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Original article

Phylogeographic analysis of *Apis cerana* populations on Hainan Island and southern mainland China, based on mitochondrial DNA sequences

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Abstract – The phylogeography of the eastern honeybee (*Apis cerana*) in southern China is poorly understood. In the present study, we investigated phylogenetic relationships among *A. cerana* populations on Hainan island and southern mainland China. Analyses were based on the partial cytochrome oxidase subunit 1 gene of mitochondrial DNA and *A. cerana* samples representing 12 geographic populations. We identified 57 novel haplotypes (GenBank accession numbers: KC175488-KC175544) within our sampled populations and inferred two deeply divergent intraspecific lineages when previously published sequences were included, which could be further divided into six sublineages. We also found a significant phylogeographic structure within lineage A. Our results reveal that *A. cerana* populations on Hainan island and southern mainland China have undergone a long period of differentiation in isolation followed by historical demographic expansion. Although gene flow now occurs between these locations, gene flow has occurred over comparatively a short time period, and is likely to be the result of anthropogenic effects.

Apis cerana **/ mitochondrial DNA / phylogeography**

1. INTRODUCTION

As a pollinator, the eastern honeybee, *Apis cerana* (Fabricius, 1793), plays a crucial role in the functioning of ecological systems (Sasaki et al. 1991; Verma and Partap 1993; Partap and Verma 1994; Klein et al. 2007; Kremen et al. 2007). The eastern honeybee is also a significant economic resource for commercial producers of bee products (Songram et al. 2006). Honeybees of this *Apis* species are distributed throughout most of mainland Asia and many of its islands (Ruttner 1988). This vast distribution has resulted in substantial variation in morphometric characteristics among different geographic populations (Ruttner 1988; Peng et al. 1989). Extensive morphological analyses of this geographic variation have been conducted (Yang et al. 1986; Hepburn et al. 2001; Tan et al. 2003, 2008; Radloff et al. 2005a, b) and used to establish the systematics of *A. cerana* (Radloff et al. 2010). However, molecular phylogenetic studies on the eastern honeybee have only been initiated more recently and report limited data, as samples from many regional bee populations are still lacking. Nevertheless, preliminary results from these

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molecular phylogenetic studies also reveal extensive variation among populations of *A. cerana* (Smith et al. 2000). Augmenting our phylogenetic knowledge of this important bee species would not only result in more robust systematic, but also help in defining evolutionarily important populations (Moritz 1994). Such knowledge will play an important role in designing and implementing conservation strategies (Zayed 2009).

Mitochondrial DNA, by virtue of being transmitted maternally without recombination and with high rates of intraspecific polymorphism (Avise et al. 1987), has been widely used in phylogenetic studies of *Apis mellifera* (Garnery et al. 1992; Arias and Sheppard 1996; Clarke et al. 2001; Zaitoun et al. 2008; Kekecoglu et al. 2009; Magnus and Szalanski 2010; Iiyasov et al. 2011), but more rarely so for *A. cerana*. Smith and Hagen (1996) and Smith et al. (2000) used the noncoding intergenic region of the mitochondrial DNA to survey a large sample of eastern honeybees. Following this initial study, phylogenetic studies were undertaken on populations from Thailand (Sihanuntavong et al. 1999), the Philippines (de la Rua et al. 2000), Indonesia and Malaysia (Tanaka et al. 2003), Myanmar (Smith et al. 2004), and Japan (Takahashi et al. 2007). Very few samples of bees from mainland China were included in these studies. In 2006 and 2007, two studies investigating mtDNA divergence in *A. cerana* populations were conducted on samples from colonies covering a large area of mainland China and Hainan island (Tan et al. 2006, 2007). These previous studies have revealed that substantial variation exists among different geographic populations and proposed a phylogeny consisting of four major intraspecific lineages (Smith 2011). However, despite this knowledge and the larger geographic coverage, sampling has still been too sparse to elucidate the precise phylogeography of this species in this part of Asia, and many questions remain to be answered (Tan et al. 2006, 2007).

The Asian continent, particularly mainland China, is a vast area of complex topography and divergent climates, all of which are expected to result in extensive diversification in resident species. Considering past cycles of glaciation and declines in sea levels during the Pleistocene era, current populations *A. cerana* on Hainan island are expected to have colonized naturally from the Asian mainland (once or repeatedly). To date, however, there is little evidence to support this hypothesis. Hence, sampling honeybees extensively in this area should provide a more accurate phylogeographic structure for *A. cerana* inhabiting within this range.

In this study, large numbers of *A. cerana* colonies were collected from Hainan island and from southern mainland China, to elucidate patterns of divergence and phylogenetic relationships among bee populations in this area. Many previous molecular phylogenetic studies on *Apis* species have used the noncoding intergenic region of mtDNA, which lies between leucine tRNA (tRNA-leu) and cytochrome oxidase subunit 2. Despite being shown repeatedly to be highly useful for elucidating the phylogenetics of *A mellifera* (Cornuet et al. 1991; Garnery et al. 1992; Clarke et al. 2001; de la Rua et al. 2001; Franck et al. 2001; Collet et al. 2006), this region of mtDNA is unsuitable for studies on the eastern honeybee, as this region is either extremely small in size (around 89 bp; Cornuet and Garnery 1991) or completely absent in *A. cerana* (e.g., in *A. cerana* samples from Taiwan and Sangihe; Smith and Hagen 1996). Therefore, previous phylogenetic studies in *A. cerana* have used other mtDNA fragments such as cytochrome oxidase subunit 1 (*COX1*; Tanaka et al. 2003), dehydrogenase gene subunit 2 (Tan et al. 2006) and *ATPase6-ATPase8* (Songram et al. 2006). In this study, we used a partial sequence of *COX1* to investigate honeybee phylogenetics in this region.

2. MATERIALS AND METHODS

2.1. Sampling and DNA extraction

A total of 360 *A. cerana* colonies representing 12 geographic populations were sampled from natural nests or semi managed hives in Guangdong province (GD), Guangxi autonomous region (GX), and Hainan island (HN) as displayed in Figure 1. There is no migratory beekeeping of *A. cerana* in these regions. For each geographic population, 30 bee colonies were

collected. Sampled colonies within geographic populations were less than 10 km apart. Bee specimens were caught directly on the comb and immersed in 100 % ethanol. All samples were stored at −75 °C for future DNA extraction. For each bee colony, one worker bee was randomly selected and its whole thorax was used for extraction of total genomic DNA. A standard phenol–chloroform DNA extraction protocol was employed (Smith and Hagen 1996) with several slightly modified parameters. The final DNA samples were treated with ribonuclease A (Roche, Basel, Switzerland) according to the manufacturer's recommendation and quantified using a Nano-100 micro-spectrophotometer (Allsheng Instruments CO., Ltd, Hangzhou, China).

2.2. Polymerase chain reaction and DNA sequencing

Based on the published mitochondrial genome of *A. cerana* (GenBank accession number: NC014295), specific polymerase chain reaction (PCR) primers were designed using Oligo 6.0 (forward primer: 5′-CTCCAGATATAGCATTTCCTCG-3′; reverse primer: 5′-TGCAAATACTGCTCCTATTGA-3′), and synthesized by Sangon Biotech Co., Ltd, Shanghai, China. Amplification with this primer pair was expected to yield an amplicon size of about 910 bp.

PCR reactions were performed in a final volume of 50 μL: 32 μL sterile deionized water, 5 μL of 10× PCR Buffer (Mg^{2+} free), 4 μL $MgCl_2$ (25 mM), 4 μL dNTPs (2.5 mM each), 1 μL each primer (20 mM), 0.5 μL *Taq* DNA polymerase (5 U/μL, Takara), and 2.5 μL DNA template (50 ng/μL). All PCR amplifications were carried out on a Bio-Rad T100 thermocycler (Bio-Rad, California, USA) with the following conditions: initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturation at 94 °C for 30 s, annealing at 58.9 °C for 30 s, and elongation at 72 °C for 1 min with a final elongation at 72 °C for 10 min. All PCR products were examined on 1.5 % agarose gels stained with ethidium bromide and observed under an ultraviolet transilluminator. PCR products were purified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (Takara, Dalian, China) and sequencing was carried out in both directions with corresponding PCR primers by a professional sequencing service (Sangon Biotech Co., Ltd).

2.3. Dataset analysis

DNA sequences were manually edited and trimmed using DNAStar 6.0 to produce the consensus sequences, which were then aligned using the Clustal W algorithm (Thompson et al. 1994). Haplotype diversity (Hd) and nucleotide diversity (π) were computed with DnaSP v5.10 (Rozas and Rozas 1995; Rozas 2009). The neutrality tests Tajima's D (Tajima 1989), Fu's F_s (Fu 1997), and three tests of analysis of molecular variance (AMOVA; Excoffier et al. 1992) were calculated using Arlequin 3.11 (Excoffier et al. 2005). Groupings of the three AMOVA tests were as follows: (1) the mainland populations (including three GD populations and five GX populations) versus the island populations (including HNHK, HNTC, HNBS, and HNWN); (2) GD populations (including GDJL, GDLM, and GDYD) versus GX populations (including GXBH, GXCZ, GXGL, GXHZ, and GXLB); and (3) two populations HNHK + HNTC versus two populations HNBS + HNWN (seeing in Figure 1). Pairwise genetic distance "Dest" (Crawford 2010) was used as an index of population differentiation. To further explore phylogeographic substructures respectively for the mainland (GD and GX) populations and island (HN) populations, two independent Mantel tests were performed between the "Dest" matrix and the geographic distance matrix using XLSTAT program (http://www.xlstat.com/en/).

We downloaded 35 previously published *A. cerana COX1* sequences from other regions of Asia and one homologous sequence of *Apis koschevnikovi* (AY754321) from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Detailed information about the 35 reference sequences can be found in Table I. Combined with the sequences obtained in this study, a separate alignment was compiled and subjected to phylogenetic reconstructions. A neighbor-joining (NJ) tree of all the haplotypes identified from the separate alignment was reconstructed using the Kimura-2-parameter (Kimura 1980) nucleotide substitution model and 1,000 bootstrap replicates using the MEGA v5.05 program (Tamura et al. 2011). The phylogenetic relationships of these haplotypes were then reconfirmed by maximum parsimony (MP) and a Bayesian algorithm implemented respectively in PAUP v4.0 (Swofford 2001) and MrBayes v3.2 (Ronquist and Huelsenbeck 2003). The MP analysis used a heuristic search strategy

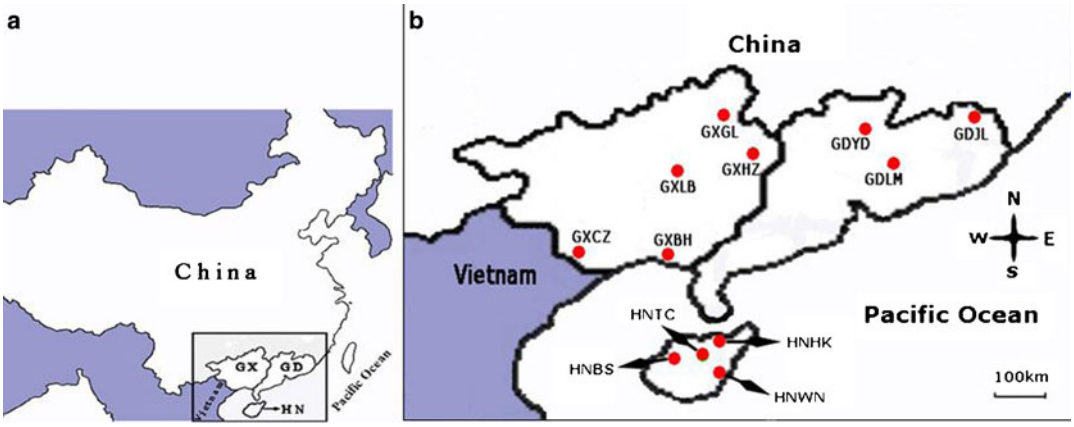

Figure 1 **a** Location of main sampling regions: *GD* Guangdong province, *GX* Guangxi autonomous region, *HN* Hainan island. **b** Location of sampling sites within the main sampling regions. *Filled circles* represent sampling sites; GDJL (N24.67°, E116.06°), Jiaoling county in GD; GDLM (N23.77°, E114.29°), Longmen county in GD; GDYD (N24.21°, E113.77°), Yingde city in GD; GXBH (N21.88°, E109.44°), Beihai city in GX; GXCZ (N22.02°, E107.52°), Chongzuo city in GX; GXGL (N24.98°, E110.42°), Guilin city in GX; GXHZ (N23.88°, E111.04°), Hezhou city in GX; GXLB (N23.87°, E109.31°), Laibin city in GX; HNBS (N19.34°, E109.46°), Baisha county in HN; HNHK (N19.95°, E110.30°), Haikou city in HN; HNTC (N19.35°, E110.07°), Tunchang county in HN; HNWN (N18.75°, E110.35°), Wanning city in HN

and 1,000 bootstrap replicates with options for collapse, character optimization, sequence addition, branch swapping, and steepest descent set to MAXBRLEN, ACCTRAN, RANDOM, TBR, and YES, respectively. Bayesian inference was based on a best-fit nucleotide substitution model selected using the jModelTest 0.1.1 program (Posada 2009) under Akaike information criterion (Akaike 1974) and 100 % confidence interval. After that, two independent runs of Markov chain Monte Carlo simulations involving four chains (three heated and one cold) with 10,000,000 iterations each were performed with the selected best-fit model, 100 generations of sampling frequency and a 30 % burn-in fraction. The final haplotype network was predicted using a median-joining algorithm using the Network v4.61 software package (http://www.fluxus-engineering.com).

3. RESULTS

3.1. Sequence variation

For this study, 357 partial *COX1* sequences with a length of 799 bp were obtained (three GXBH sequences were eliminated as they exhibited low sequencing efficiency). A total of 57 haplotypes (GenBank accession numbers: KC175488–KC175544) were identified out of the 357 sequences by 57 variable sites. Of these, 34 were parsimoniously informative and 23 were singletons. Twenty-two haplotypes that were only present in HN populations were defined as HN + I (I denotes integers) and the remainder as CH + I (CH denotes mainland China, I denotes integers). The distribution of the 57 haplotypes among the 12 geographic populations is available in Electronic supplementary material (ESM) 1, while the variable sites among all haplotypes of *A. cerana* (including previously published sequences) are listed in ESM 2.

We observed no insertions/deletions (indels). The average nucleotide composition of A, T, C, and G in the *COX1* region was 33.2, 41.1, 13, and 12.7 %, respectively. Compared with previous data (Tan et al. 2007), we observed a notably higher Hd (0.812±0.019) and a lower π (0.00481 ±0.00022) for the total population (Table II). Values of Tajima's D and Fu's F_s varied substantially among different populations (Table II).

Table I. Reference sequences of *A. cerana* and the haplotypes they are inferred to

GenBank accession number	Geographic location	Haplotype
DQ016084	Indonesia: Kutai National Park, East Kalimantan	Indone 3
DQ016085	Indonesia: Kutai National Park, East Kalimantan	Indone 11
DQ016086	Indonesia: Loksad, South Kalimantan	Indone 3
DQ016087	Indonesia: Loksad, South Kalimantan	Indone 12
DQ016088	Indonesia: Pelaihari, South Kalimantan	Indone 6
DQ016089	Indonesia: Pelaihari, South Kalimantan	Indone 1
DQ016090	Indonesia: Palangkaraya, South Kalimantan	Indone 6
DQ016091	Indonesia: Sei Pinyuh, West Kalimantan	Indone 2
DQ016092	Indonesia: Sadap, West Kalimantan	Indone 2
DQ016093	Indonesia: Sadap, West Kalimantan	Indone 3
DQ016094	Malaysia: Tawau, Sabah	Indone 3
DQ016095	Malaysia: Sungai Baram, Sarawak	Indone 3
DQ016096	Malaysia: Sungai Baram, Sarawak	Indone 3
DQ020236	Indonesia: Kebon Kopi, Central Sulawesi	Indone 4
DQ020237	Indonesia: Parepare, South Sulawesi	Indone 5
DQ020238	Indonesia: Mt. Salak, West Java	Indone 5
DQ020239	Indonesia: Pangandaran, West Java	Indone 6
DQ020240	Indonesia: Banyuwangi, East Java	Indone 7
DQ020241	Indonesia: Kaliklatak, East Java	Indone 8
DQ020242	Indonesia: Kuta, Bali	Indone 9
DQ020243	Indonesia: Lovina, Bali	Indone 5
DQ020244	Indonesia: Mt. Agung, Bali	Indone 10
DQ020245	Indonesia: Mt. Agung, Bali	Indone 9
DQ020246	Indonesia: Senggigi, Lombok	Indone 5
DQ078750	Malaysia: Imbak Canyon, Sabah	Malay 3
AY012722	Malaysia: Crocker Range Park, Sabah	Malay 1
AF153101	Malaysia: Kinabalu, Sabah	Indone 3
AF153102	Malaysia: Kinabalu, Sabah	Malay 2
AF153104	Taiwan	Taiwan 1
AF153105	Taiwan	Taiwan 2
AF153106	Thailand	Thailand
AF153107	Russia: Primorie	Russia
AF153108	South Korea	Japan + Korea
AF153109	Japan	Japan + Korea
AY311385	Vietnam	Vietnam

3.2. Phylogenetic analysis

We obtained 21 haplotypes from previously published sequences. These were subjected to phylogenetic reconstructions together with the 57 haplotypes identified in this study and one homologous sequence of *A. koschevnikovi*. No indels were found. A phylogenetic tree with two deeply divergent lineages (lineage A and B, Figure 2a) was inferred by the neighbor-joining

Table II. Haplotype diversity, nucleotide diversity, and neutrality tests for the 12 *A. cerana* populations

Population	*N*	Nh/u	Hd ± SD	π ± SD	*D*	F_s
GDJL	30	8/5	0.823±0.040	0.00228±0.00034	−1.105	−1.610
GDLM	30	11/6	0.853±0.047	0.00200±0.00027	−1.369	−5.554 **
GDYD	30	2/1	0.508±0.034	0.00191±0.00013	2.383	4.888
GXBH	27	3/2	0.453±0.087	0.00063±0.00014	−0.048	0.021
GXCZ	30	12/10	0.710±0.088	0.00138±0.00028	−2.293 **	−9.736 **
GXGL	30	2/0	0.067±0.061	0.00017±0.00015	−1.507 *	−0.396
GXHZ	30	3/0	0.297±0.099	0.00068±0.00024	−0.660	0.234
GXLB	30	6/4	0.311±0.109	0.00058±0.00024	−2.174 **	−4.187 **
HNBS	30	10/6	0.729±0.078	0.00149±0.00028	−1.670 *	−5.854 **
HNHK	30	9/3	0.864±0.030	0.00611±0.00036	1.276	1.073
HNTC	30	10/3	0.869±0.033	0.00556±0.00032	0.861	−0.003
HNWN	30	8/4	0.749±0.069	0.00146±0.00025	−1.278	−3.298 **
Total	357	57/44	0.812±0.019	0.00481±0.00022	−1.622 **	−25.386 **

N number of individuals, *Nh* number of haplotypes, *U* number of unique haplotypes, *Hd* haploytpe diversity, π nucleotide diversity, *SD* standard deviation, *D* Tajima's D test, F_s Fu's F_s test

*$0.01<p\leq0.05$ and **$p\leq0.01$

algorithm. This tree could be further divided into several sublineages with high bootstrap values: CH, HN, and JKR (Japan + Korea + Russia), Taiwan, IM (Indonesia + Malaysia) and Indonesia. Most of the previously published haplotypes belonged to the JKR, Taiwan, IM, and Indonesia sublineages, while the other two sublineages (CH and HN) were composed of the 57 haplotypes from this study and two published haplotypes from Thailand and Vietnam. An interesting result was the genealogical positions of three HN haplotypes (HN12, HN17, and HN18), which were only present in the HN populations, clustered with the CH sublineage.

For parsimony analysis, the 100 most parsimonious trees were identified with 194 steps, a 0.758 consistency index and 0.926 retention index. The 50 % majority rule consensus tree was identical to the NJ tree. The results of Bayesian estimation showed that the general time reversible model (GTR + I + G) was selected as the best-fit nucleotide substitution model. The output phylogeny of this analysis exhibited a similar topology to the MP tree except that the position of the Taiwan sublineage clustered with lineage A in the MP tree but with lineage B in the Bayesian tree (Figure 2b). High supporting values were generated for both the MP and Bayesian analysis.

3.3. Phygeographical structure

Our results revealed that lineage A exhibited a clear phylogeographical structure consisting of four sublineages (CH, HN, JKR, and Taiwan), corresponding to four regions (southern mainland China, Hainan island, Japan–Korea–Russia district, and Taiwan). Nevertheless, we did detect evidence of gene flow occurring between the mainland (GD and GX) populations and the island (HN) populations (three CH haplotypes [CH1, CH5, and CH34] were shared among the mainland populations and two of the island populations, while three HN haplotypes [HN12, HN17, and HN18] clustered with the CH sublineage; Figures 2 and 3). Still, the results of the phylogenetic reconstructions and the AMOVA test (Table III) strongly support partition of the CH and HN sublineages. However, pairwise comparison of nucleotide differences among the six inferred sublineages provided further support for inter- and intralineage variation (Table IV).

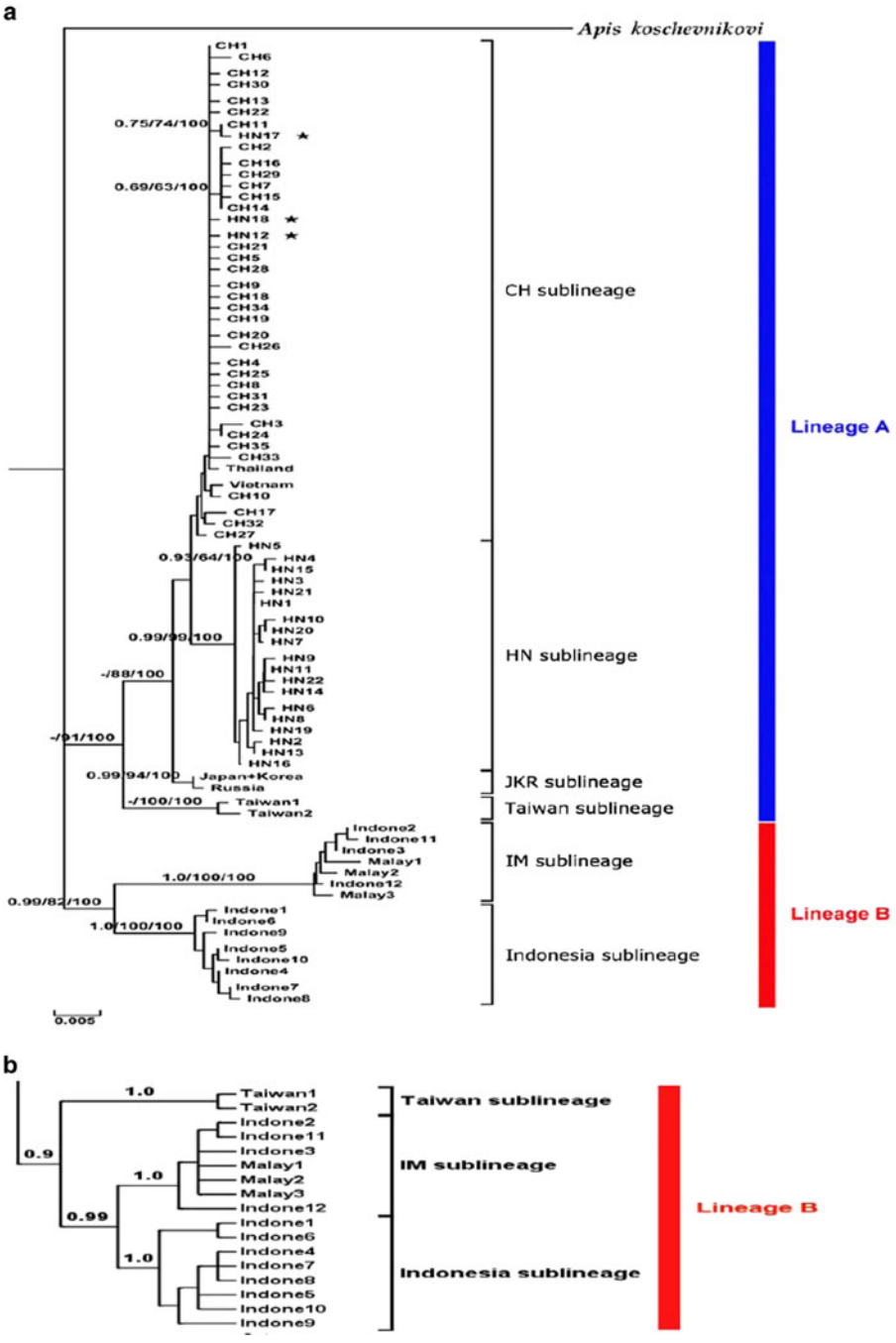

Figure 2 a The neighbor-joining tree of 78 *COX1* haplotypes of *A. cerana* and 1 *A. koschevnikovi* homologous sequence. The branch support values are the posterior probabilities of the results of the Bayesian analysis (general times reversible + I + G model), the bootstrap values from the neighbor-joining algorithm (Kimura-2-parameter model) and the bootstrap values from the maximum parsimony analysis, respectively. The three-labeled haplotypes with the pentagram are those present only in the HN population that clustered with the CH sublineage. **b** The Bayesian tree of inferred *lineage B*. The support values are the posterior probabilities of the results of the Bayesian analysis

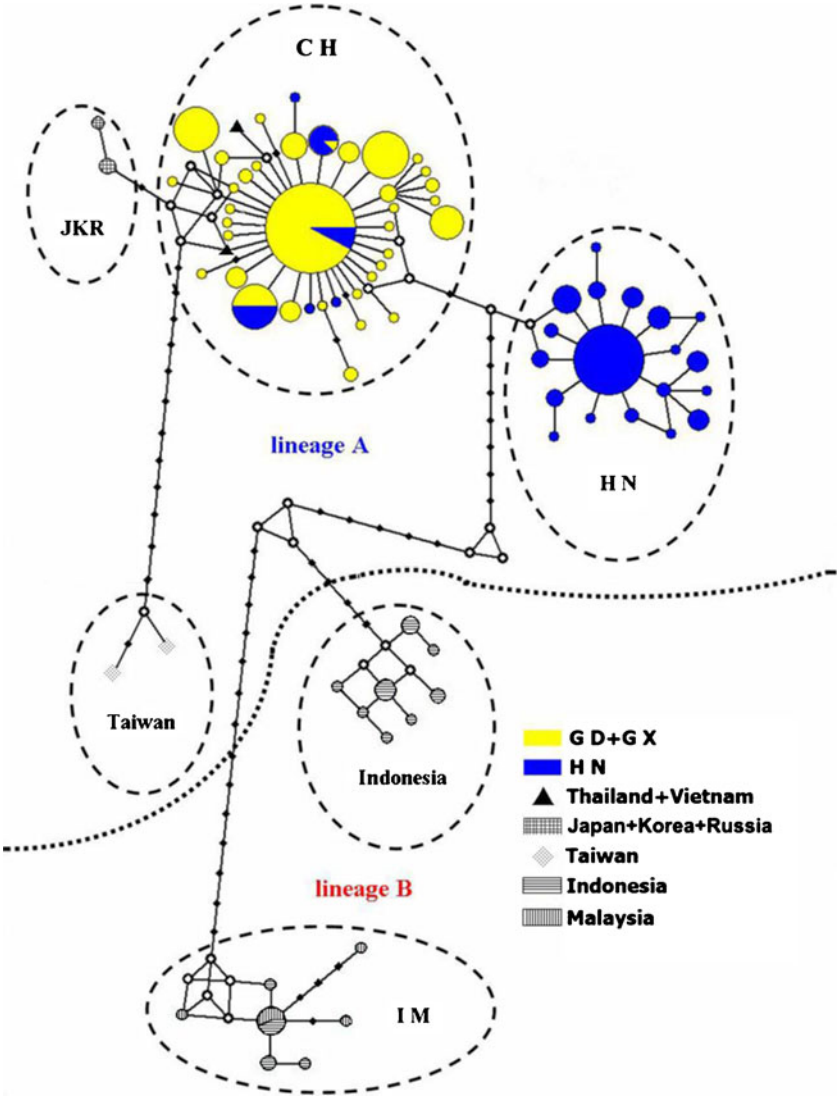

Figure 3 Median-Joining networks among the 78 COX1 haplotypes of Apis cerana. *Square nodes* represent mutation steps; *circles* represent the putative intermediate types; *circles filled with lightand* (or) *dark colors* represent newly identified haplotypes while triangles, *diamond-shaped* symbols and other circles filled with line patterns represent downloaded haplotypes

No correlation between genetic distance and geographic distance was inferred from the Mantel test performed on the island populations (r=−0.009, P=0.835) or on the mainland populations (r=−0.046, P=0.822) suggesting there are no phylogeographic substructures within either population group. AMOVA tests also supported this conclusion (Table III).

The haplotype network within the CH sublineage showed a star-like topology. The most frequent haplotype (CH1) was present in all mainland populations, while less frequent haplotypes were present in no more than three populations. This was also the case for HN1 within the island populations. Every population, except GXGL and GXHZ, possessed unique

Table III. Analysis of molecular variance for the 12 *A. cerana* populations. Grouping indicates the geographical isolation of populations or their phylogenetic differentiation pattern

Grouping	Variation (%)			Fct	Fsc	Fst
	Between groups	Among populations within groups	Within populations			
Mainland populations vs island populations	60.55	11.65	27.80	0.6055**	0.2953**	0.7220**
GD populations vs GX populations	3.46	26.08	70.46	0.0346	0.2702**	0.2954**
HNHK + HNTC vs HNBS + HNWN	40.63	0.35	59.02	0.4063	0.0060	0.4098**

The mainland populations include three GD populations and five GX populations; the island populations include HNHK, HNTC, HNBS, and HNWN; GD populations include GDJL, GDLM, and GDYD; GX populations include GXBH, GXCZ, GXGL, GXHZ, and GXLB

**$p<0.01$

haplotypes. Moreover, all less frequent and unique haplotypes were differentiated from the most common haplotypes (CH1 or HN1) by just one or two mutation steps with only one exception, indicating very recent differentiation.

4. DISCUSSION

In this study, we investigated patterns of divergence and phylogenetic relationships among eastern honeybee populations on Hainan island and southern mainland China. We found two deeply divergent intraspecific lineages when previously published sequences were included, which could be further divided into six sublineages. These results were broadly consistent with the phylogeographic structure proposed by Smith (2011), who identified four major mtDNA lineages or groups of *A. cerana* (a Mainland Asian group, a Sundaland group, an Oceanic Philippines group, and a Yellow Indian group). In this study, our "lineage A" can be considered a subset of the Mainland Asian group, while our "lineage B" is a subset of the Sundaland group. Furthermore, our data suggest a strong intralineage phylogeographic structure within the Mainland Asian group. Four genetically distinct sublineages were identified within

Table IV. Pairwise comparisons of nucleotide difference percentage of the partial *COX1* sequences among the six inferred sub-lineages of *A. cerana*

	Lineage A				Lineage B	
	CH (%)	HN (%)	JKR (%)	Taiwan (%)	Indonesia (%)	IM (%)
CH		0.302	0.305	0.433	0.652	0.695
HN	1.16		0.378	0.481	0.636	0.756
JKR	0.83	1.41		0.477	0.659	0.776
Taiwan	2.18	2.80	2.00		0.695	0.780
Indonesia	3.51	3.80	3.36	3.99		0.568
IM	4.66	4.76	4.76	4.91	3.62	

The average values of nucleotide difference percentage (lower diagonal) and standard error (upper diagonal) are shown

this group and assigned almost exclusively to four regions (mainland southern China, Hainan island, Japan–Korea–Russia district, and Taiwan).

In a previous study of mtDNA variation among eastern honeybees on Hainan island, Tan et al. (2007) reported that most *A. cerana* samples belonged to the haplotype "Japan1", which was ubiquitously distributed throughout mainland China populations. Our study identified a suit of novel local HN haplotypes, which exhibited patterns of sequence variation different to any known Mainland Asian or Sundaland haplotypes. Existence of these peculiar HN types strongly suggests that the native honeybee populations on Hainan island have been isolated for a long time. Hainan island is separated from mainland China by the Qiongzhou Strait, which is approximately 70 km long, 30 km wide, and 120 m deep (Shi et al. 2002). Over the past 0.25 Ma, the sea levels in the strait have fallen to below 100 m due to glaciation at least twice and have remained there for around 29,000 years. This most recent sea level fall was estimated to have occurred 17,000 years ago (Voris 2000). Given this, it is likely that the HN sublineage has experienced at least 17,000 years of differentiation in isolation. However, we also detected convincing evidence of gene flow between mainland China and Hainan island. We suggest that this is most likely to be a recent occurrence and to have occurred as a consequence of human activities. Indeed, we learned from personal communications with local beekeepers that *A. cerana* colonies have been transported intentionally from GD, GX district to HN island several times.

Within the populations on Hainan island, the predominant haplotype HN1 accounted for just 36.67 % (44/120) of the total samples. This result is very different to that reported in a previous population survey of *A. cerana* in Japan, which only detected three haplotypes in total, and showed that 98.9 % of samples belonged to just one haplotype (Japan1; Takahashi et al. 2007). A possible explanation for this difference was that the previous study used a shorter genetic marker that has a low resolution and power to discriminate closely related individuals.

We found that there was no significant genetic variation among the mainland (GD and GX) populations (see results of the AMOVA test Table III), suggesting that there is nearly no interpopulation phylogeographic substructure and that the eight populations essentially share the same gene pool. Similar population structures have been observed in other studies of different species, including wild yak (Wang et al. 2010), domestic goat (Naderi et al. 2007), freshwater fish (Tomas et al. 2005), and stingless bee (Henrique et al. 2010). We suggest that this lack of fine scale genetic structure reflects historical demographic expansions, whereby local colonies have apparently undergone continuous spatial dispersion or spread. The mainland population sampling regions are part of greater mainland Asia and are not surrounded by natural physical barriers. Based on the expansion model proposed by Hewitt (1999), most genetic variability was maintained within the pre-expansion populations, while a lower level of genetic diversity was restricted to the expanded populations. In our data, genetic variability was greatest within the GXCZ population (12 haplotypes and 10 unique types), and lowest within the GDYD and GXGL populations (two haplotypes). This pattern could indicate that population expansions occurred from the GXCZ population to the GXGL population. However, further data and analyses will be needed to verify this assertion.

In this study, we have shown that *A. cerana* populations on Hainan island and southern mainland China have undergone a long period of differentiation in isolation and subsequent historical demographic expansion. While gene flow does appear to have occurred between these locations, this has evidently occurred over a much shorter time period and is likely to be the result of anthropogenic effects. To date, *COX1* sequences of *A. cerana* from the Japan–Korea–Russia district and Taiwan remain scarce, and hence inferences about the genetic structure and phylogeography of honeybee populations in these additional areas should interpreted with caution. Our study and current data supports the phylogenetic positions of JKR and Taiwan as sublineages. Nevertheless,

further studies are necessary to better understand the phylogeography of the eastern honeybee within these ranges.

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Analyse phylogéographique des populations d'*Apis cerana* sur l'île de Hainan et dans le sud de la Chine continentale, basée sur les séquences d'ADN mitochondrial

Phylogéographie / ADN mitochondrial/haplotype / flux génétique

Phylogeographische Analyse von *Apis cerana* Populationen von der Insel Hainan und dem südlichen Festlandchina, auf der Basis von mitochondrialen DNA-Sequenzen

***Apis cerana* / mitochondriale DNA / Phylogeographie**

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