Original article

**New haplotypes for the non-coding region
of mitochondrial DNA in cavity-nesting honey bees
Apis koschevnikovi and *Apis nuluensis***

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Abstract – The cavity-nesting honeybees, *Apis cerana*, *A. koschevnikovi* and *A. nuluensis*, are distributed in Sabah, Borneo. We sequenced the non-coding intergenic region between the tRNA leu and COII mitochondrial genes of these three species. About 50 different haplotypes have been reported for the non-coding region of *A. cerana*, *A. koschevnikovi* and *A. nigrocincta*. This study detected new haplotypes in *A. koschevnikovi* and *A. nuluensis*, which we designated Sabah 1 and Sabah short, respectively. Sabah short is the first short sequence reported from Borneo.

Apis cerana / *Apis koschevnikovi* / *Apis nuluensis* / mitochondrial DNA

1. INTRODUCTION

Recent taxonomic studies on cavity-nesting honey bees have reported three species in addition to *Apis cerana* Fabr. – the recently rediscovered *Apis koschevnikovi* v. Buttel-Reepen (Koeniger et al., 1988; Maa, 1953; Tingek et al., 1988), and two newly described species, *Apis nigrocincta*

Smith (Hadisoesilo et al., 1996) and *Apis nuluensis* Tingek, Koeniger & Koeniger (Tingek et al., 1996), two newly described species. *A. cerana* distributed in East-Asia ranging from Afghanistan east to far eastern Russia and from Indonesia north to Japan. *A. koschevnikovi* is distributed in Malaysia and Indonesia. *A. nigrocincta* is distributed in Sulawesi and Mindanao.

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A. nuluensis is distributed in the highlands of Sabah, Borneo. The distribution of these honey bees are shown in Figure 1. These three species are partially sympatric with *A. cerana*. Three of the four Asian cavity-nesting honey bees inhabit Borneo. Morphological comparisons of these three sympatric species in Borneo indicate that *A. cerana* shares more similarities with *A. nuluensis* than with *A. koschevnikovi* (Fuchs et al., 1996). Similarly, an estimated phylogeny based on mitochondrial and nucleotide DNA sequences indicates that *A. cerana* and *A. nuluensis* are more closely related (Arias et al., 1996; Tanaka et al., 2001).

Mitochondrial DNA sequences have been used to determine intraspecific phylogenetic relationships and the genetic structure of insect species because of the

abundance of intraspecific polymorphism. In *A. cerana*, polymorphism is often found in a mitochondrial, non-coding intergenic region between tRNA leu and COII (Smith and Hagen, 1996). At present about 50 different haplotypes have been reported for the non-coding region of *A. cerana* (De La Rua et al., 2000; Smith and Hagen, 1996; Smith et al., 2000). A total of seven haplotypes have been found for the intergenic region in *A. cerana* from Borneo (Smith and Hagen, 1996). *A. koschevnikovi* from Borneo has a single known haplotype (Smith and Hagen, 1996), and *A. nigrocincta* from Sulawesi and Sangihe have been shown to have two haplotypes (Smith et al., 2000). However, the sequence of *A. nuluensis* has not been studied. Thus, the present study aims to demonstrate the haplotype variation of three species in

Figure 1. Map of the distribution of *A. cerana* (①), *A. nuluensis* (②), *A. koschevnikovi* (③) and *A. nigrocincta* (④) in Borneo and neighboring regions.

Sabah based on sequence analysis of the non-coding intergenic region and examines whether the detected sequences match known sequences.

2. MATERIALS AND METHODS

We collected adult workers of *A. cerana* and *A. koschevnikovi* from colonies at Tenom, Sabah, Malaysia (Borneo) and of *A. nuluensis* on flowers in the highlands of Crocker Range near Tambunan, Sabah, in March 2000. The collected workers were transferred immediately to 95% ethanol for mitochondrial DNA analysis. Two adult workers of each species were used for sequence analysis. Template DNA was extracted from thoracic muscle tissue of each individual by heating the homogenized tissue in 400 µl of lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.5% SDS, 0.8 mg/ml Proteinase K) at 55 °C for 24 h. The lysate was extracted twice with water-saturated phenol and once with chloroform. The DNA was recovered from the purified lysate by isopropanol precipitation, rinsed with 70% ethanol, dried, and dissolved in 50 µl of TE solution (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) and then stored at -20 °C until the polymerase chain reaction (PCR) was performed. The total volume of the reaction mixture was 30 µl, composed of 20 µl of distilled water, 3 µl of DNA extract, 200 pM of each primer (E2 forward: 5'-GGC AAG AAT AAG TGC ATT G-3', H2 reverse: 5'-CAA TAT CAT TGA TGA CC-3') (Cornuet and Garnery 1991, 3 µl of dNTP mix (200 mM of each dNTP), 3 µl of 10 µl PCR buffer, 1.5 mM MgCl₂, and 0.5 units of Taq polymerase (Takara). The temperature profile was 3 minutes at 94 °C followed by thirty 30 cycles of seconds at 94 °C, 1 minute at 46 °C and 1 minute at 70 °C. The final elongation step was increased to 10 minutes. The amplification products were purified using QIAquick PCR purification kits (QIAGEN). The dye-terminator la-

beled cycle sequencing reaction was performed with a DNA Sequencing Kit FS (Perkin Elmer) and primers E2 and H2, at a temperature profile of 94 °C for 3 minutes followed by thirty 15-second cycles at 94 °C, 46 °C for 30 seconds and 70 °C for 2 minutes. The reaction products were analyzed using an ABI373 automated sequencer (Perkin Elmer).

3. RESULTS AND DISCUSSION

The length of the PCR product of the intergenic non-coding sequences ranged from approximately 30 bp to 100 bp, depending on the species. The sequences are shown in Figure 2 and are available from DNA Data Bank of Japan (accession numbers AB072435-AB072438 <http://www.ddbj.nig.ac.jp>). The sequences of the two samples of *A. cerana* were identified and matched the Borneo-2 haplotype described by Smith and Hagen (1996) (Fig. 2). The two individuals of *A. koschevnikovi* had different sequences; one was a new haplotype, and the other was identified as the haplotype described by Smith and Hagen (1996). The new haplotype, which we designated Sabah 1, has one G inserted between positions 27 and 28 of the haplotype from *A. koschevnikovi* of Smith and Hagen (1996) (Fig. 2). The sequences from the two individuals of *A. nuluensis* were very short and most of the intergenic non-coding sequences were lost (Fig. 2). Short haplotypes have been reported in *A. cerana* from Taiwan and Philippines, and in *A. nigrocincta* from Sulawesi and Sangihe, Indonesia but it has not been found in *A. cerana* from Malaysia nor *A. koschevnikovi* (De La Rua et al., 2000; Smith and Hagen, 1996; Smith et al., 2000). This Sabah-short sequence is the first reported for *A. nuluensis* and the first finding of a short haplotype from *Apis* in Borneo.

To summarize the short haplotypes of cavity-nesting honey bees, the Sulawesi

<i>Apis cerana</i>		
Borneo 2	<u>AAAA</u> TTTAATAAGCTTTAATTGCATTGAATTTTGAATTCAAATTTAAATAAAAAACTTTTATTAATAAATAATTTAAATTTATTATTAATAATTT	
<i>Apis koschevnikovi</i>		
Haplotype 1	ATAATTTAATAAGCAATTTATGCACT--AATTTAATCAATAAAATAGTTATATAAACTTTTATTAATATTAATAATTAATTAATTAATTAATAATTT	
Sabah 1	<u>ATAATTTAATAAGCAATTTATGCACTGAATTTAATCAATAAAATAGTTATATAAACTTTTATTAATATTAATAATTAATTAATTAATTAATAATTT</u>	
<i>Apis nigrocincta</i>	Sulawesi Short	AAAATTAATAATTT-AA-----TT-ATTATTAATAATTT
<i>Apis cerana</i>	Taiwan Short	AAAATTAATAATTTTAA-----TTT-ATTATTAATAATTT
<i>Apis cerana</i>	Philippine Short	AAGATTAATAATTT-AATAAATTTAATTATTAATAATTT
<i>Apis nuluensis</i>	Sabah Short	<u>AAAATTAATAATTT</u> -----AAATTT-ATTATTAATAATTT

Figure 2. Sequences of the non-coding intergenic region between tRNA leu and COII of mitochondrial DNA of *A. cerana*, *A. koschevnikovi*, *A. nuluensis* and *A. nigrocincta*. The sequences of *A. cerana*, *A. koschevnikovi* and *A. nigrocincta* were first reported in a previous study (Smith and Hagen, 1996). The Sabah-1 haplotype of *A. koschevnikovi* and the Sabah-short sequence of *A. nuluensis* is first reported in this study. Gaps are shown by dashes. The underlined indicate the “stem” of the hairpin structure.

<i>Apis cerana</i>	Ce 1	ATTCTACAT	GATTATATT	TATATTTCAA	GAATCAAATT	CATACTATGC	TGATAATTTA	ATTTCATTTC	ATAATATAGT
<i>Apis cerana</i>	Ce 2					...T....			
<i>Apis cerana</i>	Ce 3			...G....		...T....			
<i>Apis cerana</i>	Ce 4					...T....			
<i>Apis cerana</i>	Borneo 2		...C....	...G....		...T....		...C....	
<i>Apis nuluensis</i>	Sabah Short	C....		...C....		...G...T....		...C....	
<i>Apis koschevnikovi</i>	Haplotype 1	A....			...T....		C.....		
<i>Apis koschevnikovi</i>	Sabah 1	A....			...T....		C.....		

<i>Apis cerana</i>	Ce 1	AATAATAATT	ATTATTATAA	TTTCTACACT	AACAGTTTAT	ATTATTATAG	ATTTATTTTT	AAATAAAATT	TCAAATTTAT
<i>Apis cerana</i>	Ce 2	T.....		TT			...C·C....		
<i>Apis cerana</i>	Ce 3			TT				...C....	
<i>Apis cerana</i>	Ce 4			TT				...C....	
<i>Apis cerana</i>	Borneo 2			...A·TT			...C....		...C·
<i>Apis nuluensis</i>	Sabah Short			...A·TT	C	·A·...TC·	...C·T....		
<i>Apis koschevnikovi</i>	Haplotype 1	T.....	...C....	...A·T			...C....	...T....	
<i>Apis koschevnikovi</i>	Sabah 1	T.....	...C....	...A·T			...C....	...T....	

<i>Apis cerana</i>	Ce 1	TTTTATTAAA	AAATCATAAT	ATTGAAATTA	TTTGAACAGT	AATCCCAATT	ATTATTTTAT
<i>Apis cerana</i>	Ce 2		C	C		...C.....	
<i>Apis cerana</i>	Ce 3			C		...T.....	
<i>Apis cerana</i>	Ce 4			C		...T.....	
<i>Apis cerana</i>	Borneo 2			C		...T.....	
<i>Apis nuluensis</i>	Sabah Short	·C.....			T.....		
<i>Apis koschevnikovi</i>	Haplotype 1				...T.....	...C·T·	
<i>Apis koschevnikovi</i>	Sabah 1				...T.....	...C·T·	

Figure 3. Sequences of the non-coding intergenic region between tRNA leu and COII of mitochondrial DNA of *A. cerana*, *A. koschevnikovi*, *A. nuluensis* and *A. nigrocincta*. The four haplotypes sequences (Ce1, Ce2, Ce3 and Ce4) were reported by De la Rua et al. (2000). The common sequences are shown by dots.

short is a very short sequence found in Sulawesi and Sangihe, Indonesia and is only known in *A. nigrocincta* (Smith et al., 2000). The Philippine short is the longest of the short sequences and is limited to the Philippine islands of Negros and Panay (Smith et al., 2000). The Taiwan short is only known from Taiwan (Smith and Hagen, 1996). The sequence of the newly discovered Sabah short is most similar to the Philippine short (Fig. 2). These haplotypes have limited utility in phylogenetic analyses because the extremely short sequences are highly variable and consequently difficult to align. Therefore we suggest that phylogenetic relationships among extant populations of short haplotype individuals need to be constructed using data from other regions. Thus, we compared the number of substitutions in the nucleotide sequence of the 5'-end of the mtDNA CO II gene in *A. cerana* and *A. nuluensis* from Sabah with previous data reported from *A. cerana* from the Philippines (De La Rua et al., 2000). The nucleotide sequence of the 5'-end of the mtDNA CO II gene was slightly more similar between *A. cerana* and *A. nuluensis* from Sabah (substitution numbers 14) than among *A. cerana* from the Philippines or between *A. cerana* from the Philippines and *A. nuluensis* (substitution numbers 15–16) (Fig. 3). Short haplotypes of *A. cerana* from Philippine and *A. nuluensis* did not appear to be closely related and these data from the coding region are consistent with previous reports (Arias et al., 1996; Tanaka et al., 2001).

It is likely that new haplotypes or additional known haplotypes will be found in the *A. cerana* group from Borneo, because only a few individuals have been surveyed so far. If *A. cerana* individuals with the short haplotype of the non-coding region were found in Borneo or *A. nuluensis* individuals with normal haplotypes of the non-coding region were found, it may be useful in reconstructing the evolutionary history of *A. cerana* and *A. nuluensis*.

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Résumé – Nouveaux haplotypes pour la région non codante de l'ADN mitochondrial chez les abeilles qui nidifient dans des cavités, *Apis koschevnikovi* et *A. nuluensis*. Outre *Apis cerana*, trois autres abeilles mellifères ont été décrites : *Apis cerana*, *A. koschevnikovi* et *A. nuluensis*. La figure 1 donne leur répartition géographique. La séquence de l'ADN mitochondrial d'*A. nuluensis* n'a pas été étudiée jusqu'à présent. Cette étude a donc pour but de montrer la variation haplotypique des trois espèces à Sabah, Bornéo, Malaisie, d'après la séquence entre l'ARNt et la région intergénique non codante COII. La séquence de deux individus d'*A. cerana* concordait avec l'haplotype Bornéo-2 décrit par Smith et Hagen (1996). L'analyse moléculaire précédente montrait que *A. koschevnikovi* de Bornéo n'avait qu'un seul haplotype, mais cette étude a montré l'existence d'un nouvel haplotype nommé Sabah-1. Parmi les trois espèces, la séquence de deux individus d'*A. nuluensis* était la plus petite et ce nouvel haplotype a été nommé Sabah-short (= court). Il s'agit de la première description d'un haplotype court pour les abeilles de Bornéo nidifiant dans des cavités. La variation géographique de la région non codante sera utile pour reconstruire l'histoire évolutive d'*A. cerana* et *A. nuluensis*.

Apis cerana / *Apis koschevnikovi* / *Apis nuluensis* / ADN mitochondrial / Bornéo

Zusammenfassung – Ein neuer Haplotyp einer nichtcodierenden Region der mitochondrialen DNA bei den höhlenbrütenden Honigbienen *Apis koschevnikovi* und *Apis nuluensis*. Die mitochondriale DNA von 3 Arten der Honigbienen, *Apis cerana*, *Apis koschevnikovi* und *Apis nuluensis* von Borneo, Malaysia wurde zwischen den Genloci tRNA leu und COII sequenziert. Die Sequenzen von 2 Individuen von *A. cerana* stimmte mit dem Borneo-2 Haplotyp überein, der von Smith und Hagen (1996) beschrieben wurde. Bisherige molekulare Analysen zeigten bei *Apis koschevnikovi* von Borneo nur einen Haplotyp auf, aber bei dieser Untersuchung wurde ein neuer Haplotyp gefunden und als Sabah-1 bezeichnet. Die Sequenz der beiden Bienen von *Apis nuluensis* war die kürzeste innerhalb der 3 Arten und dieser neue Haplotyp wurde Sabah short (Sabah kurz) genannt. Dies ist die erste Beschreibung eines kurzen Haplotyps bei den höhlenbrütenden Honigbienen von Borneo. Diese geografische Variation in dieser nichtcodierenden Region wird bei einer Rekonstruktion der Evolutionsgeschichte von *Apis cerana* und *Apis nuluensis* nützlich sein.

***Apis cerana* / *Apis koschevnikovi* / *Apis nuluensis* / mitochondriale DNA**

REFERENCES

- Arias M.S., Tingek S., Kelitu A., Sheppard W.S. (1996) *Apis nuluensis* Tingek, Koeniger and Koeniger, 1996 and its genetic relationship with sympatric species inferred from DNA sequences, *Apidologie* 27, 415–422.
- Cornuet J.M., Garnery L. (1991) Putative origin of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA, *Genetics* 128, 393–403.
- de La Rua P., Simon U.E., Tilde A.C., Moritz R.F.A., Fuchs S. (2000) MtDNA variation in *Apis cerana* populations from the Philippines, *Heredity* 84, 124–130.
- Fuchs S., Koeniger N., Tingek S. (1996) The morphometric position of *A. nuluensis* (Tingek, Koeniger and Koeniger) within cavity-nesting honey bees, *Apidologie* 27, 397–406.
- Hadisoeso S., Otis G.W., Meixner M. (1996) Two distinct populations of cavity-nesting honey bees (Hymenoptera: Apidae) in south Sulawesi, Indonesia, *J. Kans. Entomol. Soc.* 68, 399–407.
- Koeniger N., Koeniger G., Tingek S., Rinderer T.E. (1988) Reproductive isolation by different time of drone flight between *Apis cerana* Fabricius 1793 and *Apis vechti* Maa 1953, *Apidologie* 19, 103–106.
- Maa T.C. (1953) An inquiry into the systematics of the tribus Apidini or honeybees (Hym), *Treubia* 21, 525–640.
- Smith D.R., Hagen R.H. (1996) The biogeography of *Apis cerana* as revealed by mitochondrial DNA sequence data, *J. Kans. Entomol. Soc.* 69, 294–310.
- Smith D.R., Villafuerte L., Otis G., Palmer M.R. (2000) Biogeography of *Apis cerana* F. and *A. nigrocincta* Smith: insights from mtDNA studies, *Apidologie* 31, 265–280.
- Tanaka H., Roubik D.W., Kato M., Liew F., Gunsalam G. (2001) Phylogenetic position of *Apis nuluensis* of northern Borneo and phylogeography of *A. cerana* as inferred from mitochondrial DNA sequences, *Insectes Soc.* 48, 44–51.
- Tingek S., Mardan M., Rinderer G., Koeniger N., Koeniger G. (1988) Rediscovery of *Apis vechti* (Maa, 1953): the Sabah honey bee, *Apidologie* 19, 97–102.
- Tingek S., Koeniger N., Koeniger G. (1996) Description of a new cavity-dwelling species of *Apis* (*Apis nuluensis*) from Sabah, Borneo with notes on its occurrence and reproductive biology (Hymenoptera, Apoidea, Apini), *Senckenbergiana Biol.* 76, 115–119.

Arias M.S., Tingek S., Kelitu A., Sheppard W.S. (1996) *Apis nuluensis* Tingek, Koeniger and Koeniger, 1996 and its genetic relationship with