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Original article

Mitochondrial DNA diversity of Chinese *Apis cerana**

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Abstract – DNA sequence diversity in a non-coding portion of the mitochondrial genome was investigated in samples of *Apis cerana* from 47 locations in China. Nine haplotypes (mitochondrial genotypes) were found: Japan1, Japan2, Korea4, and Cambodia2, which were previously reported from other populations, and China1−5, which are new. All nine sequences belong to the Mainland mitochondrial lineage, and none differs from the Japan1 haplotype by more than a single base substitution and/or a single insertion/deletion. Japan1 is the most common haplotype, making up 39 of 49 sequences. Haplotype diversity was 0.4 (s.d. 0.089) and nucleotide diversity was 0.00569 (s.d. 0.00154). By both measures the Chinese samples were more diverse than those from Japan and Thailand, similar to populations from Pakistan, Burma and Korea, and less diverse than samples from Indochina (Laos-Cambodia-Vietnam).

mitochondrial DNA / *Apis cerana* / China / phylogeography

1. INTRODUCTION

Apis cerana Fabricius is the most widely distributed of the Asian honey bees. A major portion of its range lies within China, which encompasses diverse habitats and plant communities. The terrain is also dissected by potential barriers to dispersal such as mountain ranges, rivers and deserts. This would appear to provide ideal conditions for the evolution of geographic variation among *A. cerana* populations. Despite this, relatively little work has been devoted to the study of geographic variation in Chinese *A. cerana*. This is particularly true of studies of genetic variation. Here we present data on variation in non-coding mitochondrial DNA sequences in Chinese *A. cerana.*

Hou (1983) recognized eight major vegetational zones in China: cold-temperate deciduous needle-leaved forest, temperate deciduous broad-leaved forest, subtropical evergreen broad-leaved forest, tropical seasonal rain forest, temperate steppe, temperate desert, high-cold meadow and steppe, and high cold semi-desert and desert. Peng et al. (1989) undertook a review and reanalysis of geographical races of *A. cerana* in China covering both English and Chinese language publications – a major contribution, since most western biologists neglect publications in non-European languages. In China, *A. cerana* was found mainly in temperate, broad-leaved deciduous forest, subtropical broad-leaved evergreen forest, tropical seasonal rainforest and the southern parts of the temperate steppe habitat, and was not found in deserts or cold high meadow and

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steppe (Peng et al., 1989; citing work of Yang, 1984a).

Yang (1984a, b, cited in Peng et al., 1989) recognized five geographical races on the basis of morphometric variation: *Eastern* (in southern edge of the eastern temperate steppes and in temperate deciduous, subtropical and tropical evergreen forest of eastern and southeastern China), *Southern Yunnan* (in subtropical and tropical forest south of 24° 30′ N), *Hainan* (in tropical seasonal rain forest on Hainan island), *Aba* (along river valleys of western Sichuan province, at the north western extreme of the subtropical evergreen broad-leafed forest), and *Xizang* or *Tibet* at elevations of 2000 to 4000 m. He further recognized five biotypes within the Eastern race and two within the Hainan race. The ranges of Yang's proposed geographical races and biotypes are illustrated in Peng et al. (1989) and Hepburn et al. (2001).

A recent analysis of morphometric and mitochondrial DNA variation of honey bee populations across China revealed a high degree of variation, strongly associated with ecological zones and correlated with geographical and climatic parameters (Tan et al., 2006). Recent morphometric studies by Radloff, Hepburn and Tan (unpubl. data) indicate a single large "morphocluster" over most of mainland Asia, within which they recognize five biometrically definable subclusters: the Indus and Himachali subclusters west of the Tibetan plateau, and three primarily Chinese subclusters east of the plateau — Aba, Southeastern and Japonica. The northerly Aba population corresponds to the Aba subspecies of Yang. The Southeastern subcluster occurs in central and south China, and the Japonica subcluster occurs in north eastern China. Most of the races and ecotypes recognized by Yang (1984a, b, cited in Peng et al., 1989) were not detected.

A non-coding region of the *Apis* mitochondrial genome (Cornuet et al., 1991) has been used extensively in studies of honey bee biogeography and population biology (for example, Garnery et al., 1992, 1995, 1998; Oldroyd et al., 1995; Moritz et al., 1994; Deowanish et al., 1996; Smith and Hagen, 1996; de la Rúa et al., 1998, 2000; Deowanish et al., 1998; Franck et al., 1998, 2000a,b, 2001; Sheppard et al., 1999; Sihanuntavong et al., 1999; Smith and Hagen, 1999; Palmer et al., 2000; Smith et al., 2000, 2003, 2004, 2005; Warrit et al., 2006, and many more). Few data of this nature are available for Chinese *A. cerana*. To address this we carried out a survey of non-coding mitochondrial DNA (mtDNA) sequence variation in samples of Chinese *A. cerana*.

2. METHODS

Samples of *A. cerana* were collected from 46 locations in 16 provinces and two samples from Hong Kong were provided by M. Crosland (see Tab. I and Fig. 1); a total of 49 colonies were sampled. Workers were collected from managed or semi-managed colonies into 75–90% ethanol, and transported to University of Kansas for DNA analysis.

Total DNA was extracted from one worker from each colony using Qiagen DNEasy binding columns (Qiagen, Valencia, CA, USA) and the protocol recommended for animal tissue. A non-coding region of the mitochondrial genome (Cornuet et al., 1991) was amplified using primers (Hall and Smith, 1991) located in Cytochrome Oxidase I (5'-TCTATACCACGACGTTATTC-3') and Cytochrome Oxidase II (5'-GATCAATATCATTGATGACC-3') and PCR conditions described in Smith and Hagen (1996, 1999). The amplification products were prepared for sequencing by gel purification using the Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Sequencing was carried out at the Idaho State University Molecular Research Core Facility, using an internal primer (5'-GGCAGAATAAGTGCATTG-3') located in the tRNA adjacent to the non-coding region (Cornuet et al., 1991). Sequences were manually aligned with previously published sequences.

Haplotype and nucleotide diversity (π) (Nei, 1987; Nei and Miller, 1990), were calculated for the China samples and for samples from previously studied populations from the Asian mainland (Tab. II), using the program DnaSP (Rozas et al., 2003). Haplotype diversity considers the number and frequency of observed haplotypes, while nucleotide diversity considers sequence divergence among haplotypes as well as the frequency of each haplotype to estimate the average number of nucleotide differences per site between two sequences in a population. Because we consider insertion/deletion events as well as base substitutions

Table I. Collection sites and mitochondrial DNA haplotypes of Chinese samples of *Apis cerana*.

	City	Province	Latitude	Longitude	Haplotype
1	Jianfeng	Hainan	18° 45′ N	108° 38′ E	Japan1
2	Qiongzhong	Hainan	19° 05′ N	109° 55′ E	Japan1
3	Wenchang	Hainan	19° 35′ N	110° 45′ E	Japan1
4	Yongxin	Hainan	19° 50′ N	110° 21′ E	Japan2
5	Jinhong	Yunnan	21° 55′ N	100° 23′ E	Japan1
6	Hekou	Yunnan	22° 20′ N	103° 55′ E	Japan1
7	Pinbian	Yunnan	22° 30′ N	103° 43′ E	Japan1
8	Dehong	Yunnan	24° 10′ N	97° 50′ E	Japan1
9	Jinglin	Yunnan	24° 50′ N	102° 33′ E	Japan1
10	Baoshan	Yunnan	25° 08′ N	99° 20′ E	Japan1
11	Lufeng	Yunnan	25° 10′ N	102° 20′ E	Japan1
12	Wuding	Yunnan	25° 36′ N	102° 47′ E	Japan1
13	Binchuan	Yunnan	25° 55′ N	100° 31′ E	Japan1
14	Yongren	Yunnan	26° 07′ N	101° 44′ E	Japan1
15	Nanhai	Guangdong	23° 00′ N	112° 45′ E	Japan1
16	Laibin	Guangxi	23° 55′ N	109° 22′ E	Japan1
17	Pinle	Guangxi	24° 24′ N	110° 44′ E	Japan1
18	Huichang	Jiangxi	25° 20′ N	115° 45′ E	Japan1
19	Hunzhou	Hujiang	26° 15′ N	119° 20′ E	China1
20	Jianghua	Hunan	26° 80′ N	110° 85′ E	Japan1
21	Shaoyang	Hunan	27° 02′ N	111° 29′ E	Japan1
22	Hengdong	Hunan	27° 08′ N	112° 54′ E	Japan1
23	Youxian	Hunan	28° 53′ N	113° 56′ E	Japan1
24	Huangshan	Anhui	30° 10′ N	118° 21′ E	Japan1
25	Zhongxian	Chongqing	30° 32′ N	108° 10′ E	Japan1
26	Shengnongjia	Hubei	31° 32′ N	109° 23′ E	Japan1
27	Shengnongjia	Hubei	31° 32′ N	109° 43′ E	Japan1
28	Nanchuan 1	Chongqing	31° 05′ N	107° 02′ E	Japan1
28	Nanchuan 2	Chongqing	31° 05′ N	107° 02′ E	Japan1
29	Anyue	Sichuan	30° 12′ N	105° 22′ E	Japan1
30	Jiuzaigou 4	Sichuan	32° 55′ N	103° 32′ E	Japan1
31	Jiuzaigou 3	Sichuan	33° 05′ N	103° 42′ E	Korea4
32	Jiuzaigou 2	Sichuan	33° 18′ N	103° 58′ E	Korea4
33	Jiuzaigou 1	Sichuan	33° 22′ N	104° 08′ E	China4
34	Xinyang	Henan	32° 10′ N	114° 07′ E	Japan1
35	Zhumadian	Henan	32° 57′ N	114° 01′ E	Japan1
36	Luoyang	Henan	34° 38′ N	112° 28′ E	Japan1
37	Mengjing	Henan	34° 50′ N	112° 28′ E	China5
38	Tianshui 2	Gansu	34° 46′ N	105° 30′ E	Japan1
39	Tianshui	Gansu	34° 48′ N	105° 32′ E	Japan1
40	Mianxian	Gansu	34° 48′ N	105° 32′ E	China2

Table I. Continued.

	City	Province	Latitude	Longitude	Haplotype
41	Qingjian	Shanxi	37° 12′ N	110° 18′ E	China3
42	Beijing		40° 25′ N	116° 38′ E	Cambodia2
43	Jian	Jilin	41° 08′ N	126° 09′ E	Japan1
44	Jingyu	Jilin	41° 47′ N	126° 56′ E	Japan1
45	Linjing	Jilin	42° 05′ N	126° 37′ E	Japan1
46	Jiangyuan	Jilin	42° 27′ N	126° 42′ E	Japan2
47	Hong Kong		22° 17′ N	114° 08′ E	Japan1
47	Hong Kong		22° 17′ N	114° 08′ E	Japan1

Figure 1. *Apis cerana* collection sites in China. Symbols indicate mitochondrial non-coding sequence, numbers correspond to those in Table I.

to be valid characters, alignment gaps were replaced with "dummy bases" so they would be included in the calculations.

3. RESULTS AND DISCUSSION

Mitochondrial diversity is low, and the Japan1 haplotype is the most common across all of our Chinese samples. There is no striking geographic pattern to the distribution of mitochondrial haplotypes, although samples from the Minshan Mountain region (corresponding to Aba bees of other authors; samples 30−33 from Sichuan province, 38−40 from Gansu province) include more haplotype variants than other sampled regions.

Nine haplotypes were found among the samples. Japan1, Japan2, Korea4, and Cambodia2 have been reported previously, while

Table II. Location, sample size (number of colonies), number of different mitochondrial haplotypes observed, and mtDNA haplotype lineages of *Apis cerana* populations from mainland Asia. Northern Thailand is north of 11° N, Southern Thailand is south of 11° N (Warrit, 2002; Warrit et al., 2006); Japan samples are described in Smith and Hagen (1996); Korean samples in Smith et al. (2000); Burma samples in Smith et al. (2004); Indochina includes samples from Laos, Cambodia and Vietnam (Smith et al., 2005); Pakistan samples (unpublished data) are from the vicinity of Islamabad.

Location	Sample Size	No. Mainland Haplotypes	No. Sundaland Haplotypes	Freq. Japan1 Haplotypes
N. Thailand	40	3	0	0/40
S. Thailand	37	0	3	0/37
Japan	15	2	0	14/15
China	49	9	0	39/49
Korea	11	4	0	8/11
Pakistan	15	5	0	13/15
Burma	23	5	1	11/23
Indochina	36	10	0	21/36
Combined	226	25	3	106/226

China1–5 were new. The new non-coding sequences are deposited in Genbank (http://www.ncbi.nlm.nih.gov/, accession numbers DQ269024-DQ269028, respectively). The geographic distribution of haplotypes is shown in Figure 1.

All nine sequences are 96–98 bases long and belong to the Mainland Asian mitochondrial lineage (Smith and Hagen, 1996; Smith et al., 2000). None differs from the Japan1 haplotype by more than a single base substitution and/or a single insertion/deletion. Japan1 is by far the most commonly encountered haplotype, making up 39 of the 49 sequences. Japan2 and Korea4 each appeared twice, and the others only once. Haplotype diversity among our samples was 0.4 (s.d. 0.089) and nucleotide diversity was 0.00569 (s.d. 0.0015) (Figs. 2, 3).

Several mitochondrial lineages have been observed within *A. cerana*; these include the Mainland, Sundaland, Palawan, Oceanic Philippine and "yellow Indian" lineages (Smith and Hagen, 1996, 1999; Smith et al., 2000). Both the "Mainland" and "Sundaland" *A. cerana* mitochondrial lineages occur in mainland Asia. The "Mainland" lineage is found over most of mainland Asia and in Japan (Smith and Hagen, 1996; Warrit et al., 2006). The Sundaland lineage occurs primarily in bees from Indonesia and Malaysia, but it is also found in the Thai-Malay peninsula as far north as ~11° N latitude, and, unexpectedly, in east-central Burma (Smith et al., 2004). A high frequency of the Japan1 haplotype is typical of all populations with Mainland lineage mtDNA except India (which is not considered here), and northern Thailand (Tab. II).

Figures 2 and 3 compare Chinese samples to other populations on the Asian mainland. Haplotype diversity is extremely low in Thai and Japanese samples; it is higher in the Chinese samples, similar to that observed among samples from Korea and Pakistan, despite the fact that the samples from China are more numerous and represent a much greater geographic area. The highest haplotype diversities are observed in "Indochina" (Laos, Cambodia and Vietnam) and Burma. The high diversity in Burma is partly attributable to the pocket of bees with Sundaland haplotypes amidst the Mainland haplotypes. (Compare haplotype diversity in Burma with and without inclusion of the Sundaland haplotypes; Fig. 2). Nucleotide diversity shows the same trends, though in a more exaggerated form; removal of the Sundaland samples has a more dramatic effect on nucleotide diversity than on haplotype diversity

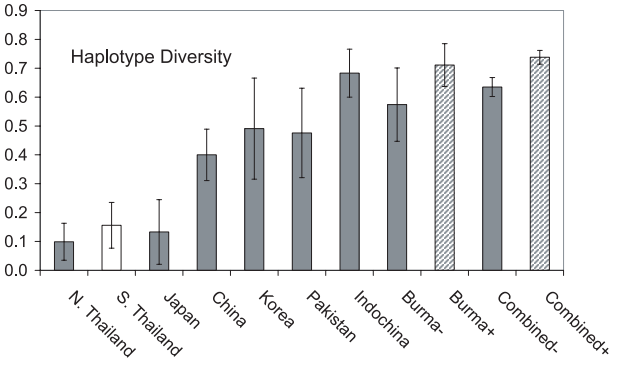

Figure 2. Mitochondrial haplotype diversity in local populations of *Apis cerana* and in all samples combined. Whiskers indicate standard deviation (square root of the variance). Gray bars indicate all haplotypes belong to the Mainland mitochondrial lineage, white bar indicates all haplotypes belong to the Sundaland mitochondrial lineage, and hatched bars indicate presence of both Mainland Asian and Sundaland mitochondrial haplotypes. In most locations only a single mitochondrial lineage is found; the exception is Burma, where both Mainland and Sundaland lineages have been found. Haplotype diversity for Burma and the combined samples are calculated with (+) and without (−) inclusion of Sundaland haplotypes. Sample sizes and numbers of haplotypes detected are shown in Table II.

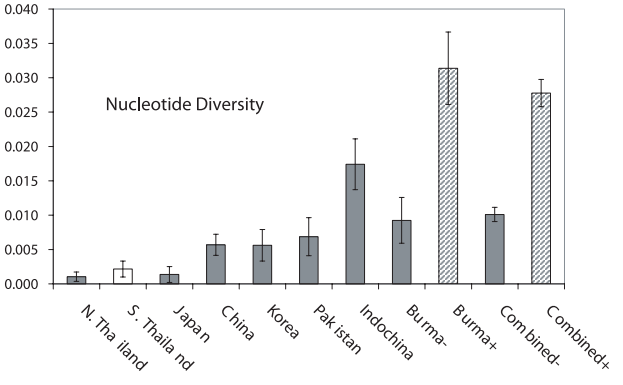

Figure 3. Mitochondrial nucleotide diversity in local populations of *Apis cerana* and in all samples combined. Whiskers indicate standard deviation (square root of the variance). Bar shadings and sample sizes as in Figure 2.

because of the relatively high sequence divergence between the Mainland and Sundaland lineages (Fig. 3).

It is somewhat surprising that haplotype and nucleotide diversity in mitochondrial non-coding sequences are so low, given that the geography of China seems to present ample opportunity for genetic differentiation of semi-isolated populations. However variation in morphological and mitochondrial characters may provide information about different time scales. Mitochondrial non-coding sequences evolve rapidly, but they are (presumably) not directly affected by natural selection. In contrast, morphological characters may respond to selective pressures imposed by environmental conditions. Thus it is not unreasonable to conclude that the mitochondrial data provide information on biogeographic patterns resulting from mutation, migration and genetic drift, while morphology responds to current environmental conditions.

These results pose several intriguing questions: (1) Why is Japan1 haplotype

so widespread and common among most populations of *A. cerana*? (2) Why is this haplotype absent from northwestern Thailand, and (on the basis of very limited data) from India? (3) Why are haplotype and nucleotide diversity lowest in Japan and Thailand and highest in Indochina? Further studies will address these issues, but we suggest that all three may stem from post-Pleistocene migration and population expansion into north Asia, when climates unfavorable for honey bees were followed by more favorable climate and habitat.

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Diversité de l'ADN mitochondrial chez l'abeille chinoise *Apis cerana*.

***Apis cerana* / ADN mitochondrial / phylogéographie / Chine**

Zusammenfassung – Diversität mitochondrialer DNA bei chinesischen *Apis cerana*. Obwohl ein großer Anteil des Verbreitungsgebietes von *Apis cerana* innerhalb von China liegt, wurden dem Studium der geografischen und genetischen Variation dieser Bienen bislang nur wenig Arbeiten gewidmet. Wir präsentieren hier Daten über die Variation von 49 nichtkodierenden mitochondrialen DNA-Sequenzen chinesischer *A. cerana* von 47 Orten in 16 verschiedenen Provinzen (Abb.1, Tab. I). In diesen Proben wurden neun verschiedene Haplotypen gefunden (Abb. 1, Tab. I). Vier von diesen – Japan1, Japan2, Korea4 und Kambodscha 2 – waren bereits zuvor bekannt, während fünf weitere – China1, China2, China3, China4 und China5 – neu waren. Die nichtkodierenden Sequenzen wurden in der Genbank (http://www.ncbi.nlm.nih.gov/) hinterlegt (Zugangsnummern DQ269024 bis DQ269028). Alle gehören der mitochondrialen Festlandslinie an (Smith und Hagen, 1996; Smith et al., 2000), und keine unterscheidet sich von dem Japan1 Haplotyp in mehr als einer einzigen Basensubstitution und/oder einer einzigen Insertion oder Deletion.
Wir untersuchten die Diversität der Haplotypen oder Nukleotiden mit dem Computerprogramm DnaSP (Rozas et al., 2003), und verglichen die chinesischen Proben mit anderen Populationen vom asiatischen Festland. In den meisten Festlandspopulationen stellt Japan1 den am häufigsten angetroffenen Haplotyp dar (Tab. II). 39 der 49 chinesischen Sequenzen waren Japan1. Japan2 und Korea4 kamen jeweils zweimal vor, die anderen nur einmal. Die Diversität zwischen unseren chinesischen Proben betrug 0.4 (s.d. 0.089), die Diversität der Nukleotide 0.00569 (s.d. 0.00154).
In Abb. 2 und 3 wird die Haplotyp- und Nukleotiddiversität der chinesischen Proben mit der anderer asiatischer Festlandpopulationen verglichen. Die Haplotypdiversität innerhalb der thailändischen und japanischen Proben ist extrem gering, mittelmäßig hoch in chinesischen, koreanischen und pakistanischen Proben, und am höchsten in „Indochina" (Proben aus Laos, Kambodscha und Vietnam) und in Proben aus Burma. Die hohe Diversität in Burma ist teilweise auf das Vorkommen von Bienen des Sundalandhaplotyps zusammen mit Festlandshaplotypen zurückzuführen (vergl. hierzu die Haplotypdiversität in Burma mit und ohne die Sundaland Haplotypen; Abb. 2).
Die Nukleotiddiversität zeigt die gleichen Trends in gesteigerter Form. Die Nukleotiddiversität war in Japan und Thailand extrem niedrig, höher in China, Korea und Pakistan, und am höchsten in Indochina und Burma. Wenn allerdings die Haplotypen der Sundalandlinie aus dem Datenset von Burma entfernt werden (Abb. 3), ist die Nukleotiddiversität zwischen den verbleibenden burmesischen Festlandlinien deutlich vermindert und der von China, Pakistan und Korea ähnlich. Der Ausschluss der Sundalandlinien wirkt sich wegen der relativ großen Sequenzdiversität zwischen Sundalandlinien und Festlandlinien auf die Nukleotiddiversität dramatischer aus als auf die Haplotypdiversität.

***Apis cerana* / mitochondriale DNA / China / Phylogeography**

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