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Genetic Diversity and Relationship Between Genetic Distance and Geographical Distance of 6 *Apis Cerana Cerana* Populations in China

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Abstract: Genetic diversity and the relationship between genetic distance and geographical distance of 6 *Apis cerana cerana* populations in China were evaluated with 11 microstaellite loci, the results showed that the number of alleles per locus ranged from 7-21, the average expected heterozygosity and PIC of all loci were 0.7339 and 0.7082, respectively. Average number of alleles/locus was ranged from 2.3636 in Guangxi bee and Jiling bee to 6.4545 in Zhejiang bee. The overall expected heterozygosity of 6 *Apis cerana cerana* populations was 0.7339±0.0344, all populations showed relative large heterozygosity. In the whole population, the average of genetic differentiation among population was 32.4% ($p<0.001$). The geographical elements may own to the close relationship for particular population pairs, however, the equation $F_{ST}/(1-F_{ST}) = -1.5904 + 1.0604 \ln (d)$ and the result from Mantel's test ($p = 0.130$) did not provide enough support for a significant correlation between the genetic and geographical pair wise distances.

Key words: Genetic diversity, microsatellite, genetic distance, geographical distance, *Apis cerana cerana*

INTRODUCTION

Apis cerana cerana is a proper honeybee resource of China and there is a variety of ecotypes with China's long history of honeybee husbandry and diversified geographical conditions. However, as a result of the introduction of Apis mellifera and the limitation for conservation measures, some populations have decreased rapidly in population sizes. Some *Apis cerana cerana* populations are even facing extinction.

Though, decisions on conservation rely upon a range of information including the degree of endangerment, adaptation to a specific environment, possession of traits of current or future economic importance, possession unique traits of scientific interest and the cultural or historical value of the population, accurate assessment of populations with regard to their contribution to national and overall genetic diversity is an important step in determining priorities for conservation. Some centers of honeybee resource in China were set up according to their geographical distribution in last decades, however, in the process of developing strategies to conserve genetic diversity in *Apis cerana cerana*, it is important to assess quantitatively the genetic uniqueness of a given population, which may be deduced from genetic distances and molecular markers may serve as an important initial guide to evaluate ecotypes as genetic resources (Barker, 1994; Ruane, 1999; Weigend and Romanov, 2001).

With the characteristics of high polymorphism, locus specificity, abundance and random distribution over the genome and their co-dominant inheritance, microsatellites are currently most commonly used to assess population structure and diversity (Romanov and Weigend, 2001; Chen *et al*., 2004b; Du *et al*., 2004). According to FAO, recommendations, determining classic genetic distances using neutral, highly polymorphic microsatellite markers is the method of choice for investigating genetic relationships and breed differentiation. This methodology also provides information for establishing preservation priorities for livestock breeds (Barker, 1999).

The aim of this study, was to evaluate genetic diversity in 6 *Apis cerana cerana* populations in China with 11 microsatellite markers and to analyze the relationship between all pairs of geographical distance and genetic distance. The results may be useful to understand genetic differentiation of this important genetic resource and contribute to a more efficient conversation.

MATERIALS AND METHODS

Experimental populations: A total 191 individuals from Apis mellifera ligustica and *Apis cerana cerana* in China were genotyped. All populations are from China, The main original area and the number of individuals studied was presented in Table 1.

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Table 1: Main original area and number of animals studied of 6 populations

Name	Number	Time	Main original area	No. of animals studied
Apis cerana cerana of Guangxi	GX	5/22/2007	Guangxi, China	33
Apis cerana cerana of Guangdong	GD	7/18/2007	Guangdong, China	30
Apis cerana cerana of Beijing	BJ	9/16/2007	Beijing, China	33
Apis cerana cerana of Jiling	JL	9/12/2007	Jiling, China	30
Apis cerana cerana of Zhejiang	ZJ	7/19/2007	Zhejiang, China	35
Apis cerana cerana of Shandong	SD	8/20/2007	Shandong, China	30

Table 2: The location of 11 microsatellite loci in chromosome or linkage group and condition of PCR

Locus	Gen bank accession	Chromosome or linking group	Mg^{2+} (mmol L^{-1})	Annealing temperature (°C)
AP243	AJ509466	Chr LG1	2.2	57.5
Ap043	AJ509329/AJ509667	Chr LG3	2.2	56.5
AP313	AJ509504	Chr LG4	2.0	57.0
A113	AJ509290	Chr LG6	2.0	58.2
A024d	AJ509241	Chr LG7	2.0	57.0
AC011	AJ509637	Chr LG9	1.8	57.0
Ap085	AJ509359	Chr LG12	2.0	56.5
At003	AJ509505	Chr LG13	2.0	55.6
A035	AJ509251	Chr LG14	2.0	53.4
A028	AJ509244	Chr LG14	2.0	55.0
Ap068	AJ509351	Chr LG15	2.0	55.6
AG005C	AJ509723	Chr LG16	2.0	55.0

Genotyping: Eleven microsatellite markers (Table 2) spread across the honeybee genome were used for genotypes. The primers are selected according to NCBI and report by Solignac *et al.* (2003). PCR products were obtained in a 20 μL volume using thermal cycler. Each PCR tube contained 50 ng of genomic DNA, 2.0 μL of 10× buffer, 1.2~2.0 μL of 25 mmol L^{-1} $MgCl_2$, 0.5 μL of 10 mmol μL^{-1} dNTP, 1 μL of both 10 pmol μL^{-1} forward primer and reverse primer, 5 U μL^{-1} Taq DNA Polymerase 0.2 μL. The amplification involved initial denaturation at 95°C (5 min), 35 cycles of denaturation at 95°C (50 sec), annealing temperature varying between 50 and 60°C (50 sec) and extension at 72°C (50 sec), followed by final extension at 72°C (10 min). DNA fragments were scored on 8% polyacrylamide gel using a LI-COR automated DNA analyzer (LI-COR Biotechnology Division, Lincoln, NE68504). Electrophoregram processing and allele-size scoring was performed with the RFLP scan package (Scanalytics, Division of CSP, Billerica, MA). The primers are selected according to NCBI and report by Solignac *et al.* (2003).

Statistical analysis

Genetic diversity: Allele frequencies, The observed and expected heterozygosity (Ho and He) (Nei, 1987) for each population across the loci and that for each locus across populations were estimated with Microsatellite-Toolkit for Excel. Polymorphism Information Content (PIC) for each locus and each breed was obtained according Botstein *et al.* (1980):

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2$$

Where:

n = The number of alleles.

p_i = Frequency of the allele I.

p_j = Frequency of the allele j.

Genetic differentiation: The F-statistics indices (Wright, 1978), were estimated in the form of F, θ and f, the sample-based, respective estimators of these parameters proposed by Weir and Cockerham (1984), as implemented in FSTAT program (Goudet, 2002). Significance of the F-statistics was determined from permutation tests with the sequential Bonferroni procedure applied over loci (Hochberg, 1988). As a measure of deviation from Hardy-Weinberg equilibrium, the F_{IS} value was calculated and type-I error probability was computed. The F_{ST} values among pairs of breeds were calculated with GENEPOP program (Raymond and Rousset, 1995). Rousset's (1997) isolation by distance was applied to these *Apis cerana cerana* populations. A linear regression was used to estimate the coefficients:

$$F_{ST}/(1-F_{ST}) = \alpha + \beta \ln(d)$$

where, d represents the pair wise geographical distance between populations. Gene flow between populations, defined as the number of reproductively successful migrants per generation (Nm), was estimated by the methods based on the n island model of population structure. The estimate was based on the relationship $F_{ST} = 1/(4Nm+1)$, where N is the effective population size, m is the migration rate and F_{ST} is the mean F_{ST} value calculated over all loci (Slatkin and Barton, 1989). The Reynolds' genetic distance (Reynolds *et al.*, 1983) between populations was calculated, based on F_{ST} values.

RESULTS

Genetic variability within popualtions: A total of 142 were detected in alleles 6 *Apis cerana cerana* populations in China with 11 microsatellite markers. Total number of alleles, size of alleles, expected heterozygosity (He) and mean Polymorphic Information Content (PIC) for each locus across 6 populations were listed in Table 3.

The number of alleles per locus ranged from 7 (Ap313) to 21 (AC011) and the average number of the alleles observed in 11 microsatellite loci was 12.9091±4.5267. Across populations, locus Ap313 had the lowest He, 0.4648 and the lowest PIC, 0.4418, however, the locus Ap043 had the highest He and PIC value, 0.8552 and 0.8405, respectively.

The average number of alleles per locus, expected and observed heterozygosity and for each population across 11 loci were shown in Table 4. Average number of alleles/locus ranged from 2.3636 in Jiling population to 6.4545 in Zhejiang population. All populations showed relative large heterozygosity. Across 11 loci, the lowest value 0.3116 of heterozygosity was obtained for the Jiling population and the highest 0.7028 was found for Zhejiang population. The overall expected heterozygosity of 6 *Apis cerana cerana* populations in China was 0.7339±0.0344.

The results from F-statistics analysis and number of populations deviation from Hardy-Weinberg equilibrium were shown in Table 5.

Table 3: Names, total number of alleles, size of alleles, expected heterozygosity and PIC of 11 microsatellite markers

Locus	No. of alleles	Size of alleles (bp)	Expected heterozygosity	PIC
AP243	11	98-315	0.7932	0.7686
Ap043	20	120-217	0.8552	0.8405
AP313	7	332-400	0.4648	0.4418
A113	11	172-240	0.7564	0.7255
A024d	8	64-98	0.6263	0.5940
AC011	21	91-149	0.8223	0.8043
Ap085	14	58-220	0.8072	0.7796
At003	14	78-227	0.7999	0.7774
A035	9	87-234	0.6387	0.6012
A028	12	102-136	0.7520	0.7225
Ap068	15	124-173	0.7571	0.7351
Mean	12.9091		0.7339	0.7082
	(4.5267)		(0.1441)	(0.1170)

Table 4: Mean number of alleles per locus, mean heterozygosity (He and Ho) for 6 populations

Population	Number of alleles (mean±SD)	Average He (mean±SD)	Average Ho (mean±SD)
GX	3.3636±1.6895	0.4960±0.0888	0.2160±0.0218
GD	4.2727±2.4531	0.5023±0.0884	0.1190±0.0179
BJ	2.4545±0.8201	0.4225±0.0623	0.2726±0.0237
JL	2.3636±1.1200	0.3116±0.0632	0.2916±0.0252
ZJ	6.4545±1.8635	0.7028±0.0279	0.2532±0.0237
SD	5.7272±2.1950	0.6996±0.0347	0.3481±0.0237
Total	12.9091±4.5266	0.7339±0.0344	0.2483±0.0096

In the exact test for deviation from Hardy-Weinberg equilibrium, more or less populations showed significant deviation for all loci (data not shown). The negative FIS values of some populations indicated an excess of heterozygous genotypes with respect to the expected value.

Genetic differentiation: Genetic differentiation was examined by fixation indices F_{IT}, F_{ST}, F_{IS} on each locus. Results of the F-statistics analysis for 11 microsatellite markers in 6 *Apis cerana cerana* populations in China were presented in Table 5.

The fixation coefficients of subpopulations within the total population, measured as F_{ST} value, for the 11 loci varied from 0.211 (AC011) to 0.441 (A024d), with a mean of 0.324 ($p<0.001$), all loci contributed significantly to this differentiation.

Estimated of gene flow (Nm) and Reynolds' genetic distances (D_R) between each population pair are presented in Table 6. Reynolds' distance values varied between 0.0697 (Guangdong-Guangxi population pair) and 0.5924 (Shandong-Jiling population pair). The Nm value ranged from 0.3093 (between Shandong-Jiling population pair) to 3.4647 (between Guangdong-Guangxi population pair). And only Nm values between pair of Guangdong-Guangxi population was over 3.0.

The application of Rousset's isolation by distance method, as implemented in GENEPOP program, yielded the

Table 5: The results from F-statistics analysis and number of populations deviation from Hardy-Weinberg equilibrium

Locus	$F_{IT} = F$	$F_{ST} = \theta$	$F_{IS} = f$	No of population deviation from Hardy-Weinberg equilibrium
AP243	0.810***	0.413***	0.676***	5
Ap043	0.562***	0.316***	0.360***	5
AP313	0.890***	0.251***	0.854***	5
A113	0.676***	0.422***	0.439***	2
A024d	0.467***	0.441***	0.046	4
AC011	0.798***	0.211***	0.745***	6
Ap085	0.755***	0.342***	0.628***	5
At003	0.762***	0.215***	0.697***	6
A035	0.165***	0.283***	-0.165	3
A028	0.800***	0.330***	0.702***	5
Ap068	0.752***	0.330***	0.63***	5
Mean	0.681***	0.324***	0.528***	5
	0.055	0.024	0.086	

Mean estimates from jack-knife over loci, standard deviations are given in parentheses; *p<0.05,**p<0.01,***p<0.001

Table 6: Reynolds genetic distances, DR (upper triangle) and the gene flow, Nm (lower triangle) between populations

Population	GX	GD	BJ	JL	ZJ	SD
GX		0.0697	0.2930	0.4458	0.3936	0.3919
GD	3.4647		0.2950	0.5534	0.3778	0.3801
BJ	0.7343	0.7285		0.3099	0.4483	0.4700
JL	0.4450	0.3382	0.6881		0.5708	0.5924
ZJ	0.5183	0.5447	0.4419	0.3248		0.2611
SD	0.5211	0.5406	0.4167	0.3093	0.8379	

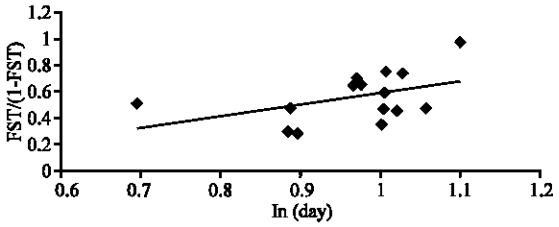

Fig. 1: Plot of relationship between geographical distance. ln(d). and pair wise $F_{ST}/(1-F_{ST})$ for all pairs of 6 *Apis cerana cerana* populations in China

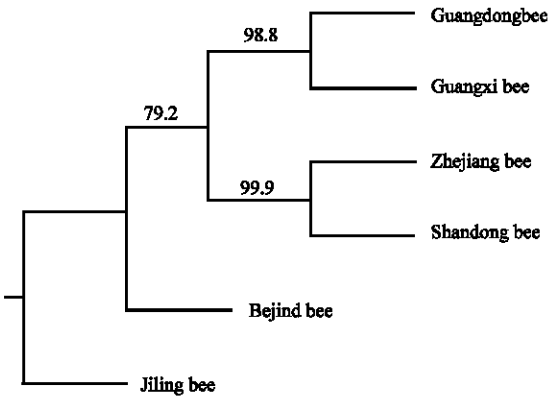

Fig. 2: Dendogram of phylogenetic relationships among 6 *Apis cerana cerana* populations a based on Nei's genetic distance, using UPGMA method, numbers at the nodes are percentage bootstrap values from 1000 replications with resampled loci

parametersα and β in the regression, $F_{ST}/(1-F_{ST})$ = -1.5904 + 1.0604ln (day) (Fig. 1). However, regression failed to provide enough support for a significant correlation between the genetic and geographical pair wise distances, as indicated by Mantel's test (p = 0.130).

Clustering of populations: The Neighbour-Joining (NJ) tree derived from the kinship distances is given in Fig. 2. The tree topology revealed the relationships between populations were always supported by high bootstrap values. There was a close genetic relationship between Guangdong bee-Guangxi bee pair and Zhejiang bee-Shandong bee pair, these 2 pairs fell together with the 98.8% bootstraps and 99.9% bootstraps, respectively. Beijing bee then formed a branch with that cluster, in the last, Jiling bee fell together with the branch.

DISCUSSION

Genetic diversity within populations: The average PIC was 0.7082. Fairly, high PIC values for majority of the markers employed are suggestive of their use in biodiversity evaluation of *Apis cerana cerana* populations. The results also revealed a relatively high level of genetic diversity in *Apis cerana cerana* populationsand in China (He = 0.4648-0.8552, average number of alleles per locus = 7-21) compared with Apis dorsata (He = 0.68-0.74, average number of alleles per locus = 6.0-9.0) distribution in Thailand using DNA pools typed at 3 microsatellite loci (Insuan *et al*., 2007).

Among 6 *Apis cerana cerana* populations, Zhejiang bee had the highest genetic variability and Shandong bee had a very close value to it and Guangdong bee and Guangxi bee which had very close value were next to them. Jiling bee had the lowest one, which was lower than Beijing bee. The reason may be that the Zhejiang and Shandong located in Eastern China where there were a heterogeneous environment for honeybee that included sufficient food and habitat resources needed for survival, growth and reproduction. So, *Apis cerana cerana* spread quickly there. Contrarily, Jiling and Beijing located in Northern China, where was poor in diversity and abundance of successional flowering plants because of the cold weather, which can not provide food and cavity sites needed by honeybee, in add of rapidly spreading of Apis mellifera, so the amount of *Apis cerana cerana* there deceased rapidly. Recently, as the fast development of economy in Southern China, management practices and other human activities have altered the landscape and thereby decreased the mount and the diversity of the *Apis cerana cerana* in Guangdong and Guangxi.

Genetic differentiation among populations: In our study, on average, the genetic differentiation (F_{ST}) among populations was 32.4% (Table 4), a relative high value and extremely significant ($p < 0.001$), which indicated that there is a great differentiation (Wright, 1978; Hartl and Clark, 1997) among 6 *Apis cerana cerana* populations. It is clear that about 32% of the total genetic variation corresponds to differences of breeds and the remaining 68% is the result of differences among individuals. All loci contribute to this differentiation significantly.

The coefficient F_{IS}, which indicates the degree of departure from random mating, positive F_{IS} values mean a significant deficit of heterozygotes, while the negative F_{IS} values indicate an excess of heterozygous genotypes with respect to the expected value. In this study, high average of F_{IS} was 0.528. In addition, 9 loci showed significant deficit of heterozygotes. Two reasons maybe contribute to the deficit of heterozygotes for these nine loci: first, the locus may be under selection (genetic hitchhiking effect) with some morphological or productive traits of selective interest; secondly, null alleles may be present (Nei, 1987).

Relationships among breeds: Guangdong bee and Guangxi bee had a close genetic relationship. From geographical locations, Guangdong province and Guangdong province are neighbours to each other in Eastern China, it was convenient for these populations there to communicate with each other. The very high gene flow, Nm (3.4647), Guangdong bee and Guangxi bee, also supported that there may be genetic migration between these 2 populations.

Geographical elements may owe to the close relationship for particular population pairs, for instance, Zhejiang bee and Shandong bee. Though, Beijing bee and Jiling bee also had very near geographical distance, these 2 breeds had not showed close genetic relationship. The result from Mantel's test failed to support a significant correlation between genetic and geographical pair wise distances for the whole dataset, all these results indicated that the geographical distribution was not a decisive factor to influence the genetic structure of *Apis cerana cerana* populations during their cultured history. So, the geographical condition was only a reference when we set up the program of *Apis cerana cerana* conservation, the genetic distance should be served as the most important guide in determining priorities for conservation of *Apis cerana cerana*.

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