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Study on Genetic Diversity of *Apis cerana cerana* and *Apis mellifera ligustica* in China with Microsatellite Markers

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Abstract: Analysing genetic diversity of *Apis cerana cerana* and *Apis mellifera ligustica* in China could provide a theoretical foundation for the research on the conservation and reasonable utilization of honey bee populations, but there have not public reports on this topic by using microsatellite markers until now. Genetic diversity of Changbaishan bee (*Apis cerana cerana*) and Pinghu royal jelly bee (*Apis mellifera ligustica*) in China were evaluated with 21 microstaellite loci, the genetic variability within breeds and genetic differentiation between breeds were estimated, the results showed that in 21 microstaellite loci, 171 alleles were found, the number of alleles per locus ranged from 3-13, the average expected heterozygosity and PIC of all loci were 0.7175 and 0.6755, respectively. Mean numbers of alleles of Changbaishan bee and Pinghu royal jelly bee were 1.86 and 6.27, the average of genetic differentiation measured as FST value, was 51.9% ($p<0.001$) and all loci contributed significantly ($p<0.001$) to this differentiation. It can also, be seen that the deficit of heterozygotes was very high (0.574) ($p<0.001$). Reynolds' distance values between 2 populations were 0.734.

Key words: *Apis cerana cerana*, *Apis mellifera ligustica*, microsatellite, genetic diversity

INTRODUCTION

China has a very long history of apiculture, at the end of the 19th centry, *Apis mellifera* was invited to China, before which there were only *Apis cerana cerana* in China (Chen, 2001). Since, the population has some excellenter characters than *Apis cerana cerana* who is aboriginal, *Apis mellifera* spread quickly across China, meanwhile, some *Apis cerana cerana* populations have decreased rapidly in population sizes because of *Apis mellifera* population rapid spread and some other reasons, some populations have decreased rapidly in population sizes, Some *Apis cerana cerana* populations are even facing extinction. Analysing genetic diversityof *Apis cerana cerana* and *Apis mellifera ligustica* in China could provide a theoretical foundation for the research on the conservation and reasonable utilization of honeybee populations, but there are less public reports on this topic by using microsatellite markers until now.

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 (Weigend and Romanov, 2001). 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).

Pinghu royal jelly bee was cultivated in Pinghu of ZheJiang province, which was bred by hybridized *Apis mellifera* L. from Italy and *Apis mellifera* L. in ZheJiang. This breed has many fantastic peculiarities and the most outstanding one is higher royal production (Sun *et al*., 2003). Changbaishan bee is *Apis cerana cerana* distributed in Changbai Mountain, which has special morphological and biological characters. This population experienced thousands years of golden period, but its survival area are shrinking day by day because of introduction of *Apis mellifera* and the limitation for conservation measures (Li and Wang, 2004).

The aim of this study, was to analyze genetic diversity in Changbaishan bee (*Apis cerana cerana*) and Pinghu royal jelly bee (*Apis mellifera ligustica*) in Chinawith 21 microsatellite markers and to evaluate their genetic strcture. The results may be useful to understand genetic differentiation of this important genetic resource and contribute to a more efficient conversation.

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Table 1: The location of 21 microsatellite loci in chromosome or linkage group and condition of PCR

Locus	GenBank accession number	Chromosome or linkage group	Concentration of Mg^+	Annealing temperature (°C)
AP243	AJ509466	Chr LG1	2.2	57.5
Ap049	AJ509334	Chr LG1	2.2	55.6
AP226	AJ509455	Chr LG1	2.0	55.6
AC306	AJ509721	Chr LG2	2.0	55.6
AP274	AJ509486	Chr LG3	2.0	55.0
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
A107	AJ509287	Chr LG7	2.0	57.0
A024d	AJ509241	Chr LG7	2.0	57.0
A014	AJ509239	Chr LG8	2.0	55.6
A088	AJ509283	Chr LG8	1.6	58.0
AC011	AJ509637	Chr LG9	1.8	57.0
BI366	BI516839	Chr LG11	1.6	57.0
AT101	AJ509549	Chr LG12	2.0	55.6
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

MATERIALS AND METHODS

Experimental populations: The 92 individuals from Pinghu royal jelly bee and 30 individuals from Changbaishan bee were genotyped. DNA was extracted according to the reports by Ji *et al.* (2005) and then preserved in -20°C after tested content and purity.

Genotyping: Twenty one microsatellite markers (Table 1) spread across the honeybee genome were used for genotypes. The primers are selected according to NCBI and report by Michel *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).

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. 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). The Reynolds *et al.* (1983) genetic distance between populations was calculated, based on F_{ST} values.

RESULTS

A total of 171 alleles were detected in 2 populationsin China with 21 microsatellite markers. Total number of alleles, size of alleles, Expected Heterozygosity (He) and mean Polymorphic Information Content (PIC) for each locus across 2 populations were listed in Table 2.

Table 2: Names, total number of alleles, size of alleles, expected heterozygosity and *PIC* of 21 microsatellite markers

Locus	Chromosome	No.of alleles	Size of alleles(bp)	Expected heterozygosity	PIC
AP243	AJ509466	7	253-366	0.5140	0.4801
Ap226	AJ509455	10	234-260	0.8463	0.8243
Ap049	AJ509334	7	90-166	0.7649	0.7235
AC306	AJ509721	10	150-180	0.7969	0.7649
AP274	AJ509486	10	102-209	0.6364	0.5891
Ap043	AJ509329/AJ509667	7	106-160	0.5752	0.5180
AP313	AJ509504	4	239-400	0.5164	0.4053
A113	AJ509290	10	186-226	0.7651	0.7276
A107	AJ509287	13	127-188	0.8803	0.8644
A024d	AJ509241	6	68-98	0.7117	0.6703
A014	AJ509239	4	178-247	0.6981	0.6423
A088	AJ509283	10	116-147	0.7894	0.7554
AC011	AJ509637	12	98-136	0.8357	0.8135
BI366	BI516839	3	116-172	0.4830	0.4195
AT101	AJ509549	7	253-281	0.6909	0.6300
Ap085	AJ509359	8	180-198	0.8121	0.7821
At003	AJ509505	10	87-234	0.8114	0.7846
A035	AJ509251	11	82-250	0.8607	0.8408
A028	AJ509244	3	114-118	0.5045	0.4404
Ap068	AJ509351	10	124-213	0.7323	0.6909
Ag005C	AJ509723	9	102-148	0.8412	0.8181
Mean		8.1429 (2.9023)		0.7175 (0.1298)	0.6755 (0.1475)

Standard deviations for mean number of alleles, He and PIC, were given in parentheses

Table 3: Mean number of alleles per locus, mean heterozygosity (He and Ho) for 2 populations

Population	Number of alleles (mean±SD)	Average He (mean±SD)	Average Ho (mean±SD)
Changbaishan bee	1.86±1.06	0.4356±0.0695	0.1528±0.0314
Pinghu royal jelly bee	6.27±3.20	0.6085±0.0222	0.2378±0.0232

Table 4: The results from F-statistics analysis

Locus	F_{IT} = F	F_{ST} = θ	F_{IS} = f
AP243	0.762***	0.713***	0.171
Ap226	0.955***	0.555***	0.899***
Ap049	0.921***	0.475***	0.849***
AC306	0.806***	0.431***	0.659***
AP274	0.743***	0.685***	0.185**
Ap043	0.906***	0.665***	0.719***
AP313	0.764***	0.405***	0.603***
A113	0.686***	0.470***	0.408***
A107	0.599***	0.192***	0.504***
A024d	0.557***	0.472***	0.160*
A014	0.844***	0.623***	0.586***
A088	0.860***	0.463***	0.739***
AC011	0.860***	0.373***	0.777***
BI366	1.000***	0.858***	1.000***
AT101	0.925***	0.629***	0.798***
Ap085	0.676***	0.501***	0.352***
At003	0.864***	0.169***	0.836***
A035	0.078*	0.320***	-0.356
A028	1.000***	0.834***	1.000***
Ap068	0.869***	0.516***	0.73***
Ag005C	0.965***	0.478***	0.934***
Mean	0.796*** (0.046)	0.519*** (0.037)	0.574*** (0.086)

F, total inbreeding estimate; F_{ST}, measure of population differentiation; f, within-population inbreeding estimate, Mean estimates from jack-knife over loci, standard deviations are given in parentheses. Significance of F statistics was done using Bonferroni permutations based on 1000 resamplings *p<0.05; **p<0.01; ***p<0.001

The number of alleles per locus ranged from 3 (BI366, A028) to 13 (A107) and the average number of the alleles observed in 21 microsatellite loci was 8.1429±2.9023. Across populations, locus BI366 had the lowest He, 0.4830 and the lowest PIC, 0.4192, however the locus A103 had the highest He and PIC value, 0.8803 and 0.8644, respectively.

The average number of alleles per locus, expected and observed heterozygosity and for each population across 21 loci were shown in Table 3.

Average number of alleles/locus was 6.27 in Pinghu royal jelly bee and 1.86 in Changbaishan bee. Both 2 populations showed relative large heterozygosity. Across 21 loci, the value 0.6085 of heterozygosity was obtained for the Pinghu royal jelly bee and the 0.4356 was found for Changbaishan bee.

The results from F-statistics analysis was shown in Table 4. The negative FIS values of some populations indicated an excess of heterozygous genotypes with respect to the expected value. For the 2 populations, inbreeding index (FST) was 51.9% ($p<0.001$), all loci supported this result. Reynolds' genetic distance between 2 populations was 0.734.

DISCUSSION

The 21 microsatellite markers used in the present study are randomly distributed across 14 chromosomes or

linkage groups in the honeybee genome, so the data had certain commparability and representativeness. The Polymorphism Information Content (PIC) value is a good measure of the polymorphisms of gene fragment, while PIC>0.5, the locus is a highly polymorphic locus; while 0.25<PIC<0.5, the locus is a medium polymorphic locus; while PIC<0.25, the locus is a low polymorphic locus (Vanhala *et al*., 2001). Meanwhile, PIC value is related to the availability and utilization efficiency of a marker, the higher PIC value of the marker, the higher heterozygote frequency in one population, as well as the more genetic information it provides. In this study, 20 loci among 21 microsatellite loci exhibited high polymorphic, while 1 loci showed medium polymorphic, mean PIC value across all loci exceeded 0.5, which could provide enough information for the assessment of genetic diversity.

Effective number of alleles is also a good measure of the genetic variation, especially in conservation genetics study. Sometimes its effect on populations is put more emphasis, but effective number of alleles is easy to be affected by sample size (Maudet *et al*., 2002). The average number of the alleles was 8.1429 across 21 microsatellite loci in the present study, which indicated that the sample size was enough. On the other hand, this result also indicated that the polymorphism information content provided by these 21 microsatellite loci in 2 populations was rich and the distribution of the allelic frequency was rather even. Therefore, using effective number of alleles to analysis genetic diversity is more effective and reliable.

Gene heterozygosity, also called gene diversity, is a suitable parameter for investigating genetic variation. Ott (2001) gave a definition that a polymorphic locus must have at least 0.10 heterozygosity. All 21 microsatellite loci in this study had high polymorphism with a mean expected heterozygosity, 0.7175, showing a high degree of genetic diversity and relative high selection potential. Mean expected heterozygosity can approximately reflect the variation of genetic structure, Pinghu royal jelly bee had much higher genetic variability (0.6085) than Pinghu royal jelly bee (0.4356).

In our study, on average, the genetic differentiation (F_{ST}) among breeds was 51.9% (Table 4), high value and extremely significant ($p<0.001$), which indicated that there is a great differentiation between Changbaishan bee and Pinghu royal jelly bee, this result strongly supported that *Apis cerana cerana* and *Apis mellifera ligustica* are 2 different subspecies. It is clear, that about 51.9% of the total genetic variation corresponds to differences of populations and the remaining 48.1% 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, average of F_{IS} was 0.574. In addition, all loci showed significant deficit of heterozygotes except AP297 and A035. Two reasons maybe contribute to the deficit of heterozygotes for these 19 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.

CONCLUSION

All 21 microsatellite loci in this study had high polymorphism with a mean expected heterozygosity, 0.7175, showing a high degree of genetic diversity and relative high selection potential. Pinghu royal jelly bee had much higher genetic variability than Pinghu royal jelly bee, there is a great differentiation between Changbaishan bee and Pinghu royal jelly bee, this result strongly supported that *Apis cerana cerana* and *Apis mellifera ligustica* are two different subspecies.

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