
Research article

New mitochondrial CO1 haplotypes and genetic diversity in the honeybee *Apis koschevnikovi* of the Crocker Range Park, Sabah, Malaysia**Takeshi SUKA^{1*} and Hiroyuki TANAKA^{2*}**¹*Nagano Environmental Conservation Research Institute, Kitago, Nagano 381-0075, Japan*²*Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan*

ABSTRACT. The distribution of the Saban honeybee, *Apis koschevnikovi*, is considered to be restricted to the humid primary forests of Sundaland. A biogeographical uniqueness of the Crocker Range had been suggested in our previous phylogenetic analyses of this species. To examine this uniqueness, we analyzed additional specimens collected from the Crocker Range in the present study. Four new haplotypes of the mitochondrial cytochrome oxidase I (CO1) gene were found. Phylogenetic analyses including the new haplotypes supported a previous finding that local groups of *A. koschevnikovi* in Borneo have derived from three distinct lineages. All the haplotypes from the Crocker Range belonged to either of two of the three lineages: one consisted of only the haplotypes from the Crocker Range, and the other comprised relatively less divergent haplotypes from a wider region including the Crocker Range. Nevertheless, the lineage consisting of only the Crocker Range types is largely divergent from the two other lineages. It indicates that a forest around the Crocker Range has been isolated for a long period on a geological time scale, suggesting the importance of preserving primary forests as a source of genetic diversity in this species.

Key words: Borneo, cytochrome oxidase I, genetic differentiation, mtDNA, phylogeography

*: Equal contributions to this work.

INTRODUCTION

The island of Borneo holds five species of honeybees (genus *Apis*) among the nine recognized at present (Engel, 1999; Otis, 1996; Tingek *et al.*, 1996): the highest concentration of the genus in the world. Of the five species, the dwarf honeybee, *Apis andreniformis*, the giant honeybee, *A. dorsata*, and the Asian honeybee, *A. cerana*, are widely distributed over Borneo as well as in mainland Asia (Otis, 1996), whereas the montane honeybee, *A. nuluensis*, has restricted ranges in mountain areas of northern Borneo (Tingek *et al.*, 1996). The Saban honeybee, *A. koschevnikovi*, is considered to be adapted to humid primary forest environments in Sundaland (the Malay Peninsula, Borneo, Sumatra and the western part of Java; Otis, 1996). The honeybees on Borneo probably function as effective pollinators for many tropical plants (Kenta, 2002; Momose *et al.*, 1998; Roubik *et al.*, 1995; Sakai, 2002). Therefore, detailed phylogeographic inquiries into the Bornean honeybees will contribute to clarify the evolutionary history of tropical forests as well as to provide crucial information for conservation of plant-pollinator mutualisms on the island.

In a previous study (Tanaka *et al.*, 2001b), we sequenced the mitochondrial cytochrome

oxidase subunit 1 (CO1) gene of three honeybee species, and found that genetic differentiation among local groups within northern part of Borneo (Sabah, Sarawak and Brunei) was much larger in *Apis koschevnikovi* than in *A. cerana* and *A. dorsata*. The CO1 sequences in *A. koschevnikovi* exhibited a large divergence between samples from the Crocker Range Park and elsewhere. Only the sampling sites in the Crocker Range were located at the eastern side of the mountain range, whereas other sampling sites were spread over the western side. Accordingly, we proposed a possible scenario to explain the large divergence in *A. koschevnikovi* that mountain ranges in northern Borneo would have been a barrier preventing the dispersal of this species. We also discussed about migratory nesting behavior and cold tolerance of each honeybee species as well as the paleoclimates of the Southeast Asian tropics, as factors that produced those pattern of the genetic marker.

In the other preceding study (Tanaka *et al.*, 2003), we extended the analyses by using samples from a broader area of the island including Indonesia (Kalimantan) and the eastern side of Sabah (Tawau). The results supported the previous finding about the geographical uniqueness of the Crocker Range for the CO1 divergence in *A. koschevnikovi*. The local groups of this species in Borneo were divided into three distinct lineages. One of the lineages consisted of only the one haplotype found in the Crocker Range. However, another lineage was distributed over a broader area inclusive of Tawau as well as the western side of the mountain ranges in northern Borneo. Therefore, the sole scenario of the mountain ranges as a barrier is not enough to explain the whole pattern. More extensive surveys are required to reconstruct the process of genetic divergences in *A. koschevnikovi*.

In the present study, we added twelve individuals of *A. koschevnikovi* collected from both western and eastern side of the Crocker Range to the

analyses. Four sampling sites of the specimens were located along a road passing across the mountain range. We report the results of the phylogenetic investigations and present their implications for conservation of genetic diversity in this species.

MATERIALS AND METHODS

Honeybee samples

Samples of *A. koschevnikovi* for the present study were collected in the Crocker Range Park in September 2002. Figure 1 shows sampling sites located along a road that is crossing over the mountain range from Ulu Kimanis to the TBC station: at altitudes of 500 m above sea level (n=3; number of individuals examined in DNA analysis, see below) and 800 m a.s.l (n=5) on the western side and at altitudes of 900 m (n=3) and 1,100 m a.s.l (n=1) on the eastern side, respectively. All of the sites were adjacent to primary forests. Soon after the collection, bee samples were fixed with 99.5% ethanol until DNA extraction.

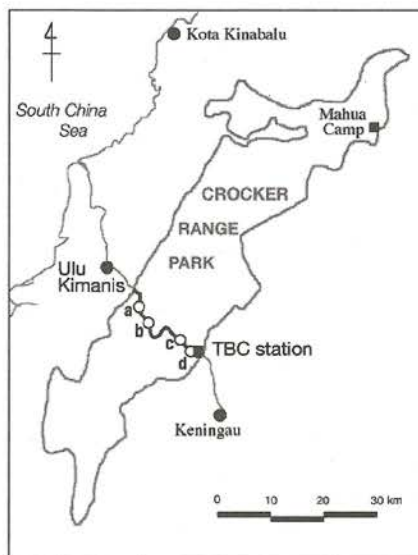


Fig. 1. Map of the sampling sites in the Crocker Range Park, a: western side of the mountain range, altitude 500 m, b: western side, altitude 800 m, c: eastern side, altitude 1100 m, d: eastern side, altitude 900 m.

DNA extraction, PCR and DNA sequencing

A total of twelve specimens comprising one to five individuals of bees per sampling site were used for DNA analysis. Preparation of total DNA was done using the thoracic muscle of the bees by the conventional phenol-chloroform extraction method. The 1064 base-pair regions of the CO1 were amplified by the polymerase chain reaction (PCR) using the following primers: forward 5'-ATAATTTTTTTTATAGTTATAC-3', and reverse 5'-GATATAATCCTAAAAAATGTTGAGG-3' (Tanaka *et al.*, 2001a). The condition of PCR was: initial denaturing at 93°C for 1 min, 30 cycles of denaturing at 93°C for 45 sec, annealing at 45°C for 1 min and extension at 60°C for 3 min, and final extension at 60°C for 4 min (Tanaka *et al.*, 2001a). The PCR products were purified with a QIAquick PCR purification kit (QIAGEN). Sequencing reaction was conducted for the purified DNA using a BigDye Terminator Ver.3.1 Cycle sequencing Kit (Applied Biosystems) with the primers mentioned above and the following internal primers: forward 5'-ATTTGATCGAAATTTTAATAC-3' and reverse 5'-CCAGAAGTTTATATTTTAAT-3' (Tanaka *et al.*, 2001a). DNA sequences were determined with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and subsequently confirmed using the software, Sequence Navigator (Applied Biosystems). Voucher specimens are at the Primate Research Institute, Kyoto University.

Phylogenetic analyses

We used the 1041 base-pair sequences of the CO1 region in the phylogenetic analyses because the nucleotide sequences near the primers sometimes could not be clearly determined. The CO1 sequences obtained for the samples of this study (GenBank accession Nos. AY754729 - AY754732; Table 1) were aligned together with the sequence data of *A. koschevnikovi* that had been examined in the previous studies (Tanaka *et al.*, 2001b; Tanaka *et al.*, 2003; Table 1). Data of another honeybee species, *Apis florea* from

Thailand (Tanaka *et al.*, 2003), was also included into the analysis as an out-group. PAUP* 4.0b10 (Swofford, 2003) was utilized to perform neighbor-joining (NJ) analysis. Evolutionary distances in the NJ tree were estimated by a general time-reversible model (Yang, 1994). A bootstrap test was implemented with 1,000 replications. For geographic mapping of the gene genealogy network (Templeton *et al.*, 1992) of CO1 haplotypes, the cladistic program TCS (Clement *et al.*, 2000) was applied to the sequence data. To estimate genetic diversity among the haplotypes or geographic groups of *A. koschevnikovi*, Tamura-Nei evolutionary distance (Tamura & Nei, 1993) was measured and the number of substitutions was counted based on pairwise comparison of CO1 haplotypes using PAUP*.

RESULTS

Four haplotypes of the CO1 gene were identified from the specimens of *Apis koschevnikovi* collected for the present study in the Crocker Range, all of which were new and different from the one obtained at Mahua camp (Crocker Range type 1), a northeastern site of the Crocker Range, in 1999 (Tanaka *et al.*, 2001b; Fig. 1). Each haplotype was named as Crocker Range type 2 to type 5 (Table 1). The localities where the specimens of the new haplotypes were collected are given in Table 1. One of the new haplotypes, Crocker Range type 3, was at both the western (500 m, 800 m) and the eastern (1,100 m) sides of the mountain range.

Figure 2 shows phylogenetic relationships among CO1 haplotypes of *A. koschevnikovi* in Borneo obtained by the NJ analysis. The topology of the figure is congruent to that of the preceding study (Tanaka *et al.*, 2003) and the three major clusters were observed. The four new haplotypes from the Crocker Range were separated into two major clusters: one of the new haplotypes, Crocker Range type 2, was connected to the haplotype of Mahua camp (type1) which had been identified in the previous study, and the other new haplotypes, Crocker

Table 1. The COI haplotypes of *A. koschevnikovi* used in phylogenetic analyses

COI Haplotype	Locality	GenBank accession No. or reference
Crocker Range type 1	Mahua Camp, Crocker Range Park, Sabah, Malaysia	AY012723
Crocker Range type 2	Western side 800 m alt., Crocker Range Park, Sabah, Malaysia	AY754729
Crocker Range type 3	Western side 500 m alt. and 800 m alt. and eastern side 1,100 m alt., Crocker Range Park, Sabah, Malaysia	AY754730
Crocker Range type 4	Eastern side 900 m alt., Crocker Range Park, Sabah, Malaysia	AY754731
Crocker Range type 5	Western side 500 m alt., Crocker Range Park, Sabah, Malaysia	AY754732
Tawau 1	Tawau, Sabah, Malaysia	Tanaka <i>et al.</i> (2003)
Tawau 2	Tawau, Sabah, Malaysia	Tanaka <i>et al.</i> (2003)
Tawau 3	Tawau, Sabah, Malaysia	Tanaka <i>et al.</i> (2003)
Lambir	Lambir Hills National Park, Sarawak, Malaysia	AF153111
Brunei	Brunei	AF153110
Upper Baram C1	Coupe 1, near from Baram River, Sarawak, Malaysia	Tanaka <i>et al.</i> (2003)
Upper Baram C5	Coupe 5, near from Baram River, Sarawak, Malaysia	Tanaka <i>et al.</i> (2003)
Kutai	Kutai National Park, East Kalimantan, Indonesia	Tanaka <i>et al.</i> (2003)
Loksad	Loksad, South Kalimantan, Indonesia	Tanaka <i>et al.</i> (2003)
Upper Kapuas	Field site near from Sadap, West Kalimantan, Indonesia	Tanaka <i>et al.</i> (2003)

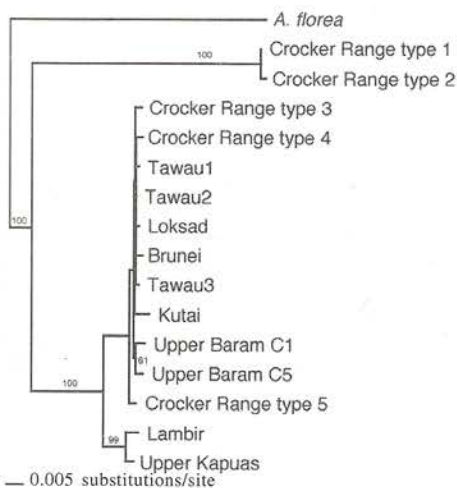


Fig. 2. Phylogenetic relationships of the CO1 haplotypes of *A. koschevnikovi* estimated by the neighbor-joining method using evolutionary distance measured by the general time-reversible model. The number above the branch indicates the bootstrap probability with 1,000 replicates. *Apis florea* was used as the out-group.

Range types 3, 4 and 5, were placed in a cluster that includes haplotypes from various localities. The cluster of Crocker Range types 1+2 was diverged greatly from the other clusters, as indicated by the deep branching in the figure.

Figure 3 presents a phylogeographic network of CO1 haplotypes in *A. koschevnikovi* obtained by the cladistic program TCS. Three mtDNA lineages are clearly distinguished. The five haplotypes from the Crocker Range are divided into two different lineages: one of them is comprised of only the two haplotypes from the Crocker Range (types 1+2) and the other is composed of haplotypes from a considerably broader area, including the other haplotypes from the Crocker Range (types 3, 4 and 5), with the haplotype of Tawau 2 at the basal position.

Sequence divergence among the CO1 haplotypes of *A. koschevnikovi* is given in Table 2. Of the four haplotypes newly found in the Crocker Range, the haplotype Crocker Range type 2 was closely related to type 1 (0.001 in Tamura-Nei distance, 1 nucleotide substitution) and the remaining 3 haplotypes (types 3, 4 and 5) were close to one another (0.004-0.006 in Tamura-

Nei distance, 4-6 substitutions). The sequence divergence was large between the haplotypes of Crocker Range types 1+2 and those of types 3+4+5 (0.078-0.082 in Tamura and Nei distance, 75-79 substitutions).

DISCUSSION

The results of the present study suggest that the forest of the Crocker Range would hold a substantial genetic diversity in the population of *Apis koschevnikovi*. At least two different lineages inhabit this area and one of them is distinctly unique among local groups of this species in Borneo (Figs. 2 and 3). Because *A. koschevnikovi* prefers wet primary forests (Otis, 1996; Roubik, Suka and Tanaka, *pers. obs.*), it also implies the importance of conserving such habitats.

From the twelve individuals of *A. koschevnikovi* examined in this study, four haplotypes of CO1 gene were identified and all of them were previously unknown. One of the new haplotypes (type 2) comprises the lineage restricted to the Crocker Range with the one from the Mahua camp (type 1), whereas the other haplotypes (types 3+4+5) are derived from another lineage which had dispersed from an area in or around Tawau (Fig. 3, as for the genetic differentiation between these lineages, see Table 2). Thus there are multiple origins of mtDNA lineages of *A. koschevnikovi* in the Crocker Range. Both the Crocker Range and the Tawau Hills are considered to have retained wet primary forests as suitable habitats for this species, although those territories are more than 200km apart at present. Therefore, to understand the pattern of derivations of *A. koschevnikovi* in the Crocker Range, it is necessary to deliberate on the historical background of changing distribution of the vegetations.

According to a phylogenetic analysis (Tanaka *et al.*, 2001b), *A. koschevnikovi* have probably occurred in the Southeast Asian tropics in a period of warmer climates in the Tertiary. Then its range may have been fragmented along with reduction of humid tropical forests caused by

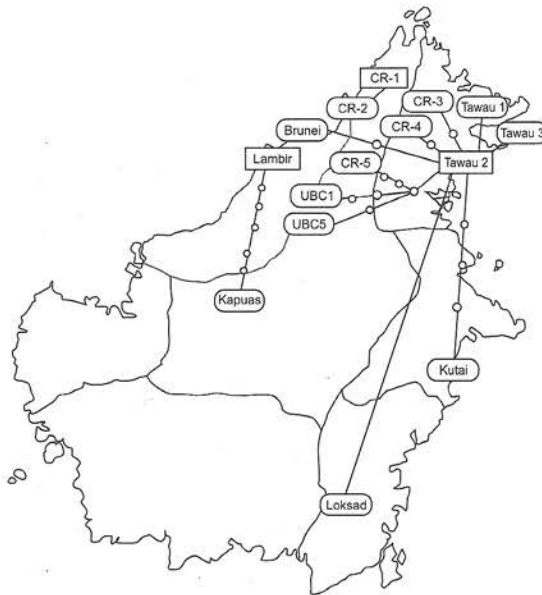


Fig. 3. Networks of the CO1 haplotypes of *A. koschevnikovi* showing the different mitochondrial lineages on the map representing the seven biogeographic divisions in Borneo (MacKinnon *et al.* 1997). The haplotypes determined to be placed at the basal position by the cladistic analysis are displayed with squares (CR-1, Tawau2 and Lambir). Each bar shows a single-step nucleotide substitution and "o" between any two haplotypes represents an intermediate haplotype not found in the samples examined. Abbreviations of haplotypes: CR-1, -2, -3, -4, and -5: Crocker Range type 1, 2, 3, 4 and 5, respectively; UBC1 and UBC5: Upper Baram C1 and C5, respectively; Kapuas: Upper Kapuas.

Table 2. Sequence divergence between the CO1 haplotypes of *A. koschevnikovi*, measured by the Tamura and Nei's evolutionary distance (upper diagonal) and absolute number of substitutions in 1041 nucleotide sequences (lower diagonal) based on pairwise comparison

CO1 Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Crocker Range type 1	-	0.001	0.078	0.080	0.081	0.081	0.080	0.079	0.082	0.083	0.081	0.082	0.079	0.080	0.078
2. Crocker Range type 2	1	-	0.079	0.081	0.082	0.082	0.081	0.080	0.083	0.084	0.082	0.083	0.080	0.081	0.079
3. Crocker Range type 3	75	76	-	0.004	0.006	0.003	0.002	0.003	0.006	0.005	0.004	0.006	0.003	0.019	0.017
4. Crocker Range type 4	77	78	4	-	0.005	0.003	0.002	0.003	0.006	0.005	0.004	0.006	0.003	0.021	0.019
5. Crocker Range type 5	78	79	6	5	-	0.005	0.004	0.005	0.006	0.005	0.006	0.008	0.005	0.019	0.019
6. Tawau 1	78	79	3	3	5	-	0.001	0.002	0.005	0.004	0.003	0.005	0.002	0.020	0.018
7. Tawau 2	77	78	2	2	4	1	-	0.001	0.004	0.003	0.002	0.004	0.001	0.019	0.017
8. Tawau 3	76	77	3	3	5	2	1	-	0.005	0.004	0.003	0.005	0.002	0.020	0.018
9. Upper Baram C1	79	80	6	6	6	5	4	5	-	0.005	0.006	0.008	0.005	0.021	0.021
10. Upper Baram C5	80	81	5	5	5	4	3	4	5	-	0.005	0.007	0.004	0.020	0.020
11. Brunei	78	79	4	4	6	3	2	3	6	5	-	0.006	0.003	0.020	0.018
12. Kutai	79	80	6	6	8	5	4	5	8	7	6	-	0.005	0.021	0.019
13. Losad	76	77	3	3	5	2	1	2	5	4	3	5	-	0.020	0.018
14. Lambir	77	78	19	21	19	20	19	20	21	20	20	21	20	-	0.006
15. Upper Kapuas	75	76	17	19	19	18	17	18	21	20	18	19	18	6	-

drier climates during 'glacial' stages through the Pleistocene (Morley, 2000), and after that it would have recovered partially accompanied by regaining moist climate regions in the Holocene. Recently, deforestation has occurred in broad areas in the north of Borneo as well as other regions of the Southeast Asian tropics by human activities. Such a sequence of events would explain the derivation of the genetic compounds in *A. koschevnikovi* collected from the Crocker Range at the present study.

The primary forests around Tawau would have

served as a 'refuge' and a source for the wide-ranging lineage of *A. koschevnikovi* (Fig. 3). On the other hand, the lineage peculiar to the Crocker Range should have been derived from another 'refuge' that had been isolated for a long period on a geological time scale. This insight is supported by the fact that the cluster consisting of the Crocker Range types 1+2 is divergent largely from the other two clusters in the phylogenetic tree (Fig. 2). Nowadays the forests of the Crocker Range form invaluable habitats for both the lineages because the

environment in surrounding areas has been vastly changed by deforestation.

Environmental changes in the range of *A. koschevnikovi* ought to be accompanied by movements of other species of plants and animals, including angiosperms pollinated by this species of honeybee. Thus ecological relationships among the species also have their own phylogeographic backgrounds. Therefore, making efforts to preserve the genetic diversity of *A. koschevnikovi* in the Crocker Range, with insights into its historical setting, will contribute to conserve priceless items of this natural heritage persisting in a "biodiversity hotspot" of Borneo, where the species endemism is rich, but highly vulnerable to devastation by human activities (Myers *et al.*, 2000).

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