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CUTICULAR HYDROCARBONS AND AGGRESSION IN THE TERMITE *Macrotermes Subhyalinus*

MANFRED KAIB,[1]* PATRICK JMHASLY,[2] LENA WILFERT,[1] WALTER DURKA,[3] STEPHAN FRANKE,[4] WITTKO FRANCKE,[4] REINHARD H. LEUTHOLD,[2] and ROLAND BRANDL[5]

[1]*Department of Animal Physiology, University of Bayreuth, D-95440 Bayreuth, Germany*

[2]*Zoological Institute, University of Berne, Balzerstrasse 9a, CH-3012 Berne, Switzerland*

[3]*Department of Community Ecology, UFZ-Centre for Environmental Research, Theodor-Lieser-Strasse 4, D-06120 Halle/Saale, Germany*

[4]*Institute for Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany*

[5]*Department of Animal Ecology, Faculty of Biology, Philipps-University of Marburg, Karl-von-Frisch-Str., D-35032 Marburg, Germany*

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Abstract—Cuticular hydrocarbons are among the prime candidates for nestmate recognition in social insects. We analyzed the variation of cuticular hydrocarbons in the termite species *M. subhyalinus* in West Africa (Comoë National Park) on a small spatial scale (<1 km). We found considerable variation in the composition of cuticular hydrocarbons among colonies, with four distinct chemical phenotypes. Different phenotypes occurred within each of the four habitats. The difference between these phenotypes is primarily due to unsaturated compounds. A clear correlation between the difference of the hydrocarbon composition and the aggression between colonies was found. This correlation also holds in a multivariate analysis of genetic similarity (measured by AFLPs), morphometric distances (measured by Mahalanobis-distances), as well as geographic distances between colonies. In a more detailed analysis of the correlation between the composition of cuticular hydrocarbons and aggression, we found that no single compound is sufficient to explain variation in aggression between pairings of colonies. Thus, termites seem to use a bouquet of compounds. Multiple regression analysis suggested that many of these compounds are unsaturated hydrocarbons and, thus, may play a key role in colony recognition.

Key Words—Nestmate recognition, cuticular lipids, AFLP, DNA-fingerprinting, relatedness, morphometrics, speciation, chemical ecology.

* To whom correspondence should be addressed. E-mail: manfred.kaib@uni-bayreuth.de

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INTRODUCTION

All social insects have the need to distinguish nestmates from nonnestmates (reviewed in Hölldobler and Michener, 1980; Breed and Bennett, 1987; Hölldobler and Wilson, 1990; Breed, 1998). For nestmate recognition, individuals search for a label when comparing one another and compare this label with a template. If label and template match, the two individuals will recognize each other as nestmates. This implies the acquisition, expression, or transfer of a label, and the learning of a template. Different models of nestmate recognition have been proposed (Carlin and Hölldobler, 1986; Hölldobler and Wilson, 1990; Smith and Breed, 1995). (1) The individualistic model assumes that labels are genetically determined and nontransferable. (2) The labels are acquired from the environment. (3) The Gestalt model assumes that the labels are a mixture of substances transferred among nestmates. (4) Finally, labels may be distributed from the queen to all nestmates.

Intraspecific nestmate recognition and associated mechanisms have been thoroughly studied in Hymenoptera. Intraspecific recognition cues, including cuticular hydrocarbons, are thought to be transferred among nestmates via intermediary nest structures such as comb wax in the honey bee *Apis mellifera* (Smith and Breed, 1995; Breed et al., 1998) or via the paper nest in *Polistes* (Gamboa et al., 1986; Dani et al., 1996; Gamboa et al., 1996). Thus, *Apis* and *Polistes* seem to follow the Gestalt model. Although cues for colony recognition have not yet been clearly identified in the sweat bee *Lasioglossum zephyrum*, they are not transferred among nestmates, which corresponds to the individualistic model (Greenberg, 1979; Buckle and Greenberg, 1981; Smith and Breed, 1995). In various ant species, recognition mechanisms may involve cues collected from the environment (e.g., Obin, 1986). However, evidence supporting the individualistic model (e.g., Mintzer and Vinson, 1985), the Gestalt model (e.g., Soroker et al., 1994), or recognition being mediated by queen derived cues (e.g., Carlin and Hölldobler, 1986) has also been found in ants.

It is generally believed that chemical cues are the labels involved in nestmate recognition. Cuticular hydrocarbons are assumed to be of major importance (reviewed e.g., in Smith and Breed, 1995). In ants, the search for recognition cues has almost exclusively concentrated on cuticular hydrocarbons (Bonavita-Cougourdan et al., 1987; Henderson et al., 1990; Nowbahari et al., 1990; Soroker et al., 1994). In termites, research on nestmate recognition has focused on behavioral aspects (reviewed by Thorne and Haverty, 1991; Shelton and Grace, 1996; Clément and Bagnères, 1998), but chemical cues have rarely been investigated. Nevertheless, it was repeatedly shown that cuticular hydrocarbon composition can vary considerably between termite colonies in close proximity (e.g., Haverty et al., 1988, 1996b,c; Bagine et al., 1994; Brown et al., 1996; Kaib et al., 2002). Some studies have combined the analysis of cuticular hydrocarbons with behavioral tests. These studies provide some evidence that cuticular hydrocarbons are correlated with the

recognition process (Howard et al., 1982; Haverty and Thorne, 1989; Bagnères et al., 1991; Takahashi and Gassa, 1995; Kaib et al., 2002). However, most of these studies concentrated on species recognition (but see Kaib et al., 2002). Finally, three studies suggested that the cues for nestmate recognition have a genetic basis, although the chemical nature of the labels themselves were not analyzed (Adams, 1991; Husseneder et al., 1997, 1998).

In the present study, we combine results derived from the chemical analysis of cuticular hydrocarbons, aggression tests between major workers from different colonies, genetic relatedness between colonies (AFLP-fingerprinting), and morphometric differences between minor soldiers from colonies in the higher termite *Macrotermes subhyalinus* (Isoptera, Termitidae). We include morphometric differences in our study, as individuals may recognize nestmates from their morphological similarity, which is generated by the similar genetics of nestmates, but also by the common environment they share. With these data sets we want to answer the following questions:

1. Is there a sufficient difference in the composition of cuticular hydrocarbon mixtures among colonies to allow for nestmate recognition? 2. Is the difference in the composition of cuticular hydrocarbons correlated with the behavioral interaction between colonies? 3. Are genetic or environmental factors more important than the cuticular hydrocarbon composition for predicting the level of aggression between colonies?

METHODS AND MATERIALS

Species and Study Site. According to Ruelle (1970), *M. subhyalinus* occurs across the northern savannah region from West to East Africa. In West Africa, its distribution stretches from coconut plantations along the coast to the semiarid northern regions. The species builds large earthen mounds (up to 1 m high) that, in contrast to East African *M. subhyalinus*, do not have open ventilation chimneys. It may be that the western and eastern populations are different species, a conclusion that is supported by mt-DNA sequences (M. Kaib, unpublished results). Hence, in this study the species name is provisional and may change when a revision of the genus *Macrotermes* becomes available. Colonies of the investigated species are monocalic and, in most cases, monogynous. *M. subhyalinus* feeds on rotten wood, dead leaves, and dry grass.

The study was carried out in the southeastern part of Comoë National Park ($8^\circ 45' N - 3^\circ 47'$ W; Ivory Coast, West Africa), an area characterized by a wet Guinea Savannah with 1100–1700 mm precipitation per year (Korb, 1997). The sampling area was situated in loose island forests. We selected 10 colonies within 4 different focus locations (Figure 1). Distances between adjacent locations ranged from 190 m–790 m. As an outgroup, one colony (R1; not shown in Figure 1) was

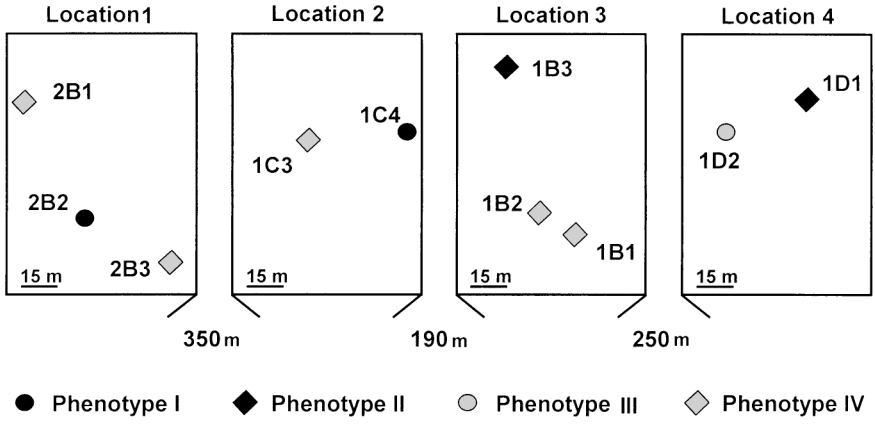

FIG. 1. Map of the 10 colonies within the 4 sampled locations at the focus area. Note that phenotypes are not clustered according to location.

collected about 20 km away from the main sampling area. This colony was included in the chemical analysis (Figure 2), but not in the evaluation of the relationship between aggression and hydrocarbons.

Chemical Analysis of Cuticular Hydrocarbons. In March 1997, 12 major workers were collected from each of the 11 colonies (except for 1D1: 6 major workers; for colony labels and distribution see Figure 1). Individuals were washed immediately after collection for 15 min in 100 μl *n*-hexane (Merck 1.04391). Cuticular rinses were evaporated to dryness in order to eliminate volatile constituents. For chemical analysis, the rinses were reconstituted with *n*-hexane, and aliquots were assayed by gas chromatography (Hewlett Packard 5890, equipped with a flame ionization detector). Separation of components was performed on a fused silica capillary column (DB-1; 30 m by 0.32 mm i.d.; 0.11 μm film thickness). The carrier gas was helium at a flow rate of 1.5 ml/min. The injection mode was splitless for the first 60 sec after injection. For separation of hydrocarbons, the oven temperature was programmed from 160 to 260°C at 2°C/min, thereafter to 300°C at 5°C/min, and then held isothermally for 15 min. FID signals were acquired and quantified by HP ChemStation. Peaks from different chromatograms were classified by comparison of linear retention indices calculated on the basis of an *n*-alkane series from eicosane to hexatriacontane.

We used 40 gas chromatographic peaks that were derived from hydrocarbons. The percentages of each peak were calculated on the basis of the total counts of all 40 peaks. The matrix resulting from the percentages of the peaks from all analyzed individuals was subjected to further statistical analyses. We are aware of the fact that in some cases, peaks assigned to a retention index may include more than one hydrocarbon. Therefore, our distance measures are conservative estimates.

To quantify differences of chemical compositions between sampled individual major workers, we calculated a matrix of Nei-distances as described by Kaib et al. (1991). Other methods of quantifying the differences between compositions (see for example Kaib et al., 2002) generated the same patterns and, thus, we report only the analyses of the Nei-distances. Chemical distances between colonies were estimated by the mean of all possible pairwise combinations of the distances between individual major workers of the different colonies.

We identified individual hydrocarbons by coupled GC/MS (Blomquist et al., 1987; Nelson et al., 1980; Page et al., 1990a, b). For selected samples, structural analysis was performed on a VG 70-250 SE mass spectrometer connected to a Hewlett Packard HP 5890 GC. Aliquots containing 1–2 μl of an extract were applied to the column under the conditions stated above, apart from the oven temperature program (80°C; 1.5 min hold; at 40°C/min to 160°C; then at 2.5°C/min to 280°C). The mass spectrometer was operated in electron impact ionization mode (70 eV, 500 μA ionization current, 200°C source temperature) and scanned from *m/z* 600 to *m/z* 35 at a rate of 0.7 sec/decade with an interscan time of 0.2 sec. For the determination of the double bond positions of unsaturated hydrocarbons, dimethyl disulfide (DMDS) derivatives were prepared following the protocol provided by Kaib et al. (2000).

Aggression Among Colonies. Jmhasly and Leuthold (1999) report behavioral data (aggression indices) derived from the same colonies that we sampled for the analyses of chemical, morphometric, and genetic data. These behavioral data, which were obtained in 1996, were also used in the present study. Quantitative behavioral tests were done by pairing two groups of five major workers each from different colonies. During a period of 5 min, aggressive interactions were scored in 30-sec intervals. Aggression comprised grasping, nipping, and biting. For each colony, 10 replicates were done. An average of the 10 scores per test and the 10 replicates was used to calculate the aggression index that had a maximum range from 0 to 10. See Jmhasly and Leuthold (1999) for further details. They were not able to carry out experiments for all 45 possible combinations of the 10 colonies, thus, only 20 combinations are available.

Morphometrics. A total of 15 linear parameters of minor soldiers (collected in March 1997 and stored in 70% ethanol) were measured in 10 individuals from each of the 10 investigated colonies: head capsule length, distance from the fontanelle to the anterior margin of the head capsule, distance from the fontanelle to the posterior margin of the head capsule, distance between left and right mandible bases, distance from the fontanelle to the left mandible basis, maximal head capsule width, maximal pronotum width, pronotum length, maximal mesonotum width, maximal metanotum width, maximal head capsule height, left mandible length, length of left hind femur, length of left hind tibia, and length of left hind tarsus. Prior to analysis, all parameters were log-transformed. The data sets were subjected to canonical discriminant analysis by defining all individuals measured

from a colony as a group. Morphometric distances between termites from different groups (colonies) were calculated as Mahalanobis-distances.

AFLP-Fingerprinting. From each of the 10 colonies, four major workers (collected in March 1997 and stored in 100% ethanol) were used for genetic analysis. DNA was extracted from the heads of individual termites using DNeasy Tissue Kit (QIAGEN), following manufacturer's instructions applying 50-μl H_2O at the final elution step. The use of heads only minimizes the risk of contamination from gut symbionts of the termites. Amplified fragment length polymorphisms (AFLP; Vos et al., 1995) were generated as follows: First, in a total of 11 μl, 5.5 μl of DNA solution were restricted with 5 U *Eco*RI and 1 U *Mse*I, and ligated to respective adapters by 67 U T4 DNA ligase in the presence of 1 $\times$ T4 DNA ligase buffer, 0.05 mg/ml BSA, and 50 mM NaCl at 37°C for 2 hr. Second, after 1:2 dilution, 5 μl of the restriction-ligation reaction were used for preselective amplification in a total volume of 20 μl with 0.8 U *taq* polymerase (MBI Fermentas), 1x PCR buffer with $(NH_4)_2SO_4$ provided by the manufacturer, 1.5 ng/μl of *Eco*RI and *Mse*I preselective primers, 200 μM dNTPs, and 1.5 mM $MgCl_2$. PCR was performed in a Primus 96 thermocycler (MWG Biotech) programmed for 72^{o}C, 2 min followed by 30 cycles of (94^{o}C, 20 sec; 56^{o}C, 30 sec; 72^{o}C, 2 min) and 60^{o}C, 30 min. Third, after 1:20 dilution, 6 μl of the diluted amplification reaction were used for selective amplification with the same reaction conditions using 0.25 ng/μl *Eco*RI selective primer labeled with a fluorescent dye and 1.5 ng/μl *Mse*I selective primer and a "touch down" PCR protocol of 94°C, 2 min, followed 10 cycles of (94°C, 20 sec; 66°C, 30 sec, decrease 1°C per cycle; 72°C, 2 min), 20 cycles of (94°C, 20 sec; 56°C, 30 sec; 72°C, 2 min), and 60°C, 30 min. Subsequently, fragments were separated on an ABI 310 genetic analyzer. We tested 64 different primer combinations in four individuals, from which four primer combinations were chosen for further analysis according to number and size distribution of polymorphic fragments: *Eco*RI-ACT/*Mse*-CTT, and *Eco*RI-ACT/*Mse*-CAA, *Eco*RI-ACG/*Mse*-CTT, *Eco*RI-ACC/*Mse*-CAA. In total, these primers yielded 403 dominant loci, 92 of which were monomorphic. Only fragments that amplified consistently across samples were scored in a presence–absence matrix. From this matrix, genetic similarities among individuals were calculated using the Jaccard-index (Rohlf, 1990; other indices for presence/absence data produce the same results). For comparisons of colonies, the mean of all possible pairwise combinations between individuals from all combinations of colonies were calculated.

Statistical Analyses. To summarize the patterns in the 114 $\times$ 114 matrix of chemical distances between individuals, we performed a principal coordinate analysis. Cuticular hydrocarbon compositions, the aggression tests, morphometrics, and fingerprinting, as well as geographical distance between colonies were compared by matrix correlations. One-tailed error probabilities were estimated via permutations (5000 permutations) by a program that takes missing values into account (see also Manly, 1997).

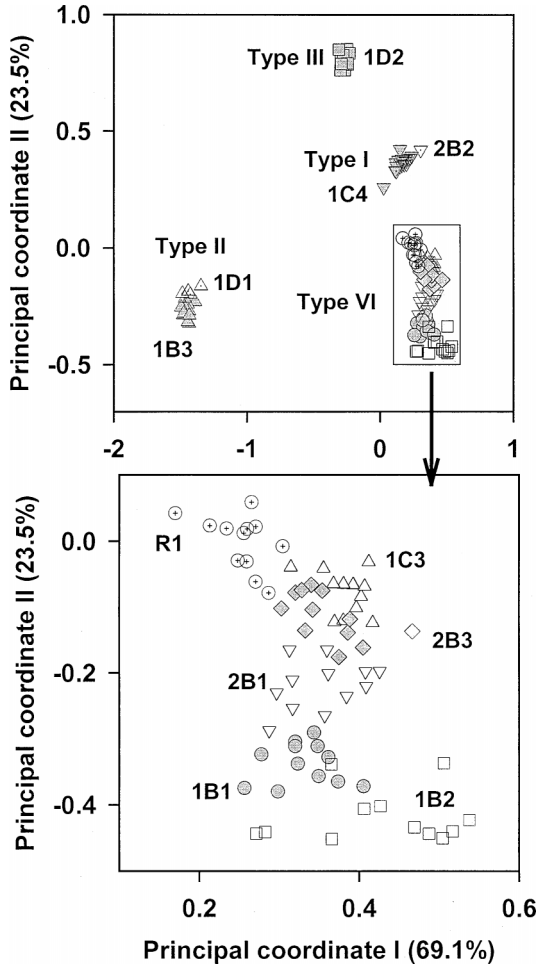

FIG. 2. Principal coordinate analysis of the differences among hydrocarbon compositions of 126 individuals from 11 colonies (including the outgroup colony R1, see Figure 1; differences measured by Nei-distances; Rohlf, 1990). Individuals from the same colony have the same symbol. Note the four distinct chemical phenotypes. The lower plot zooms the ordination of phenotype IV (rectangle in the upper plot). Individuals from different colonies form more or less distinct groups.

We used hierarchical partitioning (Chevan and Sutherland, 1991) to estimate the importance of dissimilarity in hydrocarbon compositions, morphological distance, genetic similarity, and geographic distance between colonies for the aggression between colonies. Hierarchical partitioning is a protocol in which all

possible models in a multiple regression setting are jointly considered to identify the most likely causal factor. This is done by averaging the increase in the goodness-of-fit caused by each independent variable across all possible models (for details see Chevan and Sutherland, 1991). This averaging is likely to solve the problem of correlations among independent variables, which severely influences all one-model techniques, such as stepwise protocols (e.g., see MacNally, 2000; see also Kaib et al., 2002).

RESULTS

Chemical Analysis of Cuticular Hydrocarbons. Mean chemical distances (Nei-distance) between nestmates (major workers) were always low and ranged from 0.003 (standard deviation SD = 0.002, $N = 66$) in colony 1B1 to 0.022 (SD = 0.022, $N = 66$) in colony 1C4. In contrast, mean chemical distances between major workers from different colonies differed considerably and ranged from 0.013 (SD = 0.007, $N = 144$) between the colonies 2B1 and 1B1 to 3.566 (SD = 0.362, $N = 144$) between the colonies 1B2 and 1B3. In only two cases out of 45 (1B1–2B1, 1B1–1B2), were the intercolonial distances as low as the intracolonial differences. The principal coordinate analysis of the similarity patterns between individuals suggested four discrete hydrocarbon phenotypes (Figure 2). Within one phenotype, the variation between colonies was small. Nevertheless, individuals from one colony clustered consistently together (Figure 2).

Phenotype I (2B2, 1C4) as well as phenotype II (1B3 and 1D1) were each found at two different locations. Phenotype III was found in one colony only (1D2). Phenotype IV was most frequent and was found in five colonies (1B1, 1B2, 2B1, 2B3, 1C3) collected from three of the four focus locations. The same phenotype was also found in a colony (R1) over 20 km distant from the 10 focus colonies (this colony was not included in the analyses presented later). In each of the four focus locations, different hydrocarbon phenotypes were found.

The hydrocarbons identified ranged from C_{20} to C_{33} in chain length and were *n*-alkanes, alkenes, and alkadienes, as well as trace amounts of methylalkanes (Table 1). Chemical compositions were dominated by hydrocarbons with an uneven chain length (Table 1). The phenotypes can be chemically characterized by *n*-alkanes and alkenes, which together make up 88.8% (type I) to 98.0% (type III) of the total hydrocarbons (Table 2). *n*-Alkane compositions were largely congruent in phenotypes I and IV with a high abundance of *n*-heneicosane and *n*-tricosane. Comparing chemical phenotypes, classes of substances differ in chain length. Such a pattern in the change of chain length became apparent in the alkenes with high abundance of 5-tricosene and 11-tricosene in phenotypes I and IV, respectively. 9-Hentriacontene was the most abundant compound in phenotype II, as was 9-heptacosene in phenotype III, and both alkenes were restricted to the respective phenotype.

TABLE 1. PERCENTAGES OF THE TOTAL HYDROCARBON FRACTION IN THE CUTICLE OF MAJOR WORKERS IN THE TERMITE *Macrotermes subhyalinus*

		Type I		Type II		Type III		Type IV	
RI	Component	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2060	10-Heneicosene	0	0	0	0	0	0	0.20	0.05
2100	*n*-Heneicosane	13.21	0.31	0	0	0	0	18.20	0.57
2160	10-&11-Docosene	0	0	0	0	0	0	0.62	0.09
2200	*n*-Docosane	1.67	0.03	0	0	0.03	0.03	1.20	0.05
2252	6,9-Tricosadiene	4.88	0.65	0	0	0	0	0.48	0.09
2261	11-Tricosene	3.00	0.19	0	0	0	0	39.49	0.62
2272	9-Tricosene	2.06	0.43	0	0	0	0	0	0
2284	5-Tricosene	16.06	0.41	0	0	0	0	0	0
2300	*n*-Tricosane	40.70	0.90	0	0	24.86	0.60	23.07	0.85
2336	11-&13-Methyltricosane	0	0	0	0	0	0	0.81	0.14
2351	6,9-Tetracosadiene	0	0	0	0	0	0	0.11	0.04
2400	*n*-Tetracosane	0	0	0	0	0.37	0.09	0	0
2409	Unknown	0	0	0	0	0	0	0.02	0.01
2450	Unknown	0	0	0	0	0	0	0.13	0.03
2453	6,9-Pentacosadiene	3.97	0.51	0	0	0	0	0	0
2459	11-Pentacosene	0.13	0.13	0	0	0	0	4.04	0.29
2465	9-Pentacosene	0.00	0.00	0	0	0	0	1.18	0.31
2474	7-Pentacosene	1.08	0.23	0	0	0.48	0.08	0	0
2485	5-Pentacosene	1.18	0.03	0	0	0.00	0.00	0	0
2500	*n*-Pentacosane	2.18	0.05	11.17	0.63	9.22	0.26	0.95	0.04
2513	Unknown	0	0	0	0	0	0	0.01	0.01
2566	9-Hexacosene	0	0	0	0	0.81	0.03	0	0
2661	11-Heptacosene	0	0	0	0	0.11	0.06	0.28	0.05
2669	9-Heptacosene	0	0	0	0	50.55	0.92	0	0
2676	7-Heptacosene	0	0	0	0	5.40	0.25	0	0
2700	*n*-Heptacosane	2.79	0.10	11.15	0.49	2.63	0.09	2.93	0.08
2733	11-&13-Methylheptacosane	0	0	0	0	0.95	0.05	0	0
2749	Unknown	0	0	0	0	0.38	0.14	0	0
2800	*n*-Octacosane	0.17	0.06	0	0	0.04	0.04	0.26	0.04
2868	9-Nonacosene	0	0	0.94	0.13	0.84	0.03	0	0
2877	7-Nonacosene	0	0	8.05	0.24	0	0	0	0
2900	*n*-Nonacosane	4.13	0.14	5.25	0.20	2.36	0.09	3.96	0.10
3043	8,22-Hentriacontadiene	0	0	1.53	0.12	0	0	0.01	0.01
3052	7,23-Hentriacontadiene	0	0	0.95	0.15	0	0	0	0
3069	9-Hentriacontene	0	0	25.60	0.56	0	0	0	0
3074	7-Hentriacontene	0	0	4.17	0.21	0	0	0.01	0.01
3100	*n*-Hentriacontane	0.32	0.08	2.09	0.19	0.31	0.14	0.64	0.14
3231	Tritriacontadiene	0.11	0.08	15.47	0.40	0	0	0.06	0.03
3238	Tritriacontadiene	0	0	7.27	0.25	0	0	0	0
3262	Tritriacontene	0	0	1.37	0.17	0	0	0	0

Note. The table gives the mean (±SE) across all major workers within one phenotype (Type I: $N = 18$, Type II: $N = 24$, Type III: $N = 12$, Type IV: $N = 60$). RI = retention index. The means may not sum up to exactly 100% (rounding errors).

TABLE 2. HYDROCARBON COMPOSITION IN THE CUTICLE OF MAJOR WORKERS IN THE TERMITE *M. subhyalinus*

	-ane	-ene	-di	Me-	Unknown
Total number of peaks	9	18	8	4	4
Type I	65.17	23.63	8.85	0.00	0.00
Type II	29.66	62.82	2.48	0.00	0.00
Type III	39.83	58.19	0.00	0.95	0.38
Type IV	51.21	45.89	0.61	0.81	0.15

	5-ene	7-ene	9-ene	11-ene
Type I	72.90	4.57	8.72	13.29
Type II	0.00	19.44	41.71	2.18
Type III	0.00	10.10	89.71	0.19
Type IV	0.00	0.02	2.57	95.53

Note. (Type I: $N = 18$, Type II: $N = 24$, Type III: $N = 12$, Type IV: $N = 60$). The upper part of the table gives the sum of all saturated (-ane), unsaturated (-ene, -di), as well as branched hydrocarbons (Me-) (in %). The lower part gives a more detailed analysis of the differences in alkenes (percentage of the total alkenes) between the phenotypes. Note the shift by two CH_2-groups from phenotype I to phenotype IV. The means may not sum up to exactly 100% (rounding errors).

Aggression and Hydrocarbons—A Search for Correlations. As already discussed in detail by Jmhasly and Leuthold (1999), *M. subhyalinus* shows discriminatory behavior and aggression that varies according to the specific combinations of colonies sampled within a rather small spatial scale (for the data see Table 3). In pairings of individuals from the same colony, no aggression was found, whereas in pairings from different colonies, the aggression index may range from 0 to more than 6. The observation of multiple chemical phenotypes, as well as variation in aggression among pairings of colonies, calls for an analysis of the correlation between these two data sets. However, aggression may be based on other cues than cuticular hydrocarbons, e.g., cues collected from the environment, morphological differences, or cues based on genetic differences. Thus, we include in our search of patterns, the genetic similarity between colonies (measured by the Jaccard-index from AFLP-fingerprints), the morphometric distance, as well as the geographic distance. The latter served as an indirect measure of environmental similarity.

Among colonies, mean chemical distances, the level of aggression, morphometric distances, and genetic similarity, were all correlated (Figure 3). Only geographic distance shows no correlation with any of the other parameters (Table 4). Most importantly, chemical distance correlates positively with morphological distance, and negatively with genetic similarity (Figure 3, note that this implies that genetically similar colonies have similar hydrocarbon compositions). In turn,

TABLE 3. ALL POSSIBLE COLONY PAIRINGS (45) AND ASSOCIATED MEASURES OF DIFFERENCE

Colony combinations		Pairing of phenotype	Aggression	Hydrocarbons	Morphology	Genetics	Geography (m)
1B1	2B1	IV-IV		0.01	15.9	0.683	577.2
1B1	1B2	IV-IV	0.00	0.01	14.9	0.792	16.8
2B2	1C4	I-I		0.03	16.6	0.800	387.6
1B3	1D1	II-II	0.00	0.03	120.0	0.677	268.8
2B1	2B3	IV-IV	0.00	0.03	29.4	0.677	86.4
1B2	2B1	IV-IV		0.03	12.6	0.675	562.8
2B1	1C3	IV-IV	0.40	0.04	12.7	0.679	374.4
1B1	2B3	IV-IV		0.05	35.3	0.799	518.4
2B3	1C3	IV-IV	0.02	0.06	21.2	0.737	316.8
1B1	1C3	IV-IV		0.06	11.3	0.724	206.4
1B2	2B3	IV-IV		0.06	32.2	0.799	504.0
1B2	1C3	IV-IV		0.10	10.9	0.714	192.0
2B2	2B3	I-IV	1.20	0.39	26.7	0.798	38.4
2B3	1C4	IV-I		0.42	36.8	0.819	355.2
2B2	1C3	I-IV		0.43	12.0	0.747	350.4
1C3	1C4	IV-I	0.04	0.47	13.7	0.742	38.4
2B1	2B2	IV-I	3.31	0.54	12.6	0.703	54.0
2B1	1C4	IV-I		0.58	8.4	0.687	410.4
1B1	2B2	IV-I		0.70	21.4	0.779	552.0
1B1	1C4	IV-I		0.74	10.2	0.796	170.4
1B2	2B2	IV-I		0.80	12.9	0.779	538.8
1B2	1C4	IV-I	2.17	0.90	16.1	0.783	84.0
2B2	1D2	I-III		0.95	11.6	0.757	765.6
1C4	1D2	I-III	1.59	0.96	17.6	0.791	379.2
1C3	1D2	IV-III		1.31	7.1	0.735	392.4
2B3	1D2	IV-III		1.40	12.7	0.794	734.4
2B1	1D2	IV-III		1.55	15.3	0.684	788.4
1B1	1D2	IV-III	3.21	1.77	18.4	0.777	216.0
1B2	1D2	IV-III		2.09	17.0	0.769	228.2
1D1	1D2	II-III	2.83	2.53	52.6	0.678	33.6
1B3	1D2	II-III	2.49	2.77	80.1	0.666	240.0
1C4	1D1	I-II	5.04	2.91	80.4	0.677	411.6
1B3	1C4	II-I	6.34	3.02	85.12	0.673	142.8
2B2	1D1	I-II	4.89	3.10	55.6	0.704	799.2
1B1	1D1	IV-II		3.14	96.3	0.668	249.6
1B1	1B3	IV-II	3.65	3.21	77.7	0.664	76.8
1B3	2B2	II-I		3.22	59.2	0.680	530.4
2B1	1D1	IV-II		3.23	56.0	0.674	820.8
2B3	1D1	IV-II	3.49	3.25	68.4	0.675	765.6
1B3	2B1	II-IV		3.28	71.4	0.754	550.8
1B3	2B3	II-IV	6.38	3.34	129.1	0.667	496.8
1C3	1D1	IV-II		3.35	60.6	0.697	448.8

TABLE 3. CONTINUED

Colony combinations		Pairing of phenotype	Aggression	Hydrocarbons	Morphology	Genetics	Geography (m)
1B3	1C3	II-IV		3.45	77.6	0.656	180.0
1B2	1D1	IV-II		3.54	90.6	0.655	261.6
1B2	1B3	IV-II	5.88	3.57	56.6	0.646	40.8

Note. In aggression (aggression; from Jmhasly and Leuthold, 1999), in the composition of hydrocarbons (mean Nei-distance), in morphometric dissimilarity (morphology; measurements from minor soldiers; Mahalanobis-distance), in genetic similarity (genetics, Jaccard-index from AFLP-fingerprints), and geographic distance between colonies (geography; see Figure 1). Note that the aggression data do not cover all possible combinations. Furthermore we indicate for each colony pairing also the pairing of the phenotypes (see Figure 2).

chemical distance, morphological distance, and genetic similarity are all significantly correlated with the level of aggression. However, the independent contributions (Figure 3) suggest that the difference of the hydrocarbon compositions among colonies is the data set that explains most of the variation in aggression among individuals from different colonies.

The difference in the hydrocarbon compositions among colonies as measured by the Nei-index is anonymous and provides no information about possible correlations of the difference in the relative amount of individual peaks to the aggression of pairings of colonies. Thus, we used individual peaks for a more detailed search. First of all, we found that differences in the relative amount of individual peaks never reached the predictive power of the Nei-distance ($r^2 = 0.79$; Table 4). Squared correlation coefficients exceeded 0.1 in only a few cases, and the chain lengths of these compounds were 23 and 25. Overall, we found a negative correlation between chain length and squared correlation coefficients (Figure 4). In a further step, we used multiple regression coefficients to predict the level of aggression between pairings of colonies from the differences in the relative amount of individual peaks. Using 10 compounds, we were able to explain almost 100% of the variation in aggression between colonies (Figure 5). Most of these compounds were unsaturated and/or merely minor compounds. Note that we present no formal statistical tests. This has three reasons. First, with differences in the relative amount of peaks among colonies being measured as Nei-distances, the data set is a matrix that calls for special test procedures (Manly, 1997). Second, we calculated about 40 such correlations with the risk of chance correlations, and third, the individual peaks are not independent as the sum of all peaks is always 100%.

DISCUSSION

Our results provide the following answers to the questions posed within the introduction: 1. There is sufficient variation in the hydrocarbon composition among

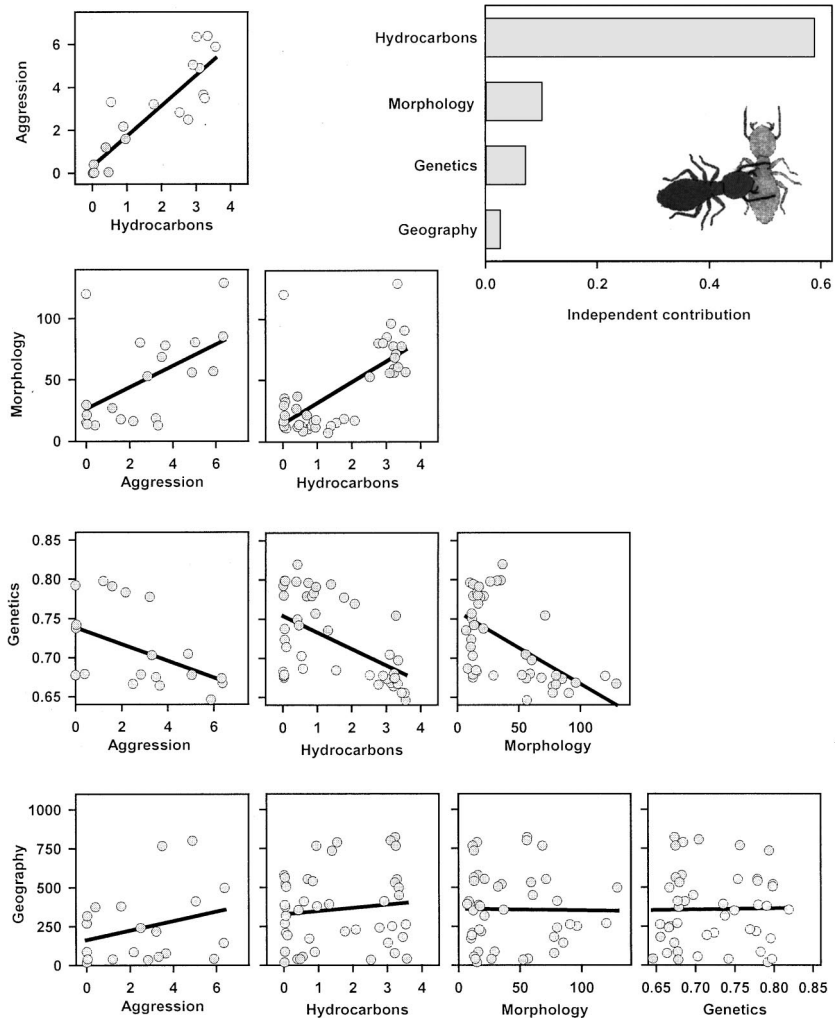

FIG. 3. All possible matrix correlations of distances or similarities among colonies (data see Table 3). The distance (or similarity) matrices measure aggression among colonies (aggression), differences in the composition of hydrocarbons (mean Nei-distance), morphometric dissimilarity among colonies (morphology; Mahalanobis-distance between colonies), genetic similarity among colonies (genetics, Jaccard-index from AFLP fingerprints), and geographic distance among colonies (geography; see Figure 1). The correlation coefficients and the associated error probabilities are tabulated in Table 4. Note the close correlation between aggression and hydrocarbons. The independent partitions also show that differences in the composition of cuticular hydrocarbons among colonies form the most important variable explaining variation in aggression between colonies.

TABLE 4. MATRIX CORRELATIONS (LOWER TRIANGLE) AND ASSOCIATED ERROR PROBABILITIES (999 PERMUTATIONS; ONE-TAILED PROBABILITIES; UPPER TRIANGLE) FOR THE DIFFERENT DISTANCE MATRICES (DATA SEE TABLE 3: PLOTS FIGURE 3)

	Aggression	Hydrocarbons	Morphology	Genetics	Geography
Aggression	—	0.002	0.008	0.038	0.10
Hydrocarbons	**0.89**	—	0.034	0.039	0.15
Morphology	**0.53**	**0.70**	—	0.017	>0.3
Genetics	**−0.46**	**−0.54**	**−0.58**	—	>0.3
Geography	0.29	0.12	−0.14	0.02	—

Note. Significant values are given in bold. The distance (or similarity) matrices measure aggression between colonies (aggression), difference in the composition of hydrocarbons (mean Nei-distance), morphometric dissimilarity between colonies (morphology; Mahalanobis-distance between colonies), genetic similarity between colonies (genetics, Jaccard-index from AFLP-fingerprints), and geographic distance between colonies (geography; see Figure 1). Note that the aggression data do not cover all possible combinations and, thus, the number of data points is 20 for all matrix correlations in which aggression is involved. All other matrix correlations are based on 45 data points (all possible combinations of 10 colonies).

colonies to allow for nestmate recognition. However, among some colonies the differences are small. 2. The level of aggression among colonies increases with an increase in the difference of cuticular hydrocarbons. Thus, individuals seem to adjust their behavior according to the differences in the composition of cuticular hydrocarbons. 3. Genetic, morphometric, and especially environmental differences, are of minor importance in predicting aggression relative to cuticular hydrocarbons.

Our analysis provides evidence that hydrocarbon compositions represent, at least in part, the cues for nestmate recognition. Furthermore, analysis of single peaks suggests that termites use a bouquet of compounds to recognize nestmates. This discrimination seems to be based not on the major components, but instead on components present only in lower concentrations and mostly unsaturated hydrocarbons. Of course, correlation does not prove causality. Thus, further experimental work should concentrate on unsaturated minor compounds in the hydrocarbon mixtures to increase the mechanistic understanding of nestmate recognition.

In the present study, variation of cuticular hydrocarbons was always low within colonies and was pronounced among colonies. Comparable chemical analyses in termites have been performed in different species of *Zootermopsis* (Haverty et al., 1988), in *Drepanotermes perniger* (Brown et al., 1996), in *Nasutitermes acajutlae* (Haverty et al., 1996c), in *Coptotermes formosanus* (Haverty et al., 1996b), and in *Macrotermes falciger* (Kaib et al., 2002). The most extensive work has been done in *Reticulitermes* (Haverty et al, 1996a, 1999; Haverty and Nelson, 1997; Page et al., 2002) and in *Heterotermes* (Watson et al., 1989). There is no reason to believe that the four chemical phenotypes we found correspond to subspecies

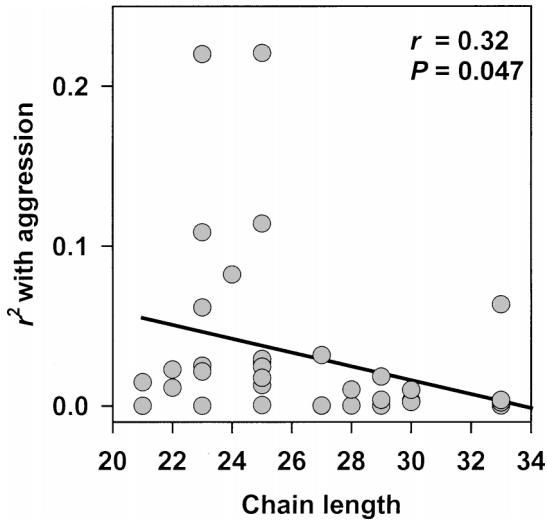

FIG. 4. Plot of squared correlation coefficients between the squared difference of individual compounds and aggression among colonies (see also insert in Figure 5) versus the chain length of the compounds. Note that most of the higher correlations ($r^2 > 0.1$) occur in compounds with a chain length of 23 and 25.

or cryptic species. No morphological, behavioral, or ecological differences were found among the 10 investigated colonies. Note also that the chemical phenotypes occur sympatrically. Haverty and Thorne (1989) distinguished two subspecies of *Zootermopsis nevadensis* on the basis of hydrocarbon phenotypes. Their investigation, however, covered the whole state of California and they did not find more than one hydrocarbon phenotype in any one specific location (see also discussion in Kaib et al., 2002).

Interestingly, the variation among colonies is not continuous. Phenotypes do not cover the whole range of possible hydrocarbon compositions, but seem to follow discrete patterns. This was also reported for *M. falciger* (Kaib et al., 2002). Chemical phenotypes differ in chain length of the hydrocarbons (elongation or shortening by one or several acetyl groups) or by a shift of the double bond along the chain, again by an even number of carbon atoms. The patterns of chain length and double bond positions in *M. subhyalinus* and *M. falciger* suggests that chain elongation or shortening of alkenes in termites may occur at the long end, as well as at the short end, of the carbon chain. These structural changes are in good agreement with the biosynthesis of insect cuticular hydrocarbons in which ethyl groups are supposed to be the "units" affected by enzymes (Blomquist et al., 1998). Hence, only few changes in the biochemical pathway are needed to generate such differences. This is a further hint that the phenotypes belong to the same species.

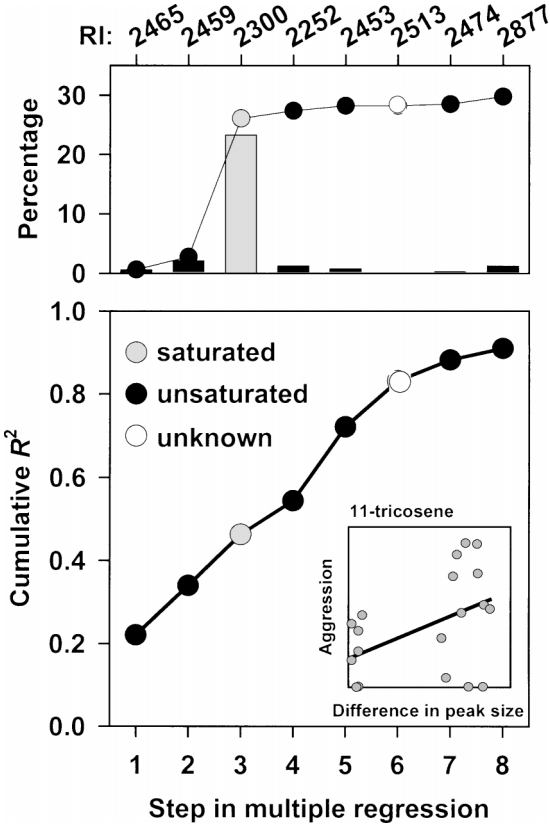

FIG. 5. Step-wise multiple correlation coefficient (R^2) of aggression among colonies versus squared differences of individual peaks. The lower graph plots the increase of the squared multiple correlation coefficient with each step. The upper graph gives the mean cumulative percentage of these peaks (%). The mean percentage of each peak was calculated as the mean across all individuals. Note that with only one exception, all selected compounds (retention indices RI according to Table 1 are given on the top of the upper graph) are only minor peaks that contribute less than 3% to the total hydrocarbon composition. The inset shows an example of the correlation of the squared difference in one selected compound versus aggression among colonies.

Also note that the genetic differences between colonies (measured by AFLPs, see Figure 3) are rather small. Colonies have between 60 and 80% of bands in common (Table 3).

In *Reticulitermes*, experiments with washed and retreated lures (Howard et al., 1982; Bagnères et al., 1991; Takahashi and Gassa, 1995) also suggested that cuticular hydrocarbons are involved in species and nestmate recognition. Furthermore,

in *Zootermopsis*, correlations between agonistic behavior and hydrocarbon phenotypes were found (Haverty and Thorne, 1989). However, these studies referred mostly to species recognition and not to intraspecific colony recognition, and the origin of labels is unknown. On the other hand, some studies in ants (Obin, 1986; Kaib et al., 1993), as well as in the termite *Coptotermes formosanus* (Su and Haverty, 1991), showed no evidence that cuticular hydrocarbons are involved in colony recognition. As alternative recognition cues, fatty acids or esters have been proposed (Heinze et al., 1996).

Altogether, convincing evidence has accumulated showing that cuticular hydrocarbons are involved in the recognition process. If we accept this, a further question arises: Are the hydrocarbons acquired from the environment, or are the differences in the hydrocarbon composition based on genetic differences? Our study favors the second possibility, but other authors provide evidence for the former (e.g., Gamboa et al., 1986). Our conclusion is based on the positive correlation of the differences in hydrocarbon composition (and of the aggression index) with morphometric differences, as well as the negative correlation with genetic similarity.

In this study, morphologic data were based on minor soldiers, whereas the other approaches used major workers. This has technical reasons. Soldiers' cuticle is highly sclerotized and does not shrink during alcohol fixation. In contrast, workers do not possess frontal gland secretion that may hamper chemical analysis or the isolation and amplification of genomic DNA. Nevertheless, the data sets from the different castes can be correlated, as in *M. subhyalinus* soldiers show the same differential response to nonnestmates as workers (Jmhasly and Leuthold, 1999). Furthermore, cuticular hydrocarbon compositions do not differ between workers and soldiers from the same colony (M. Kaib, unpublished data).

Husseneder et al. (1998) found a negative correlation between morphometric distances or the level of aggression, and genetic similarity in *Schedorhinotermes lamanianus*. Hence, in *S. lamanianus* and *M. subhyalinus* morphological differences among colonies seem to be based on genetic differences between colonies and are not the result of environmental differences faced by each colony. We sampled colonies from four different locations. However, none of the other data sets correspond with the geographic population structure (geographic distance). Thus, correlations found among chemistry, behavior, genetics, and morphology are not due to local adaptations of the colonies.

The good correlation of morphology and genetics in termites may be explained by the fact that termites live within closed colonies and under fairly constant climatic conditions, at least during their development within the colonies. Environmental variations and differences do not have as much influence on morphology as in species exposed to fluctuations of the environment during their development. The clear correlations of morphology and genetics with hydrocarbons suggest that the composition of cuticular hydrocarbons has a genetic basis

(see also Husseneder et al., 1997, 1998). A genetic basis for recognition cues was found in several species of *Hymenoptera* (see reviews in Breed and Bennett, 1987; Hölldobler and Wilson, 1990). In termites, the heritability of recognition cues was established for *Microcerotermes arboreus* (Adams, 1991). Genetically based recognition cues might facilitate the acceptance of kin and may help to increase the inclusive fitness (Gamboa et al., 1991). Alternatively, as Beye et al. (1997) note, such cues might have evolved to function as a mechanism to permit the segregation of colonies within the same environment.

Among colonies with the same chemical phenotypes, we found little or no aggression. Thus, the colony recognition system is not perfect (see Table 3). Why is the system of colony recognition imperfect? If there is a need to guarantee colony integrity, individuals should always show some aggression towards nonnestmates. There are several possible explanations. First, the experimental situation in neutral arenas might be too artificial to measure all the details of behavioral interactions among colonies. Second, colonies may use additional cues to distinguish nestmates from nonnestmates. For example, in *M. falciger*, individuals from neighboring colonies were not aggressive to each other, although they had different compositions of cuticular hydrocarbons (dear-enemy phenomenon; Kaib et al., 2002). This provides evidence that termites use additional information in their decision-making to become aggressive or not (see also Husseneder et al., 1997). In *M. subhyalinus*, there was no indication of a dear-enemy phenomenon (see Jmhasly and Leuthold, 1999). At present, we have no conclusive answer to explain the imperfect system of nestmate recognition. It may be that aggression among phenotypes is only a nonadaptive by-product of small evolutionary differences among colonies. This may have a substantial impact on other processes during the life cycle of the termites. If alates also use hydrocarbons to recognize possible partners to found a colony and hydrocarbon composition of alates is the same as of the parent colony, phenotypes would lead to assortative mating and to independent evolutionary lineages, a possibility for sympatric speciation.

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