Article

Genetic differentiation among geographic groups of three honeybee species, *Apis cerana, A. koschevnikovi* and *A. dorsata*, in Borneo

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Abstract

We sequenced the mitochondrial cytochrome oxidase 1 (CO1) gene of three honeybee species of Borneo, *Apis cerana, A. koschevnikovi* and *A. dorsata,* and carried out phylogenetic analyses considering geographic genetic variation. We also compared the sequence divergence-geographic distance relationships among the three species. Estimated genetic differentiation was an order of magnitude larger in *A. koschevnikovi* than in *A. cerana and A. dorsata*. Migratory nesting behavior and cold tolerance of each honeybee, and the paleoclimate of the Southeast Asian tropics, are discussed as factors that produced these characteristics for mitochondrial genetic markers, and conservation priorities are recommended.

Key words: *Apis,* Borneo, cytochrome oxidase 1, genetic differentiation, migratory nesting behavior, paleoclimate

Introduction

Of the nine species and/or candidate of species recognized at present in honeybees, genus *Apis* (Engel, 1999; Otis, 1996), five species inhabit Borneo; the dwarf honeybee (*A. andreniformis*), the giant honeybee (*A. dorsata*), the Asian honeybee (*A. cerana*), the Saban honeybee (*A. koschevnikovi*) and the Bornean montane honeybee (*A. nuluensis*). The dwarf honeybees and the giant honeybees make a single-comb nest in the open (Ruttner, 1988), whereas the others are cavity-nesting honeybees which construct multiple parallel combs in hollows (Ruttner, 1988; Tingek et al., 1988, 1996).

Recent studies reveal that eusocial bees such as honeybees and stingless bees (Apini and Meliponini, Apidae) are important as pollinators for many tropical plant species (e.g., Momose et al., 1998). Hence, detailed geographic studies on phylogenetic relationships and genetic divergence among honeybees in the Southeast Asian tropics will not only contribute to understanding evolution of plant-pollinator interaction but also present essential knowledge to conservation planning of tropical forests (see Lee et al., 2000; Rincón et al., 1999). We suggest that genetic variation within plant-pollinator assemblages clarifies not only phylogenetic concepts associated with biological species, but may also indicate evolutionary potential and adaptive diversity in mutualisms (Thompson, 1994).

Smith (1991) demonstrated that mitochondrial DNA (mtDNA) was a powerful tool to estimate genetic differentiation and geographic variations within species of *Apis*. For *A. mellifera*, the western cavity-nesting honeybee, intensive studies have been carried out to clarify the phylogeny of subspecies or geographic races by employing the CO1-CO2 region (the intergenic non-coding region between cytochrome oxidases 1 and 2) (Cornuet and Garnery, 1991; Franck et al., 1998; Garnery et al., 1992) and NADH dehydrogenase 2 (Arias and Sheppard, 1996) of mtDNA. Geographic

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relationships of *A. cerana* in its range (Deowanish et al., 1996; Smith and Hagen, 1996; Smith et al., 2000) and population structure of this species in Thailand (Sihanuntavong et al., 1999) and in the Philippines (De La Rúa et al., 2000) were also investigated by using the CO1-CO2 region. However, such a genetic study has been rarely conducted for other *Apis* since Smith's (1991) work, and never for more than one co-occurring species. Tanaka et al. (2001) suggested that mitochondrial cytochrome oxidase 1 (CO1) is useful for intra-specific genetic studies in *Apis* in the previous work that examined the phylogenetic position of *A. nuluensis* of northern Borneo in genus *Apis*.

In the present study, we sequence the CO1 gene of three honeybee species living in Borneo (*A. cerana, A. koschevnikovi* and *A. dorsata*) and carry out phylogenetic analyses in order to examine genetic relationships among geographic groups of each species. Then we compare the sequence divergence-geographic distance relationships in Borneo among the three species. Finally, we discuss possible roles of different factors producing geographical genetic divergences among those species.

Materials and Methods

Collection of bees and experimental procedures

Bee samples of Borneo were collected from localities shown in Fig. 1. In Crocker Range Park, Sabah, Malaysia, *A. cerana* and *A. koschevnikovi* were collected at Mahua camp and *A. dorsata* was caught at Ulu Senagang in a field survey as a part of the CROCKER XPDC '99 organized by The Sabah Parks, Universiti Malaysia Sabah and Universiti Malaysia Sarawak. Both Mahua and Ulu Senagang are located on the eastern side of the mountain range. The specimens of *A. dorsata* were also collected in Bandar Sri Aman, Sarawak, Malaysia. After the collection, bee samples were fixed with 99.5 % ethanol until DNA extraction. Samples examined in a previous study (Tanaka et al., 2001) were also included in phylogenetic analyses (see below). Those specimens comprise *A. cerana* from Kinabalu Park (Sabah), Brunei, Taiwan, Thailand, Russia, Japan, South Korea, *A. nuluensis* from Mt. Kinabalu, *A. koschevnikovi* from Brunei, Lambir (Sarawak), *A. mellifera* from Uzbekistan, and *A. dorsata* from Lambir (Table 1).

Extraction of total DNA was done according to the method of Hillis et al. (1996). A partial sequence of the CO1 was amplified by polymerase chain reaction (PCR). The primers used in amplification and the condition of PCR were described elsewhere (Tanaka et al., 2001). Amplified fragments were purified by a QIAquick PCR purification kit (QIAGEN) and then used in sequencing reaction with a Dye terminator cycle sequencing kit (Applied Biosystems). The DNA

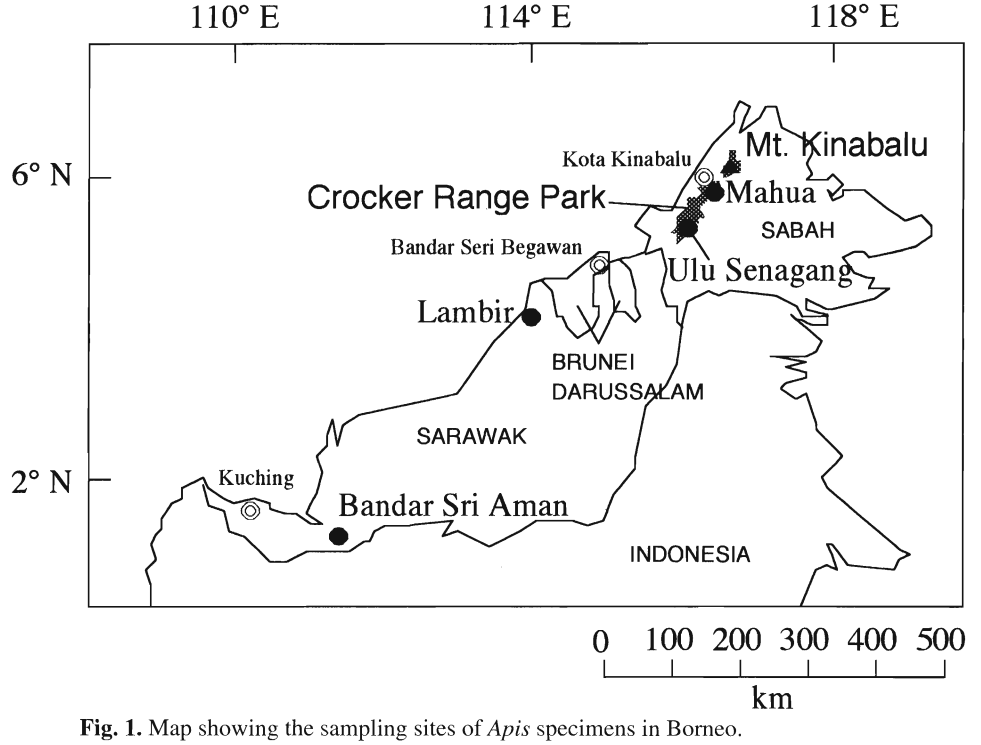

Fig. 1. Map showing the sampling sites of *Apis* specimens in Borneo.

Table 1. List of *Apis* samples used in phylogenetic analyses in the present study

Species	Locality	No. of CO1 haplotypes obtained	Genbank accession No.	Reference
Apis cerana	Kinabalu Park, Sabah, Malaysia	2	AF153101, AF153102	Tanaka et al. (2001)
	Brunei	1	AF153103	Tanaka et al. (2001)
	Crocker Range Park, Sabah, Malaysia	1	AY012722	Present study
	Taiwan	2	AF153104, AF153105	Tanaka et al. (2001)
	Bangkok, Thailand	1	AF153106	Tanaka et al. (2001)
	Primorye, Russia	1	AF153107	Tanaka et al. (2001)
	Japan	1	AF153109	Tanaka et al. (2001)
	South Korea	1	AF153108	Tanaka et al. (2001)
A. nuluensis	Mt. Kinabalu, Sabah, Malaysia	2	AF153099, AF153100	Tanaka et al. (2001)
A. koschevnikovi	Brunei	1	AF153110	Tanaka et al. (2001)
	Lambir Hills National Park, Sarawak, Malaysia	1	AF153111	Tanaka et al. (2001)
	Crocker Range Park, Sabah, Malaysia	1	AY012723	Present study
A. mellifera	Tashkent, Uzbekistan	1	AF214668	Tanaka et al. (2001)
A. dorsata	Lambir Hills National Park, Sarawak, Malaysia	2	AF153112, AF153113	Tanaka et al. (2001)
	Crocker Range Park, Sabah, Malaysia	2	AY012724, AY012725	Present study
	Bandar Sri Aman, Sarawak, Malaysia	2	AY012726, AY012727	Present study

sequencing was carried out with an auto-sequencer (Applied Biosystems 373 DNA sequencer). Voucher specimens are at the Center for Ecological Research, Kyoto University.

Phylogenetic analyses

The honeybee sequences listed in Table 1 were aligned with an outgroup sequence of a stingless bee (*Trigona amalthea*; Genbank accession No. AF214669, Tanaka et al., 2001) using CLUSTAL W (Thompson et al., 1994). The 1041 base-pair sequences were analyzed by maximum parsimony (MP) and neighbor-joining (NJ) methods using PAUP*4.0 beta version (Swofford, 1999). In the MP analysis, a character-weighting method was performed with transversions weighted 5 times as much as transitions, according to our previous study (Tanaka et al., 2001). The heuristic search option with the simple sequence addition and TBR branch-swapping algorithm were selected. For the NJ analysis, evolutionary distance was measured by a general time-reversible model (Yang, 1994). To evaluate the confidence of internal branches, a bootstrap test was implemented with 1,000 replicates in both analyses.

To estimate the degree of genetic differentiation among geographic groups of *A. cerana, A. koschevnikovi* and *A. dorsata*, sequence divergence was calculated using the Tamura and Nei (1993) distance, based on pairwise comparisons of CO1 haplotypes.

Results

Phylogenetic analyses

Sequence data obtained here have been entered in GenBank (Table 1). The common CO1 haplotypes were shared between *A. cerana* of Kinabalu 1 (sample No. 1 collected at Kinabalu Park) and of Brunei and also between *A. cerana* of Japan and of South Korea, respectively. Therefore, a total of 20 different honeybee sequences were analyzed. Of the 1041 nucleotide positions, 277 characters were variable, which included 180 parsimony-informative characters. Figures 2 and 3 present phylogenetic relationships among *Apis* species and geographic groups of *A. cerana, A. koschevnikovi* and *A. dorsata* obtained by the weighted MP and NJ analyses, respectively. The topologies of both phylogenetic trees were nearly congruent with each other. The species relationships depicted in these trees coincided with the *Apis* phylogeny proposed by Engel (1998) and Engel and Schultz (1997), also with that reconstructed using morphological data (Alexander, 1991a, 1991b), except for including *A. nuluensis* (sister to *A. cerana*) and excluding dwarf honeybees (basal to *A. dorsata*).

In the phylogenetic trees, *A. cerana* from Crocker Range was placed in a cluster of Bornean group. *Apis koschevnikovi* from Crocker Range was connected to the cluster consisting of Lambir and Brunei but diverged from each, as indicated by the deep branching pattern in the NJ tree (Fig. 3). For *A. dorsata*, two slightly

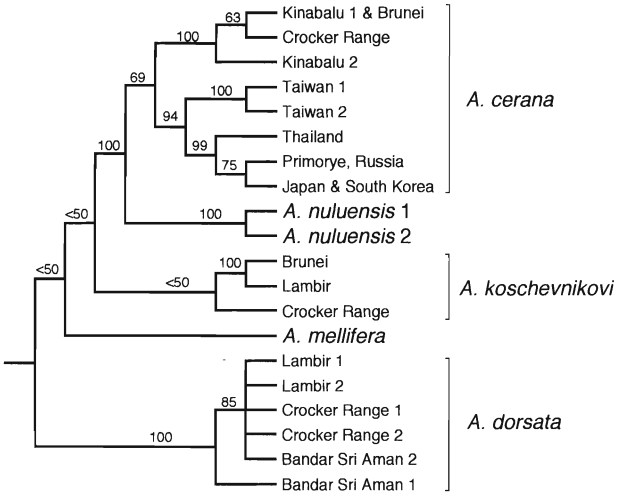

Fig. 2. Phylogenetic relationships of *Apis* species and the geographic groups of *A. cerana, A. koschevnikovi* and *A. dorsata* studied. The tree is strict consensus of 3 equally parsimonious trees obtained by weighted maximum parsimony analysis with transversions weighted five times as much as transitions. Length=2205, CI=0.634 and RI=0.793. Numbers above the branches indicate bootstrap probabilities. A stingless bee (*Trigona amalthea*) was used as an outgroup.

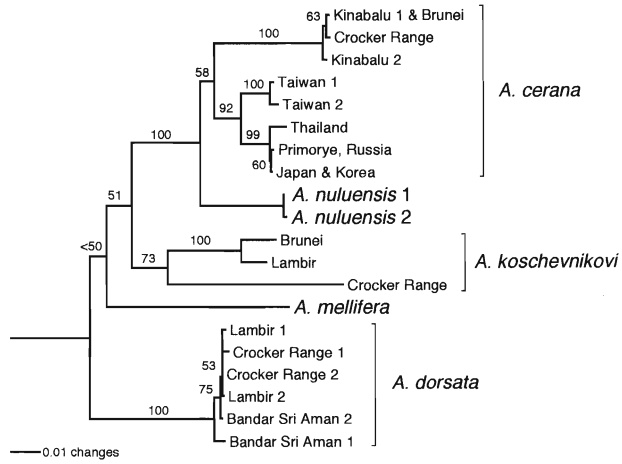

Fig. 3. The neighbor-joining tree showing phylogenetic relationships of *Apis* species and the geographic groups of *A. cerana, A. koschevnikovi* and *A. dorsata* studied. The evolutionary distance was measured by a general time-reversible model (Yang, 1994). Numbers above the branches indicate bootstrap probabilities. A stingless bee (*Trigona amalthea*) was used as an outgroup.

Table 2. Matrix showing the Tamura-Nei evolutionary distance between the geographic groups of *Apis cerana, A. koschevnikovi* and *A. dorsata* based on pairwise comparison of mitochondrial CO1 haplotypes.

Apis cerana	2	3	4	5	6	7	8
1. Kinabalu 1 & Brunei *	0.002	0.002	0.052	0.054	0.056	0.055	0.054
2. Kinabalu 2	-	0.004	0.050	0.052	0.056	0.055	0.054
3. Crocker Range		-	0.052	0.054	0.056	0.055	0.054
4. Taiwan 1			-	0.004	0.024	0.023	0.022
5. Taiwan 2				-	0.026	0.025	0.024
6. Bangkok, Thailand					-	0.007	0.006
7. Primorye, Russia						-	0.001
8. Japan & South Korea *							-

A. koschevnikovi	10	11
9. Brunei	0.020	0.081
10. Lambir	-	0.080
11. Crocker Range		-

A. dorsata	13	14	15	16	17
12. Lambir 1	0.002	0.003	0.001	0.007	0.002
13. Lambir 2	-	0.003	0.001	0.007	0.002
14. Crocker Range 1		-	0.002	0.008	0.003
15. Crocker Range 2			-	0.006	0.001
16. Bandar Sri Aman 1				-	0.007
17. Bandar Sri Aman 2					-

*Common CO1 haplotypes were shared between *A. cerana* of Kinabalu 1 and of Brunei and between *A. cerana* of Japan and of South Korea, respectively.

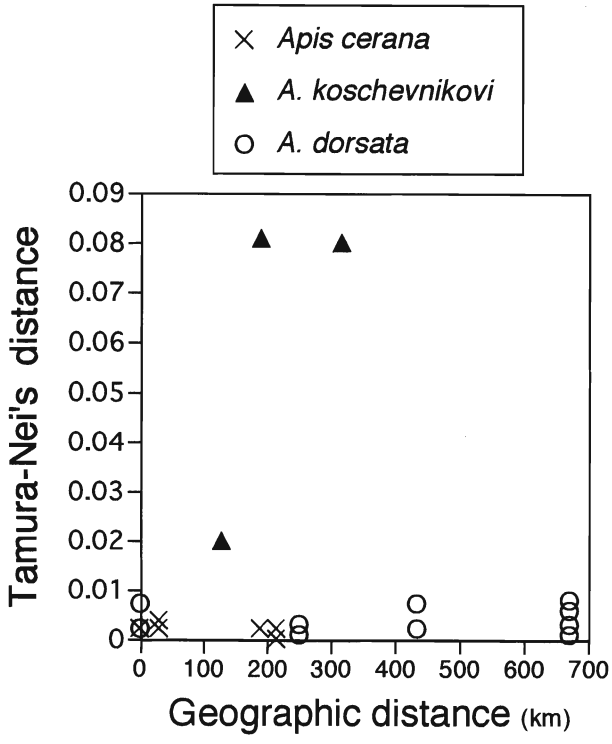

Fig. 4. Comparison of the Tamura-Nei evolutionary distance among geographic groups of *Apis cerana, A. koschevnikovi* and *A. dorsata* in Borneo.

divergent groups were recognized: Bandar Sri Aman 1 and the others.

Sequence divergence among geographic groups of *Apis cerana, A. koschevnikovi* and *A. dorsata*

Table 2 presents sequence divergence corrected by the Tamura-Nei distance between local groups within the species of *A. cerana, A. koschevnikovi* and *A. dorsata*. It ranged from 0.000 to 0.056 for *A. cerana*, from 0.020 to 0.081 for *A. koschevnikovi*, and from 0.001 to 0.008 for *A. dorsata*, respectively. We found a marked difference, one order of magnitude among the extremes, in degree of mitochondrial differentiation among the three species in Borneo. Estimated genetic differentiation among Bornean groups was large in *A. koschevnikovi* but small in *A. cerana* and *A. dorsata* when compared between similar geographic distances (see Fig. 1 and Table 2). The Tamura and Nei distance of Crocker Range-Brunei (linear geographic distance of 190 km, see Fig. 1) was much higher in *A. koschevnikovi* (0.081) than in *A. cerana* (0.002). Similarly, that of Crocker Range-Lambir (linear geographic distance of 250-320 km) was higher in *A. koschevnikovi* (0.080) than in *A. dorsata* (0.001-0.003). For *A. dorsata*, the Tamura-Nei distances of Crocker Range-Bandar Sri Aman (with haplotype 2; 670 km) and Lambir-Bandar Sri Aman (430 km) were a similar level to that of Crocker Range-Lambir. Figure 4 shows plots of Tamura and Nei distance compared to geographic distance between geographic groups of *A. cerana, A. koschevnikovi* and *A. dorsata* in Borneo.

Discussion

The present study revealed that the degree of intra-specific genetic divergence varied widely among the three species of honeybees in Borneo (Table 2 and Fig. 4). Each species may have colonized Borneo at different times and with a different amount of population-level genetic variation. In addition, variation may have been maintained or lost by ecological and demographic processes that differ among these bees.

Although both of *A. cerana* and *A. koschevnikovi* are cavity-nesting honeybees, they inhabit quite different ranges within Asia. *Apis cerana* is widely distributed from the Asian tropics to the temperate zone and also from lowland to high mountain regions (Ruttner, 1988). *Apis koschevnikovi* occurs only in humid tropical forests of limited areas in Southeast Asia (Malay Peninsula, Borneo, Sumatra, a western part of Java) (Otis, 1996). The contrasting habitat ranges of the two species, possibly a result of their speciation in the Cenozoic era during climatic fluctuations (*Apis* was present in the Oligocene, Engel, 1998), could explain such a difference in present geographical distribution. Tanaka et al. (2001) inferred that the separation of *A. nuluensis* from the *A. cerana* population in northern Borneo occurred 2.4 million years ago based on the sequence divergence of CO1. Additionally, they suggested that geographic radiation of *A. cerana* was considerable during this period because intra-specific sequence diversity of *A. cerana* was estimated at a level similar to that between *A. nuluensis* and *A. cerana* of Borneo (Tanaka et al., 2001). Coincidentally, Ruttner (1988) concluded that morphological diversification within *A. cerana* group occurred shortly before or during the Pleistocene.

Paleoclimate of the tropics from the late Tertiary to the Quaternary has been postulated to be cooler and drier than that of present days (Whitmore, 1998) while the climate during the late Pliocene until the Holocene was greatly influenced by glaciations which reduced land temperatures 5°C to 6°C below their present ranges (Colinvaux et al., 1996). During this time, humid tropical forests of Malesia were packed into two blocks, Sundaland (east Malesia) and Papuasia, by penetration

of seasonal forests toward the equator (Whitmore, 1981). The remnant of humid forest in Sundaland of this period has been considered a refuge for the humid tropical Malesian flora (Meijer, 1982). Although the cradle of *A. cerana* is still unknown, adaptation to low temperature and the seasonal forest environment would allow *A. cerana* to radiate to the temperate region. Moreover, *A. koschevnikovi* is considered as an older species among the cavity-nesting honeybees, as suggested from the phylogenetic tree of *Apis* (Figs. 2 and 3; Engel, 1998; Tanaka, unpublished). The range of this species (Otis, 1996) is confined to Sundaland. Therefore, we infer that *A. koschevnikovi* may have occurred in the Southeast Asian tropical region in a time of warmer climate in the Tertiary (Whitmore, 1981), and that its range may have been restricted along with reduction of humid tropical forests through the Pleistocene. Mountains in northern Borneo would have been a barrier preventing the dispersal of *A. koschevnikovi*, and this scenario would explain the large divergence between bees in Crocker Range and elsewhere. *Apis cerana* should maintain larger, connected populations, including mountain areas, during glaciations, due to its superior cold tolerance and larger colony size. The fact that its level of population divergence detected on Borneo is the same as that of *A. dorsata* probably means that these two species, by their contrasting adaptations, continued to maintain similar colonization and local extinction rates since the Pliocene.

Apis dorsata arose before cavity-nesting honeybees, indicated by a more ancestral position in phylogenetic trees (Figs. 2 and 3; Engel, 1998). In spite of that, the degree of genetic differentiation was low among the groups distributed over relatively larger areas of Borneo. It is noteworthy that the Tamura-Nei distance between the CO1 haplotypes, except for the type of Bandar Sri Aman 1, ranges from 0.001-0.003. These haplotypes are considered to belong to the same mitochondrial lineage distinct from a lineage of Bandar Sri Aman 1. Such a distribution of closely related haplotypes indicates that genetic contact across a larger geographic area has been maintained sufficiently more than in *A. koschevnikovi* and possibly *A. cerana*, as postulated from the shape of a mitochondrial gene tree (Avise, 2000). Seasonal long-distance migration (ca. 150-200 km) is well known for *A. dorsata* (Koeniger and Koeniger, 1980). In aseasonal forests in Borneo, Sumatra and Malay Peninsula, general flowering (or mass flowering) occurs at irregular intervals of 3-10 years (Appanah, 1993; Ashton et al., 1988; Momose et al., 1998; Sakai, 2000; Sakai et al., 1999). In a long-term monitoring conducted at Lambir Hills National Park in one general flowering period from 1992 to 1996 (Kato et al., 2000; Momose et al., 1998; Nagamitsu, 1998; Sakai et al., 1999), Nagamitsu (1998) observed that *A. dorsata* immigrated from elsewhere and nested there before general flowering and actively foraged during it. However, it is still unknown where these giant honeybees came from (Sakai, 2000). Recently, it was simultaneously demonstrated in Tenom, Sabah, Malaysia and Assam, India that *A. dorsata* colonies return to the same nesting sites where they nested two to three years prior by using microsatellite DNA markers (Neumann et al., 2000; Paar et al., 2000). The low genetic differentiation and the distribution pattern of CO1 haplotypes in *A. dorsata* (Figs. 2, 3 and 4) might indicate that they move widely searching for floral resources, such as those available during general flowering. Not all colonies return to their former sites during migration episodes, thus genetic exchange over broad areas must occur during colony reproduction. To clarify the scale of migratory range, it is necessary to examine the distribution of mitochondrial lineages in a large area within Borneo.

To confirm or refute the proposed roles of geological history and present ecology in producing the genetic differentiation among geographic groups of the Bornean honeybees, further investigations into distribution, micro-variation and behavior of the Bornean honeybees including other areas of this island are required. *Apis koschevnikovi* prefers wet primary forests whereas *A. cerana* has spread into secondary forests as well as disturbed areas. *Apis dorsata* locates and visits massive flowering dipterocarps that appear at intervals of several years (Nagamitsu, 1998). These species are thus adapted differently to tropical forests in Borneo but probably all of them function as important pollinators. Therefore, researches on the ecological and evolutionary relationships between honeybees and plants in Borneo are needed for conservation of tropical biodiversity of this area. The large evolutionary distances among local groups of *A. koschevnikovi* (Fig. 4) imply fragmentation of wet lowland forests. A high local level of differentiation in this pollinator strongly implies a need for treating each area as an independent unit in a conservation strategy. For implementation of such a multidimensional conservation effort, further collaboration among biologists, conservation planners and social scientists is necessary.

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