Genotypic variability of the queen retinue workers in honeybee colonies (*Apis mellifera*)

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The polyandrous mating behaviour of the honeybee queen increases the genetic variability amongst her worker offspring and the genetic variability within the honeybee colony can affect their polyethism. In this study, we intend to understand whether there is genetic variability in the task of the queen retinue. Microsatellite DNA analyses revealed a total of 13 and 12 subfamilies in two colonies, respectively. It shows that the subfamily proportion of the queen retinue workers significantly deviated from random distribution, which suggests that they might have a genetic preference in the task of the queen retinue.

Key words: honeybee, queen retinue workers, microsatellite, subfamily

INTRODUCTION

Honeybees are widely acknowledged as a model species for studies of social behaviour, social organisation and even neurology (Robinson *et al*. 2005; Qin *et al*. 2014; He *et al*. 2016). Previous studies showed that the task choices of honeybee workers are closely associated with their ages. In other words, the tasks of honeybees vary with their age. The honeybee workers tend to clean the cells or keep the brood warm when they are 1–3 days old. When they reach the age of 3–6 days, they start packing the pollen and honey. Later, the workers start to feed larvae or the queen with royal jelly secreted from their hypopharyngeal glands which are fully developed when they are 6–12 days old. Then, the workers do the task of wax-making, hive-cleaning or pollen-handling at the age of 12–18 days. After 18 days, workers tend to collect water, pollen, nectar, propolis for the colony or guard their colony (Lindauer 1952, 1954; Zeng 2007).

In addition, for the reason that queens mate, there are many subfamilies (patrilines) within a colony that are genetically different, and these genetic differences also have translated into different behaviours among them (Page & Robinson 1991). These authors also found that workers of one subfamily were more likely to become pollen gatherers, whereas others were more likely to gather nectar. Genetic variability has been described for several behavioural traits: grooming behaviour (Frumhoff & Banker 1988), guarding and undertaking (Robinson & Page 1988), nectar and pollen foraging (Page & Robinson 1991) and nest site scouting (Robinson & Page 1989), plant choice for pollen collection (Oldroyd *et al*. 1992), foraging distance (Oldroyd *et al*. 1993), queen rearing (Robinson *et al.* 1994), oophagy, oviposition and larval caring in queenless colonies (Robinson *et al.* 1990; Page & Robinson 1994), egg-laying in queen right colonies (Visscher 1996), water collecting and scenting (Kryger *et al*. 2000), fanning (Su *et al*. 2007), emergency queen-cell building (Xie *et al*. 2008), mite (*Varroa destructor*) parasitism rate (Liu *et al*. 2009) and survival differences (Wang *et al*. 2012) to name but a few. However, in all these studies only two or three patrilines could be distinguished, due to the low variability of allozymes in honeybees. Furthermore, the use of lines selected for specific allozyme markers may have an effect on the division of labour (Harrison *et al*. 1996).

The semiochemicals released by a honeybee queen have many effects for the colony (Winston & Slessor 1998). The most obvious effect is to attract workers around her, which are known as queen retinue workers (there is a circle consisting of queen retinue workers in Fig. S1). The age-bracket of honeybee queen retinue workers is 2–23 days, but mainly in 6–18 days (Yi *et al*. 2016). Queen retinue workers feed the queen or groom the queen with their antennae or proboscis, and then transmit the queen pheromone messages to their other nestmates throughout the colony (Keeling *et al*. 2003). It showed that there are three transmitting modes (queen bee, worker bee and air) of queen

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bee pheromones existing in a honeybee colony. And only when three kinds of mode exist in a colony, can the queen bee play a role of governing in the colony and make it disciplined (Qi *et al*. 2008). Thus, it is important to learn about the task of the queen retinue.

Previous studies indicate that the queen's mandibular gland pheromone (QMP) is the most important component of queen-produced compounds. And QMP is the main substance that inhibits the development of worker honeybee ovaries and attracts honeybee queen retinue workers (Hoover *et al*. 2003; Xuan & Chen 2005). Thus, all workers may have the will to be a member of queen retinue workers in theory. That is, workers from all partrilines may be willing to become queen retinue workers. In this study, we intended to understand whether there is a genetic variability in the task of queen retinue. Ninety-four queen retinue workers and 94 workers were collected randomly ('control workers') from two colonies respectively. After the procedure of DNA extraction, PCR amplification reaction and microsatellite DNA analyses, the Fisher exact test was used for assessing the genetic variability across these groups.

MATERIAL AND METHODS

Two honeybee (*Apis mellifera*) colonies (colony A and colony B) were used for this study and maintained at the Honeybee Research Institute of Jiangxi Agricultural University in Nanchang, China (28.46°N 115.49°E).

We sampled 94 queen retinue workers from each colony. Before sampling, we needed to find the circle of queen retinue workers first. The circle consisting of queen retinue workers was usually formed when the queen was laying eggs, standing still or being fed (Yi *et al*. 2016). We then used a pair of tweezers to catch the retinue workers when the queen stopped walking with the surrounded retinue workers. The queen retinue workers can be defined as: 1) following the queen to walk and become queen retinue workers soon when the queen stops walking, 2) tapping the queen's body fast with their antennae or 3) licking the queen with the proboscis. The samples were snap frozen in liquid nitrogen and preserved in a deep freezer at –80 °C for DNA extraction. When sampling, it was too difficult to catch all the queen retinue workers. On the one hand, the sampling action may break the balance between the queen and the queen retinue workers. Conversely, when a queen retinue worker was caught, it may release an alarm pheromone to remind or warn the queen and other workers about escaping (Pankiw 2004). Hence, we could only catch 1~3 queen retinue workers from each circle of queen retinue workers at a time. And, once we caught the sample of queen retinue workers, the next sampling time is random. For each colony, we caught 20 samples of queen workers per day for 5 days.

Additionally, we collected 94 workers ('control workers') randomly from each colony to represent the background subfamily proportion. The total number of collected 'control workers' were equal with the number of the queen retinue workers we collected each day. Thus, we first took the samples of queen retinue workers each day. After that, we used tweezers to catch the 'control workers' randomly from the same hive of the queen retinue workers.

After the sampling, a genomic DNA extraction kit (TaKaRa MiniBEST Universal Genomic DNA Extraction Kit) was used to extract DNA of each sample. Then, four microsatellite loci (Peter *et al*. 1999) were selected to determine the subfamily using Matesoft (Moilanen *et al*. 2004) (Table 1). The four microsatellite loci (Ap289, Ap043, A113 and Ap226) were based on the reports of Tian *et al*. (2013) and Zhang *et al*. (2015). The allele of each microsatellite marker was analysed with QIAxcel Advanced system (Shanghai Konecranes Co. Ltd.). The statistical evaluation of the distribution of each patriline between the queen retinue workers and 'control workers' was carried out with a Fisher exact test (Raymond & Rousset 1995). Probability values were calculated by comparing the distribution of each patriline against all other patrilines in each of the two honeybee colonies respectively with Fisher exact tests (Kryger *et al*. 2000).

RESULTS

Figure 1 shows that there are 13 patrilines in colony A and 12 patrilines in colony B (the genotypes of paternity of each drone tissue are shown in Table S1). In addition, there are four patrilines (nos 6, 8, 11 and 12) in colony A and six patrilines (nos 4, 8, 9, 10, 11and 12) in colony B which could not find workers from the group of queen retinue workers (numbers stand for the number of patrilines in each colony). By comparing the subfamily distribution between queen retinue

Table 1. Core sequences in cloned alleles and part of PCR condition for four used microsatellites.

Locus	Sequence of primers	Size (bp)	Annealing temperature (°C)	No. cycles
AP289	F 5′-AGCTAGGTCTTTCTAAGAGTGTTG-3′ R 5′-TTCGACCGCAATAACATTC-3′	174	55	30
AP043	F 5′-GGCGTGCACAGCTTATTCC-3′ R 5′-CGAAGGTGGTTTCAGGCC-3′	137	58	30
A113	F 5′-CTCGAATCGTGGCGTCC-3′ R 5′-CCTGTATTTTGCAACCT CGC-3′	220	60	30
AP226	F 5′-AACGGTGTTCGCGAAACG-3′ R 5′-AGCCAACTCGTGCGGTCA-3′	231	58	30

workers and 'control workers', a significant difference was found in both colonies (colony A, $P < 0.05$; colony B, $P < 0.01$).

Furthermore, Fisher exact tests were carried out for the distribution of each patriline against all other patrilines on the two categorised groups in both colony A and B (Table 2, last column). In colony A, two patrilines (nos 2 and 3) were significantly different at $P < 0.05$. In colony B, two patrilines (nos 4 and 6) were significantly different at $P < 0.01$ and one patriline (no. 2) at $P < 0.001$. For 11 patrilines in colony A and nine patrilines in

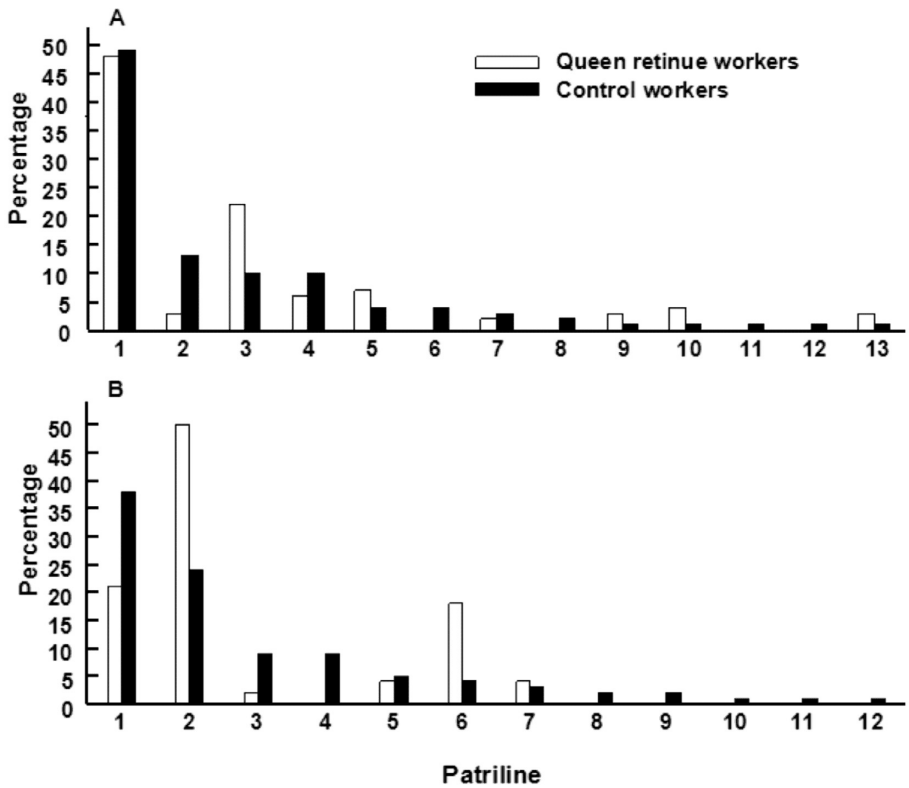

Fig. 1. Percentages of each of the 13 patrilines in colony A and 12 patrilines in colony B in the group of queen retinue workers and the group of 'control workers'. The number of workers of each subfamily was listed from the highest to the lowest in the group of 'control workers', and the group of queen retinue workers shared the same serial number of the subfamily in both colonies. It also showed that four patrilines (nos 6, 8, 11 and 12) in colony A and six patrilines (nos 4, 8, 9, 10, 11 and 12) in colony B were not found in the group of queen retinue workers.

Table 2. The number of workers between two groups in each patriline. The last column gives the *P*-values for Fisher exact tests for the distribution of each patriline against all other patrilines in the two groups.

Subfamilies	Colony A			Colony B		
	Number of queen retinue workers	Number of control workers	*P*	Number of queen retinue workers	Number of control workers	*P*
1	45	46	1.000	20	36	0.160
2	3	12	0.028	47	23	0.000
3	21	9	0.027	2	8	0.100
4	6	9	0.592	0	8	0.007
5	7	4	0.536	4	5	0.744
6	0	4	0.121	17	4	0.004
7	2	3	1.000	4	3	1.000
8	0	2	0.497	0	2	0.497
9	3	1	0.621	0	2	0.497
10	5	1	0.368	0	1	1.000
11	0	1	1.000	0	1	1.000
12	0	1	1.000	0	1	1.000
13	7	1	0.621			

colony B no significant differences were detected. However, that the number of some patrilines (colony A: nos 8, 11 and 12; colony B: nos 8, 9, 10, 11 and 12) is small may reduce the power of the Fisher exact test. In summary, we find evidence in favour of the hypothesis that there is a genotypical component for the performance of the different tasks examined in a colony headed by a naturally mated queen.

DISCUSSION

Queen retinue workers play an important role in feeding their queen and transferring queen pheromones for helping the queen to govern the whole honeybee colony under a good discipline (Qi *et al*. 2008). Our previous study showed that queen retinue workers have an age-bracket of day 6–18 (Yi *et al*. 2016). This study showed that the subfamily distribution of queen retinue workers was significantly different from the control workers (Fig. 1). Furthermore, two (nos 3 and 4) and three patriline (nos 2, 4 and 6) were strongly overrepresented or underrepresented against control workers in colony A and B respectively (Table 2). These findings suggest that there might be a genotypical component for the performance of queen retinue, not only being affected by worker ages. Accumulating evidence indicates that honeybee patrilines are strongly related to their division of labour and some special behavioural traits, such as guarding and undertaking (Robinson & Page 1988), nectar and pollen foraging (Page & Robinson 1991) and even queen rearing (Robinson *et al*. 1994). Consequently, these studies potentially support our hypothesis that queen retinue task may also require a special worker caste in terms of genetic variability.

There are two transmitting modes for workers to obtain the information that there is a queen around: by airborne transmission or touching with the queens' body (Huang 1994). Some queen retinue pheromones such as (2E)-9-oxodecenoic acid (9-ODA) and (2E)-9-hydroxydecenoic acid (9-HDA) are volatile chemicals (Zhang *et al*. 2008). Workers around the queen might be able to get this message through airborne transmission or by body touch and then follow the queen. In our observation, only a few workers followed their queens whereas most workers did not follow their queens even when touching their queen's body. Perhaps there is a selection of workers who are able to be a queen retinue worker after being stimulated by queen pheromones, and some subfamilies might be more willing to do this task of becoming a queen retinue worker.

In addition, Robinson *et al*. (1994) showed that there were significant genotypic biases in the relative likelihood of rearing queens. Queen rearing workers are a part of the queen retinue workers (Butler & Fairey 1964; Simpson 1966). Yi *et al*. (2016) showed that worker ages also affect the queen retinue behaviour. These findings, and our results, are consistent with the interpretation that the ontogeny of queen retinue behaviour might be

affected by the stimulation of queen bee pheromones, worker age and genotype, or by a combination of these three factors. Further investigations should focus on the mechanism of how workers respond to the queen retinue demands and how their patriline affects their behaviour.

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Compliance with ethical standards

The authors declare that they have no conflict of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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Fig. S1. A circle consisting of queen and queen retinue workers.

Table S1. Genotypes of paternity of each subfamily in colonies A and B.

Subfamilies	Colony A Genotypes of paternity (AP289/AP043/A113/AP226)	Colony B Genotypes of paternity (AP289/AP043/A113/AP226)
1	189/151/232/253	190/150/219/240
2	189/151/232/266	206/150/219/240
3	189/185/232/253	206/150/219/252
4	189/141/232/253	190/150/219/252
5	170/151/242/253	190/142/219/240
6	189/141/232/266	206/142/219/240
7	210/151/232/253	206/150/259/240
8	170/151/232/253	206/150/259/252
9	170/141/242/253	190/142/219/252
10	189/151/242/253	190/150/259/240
11	170/141/232/253	190/150/259/252
12	189/185/232/266	206/142/219/252