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Male mate recognition via cuticular hydrocarbons facilitates sexual isolation between sympatric leaf beetle sister species

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ABSTRACT

Chemical signals in insects have been documented to play an important role in mate recognition, and divergence in chemical signals can often cause sexual isolation between closely related species or populations within species. We investigated the role of cuticular hydrocarbons (CHCs), short distance chemical signals, in male mate recognition between the two sympatric elm leaf beetles, *Pyrrhalta maculicollis* and *Pyrrhalta aenescens*. Mating experiments demonstrated that strong sexual isolation between the two species was driven by CHCs divergence. Males preferred to mate with conspecific females with intact conspecific CHCs or conspecific CHCs reapplied after removal. Males also preferred heterospecific females that were treated with conspecific CHCs. Chemical analysis showed that the CHC profiles differ significantly between species. In *P. maculicollis* dimethyl-branched alkanes between C29 and C35 account for the majority of the saturated alkanes while the CHC profile of *P. aenescens* mostly consisted of monomethyl-branched alkanes between C22 and C29. Additionally, some compounds, such as 12,18-diMeC32, 12,18-diMeC34, are unique to *P. maculicollis*.

1. Introduction

Sexual isolation is characterized by assortative mating between populations or closely related species. The reduced probability of inter-population mating often underlies the evolution of pre-zygotic reproductive isolation and speciation (Coyne and Orr, 2004; Lande, 1981; Langerhans and Makowicz, 2013; Maan and Seehausen, 2011). During courtship and mating behaviors insects communicate with visual, acoustic, olfactory, gustatory, and tactile sensory signals (Greenspan and Ferveur, 2000). Thereby it is important to find out which of these signals are undergoing sexual selection and whether they contribute to behavioral isolation (Boake, 2002).

Several studies have shown the importance of chemical signaling systems in the evolution of sexual isolation in insects (Howard et al., 2003; Peterson et al., 2007). In addition to volatile chemicals affecting mating behaviors over long distances such as pheromones in moths (Hansson, 1995; Roelofs and Comeau, 1969), contact compounds such as cuticular hydrocarbons (CHCs) can also serve as recognition cues at close range (Ferveur, 2005; Singer, 1998). CHCs have been characterized to serve as multiple recognition signals in insects; for example, nestmate, fertility and task-specific cues in social insects (Greene and Gordon, 2003; Izzo et al., 2010), chemical mimicry and chemosensory self-referencing cues in crickets (Howard and Blomquist, 2005; Weddle et al., 2013) or mate recognition in *Drosophila* (Greenspan and Ferveur, 2000) and beetles (Ginzel and Hanks, 2003; Page et al., 1997; Rodstein et al., 2009). While the composition and function of CHCs in different species under various physiological phases or environmental conditions have been identified, relatively little is known about the role that CHCs play in sexual isolation of closely related species beyond a few studies in Diptera and beetles (Ferveur, 2005; Peterson et al., 2007). Here we aimed to elucidate the role of CHCs in the sexual isolation between two sister elm leaf beetles.

The elm leaf beetles *Pyrrhalta maculicollis* (PM) and *Pyrrhalta aenescens* (PA) (Chrysomelidae: Galerucinae) are serious pests of elm trees and are widely distributed in eastern Asia. They occur sympatrically and synchronously over a large geographic area, and both adults and larvae feed on the same elm species in micro-sympatry. The adults of these species are morphologically very similar, only differing in elytron color which is brown in PM and green in PA. A previous study has shown that they are not color polymorphism but distinct sibling species through life history, genital morphology and molecular sequence data (Nie et al., 2012). Synchronization of emergence phenology and significant

Abbreviations: CHCs, cuticular hydrocarbons; PM, *Pyrrhalta maculicollis*; PA, *P. aenescens*.

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overlap in ecological niche, begs the question of how species boundaries are maintained between these closely related species. Strong reproductive isolation between the two species has been confirmed by molecular markers (mtDNA and ITS2) (Nie et al., 2012); however, the factors contributing to this pattern remain unknown.

Here, we conducted a series of mating experiments to estimate the extent of sexual isolation between the two species and to examine visual and chemical signals that might have been involved in the divergence of these species. Then we analyzed cuticular hydrocarbons using GC–MS and GC–FID to compare the composition and their relative abundance not only between the two species, but also with all other studied leaf beetles.

2. Materials and methods

2.1. Insects

Both PM and PA were collected in the Northern part of the Olympic Forest Park (40.01°N, 116.39°E) in Beijing, China, in early June and mid July 2013. The specimens were collected as 3th instar larvae or as pupae and reared in plastic cups (9.0 cm diameter, 9.0 cm depth), placed in a climate box at 16 h:8 h LD and 25 °C (Nie et al., 2012). Newly emerged adults were sorted by species and gender, and kept separately in containers with fresh elm leaves. Ten days after hatching, the sexually mature beetles were either used in the mating experiments or stored in vials in a −30 °C freezer for CHCs extraction.

2.2. Mating experiment

To estimate the extent of sexual isolation between PM and PA and to determine the role of CHC profiles in mate recognition and the relative contribution of chemical and visual signals, we performed a series of two choice experiments. In each experiment, a male (PA or PM) was placed in the middle of a 90 × 15 mm Petri dish lined with filter paper and containing two females located at opposite sides of the dish. We used four treatments per species (Table 1). For each treatment, 37–127 replicates were performed. Each individual was used only once in the mating experiments.

In general, the test males would encounter a potential female and then mount and vibrate its antennae on the female. In positive responses, the males bent their abdomen and exerted their aedeagus for copulation. We regarded the exertion of the aedeagus as the key signal to record a positive mating response. Each assay lasted 3 h, if the male showed no exertion of aedeagus to either female within this timeframe we regarded it as no response. All experiments were conducted under natural lighting from 1:00 to 4:00 pm (i.e., 8 h after light on) at approximately 25 °C in the laboratory in Beijing from June to September in 2013.

To remove CHCs, the frozen (−30 °C) dead beetles were thawed for 15 min at room temperature and washed for 10 min using 0.2 ml *n*-hexane (HPLC grade, Fisher, UK) in 1.5 ml glass vials (Agilent, USA) (Geiselhardt et al., 2009a). Each individual was treated twice to ensure absolute removal of any CHCs. Afterward, the extracts were combined and concentrated to about 0.05 ml. This concentrate was painted on the elytra of a potential female using 10 μL capillaries. The solvent was allowed to evaporate before the mating bioassay.

Differences in the male responses to treatment females were analyzed with the chi-squared test (Geiselhardt et al., 2012) using IBM SPSS Statistics 19.0. Based on the mating frequency data we estimated the extent of sexual isolation (I_{PSI} index) between the two species using JMATING 1.0.8 software in Java runtime environment (Carvajal-Rodríguez and Rolán-Alvarez, 2006). I_{PSI} values between −1 and 0 indicate negative assortative mating, those between 0 and 1 represent positive assortative mating. Standard deviations and significance test for I_{PSI} were obtained by bootstrapping (10,000 bootstrap samples) (Geiselhardt et al., 2012). We calculated the response rate in each treatment as the formula: $R = n/N$, where R is the response rate, n is the response number and N is the number of replicates.

2.3. Analysis of CHC profiles

Chemical identification was performed using coupled gas chromatography–mass spectrometry (HP 6890 series GC – HP 5973 MSD; GC–MS) with MS Library NIST2005 (Agilent Technologies, Inc.). The gas chromatography (GC) was equipped with an HP5 column (30 m × 0.25 mm internal diameter × 0.25 μm flm thickness, Agilent Technologies, Inc.), used helium at 1.0 mL/min as carrier gas, and the manual injection was done at 280 °C. Afterward, the samples were treated with 40 °C for 1 min, then the temperature was raised to 300 °C with 8 °C/min and finally with 20 °C/min to 320 °C. Mass spectrometry was performed in electron impact mode with 70 eV. Three microliters of each extract was injected in the splitless mode. Three replicates were conducted for each sex of both species for a total of 12 samples. The *n*-alkane (C6–C40) standards were used for calculating the Retention Indices (RI) (Kováts, 1965). Individual compounds were identified by integrative analysis of their MS (Doolittle et al., 1995; Nelson et al., 1972; Pomonis et al., 1980), their retention indices (RI) and Carlson et al. (1998).

For detailed chemical quantification we used gas chromatography–flame ionization detector (GC–FID) which provides a better result in quantification than GC–MS (Dodds et al., 2005). We used the same settings and conditions as for GC–MS with the exception that the injection was automatic and we applied ca. 40 replicates for each sex of each species.

The peak areas relative to the total peak area was calculated for each compound. To avoid limitations inherent to the analysis of compositional data, each peak area was transformed according to this formula: $Z_{ip} = \ln[A_{ip}/g(A_p)]$, whereas A_{ip} is the peak area of i for sample p, $g(A_p)$ the geometric mean of all peaks for sample p and Z_{ip} is the transformed area of peak i for sample p (Aitchison,

Table 1
The description of four treatments per species in mating experiments.

Treatments	Aims	Descriptions of the two potential mates	Abbreviates
1	To test the extent of the sexual isolation between PA and PM	PA and PM living females with intact CHCs	LI
2	To test the role of female behavior in mate recognition (if the response of female play the crucial role in mating decision, the males may not give a correct choice in this treatment)	PA and PM frozen dead females with intact CHCs	DI
3	To determine the role of CHCs in mate recognition in PA or PM	Two frozen dead conspecific females, one with CHCs stripped and then reapplied, the other CHCs stripped but not reapplied	DCSR
4	To determine the relative contribution of chemical and visual signals to the mate recognition	PA and PM dead females with CHCs exchange	DE