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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

1986). To apply the formula also to the undetected peaks, we added 0.01 to each relative peak area (Aitchison, 1986). Finally, we used the transformed data to conduct a principal components analysis (PCA) with varimax rotation. To determine whether there were significant differences between species or the sexes as well as the potential interactions between them, we used a multivariate ANOVA on the first three principal components (Blows and Allan, 1998; Peterson et al., 2007; Simmons et al., 2003).

3. Results

3.1. Mating experiment and sexual isolation

In the choice experiments, adult males of PM and PA showed a significant mating preference for conspecific females in both treatment 1 (LI: $P < 0.001$ for both PA and PM males; Table 2a) and treatment 2 (DI) (DI: $P < 0.001$ for both PA and PM males; Table 2b) (chi-squared test). Additionally, the index of sexual isolation also showed significant sexual isolation between the two species (LI: $I_{psi} = 0.93 \pm 0.04$, $P < 0.001$; DI: $I_{psi} = 0.91 \pm 0.05$, $P < 0.001$).

To assess the role of CHCs in male mating preferences we performed treatments 3 (DCSR) and 4 (DE). In the DCSR treatment, the males significantly preferred conspecific females where CHCs were reapplied after removal over those without reapplication. ($P < 0.001$ for both PA and PM males; Fig. 1a; Table 2c). In the DE treatment, males preferred heterospecific females when the conspecific CHC was applied on them. PM males preferred PA females ($P < 0.001$) and PA males preferred PM females (Fig. 1b; Table 2d), thus showing disassortative mating with respect to species designation and elytra color ($I_{psi} = -0.89 \pm 0.07$, $P < 0.001$). However, when considering the CHC profiles and not the species, males showed a high level of positive assortative mating and sexual isolation ($I_{psi} = 0.90 \pm 0.07$, $P < 0.001$). In addition, mate response rate ($R = n/N$) in PA males was about three times lower than in PM males in all treatments (Table 2). We also observed that females often contracted their abdominal ends to reject the mounting males during the mate choice experiments.

Table 2
Response of males and isolation index between the two species in the mating experiments. N is the number of replicates, n is the response number and R is the response rate ($R = n/N$). I_{psi} is the isolation index between *Pyrrhalta maculicollis* and *P. aenescens*. Significant differences in response and significance of isolation index are indicated by the asterisks (***$P < 0.001$).

(A) Treatment 1							
	N	*n*	*R* (%)	PA♀	PM♀	*P*	I_{psi}
PA♂	125	25	20.0	24	1	***	0.93 ± 0.04***
PM♂	80	52	65.0	2	50	***	
(B) Treatment 2							
	N	*n*	*R* (%)	PA♀	PM♀	*P*	I_{psi}
PA♂	100	31	31.0	30	1	***	0.91 ± 0.05***
PM♂	37	33	89.2	3	30	***	
(C) Treatment 3							
	N	*n*	*R* (%)	+	–	*P*	I_{psi}
PA♂	95	24	25.3	23	1	***	\
PM♂	48	35	72.9	34	1	***	
(D) Treatment 4							
	N	*n*	*R* (%)	PA♀	PM♀	*P*	I_{psi}
PA♂	127	9	7.1	0	9		−0.89 ± 0.07***
PM♂	57	38	66.7	35	3	***	

Note: In treatment 3, " + " represents the conspecific female with CHCs stripped and then reapplied, "–" is the conspecific female with CHCs stripped.

3.2. CHC profiles

GC–MS showed that the CHC profile of PM contained 43 compounds and that both sexes had all of these compounds. In PA, we identified 30 compounds, all included in PM and also present in both sexes (Fig. 2; Table 3). The 40 compounds we were able to identify were all saturated alkanes with straight chains and one, two or three methyl branches. They ranged in length between C22 and C35 (Table 3).

The CHC profiles were dominated by dimethyl alkanes (PM♀: 56.84 ± 8.25%, PM♂: 50.81 ± 5.71%, PA♀: 30.25 ± 5.17%, PA♂: 33.91 ± 6.63%) and monomethyl alkanes (PM♀: 34.93 ± 6.23%, PM♂: 45.44 ± 7.78%, PA♀: 56.03 ± 5.43%, PA♂: 52.63 ± 9.01%), whereas *n*-alkanes (PM♀: 1.92 ± 0.33%, PM♂: 3.64 ± 0.76%, PA♀: 13.32 ± 3.04%, PA♂: 12.90 ± 3.23%) and trimethyl alkanes (PM♀: 4.55 ± 0.74%, PM♂: 1.46 ± 0.43%, PA♀: not detected, PA♂: not detected) represented only a small amount of the total hydrocarbons (Supplemental Table S1; Table 3). Overall, the CHC profiles were qualitatively similar within a species, while qualitatively different between species (Fig. 2). While their relative abundance varied between species and the sexes (Table 3).

The monomethyl alkanes between C22 and C29 and the dimethyl alkanes between C29 and C35 had extremely opposite ratios between species (Fig. 3), e.g. 11,17-diMeC29, 13,17-diMeC31 and 11,15-diMeC33 (total relative areas of females and males: 36.87%, 34.72%, respectively) were the most prominent peaks in PM, but were nearly undetectable in PA. Likewise 11/13-MeC27, 2-MeC28, 13-MeC29 (42.32%, 40.15%, respectively) were dominant in PA (Table 3), but much lower in PM. Between the sexes we could not find any compositional differences in the CHC profiles; however, they differed in relative abundance: for example, 11,17-diMeC29 (female: 5.77%; male: 17.82%) and 11,15-diMeC33 (female: 18.03%; male: 4.87%) had opposing ratios for PM males and females (Table 3).

We extracted three principal components with eigenvalues over one which together explained 92.03% of the total variation in CHC profiles (PC1: 77.42%, PC2: 9.70%, PC3: 4.91%). The multivariate ANOVA on the three PCs revealed significant effects for species ($F_{3,154} = 138.82$, $P < 0.001$), sex ($F_{3,154} = 73.83$, $P < 0.001$) and their interaction ($F_{3,154} = 61.56$, $P < 0.001$). The principal components

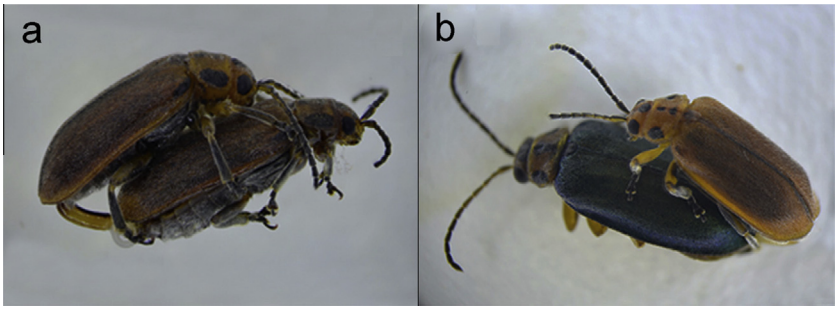

Fig. 1. Response of *Pyrrhalta maculicollis* males to potential mates. (a) A *P. maculicollis* male response to a dead *P. maculicollis* female after removal and reapplication of *P. maculicollis* CHCs; (b) a *P. maculicollis* male response to a dead *P. aenescens* female after removal and reapplication of *P. maculicollis* CHCs.

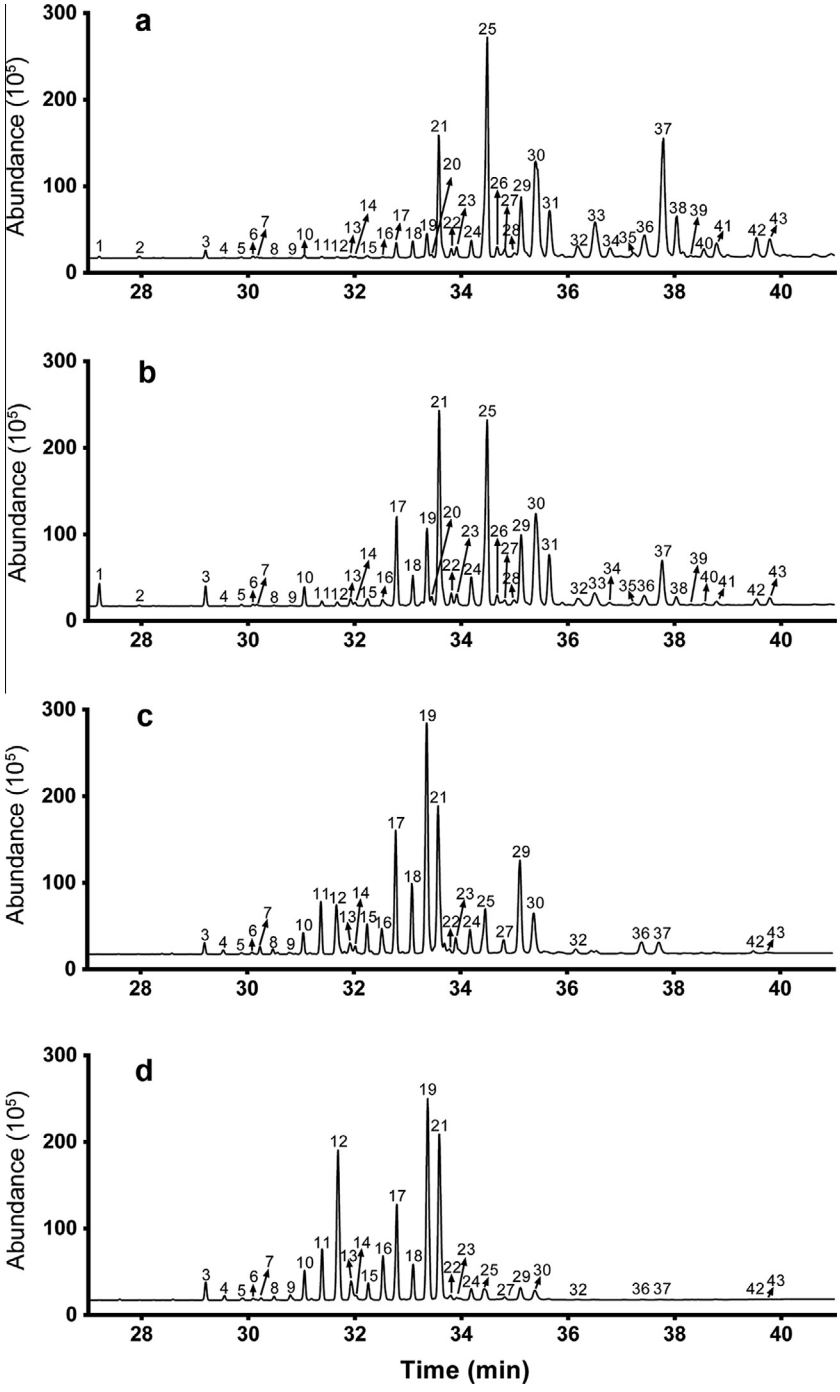

Fig. 2. Representative GC–MS profiles of cuticular hydrocarbons from *Pyrrhalta maculicollis* ((a) female; (b) male) and *P. aenescens* ((c) female; (d) male) extracted in hexane. The numbered peaks correspond with Table 3.

plot (Fig. 4a and b) also shows the apparent separation of the CHC profiles between both species and sexes.

4. Discussion

4.1. CHC profiles mediate male mate recognition

Communication between animals can be visual, acoustic, gustatory, or tactile (Greenspan and Ferveur, 2000). Among these, chemical signals have been considered as the most primitive form of communication (Howard and Blomquist, 2005; Hunt et al., 2012; Johansson and Jones, 2007) and play an essential role in mate recognition (Howard et al., 2003; Peterson et al., 2007). The importance of cuticular hydrocarbons (CHCs) as contact sex pheromones in beetles in general (Ginzel and Hanks, 2003; Page et al., 1997; Rodstein et al., 2009) and in leaf beetles (Chrysomelidae) in particular (Geiselhardt et al., 2009a,b; Jermy and Butt, 1991; Nelson et al., 2002; Nelson and Charlet, 2003; Peterson et al., 2007; Sugeno et al., 2006) has been shown previously. Although CHCs are pivotal in mate recognition, Nie et al. (2012) assumed that in the case of the two sympatric and closely related leaf beetles species studied here, that elytra coloration might serve a role in mate recognition. Males of both species significantly preferred conspecific females with intact CHCs over those where they were

Table 3
Cuticular hydrocarbon components and their relative peak areas of each sex of the two closely related elm leaf beetles, *Pyrrhalta maculicollis* and *P. aenescens*.

Peak no[a]	Retention index (RI)	Hydrocarbons	PM		PA	
			Female (N = 38)	Male (N = 42)	Female (N = 40)	Male (N = 40)
1	2258	2-MeC22	tr[b]	1.16 ± 0.57	nd[b]	nd
2	2330	13-MeC23	tr	0.12 ± 0.04	nd	nd
3	2457	2-MeC24	0.29 ± 0.16	2.28 ± 0.76	0.63 ± 0.32	0.83 ± 0.36
4	2500	n-C25	tr	tr	0.26 ± 0.06	0.30 ± 0.08
5	2528	13-MeC25	tr	0.26 ± 0.15	tr	0.11 ± 0.07
6	2558	2-MeC25	0.11 ± 0.04	0.15 ± 0.07	0.15 ± 0.04	0.17 ± 0.04
7	2567	3-MeC25	tr	tr	0.22 ± 0.11	0.19 ± 0.04
8	2600	n-C26	tr	tr	0.28 ± 0.09	0.28 ± 0.07
9	2627	13-MeC26	tr	tr	0.26 ± 0.06	0.35 ± 0.10
10	2658	2-MeC26	0.21 ± 0.12	1.79 ± 0.60	2.29 ± 0.83	3.03 ± 0.71
11	2700	n-C27	tr	0.41 ± 0.08	4.79 ± 0.95	4.95 ± 1.21
12	2730	11/13-MeC27	tr	0.37 ± 0.09	9.06 ± 1.65	10.36 ± 3.40
13	2755	11,15-diMeC27	tr	0.60 ± 0.11	1.94 ± 0.99	4.15 ± 1.80
14	2764	9,15-diMeC27	tr	0.25 ± 0.04	0.70 ± 0.18	tr
15	2800	n-C28	tr	0.66 ± 0.12	2.37 ± 0.64	2.14 ± 0.52
16	2827	11/13-MeC28	tr	0.42 ± 0.06	3.21 ± 0.26	3.50 ± 0.65
17	2857	2-MeC28	0.93 ± 0.48	5.86 ± 0.86	10.61 ± 1.85	13.01 ± 3.19
18	2900	n-C29	1.05 ± 0.32	1.68 ± 0.35	4.98 ± 1.05	4.79 ± 1.20
19	2930	13-MeC29	1.04 ± 0.59	4.38 ± 0.76	22.65 ± 4.48	16.78 ± 5.35
20	2935	11-MeC29	tr	0.52 ± 0.16	nd	nd
21	2955	11,17-diMeC29	5.77 ± 2.51	17.82 ± 2.17	21.74 ± 4.66	25.23 ± 4.71
22	2980	5,17-diMeC29	0.48 ± 0.22	1.05 ± 0.19	0.73 ± 0.40	1.31 ± 0.76
23	3000	n-C30	0.87 ± 0.21	0.89 ± 0.21	0.64 ± 0.25	0.44 ± 0.15
24	3021	12-MeC30	0.93 ± 0.30	2.13 ± 0.24	1.57 ± 0.25	1.24 ± 0.10
25	3056	2-MeC30	18.24 ± 2.42	17.67 ± 2.01	2.75 ± 0.59	2.74 ± 0.68
26	3079	5,17-diMeC30	0.89 ± 0.39	1.12 ± 0.20	nd	nd
27	3092	2,12-diMeC30	0.79 ± 0.19	0.44 ± 0.13	tr	tr
28	3114	Unresolved	0.38 ± 0.15	0.48 ± 0.12	nd	nd
29	3124	13-MeC31	4.11 ± 1.18	5.61 ± 0.79	3.87 ± 1.85	1.56 ± 0.46
30	3156	13,17-diMeC31	13.07 ± 3.17	12.03 ± 1.08	3.13 ± 0.80	1.98 ± 0.34
31	3178	5,13-diMeC31	4.96 ± 1.63	5.52 ± 0.72	nd	nd
32	3227	16-MeC32	1.70 ± 0.28	1.15 ± 0.17	tr	nd
33	3252	12,18-diMeC32	5.73 ± 0.88	2.39 ± 0.40	nd	nd
34	3277	?, ?-diMeC32	0.93 ± 0.33	0.49 ± 0.12	nd	nd
35	3312	Unresolved	0.79 ± 0.34	0.38 ± 0.09	nd	nd
36	3327	11/13/17-MeC33	3.89 ± 1.48	1.39 ± 0.35	0.33 ± 0.12	nd
37	3353	11,15-diMeC33	18.03 ± 3.65	4.87 ± 1.55	0.44 ± 0.29	tr
38	3377	7,17,21-triMeC33	3.46 ± 0.46	1.20 ± 0.37	nd	nd
39	3388	Unresolved	0.56 ± 0.23	0.14 ± 0.06	nd	nd
40	3423	11/13/15/17-MeC34	1.09 ± 0.28	0.26 ± 0.06	nd	nd
41	3454	12,18-diMeC34	2.61 ± 0.73	0.73 ± 0.51	nd	nd
42	3493	2,16-diMeC34	2.65 ± 1.37	1.37 ± 0.68	tr	nd
43	3527	11/13/15/17-MeC35	4.41 ± 2.31	2.31 ± 0.67	tr	nd

[a] Peaks number correspond to those in Fig. 2.
[b] Percent of total hydrocarbons area; mean ± sd; tr, trace; nd, not detected.

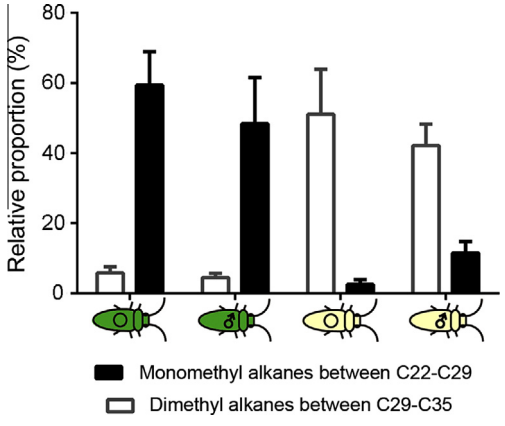

Fig. 3. Relative proportion of monomethyl alkanes between C22 and C29 and dimethyl alkanes between C29 and C35 of both sexes of *Pyrrhalta maculicollis* and *P. aenescens*. Green and yellow beetles represent *P. aenescens* and *P. maculicollis*, respectively. Beetles with "O" and "♂" are females and males, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

removed (Fig. 1a; Table 2c). They even attempted to mate with heterospecific females after the conspecific CHCs were applied to them (Fig. 1b; Table 2d), thus ignoring the coloration of the elytra. Therefore, our results clearly reject the hypothesis that the coloration is the reason for this isolation but rather different CHC profiles.

In general, monomethyl and dimethyl alkanes are two potential active compounds of contact pheromones in insects (Blomquist and Bagnères, 2010). For example, in the chrysomelid *Gastrophysa atrocyanea*, C27- and C29-monomethyl alkanes are the active components (Sugeno et al., 2006). In the two species studied here, the major component of the CHC profile were monomethyl and dimethyl alkanes, but their ratios changed; in PA, monomethyl alkanes were the dominant component followed by dimethyl alkanes whereas PM, was dominated by dimethyl alkanes (Supplemental Table S1; Table 3). Additionally, we also found differences in specific compounds such as 12,18-diMeC32, 12,18-diMeC34 which are present in PM but absent in PA (Table 3), providing yet another possible explanation for their divergence in recognition. In some other studies, the quantitative but not qualitative difference can account for the chemical divergence, such as,

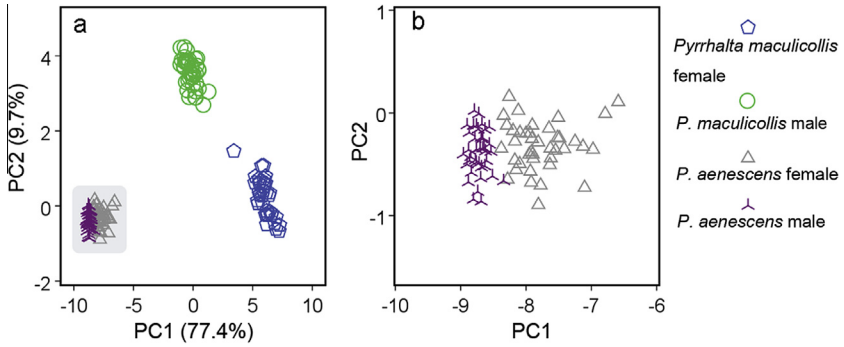

Fig. 4. Principal component plots of CHCs profiles between *Pyrrhalta maculicollis* and *P. aenescens*. Each symbol represents a single beetle individual, PC1 and PC2 accounted for 77.4% and 9.7% of the total variance, respectively. (b) Enlarged from the highlighted square in the lower left corner of (a).

Drosophila (Coyne et al., 1994; Howard et al., 2003) and *Chrysochus* leaf beetles (Peterson et al., 2007). In these examples, mate recognition is based on differences in the ratios of compounds rather than on CHC composition. Further study should be done to determine whether quantitative or qualitative difference play a critical role in sexual isolation in our system.

An interesting result of the choice experiments was that the mate response rate ($R = n/N$) in PA males was about three times lower than in PM males across all treatments (Table 2). This suggests that PA males have a lower discrimination threshold than those of PM. A similar phenomenon was also observed in two other closely related leaf beetles, *Chrysochus cobaltinus* and *Chrysochus auratus* (Peterson et al., 2007). In this case *C. cobaltinus* males are more discriminating than *C. auratus* males. In view of the same designation of potential mates for both PM and PA males, we infer that the divergence in males discrimination ability lies in their different procedures of perception and processing for chemical signals, however, these mechanisms remain poorly known in insects (Geiselhardt et al., 2012).

In addition to the male mate recognition based on CHC profiles, we also observed that females often contracted their abdominal tip to reject the mounting males during the mate choice experiments. This indicates the presence of either intra- or inter-specific female mate choice, another possible important behavior with regard to sexual isolation in this system. However, whether heterospecific females exhibit more of these behaviors would require further study. Anyhow, we can draw the following conclusion at least: the response of female do not play the crucial role in mating decision, or the males may not give a correct choice in treatment 2.

Between the sexes, CHC profiles of PM and PA showed no difference in their composition. Nevertheless, the abundance of the compounds differs between the sexes (Fig. 2). This is also observed in other leaf beetles such as the Japanese dock leaf beetle, *G. atrocyanea* (Sugeno et al., 2006), the blue milkweed beetle, *C. cobaltinus* (Peterson et al., 2007), and the mustard leaf beetle, *Phaedon cochleariae* (Geiselhardt et al., 2009b). It is likely that these differences are used for sex recognition by males as observed in *C. cobaltinus* (Peterson et al., 2007).

4.2. CHC profiles in Chrysomelidae

So far the CHC profiles have been examined in 29 chrysomelid species (Supplemental Table S1; Dubis et al., 1987; Geiselhardt et al., 2009a,b, 2011; Jacob et al., 1979; Jacob and hanssen, 1986; Nelson et al., 2002; Nelson and Charlet, 2003; Peterson et al., 2007; Sugeno et al., 2006). In general, the major compounds in leaf beetles are saturated alkanes, which account for over 80% of their CHC profiles in most (25 out of 29) of reported species. In the species examined here, saturated alkanes account for 100% of the chemical composition as *Gastrophysa atrocyanea* female (Sugeno et al., 2006) and a *Gonioctena (Phytodecta)* species (Jacob and hanssen, 1986).

Geiselhardt et al. (2009a) assumed that a high percentage of monomethyl alkanes, especially 2-methyl alkanes might be typical for leaf beetles. In fact, in 75.9% (22 out of 29) of chrysomelid species, monomethyl alkanes provide more than 30% and 2-methyl alkanes make up at least 15% of the saturated alkanes. In our studied species, monomethyl (PA♀: 56.0%, PA♂: 52.6%, PM♀: 34.9%, PM♂: 45.4%) and 2-methyl-alkanes (PA♀: 16.4%, PA♂: 19.8%, PM♀: 19.8%, PM♂: 28.9%) also take up a large proportion of their CHCs (Supplemental Table S1; Table 3). Thus, the hypothesis of Geiselhardt et al. (2009a) is mainly supported.

In addition to the saturated alkanes, some unsaturated hydrocarbons also include in some leaf beetles, such as olefines, which could serve as active components of sex pheromones in moths (Francke et al., 2000) and *Drosophila* (Etges and Jackson, 2001). However, the absence of olefines is not unusual in chrysomelids (Supplemental Table S1). Therefore, olefines may not the indispensable active components in leaf beetles.

In conclusion, our study shows that strong differences in the CHC profiles can mediate male mate recognition which underlies the sexual isolation between these two closely related species, PM and PA. In fact, the great divergence, which would have accelerated the process of diversification, is expected if the chemical signals are really selected forces during speciation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2014.08.006.

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