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Genetic structure of Chinese *Apis dorsata* population based on microsatellites

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Abstract – We investigated the genetic structure of Chinese *Apis dorsata* population based on DNA microsatellites. The results show that populations from different geographical regions are significantly differentiated. Furthermore, the *A. dorsata* population from Hainan Island very probably diverged from that of the China mainland and also exhibits a lower level of genetic diversity, which is probably the result of founder effects and genetic drift. The results provide useful information for effective management and conservation of *A. dorsata* in China, particularly with regard to the population of Hainan Island.

Apis dorsata / genetic structure / China / microsatellites

1. INTRODUCTION

Apis dorsata Fabricius, the common giant honeybee, is found throughout South and Southeast Asia (Oldroyd and Wongsiri 2006; Hepburn and Radloff 2011). *A. dorsata* plays an important role as a pollinator of tropical rainforest and various crops (Wongsiri et al. 2001; Corlett 2011; Partap 2011). Most honey (70–80 %) produced in Nepal, India, and some other Southeast Asian countries originate from the giant honeybees (Woyke et al. 2008). Honey harvested from *A. dorsata* nests also provides an important source of income for people in remote mountain areas. However, many threats are severely affecting this important native species, the main ones being deforestation, hunting with destructive methods, and the loss of nest sites (Oldroyd and Wongsiri 2006; Oldroyd and Nanork 2009). Nath et al. (1994)

reported a decrease in the number of colonies of *A. dorsata* in Tamil Nadu, India. Our own experiences also suggest that the number of *A. dorsata* colonies in China has declined, and in some regions, *A. dorsata* is on the verge of disappearing. For the conservation of *A. dorsata*, some strategies such as sustainable hunting based on harvesting in a more sustainable manner have been proposed (Oldroyd and Wongsiri 2006; Oldroyd and Nanork 2009).

Knowledge of genetic population structure can be of great use in the construction of natural resource management and conservation programs for *A. dorsata*, especially considering its wide distribution and great variation. *A. dorsata* can be classified into three subspecies (*Apis dorsata dorsata*, *Apis dorsata binghami*, and *Apis dorsata breviligula*) (Ruttner 1988; Oldroyd and Wongsiri 2006), and more recently, *A. breviligula* species was suggested by Lo et al. (2010). Furthermore, an extensive sequence divergence has been found between *A. dorsata dorsata* samples from the Indian subcontinent and other mainland populations (Smith 1991; Arias and

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Sheppard 2005). However, the genetic relationship among *A. dorsata* subspecies has not been clarified, and the genetic divergence within *A. dorsata* across its range is little known. In China, *A. dorsata* is mainly distributed in the south, including Yunnan and Guangxi Provinces and Hainan Island. In a previous study (unpublished data), Chinese *A. dorsata* populations were classified as *A. dorsata dorsata* based on mitochondrial DNA sequences. However, the *A. dorsata* of Hainan Island have never been included in comparative studies on the worldwide distribution of *A. dorsata* (Oldroyd and Wongsiri 2006; Hepburn and Radloff 2011). The population structure of Chinese *A. dorsata* has not been previously investigated.

Microsatellites are valuable tools for population structure studies due to their high levels of polymorphism as well as being selectively neutral and displaying codominance and Mendelian inheritance (Goldstein and Schlötterer 1999). Using microsatellites, Paar et al. (2004) showed that there was a significant population differentiation between different geographical regions of *A. dorsata* in northern Indian. Insuan et al. (2007) also found significant differentiation among *A. dorsata* populations from geographically different regions in Thailand using three microsatellite loci, which was not detectable by mtDNA sequences. Based on microsatellite analysis, significant genetic differentiation was observed among three sampling days in a drone congregation area of *A. dorsata* (Kraus et al. 2005). DNA microsatellites can also be used to estimate the extent of worker drifting between nests in *A. dorsata* aggregations (Paar et al. 2002). Few data on the variation of Chinese *A. dorsata* are available. To address this, we examined the population structure of Chinese *A. dorsata* using microsatellite markers.

2. MATERIALS AND METHODS

2.1. Sample collection

A. dorsata samples were collected from Yunnan, Guangxi, and Hainan provinces of China in May 2010. The localities and the number of colonies

sampled are given in Figure 1. Samples from the same locality were collected randomly and not from aggregations. Specimens were placed in 95 % ethanol and kept at -20°C .

2.2. DNA extraction and microsatellite amplification

DNA was extracted from the thorax of each worker bee with proteinase K followed by the standard phenol–chloroform method (Sambrook et al. 1989). DNA was then diluted to 10 ng/ μL with distilled water for amplification. The microsatellite loci used were A14, A88, A76, A24, and B124 (Estoup et al. 1993). These loci were amplified using the polymerase chain reaction (PCR). The reverse primers were labeled with the fluorescent dyes FAM or HEX. The PCRs were carried out in a total volume of 10 μL . Each reaction contained 0.4 μM of each primer, 100 μM of dNTP, 0.4 units of Taq polymerase, 1 \times reaction buffer, 1.2–1.7 mM of MgCl_2 , and 1 μL of sample DNA. PCRs profiles consisted of 1 cycle at 94°C for 4 min; 35 cycles of 94°C for 30 s, $55\text{--}58^{\circ}\text{C}$ for 30 s, and 72°C for 30 s; and 1 cycle at 72°C for 10 min. The PCR products were visualized on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA). Genotypes were scored using GeneMarker software (Softgenetics, State College, PA).

2.3. Data analysis

Because workers within social insect colonies are typically related and should not be considered independent of each other in population analyses, we followed the methods of Paar et al. (2004) and Berghoff et al. (2008). Twenty-four workers per colony were subjected to microsatellite analysis. The genotypes of the mother queen were derived from the genotypes of the sampled workers for each colony using the program MATESOFT 1.0 (Moilanen et al. 2004), according to Kronauer et al. (2006). After the genotypes of the mother queens were determined, the genotypes of each worker's father were derived by subtracting the queen allele of each worker (Oldroyd et al. 1996). The male genotypes were then diploidized and for the following analyses. For the male genotypes that could not be determined exactly, alternative male genotypes were entered as heterozygote for analyses.

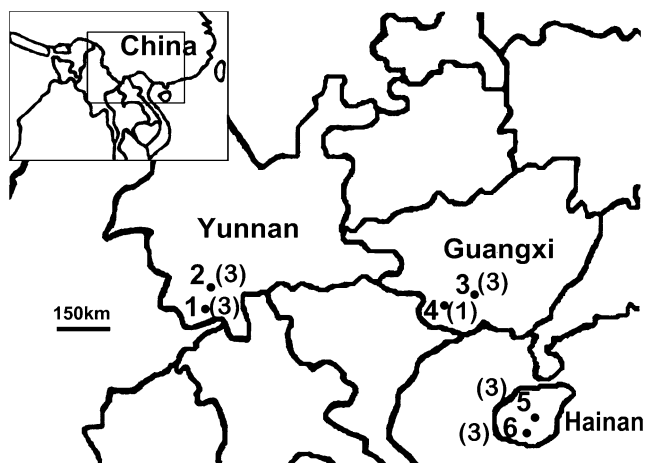


Figure 1. Collection sites and number of *A. dorsata* in China. Symbols indicate localities from which samples were collected. The number of colonies collected is given in *bracket*. 1 Xishuangbanna, 700 m (altitude), 21° 50'N, 101°10'E; 2 Puer, 1,300 m, 22°53'N, 100°10'E; 3 Nanning, 100 m, 22°40'N, 108°20'E; 4 Chongzuo, 500 m, 22°30'N, 107°20'E; 5 Qiongzong, 300 m, 19°16'N, 109°47'E; 6 Baoting, 100 m, 18°30'N, 109°40'E.

Three *A. dorsata* populations (Yunnan, Guangxi, and Hainan) were defined in our study according to geographical regions. Exact tests for Hardy–Weinberg equilibrium were conducted for each locus–population combination (Paar et al. 2004), and genotypic linkage disequilibrium for all pairs of loci in each population. Analyses were carried out with the mother queen genotypes using the software package GENEPOP 4.1 (Rousset 2008). The overall *A. dorsata* population was also tested.

Allelic richness (*A*) and private allelic richness (*pA*) were calculated with the diploidized male genotypes and corrected for differences in sample size using the rarefaction method (Kalinowski 2004) for each locus–population combination in the program HP-Rare (Kalinowski 2005). Nei's (1987) unbiased estimates of expected heterozygosities (*Hs*) were estimated with the program FSTAT 2.9.3 (Goudet 2001). Because workers in social insect colonies are related, *Hs* would be biased when estimated from the worker genotypes. Unequal sampling of spermatozoa during fertilization may also influence the allele frequency. The differences in allele frequencies between the sexes in the parental generation (Berghoff et al. 2008) were tested by calculating pairwise F_{ST} for each population in FSTAT 2.9.3.

For examining population structure of *A. dorsata* in China, the unbiased multilocus estimate of F_{ST}

(Weir and Cockerham 1984) was calculated for overall population and population pairs across loci based on the derived male genotypes with Program FSTAT 2.9.3 (Goudet 2001). The F_{ST} estimates were tested for significant differences from zero by permuting multilocus genotypes among samples. Allele frequency differences (genetic differentiation) for population pairs were also tested with GENEPOP 4.1 (Rousset 2008) using the derived male genotypes. The genetic differentiation between Hainan Island and China mainland was also analyzed. Further, an analysis of molecular variance (AMOVA) was carried out to analyze the variation within and between populations. The AMOVA analysis and significance tests are performed using GenAlEx version 6.4 (Peakall and Smouse 2006).

Allele frequencies were used to generate the D_A genetic distance (Nei et al. 1983) between each pair of colonies, and distance matrices were used to build phylogenetic trees using the unweighted pair group method with arithmetic mean (UPGMA) and neighbor-joining (NJ) algorithms with the DISPAN program (Ota 1993). The robustness of the phylogenetic relationships was assessed using bootstrap analysis with 1,000 replications. Principal component analysis (PCA) was also performed using GenAlEx version 6.4 to plot the genetic distance between individual colonies.

To assess whether the Hainan Island *A. dorsata* population exhibited detectable genetic deterioration in comparison with the mainland population (Yunnan and Guangxi), the difference of *A*, *pA*, and *Hs* between Hainan population and mainland population was tested using one-way ANOVAs.

3. RESULTS

The genotypes of the mother queens are shown in Table I. No population showed deviation from Hardy–Weinberg equilibrium (overall *A. dorsata* population, $\chi^2=4.905$, $P=1.000$; Yunnan, $\chi^2=2.779$, $P=0.986$; Guangxi, $\chi^2=1.222$, $P=1.000$; Hainan, $\chi^2=0.904$, $P=0.989$). We did not detect significant linkage disequilibrium between any pair of loci ($P>0.05$ in all cases).

For all *A. dorsata* populations in our study, the total number of alleles detected per locus ranged from 7 at locus A14 to 14 at locus A88, and average allelic richness and expected heterozygosity were 9.208 ± 2.101 and 0.745 ± 0.097 (Table II). For each *A. dorsata* population, the mean number of alleles varied from 6.4

(Hainan) to 9.6 (Yunnan). Allelic richness (*A*), expected heterozygosities (*Hs*), and private allelic richness (*pA*) for each locus and each population are also shown in Table II. Allelic richness estimates in the three *A. dorsata* geographical populations ranged from 4.000 (A14 in Guangxi population) to 13.665 (A88 in Yunnan population). Mean allelic richness across loci were 6.266 ± 1.127 (Hainan), 6.800 ± 1.924 (Guangxi), and 9.461 ± 2.509 (Yunnan). Heterozygosity estimates ranged from 0.359 (A14 in Hainan population) to 0.878 (A88 in Yunnan population). Mean heterozygosity estimates were 0.578 ± 0.136 (Hainan), 0.714 ± 0.138 (Guangxi), and 0.786 ± 0.072 (Yunnan). Average private allelic richness for the three populations were 0.200 ± 0.447 (Guangxi), 0.203 ± 0.452 (Hainan), and 1.995 ± 1.592 (Yunnan). There was no significant genetic differentiation between parental queens and males in any of the populations by pairwise F_{ST} estimates ($P>0.05$ in all cases).

Population structure analysis based on F_{ST} showed that *A. dorsata* populations were significantly differentiated at the nuclear microsatellite loci (overall $F_{ST}=0.107\pm 0.030$, $P=0.000$). F_{ST}

Table I. Inferred queen genotypes of each colony at each locus.

Colony/locus		A14	A88	A76	A24	B124
Yunnan	1	207/211	137/141	207/215	99/99	215/219
	2	205/209	131/141	203/207	99/101	215/217
	3	205/207	131/143	203/203	97/101	215/217
	4	205/215	129/135	203/215	99/99	215/217
	5	209/209	129/139	207/215	95/101	215/217
	6	207/207	131/135	203/203	95/99	215/215
Guangxi	1	205/207	131/139	203/213	93/99	215/217
	2	207/209	131/146	207/217	93/95	215/217
	3	207/209	129/137	203/205	99/101	215/217
	4	205/207	135/139	205/207	99/99	215/215
Hainan	1	209/209	135/137	203/213	95/99	223/223
	2	209/209	131/135	203/213	95/99	223/223
	3	209/209	135/141	213/213	95/105	223/223
	4	209/209	135/139	213/213	95/99	223/223
	5	209/209	135/135	213/215	95/99	223/223
	6	209/209	135/135	203/213	95/95	223/223

Table II. Genetic diversity of *A. dorsata* populations.

Locus/subpopulation		Yunnan (144)	Guangxi (96)	Hainan Island (144)	Total population (384)
A14	<i>N</i>	7	4	6	7
	<i>A</i>	6.892	4.000	5.784	6.344
	<i>Hs</i>	0.742	0.539	0.359	0.591
	<i>pA</i>	1.107	0.000	0.001	
A88	<i>N</i>	14	9	5	14
	<i>A</i>	13.665	9.000	4.999	11.958
	<i>Hs</i>	0.878	0.859	0.718	0.842
	<i>pA</i>	4.667	0.001	0.000	
A76	<i>N</i>	9	8	8	10
	<i>A</i>	8.890	8.000	7.888	9.124
	<i>Hs</i>	0.837	0.822	0.658	0.811
	<i>pA</i>	0.890	1.000	0.000	
A24	<i>N</i>	9	7	6	9
	<i>A</i>	8.880	7.000	5.779	8.339
	<i>Hs</i>	0.776	0.745	0.579	0.736
	<i>pA</i>	1.003	0.000	0.001	
B124	<i>N</i>	9	6	7	11
	<i>A</i>	8.977	6.000	6.878	10.275
	<i>Hs</i>	0.699	0.603	0.578	0.745
	<i>pA</i>	2.110	0.000	1.011	
Average	<i>N</i>	9.6	6.8	6.4	10.2
	<i>A</i>	9.461	6.8	6.266	9.208
	<i>Hs</i>	0.786	0.714	0.578	0.745
	<i>pA</i>	1.955	0.200	0.203	

Workers used for analyses are given in bracket. Minimum sample size for *A* and *pA* was 192 genes
N the number of alleles, *A* allelic richness, *Hs* expected heterozygosities, *pA* private allelic richness

values in any pair of the *A. dorsata* populations (Yunnan, Guangxi, and Hainan) were significantly different from zero (Table III). The Hainan Island population had higher F_{ST} scores (0.138 (vs. Yunnan) and 0.150 (vs. Guangxi)) in comparison with the F_{ST} between Yunnan and Guangxi (0.022). Allele frequency differences (genetic differentiation) in the three pairs of populations were also highly significant. The AMOVA revealed that differences between indi-

Table III. F_{ST} values (Weir and Cockerham 1984) among *A. dorsata* populations.

Populations compared		F_{xy}	Standard error	<i>P</i>
Yunnan	Hainan Island	0.138	0.038	<0.00007
Guangxi	Hainan Island	0.150	0.050	<0.00007
Yunnan	Guangxi	0.022	0.009	<0.00007

Significant differences from zero are indicated by *P* values

viduals within populations and within-individual differences were large (74 % and 20 %, respectively; $P < 0.01$), while a low but significant proportion of variation was detected among populations (6 %; $P < 0.01$).

Phylogenetic trees were constructed using the UPGMA and NJ algorithms for individual colonies. As both trees retained similar structure, only the NJ tree constructed from a matrix of D_A distances is presented in Figure 2. The Hainan Island population was divergent from the two China mainland populations (Yunnan and Guangxi) significantly. The grouping of samples in the PCA plot (Figure 3) is consistent with the clustering pattern of the NJ tree.

Due to the clear geographic isolation and genetic structure between the Hainan Island *A. dorsata* population and the two China mainland populations, we further investigated the genetic differentiation between Hainan Island *A. dorsata* and China mainland *A. dorsata*. Results showed that they were also highly significant ($F_{ST} = 0.131 \pm 1.042$, $P = 0.000$). Allelic richness (A), expected heterozygosities (H_s), and private allelic richness (pA) were all lower in the Hainan population than the mainland population

using one-way ANOVAs (A , $F_{1,8} = 7.954$, $P = 0.022$; H_s , $F_{1,8} = 6.360$, $P = 0.036$; pA , $F_{1,8} = 6.551$, $P = 0.034$).

4. DISCUSSION

Estoup et al. (1994) compared the genetic structure within an *Apis mellifera* colony using derived paternal DNA microsatellites with the genetic structure of the local honeybee population and found that the genetic diversity within the colony could provide a good estimate of that of the local honeybee population. In our study, we used the genotypes of derived workers' fathers for estimating genetic diversity and population structure. This method has also been used in the genetic population structure analyses of Indian *A. dorsata* populations (Paar et al. 2004) and another social insect army ant, *Eciton burchellii* (Berghoff et al. 2008).

Microsatellites confirmed their discriminating power for population structure analysis in our study. Significant genetic differentiation was found among *A. dorsata* populations from different regions in China (F_{ST} and AMOVA). The sampling regions in our study encompass

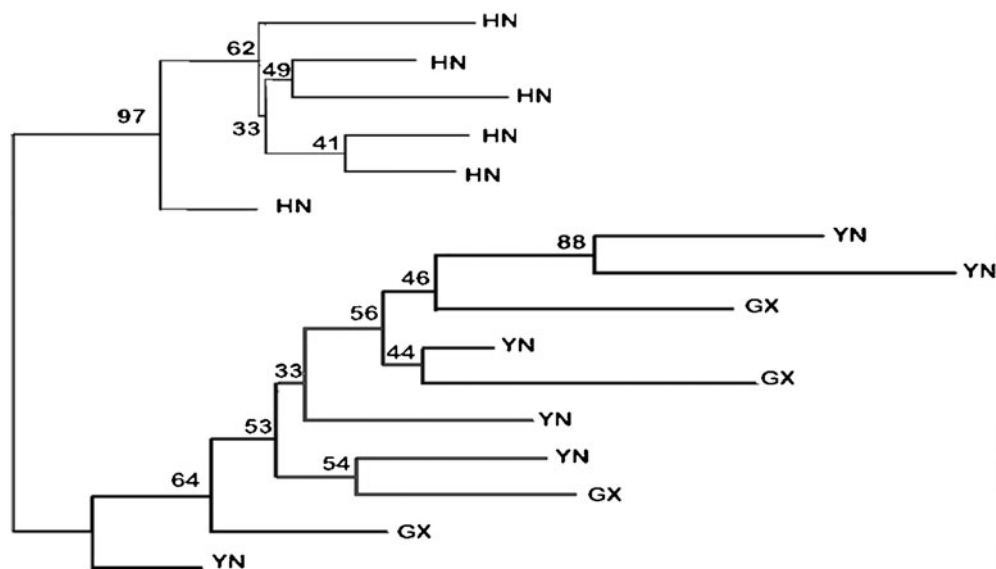


Figure 2. Neighbor-joining tree based on D_A distances among 16 samples from three populations of *A. dorsata* in China. *HN* Hainan, *YN* Yunnan, *GX* Guangxi.

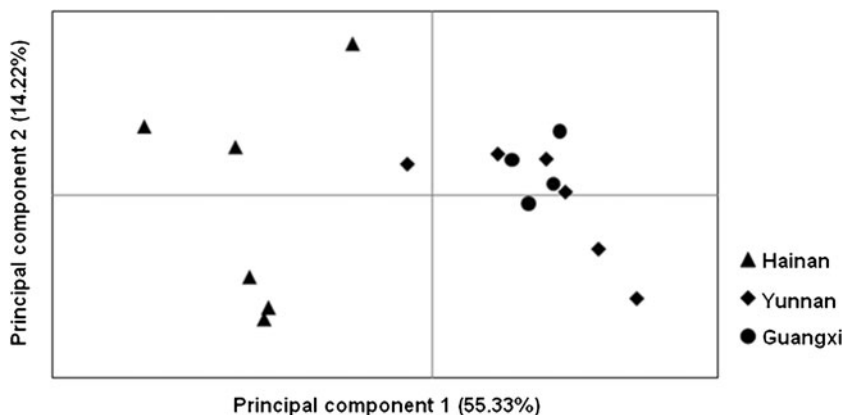


Figure 3. Principal coordinate analysis of genetic distance among 16 samples from three populations of *A. dorsata* in China.

diverse climatic zones and plant communities, which are located hundreds of kilometers apart and at different altitudes. The different climates and geographical environments may have provided ideal conditions for the divergence of *A. dorsata* in China. Significant differentiation of *A. dorsata* from different geographical regions was also found in northern India (Paar et al. 2004) and Thailand (Insuan et al. 2007).

Furthermore, we found higher pairwise F_{ST} scores between the Hainan Island population and each of the two mainland populations than between the two mainland populations. NJ tree and PCA analysis which based on genetic distance also revealed the significant differentiation between the Hainan Island population and the mainland populations. Qiongzhou Strait, which is located between Hainan Island and the China mainland, is about 80 km long and on average 30 km wide, and formed about 10,000 years ago in the middle Holocene (Zhao et al. 2007). The broad sea between Hainan Island and the China mainland may have been an important barrier to gene flow for *A. dorsata* and other honeybees. In a previous study which was based on mitochondrial DNA gene polymorphism (unpublished data), the Hainan Island population of *A. dorsata* was also found to be very probably diverged from the mainland populations. Based on morphological analysis and mitochondrial DNA analysis, *Apis cerana*

from Hainan Island was also shown to be a distinct group from those of the China mainland (Yang 1984a, b; Jiang et al. 2007; Gao et al. 2008). Similarly, significant differentiation of *A. dorsata* was found between the Thailand mainland and its islands (Insuan et al. 2007).

It is noteworthy that island populations usually have lower levels of genetic variation than their equivalent continental populations (Frankham 1997; Estoup et al. 1996; Widmer et al. 1998). In our study, the Hainan Island population of *A. dorsata* also showed a lower level of genetic diversity compared to the mainland population. This indicates that the Hainan Island population of *A. dorsata* probably diverged from the mainland populations essentially by founder effects and genetic drift. The lower level of genetic diversity may also reflect recent bottlenecks in the *A. dorsata* population of Hainan Island which needs further investigations.

Our study suggests that *A. dorsata* from Hainan Island might have diverged from the China mainland and suffers genetic deterioration. Therefore, *A. dorsata* populations from Hainan Island and China mainland should be treated separately during the conservation of this natural resource. Considering that much of the world's threatened biodiversity exists in small island populations which are particularly susceptible to changes in habitat and disruption of system interactions (Kaiser-Bunbury et al.

2010; Groom and Schwarz 2011), the lower level of genetic diversity in the Hainan Island population suggests that more attention should be given to Hainan Island *A. dorsata*.

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Structure génétique de la population chinoise d'*Apis dorsata*, basée sur les microsatellites

Apis dorsata / structure génétique / Chine / microsatellites / diversité génétique

Genetische Struktur von *Apis dorsata* Populationen in China auf der Basis von Mikrosatelliten

Apis dorsata / genetische Struktur / China / Mikrosatelliten

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