Behavioral Ecology

The official journal of the
ISBE
International Society for Behavioral Ecology

Behavioral Ecology (2018), 29(6), 1462–1471. doi:10.1093/beheco/ary105

Original Article

Chemically mediated sexual signals restrict hybrid speciation in a flea beetle

Huai-Jun Xue,[a,#] Kari A. Segraves,[b,c,#] Jing Wei,[a,d] Bin Zhang,[a] Rui-E Nie,[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]Department of Biology, Syracuse University, 107 College Place, Syracuse, NY 13244, USA, [c]Archbold Biological Station, 123 Main Drive, Venus, FL 33960, USA, and [d]University of Chinese Academy of Sciences, Beijing 100049, China

Received 11 August 2017; revised 1 July 2018; editorial decision 2 July 2018; accepted 12 July 2018; Advance Access publication 28 July 2018.

The evolution of reproductive isolation following hybridization is a major obstacle that may limit the prevalence of hybrid speciation among specific groups of organisms. Here, we use a flea beetle system to offer a behavioral hypothesis for why there are so few examples of homoploid hybrid speciation among insects. Specifically, we examined cuticular hydrocarbon (CHC) mating signals and mate-choice decisions of *Altica fragariae* and *A. viridicyanea* to test whether the signals produced by hybrids cause prezygotic reproductive isolation. Although hybrids of *A. fragariae* and *A. viridicyanea* had unique CHC profiles as compared to the parental species, mate-choice trials indicated that these differences were insufficient to prevent gene flow between hybrids and parental species. We found that mate-choice decisions and CHC signals were not correlated. Considering the ubiquity of CHC signaling molecules in insects, we propose that decoupling of CHC signals and mate choice may be a general mechanism limiting hybrid speciation in insects.

Key words: ***Altica*, cuticular hydrocarbon, hybrid speciation, interspecific hybridization, mate choice.**

INTRODUCTION

Hybridization is an important evolutionary force that can serve as a source of adaptive genetic variation (e.g. Stebbins 1959; Arnold 1997; Mallet 2005; Grant and Grant 2008; Hedrick 2013; Soltis et al. 2014). Gene flow between divergent populations or species introduces new alleles that can produce a wider range of phenotypic trait values than expressed in either parental population (e.g. Rieseberg et al. 1999; Dittrich-Reed and Fitzpatrick 2013; Goulet et al. 2017). These phenotypes provide the raw material for evolution and can allow hybrids to invade novel ecological niches or expand in range (e.g. Arnold 2004; Seehausen 2004; Gross and Rieseberg 2005; Hedrick 2013; Soltis et al. 2014; Pfennig et al. 2016). Moreover, if hybrids become reproductively isolated from their parental species, the process of hybridization can also contribute directly to diversification via the formation of hybrid species.

Hybrid speciation is particularly common among plants undergoing speciation via whole genome duplication, but is comparatively rare in the absence of polyploidy (Mallet 2007; Soltis and Soltis 2009). For example, homoploid hybrid speciation events in which hybrid species instantaneously form without changes in ploidy level have only been fully documented in a handful of study systems (reviewed by Schumer et al. 2014; Yakimowski and Rieseberg 2014; Lamichhaney et al. 2018). Although a number of studies suggest that hybrid speciation is likely to be an important evolutionary force (Mallet 2005; Mavárez and Linares 2008), the current evidence suggests that the mechanisms leading to homoploid hybrid speciation are restrictive (Schumer et al. 2014). For instance, a key obstacle of hybrid speciation is that hybrids must become reproductively isolated from the parental species (Stebbins 1959; Coyne and Orr 2004). This is a challenge because reproductive isolation must either be an outcome of the hybridization event itself or it must evolve in the face of gene flow from co-occurring parental taxa. Furthermore, if hybrids are intermediate to the parents, then the formation of reproductive barriers may be less likely as mating signals may overlap (Christophe and Baudoin 1998; Velthuis et al. 2005). As a consequence, understanding how reproductive isolation is promoted or blocked in hybrids is critical to resolving the debate on the role of hybrid speciation in diversification.

One group of organisms that may provide insightful clues about hybrid speciation is plant-feeding insects. Phytophagous insects are exceedingly species rich, and would seem to be strong candidates for diversification via hybrid speciation because they offer some of the

[#]These authors contributed equally to this work.
Address correspondence to X.K. Yang. E-mail: yangxk@ioz.ac.cn.

best examples of sympatric and parapatric speciation (e.g. Berlocher and Feder 2002; Bush and Butlin 2004). Furthermore, divergence in phytophagous insects is often associated with shifts in host-plant use in which host-associated differentiation creates host races or species that are specialized to feed on different plant species (e.g. Drés and Mallet 2002; Stireman et al. 2005; Matsubayashi et al. 2010). Given that many plant-feeding insects need to overcome mechanical and chemical plant defenses, secondary contact of assortatively mating host races or sister species could lead to the formation of hybrid populations that differ in the ability to use the ancestral hosts and potentially produce combinations of traits that allow them to colonize new host-plant species (Schwarz et al. 2005; Schwarz et al. 2007). Indeed, many sibling insect species can hybridize, suggesting the potential for hybrid speciation as a mechanism of diversification in phytophagous insects (Helms Cahan and Keller 2003; Schwarz et al. 2005; Scriber and Ording 2005; Gompert et al. 2006; Cáceres et al. 2009; Kunte et al. 2011; Nice et al. 2013). Although there are a number of accounts of hybridization between closely related insect species (Scriber 2011), there are only a few documented instances of hybrid speciation in phytophagous insects (Schwarz et al. 2005; Gompert et al. 2006; Mavárez et al. 2006; Schwarz et al. 2007; Melo et al. 2009; Kunte et al. 2011; Nice et al. 2013; Schumer et al. 2014). This raises the question of why hybrid speciation appears to be relatively rare among phytophagous insects despite the opportunity for hybridization, ecological divergence, and reproductive isolation.

A relatively unexplored hypothesis for the paucity of hybrid speciation in insects is that mate choice of hybrids and parental species will dictate whether hybrid individuals become reproductively isolated. Although host-plant choice can impact mating decisions (e.g., Anderson et al. 2013; Anderson and Anton 2014; Otte et al. 2016), other cues such as cuticular hydrocarbon (CHC) profiles will also play a significant role in mate choice (Coyne et al. 1994; Dietemann et al. 2003; Peterson et al. 2007; Kather and Martin 2012; Zhang et al. 2014). CHCs are common signaling molecules used by a broad array of insects to attract and identify conspecific mates (Dietemann et al. 2003; Steiger et al. 2009; Kather and Martin 2012; Otte et al. 2016; Keppner et al. 2017). In addition to species identification, these chemicals are costly to make and thus may also serve as intraspecific signals of an individual's quality as a mate (e.g. Blows 2002; Ferveur 2005; Sorvari et al. 2007; Van Homrigh et al. 2007; Izzo et al. 2010; Ingleby 2015). This signaling system is controlled by a large genetic network (Niehuis et al. 2011; Diao et al. 2016), suggesting that recombination during hybridization may alter both CHC profile and mate choice. Recombination in hybrids may produce intermediate or transgressive phenotypes. Transgressive phenotypes are outside of the range of the phenotypic values of either parental species, often caused by epistasis or additivity of more than one gene (Dittrich-Reed and Fitzpatrick 2013). If hybrids exhibit intermediate chemical signals (e.g. Coyne et al. 1994), this may increase the likelihood of backcrosses with one or both parental species, and may block reproductive isolation of hybrids and hybrid speciation. Alternatively, if hybrids express a transgressive CHC phenotype, this may promote immediate reproductive isolation of hybrid individuals and act as a key first step in hybrid speciation. Because of their prevalence as mating signals, CHCs are likely to serve as a general mechanism that promotes or breaks down prezygotic reproductive isolation of hybrid insects.

A good system in which to examine how mating signals and mate choice are altered by hybridization is the species rich flea beetle genus *Altica* Geoff. (Insecta: Coleoptera: Chrysomelidae). *Altica* is a taxonomically challenging genus with about 235 valid species that use a wide range of host plants (Reid and Beatson 2015). Speciation in this group is strongly tied to host-plant use (Laroche et al. 1996; Jenkins et al. 2009; Xue et al. 2014; Reid and Beatson 2015), and many closely related species co-occur sympatrically. There is also evidence of interspecific hybridization between species, and hybrids can be easily generated in the lab (Xue et al. 2009b; Xue et al. 2014; Xue et al. 2016a). Even these rare hybridization events could offer sufficient opportunity for hybrid speciation. For example, hybrid speciation has been demonstrated in *Heliconius* butterflies, and the rate of hybridization in natural populations is rare, occurring at about 0.05% (Mallet et al. 2007). Together, these ecological and life history traits in *Altica* set up conditions that would favor the formation of hybrids and the opportunity for hybrid speciation.

In this study, we focus on 2 closely related *Altica* species that form natural hybrids in the field. *Altica fragariae* Nakane (hereafter "AF") is an oligophagous species that primarily feeds on *Duchesnea indica* (Andrews) Focke and *A. viridicyanea* (Baly) (hereafter "AV") is a monophagous species on *Geranium nepalense* (Sweet). AF and AV are distributed sympatrically across their range in eastern Asia and can also co-occur locally within sites. There is little phenological isolation of AF and AV as the timing of emergence overlaps broadly between the species and both sexes mate several times throughout adulthood (Xue et al. 2014). These species are also highly specialized to their host plants, only feeding and ovipositing on their natal host even under no-choice laboratory conditions. This host specificity limits mating opportunities between the species due to decreased encounter rates, coupled with strong behavioral isolation (Xue et al. 2014). Sexual isolation between AF and AV appears to be predominantly determined by species-specific CHCs that allow males to discriminate conspecific from heterospecific females (Xue et al. 2016a; Xue et al. 2016b), and CHC profiles are also sex and age specific, whereby male *Altica* beetles can use chemical cues to distinguish conspecific males and females and to distinguish sexually mature females from immature ones (Xue et al. 2016b).

In this system, males are the choosier sex and are the dominant partner controlling mate choice. Despite the presence of prezygotic isolating barriers, interspecific gene flow has been detected with molecular markers, suggesting that occasional hybrids arise in the field (Xue et al. 2014). Since the 2 host plants are sympatric at many sites and grow within close proximity to one another, there is ample opportunity for interspecific encounters between AF and AV. These encounters could result in hybridization events as mating mistakes occur in laboratory mating trials. About 15% of AF males mated with AV females in no choice tests and 2.3% of AF males mated disassortatively when given a choice between conspecific and heterospecific females (Xue et al. 2014). Furthermore, crosses can be generated in the laboratory indicating that postzygotic isolation is incomplete. For example, although crosses between AF females and AV males are inviable, the reciprocal cross generates viable F_1 offspring and subsequent backcrosses are also viable (Xue et al. 2009a; Xue et al. 2009b; Xue et al. 2011). Interestingly, host-plant preference breaks down in F_1 offspring as F_1 hybrids willingly feed on both of the hosts of the parental species (Xue et al. 2009a). Compared with parental species, F_1 hybrids (AV♀×AF♂) possess a unique CHC profile (Xue et al. 2016a). Furthermore, the CHC profile and mating preference of F_1 males is affected by their larval feeding substrate. The relative amount (i.e. percentage) of CHCs is affected by feeding experience; hybrid males raised on one host plant preferred females with a matching profile. These results suggest that plasticity in CHC expression may have contributed to the original speciation process between the parental species (Xue et al. 2016a).

Given the previous work showing assortative mating of the species (e.g. Xue et al. 2009b), we predicted that reproductive isolation is being directed by CHC-mediated mating preference. If so, the CHC profiles of hybrids could create a bridge between parental species by presenting chemical cues that are recognized by one or both parent species, or if the CHC profiles are unique, this could offer a route to hybrid speciation. Because the F_1 hybrid was previously shown to possess a unique CHC profile (Xue et al. 2016a), we expected to observe assortative mating of parental species and hybrids. Our goals in this study were to expand on previous work by 1) using gas chromatography to compare an expanded set of CHC profiles for the parental species, F_1 hybrids, F_2 hybrids, and several backcrosses. Previously, we have only examined the CHC profiles of F_1 hybrids, and by expanding the set of hybrid crosses, we hoped to better understand the genetic basis of this complex trait. 2) We assessed mate choice decisions of parental species and hybrids using a series of 2-choice mating trials. Although we know how interspecific and intraspecific variation contribute to mate choice in the parental species, we lack an understanding of the mate choice decisions of hybrids. 3) We directly tested whether CHCs determine mate choice decisions of parental species and hybrids by manipulating CHC profiles of the beetles.

MATERIALS AND METHODS

Genetic lines

To create laboratory colonies of beetles, approximately 30 overwintered adults (~15 per sex) of both AF and AV were collected in Mentougou (40.09°N, 115.95°E), Beijing, China, a representative sympatric population. We chose to focus our analysis on only one population due to space and time constraints, and past work suggests that there is limited intraspecific variation in CHC profile. For example, comparisons of CHC profiles of 2 populations show little interpopulation variation in either AF or AV (Xue et al. 2016b; present study) and intraspecific variation is much smaller than the differences observed between the species (Xue et al. 2016b). Furthermore, mating preference experiments suggest that both *Altica* species exhibit reliable interspecific assortative mating, suggesting that low variation in these traits are unimportant for mate choice decisions (Xue et al. 2014; Xue et al. 2016a).

Initial lab colonies were started at approximately equal sex ratios. The 2 species were maintained separately in growth chambers held at 25 °C with 16:8 h light:dark and were fed fresh leaf tissue of their natal host plants (AF: *D. indica*; AV: *G. nepalense*). Beetles were allowed to mate and lay eggs to generate stocks of virgin AF and AV for subsequent crossing and mating experiments. More than 2000 adult individuals per species were obtained for the subsequent experiments.

We mimicked field conditions to determine how beetles would respond to mates and CHC profiles under realistic conditions. This allowed us to assess mate choice decisions of parental species and an array of hybrids in an effort to explain patterns observed in the field. To accomplish this, we used virgin males and females from the first lab generation to create interspecific crosses and backcrosses. Because postmating isolation between AF and AV is incomplete and asymmetric, we were unable to use a completely balanced design. Crosses using AV as the female produce viable F_1 progeny; however, AF females crossed with AV males produce eggs with extremely low survival (Xue et al. 2009a; Xue et al. 2009b). Consequently, all F_1 individuals tested in this study were derived from the AV♀×AF♂ cross. Production of the hybrid F_1 individuals was accomplished by placing 5 AV females and 5 AF males in a glass jar (11.5 cm tall × 12 cm in diameter) with host tissue and allowing them to mate and lay eggs. To obtain sufficient sample sizes, 6 sets of crosses were conducted; this resulted in more than 1500 F_1 adult individuals. We transferred the resulting eggs into Petri dishes (11.5 cm diameter) containing moistened filter paper. Upon hatching, the F_1 larvae were transferred to new Petri dishes containing moistened filter paper and fresh leaf material of *G. nepalense* (Xue et al. 2009a; Xue et al. 2009b). We used *G. nepalense* host-plant tissue to simulate field conditions. F_1 larvae feed on *G. nepalense* in the field because female AV will only oviposit on *G. nepalense* (Xue et al. 2009a, 2009b). Since adult hybrids can and will eat both plant species, newly emerged hybrid adults were provided both *G. nepalense* and *D. indica* host tissue (Xue et al. 2009a; Xue et al. 2009b). By allowing hybrids to feed on both host plants, we hoped to avoid missing potential extreme phenotypes. If the genes controlling survival segregated in the hybrid crosses, there could be death of extreme phenotypes if only one host-plant species was offered.

Once the hybrid generation was established, we created a second generation using the same methods as those for production of the F_1. We created F_2 (F_1♀×F_1♂) progeny as well as 3 backcrosses between the pure species and the F_1 hybrids (BC1: AV♀×F_1♂, BC2: AF♀×F_1♂, and BC3: F_1♀×AF♂). Due to the low hatching success of the F_1♀×AV♂ cross, we did not include this treatment in our experiments. These second generation crosses were provided fresh leaf material of both *D. indica* and *G. nepalense*. In the end, we collected more than 1000 adults of both BC1 and F_2 for the mating experiments and CHC analysis, and more than 100 adults of both BC2 and BC3 for CHC analysis.

Chemical analysis of CHCs

Chemical analysis was used to compare the CHC profiles of the pure species and all of the first and second generation crosses. We obtained approximately 30 replicate CHC extracts from each of the genetic lines by dipping single beetles in 40 µL hexane for 15 min. Prepared extracts were placed into vial inserts (Agilent Technologies Inc.; 250 µL glass with polymer feet) and were then transferred to chromatography vials (Agilent Technologies Inc., screw cap vials, 1.5 mL) for gas chromatography-flame ionization detection (GC-FID) analysis (HP 7890 series). We used a HP5 column (30 m × 0.32 mm internal diameter × 0.25 mm film thickness, Agilent Technologies, Inc.) with helium (1.0 mL/min) as a carrier gas. The injector was set to 280 °C as we injected a 2-µL volume of the sample. The oven temperature was set to 40 °C for 1 min, 8 °C/min from 40 °C to 300 °C, then 20 °C/min to 320 °C. We used the GC-MS data from our previous studies of these beetles to identify the individual compounds (Xue et al. 2016a; Xue et al. 2016b) via integrative analysis of their mass spectra (Nelson et al. 1972; Doolittle et al. 1995; Pomonis et al. 1980) and the retention indices (Carlson et al. 1998). We also ran a set of reference compounds to further confirm the identity of the CHCs. An *n*-alkane (C6–C40) standard was injected to adjust the retention time. A blank hexane sample was also analyzed to test for potential contamination of samples.

From this analysis, we only retained the peaks that had a mean relative proportion of more than one-half percent in at least one of the groups being compared. Permutational multivariate analysis of variance (perMANOVA) was used to determine if there were quantitative differences among the CHC profiles of the different groups (Anderson 2001, 2005) using "genetic line" and "sex" as main effects.

We performed a set of canonical discriminant analyses to determine whether genetic line and sex affected the CHC profiles. A previous study showed segregation of feeding preferences in F_2 individuals, such that some individuals preferred *G. nepalense*, some individuals preferred *D. indica*, whereas others ate both plant species (Xue et al. 2009a); thus, we examined whether there was a relationship between this divergence in feeding preference and CHC profiles. The quality of the resulting classifications obtained by the discriminant function analyses were tested using the "leaving one-out cross validation" procedure as recommended by Efron (1983). Prior to analysis, the CHC peak area results were log-ratio transformed: $z_{ip} = \ln[A_{ip}/g(A_p)]$, 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; Geiselhardt et al. 2012). Because the logarithm is undefined for zero values, the value 0.01 was added to each relative peak area to uniformly apply the transformation across samples that lacked some compounds (Geiselhardt et al. 2009). The perMANOVA analyses were implemented in PAST version 3.14 (Hammer et al. 2001) and canonical discriminant analyses were implemented in SPSS 18.0 (IBM, Armonk, NY, USA).

Mating bioassays

Because mating behavior of *Altica* is determined by males, we assessed the potential for behavioral isolation between hybrids and parental species using a series of independent 2-choice mating experiments where males were allowed to choose between 2 partners. Initially, we compared mate choice of the pure parental species and F_1 hybrids; and based on these results, we inferred that F_2 hybrids and one backcross (BC1: AV♀ × F_1♂) would be the most likely second generation hybrid produced in the field. Thus, we decided to focus on these second generation hybrids for additional mating trials (see Results). This approach allowed us to narrow the pool of total combinations given the limited sample of beetles produced in second generation crosses. Mating tests were carried out in a temperature-controlled room held at 25–27 °C under natural light conditions using sexually mature beetles (>10 days after eclosion). We constructed mating arenas using petri dishes (9.0 × 1.2 cm) lined with moistened filter paper. At the beginning of the trial, a test male was placed in the center of an arena containing 2 living females. A mating was considered successful when the male inserted his aedeagus for longer than 5 min (c.f., Xue et al. 2014). Males were given a choice of 2 females; one female was a pure AF or AV and the second was one of the first or second generation crosses. To distinguish the females in the arena, we marked their elytra with enamel paint of different colors (Wood et al. 1999; Xue et al. 2014). For each of the mating bioassays, 70–120 replicates were conducted. Mate choice and the number of copulating pairs were recorded over a period of 3 h (c.f., Xue et al. 2014).

To test the role of CHCs versus other mating cues in male mate choice, we conducted a second mating assay using dead females where we had exchanged their CHC profiles (c.f., Xue et al. 2016a; Xue et al. 2016b). To reduce the number of comparisons, we focused on 2 contrasts that were significant in the 2-choice mating trials: AV males selecting between AV females and F_1 females and F_1 males choosing between F_1 and AF females. Before the trials, we exchanged the CHCs of females that were killed by freezing at −30 °C for 20 min. Each female was individually dipped in 40 µL hexane for 15 min to obtain the cuticular extracts, then the same female was subsequently submerged six times in 400 µL fresh hexane for 15 min to rinse off any remaining CHCs. Previous work has confirmed that this method creates beetles free of cuticular chemical compounds (Xue et al. 2016b). Once the females were washed, the beetles were submerged in the cuticular extracts of the other female, allowing the solvent to evaporate in a chemical fume hood. The dead specimen was glued to a small piece of triangular filter paper (length = 1 cm), and then placed on the wall of a Petri dish (9.0 × 1.2 cm) containing moistened filter paper (c.f., Xue et al. 2016a; Xue et al. 2016b)). Mating success was assessed as above.

Because each experiment involved different beetles and the experiments were conducted independently, we assessed male mate choice using chi-square (χ^2) tests in SPSS 18.0 (IBM, Armonk, NY). To further assess the degree of sexual isolation between parental species and hybrids, we also estimate the index of sexual isolation (I_{PSI}) (Rolán-Alvarez and Caballero 2000; Carvajal-Rodriguez and Rolan-Alvarez 2006) with JMATING 1.0.8. Value of I_{PSI} ranges from −1 to 1, where −1 indicates 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 10000 bootstrap iterations.

RESULTS

Chemical analysis of CHCs

Twenty-eight CHC compounds with a mean relative proportion greater than 0.5% in at least one treatment group were identified (Supplementary Table S1). Two-way perMANOVA indicated that the CHC profiles were significantly different among the crosses ($F = 77.297$, $P < 0.001$) and between the sexes ($F = 20.965$, $P < 0.001$). A series of discriminant analyses based on the 28 compounds were able to clearly separate AV and BC1 (Supplementary Figure S1), BC2 and BC3 (Supplementary Figure S2), females of the seven genetic lines (AF, AV, F_1, F_2, BC1, BC2, BC3; Figure 1), all 14 groups (genetic lines × sex; Supplementary Figure S3) and F_2 with different feeding preferences (Figure 2; Table 1).

Mating bioassays

The 2-choice mating tests where males were offered a choice between 2 living females showed that the AF and F_1 hybrid males can distinguish between each others' females (chi-square tests, F_1: $G = 14.286$, $P < 0.001$; AF: $G = 13.298$, $P < 0.001$, Figure 3a; $I_{PSI} = 0.6229 \pm 0.0897$, $P < 0.0001$). A parallel test of mate choice by AV and F_1 males showed that AV males were also able to distinguish their own females from F_1 females; however, the F_1 hybrids mated at the same frequency with both types of females when given these 2 mate choices (AV: G=27.129, $P < 0.001$; F_1: $G = 1.089$, $P = 0.297$, Figure 3b; $I_{PSI} = 0.5295 \pm 0.0771$, $P < 0.0001$). For the backcross to AV (BC1: AV♀×F_1♂), we compared mate choice by BC1 males to pure AF or AV males. The results indicated that the males of BC1 and AF were able to distinguish each others' females very well (BC1: $G = 16.030$, $P < 0.001$; AF: $G = 33.618$, $P < 0.001$, Figure 3c; $I_{PSI} = 0.7496 \pm 0.0720$, $P < 0.0001$). In contrast, BC1 and AV males mated with both BC1 and AV females (BC1: $G = 0.034$, $P = 0.853$; AV: $G = 3.500$, $P = 0.061$, Figure 3d; $I_{PSI} = 0.1462 \pm 0.1165$, $P = 0.2112$). Similarly, the F_2 males mated with females of either parental species and F_2 hybrids (AF: $G = 1.581$, $P = 0.209$, Figure 3e; AV: $G = 2.455$, $P = 0.117$, Figure 3f), whereas the pure AV and AF males both avoided mating with F_2 hybrids (AF: $G = 18.778$, $P < 0.001$, Figure 3e; AV: $G = 29.121$, $P < 0.001$, Figure 3f). For comparisons of F_2 to AF and F_2 to AV, the overall sexual isolation values were significant (F_2-AF:$I_{PSI} = 0.3528 \pm 0.0216$, $P = 0.0216$; F_2-AV: $I_{PSI} = 0.5310 \pm 0.0954$, $P = 0.0002$).

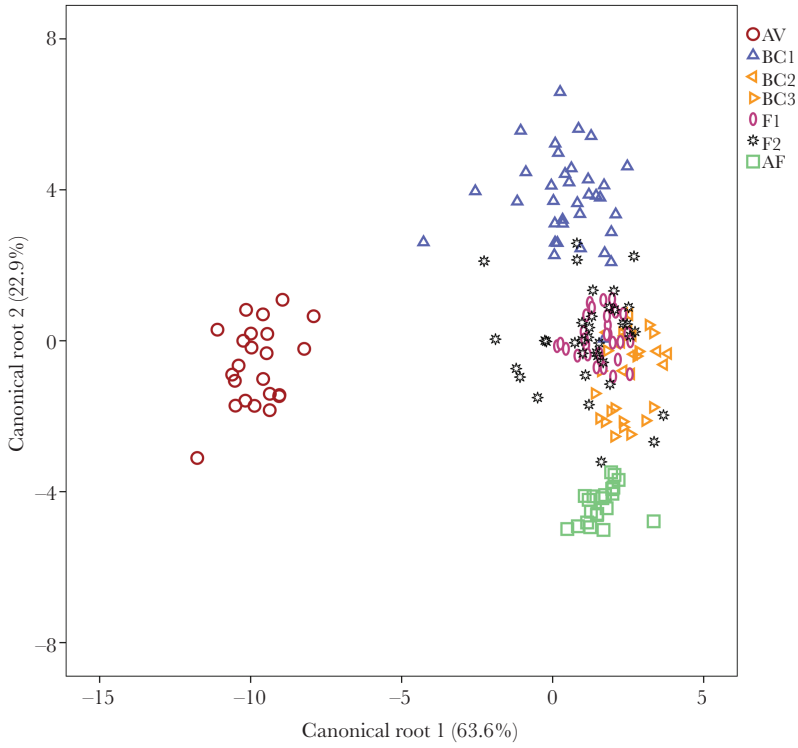

Figure 1
Discriminant analysis of relative amounts of cuticular hydrocarbons of female *Altica* from 7 genetic lines. *Altica fragariae* (AF), *A. viridicyanea* (AV), F_1 hybrids (AV♀×AF♂), F_2 hybrids (F_1♀×F_1♂), backcross BC1 (AV♀×F_1♂), backcross BC2 (AF♀×F_1♂), and backcross BC3 (F_1♀×AF♂).

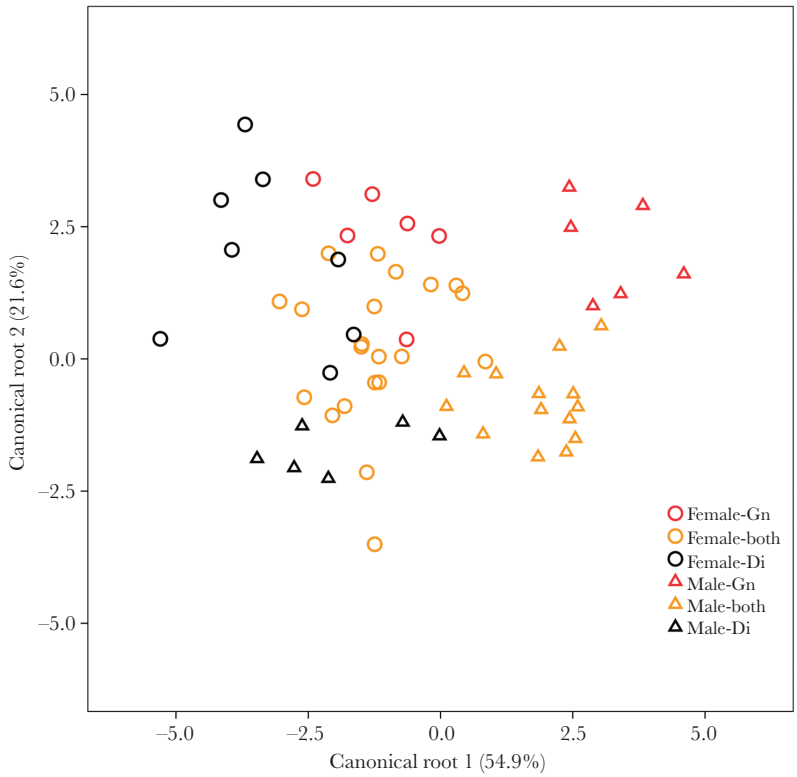

Figure 2
Discriminant analysis of the cuticular hydrocarbons of F_2 hybrids with different feeding preference. Female-Gn: F_2 females that preferred *Geranium nepalense*; Female-both: F_2 females that ate both plant species; Female-Di: F_2 females that preferred *Duchesnea indica*; Male-Gn: F_2 males that preferred *G. nepalense*; Male-both: F_2 males that ate both plant species; Male-Di: F_2 males that preferred *D. indica*.

Table 1
Results of the canonical discriminant analysis.

Comparison	Wilks's λ	χ^2	*P*	First canonical root	Second canonical root	Third canonical root	Correct classification by the original discriminant function	Correct classification by cross-validated cases
AV versus BC1	0.003	413.696	<0.001	70.8%	16.0%	13.2%	96.6%	89.8%
BC2 versus BC3	0.002	374.754	<0.001	72.0%	24.1%	3.9%	100%	88.2%
7 female lines	0.001	1098.990	<0.001	63.6%	22.9%	6.5%	83.3%	73.9%
14 genetic lines	0.00018	2476.463	<0.001	45.8%	24.9%	9.9%	77.6%	66.7%
F_2 with different feeding preference	0.013	800.028	<0.001	54.9%,	21.6%	14.1%	93.1%	63.9%

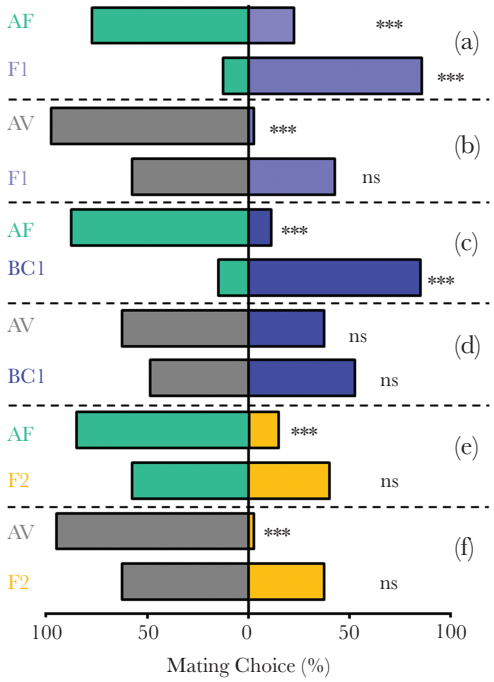

Figure 3
Mate choice decisions of *Altica fragariae* (AF), *A viridicyanea* (AV) and the hybrid crosses in 2-choice mating tests. (a) AF (n = 85, 47 mated) and F_1 hybrid (AV♀×AF♂; n = 94, 28 mated) males were allowed to choose between AF and F_1 hybrid females; (b) AV (n = 72, 31 mated) and F_1 hybrid (n = 120, 45 mated) males were allowed to choose between AV and F_1 hybrid females; (c) AF (n = 80, 55 mated) and BC1 backcross (AV♀ × F_1♂; n = 98, 33 mated) males were allowed to choose between AF and BC1 females; (d) AV (n = 80, 56 mated) and BC1 backcross (AV♀ × F_1♂; n = 70, 29 mated) males were allowed to choose between AV and BC1 females; (e) AF (n = 73, 36 mated) and F_2 hybrid (F_1♀ × F_1♂; n = 80, 31 mated) males were allowed to choose between AF and F_2 hybrid females; (f) AV (n = 75, 33 mated) and F_2 hybrid (F_1♀ × F_1♂; n = 88, 33 mated) males were allowed to choose between AV and F_2 hybrid females. Colors on bars represent male mating preference for females: green AF; gray AV; purple F_1 hybrid; blue BC1; yellow F_2. ***P < 0.001; ns is not significant.

The second round of mating bioassays where males were provided with a choice of 2 females with exchanged CHCs confirmed the results of the previous assay with live females. When we exchanged the CHCs of AV and F_1 females, males of AV reversed their mate choice decision: they chose to mate with F_1 females over their own (G = 10.000, P = 0.002; Figure 4a). Likewise, in the trials allowing F_1 males to choose between AF and F_1 females, males

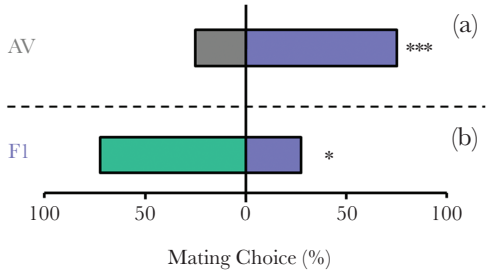

Figure 4
Mate choice as a function of exchanged cuticular hydrocarbons (CHCs) (a) males of *Altica viridicyanea* (AV), (n = 87, 40 mated) were allowed to choose between AV and F_1 hybrid females with exchanged CHCs. Gray bar: AV females with CHCs of F_1 hybrid females; purple bar: F_1 hybrid females with CHCs of AV females; (b) male F_1 hybrids (n = 90, 25 mated) were allowed to choose between F_1 hybrid and *A. fragariae* (AF) females with exchanged CHCs. Green bar: AF females with CHCs of F_1 hybrids; purple bar: F_1 hybrid females with CHCs of AF females. ***P < 0.001; *P < 0.05.

mated more frequently with AF females when the CHCs were exchanged (G = 4.840, P = 0.028; Figure 4b).

DISCUSSION

Hybrid speciation is often posited as an important mechanism of speciation that may occur as a result of the hybridization event or by subsequent evolutionary forces that create reproductively isolated hybrid populations (Mallet 2005; Mavárez and Linares 2008). Although both of these pathways are important, forming a predictive framework for hybrid speciation necessitates understanding how often these contrasting processes lead to hybrid species formation. In particular, the question remains as to the rate at which hybridization itself directly leads to reproductive isolation and speciation. There are currently only three examples from plants, one in birds, and one example in insects that have met this restrictive definition of hybrid speciation (Schumer et al. 2014; Lamichhaney et al. 2018). The paucity of examples among insects in particular is somewhat surprising since the opportunities for hybrid speciation would seem to abound. For example, phytophagous insects possess life histories that favor divergence of host races (e.g. Drés and Mallet 2002; Stireman et al. 2005; Matsubayashi et al. 2010) that in turn could subsequently hybridize and form hybrid species. The apparent rarity of hybrid species among insects suggests that there may be common barriers to reproductive isolation of hybrids. Here, we hypothesize that a decoupling of chemical mating signals and associated mate choice decisions could be a key factor that limits the prezygotic isolation of hybrid species in insects.

In insects, one of the first steps required for hybrid speciation is divergence in the mating signals of hybrids such that hybrids emit mating signals easily distinguished from pure species (Coyne and Orr 2004). Indeed, in *Altica* we found 28 CHC compounds that defined distinctive chemical signatures among the genetic lines (Supplementary Table S1). There were significant differences in CHC profiles between the parental species and all classes of hybrids, as well as between the sexes (Table 1). Females of the parental species were easily distinguished from one another by 4 unique compounds found in *A. fragariae* in addition to 8 additional compounds that had substantive differences in expression where they were present at only trace levels (≤0.1%) in one of the species. Each of the hybrid classes formed distinct clusters that were significantly different from one another and from the pure species (Figure 1), and their profiles contained all 28 of the CHCs and all of these were expressed at levels greater than 0.1%. Together, the unique profiles of the species and hybrids suggest that hybrids present signals that could favor assortative mating, yet the question remains whether these differences cause marked changes in mating behavior.

Although the chemical analysis shows that hybrid CHC phenotypes are distinct, the results from the mating trials suggest that the differences in CHC profiles are insufficient to result in positive assortative mating of hybrids. We showed that while both parental species mated with conspecific partners over F_1 hybrids, F_1 males readily paired with *A. viridicyanea* but not *A. fragariae* (Figure 3a,b). In nature, F_1 hybrids would develop on the maternal plant because *A. viridicyanea* females only oviposit on the maternal plant, *G. nepalense*, under choice and even no-choice laboratory conditions. As a consequence of their host and mate preference, then, *A. viridicyanea* males would be unlikely to mate with F_1 females whereas F_1 males would mate with both F_1 and *A. viridicyanea* females. Due to these combined host-plant and mate preferences, we would predict that there is a low chance of reproductive isolation of hybrids in *Altica*. For reproductive isolation to occur, the F_1 hybrids would have to develop on the paternal host-plant species with *A. fragariae*. If this were the case, we would predict positive assortative mating of *A. fragariae* and the hybrids. Because *A. fragariae* males prefer *A. fragariae* females over F_1 hybrids and F_1 males prefer to mate with F_1 females, there would be strong reproductive isolation of hybrids. However, due to the oviposition preference of *A. viridicyanea* females being strictly limited to *G. napelense*, reproductive isolation is unlikely to occur.

As has been shown previously (e.g. Coyne et al. 1994; Noor and Coyne 1996), we also observed an asymmetry in mating preference of hybrids. Assuming that our laboratory experiments accurately reflect mate choice under field conditions, the results suggest that we should predict the formation of F_2 hybrids and F_1 backcrosses with *A. viridicyanea* females (BC1). To further examine how these expected crosses would behave, we conducted additional mating trials using these backcrosses (Figure 3c–f). This second set of experiments revealed a similar asymmetry in hybrid mate choice. BC1 and *A. fragariae* mated assortatively (Figure 3c), whereas BC1 and *A. viridicyanea* mated randomly (Figure 3d). In addition, the F_2 hybrids mated randomly, pairing with both the parental species and F_2 females. In contrast, the pure species mated with conspecific partners over F_2 hybrids (Figure 3e–f). These combined mate-choice results suggest the potential for ongoing gene flow between hybrids and parental species, making hybrid speciation an unlikely outcome of the hybridization event. Instead, hybrid individuals may act as a bridge for gene flow between the parental species, providing a source of adaptive genetic variation (Mallet 2007; Abbott et al. 2010; Abbott et al. 2013). One possible outcome is that selection may favor reproductive isolation of the hybrids, but in *Altica*, it seems unlikely that reproductive isolation would occur from the hybridization event itself. Moreover, the results point to a definitive role of CHCs in mediating mate choice in both parental species and hybrids. Past work in this system has shown that CHCs determine mate choice in *A. fragariae* and *A. viridicyanea* (Xue et al. 2016b), and here we show that this also extends to hybrids. Here we demonstrate that mate choice decisions reverse when we exchanged the CHCs between the parental species and F_1 hybrid females (Figure 4), indicating that males are making mating decisions based on the chemical signatures presented on the cuticle of females.

The chemical analysis also revealed interesting information about the genetics underlying CHC biosynthesis. For instance, the chemical profiles of the F_1 hybrids suggested a pattern of codominant inheritance, similar to the patterns observed by Cáceres et al. (2009). The F_1 hybrid profiles had all 28 compounds and these were found in similar quantities to one parental species or were intermediate to the parent phenotypes (Supplementary Table S1). There was also some indication that maternal effects may play a role in determining CHC biosynthesis. For example, there was a significant difference in CHCs between the reciprocal BC2 and BC3 crosses between *A. fragariae* and the F_1 hybrids. Because these beetles have similar host-plant feeding preferences (Xue et al. 2009b), the differences in CHC phenotypes are unlikely to be driven by changes in larval or adult substrate. Feeding preference, however, may have affected the CHC profiles in F_2 offspring. Canonical discriminant function analyses of F_2 individuals showed a correlation between feeding preference and CHC profile (Table 1). This correlation could be an outcome of plasticity in CHCs caused by feeding experience (Geiselhardt et al. 2012; Stojković et al. 2014; Xue et al. 2016a) or linkage of genes controlling feeding preference and CHC biosynthesis. Unfortunately, it is difficult to distinguish these hypotheses given the current data. Interestingly, the differences in the CHC profiles among the feeding groups in the F_2 hybrids (Figure 2) were of smaller magnitude than the differences observed between parents and F1 hybrids (Figure 1).

If codominant inheritance is common for CHC biosynthesis genes, then we would predict that mating signals of hybrid insects may often combine characteristics of both parental species. In some cases, hybrids may be unattractive to either parental species if their mating signals are intermediate or divergent (e.g. El-Shehaby et al. 2011; Segura et al. 2011), and this could cause reproductive isolation upon hybrid formation. Alternatively, an intermediate blend may make hybrids appealing to one or both parental species (e.g., Vander Meer and Lofgren 1985; Coyne et al. 1994). In the present study, the results suggest that hybrids were generally unattractive to the parental species, but this was not absolute. For example, male *A. viridicyanea* mated randomly when offered a choice between conspecific and BC1 females (Figure 3d), despite the differences observed in CHC profiles between these groups. Conversely, hybrids mated readily with the parental species as well as with other hybrids suggesting that there would be little prezygotic isolation. Because we detected all 28 compounds in each class of hybrids, the results indicate that mate choice in *Altica* is likely dosage dependent. Since the matings involving parental species did not strictly occur between conspecifics, hybrids are unlikely to become reproductively isolated without the evolution of strong reinforcement mechanisms.

Surprisingly, there are relatively few studies that have examined the CHC profiles of hybrid insects along with mating preference, so we have limited information on how this signaling mechanism promotes

or inhibits the speciation process. Studies have demonstrated that sometimes CHCs do not determine mating preference of hybrids (Noor and Coyne 1996). There can also be an asymmetry in mating success where hybrids are more successful mating with one parental species (Coyne et al. 1994; Noor and Coyne 1996; Pike et al. 2003) or hybrids mate with both parent species, but at an intermediate level to pure species pairings of conspecific versus heterospecific combinations (Liimatainen and Jallon 2007). Frequency of mating has also been shown to be connected to the blend of CHCs presented by hybrid individuals (Blows and Allan 1998; Liimatainen and Jallon 2007). Much of this work is restricted to *Drosophila* hybrids, and generally uses no-choice mating tests which are less informative about how hybrids might behave in the field when given an opportunity to select among mates. For these reasons, we feel that the present study adds an important advance in testing hybrid mating preference.

Together, the results suggest that decoupling of CHC mating signals and mating decisions based on these chemical signatures might make hybrid species formation restrictive in *Altica*, and we propose that this is a viable hypothesis for other species that employ CHCs as mating signals. Because CHCs are a dominant form of mate recognition and signaling that have been described in at least 7 insect orders (e.g. Peschke 1987; Jurenka et al. 1989; Blows and Allan 1998; Fukaya et al. 2000; Howard et al. 2003; Peterson et al. 2007; Geiselhardt et al. 2009, 2012; Silk et al. 2009; Ruther et al. 2011; Olaniran et al. 2013; Schwander et al. 2013; Simmons et al. 2014), segregation of the genes underlying CHC biosynthesis and preference for these chemicals may ultimately limit when hybridization causes reproductive isolation. Thus far, only the genetic and behavioral evidence for *Heliconius* butterflies and Darwin's finches convincingly demonstrates that hybrids are reproductively isolated from the parental species and that hybridization itself is the cause of reproductive isolation (Schumer et al. 2014; Lamichhaney et al. 2018). In *Heliconius*, hybrids have unique wing patterns that are used in mate choice, and hybrids have strong preferences to mate with butterflies bearing wing patterns that match their own (Mavárez et al. 2006; Melo et al. 2009). In Darwin's finches, hybrids were immediately reproductively isolated due to divergence in morphology and song that form the basis for mating decisions (Lamichhaney et al. 2018). For both of these examples, hybridization is directly linked to assortative mating and there is a tie between mate attraction signals and mate choice decisions. Although chemically mediated mating systems could also have strong linkages between an individual's phenotype and choice in mates ("self-referent phenotype matching") as has been shown in noninsect animals and birds (Hauber and Sherman 2001) and has been proposed in insects (Geiselhardt et al. 2012; Weddle et al. 2013), our results instead point to a decoupling of CHC mating signals and mate choice. This decoupling may foster backcrossing rather than reproductive isolation of hybrids.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

FUNDING

This study was supported by National Natural Science Foundation of China (Grant No. 31272302).

We thank David Althoff for comments on the manuscript, and Sara Magalhãs for earlier discussions.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by Huai-Jun Xue et al. (2018).

Handling editor: Leigh Simmons

REFERENCES

Abbott R, Albach D, Ansell S, Arntzen JW, Baird SJ, Bierne N, Boughman J, Brelsford A, Buerkle CA, Buggs R, et al. 2013. Hybridization and speciation. J Evol Biol. 26:229–246.

Abbott RJ, Hegarty MJ, Hiscock SJ, Brennan JL. 2010. Homoploid hybrid speciation in action. Taxon. 59:1375–1386.

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

Anderson M. 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26:32–46.

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

Anderson P, Anton S. 2014. Experience-based modulation of behavioural responses to plant volatiles and other sensory cues in insect herbivores. Plant Cell Environ. 37:1826–1835.

Anderson P, Sadek MM, Larsson M, Hansson BS, Thöming G. 2013. Larval host plant experience modulates both mate finding and oviposition choice in a moth. Anim Behav. 85:1169–1175.

Arnold ML. 1997. Natural hybridization and evolution. New York (NY): Oxford University Press.

Arnold ML. 2004. Transfer and origin of adaptations through natural hybridization: were Anderson and Stebbins right? Plant Cell. 16:562–570.

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

Blows MW. 2002. Interaction between natural and sexual selection during the evolution of mate recognition. Proc Biol Sci. 269:1113–1118.

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

Bush GL, Butlin RK. 2004. Sympatric speciation in insects. In: Dieckmann U, Doebeli M, Metz JAJ, Tautz D, editors. Adaptive speciation. Cambridge (UK): Cambridge University Press. p. 229–248.

Cáceres C, Segura DF, Vera MT, Wornoayporn V, Cladera JL, Teal P, Sapountzis P, Bourtzis K, Zacharopoulou A, Robinson AS. 2009. Incipient speciation revealed in *Anastrepha fraterculus* (Diptera: Tephritidae) by studies on mating compatibility, sex pheromones, hybridization, and cytology. Biol J Linn Soc. 97:152–165.

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

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.

Christophe N, Baudoin C. 1998. Olfactory preferences in two strains of wild mice, *Mus musculus musculus* and *Mus musculus domesticus*, and their hybrids. Anim Behav. 56:365–369.

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. Sunderland (MA): Sinauer & Associates.

Diao W, Mousset M, Horsburgh GJ, Vermeulen CJ, Johannes F, van de Zande L, Ritchie MG, Schmitt T, Beukeboom LW. 2016. Quantitative trait locus analysis of mating behavior and male sex pheromones in *Nasonia* wasps. G3 (Bethesda). 6:1549–1562.

Dietemann V, Peeters C, Liebig J, Thivet V, Hölldobler B. 2003. Cuticular hydrocarbons mediate discrimination of reproductives and non-reproductives in the ant *Myrmecia gulosa*. Proc Natl Acad Sci USA. 100:10341–10346.

Dittrich-Reed DR, Fitzpatrick BM. 2013. Transgressive hybrids as hopeful monsters. Evol Biol. 40:310–315.

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

Drès 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.

El-Shehaby M, Salama MS, Brunner E, Heinze J. 2011. Cuticular hydrocarbons in two parapatric species of ants and their hybrid. Integr Zool. 6:259–265.
Ferveur JF. 2005. Cuticular hydrocarbons: their evolution and roles in Drosophila pheromonal communication. Behav Genet. 35:279–295.
Fukaya M, Akino T, Yasuda T, Wakamura S, Satoda S, Senda S. 2000. Hydrocarbon components in contact sex pheromone of the white-spotted longicorn beetle, *Anoplophora malasiaca* (Thomson) (Coleoptera: Cerambycidae) and pheromonal activity of synthetic hydrocarbons. Entomol Sci. 3:211–218.
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.
Gompert Z, Fordyce JA, Forister ML, Shapiro AM, Nice CC. 2006. Homoploid hybrid speciation in an extreme habitat. Science. 314:1923–1925.
Goulet BE, Roda F, Hopkins R. 2017. Hybridization in plants: old ideas, new techniques. Plant Physiol. 173:65–78.
Grant BR, Grant PR. 2008. Fission and fusion of Darwin's finches populations. Philos Trans R Soc Lond B Biol Sci. 363:2821–2829.
Gross BL, Rieseberg LH. 2005. The ecological genetics of homoploid hybrid speciation. J Hered. 96:241–252.
Hammer Ø, Harper D, Ryan P. 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electron. 4:1–9.
Hauber ME, Sherman PW. 2001. Self-referent phenotype matching: theoretical considerations and empirical evidence. Trends Neurosci. 24:609–616.
Hedrick PW. 2013. Adaptive introgression in animals: examples and comparison to new mutation and standing variation as sources of adaptive variation. Mol Ecol. 22:4606–4618.
Helms Cahan S, Keller L. 2003. Complex hybrid origin of genetic caste determination in harvester ants. Nature. 424:306–309.
Howard RW, Jackson LL, Banse H, Blows MW. 2003. Cuticular hydrocarbons of *Drosophila birchii* and *D. serrata*: identification and role in mate choice in *D. serrata*. J Chem Ecol. 29:961–976.
Ingleby FC. 2015. Insect cuticular hydrocarbons as dynamic traits in sexual communication. Insects. 6:732–742.
Izzo A, Wells M, Huang Z, Tibbetts E, 2010. Cuticular hydrocarbons correlate with fertility, not dominance, in a paper wasp, Polistes dominulus. Behav Ecol Sociobiol. 64:857–864.
Jenkins TM, Braman SK, Chen Z, Eaton TD, Pettis GV, Boyd DW. 2009. Insights into flea beetle (Coleoptera: Chrysomelidae: Galerucinae) host specificity from concorndant mitochondrial and nuclear DNA phylogenies. Ann Entomol Soc Am. 102:386–395.
Jurenka RA, Schal C, Burns E, Chase J, Blomquist GJ. 1989. Structural correlation between cuticular hydrocarbons and female contact sex pheromone of German cockroach *Blattella germanica* (L.). J Chem Ecol. 15:939–949.
Kather R, Martin SJ. 2012. Cuticular hydrocarbon profiles as a taxonomic tool: advantages, limitations and technical aspects. Physiol Entomol. 37:25–32.
Keppner EM, Prang M, Engel KC, Ayasse M, Stökl J, Steiger S. 2017. Beyond cuticular hydrocarbons: chemically mediated mate recognition in the subsocial burying beetle *Nicrophorus vespilloides*. J Chem Ecol. 43:84–93.
Kunte K, Shea C, Aardema ML, Scriber JM, Juenger TE, Gilbert LE, Kronforst MR. 2011. Sex chromosome mosaicism and hybrid speciation among tiger swallowtail butterflies. PLoS Genet. 7:e1002274.
Lamichhaney S, Han F, Webster MT, Andersson L, Grant BR, Grant PR. 2018. Rapid hybrid speciation in Darwin's finches. Science. 359:224–228.
Laroche A, DeClerck-Floate R, LeSage L, Floate K, Demeke T. 1996. Are *Altica carduorum* and *Altica cirsicola* (Coleoptera: Chrysomelidae) different species? Implications for the release of *A. cirsicola* for the biocontrol of Canada thistle in Canada. Biol Control. 6:306–314.
Liimatainen JO, Jallon JM. 2007. Genetic analysis of cuticular hydrocarbons and their effect on courtship in *Drosophila virilis* and *D. lummei*. Behav Genet. 37:713–725.
Mallet J. 2005. Hybridization as an invasion of the genome. Trends Ecol Evol. 20:229–237.
Mallet J. 2007. Hybrid speciation. Nature. 446:279–283.
Mallet J, Beltrán M, Neukirchen W, Linares M. 2007. Natural hybridization in heliconiine butterflies: the species boundary as a continuum. BMC Evol Biol. 7:28.
Matsubayashi KW, Ohshima I, Nosil P. 2010. Ecological speciation in phytophagous insects. Entomol Exp Appl. 134:1–27.
Mavárez J, Linares M. 2008. Homoploid hybrid speciation in animals. Mol Ecol. 17:4181–4185.
Mavárez J, Salazar CA, Bermingham E, Salcedo C, Jiggins CD, Linares M. 2006. Speciation by hybridization in *Heliconius* butterflies. Nature. 441:868–871.
Melo MC, Salazar C, Jiggins CD, Linares M. 2009. Assortative mating preferences among hybrids offers a route to hybrid speciation. Evolution. 63:1660–1665.
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.
Nice CC, Gompert Z, Fordyce JA, Forister ML, Lucas LK, Buerkle CA. 2013. Hybrid speciation and independent evolution in lineages of alpine butterflies. Evolution. 67:1055–1068.
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.
Noor MA, Coyne JA. 1996. Genetics of a difference in cuticular hydrocarbons between *Drosophila pseudoobscura* and *D. persimilis*. Genet Res. 68:117–123.
Olaniran OA, Sudhakar AV, Drijfhout FP, Dublon IA, Hall DR, Hamilton JG, Kirk WD. 2013. A male-predominant cuticular hydrocarbon, 7-methyltricosane, is used as a contact pheromone in the western flower thrips Frankliniella occidentalis. J Chem Ecol. 39:559–568.
Otte T, Hilker M, Geiselhardt S. 2016. Phenotypic plasticity of mate recognition systems prevents sexual interference between two sympatric leaf beetle species. Evolution. 70:1819–1828.
Peschke K. 1987. Cuticular hydrocarbons regulate mate recognition, male aggression, and female choice of the rove beetle, *Aleochara curtula*. J Chem Ecol. 13:1993–2008.
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.
Pfennig KS, Kelly AL, Pierce AA. 2016. Hybridization as a facilitator of species range expansion. Proc R Soc Lond B. 283:20161329.
Pike N, Wang WY, Meats A. 2003. The likely fate of hybrids of *Bactrocera tryoni* and *Bactrocera neohumeralis*. Heredity (Edinb). 90:365–370.
Pomonis J, Nelson D, Fatland C. 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.
Reid CA, Beatson M. 2015. Disentangling a taxonomic nightmare: a revision of the Australian, Indomalayan and Pacific species of *Altica* Geoffroy, 1762 (Coleoptera: Chrysomelidae: Galerucinae). Zootaxa. 3918:503–551.
Rieseberg LH, Archer MA, Wayne RK. 1999. Transgressive segregation, adaptation and speciation. Heredity (Edinb). 83:363–372.
Rolán-Alvarez E, Caballero A. 2000. Estimating sexual selection and sexual isolation effects from mating frequencies. Evolution. 54:30–36.
Ruther J, Döring M, Steiner S. 2011. Cuticular hydrocarbons as contact sex pheromone in the parasitoid Dibrachys cavus. Entomol Exp Appl. 140:59–68.
Schumer M, Rosenthal GG, Andolfatto P. 2014. How common is homoploid hybrid speciation? Evolution. 68:1553–1560.
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.
Schwarz D, Matta BM, Shakir-Botteri NL, McPheron BA. 2005. Host shift to an invasive plant triggers rapid animal hybrid speciation. Nature. 436:546–549.
Schwarz D, Shoemaker KD, Botteri NL, McPheron BA. 2007. A novel preference for an invasive plant as a mechanism for animal hybrid speciation. Evolution. 61:245–256.
Scriber JM. 2011. Impacts of climate warming on hybrid zone movement: geographically diffuse and biologically porous "species borders". Insect Sci. 18:121–159.
Scriber JM, Ording GJ. 2005. Ecological speciation without host plant specialization; possible origins of a recently described cryptic *Papilio* species. Entomol Exp Appl. 115:247–263.

Seehausen O. 2004. Hybridization and adaptive radiation. Trends Ecol Evol. 19:198–207.
Segura DF, Vera MT, Rull J, Wornoayporn V, Islam A, Robinson AS. 2011. Assortative mating among *Anastrepha fraterculus* (Diptera: Tephritidae) hybrids as a possible route to radiation of the *fraterculus* cryptic species complex. Biol J Linn Soc. 102:346–354.
Silk PJ, Ryall K, Barry Lyons D, Sweeney J, Wu J. 2009. A contact sex pheromone component of the emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). Naturwissenschaften. 96:601–608.
Simmons LW, Thomas ML, Gray B, Zuk M. 2014. Replicated evolutionary divergence in the cuticular hydrocarbon profile of male crickets associated with the loss of song in the Hawaiian archipelago. J Evol Biol. 27:2249–2257.
Soltis PS, Liu X, Marchant DB, Visger CJ, Soltis DE. 2014. Polyploidy and novelty: Gottlieb's legacy. Philos T Roy Soc B. 369:20130351.
Soltis PS, Soltis DE. 2009. The role of hybridization in plant speciation. Annu Rev Plant Biol. 60:561–588.
Sorvari J, Theodora P, Turillazzi S, Hakkarainen H, Sundstrom L. 2007. Food resources, chemical signaling, and nest mate recognition in the ant Formica aquilonia. Behav Ecol. 19:441–447.
Stebbins GL. 1959. The role of hybridization in evolution. P Am Philos Soc. 103:231–251.
Steiger S, Whitlow S, Peschke K, Müller JK. 2009. Surface chemicals inform about sex and breeding status in the biparental burying beetlenicrophorus vespilloides. Ethology. 115:178–185.
Stireman JO III, Nason JD, Heard SB. 2005. Host-associated genetic differentiation in phytophagous insects: general phenomenon or isolated exceptions? Evidence from a goldenrod-insect community. Evolution. 59:2573–2587.
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.
Vander Meer R, Lofgren C. 1985. Biochemical evidence for hybridization in fire ants. Fla Entomol. 68:501–506.
Van Homrigh A, Higgie M, McGuigan K, Blows MW. 2007. The depletion of genetic variance by sexual selection. Curr Biol. 17:528–532.
Velthuis B-J, Yang W, Van Opijnen T, Werren JH. 2005. Genetics of female mate discrimination of heterospecific males in Nasonia (Hymenoptera, Pteromalidae). Anim Behav. 69:1107–1120.
Weddle CB, Steiger S, Hamaker CG, Ower GD, Mitchell C, Sakaluk SK, Hunt J. 2013. Cuticular hydrocarbons as a basis for chemosensory self-referencing in crickets: a potentially universal mechanism facilitating polyandry in insects. Ecol Lett. 16:346–353.
Wood T, Tilmon K, Shantz A, Harris C. 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.
Xue HJ, Segraves KA, Wei J, Zhang B, Nie RE, Li WZ, Yang XK. 2018. Data from: cuticular hydrocarbon mating signals block hybrid speciation in a flea beetle. Dryad Digital Repository. http://dx.doi.org/10.5061/dryad.3s58201.
Xue HJ, Wei JN, Magalhães S, Zhang B, Song KQ, Liu J, Li WZ, Yang XK. 2016a. Contact pheromones of 2 sympatric beetles are modified by the host plant and affect mate choice. Behav Ecol. 27:895–902.
Xue HJ, Zhang B, Segraves KA, Wei JN, Nie RE, Song KQ, Liu J, Li WZ, Yang XK. 2016b. Contact cuticular hydrocarbons act as a mating cue to discriminate intraspecific variation in *Altica* flea beetles. Anim Behav. 111:217–224.
Yakimowski SB, Rieseberg LH. 2014. The role of homoploid hybridization in evolution: a century of studies synthesizing genetics and ecology. Am J Bot. 101:1247–1258.
Zhang B, Xue HJ, Song KQ, Liu J, Li WZ, Nie RE, Yang XK. 2014. Male mate recognition via cuticular hydrocarbons facilitates sexual isolation between sympatric leaf beetle sister species. J Insect Physiol. 70:15–21.