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Original article

Geographic variation in the Japanese islands of *Apis cerana japonica* and in *A. cerana* populations bordering its geographic range*

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Abstract – Genetic variation among Apis cerana japonica isolates from Japan and Apis cerana isolates from the neighboring areas of Russia, South Korea, and Taiwan was determined from DNA sequences of the mitochondrial DNA non-coding region (between tRNA leu and COII). Three haplotypes were identified among 470 colonies samples at 47 Japanese sites. All isolates from the main Japanese Islands of Honshu, Shikoku, and Kyushu belonged to a single haplotype, a previously reported Japan 1 haplotype. Two new haplotypes were found on the far southern Japanese islands of Amami-Oshima and Tsushima (the Japan 3 and Japan 4 haplotypes, respectively). The A. cerana from Russia and South Korea were the Japan 1 isolate, the A. cerana from Taiwan was the previously known Taiwan haplotype. Our studies showed little genetic variation in the mtDNA of A. cerana japonica, indicating that this genomic region is of limited use for detecting genetic variation among closely related populations of A. cerana.

***Apis cerana* / geographic variation / mitochondrial DNA / Japan / biogeography / population genetics**

1. INTRODUCTION

Apis cerana Fabricius is a cavity-nesting honey bee that is widely distributed from Afghanistan to Ussuria in Russia and from Indonesia to Japan (Ruttner, 1988; Hepburn et al., 2001). Maa (1953) recognized 11 different subspecies in his revision of the *A. cerana* group. In a comprehensive analysis of the bee's morphological characteristics, Ruttner (1988) further reviewed and distinguished four subspecies: *Apis cerana cerana* in Afghanistan, northern Pakistan, northern India, northern China, Taiwan, Korea and Russia (Ussuria); *A. cerana indica* in southeast Asia and southern India; *A. cerana himalaya* from the Himalaya region to Yunnan in China; and *A. cerana japonica* in Japan. In Japan, the species is not present on Hokkaido Island,

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while it is generally distributed over Honshu Island including Shikoku and Kyushu Islands. The species' most southern distribution is Uke Island in the Amami-Oshima Island region (Takahashi and Yoshida, 2002). Okada (1970) reported the most northern distribution to be the Shimokita Peninsula of Aomori in Honshu Island (Fig. 1).

Detailed information on the geographical differentiation of the *A. cerana* group is incomplete (Ruttner, 1988; Smith and Hagen, 1996; Damus and Otis, 1997; Smith et al., 2000; Hepburn et al., 2001; Radloff et al., 2005). In particular, a morphometric analysis of *A. cerana japonica* has been based on limited population numbers and small sample sizes. Akahira and Sakagami (1959a–c) analyzed morphological variance among 1 population on Honshu Island and 8 populations on Kyushu Island and revealed that the Kyushu Island populations were larger in almost all characteristics than the Honshu Island population. Ruttner (1988) compared the morphometric characteristics of the Honshu and Tsushima Island population using multivariate analyses, and showed that bees from both populations are morphologically distinct. Ruttner's study, however, was an analysis based on a small sample size that included few localities.

Cornuet et al. (1991) showed that *A. mellifera* exhibits variation in the non-coding region of mitochondrial DNA (mtDNA), between the tRNA leu gene and the second subunit of the cytochrome oxidase subunit II (COII) genes. Smith and Hagen (1996) used mtDNA sequence analysis of the non-coding region of *A. cerana* samples over almost the entire distribution range of this species and found two Japanese haplotypes (bee with distinct mtDNA sequences) from 5 populations, which they designated as the Japan 1 and Japan 2 haplotypes. Deowanish et al. (1996) used restriction fragment length polymorphism (RFLP) analysis of the mtDNA of *A. cerana cerana* (Korea, Taiwan), *A. cerana indica* (Thailand and Philippines), and *A. cerana japonica* (Tokyo, Nagano, Kyoto, Wakayama and Tsushima Island in Japan) to study intraspecific geographic variations among 22 populations. Their results suggested that the Tsushima population in Japan has a closer relationship to the Korean population than to other Japanese populations. However, the origin of *A. cerana japonica* and the distribution of genetic variation throughout its range are largely unknown. In the present paper, we investigated genetic variation in *A. cerana japonica* using mtDNA sequences obtained from a region of the non-coding gene of 47 populations. We also determined how closely related these populations were to *A. cerana* populations from neighboring Russia, Korea and Taiwan.

2. MATERIALS AND METHODS

Adult workers from 550 *A. cerana japonica* colonies from 47 different sites in Japan and from 15 *A. cerana* colonies located in either Russia, South Korea, or Taiwan (see Fig. 1). Collected bees were transferred immediately to 99% ethanol and stored for DNA sequencing analysis. DNA was extracted from thoracic muscles using the DNeasy Mini Kit (QIAGEN). A non-coding region between the tRNA leu and COII gene was amplified and sequenced. Primers used for amplification were those designed by Cornuet et al. (1991): E2: 5′-GGC AAG AAT AAG TGC ATT G-3′ and H2: 5′-CAA TAT CAT TGA TGA CC-3′. Polymerase chain reaction (PCR) amplifications were performed in a final volume of 30 μL of PCR buffer (1 × reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 400 nM of each primer, 50 ng of total DNA, and 0.15 units of *Taq* polymerase (TaKaRa). DNA was amplified (TaKaRa thermal cycler Dice) using the following program: 3 min denaturation at 94 °C; 30 s at 94 °C, 30 s at 46 °C, and 45 s at 72 °C for thirty cycles and a final extension period of 5 min at 72 °C for one terminal cycle. PCR products were purified using an QIAqucik PCR purification kit (QIAGEN). The PCR products were sequenced directly using a BigDye terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems) as per the manufactures instructions. To obtain reproducibility of base substitution, from PCR to sequencing, analysis was performed 3 times per sample.

Sequences were aligned using the CLUSTAL W program (Thompson et al., 1994) and compared with reported haplotypes from the non-coding region of the mitochondrial DNA (Smith and Hagen, 1996; De La Rúa et al., 2000; Smith et al., 2000; Takahashi et al., 2002; Smith et al., 2004).

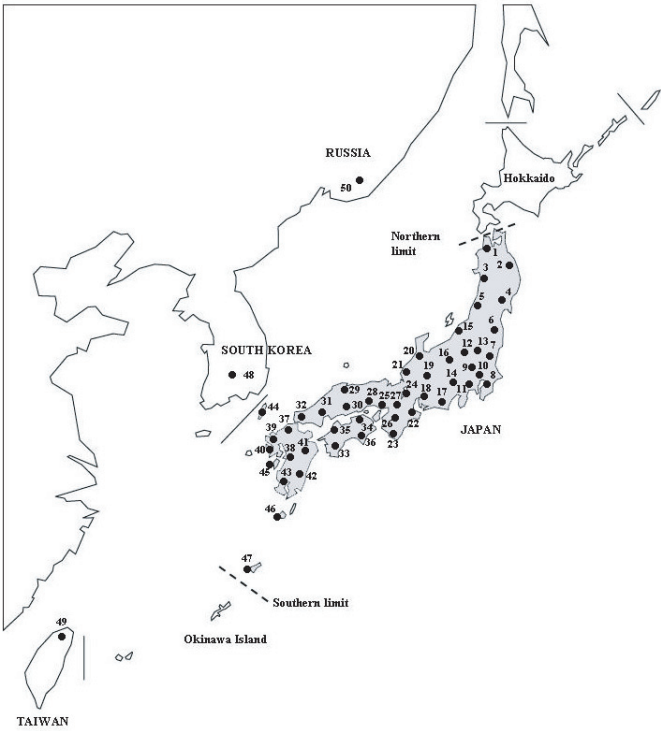

Figure 1. *Apis cerana* collection sites in Japan and neighboring regions. Symbols indicate actual sites from which samples were collected. Dotted line indicates the approximate range of *A. cerana japonica*. Sites (Latitude and Longitude): **Japan** [1- Aomori, Tokyo (40° 49' N, 140° 46' E), 2- Morioka, Iwate (39° 43' N, 141° 13' E), 3- Noshiro, Akita (40° 13' N, 140° 05' E), 4- Sendai, Miyagi (38° 17' N, 140° 50' E), 5- Yamagata, Yamagata (38° 17' N, 140° 50' E), 6- Iwaki, Fukushima (37° 03' N, 140° 57' E), 7- Tsukuba, Ibaraki (36° 12' N, 140° 08' E), 8- Sakura, Chiba (35° 19' N, 139° 51' E), 9- Kuki, Saitama (36° 04' N, 139° 40' E), 10- Machida, Tokyo (36° 12' N, 140° 09' E), 11- Hatano, Kanagawa (35° 22' N, 139° 10' E), 12- Isesaki, Gunma (36° 19' N, 139° 10' E), 13- Oyama, Tochigi (36° 23' N, 139° 51' E), 14- Yamanashi, Yamanashi (35° 41' N, 138° 40' E), 15- Joetsu, Niigata (37° 08' N, 138° 08' E), 16- Nagano, Nagano (36° 37' N, 138° 10' E), 17- Shizuoka, Shizuoka (34° 57' N, 138° 25' E), 18- Nagoya, Aichi (35° 14' N, 136° 54' E), 19- Gifu, Gifu (35° 27' N, 136° 49' E), 20- Toyama, Toyama (36° 42' N, 137° 17' E), 21- Komatsu, Ishikawa (36° 23' N, 136° 25' E), 22- Ise. Mie (34° 27' N, 136° 42' E), 23- Wakayama, Wakayama (34° 12' N, 135° 09' E), 24- Hikone, Shiga (35° 18' N, 136° 18' E), 25- Osaka, Osaka (34° 28' N, 136° 43' E), 26- Nara, Nara (34° 41' N, 135° 50' E), 27- Kyoto, Kyoto (35° 04' N, 135° 49' E), 28- Koube, Hougo (34° 21' N, 135° 41' E), 29- Himane, Shimane (35° 26' N, 133° 04E), 30- Kurashiki, Okayama (34° 34' N, 133° 40' E), 31- Hiroshima, Hiroshima (34° 32' N, 132° 33' E), 32- Yamaguchi, Yamaguchi (34° 12' N, 131° 25' E), 33- Kouchi, Kouchi (33° 34' N, 133° 34' E), 34- Takamatsu, Kagawa (34° 18' N, 134° 03' E), 35- Matsuyama, Ehime (33° 48' N, 132° 51' E), 36- Tokushima, Tokushima (34° 01' N, 134° 34' E), 37- Kasuga, Fukuoka (33° 31' N, 130° 27' E), 38- Yatsushiro, Kumamoto (32° 28' N, 130° 42' E), 39- Saga, Saga (33° 23' N, 130° 25' E), 40- Nagasaki, Nagasaki (32° 43' N, 129° 54' E), 41- Oita, Oita (33° 14' N, 131° 35' E), 42- Nobeoka, Miyazaki (32° 33' N, 131° 34' E), 43- Kagoshima, Kagoshima (31° 37' N, 130° 34' E), 44- Kamitsushima, Tsushima Island (34° 09' N, 129° 13' E), 45- Amakusa Island (32° 23' N, 130° 02' E), 46- Yakushima Island (30° 18' N, 130° 34' E), 47- Uken, Amami-Oshima Island (28° 20' N, 129° 07' E); **South Korea** 48- Nawmon, South Korea (35° 06' N, 129° 01' E); **Taiwan** 49- Taipei, Taiwan (25° 02' N, 121° 10' E); **Russia** 50- Ussria (44° 53' N, 132° 26' E). Samples from Aomori, Tokyo, Tsushima and Amami-Oshima sites (n = 30/site), other Japan sites (n = 10/site) and other country sites (n = 5/site) were analyzed.

Figure 2. Sequences of the non-coding region between leucine tRNA and cytochrome oxidase II genes of *Apis cerana* found in Japan and in neighboring area. Dots indicate the nucleotides identical to the Japan 1 haplotype. Gaps are shown by dashes. The underlined indicate the "stem" of the hairpin structure.

3. RESULTS

We found two new haplotypes, Japan 3 on Tsushima Island and Japan 4 on Amami-Oshima Island. Among ten individuals from Tsushima Island, we found one with the Japan 3 haplotype and nine with Japan 1. Among ten individuals from Aamami-Oshima Island, we found one with the Japan 4 haplotype and nine with Japan 1 (Fig. 2). Three haplotypes based on intergenic non-coding sequences were found: haplotype Japan 1 and two new haplotypes (Japan 3 and Japan 4, Fig. 2). The length of the intergenic non-coding region was equal to that reported by Smith and Hagen (1996) depending on the samples studied. *A. cerana* from Russia and South Korea were found to be the Japan 1 haplotype. The Taiwan sample was consistent with the Taiwan short haplotype of Smith and Hagen (1996). The nucleotide sequence data are available in the DDBJ GeneBank nucleotide sequence databases with the accession numbers AB078733-AB078735.

4. DISCUSSION

The northern breakpoint in the distribution of Japanese honey bees, *Apis cerana japonica*, was determined to be the Shimokita peninsula of Aomori, while the southern breakpoint was found to be Uke Island in the Amami-Oshima Islands region (Takahashi and Yoshida, 2002). The present study concerned an investigation of the subspecies across almost the entire distribution area, since 47 populations from Aomori to Amami-Oshima Island (Fig. 1) were collected. Three haplotypes were found in our analyses of 550 individuals from 47 populations in Japan. One of the three haplotypes was major (98.9%, 544/550) and consistent with the Japan 1 haplotype described by Smith and Hagen (1996). The others were new haplotypes. These haplotypes were designated as Japan 3 and 4 because Smith and Hagen (1996) had already reported another haplotype, Japan 2, from the Miyagi population, which we did not find in this study (the base sequence is also shown in Fig. 2 for comparison). The new Japan 3 haplotype was found in four samples from Tsushima Island. Similarly, the new haplotype, Japan 4, was found in only one of 30 samples from Amami-Oshima Island. These sequences show that only one out of the ten samples analyzed from Tsushima Island had a unique haplotype, and similarly only one other sample, from Amami-Oshima Island, had a different and unique haplotype. A previous study using morphometric analysis by Ruttner (1988) indicated that the Tsushima Island population was derived from Honshu populations. Similarly, Deowanish et al. (1996) revealed that Tsushima Island and other Japanese honey bees showed differences in their mitochondrial DNA RFLP patterns, but that the Honshu and Kyushu populations did not show genetic variation. The genetic variation in the Amami-Oshima population has not been described until now, but morphometric analysis of these other populations have shown strong differences between samples from Honshu and those from Kyushu (Takahashi and Yoshida, 2002), indicating that these two island populations are distinct from populations on the Japanese mainland.

The Japan 3 and Japan 4 haplotypes have not been found in any other population in East Asia, including Korea, China (Smith and Hagen, 1996; Smith et al., 2000), Thailand (Warrit et al., 2006), and Myanmar (Smith et al., 2004), or in Japan, although the Japan 1 haplotype is widespread. Therefore, we suggest that these haplotypes are indigenous to

these Islands. On the other hand, the Honshu, Shikoku, and Kyushu populations were clustered in a single genetic group and showed no variation either between or within the populations.

The sequence analysis of the non-coding region found two new haplotypes in addition to the two previously reported and confirms the potential for the use of this method as an effective tool in investigating the genetic variation and phylogenetic relationship of mtDNA among populations in *A. cerana* (Smith and Hagen, 1996; Smith et al., 2000; De La Rúa et al., 2000; Takahashi et al., 2002; Smith et al., 2004). However, it was difficult to analyze the genetic diversity of population in *A. cerana japonica* by the intergenic non-coding region. DNA microsatellite genotyping has been used to investigate the genetic structure of population of *A. mellifera*, because of the higher mutation at microsatellite loci compared to mtDNA (Estoup et al., 1995). Japan consists of many large and small islands. Therefore, sampling at several locations and the collection of a large number of samples provide an advantage in the investigation of genetic variance by microsatellite analysis. We expect that genetic variation of *A. cerana* could be found in Japan and in neighboring areas with this method.

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Variation géographique d'*Apis cerana japonica* dans les îles japonaises et des populations d'*Apis cerana* à la limite de son aire de répartition.

***Apis cerana japonica* / Japon / variation géographique / ADNmt / biogéographie / génétique des populations.**

Zusammenfassung – Geographische Variation von *Apis cerana japonica* auf den japanischen Inseln und von angrenzenden *A. cerana* Populationen. Die genetische Variation von *A. cerana japonica* in Japan und *A. cerana* in den benachbarten Arealen wurde anhand von mitochondrialen DNA Sequenzdaten einer nichtkodierenden Region untersucht. Aus 470 *A. cerana japonica* Völkern von 47 Standorten in Japan und von 5 *A. cerana* Völkern einzelner Orte in Russland, Südkorea und Taiwan wurden im Jahr 1997 und 2002 Proben von adulten Arbeiterinnen entnommen. Bei den japanischen Populationen wurden 3 Haplotypen identifiziert, der bereits vorher bekannte Japan 1 Haplotyp und zwei neue Haplotypen (Japan 3 und Japan 4). Der Japan 1 Haplotyp wurde an 43 Lokalitäten auf Honshu, Shikoku und den Kyushu Inseln gefunden, während die Japan 3 und 4 Haplotypen auf den Inseln Amami-Oshima beziehungsweise Tsushima gefunden wurden. Der neue Japan 3 Haplotyp enthielt eine Basensubstitution (T → A) an der einundzwanzigsten Position, der Japan 4 Haplotyp eine an der dreizehnten Position (G → A). Die Ergebnisse zeigten, dass *A. cerana japonica* eine sehr geringe genetische Variation in der nichtkodierenden Region der mtDNA aufweist

***Apis cerana* / geographische Variation / mitochondriale DNA / Japan / Biogeographie**

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