Identification and Characterization of Cuticular Hydrocarbons from a Rapid Species Radiation of Hawaiian Swordtailed Crickets (Gryllidae: Trigonidiinae: *Laupala*)

Sean P. Mullen · Jocelyn G. Millar · Coby Schal · Kerry L. Shaw

Abstract A previous investigation of cuticular hydrocarbon variation among Hawaiian swordtail crickets (genus *Laupala*) revealed that these species differ dramatically in composition of cuticular lipids. Cuticular lipid extracts of *Laupala* species sampled from the Big Island of Hawaii also possess a greatly reduced number of chemicals (as evidenced by number of gas chromatography peaks) relative to ancestral taxa sampled from the geologically older island of Maui. One possible explanation for this biogeographic pattern is that reduction in chemical diversity observed among the Big Island taxa represents the loss of ancestral hydrocarbons found on Maui. To test this hypothesis, we characterized and identified the structures of cuticular hydrocarbons for seven species of Hawaiian *Laupala*, two from Maui (ancestral) and five from the Big Island of Hawaii (derived) by using gas chromatography-mass spectrometry. Big Island *Laupala* possessed a reduced number of alkenes as well as a reduction in the diversity of methyl-branch positions relative to species sampled from Maui (ancestral), thus supporting our hypothesis of a founder-induced loss of chemical diversity. The reduction in diversity of ancestral hydrocarbons was more severe within one of the two sister lineages on the Big Island, suggesting that post-colonizing processes, such as drift or selection, also have influenced hydrocarbon evolution in this group.

Keywords Chemical communication · Speciation · Mate recognition · Pheromones · *Laupala*

S. P. Mullen
Department of Biological Sciences, Lehigh University, Bethlehem, PA 18015, USA

S. P. Mullen (✉) · K. L. Shaw
Department of Biology, University of Maryland, College Park, MD 02742, USA
e-mail: sem307@lehigh.edu

J. G. Millar
Department of Entomology, University of California, Riverside, CA 92521, USA

C. Schal
Department of Entomology, North Carolina State University, Raleigh, NC 27695, USA

K. L. Shaw
Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14850, USA

Introduction

Endemic Hawaiian swordtail crickets of the genus *Laupala* are a well-characterized species radiation that has been intensively studied as a model system for the evolution of acoustic communication and speciation (Otte 1994; Shaw 1996, 1999, 2000; Shaw and Herlihy 2000; Mendelson and Shaw 2002; Shaw et al. 2007). Phylogenetic evidence suggests that speciation in *Laupala* has proceeded by colonization of newly emerged volcanic islands and subsequent rapid intra-island radiations (Mendelson and Shaw 2005). Unlike examples of ecologically-driven speciation (Funk 1998; Via 1999; Rundle et al. 2000; Nosil et al. 2002), closely related species of *Laupala* have diversified in secondary sexual traits but remain morphologically and ecologically similar.

Species boundaries in *Laupala* have been hypothesized based on differences in acoustic signaling and, to some extent, small metric differences in male genitalia (Otte 1994; Shaw 2000). Molecular evidence supports these delineations (Shaw 1999, 2002; Parsons and Shaw 2001;

Mendelson and Shaw 2002, 2005). Multiple species often occur sympatrically (Otte 1994; Shaw 2000), but males of sympatric species always call with distinct pulse rates (Otte 1994; Shaw 1999, 2000; Parsons and Shaw 2001). Evidence from phonotaxis trials has established that females are preferentially attracted to conspecific pulse rates (Shaw 2000; Shaw and Herlihy 2000; Mendelson and Shaw 2002), thereby contributing to the maintenance of barriers to gene flow in sympatry.

These data suggest that divergence among species of *Laupala* is driven by evolution of male calling songs and that sexual selection on this trait may be responsible for the rapid speciation rates observed among these crickets. Although recent behavioral work suggests that female preference for conspecific pulse rates operates as a long-range signal, females that are not given an opportunity to choose males based on long-range acoustics evidently rely upon additional sexual recognition signals during close-range courtship (Mendelson and Shaw 2006). Reliance upon multiple mate-recognition signals during sexual communication is widespread among animals (Johnstone 1996; Rowe 1999; Candolin 2003), and therefore, it is possible that *Laupala* utilize diverse chemical, tactile, or vibratory cues during courtship and mating (e.g., *Drosophila*, Boake 2005). Specifically, the use of chemical mate-recognition signals has been demonstrated in other cricket species (Balakrishnan and Pollack 1997; Treganza and Wedell 1997), and cuticular compounds may function as close-range sex pheromones in the *Laupala* system as well.

To investigate the possible importance of contact pheromones in *Laupala*, Mullen et al. (2007) examined patterns of chemical variation in cuticular lipids among species of *Laupala* from Maui and the Big Island of Hawaii. Their results demonstrated that (1) significant differences in cuticular lipids exist between males and females, (2) there has been rapid and dramatic evolution of cuticular lipid composition among species in this genus, and (3) there has been a significant reduction in the complexity of cuticular lipid profiles among closely related species from the Big Island of Hawaii, as compared to two outgroup species from Maui. Detailed knowledge of the chemical differences between these two island faunas is important to our understanding of the evolution of cuticular hydrocarbon (CHC) biosynthesis in these and other insects and, furthermore, may provide insights into the evolutionary processes that shape patterns of lipid diversity among species. Specifically, chemical identification will determine whether the reduced number of gas chromatography peaks observed for species on the Big Island, relative to outgroup taxa on Maui (Mullen et al. 2007), reflects a reduction in the chain-length diversity of saturated or unsaturated hydrocarbons, differences in methylation patterns for specific hydrocarbons, or both. This would allow us to test

the hypothesis that Big Island *Laupala* species have experienced a founder-induced loss of ancestral cuticular lipid variation.

Methods

Sampling To identify the major cuticular lipid components identified by Mullen et al. (2007) and to test biogeographic hypotheses about patterns of chemical variation, extracts of cuticular lipids of males and females of seven *Laupala* species ($N=14$) were analyzed by coupled gas chromatography-mass spectrometry (GC-MS). Specifically, we sampled two ancestral species of these crickets from the island of Maui (*Laupala makaio* and *Laupala orientalis*) and five derived species from two distinct clades on the Big island of Hawaii (*Laupala pruna* and *Laupala kohalensis* vs *Laupala nigra*, *Laupala paranigra*, and *Laupala kona*; see Mendelson and Shaw 2005). Individual crickets used for GC-MS analysis were collected during 2005 and stored frozen until used for sample preparation.

Extraction and Analysis of Cuticular Lipids Lipids were extracted with high-performance liquid chromatography-grade hexane. All glassware was pre-rinsed three times with hexane to remove possible contaminants and allowed to dry in a fume hood for 5–10 min before beginning extractions. Between each extraction, forceps were rinsed in three separate containers of hexane to avoid cross-contamination of samples. Each cricket carcass was wiped with a wet Kimwipe® to remove any external debris and then submerged in 1 ml hexane for 5 min in a glass vial with a Teflon-lined cap. Extracts were concentrated to ~200 μl under a stream of nitrogen, and 1 μl aliquots were analyzed by GC-MS with an Agilent 6890 GC interfaced to an Agilent 5973 mass selective detector operated in electron impact ionization mode (70 eV). The GC was fitted with a 30 m×0.25 mm I.D. DB-5MS column (J &W Scientific, Folsom, CA, USA), programmed from 100°C for 1 min, 10°C/min to 280°C and held for 20 min. Helium was the carrier gas. The injector and transfer line temperature was 280°C, the ion source temperature was 250°C, and the quadrupole temperature 200°C. Injections were made in splitless mode, with purge on at 0.5 min.

Straight-chain saturated alkanes were identified by their molecular ions and from comparisons of retention times and mass spectra with those of authentic standards. Methyl-branched compounds were identified from their Kovat's retention indices relative to straight chain hydrocarbons, in combination with diagnostic ions from enhanced fragmentations at methyl branch points (Nelson 1993; Nelson and Blomquist 1995; Carlson et al. 1998). Unsaturated alkenes were identified by molecular weight, from retention times

Table 1 Cuticular hydrocarbons present in various *Laupala* species

Retention Time	Compound I.D.	*L. orientalis*	*L. makaio*	*L. pruna*	*L. kohalensis*	*L. paranigra*	*L. nigra*	*L. kona*	Diagnostic Fragments
16.82	2Me-C22	+							281, 309 (324)
17.15	C23	+							324
17.79	3Me-C23	+							309 (338)
18.02	C24	+							338
18.55	C25 monoene						+		350
18.56	2Me-C24	+	+	+	+	+	+	+	309, 337, 352
18.68	C25 monoene	+	+					+	350
18.66–18.68	C25 di- and triene							++	346, 348
18.74	C25 monoene	+	+						350
18.83	C25 diene							+	348
18.86	C25	+	+	+	+			+	352
19.17	9Me-C25	+							140/252 (366)
19.21	7Me-C25	+							112/280, 351 (366)
19.28	5Me-C25	+	+	+					85/309, 351 (366)
19.39	2Me-C25	+		+					323, 351 (366)
19.49	3Me-C25	+	+	+	+				337, 351 (366)
19.49	C26 mono- and diene	+						+	364, 362
19.72	C26			+					366
20.21	C27 triene	+	+			+		+	374
20.31	C27 monoene						+		378
20.33	2Me-C26	++	++	++	++	+	++	+	337, 365, 380
20.36	C27 diene							+	376
20.48–20.54	C27 diene	+++	++		+			++	376
20.53	C27 monoene		++	+	+				378
20.63	C27 monoene		+	+	+				378
20.68	C27		+	+					380
21.09	7Me-C27	+							112/309 (394)
21.20	5Me-C27		+						85, 337 (394)
21.46	3Me-C27		+	+	+				365 (394)
22.36	C29 tetraene		++						400
22.42	C29 diene	+							404
22.47	C29 triene					+			402
22.52	2Me-C28	+	+	+	+	+	+	+	365, 393 (408)
22.59	C29 diene							+	404
22.63	C29 monoene	+							406
22.74	C29 diene	+	++	+		+	+		404
22.86	C29 monoene		+	+					406
23.64	7Me-C29		+	+					112, 337 (422)
23.80	C30 triene					+			416
25.28	C31 monoene				+				434
25.40	C31 monoene				+				434
25.44	C31 tetraene		+	+					428
25.51	C31 trienes					+++	+		430
25.61	C31 diene		+	+			+		432
25.69	C31 monoene				+				434
25.87	C31 triene				+				430
26.01	C31 diene				+				432
27.65	Polyene?			+					
30.02	Polyene?		+						
30.09	C33 triene					++	+		458
30.09	C33 tri-, tetra-, and pentaene						+++		454, 456, 458
30.28	C33 tri- and tetraene			+++	++				456, 458

+ Peaks contributing <10% of total area; ++, 10–50%; and +++, >50%.

slightly shorter than those of the corresponding straight-chain saturated alkanes, and their characteristic patterns of ions with masses 2, 4, or 6 mass units less than the corresponding ions in the spectra of straight-chain alkanes, for monoenes, dienes, and trienes, respectively. The positions and geometries of double bonds were not determined.

Results

Mass spectral analyses indicated that the cuticular lipids of *Laupala* species consisted of a relatively small number of saturated and unsaturated hydrocarbons (c.f., other insects; Howard and Blomquist 2005), varying in chain length from C_{22} to C_{33}. We found considerable variability among species (Table 1, Fig. 1), with the greatest diversity in chain length observed for *L. orientalis* (Maui). In particular, this species possessed a large variety of differentially methylated pentacosanes as well as several different unsaturated C_{27} and C_{29} alkenes. *L. makaio*, the other species sampled from Maui and the sister taxon to *L. orientalis* (Mendelson and Shaw 2005), similarly showed a large variety of hydrocarbons but differed from *L. orientalis* in possessing longer-chain alkenes (C_{29} and C_{31}), and lacking C_{22}–C_{24} alkanes. These two species also showed radically different relative abundances of particular hydrocarbons, with the CHC profile of *L. orientalis* predominantly (~66%) composed of C_{27} dienes.

Patterns of hydrocarbon diversity among the Big Island species were similar to *L. makaio* but typically displayed a marked reduction in hydrocarbon diversity. We found fairly similar hydrocarbon compositions for *L. kohalensis* and *L. pruna*, but these two species varied in the relative compositions of C_{31}–C_{33} tri- and tetraenes. In contrast, the remaining three species, *L. kona*, *L. paranigra*, and *L. nigra*, each showed a drastic reduction in both the number of hydrocarbon-chain lengths and the variety of unsaturated alkenes. The two most closely related species, *L. paranigra* and *L. nigra*, differed mainly in the relative abundance of C_{31} vs C_{33}. C_{33} alkenes made up ~70% of the CHC abundance for *L. nigra*, whereas a single C_{31} triene represented ~65% of the CHC observed for *L. paranigra*. A slightly larger number of *n*-alkenes were observed for *L. kona*, with no single-hydrocarbon type comprising more than 30% of the total.

Differences between Males and Females Our previous GC analyses of CHC variation (Mullen et al. 2007) demonstrated that significant qualitative differences existed among all species sampled. In addition, we showed that males and females of *L. makaio* had different cuticular lipid compo-

sitions. One of the goals of the current study was to assay each species for both qualitative and quantitative differences in individual hydrocarbons between males and females. In line with our expectations, we found that *L. makaio* males possessed more C_{31} tetraene than females (Fig. 2; males=48% total abundance vs 5% in females; $P<0.001$). Males and females of this species also appeared
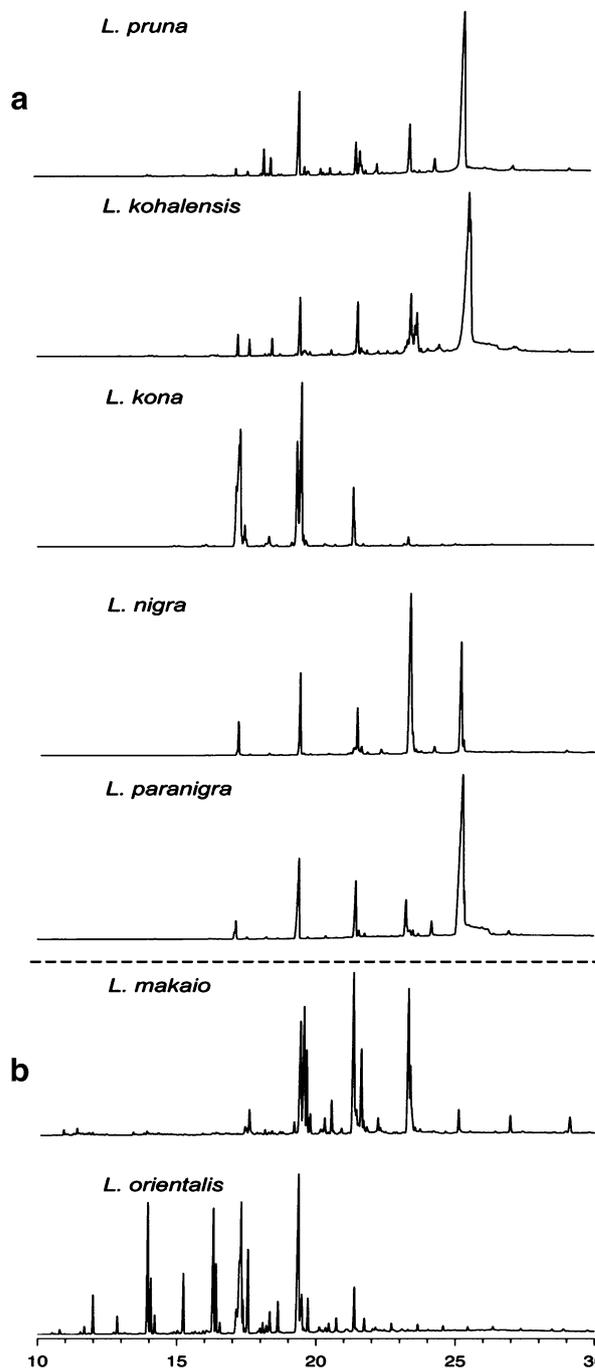

Fig. 1 Gas chromatograms showing differences in epicuticular lipids among Hawaiian *Laupala* sampled from the **a** Big Island of Hawaii (five species) and **b** Maui (two species); adapted with permission from Mullen et al. (2007)

Fig. 2 Total ion chromatograms of extracts of cuticular hydrocarbons for various species of *Laupala*. Chromatograms from females are shown on the *top* and from males on the *bottom*
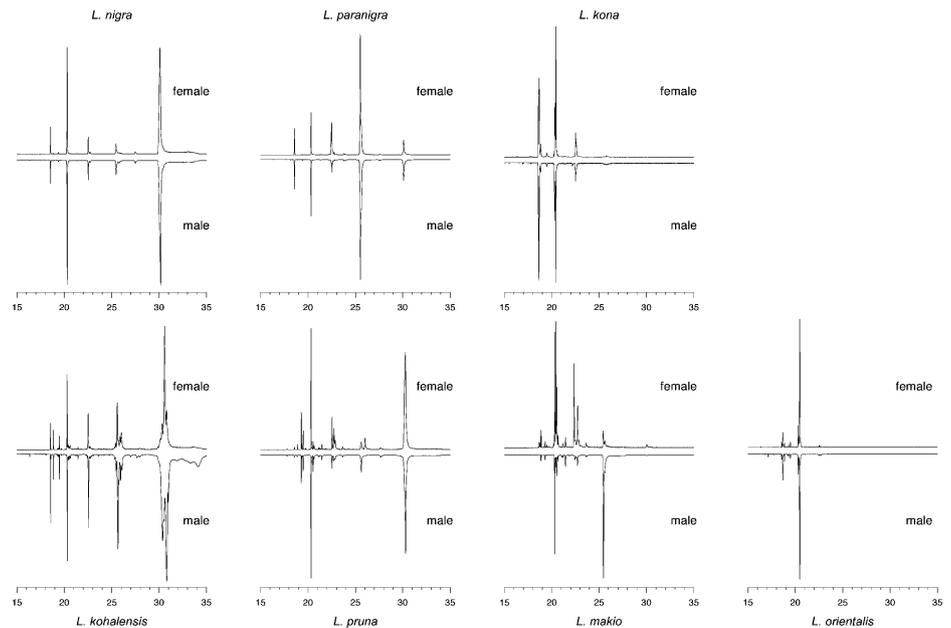
to differ in the relative ratios of C_{27} alkenes. Surprisingly, males and females of the other species sampled showed little qualitative variation (Fig. 2), even though our previous statistical analysis found significant quantitative variation between males and females when grouped by sex (Mullen et al. 2007).

Discussion

We tested our a priori hypothesis about hydrocarbon evolution in this group, namely, that the reduction in chemical diversity among the Big Island taxa represents the loss of modifications to the ancestral hydrocarbons found on the Maui taxa rather than the de novo origin of chain-length diversity. The chemical identifications supported this hypothesis and indicated that the differences between these two island faunas were due primarily to a

decrease in the number of dienic and trienic hydrocarbons on the Big Island taxa compared to the crickets on Maui (Figs. 1 and 2, Table 1). Furthermore, the two ancestral species from Maui also showed a greater diversity of methyl branch positions for a given chain-length hydrocarbon than did species from the Big Island. The differences among species are highlighted by the fact that we identified only two hydrocarbons in common for all the *Laupala* species: 2-Me-C_{26} and 2-Me-C_{28}.

The striking differences between these two lineages in their hydrocarbon composition must reflect modifications in the biosynthesis of hydrocarbon. Straight-chain alkanes and *n*-alkenes are formed via elongation of fatty acyl-CoAs, which are converted to hydrocarbons one carbon shorter in length by decarboxylation (Blomquist 2003; Howard and Blomquist 2005). Chain length is regulated by specific fatty acyl-CoA elongases. Furthermore, methyl-branched hydrocarbons are derived from substitution of methylmalonyl-CoA for malonyl-CoA at specific chain positions during
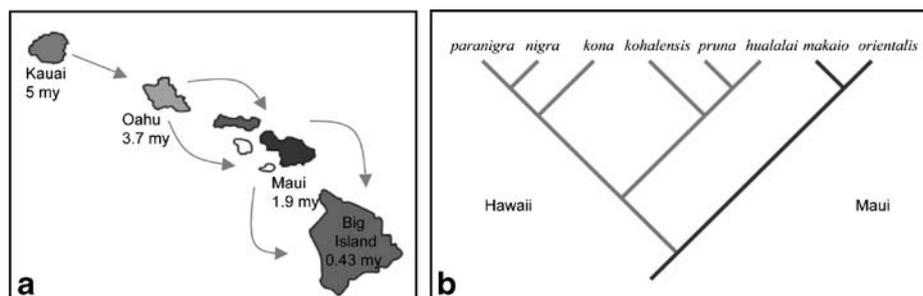

Fig. 3 **a** History of lineage splitting and intra-island radiations within the Hawaiian *Laupala* genus. **b** Simplified cladogram of the evolutionary relationships among the 'pacifica' group species sampled for this study based on Mendelson and Shaw's (2005) amplification fragment length polymorphism phylogeny

elongation (Howard and Blomquist 2005). Although it is unclear how the number and position of methyl-branching units is regulated, work on houseflies (*Musca domestica*, Gu et al. 1997) and the German cockroach (*Blattella germanica*, Gu et al. 1993) suggests that microsomal fatty-acid synthase (FAS) is involved in the production of methyl-branched fatty-acid precursors (Blomquist and Vogt 2003). Thus, the relatively greater diversity of methyl-branched alkanes and alkenes among the Maui species may involve differential regulation of FAS in this lineage.

Differences in Cuticular Composition among Laupala Species on the Big Island of Hawaii A previously unrecognized pattern that emerged as a result of the current analysis was the difference in the composition of cuticular hydrocarbons among the taxa of the Big Island 'pacifica' species group. This lineage is the most rapidly diverging clade in a rapid species radiation (Mendelson and Shaw 2005). Previous phylogenetic work on these species supports close evolutionary relationships among *L. kohalensis*, *L. pruna*, and *L. hualalai* (Fig. 3). These three species form a recently diverged lineage that is most closely related to the remaining Big Island 'pacifica' group species (*L. kona*, *L. paranigra*, and *L. nigra*). Our results indicate that the *L. kohalensis–L. pruna* clade (*L. hualalai* was not sampled) is more diverse with respect to methyl-branch positions than the *L. kona–L. paranigra–L. nigra* clade. In fact, the former two species possess hydrocarbons that are similar to those found on *L. makaio* on Maui, although the relative amounts of each hydrocarbon vary considerably. The lesser hydrocarbon diversity in the latter species clade suggests that a dramatic reduction in the complexity and diversity of cuticular lipids has occurred in this lineage relative to the remaining Big Island species of the 'pacifica' group.

It is commonly thought that the primary function of insect cuticular hydrocarbons is to regulate water balance (Hadley 1984). Therefore, one possible explanation for the differences in cuticular lipid composition among species on the Big Island is natural selection acting in different environments (e.g., wet vs dry). However, this does not appear to be the case because each of these species occurs in a range of habitats on the Big Island. Furthermore, previous work suggested that closely related, allopatrically distributed species are more similar in their hydrocarbon profiles than are sympatric taxa that share overlapping habitats and ranges (Mullen et al. 2007). An alternative possibility for the variation in cuticular hydrocarbons is that some of these compounds serve as chemical mate-recognition signals and that differences among species reflect lineage-specific adaptations to sexual selection driven by female choice. It is well-recognized that cuticular hydrocarbons serve as recognition signals in arthropods (Howard and Blomquist 2005). While direct behavioral assays have not yet been completed, our preliminary behavioral observations (unpublished data) suggest that courtship between males and females of the species described here involves chemical communication.

Courtship in *Laupala* takes place over the course of several hours and involves a complex series of behavioral elements that are repeated multiple times (Shaw and Lugo 2001). Each courtship unit culminates in the passage of a spermless "microspermatophore" to the female, which is consumed by the female before the next courtship bout. Mating terminates only after the transfer of a much larger "macrospermatophore", that contains all of the sperm for a given mating (Shaw and Khine 2004; de Carvalho and Shaw 2005). Throughout courtship, males emit the same song used for long-range calling. Behaviorally, male singing during courtship is always preceded by antennal contact, and production of each spermatophore occurs while males and females "face" each other and engage in extensive antennal interactions (Shaw and Lugo 2001; Shaw and Khine 2004). Thus, chemical and/or tactile signals are likely exchanged during courtship, and mate choice may be a function of multiple mating signals, rather than simply a byproduct of long-range attraction of females to conspecific male songs.

In summary, the biogeoraphic differences in CHC diversity among lineages of Hawaiian *Laupala* is broadly consistent with a founder-induced loss of biochemical variation resulting from the colonization history of the Big Island taxa. However, the differences observed between the two major clades of these crickets on the Big Island suggest that post-colonization processes, such as drift or selection, have also played an important role in the evolution of CHC diversity in this system. Further work will focus on complete identification of the hydrocarbons along with behavioral assays to test whether these hydrocarbons function as mate-recognition cues.

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