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Original Article

Contact pheromones of 2 sympatric beetles are modified by the host plant and affect mate choice

Huai-Jun Xue,[a] Jia-Ning Wei,[b] Sara Magalhães,[c] Bin Zhang,[a,d] Ke-Qing Song,[a,d] Jie Liu,[a] Wen-Zhu Li,[a] and Xing-Ke Yang[a]

[a]Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China, [b]State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China, [c]CE3C: Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciencias da Universidade de Lisboa, Edificio C2, 3° Piso Campo Grande, 1749016 Lisbon, Portugal, and [d]University of Chinese Academy of Sciences, 19 Yuquan Road, Shijingshan District, Beijing 100049, China

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Host-plant shifts have significantly contributed to the diversification of phytophagous insects. The contact sex pheromones of such insects may be modified by the plant they feed on, thereby contributing to the formation/maintenance of sister species on different plants. Here, we addressed this issue using 2 sister species of specialist phytophagous flea beetles *Altica fragaria* and *Altica viridicyanea*, and their oligophagous F_1 hybrids. Specifically, we tested 1) if males from these *Altica* species recognize conspecific females based on their cuticular hydrocarbon (CHC) profile, 2) if the host plant affects the CHC profile of hybrid females, and 3) whether hybrid males distinguish between hybrid females raised on different host plants. Mate choice tests revealed that males use CHCs to identify conspecific mates. We then identified different CHC profiles in females of the 2 species and showed that the profile of CHCs in hybrids is modified by the host plant in which the beetles develop. Finally, we found that hybrid males raised on one host plant choose females with a matching profile, but this is not the case for males raised on the other plant. Our results suggest that plasticity in the expression of CHCs may have contributed to the original speciation process between the parental species. This reinforces the key role of host plants in shaping the evolution of reproductive isolation among herbivore populations.

Key words: **assortative mating, cuticular hydrocarbons, ecological speciation, interspecific hybridization, reproductive isolation.**

INTRODUCTION

Small phytophagous arthropods account for 25–40% of all animal species (Berlocher and Feder 2002). About half of the speciation episodes in phytophagous insects are accompanied by a host shift, suggesting a causal link between these events (Winkler and Mitter 2008). Indeed, there is currently compelling evidence for the role of host plants in promoting divergence between phytophagous populations, potentially leading to speciation (Dres and Mallet 2002; Nosil et al. 2002; Rundle and Nosil 2005; Nosil and Crespi 2006; Magalhães et al. 2007). Therefore, the study of the mechanisms underlying speciation accompanied by a host shift may contribute to our understanding of the extreme biodiversity of phytophagous arthropods.

Several factors may account for the evolution of reproductive isolation between populations occurring on different host plants. First, reproductively active individuals from populations occurring on different host plants are more likely to encounter potential mates on the host they live in (Feder et al. 1994). Thus, "assortative meeting" due to distinct host specificity may act as a premating isolating barrier among insect populations, resulting in reproductive isolation in the absence of any other barrier (Katakura et al. 1989; Via 1999; McKinnon et al. 2004; Malausa et al. 2005; Ohshima 2010; Matsubayashi et al. 2011, 2013). Alternatively (or in addition) gene flow between populations on different hosts may be hampered due to reduced performance of migrants on the residents' habitat (Coyne and Orr 2004; Nosil et al. 2005). Moreover, residents may refrain from mating with migrants (Maan and Seehausen 2011). Both these forces can operate either on the migrants themselves or

Address correspondence to X.-K. Yang. E-mail: yangxk@ioz.ac.cn.

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on the hybrids between migrants and residents, although this possibility has been poorly explored (but see Latour et al. 2014).

Chemical signals, including volatile and contact pheromones, often underlie sexual isolation between insect species or populations (Landolt and Phillips 1997; Peterson et al. 2007; Smadja and Butlin 2009). Among such signals are nonvolatile cuticular hydrocarbons (CHCs), long-chain saturated waxes, and unsaturated nonpolar waxes (Schal et al. 1998), frequently involved in species recognition (Smadja and Butlin 2009). Although the biosynthesis of CHCs is mainly genetically determined (Coyne et al. 1994; Blows and Allan 1998; Takahashi et al. 2001; Carracedo et al. 2003; Niehuis et al. 2011; Pardy 2012), the expression of CHCs may also be affected by other factors, such as breeding status (Steiger et al. 2007, 2009), diet (Stennett and Etges 1997; Etges et al. 2006; Ming and Lewis 2010), the acoustic environment (Thomas et al. 2011), and age (Nieberding et al. 2012; Heuskin et al. 2014).

In herbivorous insects, CHCs are frequently involved in species recognition (Smadja and Butlin 2009). Moreover, their evolution has been shown to correlate with speciation events (Mullen et al. 2007). Also, in some herbivores, such as meadow grasshoppers (Neems and Butlin 1995) or *Timena* walking sticks (Schwander et al. 2013), different species inhabiting different host plants exhibit different CHC profiles, suggesting a link between ecological speciation and variation in CHC profiles. At the intraspecific levels, a few studies have shown that CHC profiles vary with the host plant in which the individuals occur (Akino et al. 2004; Etges et al. 2009; Piskorski et al. 2010). Moreover, 3 studies demonstrated that herbivores discriminate between individuals reared on different host plants on the basis of their CHC profile (Etges et al. 2006; Geiselhardt et al. 2012; Stojković et al. 2014). Finally, an experimental evolution approach showed that evolution on different host plants modified the CHC profile and mate choice of *Drosophila serrata* populations (Rundle et al. 2005). Together, these results suggest that CHC profiles play an important role in the evolution of reproductive isolation between herbivore populations specialized on different hosts (Chung and Carroll 2015).

Research on CHC involvement in ecological speciation in herbivorous arthropods has thus so far addressed host plant–dependent within-species plasticity in CHC production and differences in CHC profiles in species inhabiting different host plants. It is as yet unclear, however, whether hybrids from species occurring on different host plants also vary in their CHC profile according to the host plant, and whether this information can be used to maintain species integrity. In this paper, we aim to fill this gap using a system composed of 2 sister beetle species specialized on different host plants: *Altica viridicyanea* (Baly) (hereafter "AV") occurs exclusively on *Geranium nepalens* (Sweet) (hereafter "Gn"), whereas *Duchesnea indica* (Andrews) (hereafter "Di") is a natural host of an oligophagous species *Altica fragariae* (Nakane) (hereafter "AF"). In these species, the adults do not feed or oviposit and the larvae do not develop on the host plant of the other species (Xue et al. 2009a, 2009b). However, the 2 host plants are often close to each other, and individuals of one species are sometimes found on the host plant of the other species (Xue H-J, personal observation). Very limited interspecific gene flow was detected, suggesting occasional interspecific mating may occur in the field (Xue et al. 2014). In the laboratory, males of both species mate assortatively, whereas the rejection behavior of females is less clear (Xue et al. 2014). Finally, postmating isolation between species is incomplete under laboratory conditions (Xue et al. 2009a, 2009b, 2011): whereas few eggs produced by a cross between an AF female and an AV male hatch (Xue et al. 2009b, 2011), AV females crossed with AF males produce viable offspring, with intermediate performance on each host plant (Xue et al. 2009b), and the sex ratio of hybrid F_1 is close to 1:1. Therefore, the oligophagous F_1 hybrids offers an opportunity to test whether CHC profiles and mating behavior are host dependent.

If plasticity in CHC profiles plays a role in the maintenance of reproductive isolation among the 2 *Altica* species studied here, this can only be via the hybrids, as the parental species are unable to develop on the alternative host plant. Moreover, information on the plasticity of CHCs in the hybrids may inform on the potential role of this mechanism on the original process of speciation among these species. Therefore, in this study, we tested 1) if males from these *Altica* species recognize conspecific females based on their CHC profile, 2) if the host plant affects the CHC profile of hybrid females, and 3) whether hybrid males distinguish between hybrid females raised on different host plants. Our findings highlight the effect of hybridization on mate recognition systems and its consequences for the gene flow between closely related phytophagous species.

MATERIALS AND METHODS

Insect cultures

Overwintered adults of AF and AV were collected from field populations at Changping (40.278°N, 116.040°E) and Mentougou (40.091°N, 115.948°E) in the spring 2012. In the laboratory, cultures of these insects were maintained in their native host plants in growth chambers at 16:8 LD and 25 °C (Xue et al. 2011). Under such conditions, the flea beetles complete their life cycle within approximately 30 days. Plants were collected locally, kept in a refrigerator, and used within 1 week after collection. On eclosion, beetles were isolated by sex to obtain virgin males and females for the mating assays.

Mating bioassays

All mating tests were carried out between 13:00 and 18:00 h in an air-conditioned room at 25–27 °C under natural light conditions. The number of successfully copulating pairs was recorded over a period of 3 h. In bioassays I–IV (see below), 52–125 replicates for each combination were conducted. Each individual was used only once.

A previous study using live female beetles as potential mates showed that both AF and AV males prefer conspecific females under 2-choice and multiple-choice conditions (Xue et al. 2014). In the current study, females had to be killed in order to extract their CHCs; hence, we first tested whether male choice was maintained when dead instead of live females were offered in a 2-choice setting. To this aim, 1 AF female and 1 AV female cadavers were presented to an AF or an AV male (bioassay I). A successful mating was scored when males inserted the aedeagus into the female abdomen for longer than 5 min. In bioassay II, cadavers of an intact female and a female washed off of its CHCs were presented to a conspecific male, to test whether CHCs affect male attraction to conspecific females. In bioassay III, 1 AF and 1 AV dead females, with their CHC extract exchanged, were presented to an AF or an AV male, to test whether the CHC extract alone is sufficient to invert male choice.

In the bioassays I–III, females were killed by freezing them at −30 °C for 20 min. The dead specimen was glued to a small piece of triangular filter paper (length = 1 cm), then to the wall of a Petri

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dish (9.0 × 1.2 cm) containing moistened filter paper. In bioassay II, the CHCs were washed off from females by dipping them 4 times in 0.4 mL hexane for 30 min, a gas chromatography-flame ionization detection (GC-FID) analysis confirmed that the washed beetles were free of cuticular chemical compounds (less than 2% remained; Supplementary Table S1). In bioassay III, each female was dipped in 0.04 mL hexane for 30 min to obtain the cuticular extracts, then the same female was dipped 4 times in 0.4 mL hexane for 30 min to rinse the specimen completely. To exchange the CHCs of AF and AV females, rinsed females were dipped in cuticular extracts of females of the other species and the solvent was evaporated in a chemical fume hood.

Bioassay IV was carried out to test whether mating preference was affected by feeding experience. Because parental species cannot develop on the host plant of the other species, this experiment was done in hybrids only. F_1 (AV ♀ × AF ♂) males and females were reared either on Gn (hereafter "F_1-Gn") or on Di (hereafter "F_1-Di"). To this aim, AF males and AV females were crossed, then F_1 eggs were scraped gently and transferred into Petri dishes (9.0 cm diameter) containing moistened filter paper. On hatching, the larvae were split into 2 cohorts and placed in Petri dishes of 9.0 cm diameter containing a moistened filter paper and fresh leaf material of Di or Gn (Xue et al. 2009a, 2009b). Newly emerged adults were fed with the same host plant on which they developed until becoming sexually mature (>10 days). Subsequently, 1 female of each type (reared on either Gn or Di) was presented to an F_1 male (reared on either Gn or Di), and the choice of this male was recorded as above. To distinguish between females raised on different host plants, they were marked on elytra with enamel paint of different colors (Wood et al. 1999; Xue et al. 2014).

Male choices were analyzed using chi-square tests. To assess the degree of sexual isolation in different bioassays, we used the software JMATING 1.0.8 to estimate the index of sexual isolation (I_{PSI}) (Rolán-Alvarez and Caballero 2000; Carvajal-Rodriguez and Rolan-Alvarez 2006). I_{PSI} ranges from −1 to 1, where −1 is complete disassortative mating, 0 is random mating, and 1 is complete assortative mating. Standard deviations and tests of significance for total I_{PSI} were obtained by bootstrapping with 10 000 bootstrap iterations.

Chemical analysis

Cuticular extracts were obtained as described above (in total 8 treatments: AF ♀, $n = 35$; AF ♂, $n = 35$; AV ♀, $n = 34$; AV ♂, $n = 33$; F_1-Di ♀, $n = 20$; F_1-Di ♂, $n = 14$; F_1-Gn ♀, $n = 35$; F_1-Gn ♂, $n = 35$). Prepared extract samples were transferred into a vial insert (Agilent Technologies, Inc., 250 µL glass with polymer feet), then placed in chromatography vials (Agilent Technologies, Inc., Screw caps vials, 1.5 mL) for the gas chromatography mass spectrometry (GC-MS) analysis (HP 7890 series GC–HP 5975 MSD; GC-MS) with the MS Library NIST2005 (Agilent Technologies, Inc.). An HP5 column (30 m × 0.32 mm internal diameter × 0.25 µm film thickness, Agilent Technologies, Inc.) was used, with helium as carrier gas (1.0 mL/min). An aliquot of 2 µL per sample was injected in a splitless mode at 280 °C. The oven was programed as follows: 40 °C for 1 min, 8 °C/min from 40 to 300 °C, then 20 °C/min to 320 °C. MS was in the electron impact mode 70 eV. The *n*-alkane (C6–C40) standard was also injected to calculate retention indices (RIs) (Kováts 1965). Individual compounds were identified by integrative analysis of their MS (Nelson et al. 1972; Pomonis et al. 1980; Doolittle et al. 1995) and their RIs (Carlson et al. 1998). The FID exhibits much better precision than MS in chemical quantification (Dodds et al. 2005), so the quantification of CHCs was performed by GC-FID under the same conditions as above.

The area within each peak relative to total peak area was computed for each CHC, and peaks with a mean relative proportion of more than 0.5% at least in 1 treatment (AF ♀, ♂; AV ♀, ♂; F_1-Di ♀, ♂; and F_1-Gn ♀, ♂) were used for further analyses. Quantitative differences between the CHC profiles of AF and AV males and females and those between F_1 males and females with different feeding experiences were statistically analyzed using permutational multivariate analyses of variance (perMANOVA) (Anderson 2005).

A canonical discriminant analysis was performed to determine the effect of species (or host plant) and sex on the CHC profiles. The quality of the resulting classification was tested by "leaving one-out cross-validation" (Efron 1983). Prior to the analysis, the CHC data were centered and log-ratio transformed as follows: $z_{ip} = \ln[A_{ip}/g(A_p)]$, where A_{ip} is the area of peak i for beetle p, $g(A_p)$ is the geometric mean of all peaks for beetle p, and z_{ip} is the transformed area of peak i for beetle p (Aitchison 1986). As the logarithm is not defined for zero values, the constant 0.01 was added to each relative peak area to apply the transformation formula also to samples that did not contain all compounds (peaks) (Geiselhardt et al. 2009). Furthermore, we tested whether the magnitude of differences of CHC profiles between AF and AV was comparable with that between hybrids reared on different host plants, by analyzing differences among CHC profiles of F_1 females (F_1-Di ♀ and F_1-Gn ♀) and parental species (AF ♀ and AV ♀), also using a canonical discriminant analysis. Accordingly, the squared Mahalanobis distances of CHC profiles were calculated, and the differences of distances were analyzed using *t*-tests for the following sample pairs: (AF female to AV females) versus (F_1-Di females to F_1-Gn females); (F_1-Gn females to AF females) versus (F_1-Di females to AF females); (F_1-Gn females to AV females) versus (F_1-Di females to AV females). The perMANOVA analyses were implemented in the vegan package of R and canonical discriminant analyses were implemented in SPSS 18.0.

RESULTS

Mating bioassays

Males of both species preferred to mate with a conspecific dead female than with a dead female from the sister species (for AV, all matings were intraspecific; chi-square tests, for AF, $G = 20.167$, $P < 0.001$; $I_{PSI} = 0.9596 \pm 0.0377$, $P < 0.001$; Figure 1a). Therefore, female behavior is not a critical factor in mate recognition, and we proceeded with tests using only dead females. Males of both AF and AV significantly preferred intact conspecific females to females with their CHCs removed (chi-square tests, for AF ♂, $G = 9.966$, $P = 0.002$; for AV ♂, $G = 24.143$, $P < 0.001$; Figure 1b), indicating that CHCs are instrumental to detect the presence of a conspecific female. Finally, males significantly preferred heterospecific females with conspecific CHCs to conspecific females with heterospecific CHCs (chi-square tests, for AF ♂, $G = 6.818$, $P = 0.009$; for AV ♂, $G = 17.286$, $P < 0.001$; $I_{PSI} = -0.6374 \pm 0.0963$, $P < 0.001$; Figure 1c), implying that CHC profiles play a crucial role in species discrimination.

Hybrid F_1-Gn males preferred females feeding on the same host plant to those feeding on the alternative host (chi-square test, $G = 14.235$, $P < 0.001$), whereas F_1-Di males showed no preference between these types of females (chi-square test, $G = 0.529$, $P = 0.467$). The overall sexual isolation value (I_{PSI}) between F_1-Gn and F_1-Di was 0.2938 ± 0.1663 ($P = 0.0932$; Figure 2).

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Chemical analysis

Twenty-eight CHC compounds with a mean relative proportion of more than 0.5% at least in 1 treatment (AF ♀, ♂; AV ♀, ♂; F_1-Di ♀, ♂; and F_1-Gn ♀, ♂) were identified (Supplementary Table S2). For AF and AV, the perMANOVA analysis showed that CHC profiles were significantly different between species ($F = 6098.603$, $P < 0.001$) and sexes ($F = 10.425$, $P = 0.002$). A discriminant analysis clearly separated males and females of these 2 species (Wilks's $\lambda = 0.00009$, $x^2 = 1127.751$, $P < 0.001$).

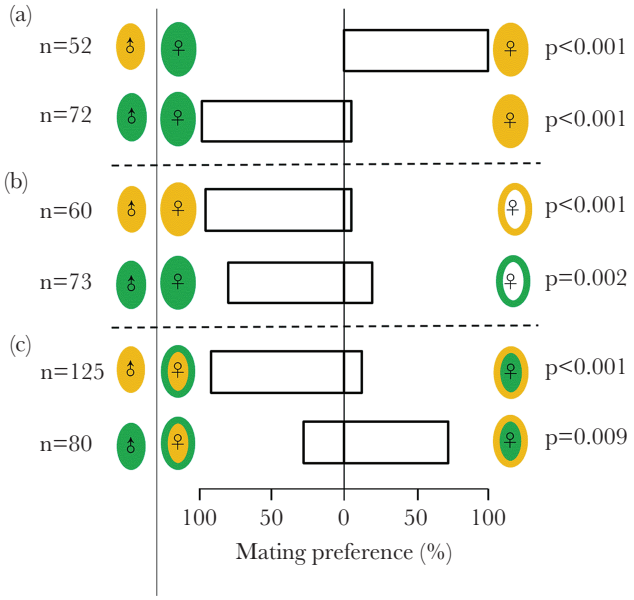

Figure 1
Mating preference of male flea beetles *Altica fragariae* (AF) and *Altica viridicyanea* (AV) in 2 choice tests. Males were offered the choice between (a) conspecific versus heterospecific dead females; (b) conspecific intact versus hexane-washed dead females; (c) conspecific versus heterospecific dead females with their CHCs exchanged. Green ellipse: AF; orange ellipse: AV; green ellipse with open filling: hexane-washed female of AF; orange ellipse with open filling: hexane-washed female of AV; green ellipse with orange filling: AF female painted with CHCs of AV female; orange ellipse with green filling: AV female painted with CHCs of AF female.

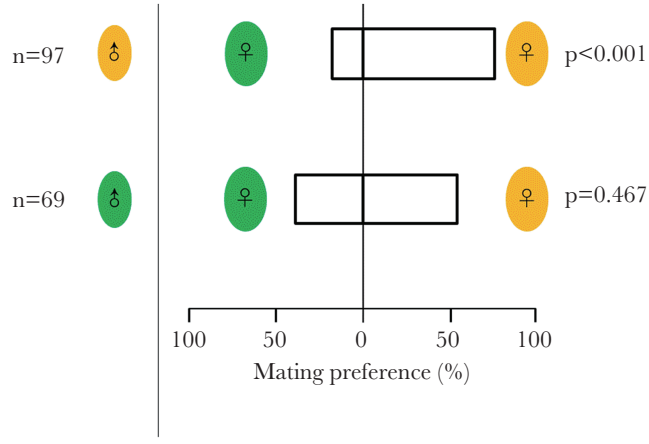

Figure 2
Mating preference of F_1 hybrid males (AV ♀ × AF ♂) reared on different host plants toward F_1 females (AV ♀ × AF ♂) with different feeding experience. Green ellipse: F_1 reared on Di; orange ellipse: F_1 reared on Gn.

The first canonical root accounted for 99.9% of the total variance of the data and separated samples according to the species. The second canonical root only explained 0.1% of the total variance but clearly separated beetles according to their sex (Figure 3). By the original discriminant function, 92.0% of the individuals were correctly classified, and 83.9% of the cross-validated cases were correctly classified.

A perMANOVA analysis in the hybrid samples showed that quantitative CHC profiles were significantly different according to the host plant ($F = 8.052$, $P < 0.001$) and sex ($F = 25.472$, $P < 0.001$). A significant host × sex interaction ($F = 13.908$, $P < 0.001$) was also found. A discriminant analysis clearly separated the 4 groups (F_1-Gn ♀, F_1-Gn ♂, F_1-Di ♀, F_1-Di ♂) (Wilks's $\lambda = 0.007$, $x^2 = 438.567$, $P < 0.001$). The first canonical root accounted for 56.2% of the total variance, and the second canonical root explained 34.3% of the total variance of the data (Figure 4). All individuals were correctly classified by the original discriminant function, and 88.7% of the cross-validated cases were correctly classified.

A discriminant analysis clearly separated the 4 female groups (i.e., AF, AV, F_1-Di, and F_1-Gn; Wilks's $\lambda = 0.000007$, $x^2 = 1270.268$, $P < 0.001$). The first and second canonical root accounted for 96.5% and 3.2% of the total variance, respectively (Figure 5). By the original discriminant function, 99.2% of the individuals were correctly classified, and 97.6% of the cross-validated cases were correctly classified. Squared Mahalanobis distances showed that CHC profiles of both female F_1-Di and female F_1-Gn were more similar to those of AF females than to those of AV females (*t*-test for dependent samples, $t = 180.111$, $P < 0.001$ and $t = 215.942$, $P < 0.001$, respectively). Also, differences between distant genetic backgrounds (AF and AV) were much higher than differences between hybrids reared on different host plants (*t*-test for dependent samples, $t = 336.305$, $P < 0.001$; Figure 5).

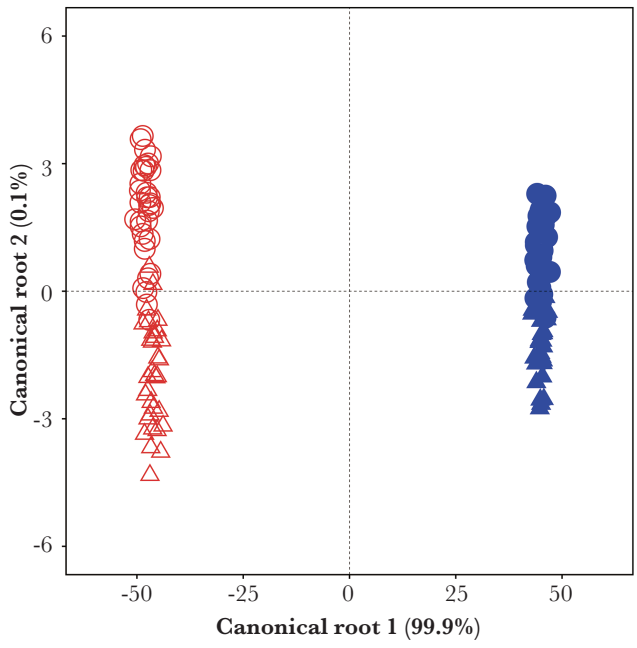

Figure 3
Scatterplot of canonical root 1 versus 2 of a discriminant analysis based on CHCs of *Altica fragariae* (AF) and *Altica viridicyanea* (AV). Circles: females; triangles: males; red: AV individuals; blue: AF individuals. The values in parentheses mean percentage of total variance.

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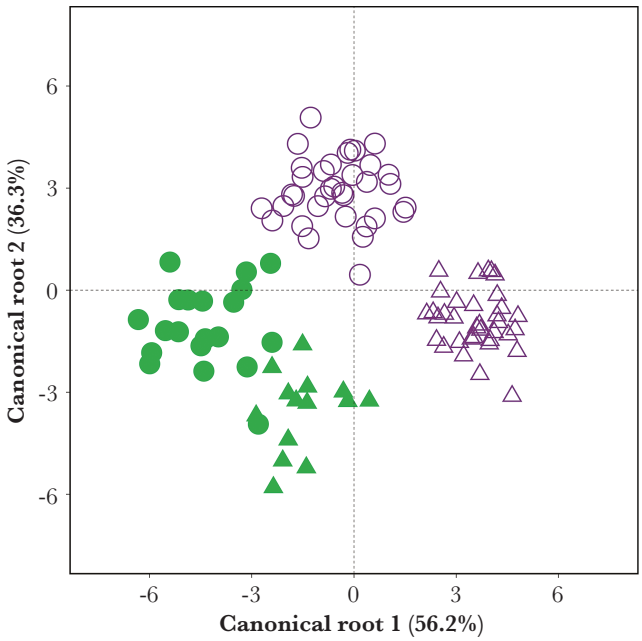

Figure 4
Scatterplot of canonical root 1 versus 2 of a discriminant analysis based on CHCs of F_1 hybrids that have fed on each host plant. Circles: females; triangles: males; purple: individuals fed Gn; green: individuals fed Di. The values in parentheses mean percentage of total variance.

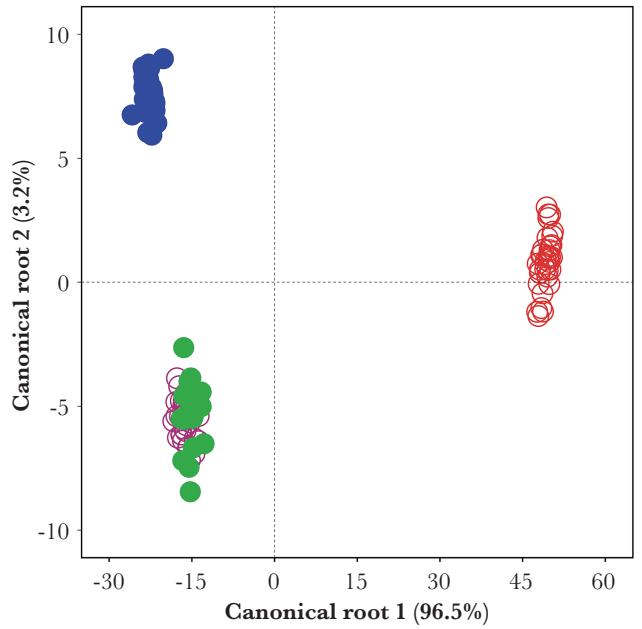

Figure 5
Scatterplot of canonical root 1 versus 2 of a discriminant analysis based on CHCs of AF (blue), AV (red), F_1-Di (green), and F_1-Gn (purple) females. The values in parentheses mean percentage of total variance.

DISCUSSION

In this study, we found that males from 2 closely related *Altica* beetles use CHCs as contact sex pheromones to distinguish conspecific from heterospecific females. Moreover, we show that, in hybrids, both the CHC profile and male mating preferences are affected by the host plant in which individuals develop. The chemical analysis showed that CHC profiles differed according to sex in both species, which suggests a role of these compounds in sexual identification. Moreover, CHC profiles differed between species, with some compounds being present in only 1 species. Finally, the facts that 1) removing CHCs from the females' cuticle is sufficient to erase male attraction and 2) exchanging CHCs between females of the 2 species is sufficient to switch male preference indicate that males are able to assess different CHC profiles and to decide whether or not to mate with a female based on that information. Therefore, we can conclude that odor cues emanating from contact pheromones are sufficient to ensure species recognition and thereby maintain reproductive isolation between species in this system.

All compounds present in either AV or AF individuals were also detected in F_1 hybrids (Supplementary Table S2). Still, the CHC profile of hybrids was clearly differentiated from that of any of the parental species. This runs counter the possibility that individuals may passively incorporate plant-derived compounds and use them as pheromones or pheromone precursors (Landolt and Phillips 1997). Indeed, the different profile of hybrids and parents reared on the same plant suggests differential biosynthesis of compounds depending on the genotype. Because F_1 individuals were reared on the same host plant as one parental species or the other, we can conclude from these differences that CHC profiles are partially genetically determined. Moreover, F_1 profiles were closer to those of AF, which suggests partial dominance of AF genes. However, AV females also exhibit small (or absent) peaks in some compounds, and this may artificially modify the geometric mean distance among peaks. To account for this, we repeated the discriminant analysis without such peaks and still obtained very similar results (Supplementary Figure S1). The fact that F_1 profiles are closer to AF profiles than to AV profiles does not correlate with patterns observed in other traits involved in specialization in this system, such as host preference and performance, which were found to be either strictly additive or AV-dominant (Xue et al. 2009b). This closer match of F_1 profiles to those of AF individuals is also not correlated with the compatibility of crosses between F_1 males and each of the parental females. Indeed, whereas some individuals do develop from crosses between F_1 males and AV females, crosses between F_1 males and AF females are fully incompatible (Xue et al. 2009b).

Although differences among CHC profiles were much clearer among genotypes than among same-genotype individuals reared on different hosts, the host plant on which individuals developed still clearly modulated their CHC profile. Moreover, F_1-Gn males preferred F_1-Gn females relative to F_1-Di females. This discrimination is only possible if these females emanate different cues, and that these cues are perceived by males. Thus, the difference between the CHC profiles of F_1-Gn and F_1-Di females, even though being orders of magnitude smaller than differences between species, is still sufficient to elicit a choice in males. Therefore, plasticity is the formation of CHC profiles can contribute to ongoing reproductive isolation between the 2 species studied here.

The host on which the males developed also affected their mate choice. Indeed, F_1 males reared on *Geranium* preferred females reared on the same host, whereas males reared on *Duchesna* showed no choice. Hence, the 2 populations of hybrids differ in their degree of choosiness, rather than in their preference function (Jennions and Petrie 1997). This asymmetry in the ability to discriminate among mates was also found in other systems, such as seed beetles (Stojković et al. 2014) or grasshoppers (Hochkirch and Lemke 2011). In others, assortative mating was found in one species or

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population, whereas the other displayed heterospecific preference. This was the case in *Papilio* butterflies (Deering and Scriber 2002) and in different populations of *Drosophila subobscura* (Barbaro et al. 2015). In the current system, this asymmetry may stem from the fact that F_1 hybrids never develop on *Duchesna* because AV females oviposit exclusively on *Geranium* (Xue et al. 2009a, 2009b, 2011). Hence, selection for such a discrimination is currently absent in this habitat. In contrast, F_1 hybrids are able to develop on *Geranium*. Thus, males may have been selected to discriminate between female hybrids with different feeding history on that host plant. Another possibility is that choosiness is condition dependent (Cotton et al. 2006), in which case F_1 males, having a higher performance on *Geranium* (Xue et al. 2009b), hence being in better condition on that host plant, would have more resources available to use in their mate choice. This suggests that the role of hybrid choice in reproductive isolation in ecological speciation hinges on their performance in the habitats of the parental species. Future studies may tell us whether this pattern is general.

In the plant where a mate choice is expressed (*Geranium*), this choice is "assortative" in that males choose females reared on that host plant. These results are aligned with other studies showing that insects discriminate between mates reared on different hosts (Rundle et al. 2005; Grace et al. 2010; Havens and Etges 2013), and even between mates reared on different clones of the same host (Rebar and Rodríguez 2014). In our system, males choose females with a CHC profile that resembles more theirs. Hence, this choice is likely to be based on self-reference phenotype matching (Hauber and Sherman 2001). Other studies have also shown that individuals choose to mate with individuals that match their CHC profile, for example, in different isofemale lines of hybrids between *D. serrata* and *D. birchii* (Blows and Allan 1998). Also, this choice is adaptive, as males choose female hybrids from *Geranium*, which perform better than those from *Duchesna* (Xue et al. 2009b). Again, the fact that this choice is "assortative" contributes to reproductive isolation, albeit on 1 plant species only.

Both the difference in CHC profiles in hybrids and F_1 discrimination ability is likely to contribute to reproductive isolation between species, as it represents yet another barrier to incoming individuals from the alternative host. F_1 males that have developed on *Duchesna*, though, did not discriminate between F_1 females reared on different host plants. This may allow individuals coming from other host plants to mate with resident individuals, possibly facilitating gene flow. Such small scale migration among plants and limited interspecific gene flow does occur occasionally in our study system (Xue et al. 2014). If this hypothesis is correct, it also implies that more introgression between species is higher on *Geranium* than on *Duchesna*, a possibility awaiting to be confirmed.

The 2 *Altica* species studied here are at an advanced stage of differentiation. Therefore, the relative contribution of CHC plasticity in hybrids for the current reproductive isolation between species is relatively low. This mechanism, however, may have been important for the original speciation process between the parental species (Fitzpatrick 2012; Geiselhardt et al. 2012). Indeed, our demonstration that CHC profiles of same genotype are altered by host plants can be extrapolated to the original unique parental species. In that case, a population that would switch from one host to another would also incur in a change in the CHC profile. Given that we also show that mate choice is based on CHC profiles in these species, plasticity in such profiles is likely to have affected reproductive isolation between populations of the same species on different hosts in the original speciation process. Our results thus reinforce the key role of host plants in the evolution of reproductive isolation underlying the formation of these 2 beetle species.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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REFERENCES

Aitchison J. 1986. The statistical analysis of compositional data. London: Chapman and Hall.

Akino T, Nakamura K, Wakamura S. 2004. Diet-induced chemical phytomimesis by twig-like caterpillars of *Biston robustum* Butler (Lepidoptera: Geometridae). Chemoecology. 14:165–174.

Anderson MJ. 2005. PERMANOVA: permutational multivariate analysis of variance. Auckland (New Zealand): Department of Statistics, University of Auckland.

Barbaro M, Mira MS, Fragata I, Simões P, Lima M, Lopes-Cunha M, Kellen B, Santos J, Varela SA, Matos M, et al. 2015. Evolution of mating behavior between two populations adapting to common environmental conditions. Ecol Evol. 5:1609–1617.

Berlocher SH, Feder JL. 2002. Sympatric speciation in phytophagous insects: moving beyond controversy? Annu Rev Entomol. 47:773–815.

Blows MW, Allan RA. 1998. Levels of mate recognition within and between two *Drosophila* species and their hybrids. Am Nat. 152:826–837.

Carlson DA, Bernier UR, Sutton BD. 1998. Evolution patterns from capillary GC for methyl-branched alkanes. J Chem Ecol. 24:1845–1865.

Carracedo MC, Asenjo A, Casares P. 2003. Genetics of *Drosophila simulans* male mating discrimination in crosses with *D. melanogaster*. Heredity (Edinb). 91:202–207.

Carvajal-Rodriguez A, Rolan-Alvarez E. 2006. JMATING: a software for the analysis of sexual selection and sexual isolation effects from mating frequency data. BMC Evol Biol. 6:40.

Chung H, Carroll SB. 2015. Wax, sex and the origin of species: dual roles of insect cuticular hydrocarbons in adaptation and mating. Bioessays. 37:822–830.

Cotton S, Small J, Pomiankowski A. 2006. Sexual selection and condition-dependent mate preferences. Curr Biol. 16:R755–R765.

Coyne JA, Crittenden AP, Mah K. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. Science. 265:1461–1464.

Coyne JA, Orr HA. 2004. Speciation. Boston: Sinauer Associates.

Deering MD, Scriber JM. 2002. Field bioassays show heterospecific mating preference asymmetry between hybridizing North American *Papilio* butterfly species (Lepidoptera: Papilionidae). J Ethol. 20:25–33.

Dodds ED, McCoy MR, Rea LD, Kennish JM. 2005. Gas chromatographic quantification of fatty acid methyl esters: flame ionization detection vs. electron impact mass spectrometry. Lipids. 40:419–428.

Doolittle RE, Proveaux AT, Alborn HT, Heath RR. 1995. Quadrupole storage mass spectrometry of mono- and dimethylalkanes. J Chem Ecol. 21:1677–1695.

Dres M, Mallet J. 2002. Host races in plant-feeding insects and their importance in sympatric speciation. Philos Trans R Soc Lond B Biol Sci. 357:471–492.

Efron B. 1983. Estimating the error rate of a prediction rule: improvement on cross-validation. J Am Stat Assoc. 78:316–331.

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Etges WJ, de Oliveira CC, Ritchie MG, Noor MA. 2009. Genetics of incipient speciation in *Drosophila mojavensis*: II. Host plants and mating status influence cuticular hydrocarbon QTL expression and G x E interactions. Evolution. 63:1712–1730.
Etges WJ, Veenstra CL, Jackson LL. 2006. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. VII. Effects of larval dietary fatty acids on adult epicuticular hydrocarbons. J Chem Ecol. 32:2629–2646.
Feder JL, Opp SB, Wlazlo B, Reynolds K, Go W, Spisak S. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proc Natl Acad Sci USA. 91:7990–7994.
Fitzpatrick BM. 2012. Underappreciated consequences of phenotypic plasticity for ecological speciation. Int J Ecol. 2012:256017.
Geiselhardt S, Otte T, Hilker M. 2009. The role of cuticular hydrocarbons in male mating behavior of the mustard leaf beetle, *Phaedon cochleariae* (F.). J Chem Ecol. 35:1162–1171.
Geiselhardt S, Otte T, Hilker M. 2012. Looking for a similar partner: host plants shape mating preferences of herbivorous insects by altering their contact pheromones. Ecol Lett. 15:971–977.
Grace T, Wisely SM, Brown SJ, Dowell FE, Joern A. 2010. Divergent host plant adaptation drives the evolution of sexual isolation in the grasshopper *Hesperotettix viridis* (Orthoptera: Acrididae) in the absence of reinforcement. Biol J Linn Soc. 100:866–878.
Hauber ME, Sherman PW. 2001. Self-referent phenotype matching: theoretical considerations and empirical evidence. Trends Neurosci. 24:609–616.
Havens JA, Etges WJ. 2013. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. IX. Host plant and population specific epicuticular hydrocarbon expression influences mate choice and sexual selection. J Evol Biol. 26:562–576.
Heuskin S, Vanderplanck M, Bacquet P, Holveck M, Kaltenpoth M, Engl T, Pels C, Taverne C, Lognay G, Nieberding CM. 2014. The composition of cuticular compounds indicates body parts, sex and age in the model butterfly *Bicyclus anynana* (Lepidoptera). Front Ecol Evol. 2:37.
Hochkirch A, Lemke I. 2011. Asymmetric mate choice, hybridization, and hybrid fitness in two sympatric grasshopper species. Behav Ecol Sociobiol. 65:1637–1645.
Jennions MD, Petrie M. 1997. Variation in mate choice and mating preferences: a review of causes and consequences. Biol Rev Camb Philos Soc. 72:283–327.
Katakura H, Shioi M, Kira Y. 1989. Reproductive isolation by host specificity in a pair of phytophagous ladybird beetles. Evolution. 43:1045–1053.
Kováts ES. 1965. Gas chromatographic characterization of organic substances in the retention index system. In: Giddings JC, Keller RA, editors. Advances in chromatography. Vol. 1. New York: Marcel Dekker. p. 229–247.
Landolt PJ, Phillips TW. 1997. Host plant influences on sex pheromone behavior of phytophagous insects. Annu Rev Entomol. 42:371–391.
Latour Y, Perriat-Sanguinet M, Caminade P, Boursot P, Smadja CM, Ganem G. 2014. Sexual selection against natural hybrids may contribute to reinforcement in a house mouse hybrid zone. Proc Biol Sci. 281:20132733.
Maan ME, Seehausen O. 2011. Ecology, sexual selection and speciation. Ecol Lett. 14:591–602.
Magalhães S, Forbes MR, Skoracka A, Osakabe M, Chevillon C, McCoy KD. 2007. Host race formation in the Acari. Exp Appl Acarol. 42:225–238.
Malausa T, Bethenod MT, Bontemps A, Bourguet D, Cornuet JM, Ponsard S. 2005. Assortative mating in sympatric host races of the European corn borer. Science. 308:258–260.
Matsubayashi KW, Kahono S, Katakura H. 2011. Divergent host plant specialization as the critical driving force in speciation between populations of a phytophagous ladybird beetle. J Evol Biol. 24:1421–1432.
Matsubayashi KW, Kahono S, Katakura H. 2013. Divergent host plant preference causes assortative mating between sympatric host races of the ladybird beetle, *Henosepilachna diekei*. Biol J Linn Soc.110:606–614.
McKinnon JS, Mori S, Blackman BK, David L, Kingsley DM, Jamieson L, Chou J, Schluter D. 2004. Evidence for ecology's role in speciation. Nature. 429:294–298.
Ming QL, Lewis SM. 2010. Pheromone production by male *Tribolium castaneum* (Coleoptera: Tenebrionidae) is influenced by diet quality. J Econ Entomol. 103:1915–1919.
Mullen SP, Mendelson TC, Schal C, Shaw KL. 2007. Rapid evolution of cuticular hydrocarbons in a species radiation of acoustically diverse Hawaiian crickets (Gryllidae: Trigonidiinae: Laupala). Evolution. 61:223–231.
Nelson DR, Sukkestad DR, Zaylskie RG. 1972. Mass spectra of methyl-branched hydrocarbons from eggs of the tobacco hornworm. J Lipid Res. 13:413–421.
Neems RM, Butlin RK. 1995. Divergence in cuticular hydrocarbons between parapatric subspecies of the meadow grasshopper, *Chorthippus parallelus* (Orthoptera, Acrididae). Biol J Linn Soc. 54:139–149.
Nieberding CM, Fischer K, Saastamoinen M, Allen CE, Wallin EA, Hedenström E, Brakefield PM. 2012. Cracking the olfactory code of a butterfly: the scent of ageing. Ecol Lett. 15:415–424.
Niehuis O, Büllesbach J, Judson AK, Schmitt T, Gadau J. 2011. Genetics of cuticular hydrocarbon differences between males of the parasitoid wasps *Nasonia giraulti* and *Nasonia vitripennis*. Heredity (Edinb). 107:61–70.
Nosil P, Crespi BJ. 2006. Ecological divergence promotes the evolution of cryptic reproductive isolation. Proc Biol Sci. 273:991–997.
Nosil P, Crespi BJ, Sandoval CP. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature. 417:440–443.
Nosil P, Vines TH, Funk DJ. 2005. Reproductive isolation caused by natural selection against immigrants from divergent habitats. Evolution. 59:705–719.
Ohshima I. 2010. Host-associated pre-mating reproductive isolation between host races of *Acrocercops transecta*: mating site preferences and effect of host presence on mating. Ecol Entomol. 35:253–257.
Pardy JA. 2012. The genetic basis of cuticular hydrocarbon production in *Drosophila melanogaster* and *D. simulans* [MS thesis]. [London (Canada): University of Western Ontario].
Peterson MA, Dobler S, Larson EL, Juárez D, Schlarbaum T, Monsen KJ, Francke W. 2007. Profiles of cuticular hydrocarbons mediate male mate choice and sexual isolation between hybridising *Chrysochus* (Coleoptera: Chrysomelidae). Chemoecology. 17:87–96.
Piskorski R, Trematerra P, Dorn S. 2010. Cuticular hydrocarbon profiles of codling moth larvae, *Cydia pomonella* (Lepidoptera: Tortricidae), reflect those of their host plant species. Biol J Linn Soc. 101:376–384.
Pomonis JG, Nelson DR, Fatland CL. 1980. Insect hydrocarbons. 2. Mass spectra of dimethylalkanes and the effect of the number of methylene units between groups on fragmentation. J Chem Ecol. 6:965–972.
Rebar D, Rodríguez RL. 2014. Genetic variation in host plants influences the mate preferences of a plant-feeding insect. Am Nat. 184:489–499.
Rolán-Alvarez E, Caballero A. 2000. Estimating sexual selection and sexual isolation effects from mating frequencies. Evolution. 54:30–36.
Rundle HD, Chenoweth SF, Doughty P, Blows MW. 2005. Divergent selection and the evolution of signal traits and mating preferences. PLos Biol. 12:e1001836.
Rundle HD, Nosil P. 2005. Ecological speciation. Ecol Lett. 8:336–352.
Schal C, Sevala VL, Young HP, Bachmann JAS. 1998. Sites of synthesis and transport pathways of insect hydrocarbons: cuticle and ovary as target tissues. Am Zool. 38:382–393.
Schwander T, Arbuthnott D, Gries R, Gries G, Nosil P, Crespi BJ. 2013. Hydrocarbon divergence and reproductive isolation in *Timema* stick insects. BMC Evol Biol. 13:151.
Smadja C, Butlin RK. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity (Edinb). 102:77–97.
Steiger S, Peschke K, Francke W, Müller JK. 2007. The smell of parents: breeding status influences cuticular hydrocarbon pattern in the burying beetle *Nicrophorus vespilloides*. Proc Biol Sci. 274:2211–2220.
Steiger S, Whitlow S, Peschke K, Müller JK. 2009. Surface chemicals inform about sex and breeding status in the biparental burying beetle *Nicrophorus vespilloides*. Ethology. 115:178–185.
Stennett MD, Etges WJ. 1997. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. III. Epicuticular hydrocarbon variation is determined by use of different host plants in *Drosophila mojavensis* and *Drosophila arizonae*. J Chem Ecol. 23:2803–2824.
Stojković B, Savković U, Đorđević M, Tucić N. 2014. Host-shift effects on mating behavior and incipient pre-mating isolation in seed beetle. Behav Ecol. 25:553–564.
Takahashi A, Tsaur SC, Coyne JA, Wu CI. 2001. The nucleotide changes governing cuticular hydrocarbon variation and their evolution in *Drosophila melanogaster*. Proc Natl Acad Sci USA. 98:3920–3925.
Thomas ML, Gray B, Simmons LW. 2011. Male crickets alter the relative expression of cuticular hydrocarbons when exposed to different acoustic environments. Anim Behav. 82:49–53.
Via S. 1999. Reproductive isolation between sympatric races of pea aphids. I. Gene flow and habitat choice. Evolution. 53:1446–1457.

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Winkler IS, Mitter C. 2008. The phylogenetic dimension of insect/plant interactions: a summary of recent evidence. In: Tillmon KJ, editor. Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. Berkeley (CA): University of California Press. p. 240–263.
Wood TK, Tilmon KJ, Shantz AB, Harris CK. 1999. The role of host-plant fidelity in initiating insect race formation. Evol Ecol Res. 1:317–332.
Xue HJ, Li WZ, Nie RE, Yang XK. 2011. Recent speciation in three closely related sympatric specialists: inferences using multi-locus sequence, post-mating isolation and endosymbiont data. PLoS One. 6:e27834.
Xue HJ, Li WZ, Yang XK. 2009a. Genetic analysis of feeding preference in two related species of *Altica* (Coleoptera: Chrysomelidae: Alticinae). Ecol Entomol. 34:74–80.
Xue HJ, Li WZ, Yang XK. 2014. Assortative mating between two sympatric closely-related specialists: inferred from molecular phylogenetic analysis and behavioral data. Sci Rep. 4:5436.
Xue HJ, Magalhães S, Li WZ, Yang XK. 2009b. Reproductive barriers between two sympatric beetle species specialized on different host plants. J Evol Biol. 22:2258–2266.

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