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Environmental factors have a strong impact on the composition and diversity of the gut bacterial community of Chinese black honeybees

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ABSTRACT

The Chinese black honeybees is an ecotype of the European honeybee that is formed by the natural hybridization of *Apis mellifera mellifera* and *A. m. carnica*. It is distributed in nature reserves in North China and has been an important breeding resource for disease resistance and other desirable traits. Compared to the areas outside of reserves, the nature reserves offer significant biodiversity benefits not only to the Chinese black honey bees but also to the other valuable plants and animals. In recent years there has been growing evidence that environmental factors including food choices play an important role in shaping the composition and activity of gut microbiota, which in turn can impact host health. In the previous studies on Chinese black honeybees, little attention has been paid to the diverse population of microbes in the gut that play a vital role in host health. In order to achieve a better understanding on the role of environmental factors in diversity and composition of gut microbiota of honey bees, in the present study, we analyzed the gut bacterial communities of Chinese black honeybees using terminal restriction fragment length polymorphism (T-RFLP). The results showed that the samples from the national nature reserves that are protected and managed so as to preserve and enrich their natural condition and resources for Chinese black honeybees had higher variety and richness of gut bacteria than that collected from unreserved regions that also harbor populations of Chinese black honeybees. The four terminal restriction fragments (T-RFs), 201, 223, 247 and 320 bp, were identified to be the dominant bacteria of Chinese black honeybees. Of which 247 and 320 bp had greater differences between bee groups sampled in different regions and therefore could be used as genetic markers to separate samples collected from the national nature reserves to samples collected from unreserved regions. The results clearly indicate that national nature reserve protects biological diversity and ecological and evolutionary processes which have had a significant influence on the diversity of gut bacteria of Chinese black honeybees. The ubiquity of gut symbiotic bacteria identified in Chinese black honeybee suggests that environmental factors could play an important role in diversity and composition of gut bacteria and warrant further investigation into the functional significance of these gut bacteria for the honeybee health.

Introduction

Chinese black honeybee is one ecotype of the European honeybee and is formed by the natural hybridization of *Apis mellifera mellifera* and *A. m. carnica* (Peng et al., 2009; Peng et al., 2015). Chinese black honeybee was introduced into China from Russia and Europe in the 1920s (Peng et al., 2015). After long-term reproduction and evolution, it has completely naturalized and adapted to the cold climate and ecological condition in North China. So far, Chinese black honeybee is mainly distributed in three provinces located in Northwest and North-eastern regions of China (Peng et al., 2009). Compared with other *A.*

mellifera subspecies, Chinese black honeybee has evolved an advantageous set of biological characteristics, including high resistance to pathogens and parasites especially resistance to ectoparasite *Varroa* mite, the single most detrimental pest of *A. mellifera* (Peng et al., 2015). Chinese black honeybee also exhibits an exceptionally high tolerance to adverse environmental factors such as cold temperature and high-elevation (Peng et al., 2012a; Peng et al., 2015; Chen et al., 2016). As a result, Chinese black honeybee has now served as an important brood resource for disease resistance, honey production and other desirable traits with tremendous economic and scientific values (Peng et al., 2009). Nevertheless, like other pollinators, the populations of Chinese

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black honeybee have also rapidly decreased because of pesticides utilization, plant diversity decline, habitats destruction, competition with non-native species, and infectious diseases by pathogens and parasites (Peng et al., 2009; Peng et al., 2012a; Peng et al., 2012b; Peng et al., 2015; Chen et al., 2016), urging the conservation efforts to reverse the population decline. Since 1997, the Chinese government has declared and maintained the national nature reserves to protect local ecological environments and to preserve and enrich the Chinese black honeybee subspecies and its important features.

The gut bacteria play vital roles in the physiology, development, immunity, digestion, and overall fitness of their eukaryotic hosts. In recent years, there has been growing evidence that environmental factors including food choices play an important role in shaping the composition and activity of gut microbiota, which in turn can impact host health. The elucidation of composition and functional diversity of complex gut bacterial communities can have important implication for evolution and conservation of animal species. Like other animals, the gut microbiota has also become a relevant aspect of honeybee health. It has been reported that gut bacteria of honeybee plays an important role in the host's metabolism, immunity, nutrition absorption, and development, thereby confers health benefits on the host (Engel et al., 2014). Previous studies have shown that the microbial community of honeybees is simple, with only nine distinct bacterial species or phylotypes (Sabaté et al., 2009; Yoshiyama and Kimura, 2009; Pettis et al., 2012; Kwong and Moran, 2016). Although simple and small, each species of honeybee gut bacteria exhibits a high level of diversity and different composition which might be closely related to nutritional and health status of their host (Engel et al., 2012). However, the composition and diversity of the gut bacteria could be influenced negatively by multiple factors, including the accelerated urbanization large-scale agriculture, pesticide applications, reduction of nectar plants, loss of habitats, interspecific competition, infection or invasion by pathogens or parasites (Babendreier et al., 2007; Mattila et al., 2012; Hroncova et al., 2015; Kwong and Moran, 2016). The analyzing the gut bacteria of *A. mellifera* at different time points showed that the composition of gut bacteria could change along with seasonal variation to some degree (Hroncova et al., 2015). Further, disease infections including American foulbrood, microsporidiosis, and chalkbrood disease were found to affect the diversity of gut bacteria (Yoshiyama and Kimura, 2009; Omar et al., 2014; Maes et al., 2016). Moreover, human activities such as feeding colonies with sugar syrup and pollen in a drought period, or moving the colonies to harvest monofloral honey were also found to affect the dynamics of gut bacterial populations in honeybee midguts (Disayathanoowat et al., 2012).

Therefore, the enriched nutrition is fundamental to the maintenance of a diverse and thriving population of beneficial gut bacteria, which, in turn, improves immune health and metabolism of honeybees (Martinson et al., 2012). Previous studies showed that health status of Chinese black honeybees in the national nature reserves was stronger than in the unreserved regions (Peng et al., 2012b). In the reserves without human disturbance, abundant and rich nectar plants could provide enough nutrient and suitable habitat for Chinese black

honeybee (Chen et al., 2016). We, therefore, hypothesized that the gut bacteria composition, diversity, and richness of the Chinese black honeybees from the national nature reserves are shaped by their unique ecological and evolutionary factors.

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a culture-independent method for profiling and comparing the composition and diversity of microbial communities (de la Fuente et al., 2014). The T-RELP method is based on the position variation of a restriction site closest to a labeled end of an amplified target gene generally the 16S rRNA and comprises the following major steps: 16S rRNA is amplified from the RNA samples using either one or both the primers with 5' end labeled with a fluorescent molecule. PCR products are purified and subjected to a four-cutter restriction enzyme digestion, and then separated by capillary- or gel-electrophoresis and detected by the fluorescence detector. The output of a T-RFLP profiling can be an electropherogram graph where X-axis marks the sizes and heights of the fragments while the Y-axis marks the fluorescence intensity of each fragment. The output of a T-RFLP profiling can be also the numerical format of the size and height of fragments which can be used for relative quantification and statistical analysis (Sjöberg et al., 2013). While no sequence information is retrieved from the T-RFLP analysis, this method has been proved highly sensitive and reproducible and has enabled the study of complex microbial communities of environmental samples in terms of their qualitative and quantitative composition (Sjöberg et al., 2013). By employing the T-RFLP technique, we compared the composition of the gut bacteria of Chinese black honeybees collected from the national nature reserve and unreserved regions and then analyzed the impacts of environmental factors on the diversity of their gut bacteria. These results could give us unique insight into the perspective on gut bacteria of Chinese black honeybee, as well as the influence of environmental changes or human activities on their gut microbiota.

Materials and methods

Sample collection

Chinese black honeybee workers were collected from a national nature reserve region and two unreserved regions during July to August 2015. Three apiaries were selected at each location and five colonies were selected in each apiary. Fifteen adult worker bees from each colony were collected at the nest entrance, a total of 675 worker bees were collected in this study. Geographical and climatic information of each sampling site (one reserved region and two unreserved regions), including longitude, latitude, altitude, rainfall and mean annual temperature were recorded during the sampling period (see Table 1). All samples were submerged in 75% ethanol immediately after collection and stored at -20°C for subsequent studies.

DNA extraction

Honeybee samples were rinsed in distilled water individually prior

Table 1
Geographical and climatic information of each sampling site.

Sample code	Sampling site	Lat. (°)	Long. (°)	Alt. (m)	Rainfall (mm)	Mean annual temperature (°C)	Sampling time
X1–X5	Unreserved region 1	83.4672	43.7068	1763	353	5.7	2015.07
X6–X10	Unreserved region 1	83.5253	43.7231	1718	353	5.7	2015.07
X11–X15	Unreserved region 1	83.5197	43.6879	1723	353	5.7	2015.07
J1–J5	Unreserved region 2	129.6564	43.7136	385	580	3.9	2015.08
J6–J10	Unreserved region 2	128.0980	43.6963	392	590	2.9	2015.08
J11–J15	Unreserved region 2	131.1247	44.0869	480	530	5.1	2015.08
H1–H5	National nature reserve	133.9229	47.0150	372	550	1.7	2015.08
H6–H10	National nature reserve	133.5459	47.1925	58	550	1.7	2015.08
H11–H15	National nature reserve	133.2979	47.0327	64	550	1.7	2015.08

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