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Biogeography of *Apis cerana* F. and *A. nigrocincta* Smith: insights from mtDNA studies

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(Invited paper)

Abstract –This study adds new data from Korea and the Philippines to earlier mitochondrial DNA (mtDNA)-based studies of the phylogeography of Asian cavity-nesting honeybees. A non-coding region that lies between the leucine tRNA gene and the cytochrome oxidase II gene of the mitochondrial genome was sequenced in bees from 153 colonies of *Apis cerana* and *A. nigrocincta*, revealing 41 different haplotypes. Five sequences could not be aligned with the others, two (from India and Sri Lanka) because the sequences were exceedingly A+T rich, and three (from Taiwan, the Philippines, and *A. nigrocincta*) because most of the non-coding sequence was absent. The remaining 36 sequences were aligned, and used in a phylogenetic analysis of *A. cerana* and *A. nigrocincta* populations. Both neighbor-joining and parsimony analyses were carried out, yielding similar results. We found five major groups of haplotypes: an Asian mainland group, a Sundaland group, a Palawan group, a Luzon-Mindanao group, and *A. nigrocincta*. The geographic distribution of these mtDNA haplotypes appears to be strongly influenced by changes in sea-level during Pleistocene glaciations.

***Apis cerana* / *A. nigrocincta* / mtDNA / biogeography / phylogeny**

I. INTRODUCTION

The Asian cavity-nesting honey bee *Apis cerana* is widespread in temperate and tropical Asia. In his 1988 monograph, Ruttner [20] summarized the data then available on morphometric variation in *A. cerana*. He recognized four subspecies: *A. c. cerana* in northern Asia; *A. c. indica* in southern Asia, *A. c. japonica* in Japan; and *A. c. himalaya* in the Himalayan region.

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However, our view of the biogeography of *A. cerana* and other Asian cavity-nesting species is undergoing changes as additional samples and data become available. For example, Peng et al. [19] summarized studies showing that morphometric variation exists among Chinese populations of *A. cerana*. Damus [2] and Damus and Otis [3] carried out a morphometric analysis of cavity-nesting bees from southeast Asia – a region from which few samples were available to Ruttner [20]. They divided Ruttner's geographically widespread *A. c. indica* into *A. c. indica* (samples from Sri Lanka), *A. c. javana* (samples from peninsular Malaysia, Borneo, Java, Bali, Lombok and Flores), a Timor population, and *A. c. philippina* from Luzon and Mindanao (subspecific names follow those proposed by Maa [15]). Smith and Hagen [23, 24] examined geographic variation of *A. cerana* collected from numerous locations, using as a source of information the sequence of a non-coding intergenic region in the mitochondrial genome [1]. Phylogenetic analysis of these data indicated a strong geographic structure in the distribution of mitochondrial DNA (mtDNA) haplotypes. The major groups recognized were mainland Asia (including samples from India, Nepal, northern Thailand, Hong Kong, Korea and Japan), Sundaland (including samples from Samui island, peninsular Malaysia, Java, Bali, Lombok, Flores, Timor and Borneo), Sulawesi, Indonesia (samples recognized as *A. nigrocincta*) and a Philippine group (based on samples from Luzon and Mindanao).

In this study, we examine the biogeography of *A. cerana* and *A. nigrocincta*, extending the work reported in Smith and Hagen [23, 24] to include new samples from Korea and the Philippines. We interpret our results in the light of Pleistocene geography and present-day climatic regions. In so doing, we hope to add to the monumental work begun by Prof. Dr. Friedrich Ruttner, and contribute to a more complete understanding of the intra- and inter-specific biogeography and phylogeny of *Apis* species.

2. MATERIALS AND METHODS

2.1. Collections

Bees for this study were collected by the authors, or donated by colleagues; Figure 1 and Table I show approximate collection sites. These samples consisted of workers frozen in liquid nitrogen or preserved in ~80% ethanol. Ten colony samples from Korea were kindly provided by Dr. Jae Choe; these were from Inje'gun and Hongchon'gun, Kangwon-do; Suwon City (2 samples), Kyonggi-do; Chechon City and Yesangun, Chungchong-buk-do; Mungyong City and Youngchon'gun, Kyongsan-buk-do; Chongju City and Sunchang'gun, Cholla-buk-do; and Kochang'gun, and Kyongsan-nam-do.

Older samples from the Philippines were collected by Otis or Reyes. New samples from the Philippines came from the collection of the University of the Philippines, Los Banos Bee Program (UPLB). As such, collection times varied, with some samples several years older than the rest. These samples were examined earlier using morphometric techniques [26], and more recently using DNA sequencing techniques [27]. Philippine *A. cerana* are from the following locations (see Fig. 2). Palawan island: 1) Puerto Princessa, 2 colonies; 2) Brooke's Point, 3 colonies; 3) Quezon, 2 colonies; 4) Roxas, 1 colony; 5) Taytay, 1 colony; 6) El Nido, 1 colony from the UPLB; and 7) Aborlan, 1 colony collected by Reyes. Luzon island, highlands: 8) Benguet Province, 2 colonies from Baguio City, 1 colony from Tublay, from the UPLB, and 1 colony collected by G. Otis. Luzon island, lowlands: 9) Ilocos Norte Province, 4 colonies, 2 from Lubbot, Batac, 1 from San Mateo, 1 from San Pedro, Lumbao; 10) Laguna Province, 3 colonies from the UPLB, 5 colonies collected by Otis; 11) Zambales, 1 colony collected by Otis. Cebu island: 12) Argao, 1 colony. Panay island: 13) Iloilo, 1 colony. Negros island:

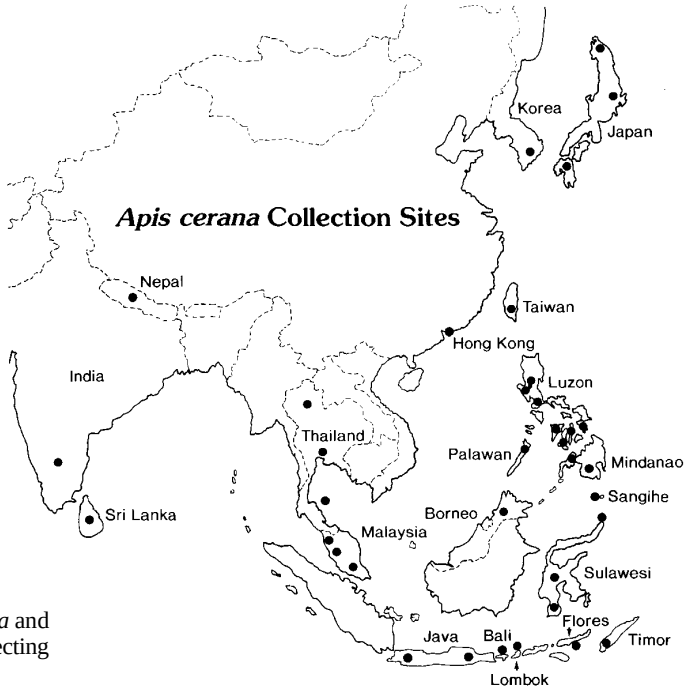

Figure 1. *A. cerana* and *A. nigrocincta* collecting sites.

14) Negros Oriental, 2 colonies from Barrio Bangbang and Bario Valencia; and 15) Negros Occidental, 1 colony from Barrio Banca, Kabangkalan. Leyte island: 16) Isabel, 1 colony. Mindanao island: 17) Davao City, 2 colonies; 18) Davao del Norte, 1 colony; 19) Davao del Sur, 1 colony; 20) North Cotabato, 2 colonies from Kidapawan; and 21) Ozamis, 1 colony.

More detailed collection information on the other samples is presented in Smith [22] and Smith and Hagen [23].

2.2. Sequencing studies

Details of DNA preparation and manual sequencing methods are provided elsewhere [18, 23]. We amplified a portion of the mitochondrial genome from the 3'end of COI to the 5' end of COII using the primers 5'-TCTATACCACGACGTTATTC-3' and 5'-GATCAATATCATTGATGACC-3' [9]. We sequenced the non-coding region using the internal primer 5'-GGCAGAATAAGTGCATTG -3' [1]. Autoradiograms were read by hand, and sequences were aligned by hand and with CLUSTAL W [14].

PAUP [25] was used for phylogenetic analysis of 36 non-coding sequences. Five sequences (IndiaY1, IndiaY2, SulawesiShort, TaiwanShort and PhilippineShort) were omitted from phylogenetic analysis because they could not be aligned with the others. Substitutions among all four bases and insertion/deletions were weighted equally. We carried out a heuristic search for the most parsimonious trees using the tree bisection-resection method, with 1 000 replicates using random addition of sequences. We then took the 50% majority consensus of all equally parsimonious trees. We also used PAUP to construct a neighbor-joining tree [21] of these 36 sequences.

Table I. *A. cerana* and *A. nigrocincta* collections used in this study.

Location	Haplotypes found (No. found)
Nepal	Nepal1 (1)
India	IndiaB1 (1), IndiaB2 (1), IndiaB3 (1), IndiaB4 (2), IndiaY1 (3)
Sri Lanka	IndiaY2 (1)
Thailand	
northern	Japan1 (2), Thai1 (3)
Samui Island	KoSamui1
Korea	Nepal1 (1), Japan1 (7), Korea4 (1), Korea7 (1), Korea8 (1)
Japan	Japan1 (14), Japan2 (1)
Hong Kong	Japan1 (2)
Taiwan	Taiwan1 (5)*
Malaysia	
peninsula	Malay1 (6), Malay2 (1), Malay3 (1)
Borneo	Borneo1 (1), Borneo2 (1), Borneo3 (1)
Indonesia	
Java	Java1 (7), Java2 (1)
Bali	Java1 (1), Bali1 (1), Bali2 (1), Bali3 (1)
Lombok	Lombok1 (5)
Flores	Java1 (4)
Timor	Java1 (4)
Sulawesi	Java1 (10), SulawesiY1 (2), SangiheY1 (4), SulawesiShort (6)*
Sangihe	SangiheY1 (2), SulwesiShort (1)*
Philippines	
Palawan	Palawan1 (4), Palawan2 (7)
Luzon	Luzon1 (13), Luzon2 (4),
Cebu	Cebu1 (1)
Negros	Negros1 (1), PhilippineShort (2)*
Leyte	Mindanao2 (1)
Mindanao	Mindanao1 (2), Mindanao2 (4), MindanaoP (1), MindanaoL (1), Luzon2 (1)

Total number of specimens sequenced: 153.

A. nigrocinta samples found on the islands of Sulawesi and Sangihe, Indonesia, are indicated in boldface small capitals. Haplotypes indicated by an asterisk* are those in which most of the non-coding region is absent. Collection information is given in the text for samples from the Philippines and Korea, and in Smith and Hagen [23] for other locations. Haplotype abbreviations are as in Figure 3.

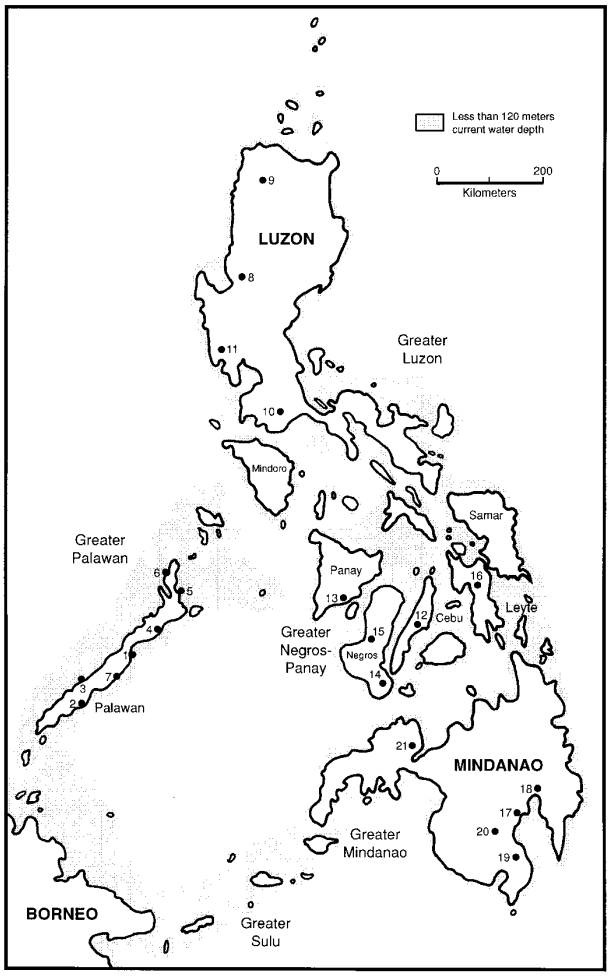

Figure 2. *A. cerana* collecting sites in the Philippines. Solid lines show modern coast lines. Shading shows inferred late Pleistocene coast lines, after Heaney [11]. Dots are approximate locations of collection sites.

3. RESULTS

Four haplotypes were found in new samples from Korea (Japan1, Korea4, Korea7, Korea9) and 11 in the samples from the Philippines (Palawan1, Palawan2, Cebu1, MindanaoP, Luzon1, Luzon2, MindanaoL, Mindanao11, Mindanao2, Negros1 and PhilippineShort). With the haplotypes published earlier [23], this totals 41 different non-coding sequences among 153 colonies sampled. Each sequence was given a short name, indicating the locality in which it was first observed.

We were able to align all but five of the 41 non-coding sequences. Sequences IndiaY1 and IndiaY2 from India and Sri Lanka (see Smith and Hagen [23]) were found in bees locally described as belonging to a 'yellow' or 'plains' race. These two sequences are slightly shorter than most of the others, and the base composition is almost 100% A+T. Although the 5' and 3' ends of these sequences can be matched with the corresponding regions of the other sequences, the mid region cannot be aligned with confidence. This does not necessarily mean that the bees carrying the IndiaY1 or IndiaY2 haplotypes

Aligned *A. cerana* haplotypes

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1)  Nepal1      AAAATTTAATAAGCTACAA-TT-GCATTGAAT-TCTGAATTCAAACTCAAAG--TAAAAAACTTTT-ATT-AAAATTAATAATCTAAATTTATTATTAAAATTT
2)  Japan1      AAAATTTAATAAGCTACAA-TT-GCATTGAAT-TCTGAATTCAAACTCAAAG--TAAAAAACTTTT-ATT-AAAATTAATAATTTAAATTTATTATTAAAATTT
3)  Korea4      AAAATTTAATAAGCTACAA-TT-ACATTGAAT-TCTGAATTCAAACTCAAAG--TAAAAAACTTTT-ATT-AAAATTAATAATTTAAATTTATTATTAAAATTT
4)  Korea7      AAAATTTAATAAGCTACAA-TT-GCATTGAAT-TCTGAATTCAAACTCAAAA--TAAAAAACTTTT-ATT-AAAATTAATAATTTAAATTTATTATTAAAATTT
5)  Korea9      AAAATTTAATAATCTACAA-TT-GCATTGAAT-TCTGAATTCAAACTCAAAG--TAAAAAACTTTT-ATT-AAAATTAATAATTTAAATTTATTATTAAAATTT
6)  Palawan1    AAAATTTAATAATCTACAA-TTTACATTGAAT-TTTAAATTCAAACTCAAAG--TAAAA--CTTT--ATT-AAAATTAATAATTTAAACTTATTATTAAAATTT
7)  Palawan2    AAAATTTAATAATCTACAA-TTTACATTGAAT-TTTGAATTCAAACTCAAAG--TAAAA--CTTT--ATT-AAAATTAATAATTTAAACTTATTATTAAAATTT
8)  Cebu1       AAAATTTAATAATCTACAA-TTTACATTGAAT-TTTGAATTCAAA------G--TAAAA--CTTT--ATT-AAAATTAATAATTTAAACTTATTATTAAAATTT
9)  MindanaoP   AAAATTTAATAATCTACAA-TTTACATTGAAT-TTTGAATTCAAACTCAAAG--TAAAA--CTTT--AT--AAAATTAATAATTTAAACTTATTATTAAAATTT
10) Luzon1      AAAATTTAATAAAATACAAAT--ACATTGAAT-TAT-AATTCAAAATTAAAGTATAA----CTTT--ATT-AAATTTAATAATTTAAA-TTATTATTAAAATTT
11) Luzon2      AAAATTTAATAAAATACAAAT--AAATTGAATCTAT-AATTCAAAATTTAAGTATAA----CTTT--ATT-AAATTTAATAATTTAAA-TTATTATTAAAATTT
12) MindanaoL   AAAATTTAATAAAATATAAAT--AAATTGAATCTAT-AATTCAAAATTTAAGTATAA----CTTT--ATTTAAATTTAATAATTTAAA-TTATTATTAAAATTT
13) Mindanao1   AAAATTTAATAAACTT-AAAT--ATATTGAAT-TTTAAATTCAAACTTAAAATAATA----TTTT--ATTTAAAATTAATAATTTAA--TTATTATTAAAATTT
14) Mindanao2   AAAATTTAATAAACTT-AAAT--ATATTGAAT-TTTAAATTCAA-CCTTAAA-ATAAAA--TTTT--ATT-AAAATTAATAATTTAAA-TTATTATTAAAATTT
15) Negros1     AA?ATTTAATAAACTTTAA-T--ATATTGAAT-TTTAAATTCAATC?TTAAA-ATAAAA--TTTT--ATT-AAAATTAATAATTTAAA-TTATTATTAAAATTT

A. nigrocincta haplotype aligned with A. cerana haplotypes
16) SulawesiY1 AAAATTTAATAAACTATA--TTTACATTGAAT-TAT-AATTCAATCCTAAAGTTTATAAA-CTTT--ATT-AAAATTAATAATTTA--CTTATTATTAAAATTT

Short haplotypes not aligned with others
17) SulawesiShort       AAAATTAATAATTT-AA-----TT-ATTATTAAAATTT
18) TaiwanShort         AAAATTAATAATTTTAA----TTT-ATTATTAAAATTT
19) PhilippineShort     AAGATTAATAATTT-AATAAATTTAATTATTAAAATTT
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Figure 3. Non-coding sequences of *A. cerana* and *A. nigrocincta.* Sequences of *A. cerana* from Korea and the Philippines, *A. nigrocincta*, and short haplotypes lacking most of the non-coding sequences are shown. See Smith and Hagen [23] for a complete listing and alignment of earlier sequences. Sequences are named after the first place they were found. Dashes indicate inferred insertion/deletion events.

are more distantly related to the other *A. cerana*; we are simply unable to interpret the historical information that might be contained in these sequences. Three haplotypes – SulawesiShort, TaiwanShort and PhilippineShort – lack most of the non-coding region. Only the 5' and 3' ends and a few bases between them are present. Figure 3 shows the sequences found in Korea and the Philippines, along with an *A. nigrocincta* haplotype, and the three haplotypes that lack most of the non-coding region.

We excluded the sequences IndiaY1, IndiaY2, and the three short sequences from the phylogenetic analyses. The neighbor-joining gene tree for the remaining 36 sequences is shown in Figure 4. This tree shows 5 major branches: a 'mainland' group of haplotypes, including IndiaB1-4, Nepal1, Japan1-2, Korea4, 7 and 9 and Thai1; a 'Sundaland' group, including KoSamui1, Malay1-3, Borneo1-3, Java1-2, Bali1-3, and Lombok1; a 'Palawan' group, including Palawan1-2, MindanaoP (so called because it was found on Mindanao, but is similar to haplotypes found on Palawan), and Cebu1; an *A. nigrocincta* group including SulawesiY1 and SangiheY1; and a Luzon-Mindanao group, including Luzon1-2, Mindanao1-2, MindanaoL (so called because it was found on Mindanao, but is similar to sequences found on Luzon), and Negros1.

The heuristic search for the most parsimonious midpoint-rooted tree of non-coding sequences produced 1 017 equally parsimonious trees of length 80. The large number of equally parsimonious trees is due to the inclusion of sets of 'nearly identical' sequences in the analysis (such as Bali1, 2, and 3, or Borneo1, 2, and 3; Smith and Hagen [23]). The 50% majority rule consensus of these trees, shown in Figure 5,

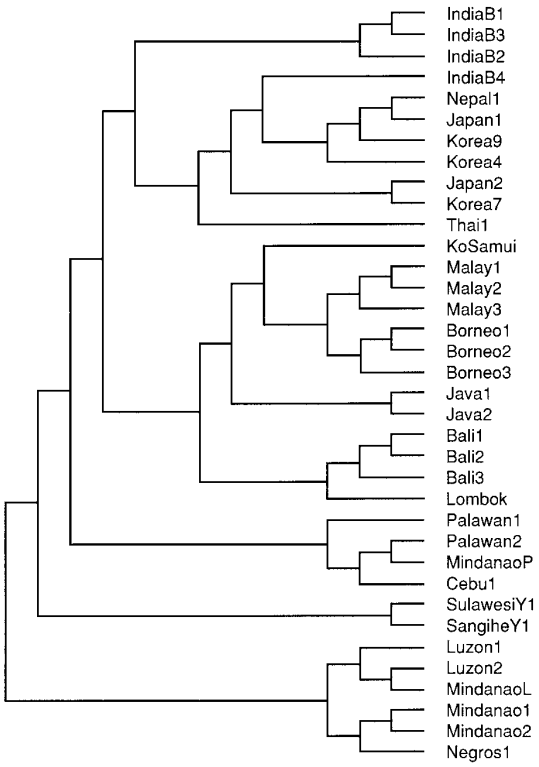

Figure 4. Neighbor-joining tree based on the sequences of 36 non-coding intergenic regions in the mtDNA of *A. cerana* and *A. nigrocincta*. Haplotype/sequence names are as in Figure 3 and in Smith and Hagen [23].

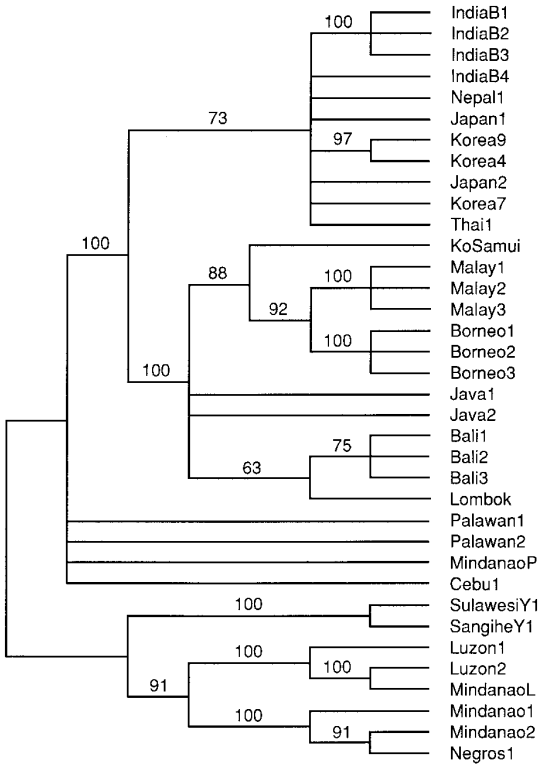

Figure 5. Fifty percent majority rule consensus of 1 017 equally parsimonious midpoint rooted trees of 80 steps. Trees were generated by a heuristic search using PAUP* [25] with the following conditions: 35 informative characters, tree bisection/resection, assignment of states to internal nodes limited to states observed in the terminal taxa; 1 000 replicates starting with random trees and random sequence addition. Numbers on branches indicate the frequency of this grouping in the 80 most parsimonious trees. Haplotype names are as in Figure 3 and in Smith and Hagen [23].

shows the same major groupings as the neighbor-joining tree. The neighbor-joining and majority-rule trees differ primarily in the placement of *A. nigrocincta* (haplotypes SulawesiY1 and SangiheY1; Smith and Hagen [23]). The neighbor-joining tree unites *A. nigrocincta* haplotypes with mainland Asia, Sundaland and Palawan *A. cerana* haplotypes, while the 50% majority-rule parsimony tree unites *A. nigrocincta* with the Luzon and Mindanao *A. cerana* haplotypes.

Figure 6a shows the neighbor-joining tree, but gives the geographic locations in which each haplotype was found. Figure 6b shows the main branches of the neighbor-joining tree and the biogeographic region (or species, in the case of *A. nigrocincta*) in which the sequences were found.

Short sequences, lacking most of the non-coding region, were found in Taiwan (sequence TaiwanShort), Sulawesi and Sangihe (sequence SulawesiShort) and in the Philippine islands of Negros and Panay (sequence PhilippineShort). Superficially, it may seem that loss of most of the non-coding region is a synapomorphy uniting these three sequences, but we feel it is more likely that these are three independent losses. Cornuet et al. [1] sequenced this non-coding region in *A. mellifera*, *A. cerana* from Sri Lanka, *A. dorsata* and *A. florea*. They showed that the non-coding sequence in the Sri Lankan *A. cerana* could be folded into a clover-leaf structure, the stem of which is made by base-pairing between the conserved 5' and 3' ends of the non-coding sequence. These stem sequences are conserved in all the *Apis* species examined by Cornuet et al. [1] and in virtually all of the *A. cerana* and *A. nigrocincta* we examined. In the short sequences, the 5' and 3' ends comprising the 'stem' are retained, but most of the

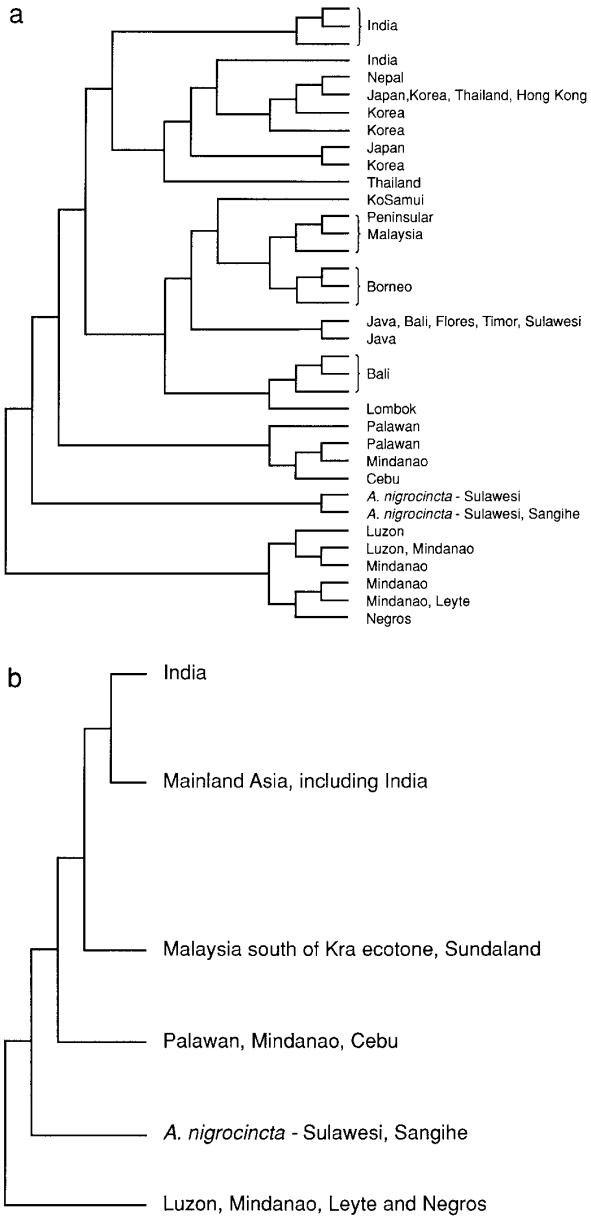

Figure 6. a: Neighbor-joining tree (from Fig. 4) showing the locations in which each haplotype was found. **b:** Main branches of neighbor-joining tree of *A. cerana* haplotypes and the geographic region to which each branch corresponds.

'leaflets' of the clover leaf have been lost (Fig. 7). The stem sequences can base pair to form a hairpin, with a stem and small loop. The exact points at which the 'leaflets' are lost, and the sequences left in the small loops differ among the three short sequences.

4. DISCUSSION

This is part of an ongoing study of the biogeography of *Apis*. Our conclusions about diversity and biogeography of *A. cerana* populations are limited by the

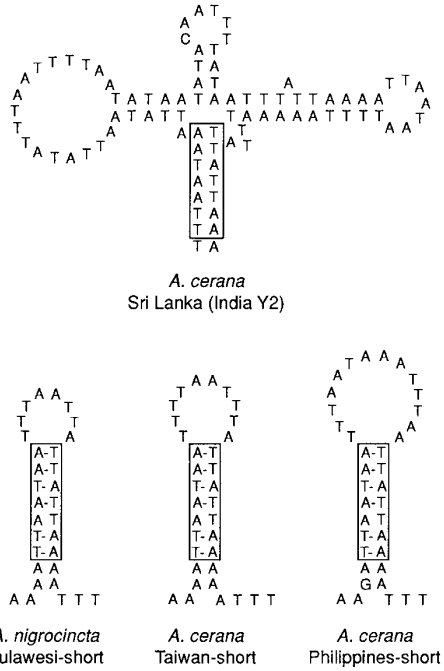

Figure 7. The non-coding sequence of Sri Lankan *A. cerana*, and the three short sequences: *A. cerana* TaiwanShort and PhilippineShort, and *A. nigrocincta* SulawesiShort, showing how they can be folded into clover-leaf and hairpin structures. The clover-leaf structure of Sri Lankan *A. cerana* is redrawn from Cornuet et al. [1].

samples available to us. Nonetheless, major biogeographic patterns within *A. cerana* are now evident, and our results indicate four groups of mtDNA haplotypes within *A. cerana*: a mainland Asia group, a Sundaland group, a Palawan group, and as Luzon-Mindanao group. A fifth group of haplotypes is found in our *A. nigrocincta* samples.

The mainland Asia and Sundaland groups are discussed in more detail in Smith and Hagen [23], but several points are worth noting here. First, India has an extremely diverse collection of *A. cerana* haplotypes, second only to the Philippines. These bees clearly merit further study. Second, the mainland Asia group of mitochondrial haplotypes is extremely widespread. It occurs over a larger geographic region than any of the other groups does, and there is little variation among mainland haplotypes outside of India. This suggests a large effective population size and gene flow among the *A. cerana* populations of mainland Asia, perhaps coupled with rapid post-glacial recolonization of the northern parts of *A. cerana's* range.

The Sundaland group includes mtDNA haplotypes found in our *A. cerana* samples from peninsular Malaysia and the islands lying on the broad Sunda continental shelf of southeast Asia: the modern islands of Sumatra, Java, Bali, Borneo and many smaller islands [10–12]. During Pleistocene episodes of glaciation, accumulation of water in glaciers and polar icecaps produced sea levels approximately 160 m lower than at present during the mid Pleistocene (160 000 years ago) and 120 m lower than at present in the late Pleistocene (16 000 to 18 000 years ago [13]. During these times, the islands on the Sunda shelf would have been united directly with the Asian mainland by dry land, forming a region known as Sundaland. Migration and gene flow between continental Asia and Sundaland was followed by isolation of Sundaland populations as sea levels rose to their present levels.

The biogeographic break between the Asian mainland group and the Sundaland group lies in the Isthmus of Kra, in the Malay Peninsula. A recent study of mtDNA restriction site polymorphisms in *A. cerana* from Thailand [4] refines this observation. The authors report that populations of *A. cerana* north of approximately 10°N latitude (in the Isthmus of Kra) show significantly different mtDNA haplotype frequencies from populations to the South. (In earlier studies by Smith [22] and Smith and Hagen [23], the samples described as being from North and South Thailand were from the Chiang Mai and Bangkok regions, respectively; both sites are within Deowanish et al.'s North Thailand region.) In the work

presented here, Samui Island is the only Thai collection site that falls into Deowanish et al.'s South group. The mtDNA haplotype from Samui Island is distinct from that of northern Thai samples and is placed with the Sundaland group.

Why does the boundary between the mainland and Sundaland mtDNA groups fall in the Malay peninsula, so that mtDNA haplotypes from southern Thailand and peninsular Malaysia group with Sundaland rather than with the mainland Asia haplotypes? The Bilauktaung mountain range, which forms part of the boundary between Myanmar (Burma) and Thailand in the Malay Peninsula, may provide a barrier to gene flow. In addition, this region of the Isthmus of Kra is known as the Kra ecotone, in which there is a shift from evergreen rainforest (South of the imaginary line joining the cities of Kangar and Pattani) to more seasonal, semi-evergreen forest [28]. In addition to marking the boundary between the mainland and Sundaland groups of *A. cerana*, the Kra ecotone also corresponds to the southern boundary of *A. florea* on the Asian mainland [16].

Sulawesi was apparently never connected by dry land to Sundaland and continental Asia. *A. nigrocincta* has been found on Sulawesi, where it is sympatric with *A. cerana*, and on Sangihe [2, 3, 5, 8]. The biology of Sulawesi cavity-nesting bees has been discussed elsewhere [5–7, 17].

The Philippines are a particularly complicated and interesting region for biogeographers. The information presented here on Philippine geology and biogeography is largely drawn from Heaney [10–12] and Heaney and Rickart [13]. Although some islands (notably Palawan) include a small amount of continental crust, most of the islands were formed de novo by volcanic and tectonic activity. The degree to which islands were connected to the mainland (via Sundaland) or to each other was influenced primarily by the changes in sea level that also affected the islands of the Sunda shelf.

Palawan may have appeared as land above the sea in the late Miocene or Pliocene. Today Palawan is separated from nearby Borneo by a trench 145 m deep. During the mid and late Pleistocene, the islands of the Palawan island chain would have been united into a larger island – Greater Palawan. Greater Palawan was united with the Asian mainland through Borneo in the mid Pleistocene, when sea levels were 160 m lower than present, but not in the late Pleistocene, when sea levels were only 120 m lower than present. The other Philippine islands have had no above-water connection to the Asian mainland. The distribution of the dwarf honey bees reflects these connections: *A. andreniformis* is found on both Borneo and Palawan, but is apparently absent from the oceanic islands of the Philippines.

During the mid and late Pleistocene periods of low sea levels many of the oceanic islands of the Philippines were joined by dry land, forming larger units, or 'mega islands': Greater Luzon, Greater Mindanao (which includes modern Mindanao, Samar, Leyte, and Bohol), and Greater Negros-Panay (which includes modern Negros, Panay, Cebu and Masbate). In the mid Pleistocene Greater Luzon and Greater Mindanao may have been joined, though this is not certain. Today the trench separating Luzon and Samar is 140 m deep, but it is also a region of geological uplift. Two island chains may form 'stepping stones' between Borneo and the oceanic islands of the Philippines: the Palawan chain between Borneo and Mindoro, and the Sulu Archipelago between Borneo and Mindanao.

As was the case with the Sundaland samples, the distribution of the 11 mtDNA haplotypes found in our Philippine *A. cerana* samples seems to be strongly influenced by Pleistocene geological history. The Palawan group of haplotypes includes Palawan1, Palawan2, Cebu1 and MindanaoP. Both our neighbor-joining and parsimony trees show the Palawan sequences to be more closely related to Sundaland and mainland Asian

sequences than to other sequences from the Philippines. Sequences Palawan1 and Palawan2 have only been found on Palawan, showing the distinctiveness of that island's *A. cerana* population. Two related sequences, Cebu1 and MindanaoP, have been found on the islands of Cebu and Mindanao, respectively.

Six mtDNA haplotypes make up the group that we have called Luzon-Mindanao (see Figs. 4 and 5). These are Luzon1, Luzon2, MindanaoL, Mindanao1, Mindanao2, and Negros1. The geographic distribution of these sequences shows there may have been gene flow or migration between Luzon and Mindanao, and between Mindanao and Negros-Panay. We have found only haplotypes Luzon1 and Luzon2 in Luzon. Haplotypes Mindanao1 and Mindanao2 were the most common in our samples from Greater Mindanao (i.e., Leyte and Mindanao), but one example of Luzon2 and one example of MindanaoL were also found there. One example of the Luzon-Mindanao haplotype 'Negros1' was found on the island of Negros, part of Pleistocene Greater Negros-Panay.

The last Philippine sequence was not included in phylogenetic analyses: this is PhilippineShort, a sequence lacking most of the non-coding region. Two examples of this were found in the islands of Greater Negros-Panay, one on Panay and one on Negros. Table I and Figure 6 show the distribution of mtDNA haplotypes in the Philippine islands.

The Pleistocene 'mega islands' of the Philippines have been shown to correspond to faunal regions, reflected in the distributions of mammal species and genera [11, 12, 13]; these regions are also applicable to *A. cerana*. Our survey of sequences also indicates that the relatively young Negros-Panay region may have received colonists from Luzon or Mindanao (evidenced by the sequence Negros1, which resembles Luzon sequences) and Palawan, perhaps via Mindanao (evidenced by sequences Cebu1 and MindanaoP, both of which resemble Palawan sequences). The haplotype PhilippineShort may be unique to the islands of Greater Negros-Panay. The complexity of the Philippines' honey bee fauna merits further study – more intensive sampling from the regions discussed here, and samples from other faunal regions such as the island of Mindoro.

In summary, our studies of *A. cerana* indicate four major groups of mtDNA lineages. These are a mainland mtDNA lineage, and three additional mtDNA lineages occurring on lands connected to the mainland for successively shorter periods of time: Sundaland (connected to the mainland during the mid and late Pleistocene), Palawan (connected only during the mid Pleistocene), and the oceanic islands of the Philippines (never connected tot the mainland). The Indonesian island of Sulawesi too, was never connected to the mainland, and here are found two exceedingly similar cavity-nesting bee species, *A. nigrocincta* and *A. cerana* with Sundaland mtDNA.

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Note added in proof

De la Rua et al. (2000) also analyzed variation in the non-coding region of mtDNA of Philippine *A. cerana*, using a slightly different survey method (De la Rua P., Simon E.U., Tilde A.C., Moritz R.F.A., Fuchs S., MtDNA variation in *Apis cerana* populations from the Philippines, Heredity 84 (2000) 124–130). Sihanuntavong et al. (1999) examined mtDNA restriction site variation in *A. cerana* of Thailand

(Sihanuntavong D., Sittipraneed S., Klinbunga S., Mitochondrial DNA diversity and population structure of the honeybee (*Apis cerana*) in Thailand, J. Apic. Res. 38 (1999) 211–219). The results of both studies agree with the data reported here.

Résumé – Biogéographie d'*Apis cerana* F. et d'*Apis nigrocincta* Smith : résultats des études d'ADNmt. Nous avons étudié la variation de la séquence d'ADN dans une région non codante des génomes mitochondriaux de 153 colonies d'*A. cerana* et d'*A. nigrocincta*. Les échantillons d'*A. cerana* provenaient d'Inde, du Sri Lanka, du Népal, de Thaïlande, de Chine (Hong Kong), de Taïwan, de Corée, du Japon, de la péninsule de Malaisie et de Bornéo, des îles indonésiennes de Java, Bali, Lombok, Timor occidental, Flores et Sulawesi (Fig. 1, Tab. I) et des îles Philippines de Palawan, Luzon, Leyte, Mindanao, Cebu, Panay et Negros (Fig. 2, Tab. I). Les échantillons de *nigrocincta* venaient des îles indonésiennes de Sulawesi et de Sangihe.

La figure 3 montre les séquences d'ADNmt trouvées dans les nouveaux échantillons de Corée (Japon1, Corée4, Corée7 et Corée9) et dans 11 échantillons des Philippines (Palawan1, Palawan2, Cebu1, MindanaoP, Luzon1, Luzon2, MindanaoL, Mindanao11, Mindanao2, Negros1 et PhilippineShort). Avec les haplotypes publiés précédemment [23] on arrive à un total de 41 séquences différentes non codantes parmi les 153 colonies échantillonnées. Deux des 6 haplotypes observés parmi les colonies d'Inde et du Sri Lanka n'ont pu être alignées avec les autres séquences d'*A. cerana*. La plus grande partie de la région non codante était absente de 3 haplotypes (TaïwanShort, SulawesiShort et PhilippineShort) (Figs. 3 et 7). La séquence non codante a probablement été perdue indépendamment à trois reprises.

L'analyse phylogénétique des 36 séquences non codantes alignées (les séquences courtes et les deux séquences non alignées mises à part) a été faite à l'aide de deux méthodes statistiques (neighbor-joining et parcimonie maximum). Il en est résulté différents dendogrammes : le dendogramme de la figure 4 est issu de l'algorithme de neighbor-joining, celui de la figure 5 de l'analyse de parcimonie. Les deux méthodes conduisent au même résultat : 5 branches principales réparties ainsi : 1) groupe d'haplotypes du continent asiatique ; 2) groupe de Sundaland ; 3) groupe de Sulawan ; 4) un groupe commun à Luzon et Mindanao ; et 5) haplotypes de *nigrocinta*. La figure 6 donne la répartition géographique de ces haplotypes. La répartition géographique des haplotypes semble avoir été fortement influencée par les changement globaux du niveau de la mer au cours du Pléistocène et par les surfaces de terres émergées qui en ont résulté dans le sud est asiatique. Au cours du Pléistocène moyen et supérieur, les îles du plateau continental Sunda ont été unies les unes aux autres (ou n'étaient séparées que par des chenaux très étroits) et unies au continent asiatique. Palawan a été relié à Bornéo et au continent asiatique au cours du Pléistocène moyen mais pas au Pléistocène supérieur. Sulawesi et les îles océaniques dénommées Philippines n'ont jamais été reliées au continent asiatique.

***Apis cerana* / *A. nigrocincta* / ADNmt / biogéographie / phylogenèse**

Zusammenfassung – Biogeographie von *Apis cerana* F. und *Apis nigrocincta* Smith: Ergebnisse von mtDNA Untersuchungen. Wir untersuchten die Variation der DNA Sequenz in einer nicht codierenden Region des mitochondrialen Genoms von 153 Völkern von *Apis cerana* und *A. nigrocincta.*

Die Proben von *A. cerana* stammten aus Indien, Sri Lanka, Nepal, Thailand, China (Hong Kong), Taiwan, Korea, Japan, Malaysia (Halbinsel und Borneo), den indonesischen Inseln Java, Bali, Lombok, West Timor, Flores und Sulawesi (Abb. 1 und Tab. I) und den philippinischen Inseln Palawan, Luzon, Leyte, Mindanao, Cebu, Panay

und Negros (Abb. 2 und Tab. I). Die Proben von *A. nigrocincta* stammten von den indonesischen Inseln Sulawesi und Sangihe.

In Abbildung 3 sind die mtDNA Sequenzen dargestellt, die in den neuen Proben von Korea (Japan1, Korea4, Korea7, Korea9) und 11 in den Proben aus den Philippinen (Palawan1, Palawan2, Ceb1, MindanaoP, Luzon1, Luzon2, MindanaoL, Mindanao11, Mindanao2, Negros1 und Philippinenshort (kurz) gefunden wurden. Zusammen mit den Haplotypen, die schon früher publiziert wurden [23], ergeben sich von den 153 beprobten Völkern 41 verschiedene nicht kodierende mitochondriale Sequenzen. Zwei von 6 Haplotypen, die in Völkern aus Indien und Sri Lanka gefunden wurden, konnten den anderen *A. cerana* Sequenzen nicht zugeordnet werden. Bei 3 Haplotypen (alle Proben von Taiwan, einige *Apis cerana* Proben von Negros und Panay, Philippinen und einige *A. nigrocincta)* fehlte das größte Stück der nicht kodierenden Region (Abb. 3 und 7). Dieser Verlust der nicht kodierenden Sequenzen ist wahrscheinlich dreimal unabhängig voneinander entstanden.

Phylogenetische Analysen von 36 zusammenhängenden nicht kodierenden Sequenzen (mit Ausnahme der kurzen Sequenzen und zwei anderen, die nicht zugeordnet werden konnten) wurden mit zwei verschiedenen statistischen Methoden durchgeführt (*neighbor-joining* und *maximum parsimony*). Es ergaben sich verschiedene Dendrogramme: das Dendrogramm der Abbildung 4 entstand durch *neighbor-joining algorithm*, das in Abbildung 5 entstand durch die 5*0% majority rule consensus of all most parsimonious trees.* Beide Verfahren führen zu 5 Hauptästen: 1) Gruppe von Haplotypen vom asiatischen Festland; 2) Sundaland Gruppe; 3) Palawan Gruppe; 4) eine gemeinsame Luzon und Mindanao Gruppe; und 5) *A. nigrocincta* Haplotypen. Die geographische Verteilung dieser Haplotypen ist in Abbildung 6 zu sehen.

Die geographische Verteilung scheint stark von den weltweiten Änderungen des Wasserspiegels während des Pleistozäns und den dadurch entstandenen Landflächen beeinflusst worden zu sein. Während des mittleren und späten Pleistozäns waren die Inseln auf dem Sundariff miteinander (oder nur durch sehr enge Kanäle getrennt) und mit dem Festland verbunden. Palawan hing mit Borneo zusammen, und während des mittleren Pleistozäns auch mit dem Festland, war aber im späten Pleistozän wieder getrennt. Sulawesi und die sogenannten Ozean Inseln der Philippinen standen mit dem Festland nie in Verbindung.

***Apis cerana* / *A. nigrocincta* / Mitochondriale DNA / Biogeographie / Phylogenie**

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