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Full Length Research Paper

Genetic characterization of three breeds of high royal jelly producing honeybee (*Apis mellifera ligustica*) in China

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There are three most famous breeds of high royal jelly producing honeybee (*Apis mellifera ligustica*) in China. After numerous rounds of selection for royal jelly production, the royal jelly production of the three breeds is much higher than the unselected. It is important to maintain genetic diversity of breeds in selective breeding and stock management. The genetic diversity and genetic differentiation of three breeds were surveyed using 18 microsatellite markers. Using Polymorphism Information Content (PIC), mean heterozygosity (H), number of effective alleles, genetic distances, gene flow (Nm) and F-statistics, we evaluated the genetic diversity and genetic differentiation. The result showed that the number of alleles per locus ranged from 2 (AP156 and A028) to 25 (AP053). All of the three breeds showed high levels of heterozygosity. Significant genetic differentiation was found among the three breeds and the average genetic differentiation coefficient of the three breeds was 0.037.

Key words: High royal jelly producing honeybee, *Apis mellifera ligustica*, genetic diversity, genetic differentiation, microsatellite marker.

INTRODUCTION

Since the consumer demand on royal jelly in Asia is much high, many honeybee breeding programs were constructed to obtain high royal jelly producing breeds in China. After several years of selection, the royal jelly production of honeybee *Apis mellifera ligustica* in China has improved a lot, and there are three most famous breeds (Xiaoshan bee, Pinghu bee, Zhenongda NO.1) cultivated in Zhejiang province, which are used widely around the country. Maintenance of genetic diversity of breeds is an important factor in selective breeding and stock management. Reductions in diversity promote susceptibility to disease outbreaks and other negative conditions associated with inbreeding (Baer and Schmid-Hempel, 2001; Tarpy and Seeley, 2006; Seeley and Tarpy, 2007). To maintain the achievement of the breeding programs, the genetic characterization of three breeds should be assessed.

With the characteristics of high polymorphism, locus specificity, abundance and random distribution over the genome, and their co-dominant inheritance, microsatellites are currently the most commonly used to assess population structure and diversity (Chapman, 2008; Bourgeois and Rinderer, 2009; Delaney et al., 2009; Zarkti et al., 2010). 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 research was to evaluate genetic diversity and genetic differentiation of the 3 high royal jelly producing breeds with 18 microsatellite markers. The results may be useful to understand genetic Characterization of the three breeds and contribute to a

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Table 1. The location of 18 microsatellite loci in chromosome and PCR conditions.

	GenBank accession NO.	Chromosome	Mg^{2} concentration (mmol/L)	Annealing temperature
AG005a	AJ509722	Chr LG1	2	55
AC306	AJ509721	Chr LG2	2	55.6
AP274	AJ509486	Chr LG3	2	55
AP043	AJ509329	Chr LG3	2	56.5
AP313	AJ509504	Chr LG4	2	57
AP053	AJ509338	Chr LG5	2.2	56.5
AP143	AJ509400	Chr LG5	2.2	56.5
A113	AJ509290	Chr LG6	2	58.2
A014	AJ509239	Chr LG8	2	55.6
AC011	AJ509637	Chr LG9	1.8	57
AP189	AJ509433	Chr LG10	1.6	57
AP156	AJ509410	Chr LG10	2	55.6
BI299	BI514528	Chr LG11	2	55.6
AP085	AJ509359	Chr LG12	2	55.6
AT101	AJ509549	Chr LG12	2	55.6
AT003	AJ509505	Chr LG13	2	55.6
A028	AJ509244	Chr LG14	2	55
AP068	AJ509351	Chr LG15	2	55.6

more efficient selection and breeding.

MATERIALS AND METHODS

Sampling

A total of 144 individual bees, 48 worker bees of each breed were analysed in this study. Xiaoshan bees (XS) were collected from Bee Breeding Farm in Xiaoshan, Zhejiang and Pinghu bees (PH) were collected from Bee Breeding Farm in Pinghu, Zhejiang and Zhenongda NO.1 (ZN) were collected from Zhejiang University.

DNA isolation

DNA was extracted from the thorax of individual bees (one bee per colony) according to the method reported by Ji et al. (2005).

Genotyping

The DNA polymorphism was assessed at 18 microsatellite loci (Table 1). These markers are randomly distributed across 14 of the 16 chromosomes of *Apis mellifera* genome. The primers are selected according to NCBI (http://www.ncbi. nlm.nih.gov). PCR products were obtained in a 20 l volume using thermal cycler. Each PCR tube contained 50 ng of genomic DNA, 2.0 l of 10×buffer 1.2 to 2.0 l of 25 mmol/l $MgCl_2$ 0.5 L of 10 mmol/ l dNTP, 1 l of both 10 pmo1/ l forward primer and reverse primer, 5 U/ l *Taq* DNA Polymerase 0.2 l. The amplification involved initial denaturation at 95ºC (5 min), 35 cycles of denaturation at 95ºC (50 s), annealing temperature varying between 50 and 60ºC (50 s), and extension at 72ºC (50 s), followed by final extension at 72ºC (10 min). DNA fragments were scored on 8% polyacrylamide gel using a ABI 377 automated DNA analyzer (P-Eapplied Biosystem, America). Electrophoregram processing was performed with GENESCAN3.1 software (P-Eapplied Biosystem, America), and allele-size scoring was analyzed by the Binthere software (P-EApplied Biosystem, America).

Statistical analysis

Genetic diversity: Total number of alleles, allele frequencies, average number of alleles per locus, observed (Ho) and expected heterozygosity (He) for each population across the loci, were estimated with Microsatellite-Toolkit for Excel (Park, 2001).

Genetic differentiation: Population differentiation was estimated by Wright's (1978) fixation indices F_{IT}, F_{ST} and F_{IS} in the form of F, , and f, respectively, for each locus across populations according to the variance based method of Weir and Cockerham (1984) using FSTAT software (Version 2.9.3, Goudet, 2002). The significance of the F-statistics was determined by permutation tests with the sequential Bonferroni procedure applied over loci (Hochberg, 1988). The extent of inbreeding was further studied with GENEPOP software (Raymond and Rousset, 1995) by estimating the F_{IS} values and their significance level within each of the populations.

Pair-wise F_{ST} values were computed for all combinations of the 3 populations using GENEPOP software. Gene flow between populations, defined as the number of reproductively successful migrants per generation (Nm), was estimated based on the n island model of population structure (Slatkin and Barton, 1989). 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 calculated as mean over loci. The Reynolds' genetic distance (Reynolds et al., 1983) between breeds was calculated, based on

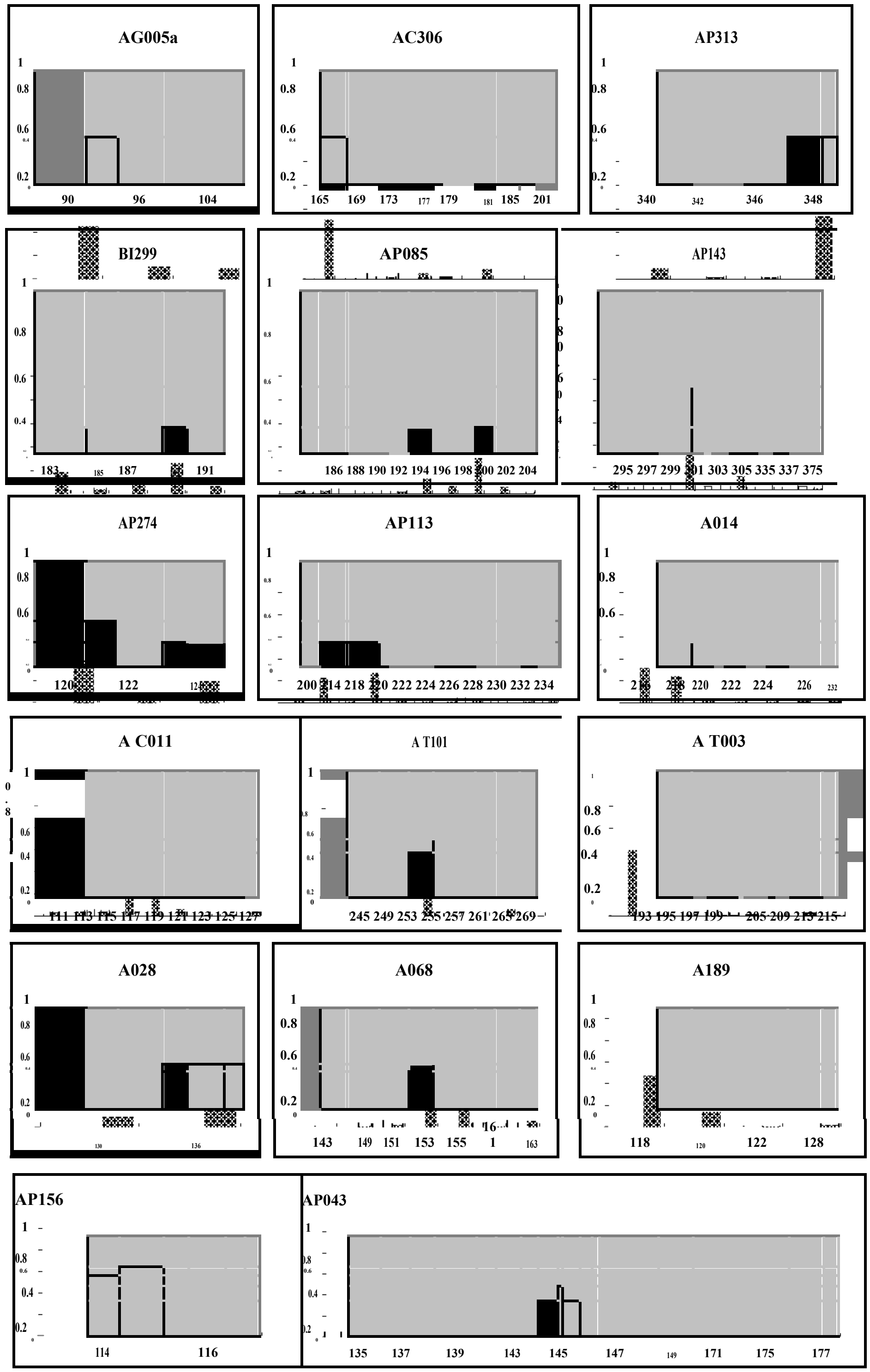

Figure 1. Graphs of microsatellite allele frequencies of 18 loci used to assess genetic diversity of 3 high royal jelly producing honeybees breeds. ■, XS; □,PH; and ▩, ZN.

F_{ST} values.

RESULTS

Genetic variability within populations

In total, 18 microsatellite loci were used for polymorphism analyses among the three breeds (Table 1). A total of 135 alleles were detected in the three breeds with varying allele frequencies by the 18 microsatellite markers (Figure 1). Among the three breeds, the number of alleles was consistent (Xiaoshan bee, 103; Pinghu bee, 107 and Zhenongda NO.1, 110). The alleles for each bread overlapped but were not identical. Unique alleles were evident within each breed (XS = 8, pH = 9, and ZN = 12).

Table 2. Total number of alleles, expected heterozygosity (He), Observed heterozygosity (Ho) and PIC.

Locus	No. of alleles	(Ho)	(He)	PIC
AG005a	3	0.529	0.583	0.467
AC306	8	0.420	0.382	0.397
AP274	3	0.577	0.458	0.499
AP043	10	0.660	0.688	0.630
AP313	4	0.393	0.451	0.330
AP053	25	0.943	0.847	0.937
AP143	9	0.341	0.361	0.325
A113	11	0.688	0.653	0.641
A014	7	0.553	0.444	0.472
AC011	9	0.782	0.813	0.748
AP189	4	0.487	0.451	0.419
AP156	2	0.214	0.174	0.191
BI299	5	0.693	0.701	0.645
AP085	10	0.756	0.764	0.719
AT101	8	0.551	0.583	0.483
AT003	8	0.196	0.188	0.192
A028	2	0.193	0.174	0.174
AP068	7	0.613	0.667	0.571
Mean	7.500 (5.2497)	0.533 (0.2100)	0.521 (0.2133)	0.491 (0.2083)

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

Table 3. Mean number of alleles per locus and the average heterozygosity (*He* and *Ho*) for three breeds of *Apis mellifera ligustica.*

Breed	Number of alleles (mean ±SD)	Average He (Mean ± SD.)	Average Ho (mean ±SD)
XS	5.722±4.586	0.519±0.057	0.536±0.017
PH	5.944±4.518	0.522±0.048	0.507±0.017
ZN	6.111±4.391	0.518±0.053	0.521±0.017
Total	7.500±5.250	0.533±0.050	0.521±0.010

Allelic richness differed between breeds but was highly varied among loci (Tables 2 and 3).

Expected heterozygosity (He) and mean polymorphic information content (PIC) for each locus across three breeds were listed in Table 2. The average number of the alleles observed in 18 microsatellite loci was 7.50. Across the three breeds, locus A028 had the lowest He, 0.193 and the lowest PIC, 0.174, however, the locus AP053 had the highest He and PIC value, 0.943 and 0.937, respectively.

Genetic differentiation among populations

The average number of alleles per locus expected and observed heterozygosity and F_{IS} for each breed across 18 loci were shown in Table 3. Zhenongda NO. 1 had the highest value of average number of alleles per locus, 6.111 and Xiaoshan bee had the lowest one with 5.722, the value of Pinhu bee was 5.944. The estimates of expected heterozygosity of the three breeds were much higher and similar to each other (XS, 0.519; PH, 0.522; ZN, 0.518). The lowest value of observed heterozygosity (0.507) was obtained for Pinhu breed, while the highest one (0.536) was found in Xiaoshan breed.

The fixation indices (F_{IT}, F_{ST}, F_{IS}) for each locus across all populations are shown in Table 4. The fixation coefficients of subpopulations within the total population, measured as F_{ST} value, for the 18 loci varied from -0.005 (AC306) to 0.284 (AT101), with a mean of 0.037 ($P<0.001$). 13 of 18 loci contributed significantly to this differentiation. The global deficit of heterozygotes across populations (F_{IT}) amounted to 0.034 ($P<0.01$). The negative F_{IS} values of some loci indicated an excess of heterozygous genotypes with respect to the expected value. Mean F_{IS} was found to be -0.004 within populations. Four loci showed significant of excess heterozygotes, while no marker showed significant of deficit heterozygotes.

Population genetic structure measures showed no evidence (P 0.05) for inbreeding but did show significant levels of diversity among breeds (Figure 2). The three breeds were differentiated significantly from each other, Xiaoshan bee differed from Pinghu bee and

Table 4. The results from F-statistics analysis.

Locus	F_{IT} = F	F_{ST} = θ	F_{IS} = f
AG005a	-0.101	0.005	-0.106
AC306	0.089	-0.005	0.094
AP274	0.213***	0.024***	0.194**
AP043	-0.023	0.053***	-0.08
AP313	-0.139	0.025*	-0.168
AP053	0.106***	0.013***	0.094***
AP143	-0.036	0.067***	-0.111
A113	0.054	0.008**	0.047
A014	0.213***	0.059***	0.164*
AC011	-0.039	-0.001**	-0.039
AP189	0.091	0.057***	0.036
AP156	0.196**	0.02	0.179*
BI299	-0.01	0.005	-0.015
AP085	-0.005	0.018***	-0.023
AT101	0.039	0.284***	-0.341
AT003	0.048	0.01*	0.038
A028	0.105	0.017	0.089
AP068	-0.087	0.004**	-0.092
Mean	0.034**(0.024)	0.037***(0.017)	-0.004(0.029)

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

Table 5. Reynolds' genetic distances, DR (upper triangle) and the gene flow, Nm (lower triangle) between three breeds.

Breed	XS	PH	ZN
XS		0.037	0.055
PH	6.714		0.022
ZN	4.405	11.011	

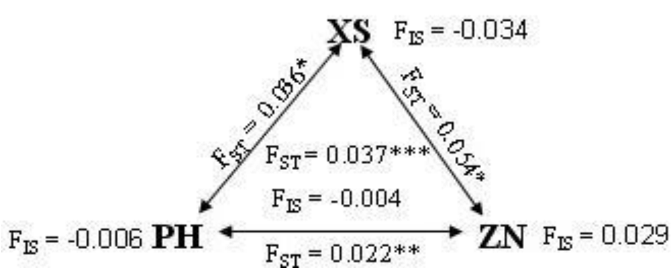

Figure 2. Graphical representation of three breeds. Genetic diversity estimates are listed along each axis. Values inside of figure represent overall diversity estimates. *P < 0.05. **P < 0.01.

Zhenongda NO.1 significantly (F_{ST} = 0.036, P = 0.013; F_{ST} = 0.054, P = 0.034, respectively), and Pinghu bee differed from Zhenongda NO.1 more significantly (F_{ST} = 0.002, P = 0.009).

Estimated of gene flow (Nm) and Reynolds' genetic distances (D_R) between each population pair are presented in Table 5. The lowest values of Reynolds' distance was 0.022 (Pinghu bee-Zhejiangnongda NO.1 pair), and the highest one was 0.055 (Xiaoshan bee-Zhejiangnongda NO.1 pair) . The Nm value ranged from 4.405 (between Xiaoshan bee- Zhejiangnongda NO.1 pair) to 11.011 (between Pinghu bee-Zhejiangnongda NO.1 pair).

DISCUSSION

Genetic variability within breeds

18 microsatellite markers used in the present study are randomly distributed across 14 chromosomes in the *Apis mellifera* genome, so the data had certain comparability 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., 1998) . 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, 11 loci among 18

microsatellite loci exhibited high polymorphic, while 4 loci showed medium polymorphic, mean PIC value across all loci exceeded 0.5, which could provide enough information for the assessment of genetic diversity.

Number of alleles is also good for measuring 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 7.50 across 18 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 18 microsatellite loci in the three breeds was rich; and the distribution of the allelic frequency was rather even.

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. Mean expected heterozygosity can approximately reflect the variation of genetic structure. All 18 microsatellite loci in this study had high polymorphism with a mean expected heterozygosity, 0.533, showing a high degree of genetic diversity. Overall levels of gene diversity, and allelic richness were high although little lower compared with levels found in a microsatellite survey of commercial Italian bee populations in the United States and Italy (Bourgeois et al., 2008), Russian honey bee stock selected for improved resistance to *Varroa destructor* (Bourgeois and Rinderer, 2009) and commercial populations surveyed in Western Australia (Chapman et al., 2008).

Genetic differentiation among breeds

In our study, on average, the genetic differentiation (F_{ST}) among breeds was 3.7% (Table 4), a relative high value and extremely significant (P <0.001), which indicated that there is a great differentiation among the three breeds. It is clear that about 3.7% of the total genetic variation corresponds to differences of breeds and the remaining 96.3% is the result of differences among individuals. Most of the loci contribute to this differentiation significantly. And the three breeds were differentiated significantly from each other (Figure 2). The values of Nm and Reynolds' genetic distances between pairs of breeds also supported the differentiation of the three breeds.

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 negative average of F_{IS} was -0.004, but not significant. In addition, four loci (AP274, AP053, A014 and AP156) showed significant excess of heterozygotes.

Many honeybee breeding programs were constructed to obtain high royal jelly producing breeds in China, and three most famous breeds Xiaoshan bee, Pinghu bee, Zhenongda NO.1 were cultivated in Zhejiang province. The estimation of characteristics of the three breeds indicated that the three breeds had been successful in maintaining heterozygosity and high levels of diversity while keeping inbreeding levels at a minimum. Genetic diversity measures should be monitored to ensure that heterozygosity and allelic richness are maintained at their current levels.

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