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Colony Fusion in Argentine Ants is Guided by Worker and Queen Cuticular Hydrocarbon Profile Similarity

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Abstract Introduced populations of the Argentine ant, *Linepithema humile*, have experienced moderate to severe losses of genetic diversity, which may have affected nestmate recognition to various degrees. We hypothesized that cuticular hydrocarbons (CHC) serve as nestmate recognition cues, and facilitate colony fusion of unrelated *L. humile* colonies that share similar CHC profiles. In this study, we paired six southeastern U.S. *L. humile* colonies in a 6-month laboratory fusion assay, and determined if worker and queen CHC profile similarity between colonies was associated with colony fusion and intercolony genetic similarity. We also compared worker and queen CHC profiles between fused colony pairs and unpaired controls to determine if worker and queen chemical profiles changed after fusion. We found that colony fusion correlated with the CHC similarity of workers and queens, with the frequency of fusion increasing with greater CHC profile similarity between colonies. Worker and queen CHC profile similarity between colonies also was associated with genetic similarity between colonies. Queen CHC profiles in fused colonies appeared to be a mix of the two colony phenotypes. In contrast, when only one of the paired colonies survived, the CHC profile of the surviving queens did not diverge from that of the colony of origin. Similarly, workers in non-fused colonies maintained their colony-specific CHC, whereas in fused colonies the worker CHC profiles were intermediate between those of the two colonies. These results suggest a role for CHC in regulating interactions among mutually aggressive *L. humile* colonies, and demonstrate that colony fusion correlates with both genetic and CHC similarities. Further, changes in worker and queen chemical profiles in fused colonies suggest that CHC plasticity may sustain the cohesion of unrelated *L. humile* colonies that had fused.

Keywords Argentine ant · *Linepithema humile* · Nestmate recognition · Cuticular hydrocarbons · Intraspecific aggression · Colony fusion · Supercoloniality

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Introduction

Cuticular hydrocarbons (CHC) play an important role in insect physiology and chemical communication. They constitute a considerable portion of insect cuticular lipids that prevent insect desiccation by reducing cuticular permeability (Wigglesworth 1945; Hadley 1980). In addition, CHC have important semiochemical functions as sex attractants, contact pheromones, aggregation and alarm pheromones, chemical defenses, kairomonal cues for parasites, and inter- and intra-specific recognition cues (Howard and Blomquist 2005). Most social insects that have been investigated, including ants, bees, wasps, and termites, have developed chemical communication systems in which CHC are used as cues that enable recognition of conspecifics (Bagnères et al. 1991a, b; Takahashi and Gassa 1995), nestmates (Liang and Silverman 2000; Wagner et al. 2000; Ruther et al. 2002), and castes (Bonavita-Cougourdan et al. 1989).

In social insect species with large colonies, queens and workers seem to be labeled by a more or less homogenous recognition "odor", or colony gestalt label, in which each colony member bears a mixture of cues representative of the variation among members of the colony (Errard and Jallon 1987; Stuart 1988). In ants, CHC represent this

colony odor, and these compounds can be homogenized through all members of the colony during trophallaxis and allogrooming. In some ant species, individual ants admix their own CHC with those of nestmates in the postpharyngeal gland (Soroker et al. 1998). Polygynous ant species are expected to bear a gestalt label unique for each colony in a population, although extreme polygyny may limit the creation of unique labels and thereby reduce intercolony variation. The broad range of intracolony odors and lack of a distinct intrinsic colony odor may facilitate formation of unicolonial populations in which colony boundaries are weak or absent, although some odor differences arising from extrinsic factors may still exist (Hölldobler and Wilson 1990).

In the unicolonial, polygynous, and widespread invasive Argentine ant, *Linepithema humile*, CHC play a central role in nestmate recognition (Liang and Silverman 2000; Greene and Gordon 2007; Torres et al. 2007). An inverse relationship between intraspecific aggression and genetic similarity between nests in both native and introduced populations suggests that recognition cues are heritable (Tsutsui et al. 2000). Because prey-derived CHC can radically affect nestmate recognition and worker-worker aggression, it is clear that exogenous, environmentally-derived hydrocarbons also significantly influence the *L. humile* recognition system (Liang et al. 2001; Liang and Silverman 2000). Interestingly, the contribution of environmentally-derived cues to nestmate recognition varies among introduced populations, suggesting that genotypic diversity can affect the expression and perception of components of the *L. humile* recognition system (Buczkowski and Silverman 2006). In addition, *L. humile* colonies from the southeastern U.S. display varying levels of intraspecific aggression and greater genetic diversity than other introduced populations (Buczkowski et al. 2004). They also exhibit a greater range of interactions, varying from highly aggressive and lethal outcomes to colony fusion (Vásquez and Silverman 2008). Colony fusion is expected to affect nestmate discrimination by changing the genetic and phenotypic composition of the fused *L. humile* colony, and thereby offers a possible mechanism for the formation of large networks of interconnected, non-aggressive, multiple-queen nests, a social organization called "supercoloniality".

Supercolony formation in introduced *L. humile* populations may result not only from lower diversity of recognition cues, due to a loss of genetic diversity (Tsutsui et al. 2000), but also from selective mixing of non-nestmates that share higher levels of phenotypic similarity (Giraud et al. 2002). It has been shown that *L. humile* colonies in southeastern U.S. differ in ratios of worker CHC, and that colony fusion at 24 h correlates with similarities of worker CHC profiles (Vásquez and Silverman 2008). Because *L. humile* colonies are extremely polygynous, often containing thousands of queens, and the CHC profile of queens is qualitatively different from that of workers (Liang et al. 2001; de Biseau et al. 2004), we hypothesize that CHC similarity of queens is relevant to colony fusion. Consequently, we would expect a higher frequency of fusion between colony pairs that share greater similarity of their respective queen CHC. The disparate colony odors of workers and queens might together, or independently, guide the outcomes of behavioral interactions between colony pairs, leading to high levels of aggression if the CHC are relatively dissimilar, or to colony fusion if the CHC are relatively more similar. Moreover, fusion of colonies could promote exchanges of CHC within the worker and queen castes, resulting in changes in their respective CHC profiles. Such an exchange could result in blending of colony recognition cues, or the predominance of the chemical profile of one of the colonies. In either case, we suggest that changes in the CHC profile will facilitate colony integration. Therefore, we hypothesize that the chemical similarities of both worker and queen CHC profiles of aggressive *L. humile* colonies determine the probability of colony fusion; as colonies fuse, ants change their CHC profiles to a homogeneous chemical recognition odor. Moreover, behavioral interactions in *L. humile* vary according to the level of CHC similarity among workers (Suarez et al. 2002; Vásquez and Silverman 2008), and also to the degree of worker genetic similarity (Tsutsui et al. 2000; Vásquez and Silverman 2008). Thus, we further hypothesize that the level of genetic similarity between colony pairs also modulates colony fusion; we expect to find a relationship between overall genetic similarity and queen and worker CHC profile similarity between colonies.

To test these hypotheses, we conducted laboratory assays in which we paired experimental *L. humile* colonies, for which we had determined levels of overall genetic similarity and worker and queen CHC profile similarity. We also investigated whether between-colony similarity of the CHC profiles of queens and workers was associated with the frequency of colony fusion at 6 months, and with genetic similarity between colonies. We further compared the CHC profiles of queens and workers from colonies that fused or did not fuse after 6 months, to the profiles of the respective unpaired control colonies (i.e., no intercolony interactions) to determine if changes in worker and queen CHC profiles occurred as a result of fusion.

Methods and Materials

Experimental Colonies and Colony Fusion Assay Argentine ants were collected from five sites in the southeastern USA: Cary (CAR), Chapel Hill (CHH), Research Triangle Park (RTP), and Winston-Salem (FOR) in North Carolina; and

Greenville (COC) in South Carolina. Experimental colonies were established and maintained as previously described (Vásquez and Silverman 2008). Briefly, experimental colonies (5 queens, ca. 100 pieces of brood, 500 workers) were established 1–2 mo after collection in individual Fluon-coated trays (17×25×11 cm), and provided an artificial nest, 25% sucrose solution, artificial diet, and a water source. All queens and 50 workers from each colony were marked with water-based paint (colony 1 = pink; colony 2 = yellow) to observe mixing of individuals and fusion events. Trays were connected through a vinyl tube (12 cm long) that was unblocked after a 24 h acclimation period. Controls consisted of experimental colonies that were not paired to any foreign colony. We recorded total number of workers fighting, and total number of dead workers in each tray for all ten pairwise colony combinations each hour for 6 h, and 24 h after colonies were allowed to interact. Colony pairs were inspected for fusion daily, from day 2 to day 30, and monthly from month 2 to month 6. Fusion was defined as the presence of queens from both colonies and all brood in the same nest (Vásquez and Silverman 2008).

Extraction, Isolation, and Chemical Analysis of Cuticular Hydrocarbons Six months after the colony fusion assay started, we collected queens (4–11) and workers (80–100) from the following colony pairs: CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, CHH-RTP. Ants were placed individually (queens) or in groups of ten (workers) in glass vials and stored at −20°C for subsequent CHC analysis. Queens were matched to their colony of origin based on the water-based paint mark. We also collected and stored queens (6–10) and workers (60) from each of the five unpaired control colonies. The CHC profiles of queens and workers from unpaired control colonies were analyzed to determine if colonies could be distinguished based on CHC, and to establish any relationship between CHC similarities between colonies and colony fusion at 6 months. We also compared queen and worker CHC profiles from colony pairs with those of their respective unpaired control colonies to determine if changes in CHC occurred after colony fusion. Cuticular lipids of thawed queens and workers were extracted, and CHC purified on silica gel as previously described (Vásquez and Silverman 2008). Capillary gas chromatography (GC) was carried out using an HP5890 gas chromatograph equipped with a DB-5 column (30 m×0.25 mm×0.5 µm film thickness). Extracts were introduced into a split-splitless inlet operated at 300°C in splitless mode (2 min purge) with helium as carrier gas at a linear velocity of 30 cm sec^{-1}. Oven temperature was held at 80°C for 2 min, increased to 270°C at 20°C min^{-1}, then to 310°C at 3°C min^{-1}, and held for 20 min. Individual queen extracts were resuspended in 20 µl hexane and 0.5 µl (0.025 queen equivalents) analyzed; extracts of ten workers were resuspended in 10 µl hexane, and 2 µl (2 worker equivalents) were injected. Peaks were integrated, and their individual percentages of the total CHC peak area calculated; only peaks with a mean percent area across all colonies of ≥1% were used in data analysis. The identity of discriminating peaks was determined by gas chromatography-mass spectrometry (GC-MS) and by comparing their retention times with those of alkane standards (*n*-C23–*n*-C38) (Liang et al. 2001; de Biseau et al. 2004). GC-MS analyses of CHC were performed on an HP6890 GC, equipped with an HP-5MS column (30 m×0.25 mm×0.25 µm film thickness), connected to an HP5973A mass selective detector. The inlet was operated at 300°C in splitless mode with helium as carrier gas at a linear velocity of 45 cm sec^{-1} (2 min purge). Data were recorded in scan mode (25–550 *m/z)* using electron impact ionization.

Genetic Similarity Among Colonies Genetic similarity among colonies used in the fusion assay was assessed by using microsatellite markers. DNA was extracted from 15 workers from each of the experimental colonies (CAR, CHH, COC, FOR, and RTP), and 7–17 queens from CAR, CHH, and RTP, using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) and analyzed at eight microsatellite loci: Lhum-11, Lhum-13, Lhum-19, Lhum-28, Lhum-35, Lhum-39 (Krieger and Keller 1999), Lihu-M1 and Lihu-T1 (Tsutsui et al. 2000). PCR reactions were performed as previously described (Buczkowski et al. 2004). Products were separated on 6.5% KB^{Plus} polyacrylamide sequencing gels with a 4300 LI-COR DNA analyzer. Microsatellite alleles were scored using GeneImagIR software (Scanalytics Inc., Billerica, MA, USA). Levels of genetic similarity among colonies were estimated based on the percentage of alleles shared between groups (Tsutsui et al. 2000). Genetic differentiation (F_{ST}) among Argentine ants from different locations was estimated with the program FSTAT v.2.9.3.2 (Goudet 1995).

Statistical Analyses Data analyses were performed with SAS 9.1.3 statistical software (SAS 2004). We performed a stepwise discriminant analysis (stepwise DA) on the transformed quantitative CHC data of control colonies by using PROC STEPDISC to identify variables (GC peaks) that differed significantly between groups of queens or workers. Peak areas were transformed following Aitchison's formula: $Z_{ij}=\ln[Y_{ij}/g(Y_j)]$, where Z_{ij} is the standardized peak area *i* for individual *j*, Y_{ij} is the peak area *i* for individual *j*, and $g(Y_j)$ is the geometric mean of all peaks for individual *j* (Reyment 1989). Only transformed variables that met the assumption of homogeneity of variance (Brown and Forsythe's test) were used in stepwise DA. We then performed a canonical DA on the selected peaks using PROC DISCRIM. Pairwise generalized square distances between group means (cent-

roids) were used as an estimate of the degree of CHC profile differentiation between colonies. To determine changes in queen and worker CHC profiles, of fused and non-fused colonies vs. their respective controls, we first estimated the linear discriminant function coefficients only for unpaired control colonies by using PROC DISCRIM, and then computed the linear discriminant function for fused and non-fused colony pairs using these coefficients.

Spearman rank correlation coefficients were used to determine relationships among percentage of colonies fused at 6 months and genetic similarity vs. queen and worker CHC similarity. The significance of the correlation coefficient was tested by Mantel correlation test in GENEPOP using 10,000 permutations. All means reported are followed by standard errors (SEM).

Results

Colony Fusion Assay The percentage of colony fusion at 6 months varied across *L. humile* colony pairs; all CAR-RTP, CHH-RTP, and COC-FOR replicates fused, none of the CAR-COC, CHH-COC, CHH-FOR, and COC-RTP replicates fused, and 40% of the CAR-FOR and FOR-RTP replicates, and 20% of the CAR-CHH replicates, fused. All queens from one of the paired colonies in replicates that did not fuse were killed. Interestingly, in CHH-FOR, unlike for all other non-fused colony pairs, we observed workers from both colonies mixing in all replicates; all CHH queens were killed by FOR workers.

Chemical Analysis of Cuticular Hydrocarbons: Cuticular Hydrocarbon Patterns of L. humile Unpaired Control Colonies GC and GC-MS analyses of queen and worker CHC showed that chemical profiles differed quantitatively and qualitatively between the castes (Fig. 1). Thirty one CHC peaks for queens and 44 CHC peaks for workers were consistently found in each colony; 21 compounds were common to both castes (Fig. 1). The stepwise DA performed on 25 queen CHC peaks that met the assumption of homogeneity of variance selected nine peaks that distinguished queens from different colonies (*Wilks'* $\lambda<0.01$, $F=13.63$, $df=36, 125.4$, $P<0.001$) (Fig. 2a), and had generalized square distances between colony centroids (i.e., chemical distance) ranging from 7.5 (CAR-RTP) to 123.2 (CAR-COC). All queen peaks that differed among colonies included the same discriminating compounds previously identified in Argentine ant queen adoption assays (Vásquez et al. 2008) as well as nonacosene (xC29:1) and *n*-hentriacontane (*n*-C31). The stepwise DA performed on 34 worker CHC peaks selected 10 peaks that grouped all workers from control colonies according to their colony of

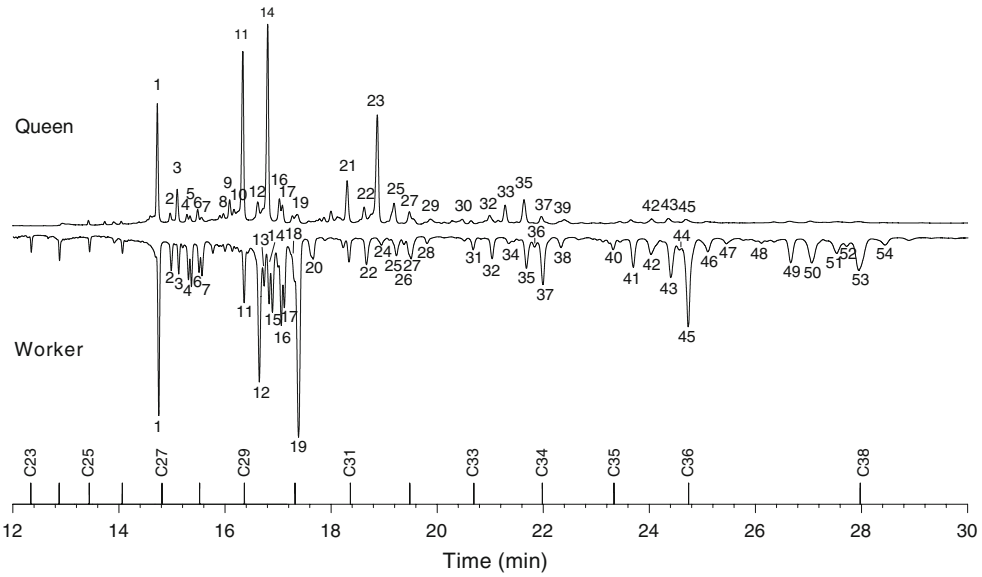

Fig. 1 Gas chromatogram of cuticular hydrocarbon profiles of *Linepithema humile* queen and worker. **1:** *n*-C27; **2:** UI; **3:** 5-MeC27; **4:** 11- and 13-MeC27; **5:** 3-MeC27; **6:** *n*-C28; **7:** UI; **8:** UI; 9: xC29:1; **10:** UI; **11**: *n*-C29; **12:** 11- and 13- and 15-MeC29; **13:** UI; **14:** 5-MeC29; **15:** UI; **16:** 3-MeC29; **17:** 5-MeC30; **18:** UI; **19:** *n*-C30; **20:** UI; **21:** *n*-C31; **22:** 11- and 13- and 15-Me C31; 23: 5-MeC31; 24: UI; **25:** x,y-diMeC31; 26: 3-MeC31; **27:** x,y,z-triMeC31; 28: UI; **29:** UI; **30:** xC33:1; 31:*n*-C33; **32:** 13- and 15- and 17-MeC33; **33:** 5-MeC33; 34: UI; **35:** 5-MeC34; 36: 5- and 15- and 19-triMeC33; **37:** UI; **38:** UI; 39: UI; **40:** UI; 41: 13- and 15- and 17-MeC35; **42:** 15,19-diMeC35; **43:** 5,15- and 5,17-diMeC35; 44: UI; **45:** 5,13,17- and 5,15,19-triMeC35; **46:** 3,13,17- and 3,15,17-triMeC35; **47:**UI; **48:** 13- and 15- and 17- and 19-MeC37; **49:** 15,19-diMeC37; **50:** 5,15- and 5,17-diMeC37; **51:** 5,15,19- and 5,13,17-triMeC37; **52:**UI; **53:**UI; **54:** UI. Numbers in *bold* indicate peaks used in stepwise discriminant analyses. Alkane standards are shown on the x-axis. UI: unidentified compound

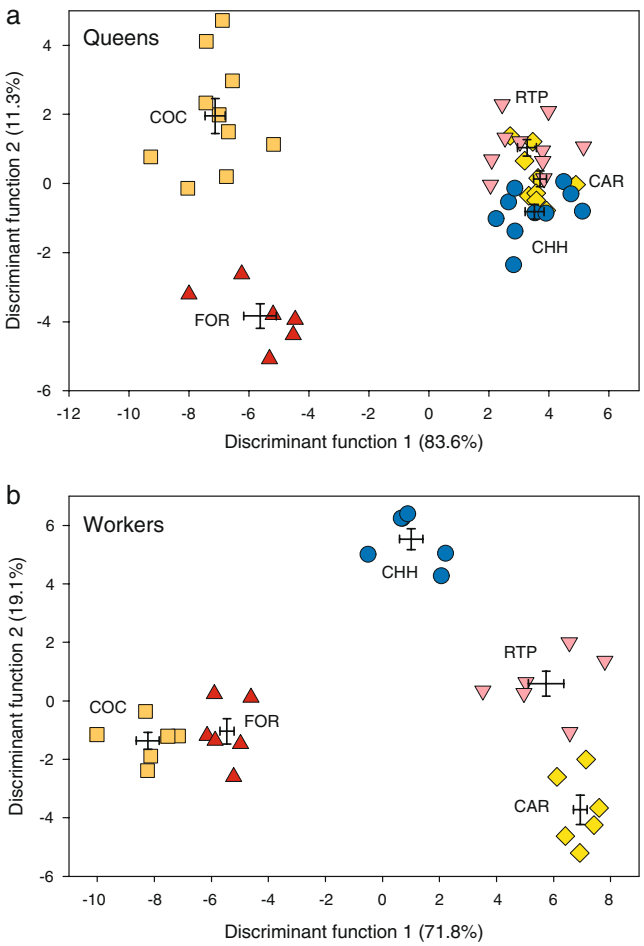

Fig. 2 Discriminant analysis of *Linepithema humile* cuticular hydrocarbons (CHC). **a** Discriminant analysis based on nine variables (CHC peaks) selected by stepwise discriminant analysis for queens from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP). **b** Discriminant analysis based on ten variables (CHC peaks) selected by stepwise discriminant analysis for workers from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP). *Bars* represent standard errors for a colony mean (centroid)

origin (*Wilks'* $\lambda<0.01$, $F=13.54$, $df=40, 62.5$, $P<0.001$) (Fig. 2b), with generalized square distances between colony centroids ranging from 24.8 (CAR-RTP) to 243.2 (CAR-COC). Five of the discriminating worker peaks were identified as 11- and 13-methylheptacosane (11- and 13-MeC27), 3-methylnonacosane (3-MeC29), 11- and 13- and 15-methylhentriacontane (11-, 13-, and 15-MeC31), 5,13,17- and 5,15,19-trimethylpentatriacontane (5,13,17- and 5,15,19-triMeC35), and 5,15- and 5,17-dimethylheptatriacontane (5,15- and 5,17-diMeC37).

Chemical Analysis of Cuticular Hydrocarbons: Cuticular Hydrocarbon Patterns of Fused and Non-fused L. humile Colonies To investigate the effects of colony fusion on queen CHC profiles, we computed discriminant functions for each colony pair by using discriminant function coefficients estimated from nine discriminating peaks derived from the control colonies. The CHC profiles of queens in colony pairs that fused (CAR-CHH, CAR-RTP, and CHH-RTP) occupied a much broader DA space and were spread across the two control groups (Fig. 3), indicating that queens in fused colonies formed a group with higher CHC variability that generally could not be distinguished unequivocally accord-

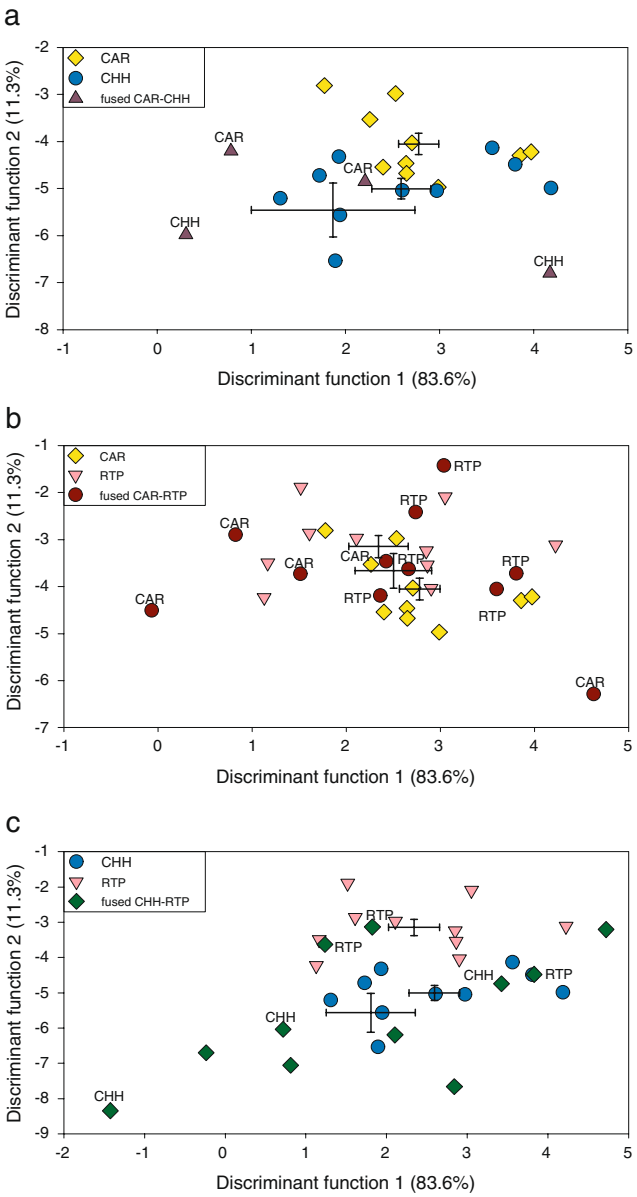

Fig. 3 Linear discriminant functions for cuticular hydrocarbons of *Linepithema humile* queens of fused colony pairs. **a** CAR-CHH (centroid: X=1.87±0.87, Y=−5.46±0.58) and unpaired controls CAR and CHH. **b** CAR-RTP (centroid: X=2.50±0.41, Y=−3.67±0.37) and unpaired controls CAR and RTP. **c** CHH-RTP (centroid: X=1.80±0.55, Y=−5.56±0.55) and unpaired controls CHH and RTP. Functions plotted based on nine variables. Bars represent standard errors for a colony mean (centroid). CAR centroid: X=2.78±0.21, Y=−4.05±0.23; CHH centroid: X=2.59±0.31, Y=−5.01±0.22; RTP centroid: X=2.34±0.32, Y=−3.15±0.24

ing to their colony of origin. This was especially apparent for the CHC profiles of five CAR queens and six RTP queens from CAR-RTP colonies that fused (Fig. 3b). The CHC profiles of two CAR and two CHH queens in fused CAR-CHH colonies made up a group with higher variability than their respective controls, but appeared more similar to CHH control queens (Fig. 3a), suggesting that CAR queens may have acquired CHH hydrocarbons. In the CHH-RTP pairings, six queens of known identity had similar CHC profiles to their controls, CHH and RTP, yet displayed higher CHC variability (Fig. 3c).

In colony pairs that did not fuse (CAR-COC and CHH-FOR), the CHC profiles of queens were more similar to the CHC profile of their respective unpaired control queens, with no overlap with the CHC of the other colony's queens (Fig. 4). In CAR-COC, the CHC profiles of the seven CAR queens and three COC queens examined were more similar to their respective unpaired controls than to those of queens of the other colony, and in CHH-FOR, the CHC profiles of ten queens known to be FOR were similar to those of the FOR control group (Fig. 4b).

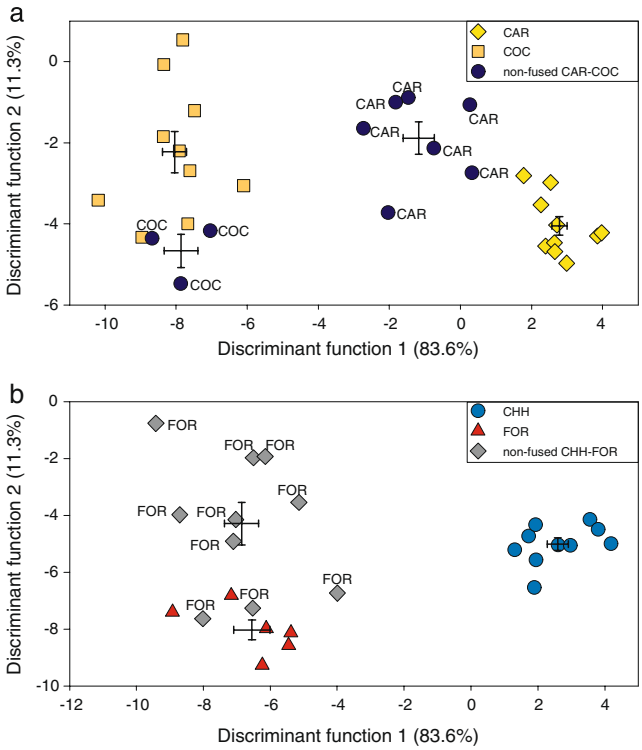

Fig. 4 Linear discriminant functions for cuticular hydrocarbons of *Linepithema humile* queens of non-fused colony pairs. **a** CAR-COC (CAR centroid: X=−1.18±0.44, Y=−1.89±0.40; COC centroid: X=−7.86±0.47, Y=−4.67±0.41) and unpaired controls CAR and COC. **b** CHH-FOR (FOR centroid: X=−6.86±0.51, Y=−4.28±0.75) and unpaired controls CHH and FOR. Functions plotted based on nine variables. *Bars* represent standard errors for a colony mean (centroid). CAR centroid: X=2.78±0.21, Y=−4.05±0.23; CHH centroid: X=2.59±0.31, Y=−5.01±0.22; COC centroid: X=−8.04±0.34, Y=−2.23±0.51; FOR centroid: X=−6.55±0.54, Y=−8.02±0.35

When we plotted worker CHC profiles of each colony pair with profiles of their respective unpaired controls, using ten discriminating peaks, we found that CHC profiles in colony pairs that fused (CAR-CHH, CAR-RTP, and CHH-RTP) occupied a much broader and relatively intermediate DA space between the unpaired control colonies, CAR, CHH, and RTP (Fig. 5). Unlike for the queens, the worker CHC profiles were not scattered throughout the control CHC profiles, probably because each sample consisted of ten randomly sampled workers of unknown colony affiliation. However, we presume that the average CHC profile of workers in fused colonies reflected a homogenized CHC composition of both colonies. In the non-fused CAR-COC pair, samples were taken from replicates that were either CAR or COC, based on colony queen identity, with CHC profiles of workers similar to CHC profiles of the workers of the respective controls (Fig. 6a). However, since CAR-COC workers were not marked, we could not rule out the possibility that these samples may have included workers from both colonies. Interestingly, in CHH-FOR pairings, all CHH queens were killed, but CHH and FOR workers apparently mixed. The CHH-FOR workers had a broad and largely distinctive CHC profile that, for some groups, was more similar to FOR workers than to CHH workers (Fig. 6b).

Genetic Similarity Among Colonies Genetic similarity among colonies (percent alleles shared) in the fusion assay varied across colony pairs, ranging from 35.3% (CHH-COC) to 74.3% (CAR-RTP) (Table 1). The overall genetic differentiation among colonies (F_{ST}) was, on average, 0.201±0.055 with pairings estimates (pairwise F_{ST}) ranging from 0.051±0.015 (CHH-RTP) to 0.431±0.090 (CHH-COC) (Table 1).

Correlations Among Cuticular Hydrocarbon Similarity, Genetic Similarity, and Colony Fusion We found a significant relationship between CHC similarities (i.e., low CHC distance) of queens from control colonies (Table 1) and the frequency of colony fusion at 6 months (*Mantel test* P=0.032) (Fig. 7). A similar relationship was evident between control worker CHC similarities (Table 1) and colony fusion at 6 months (*Mantel test* P=0.050) (Fig. 7). We found a strong relationship between percent alleles shared and queen and worker CHC profile similarity between colonies (*Mantel tests* P=0.007, and P=0.009, respectively) (Fig. 8a). Similarly, we found a relationship between pairwise F_{ST} and queen and worker CHC profile similarity between colonies (*Mantel tests* P=0.023, and P=0.040, respectively) (Fig. 8b). Also, we found a relationship between colony fusion at 6 months and percent alleles shared (*Spearman's* r=0.666, *Mantel test* P=0.042) and pairwise F_{ST} (*Spearman's* r=−0.822, *Mantel test* P=0.009).

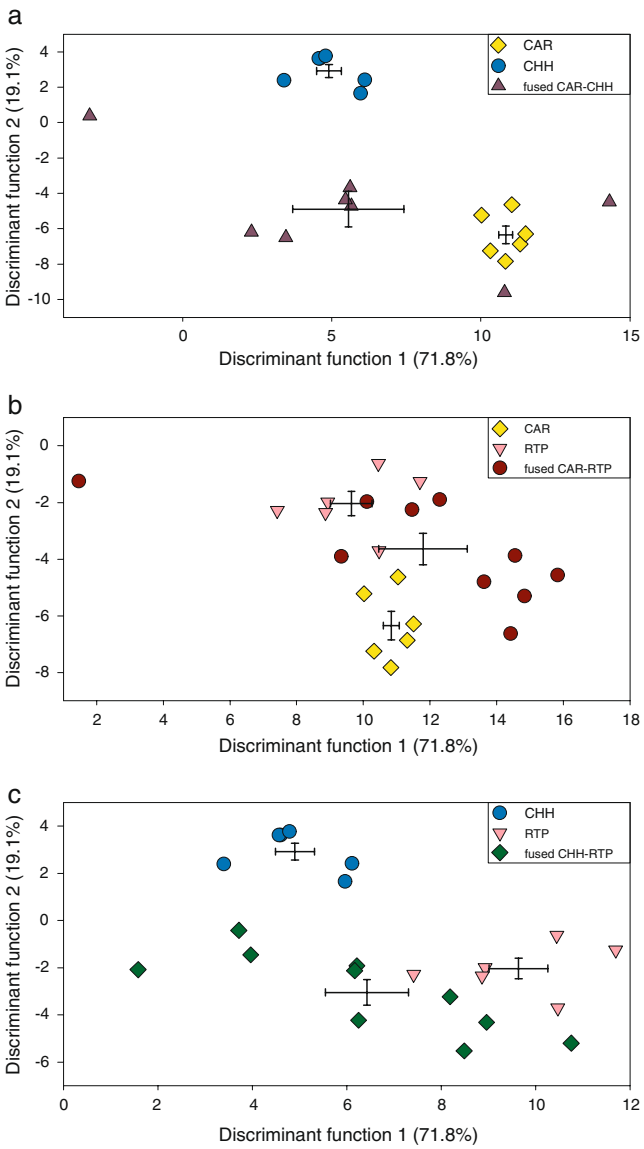

Fig. 5 Linear discriminant functions for cuticular hydrocarbons of *Linepithema humile* workers of fused colony pairs. **a** CAR-CHH (centroid: X=5.56±1.86, Y=−4.89±1.00) and unpaired controls CAR and CHH. **b** CAR-RTP (centroid: X=11.79±1.33, Y=−3.64±0.55) and unpaired controls CAR and RTP. **c** CHH-RTP (centroid: X=6.43±0.88, Y=−3.05±0.54) and unpaired controls CHH and RTP. Functions plotted based on 10 variables. *Bars* represent standard errors for a colony mean (centroid). CAR centroid: X−10.84±0.24, Y=−6.35±0.50; CHH centroid: X=4.90±0.41, Y=2.91±0.36; RTP centroid: X=9.64±0.62, Y=−2.03±0.43

Discussion

We demonstrated, for both queens and workers of *L. humile*, that similarity of CHC profiles between unrelated colonies was positively associated with colony fusion, and that the CHC profiles of fused colonies tended to be composed of a blend of CHC from both colonies. Our findings indicate that merging of unrelated *L. humile* colonies can lead to changes in the composition of recognition cues among members of the fused colonies, and possibly to expansion of the recognition template. This likely explains our earlier observation that fused colony pairs directed less aggression toward both source colonies compared to colonies that did not fuse (Vásquez and Silverman 2008). Discrimination abilities are important in structuring *L. humile* societies (Tsutsui et al. 2000; Giraud et al. 2002), and our results suggest that colony fusion can lead to more open colonies that may accept certain non-nestmates, further supporting the idea that expansive colonies in the introduced range of *L. humile* result from mixing or fusion of unrelated colonies.

Linepithema humile workers and queens differ considerably in their CHC profiles; workers have substantial amounts of dimethyl- and trimethylalkanes (diMe- and triMeC33, -C35 and -C37), whereas queens lack (or have very low amounts of) these compounds, but have monomethylalkanes (5-MeC27 to 5-MeC34) and alkenes (C29:1, C31:1, C33:1) as major components (Liang et al. 2001; de

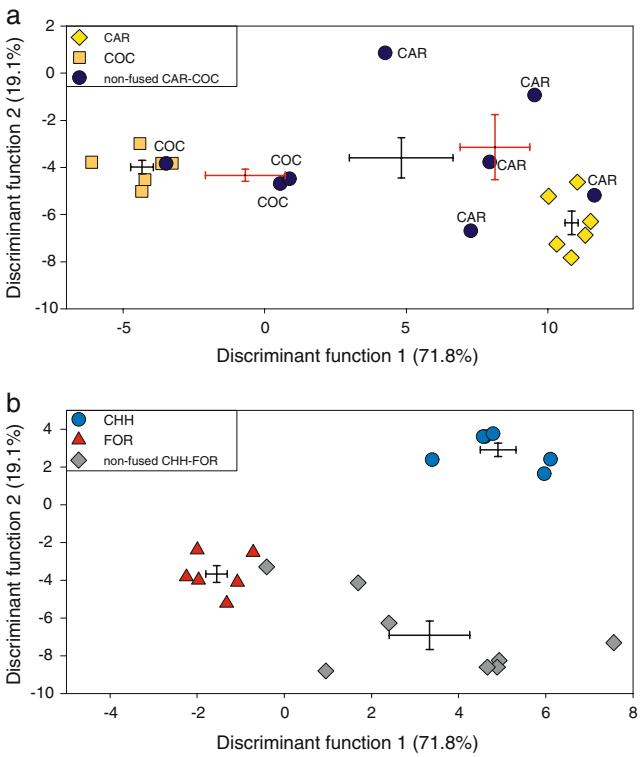

Fig. 6 Linear discriminant functions for cuticular hydrocarbons of *Linepithema humile* workers of non-fused colony pairs. **a** CAR-COC (CAR centroid: X=8.13±1.23, Y=−3.15±1.38; COC centroid: X=−0.69±1.40, Y=−4.33±0.26; or CAR-COC centroid: X=4.82±1.83, Y=−3.59±0.86) and unpaired controls CAR and COC. **b** CHH-FOR (centroid: X=3.34±0.92, Y=−6.90±0.76) and unpaired controls CHH and FOR. Functions plotted based on 10 variables. Bars represent standard errors for a colony mean (centroid). CAR centroid: X=10.84±0.24, Y=−6.35±0.50; CHH centroid: X=4.90±0.41, Y=2.91±0.36; COC centroid: X=−4.33±0.40, Y=−3.99±0.28; FOR centroid: X=−1.55±0.25, Y=−3.67±0.44

Table 1 Chemical distances and genetic similarity among *Linepithema humile* colonies. Generalized squared distances between colony centroids (i.e., chemical distance), genetic similarity (% alleles shared), and genetic differentiation (Pairwise F_{ST}) between colonies of a *L. humile* fusion assay. Chemical distances were calculated by discriminant analysis of the cuticular hydrocarbons of workers and queens from control (unpaired) colonies

Colony pair	Workers 10 variables	Queens 9 variables	Alleles shared (%)	Pairwise F_{ST}
CAR-CHH	120.96	8.52	64.50	0.160±0.046
CAR-COC	243.19	123.19	43.80	0.312±0.053
CAR-FOR	174.85	105.36	57.58	0.212±0.055
CAR-RTP	24.83	7.50	74.29	0.065±0.022
CHH-COC	140.56	122.32	35.29	0.431±0.090
CHH-FOR	98.24	96.46	64.50	0.255±0.128
CHH-RTP	51.06	8.09	66.70	0.051±0.015
COC-FOR	46.71	36.64	62.07	0.212±0.038
COC-RTP	205.91	111.14	40.00	0.312±0.115
FOR-RTP	144.86	103.63	60.53	0.200±0.125

Biseau et al. 2004). In this study, we found that quantitative differences of worker and queen CHC profiles reflect colony identity, and that a statistically derived subset of compounds may mediate colony discrimination. Worker CHC supplementation studies (Greene and Gordon 2007; Torres et al. 2007) suggest that a mixture of CHC of different structural classes, with varying ratios across colonies rather than a few compounds from a single structural class, may be used as nestmate recognition cues in *L. humile*. Our findings support this view and, in addition, suggest that alkenes and monomethylalkanes may be important in queen discrimination, while dimethyl- and trimethylalkanes and other unidentified long-chain CHC may be important in worker recognition. Methyl-branched alkanes, *n*-alkanes, and an alkene/*n*-alkane mixture have been shown to be important colony recognition cues in wasps (Dani et al. 1996; Gamboa et al. 1996). In ants, methyl-branched CHC are more colony-specific than *n*-alkanes (Astruc et al. 2001), although

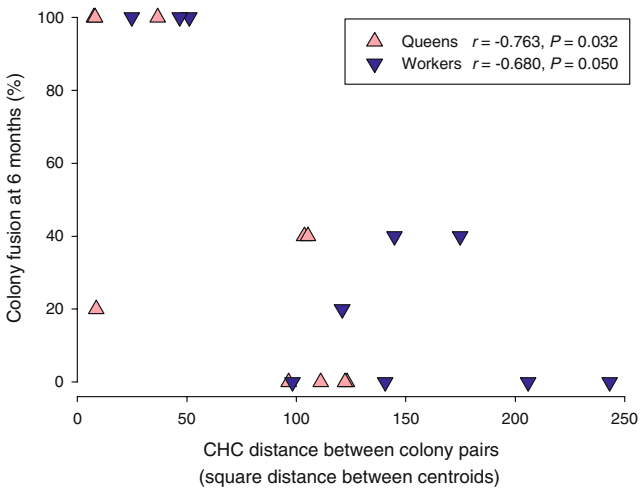

Fig. 7 Relationship (Spearman's *r*) between *Linepithema humile* colony fusion at 6 months and queen and worker cuticular hydrocarbon profile similarity based on nine and ten transformed variables, respectively

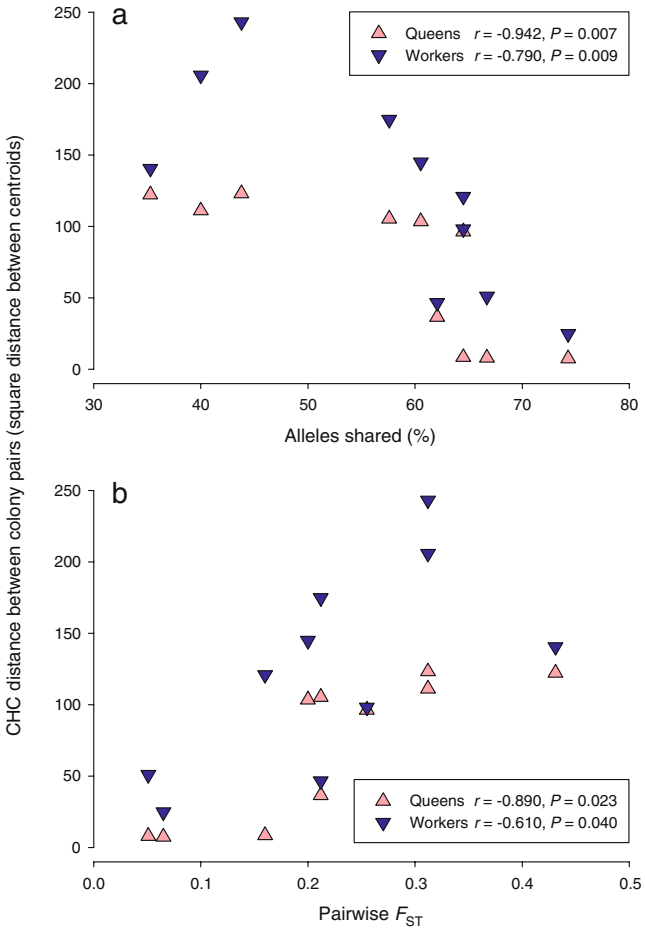

Fig. 8 Relationship (Spearman's *r*) between *Linepithema humile* cuticular hydrocarbon similarity and genetic similarity. **a** Genetic similarity (% alleles shared) between colonies versus queen and worker cuticular hydrocarbon similarity (generalized square distance between colony centroids). **b** Pairwise F_{ST} between colonies versus queen and worker cuticular hydrocarbon similarity (generalized square distance between colony centroids)

dimethylalkanes seem not to be important in nestmate recognition in *Cataglyphis* species (Dahbi et al. 1996). Chemical supplementation studies, testing the various alkanes and alkenes identified in our study, should be conducted to test the behavioral role of these compounds.

The distinct CHC profiles of queens and workers and our statistical identification of different CHC that distinguish queens and workers from different colonies suggest that *L. humile* does not form a unique colony odor distributed among all colony members (castes). Instead, individuals may have either a reproductive or sterile worker odor, that together constitutes a more complex colony template. A similar mechanism of template formation has been suggested for mixed-species groups in which individuals seem to learn and memorize allospecific cues early in adult life (Errard 1994). Two other possibilities are, that individuals bear their own endogenous cues and that these are matched with a learned Gestalt-type template, or that CHC, common to both queens and workers, are used as a single colony recognition template. In *Camponotus vagus*, dimethylalkanes are present across all castes, and are thought to be colony chemical cues, while *n*-alkanes and monomethylalkanes characterize larvae, workers, sexuals, and queens (Bonavita-Cougourdan et al. 1993), and may represent caste-specific cues.

In *L. humile*, genetically-based recognition cues appear to play a major role in nestmate discrimination among genetically diverse populations, whereas environmentally-derived CHC appear to be important in *L. humile* worker recognition in populations with reduced genetic variability (Buczkowski and Silverman 2006). In our study, CHC profiles of workers and queens are likely intrinsic because colonies were exposed to identical environmental conditions, and individuals were sampled 6 months after the start of the experiment. This explains the stronger association we found between worker CHC similarity and colony fusion at 6 months than the one found for colony fusion at 24 h (Vásquez and Silverman 2008). In the latter study, workers may have possessed both exogenous and endogenously-derived CHC. Temporal variation in worker CHC may also explain fusion events that occurred several weeks after interactions started, although factors (e.g., colony phenology, caste ratios, worker age) other than recognition cue phenotypic similarity could also govern the outcome of group interactions. In some colony pairs, CHC profiles between queens were more similar than those between workers, suggesting that workers may not be aggressive toward foreign queens but they may be aggressive toward workers from the foreign colony. Therefore, the outcome of group interactions may not exclusively reflect worker discrimination capability, or individual worker interests, but that of the whole group.

CHC profiles of queens changed after colony fusion, with the CHC profile of queens, as a group, in the fused colony not resembling the CHC profiles of either parent colony or indeed of a hybrid intermediate. Instead, profiles of queens varied across the range of profiles of both source colonies. This, together with the observation that queens could be assigned to their colony of origin based on their CHC profiles in non-fused colony pairs, suggests that by exchanging CHC, queens may match phenotypes in both colonies, thereby forming a broader queen recognition template. The collective worker CHC composition found in fused colony pairs suggests that mixing of worker CHC between colonies may have occurred. Transfer of CHC among individuals of the same colony, among mixed species, and in dulotic and inquiline species, has been well documented (Howard et al. 1980; Soroker et al. 1994). Cue exchange within castes could have occurred through direct body contact, grooming, and trophallaxis, in the same way that interactions with adult workers allow newly eclosed ant workers (callows) to acquire a colony's odor (Vander Meer and Morel 1998), or interactions with heterospecifics result in mixed hydrocarbon profiles in ants (Errard et al. 2006).

Colony fusion between aggressive *L. humile* colonies can also be predicted from the genetic similarity between colonies. This is in line with studies showing that aggression levels between colonies, or populations, of social insects are based on similarity of genetically-based CHC profiles (Dronnet et al. 2006). In *L. humile*, intraspecific aggression is based on levels of genetic similarity (Tsutsui et al. 2000), and is also guided by worker and queen hydrocarbons (Greene and Gordon 2007; Torres et al. 2007; Vásquez et al. 2008). However, the roles of chemical and genetic factors in regulating behavioral interactions among conspecifics have not been tested simultaneously. By combining behavioral, chemical, and genetic approaches, we demonstrated that both genetic and chemical factors play important roles in nestmate recognition and in shaping colony phenotypic composition, thereby offering a potential mechanism for changes in social structure in the introduced range.

While both queen and worker CHC similarity between colonies can guide fusion between genetically distinct *L. humile* colonies, changes in CHC patterns of colonies that had fused suggest homogenization of colony CHC between fused colony pairs, thus explaining reduced aggression toward unpaired control colonies (Vásquez and Silverman 2008) and fused colony cohesion. High aggression between non-fused colony pairs (winning colony) and their respective unpaired controls (defeated colony) after 6 months may be explained by maintenance of the colony chemical signatures. In line with previous studies that found an association between worker CHC similarity and intraspecific aggression in field and laboratory *L. humile* colonies (Liang and Silverman 2000; Suarez et al. 2002; Buczkowski and Silverman 2006), we found that worker and queen CHC

profile similarity is associated with fusion of unrelated colony pairs. Variation in colony genotypic composition, through mixed workers and queens, may lead to the formation of a new colony odor, implying that an updated recognition template must also be learned. It has been proposed that the greater the dissimilarity in CHC profiles between ant species that mix, the lower the aggression toward other ant species, due to a broader template (Errard et al. 2006). Similarly, increased phenotypic cue diversity in fused colonies should result in a much broader template. This may have implications at the population level, since changes in social structure may arise from changes in recognition cue diversity and/or template formation. Therefore, by increasing colony phenotypic diversity, fusion between unrelated colonies may be a proximate mechanism involved in the formation of expansive *L. humile* supercolonies in the introduced range.

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