

Apis cerana koreana Ilyasov et al., 2018a: 189, 210; 2018b: 26, unavailable name [International Commission on Zoological Nomenclature, ICZN Articles 16.1, 16.4].

Type material. Holotype, worker, South Korea, Jeollanam-do, Hakjung-ri District, Gokseong-eup Town, Gokseong-gun County, 11.05.2001, leg. J. Takahashi [Kyoto Sangyo University, Japan]. **Paratypes**: 6 workers, South Korea, Gyeongsangnam-do, Goseong; 5 workers, South Korea, Jeollabuk-do, Wanju, 23.04.2018, leg. R. Ilyasov [Incheon National University, South Korea].

Diagnosis. Based on differences of complete mtDNA analysis, the Korean, Japanese, Chinese, and Taiwanese *Apis cerana* samples can be representatives of distinct subspecies of *A. cerana* (Ilyasov et al., 2018a, Tab. 6). The levels of

genetic divergence and genetic distance among the Korean, Japanese, Chinese and Taiwanese *A. cerana* samples are matched to the range of intraspecific levels of differences in other insects - 0.80% - 8.00% (0.005 - 0.100).

Distribution. Korean Peninsula (South Korea, North Korea).

Comments. Chinese *Apis cerana* Fabricius, 1793, Japanese *A. c. japonica* Radoszkowski, 1887, and Korean *A. c. koreana* subsp. nov. are subspecies at an early stage of the sub-speciation process. Populations isolated by sea, mountains, and distance in taiga forests.

Based on transversions in mtDNA, the Korean *A. c. koreana* differs from Chinese *A. c. cerana* by nineteen SNPs (six amino acid replacements), Japanese *A. c. japonica* by thirteen SNPs (six amino acid replacements), southeastern Asia *A. cerana* by fifty-eight SNPs (sixteen amino acid replacements). On the median network and tree diagrams, the Korean, Japanese, and Chinese *A. cerana* samples were separated from one another (Ilyasov et al., 2018a, Tab. 4; Fig. 6). Analogous differentiation of the Korean, Chinese and Japanese *A. cerana* populations can be obtained through nuclear gene studies. We analyzed *VG* (Vitellogenin) (~6,000 bp) and *EF1-α* (Elongation factor 1-alpha) (~2,000 bp) genes of nuclear DNA which previously had been used in the phylogenetic studies of honeybees (Kent et al., 2011; Ilyasov, Poskryakov, & Nikolenko, 2015). On both tree diagrams (Jukes-Cantor distances, Neighbor-Joining method) constructed by CLC Genomics Workbench 11, the Korean, Japanese and Chinese *A. cerana* samples were grouped separately from one another. The species of honeybees *A. mellifera* Linnaeus, 1758 (Amel_4.5), *A. dorsata* Fabricius, 1793 (Apis dorsata 1.3) and *A. florea* Fabricius, 1787 (Aflo_1.0) were used as out-group samples (Ilyasov et al., 2018a, Fig. 8).

A similar differentiation of the Korean, Chinese, and Japanese *A. cerana* populations was obtained through morphology studies on both tree and plot diagrams (Ilyasov et al., 2018a, Fig. 7). Based on the factor and cluster analysis in Statistica 8.0 of morphological data (forewing's length and width, cubital index, hind leg length, metatarsal index, and the length of tergite 3+4th) (Ruttner, 1988) we can observe that the Korean (N=40), Chinese (N=124) and Japanese (N=8) *A. cerana* samples (Tan et al., 2003; Lee & Choi, 1986) are grouped separately from one another on the tree and plot diagrams. The Japanese *A. c. japonica* samples most differed from both Chinese and Korean samples.

***Apis cerana ussuriensis* Ilyasov, Takahashi, Proshchalykin, Lelej et Kwon, subsp. nov.**

Apis indica: Lawrjochin, 1947: 31.

Apis indica var. *japonica*: Lawrjochin, 1960: 238.

Apis indica sinensis ussuriensis Goetze, 1964: 26, unavailable name [ICZN Articles 10.2, 13.1, 15.2].

Apis cerana cerana: Pesenko et al., 1989: 529; Lelej, 1995: 580; Kuznetsov, 2002:1; Kuznetsov & Proshchalykin, 2004: 6; Kuznetsov, 2005: 9; Kuznetsov & Lelej, 2005: 83;

Apis cerana: Proshchalykin, 2012: 473; Proshchalykin et al., 2014: 295.

Apis cerana ussuriensis Ilyasov et al., 2018c: 28, unavailable name [ICZN Articles 16.1, 16.4].

Type material. Holotype, worker, Russia, Primorsky Krai, Vladivostok, 12.06.2017, leg. J. Takahashi [Kyoto Sangyo University, Japan]. **Paratypes**: twenty workers, Russia, Primorsky Krai, Vladivostok, 13.05.2018, leg. M. Proshchalykin [Federal Scientific Center of the East Asia Terrestrial Biodiversity, Vladivostok, Russia; Incheon National University, South Korea].

Diagnosis. Based on differences of complete mtDNA analysis, the Russian, Korean, Japanese, Chinese and Taiwanese *Apis cerana* samples are representatives of distinct *A. cerana* subspecies (Tab. 5, 6; Fig. 4, 5). The levels of genetic divergence and genetic distance between Russian, Korean, Japanese and Chinese *A. cerana* samples are matched to the range of intraspecific levels of differences in other insects - 0.80% - 8.00% (0.005 - 0.100).

Distribution. Russian Far East (Primorsky Krai, south of Khabarovsky Krai).

Comments. Chinese *Apis cerana cerana* Fabricius, 1793, Japanese *A. cerana japonica* Radoszkowski, 1887, and Korean *A. cerana koreana*, subsp. nov. are subspecies at an early stage of

the sub-speciation process. Populations isolated by sea, mountains, and distance in taiga forests. Based on transversions in mtDNA, the Russian *A. cerana* differs from the Chinese *A. cerana* by eighty-eight SNPs (fifteen amino acid replacements), Japanese *A. cerana* by forty-four SNPs (eight amino acid replacements), Korean *A. cerana* by sixty-nine SNPs (thirteen amino acid replacements), southeastern Asia *A. cerana* by 492 SNPs (sixty-eight amino acid replacements). On the median network graphs and tree diagrams, the Russian, Korean, Japanese, and Chinese *A. cerana* samples were separated from each other (Tab. 5; Fig. 4, 5).

The differentiation of the Russian, Korean, Chinese, and Japanese *A. cerana* populations was obtained through morphological studies. Based on the factor and cluster analysis in Statistica 8.0 and in JMP14 statistical discovery software of morphological data (forewing's length and width, cubital index, hind leg length, metatarsal index, and the length of tergite 3+4th) (Ruttner, 1988) we can observe that the Russian (N=11) Korean (N=20), Chinese (N=120) and Japanese (N=8) *A. cerana* samples (Ken et al., 2003; Lee & Choi, 1986) are grouped separately from one another on the tree and plot diagrams (Figs 8, 9). The species of honeybees *A. mellifera* Linnaeus, 1758 (N=10) were used as out-group samples (Lee & Choi, 1986). The differentiation of Russian, Korean, Chinese and Japanese *A. cerana* populations were observed through nuclear gene studies. We analyzed *VG* (Vitellogenin) (~6,000 bp) gene of nuclear DNA which previously had been used in the phylogenetic studies of honeybees (Kent et al., 2011; Ilyasov, Poskryakov, & Nikolenko, 2015). On both tree diagram and median network graph (Jukes-Cantor distances, Neighbor-Joining method) constructed by CLC Genomics Workbench 11, the Russian, Korean, Japanese and Chinese *A. cerana* samples are grouped separately from one another (Ilyasov et al., 2018a, Fig. 8).

Remarks. The *Apis indica ussuriensis* Lawrjochin, 1960 in Pesenko et al. (1989) was cited incorrectly because Lawrjochin had written about *Apis indica* var. *japonica* (see above) and had never used the name *ussuriensis*. The subsequent using of this name (Goetze, 1964) was also unavailable according to ICZN (1999).

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