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, 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 A. 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-
ngsangbukdo and Sacheong, 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, MH755831, MH755866 and MH755901 (4125 bp). For the A. c. koreana from Sancju, Gyeongsangbukdo, South Korea, they are MH755726, MH755761, MH755796, MH755831, MH755866 and MH755910 (4125 bp). For the A. c. koreana from Sancju, Gyeongsangbukdo, South Korea, they are MH755726, MH755761, MH755796, MH755831, MH755866 and MH755910 (4125 bp). For the A. c. japonica from Kitahiroshima, Hokkaido, Japan, they are MH755741, MH755777, MH755812, MH755847, MH755882, and MH755917 (4125 bp). For the second sample of A. c. japonica from Kitahiroshima, Hokkaido, Japan, they are MH755741, 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. c. 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. cerana* 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), *ApisCC1.0* (*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+4) 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).
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<sup>UA</sup> 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<sup>UUR</sup> (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.

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* - 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<sup>AGN</sup> 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<sup>CLN</sup> 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<sup>CLN</sup>, tRNA-PHE, tRNA-PRO, tRNA-TYR, and tRNA-VAL are transcribed from the heavy strand, while twenty-

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**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

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<td>6CT</td>
<td>1 (0)</td>
</tr>
<tr>
<td></td>
<td>AF153107</td>
<td>796AG(Ile-Val)</td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td>KM242596</td>
<td>6CT, 239AG(Lys-Ser)</td>
<td>2 (1)</td>
</tr>
<tr>
<td></td>
<td>KM242599</td>
<td>6CT, 239AG(Lys-Ser)</td>
<td>2 (1)</td>
</tr>
<tr>
<td></td>
<td>KM242600</td>
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<td></td>
<td>KM242602</td>
<td>6CT, 239AG(Lys-Ser)</td>
<td>2 (1)</td>
</tr>
<tr>
<td></td>
<td>KM242597</td>
<td>6CT, 239AG(Lys-Ser), 258CT</td>
<td>3 (1)</td>
</tr>
<tr>
<td></td>
<td>KM242598</td>
<td>6CT, 239AG(Lys-Ser), 258CT, 541TC</td>
<td>4 (1)</td>
</tr>
<tr>
<td></td>
<td>KM242595</td>
<td>6CT, 36GA, 249AG, 258CT, 541TC</td>
<td>5 (0)</td>
</tr>
<tr>
<td>COX2</td>
<td>AF153121</td>
<td>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, 625-627ATT-GTA (Ile-Val), 633AT, 651CT, 652TC, 654AT, 663TA, 666TC, 678CT</td>
<td>48 (4)</td>
</tr>
</tbody>
</table>
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 Chinese *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. mela-

Table 4. The transitions and transversions in the complete mtDNA and in coding sequences between all *A. cerana* samples

<table>
<thead>
<tr>
<th>Transversions in complete mtDNA / transversions in CDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transitions in complete mtDNA / transitions in CDS</td>
</tr>
<tr>
<td>AP018450, <em>A. cerana</em>, Russia, Vladivostok</td>
</tr>
<tr>
<td>KM244704, <em>A. c. cerana</em>, China, Yunnan</td>
</tr>
<tr>
<td>AP017983, <em>A. c. cerana</em>, China, Jiangsu</td>
</tr>
<tr>
<td>AP017314, <em>A. c. japonica</em>, Japan, Kyoto</td>
</tr>
<tr>
<td>AP017941, <em>A. c. japonica</em>, Japan, Amami</td>
</tr>
<tr>
<td>KX908206, <em>A. c. koreana</em>, Korea, Jeollanamdo</td>
</tr>
<tr>
<td>AP017984, <em>A. cerana</em>, Taiwan, Taipei</td>
</tr>
<tr>
<td>AP018149, <em>A. c. cerana</em>, Malaysia, Sabah, Borneo</td>
</tr>
<tr>
<td>NC001566, <em>A. m. ligustica</em>, USA, outgroup</td>
</tr>
<tr>
<td>Transitions in complete mtDNA / transitions in CDS</td>
</tr>
<tr>
<td>AP018450, <em>A. cerana</em>, Russia, Vladivostok</td>
</tr>
<tr>
<td>KM244704, <em>A. c. cerana</em>, China, Yunnan</td>
</tr>
<tr>
<td>AP017983, <em>A. c. cerana</em>, China, Jiangsu</td>
</tr>
<tr>
<td>AP017314, <em>A. c. japonica</em>, Japan, Kyoto</td>
</tr>
<tr>
<td>AP017941, <em>A. c. japonica</em>, Japan, Amami</td>
</tr>
<tr>
<td>KX908206, <em>A. c. koreana</em>, Korea, Jeollanamdo</td>
</tr>
<tr>
<td>AP017984, <em>A. cerana</em>, Taiwan, Taipei</td>
</tr>
<tr>
<td>AP018149, <em>A. c. cerana</em>, Malaysia, Sabah, Borneo</td>
</tr>
<tr>
<td>NC001566, <em>A. m. ligustica</em>, USA, outgroup</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th></th>
<th>Genetic distances Jukes-Cantor / Tamura-Nei / p-distance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP018450, <em>A.</em> cerana, Russia, Vladivostok</td>
</tr>
<tr>
<td></td>
<td>0.006/0.006/0.005</td>
</tr>
<tr>
<td></td>
<td>AP017983, <em>A.</em> cerana, China, Yunnan</td>
</tr>
<tr>
<td></td>
<td>0.002/0.002/0.001</td>
</tr>
<tr>
<td></td>
<td>KM244704, <em>A.</em> cerana, Vladivostok, China, Yunnan</td>
</tr>
<tr>
<td></td>
<td>0.002/0.002/0.001</td>
</tr>
<tr>
<td></td>
<td>AP017314, <em>A.</em> japonica, Japan, Kyoto</td>
</tr>
<tr>
<td></td>
<td>0.001/0.001/0.001</td>
</tr>
<tr>
<td></td>
<td>AP017941, <em>A.</em> japonica, Japan, Amami</td>
</tr>
<tr>
<td></td>
<td>0.001/0.001/0.001</td>
</tr>
<tr>
<td></td>
<td>KX908206, <em>A.</em> japonica, Korea, Chungcheong-bukdo</td>
</tr>
<tr>
<td></td>
<td>0.001/0.001/0.001</td>
</tr>
<tr>
<td></td>
<td>AP017941, <em>A.</em> japonica, Korea, Jeollanamdo</td>
</tr>
<tr>
<td></td>
<td>0.001/0.001/0.001</td>
</tr>
<tr>
<td></td>
<td>AP017984, <em>A.</em> cerana, Taiwan, Taipei</td>
</tr>
<tr>
<td></td>
<td>0.001/0.001/0.001</td>
</tr>
<tr>
<td></td>
<td>KX001566, <em>A. m. ligustica</em>, USA</td>
</tr>
<tr>
<td></td>
<td>0.001/0.001/0.001</td>
</tr>
<tr>
<td></td>
<td>Genetic distances Jukes-Cantor / Tamura-Nei / p-distance</td>
</tr>
<tr>
<td></td>
<td>Divergence, % / number of SNPs / Amino acid replacements</td>
</tr>
<tr>
<td></td>
<td>AP018431, <em>A.</em> japonica, Korea, Jeollanamdo</td>
</tr>
<tr>
<td></td>
<td>0.15/2.4/1.3</td>
</tr>
<tr>
<td></td>
<td>AP017984, <em>A.</em> cerana, Taiwan, Taipei</td>
</tr>
<tr>
<td></td>
<td>0.5/4.5/3.1</td>
</tr>
<tr>
<td></td>
<td>AP018149, <em>A.</em> cerana, Malaysia, Sabah, Borneo</td>
</tr>
<tr>
<td></td>
<td>0.48/5.5/6.7</td>
</tr>
<tr>
<td></td>
<td>NC001566, <em>A. m. ligustica</em>, USA</td>
</tr>
<tr>
<td></td>
<td>20.6/21.2/22.2</td>
</tr>
<tr>
<td></td>
<td>SNPs and amino acid replacements (lower triangle) based</td>
</tr>
<tr>
<td></td>
<td>on complete mtDNA sequences of all <em>A. cerana</em> samples</td>
</tr>
<tr>
<td></td>
<td>2.55% (88 SNPs, 15 amino acid replacements), from</td>
</tr>
<tr>
<td></td>
<td>Japanese samples by 1.10% (44 SNPs, 8 amino acid</td>
</tr>
<tr>
<td></td>
<td>replacements), from Korean <em>A. c. cerana</em> by 1.30%</td>
</tr>
<tr>
<td></td>
<td>(69 SNPs, 13 amino acid replacements), from</td>
</tr>
<tr>
<td></td>
<td>southeastern Asia <em>A. cerana</em> by 5.15% (492 SNPs, 68</td>
</tr>
<tr>
<td></td>
<td>amino acid replacements)</td>
</tr>
</tbody>
</table>
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 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 distinctly 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.

![Phylogenetic tree](image)

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.
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 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-
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 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

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

* 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 the 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. 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.](image-url)
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 southeastern 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 agrilli* - 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,*...
**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.

On the median networks, we can mark the most important transformations, 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

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**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.
network, Russian Far East *Apis 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 0.6 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 A. cerana ussuriensis subsp. nov. The A. 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, Poskyryakov, & 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 (Ilyasov et al., 2018a, Tab. 4; Fig. 6).

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 var. japonica* Lawrjochin, 1960: 238.

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


*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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Russian Far East *Apis cerana ussuriensis*


