Extracted Venom and Cuticular Compounds of Imported Fire Ants, *Solenopsis* spp., and Chemotaxonomic Applications Across a Persistent Hybrid Zone[1]

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Abstract Characterization of cuticular hydrocarbons permits basic distinctions among colonies of *Solenopsis invicta* Buren, *Solenopsis richteri* Forel and their hybrids (Hymenoptera: Formicidae); thus, providing opportunities to investigate details of landscape ecology for this species complex and to assess levels of invasiveness. We introduce an alternative method for the cluster analysis of cuticular molecules of imported fire ants that is complementary to a widely-used method based on calculating cuticular hydrocarbon and venom alkaloid indices. Results from this GC-MS method were analyzed using various hierarchical and normal mixtures clustering methods to test the stability of group membership. Principal component and discriminant analyses were used to produce three-dimensional views of group separation. The relative proportions of 12 intensity peaks served to differentiate imported fire ant hybrids into four assemblages – hybrids closely allied with *S. invicta*, hybrids close to *S. richteri*, a 'core' hybrid grouping, and an 'outlier' hybrid group. The most influential peaks of the assemblage (based on *F*-values) included 3 peaks with the piperidine structural motif and an alkane. Use of 3 peaks identified by stepwise linear discriminant analysis resulted in misclassification of 5% of the ant colonies, whereas use of 4 peaks resulted in the misclassification of 2.5%. Thus, this GC-MS method and multivariate assessment of biochemical data may facilitate the finer-scale distinction of hybrid colonies in terms of their surficial semiochemical complexity and 'alliance' with parental species. Application of these techniques would be especially useful in refining biological control strategies.

Key Words *Solenopsis invicta* Buren, *Solenopsis richteri* Forel, *Solenopsis invicta* x *S. richteri*, hydrocarbons, venom alkaloids

The cuticular composition of insects serves a broad spectrum of functions ranging from prevention of desiccation (Hadley 1980, 1984), protection from abrasion and microorganismal attack, to encompassing a host of other complex physiological, defensive and semiochemical functions (recently reviewed by Howard and Blomquist 2005). Insect cuticular hydrocarbon assemblages are species-specific, sex-specific, and among social insects, these suites of surficial biomolecules have also been demonstrated to be caste- and colony-specific (e.g., Hölldobler and Wilson 1990). Biomolecular characterizations of the cuticles of economically-important pests constituted the vanguard of investigations in insect chemotaxonomy. Caste-specific implications

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of cuticular hydrocarbons were first described for *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae) (Howard et al. 1978, 1982). Cuticular lipids of the imported fire ants *Solenopsis invicta* Buren and *S. richteri* Forel were initially characterized by Lok et al. (1975) and later reported as species-specific (Nelson et al. 1980).

As a consequence of the emerging linkages between comparative behavioral, taxonomic and biochemical investigations, Vander Meer et al. (1985) discovered biochemical evidence for hybridization in imported fire ants. Shortly thereafter, colony-specific hydrocarbon compositions were characterized for *S. invicta*, and dynamic changes in the hydrocarbon profile of entire colonies were observed (Obin 1986, Vander Meer et al. 1989). In addition, Obin (1986) reported that laboratory-reared colonies of *S. invicta* exhibited less distinctive colony odor suggesting that this semiochemical assemblage derived from both genotypic and epigenetic (i.e., environmental) sources.

The red imported fire ant (*S. invicta*), the black imported fire ant (*S. richteri*), as well as persistent hybrids are currently recognized in the southern United States with hybrids residing in parts of Mississippi, Alabama, and Georgia (Vander Meer et al. 1985, Vander Meer 1986, Diffie et al. 1988, Diffie et al. 2002, Graham et al. 2006, Streett et al. 2002, 2006, Gardner et al. 2008). However, *S. invicta* and *S. richteri* were originally recognized as subspecific variants (Wilson 1953, Wilson and Brown 1958). Taxonomic/systematic issues aside, imported fire ants (Hymenoptera: Formicidae) have successfully invaded over 129.5 million hectares in the United States spanning 13 states and Puerto Rico (USDA, APHIS 2006, 2007) (http://www.aphis.usda.gov/plant_health/plant_pest_info/fireants/index.shtml 2007; http://www.aphis.usda.gov/plant_health/plant_pest_info/fireants/downloads/fireant.pdf 2006) with the most recent recurrent infestations of *S. invicta* observed in Virginia (http://www.sciencedaily.com/releases/2007/05/070524101201.htm). Isolated imported fire ant colonies have also been found in Arizona, Kentucky, and Maryland; however, they are not listed as established in these states.

Current identification of imported fire ants relies on the combination of two chemotaxonomic index calculations based on GC-MS analyses of venom alkaloids and cuticular hydrocarbons (Vander Meer 1986, Ross et al. 1987, Vander Meer and Lofgren 1988). Lockey (1991) commented that epicuticular hydrocarbons of insects have many qualitative and quantitative characteristics that would make them highly discriminatory for use in taxonomy; however, at that time, this potential was not fully realized due to incomplete resolution of some isomeric mixtures in key hydrocarbon classes (e.g., olefins and methylalkanes). Improvements in analytical technologies available to evaluate chemically-complex admixtures permit more detailed investigation of the cuticular hydrocarbons and other readily extractable biomolecules of imported fire ants across a persistent hybrid zone. Molecular characterizations of cuticular hydrocarbons in tandem with the evaluation of genetic markers in imported fire ants (Ross et al. 1987) are becoming available and more affordable, but there is still the need to develop alternative methods to molecular characterization. Our objectives were to determine the relative proportions of venom alkaloids and other readily extractable cuticular hydrocarbons, identify differences among populations of imported fire ants, and evaluate patterns of variation in these predominantly semiochemical assemblages among hybrid populations. More specifically, our goal was to develop a complementary or alternative method to the combined index reported by Ross et al. (1987) that may permit rapid examination of potential degrees of introgression among red, black and hybrid imported fire ants.

Materials and Methods

Collection and maintenance of ant colonies. A total of 39 ant colony samples was used for this study. Collection sites were based on a recent imported fire ant map (Streett et al. 2006), and augmented the distribution data. *Solenopsis invicta*, *S. richteri* and hybrid ant colonies were obtained from the Delta, Loess Hills, North Central Hills, and Black Prairie physiographic provinces of Mississippi from July to September 2007 (Fig. 1). The localities in Washington, DeSoto, Carroll, Oktibbeha, and Clay counties were recorded using a global positioning system (GPS – Trimble Ag123

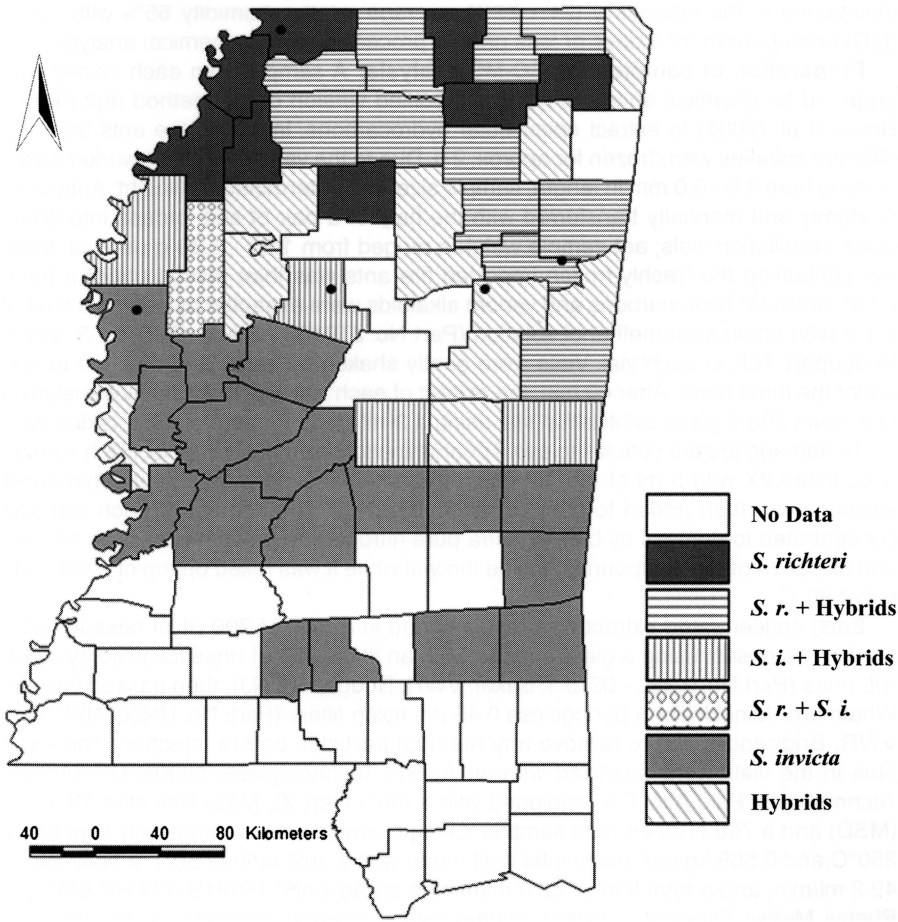

Fig. 1. Distribution of imported fire ant colonies used in this investigation (locations are depicted by black circles). The base map portrays county-wide associations of *S. invicta*, *S. richteri*, and hybrids (modified from a map produced by Streett et al., 2006). All counties in the southernmost part of Mississippi are purportedly colonized by *S. invicta*.

DGPS, Trimble Navigation Limited, Sunnyvale, CA). Parental ant colonies were identified as reported by Valles and Porter (2003) for *S. invicta* and Vander Meer et al. (1985) for *S. invicta*, *S. richteri*, and hybrid ants.

Mound soils were placed in 19-L PVC buckets; bucket rims were coated with a fine dusting of Equate® baby powder to keep ants confined during transport. Ants were separated from soil using the water-drip method (Banks et al. 1981) and placed in plastic trays (45 cm × 60 cm × 13 cm) with Fluon®-coated inside walls. Distilled water was provided to colonies *ad libitum*. Colonies were composed of eggs, larvae and workers with at least 1000 workers. Each colony was provided with 2 - 3 Petri dishes (15 cm diam × 2 cm) with 1 - 1.5 cm of hardened dental plaster filling the bottom of each receptacle and a 5 cm-diam brood chamber. All colonies used in this study were maintained in the laboratory (21 - 27°C, average relative humidity 65% with 12:12 (L:D) photoperiod) for 4 days or less prior to processing for biochemical analysis.

Preparation of samples for GC-MS analysis. A sample from each colony was prepared for chemical analyses using a modified version of the method reported by Rojas et al. (2005) to extract epicuticular hydrocarbons. Imported fire ants from the different colonies were frozen for approx. 2 h. Due to the variability in ant worker sizes, ranging from 3.0 - 5.0 mm in length, sample size was determined by weight. Ants were randomly and manually transferred with the help of a pair of soft forceps into 20-ml glass scintillation vials; ant sample weights ranged from 1.51 - 1.59 g per vial. Each vial containing the freshly-frozen imported fire ants was then placed under a fume hood. Cuticular hydrocarbons and venom alkaloids were extracted by adding 3.5 ml of a 1:2 (v/v) chloroform:methanol solution (Part No. JT9183 - 3 and JT9069 - 3, VWR, Bridgeport, NJ) to each vial. Vials were gently shaken by hand, and then left to rest under the fume hood. After 60 min, the extract of each vial was individually transferred to a clean 20-ml glass scintillation vial using a 2-ml glass Pasteur pipette, taking care not to damage the ant cuticles to avoid contamination from gut contents. Each sample was rinsed 2X with 5 ml of the 1:2 chloroform:methanol solution; these subsequent washes were then added to their corresponding vial. The content of each vial was concentrated to dryness by blowing ultra pure nitrogen (to prevent excessive heating and oxidation of the compounds) inside the vial while it was rolled on top of a hot plate set at 60°C.

Each concentrated extract was resuspended in a total of 700 µl of hexane delivered in two doses, using a glass syringe, with an initial 300 µl rinse followed by a 400 mL rinse (Part No. 9,262 - 02, J.T. Baker, VWR, Bridgeport, NJ), then passed through Whatman® Mini-UniPrep Syringeless 0.45 µm nylon filters (Part. No. UN203NPUNYL, VWR, Bridgeport, NJ) to remove any residual particles before injection. The samples in the vials were analyzed with an Agilent Technologies® 6890N GC (Agilent Technologies, Palo Alto, CA) equipped with a 5975 inert XL Mass Selective Detector (MSD) and a 7683 Series auto sampler. One µl sample was automatically injected at 250°C and 0.568 kg/cm^2 using inlet split mode with a split ratio of 50:1, a split flow of 49.2 ml/min, and a total flow of 53.0 ml/min. to an Agilent® 19091S-433 HP-5MS 5% Phenyl Methyl Siloxane capillary column with a nominal diameter of 250 µm, and nominal film thickness of 0.25 µm. Ultra pure helium was used as the carrier gas with a gas flow of 20.0 ml/min.

Preliminary GC analyses of these samples indicated that oven temperature programmed to increase from 60°C to 170°C at a rate of 8°C/min and from 170°C to 250°C at 5°C/min with a final temperature hold of 5 min were the best settings to elute the compounds of interest. The mass spectrometer was tuned using the manufacturer's

atune.u file; acquisition mode was set to scan at 280°C, with a 3 min solvent delay, and a resulting EM voltage of 1035.5. MSZones were set for MS Quad at 150°C and MS Source at 230°C. Chromatogram peaks were identified by comparing retention times and MS spectra to those reported in the Wiley® 7th Edition and NIST 2005 libraries (Agilent Technologies, Palo Alto, CA) with reference to Nelson et al. (1980) and Bagneres et al. (1991).

Statistical analysis. GC-MS chromatograms from 34 ant colonies collected during the summer season (July – September 2007) were initially rescaled using the highest peak response within each sample. In addition, replicate subsamples from 5 out of the original 34 ant colonies collected were selected and included in the analysis to gauge intracolony variability; thus, bringing the total number of chromatogram datasets evaluated to $n = 39$. Chromatogram peak intensities were subsequently normalized for each of the 12 major peaks using the weight of the corresponding ant sample.

Various clustering methods were performed on the 12-peak dataset (JMP® 7.0, SAS® Institute Inc., Cary, NC) using hierarchical and *k*-means statistical methods. Hierarchical clustering distance options used included: average linkage (the average distance between pairs of observations tends to join clusters with small variances and exhibits bias toward producing clusters with same variance); centroid (clusters defined by the squared Euclidean distance between their means); and Ward's minimum variance (where the distance between 2 clusters is the ANOVA sum of squares totaled over all of the variables and iteratively minimizing within-cluster sum of squares).

Multivariate scatterplot matrices were inspected to evaluate the separation of hierarchical clusters based on pairwise comparison of the 12 peaks. Pearson product-moment correlation coefficients were calculated for all ant colony samples ($n = 39$) across all major peaks. An inverse correlation matrix was produced, and variance inflation factors (VIF) were examined. Principal components on correlations were determined and resolved in 3-dimensions, and cluster membership was examined. In addition, higher dimensional outliers were identified for the ant colony samples using jackknifed distances. Parallel coordinate plots were inspected to identify apparent intragroup deviants as well as evaluate contrasts in peak features between clusters.

One-way analysis of variance (ANOVA) was conducted to evaluate sample distribution as well as group differences based on results from hierarchical clustering. Outliers for each group also were examined. All differences among the means were tested using Tukey-Kramer HSD.

Finally, discriminant function analysis (DA) was performed on the dataset using the nominal variables derived from the nonsupervised classification; both linear and regularized methods were used. Key peaks were identified in stepwise fashion to build each model. Canonical plots were evaluated, and the discriminant scores as well as a misclassification summary derived from the linear model have been reported.

Results

Imported fire ants from 34 colonies obtained from the Delta, Loess Hills, North Central Hills, and Black Prairie physiographic provinces of Mississippi, and distributed across a persistent hybrid zone were classified based on GC-MS analyses of 39 ant colony samples. The 12 relevant peaks resolved for *S. invicta*, *S. richteri*, and their hybrids were identified as piperidine alkaloids and hydrocarbons (Table 1, Fig. 2);

Table 1. Chemical identification for the twelve major peaks used to differentiate *S. invicta*, *S. richteri*, and hybrid imported fire ant colony samples.

Peak Number	Alkaloids	Carbon No.	Correlated	m/z	MW
1	Alkenylpiperidine	C17H35N	*S. invicta*, *S. richteri*, hybrids	98	253
2	Alkenylpiperidine	C19H37N	*S. invicta*, *S. richteri*, hybrids	98	279
3	Alkenylpiperidine	C19H37N	*S. invicta*, *S. richteri*, hybrids	98	279
4	Alkenylpiperidine, (pentyl)	C19H39N	*S. invicta*, *S. richteri*, hybrids	98	281
5	2-methyl-6-pentadecenyl-6-piperideine	C21H39N	*S. invicta* and hybrids	111	306
6	2-methyl-6-pentadecyl-6-piperideine	C21H41N	*S. invicta* and hybrids	111	308
	Hydrocarbons				
6	Nonadecane (n-C19)	C19H40	*S. richteri*	57	268
7	3-methyltricosane (3-MeC23)	C24H50	*S. invicta*, *S. richteri*, hybrids	57/309	338
8	3,11-dimethyltricosane (3,11-diMeC23)	C25H52	*S. richteri* and hybrids	57/196/323	352
9	11-methylpentacosane (11-MeC25)	C26H54	*S. richteri* and hybrids	57/196	366
10	3-methylpentacosane (3-MeC25)	C26H54	*S. richteri* and hybrids	57/337	366
11	3,11-dimethylpentacosane (3,11-diMeC25)	C27H56	*S. invicta* (monogyne), *S. richteri* and most hybrids	57/224/351	380
12	n-hexacosane (n-C26)	C26H54	No apparent association	57	366

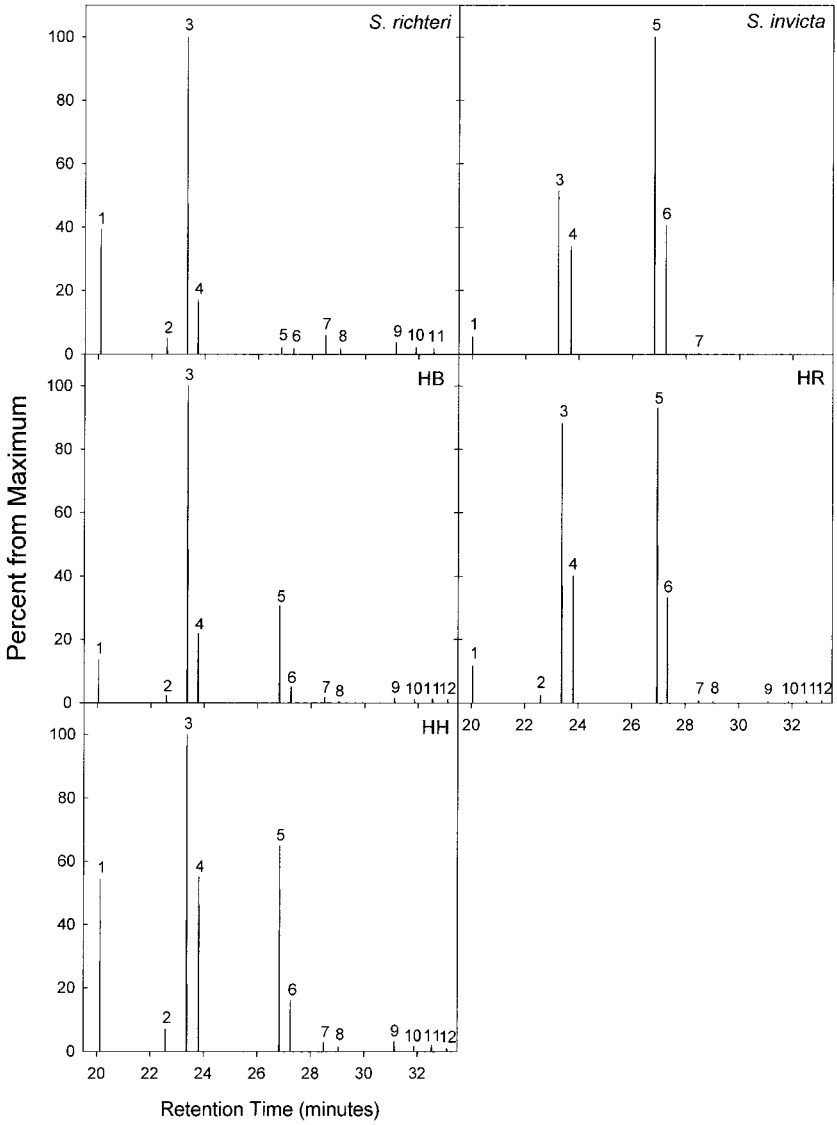

Fig. 2. Mean chromatogram intensity traces of 1:2 (v/v) chloroform:methanol extracts from *S. invicta*, *S. richteri*, and the three main hybrid groupings which include a 'core' hybrid assemblage (HH), hybrids close to *S. invicta* (HR), and hybrids close to *S. richteri* (HB).

these biomolecules were previously reported in fire ants by MacConnell et al. (1971) with detailed comparisons of alkaloidal components provided by Brand et al. (1972) and Nelson et al. (1980), respectively. A representative hierarchical clustering outcome

of all samples ($n = 39$), based on Ward's minimum variance method, depicted 5 coherent groups and identified 3 ant colonies as outliers (Fig. 3). The complex attributes of each ant colony's chemical profile were highlighted by invoking a two-way analysis using the first principal component as the ordering variable for the 12 major compounds discerned. Other hierarchical cluster methods used (i.e., average, centroid, complete linkage, and single linkage) returned similar results with minor variations reported in the identification ant colonies as *S. invicta* or hybrids close to *S. invicta.* Normal mixtures outcomes (implemented in the JMP® *k*-means clustering statistical procedure) using 3, 4, 5, and 6 groups (these trials were conducted both with and without outliers identified by jackknifed distances) facilitated the resolution of cluster overlap especially in the *S. invicta* to hybrid 'continuum'.

Relationships between chemical compounds were examined using Pearson product-moment correlation coefficients (Table 2). Ant-derived compounds linked to Peaks 1 through 6 (with retention times less than 28 min) for *S. invicta* and hybrids typically possessed the piperidine structural motif (Table 1, Fig. 2, Table 3) with the highest correlation coefficients obtained for Peaks 5 (2-methyl-6-pentadecenyl-6-piperideine, C21H39N) and 6 (2-methyl-6-pentadecyl-6-piperideine C21H41N) (r = 0.922) followed by Peaks 1 (alkenylpiperidine, C17H37N) and 2 (alkenylpiperidine, C17H37N) (r = 0.838). Peak 3 (alkenylpiperidine, C19H37N) had the lowest set of variance inflation factors among the 6 piperidine derivatives typical of each chromatogram indicating that (C19H37N) was not highly correlated with the other piperidine alkaloids.

Cuticular hydrocarbons associated with Peaks 7 through 12 were identified as alkanes (Table 1, Fig. 2, Table 3). 3-methyltricosane (3-MeC23) was linked with Peak 7, for *S. invicta, S. richteri* and all hybrid samples. Peak 8, 3,11-dimethyltricosane (3,11-diMeC23), peak 9, 11 methylpentacosane (11-MeC25), and peak 10, 3-methylpentacosane (3-MeC25) were linked to *S. richteri* samples as well as all hybrid samples. Peak 11 was identified as 3,11-dimethylpentacosane (3,11 diMeC25) for *S. richteri* and the majority of hybrids; whereas, 5 out of 6 polygyne *S. invicta* lacked this peak with the remaining *S. invicta* exhibiting the 3,11 diMeC25. Peak 12 was most commonly linked to n-hexacosane (n-C26) with no detectable pattern of association among *S. invicta, S. richteri*, and hybrids.

Among the hydrocarbon compounds with retention times greater than 28 min, Pearson product-moment correlation coefficients were highest for Peaks 8 (3,11 dimethyltricosane) and 9 (11-methylpentacosane) (r = 0.937), followed by Peaks 9 (11-methylpentacosane) and 10 (3-methylpentacosane) (r = 0.928), and finally Peaks 9 (11-methylpentacosane) and 11 (3,11-dimethylpentacosane) (r = 0.915). These relationships served to establish the nesting pattern for the two-way hierarchical clustering (Fig. 3).

Chemical compounds that served as "central organizers" to separate parental classes from the various hybrid groupings were tentatively identified by *F*-value (Table 3). Step-wise discriminant analysis revealed that peaks 3 (alkenylpiperidine, C19H37N), 11 (3,11-diMeC25), 5 (2-methyl-6-pentadecenyl-6-piperideine, C21H39N), and 1 (alkenylpiperidine, C17H35N) were the most important determinants of cluster membership (in that order). A three-dimensional scatterplot depicts clustering relationships as defined by the chemical compounds represented by peaks 3, 5, and 11 (Fig. 4A) and highlights an area where members from 2 different groups overlap (based on Ward's minimum variance method). In this particular assemblage of peaks, ant colonies 36 and 37 (identified as hybrids close to *S. invicta* by Ward's method and denoted by the small triangular symbols) appear to be more closely associated with

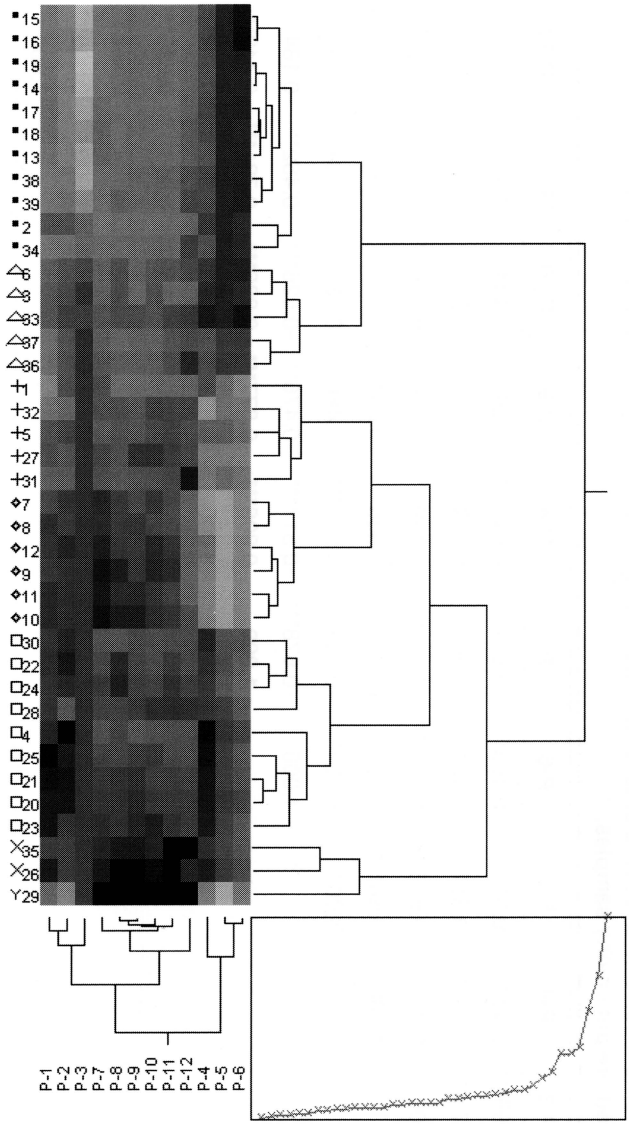

Fig. 3. Hierarchical clustering of ant colony samples (*n* = 39), based on a 12-peak assemblage of hydrocarbons, using two-way Ward's minimum variance method (with standardization) and the first principal component as an ordering variable. Grayscale depicts relative peak intensity with light gray representing low and black representing high. Vertical dendrogram specifies ant colony samples (1 - 39): solid squares = *S. invicta*; diamonds = *S. richteri*; triangles = hybrids close to *S. invicta* (HR); crosses = hybrids close to *S. richteri* (HB); open squares = core hybrid assemblage (HH); and X and Y are the outliers. Horizontal dendrogram groups peaks (P1 to P12).

Table 2. Pearson product-moment correlation coefficients for the twelve major peaks used to differentiate *S. invicta, S. richteri,* and hybrid imported fire ant colony samples.

	P-1	P-2	P-3	P-4	P-5	P-6	P-7	P-8	P-9	P-10	P-11	P-12
P-1	1.000	0.838***	0.595***	0.490**	-0.314	-0.465**	0.521***	0.546***	0.568***	0.444**	0.431**	0.096
P-2		1.000	0.675***	0.494**	-0.327*	-0.464**	0.360*	0.389*	0.304	0.210	0.254	-0.018
P-3			1.000	0.062	-0.665***	-0.786***	0.587***	0.559***	0.508***	0.525***	0.527***	0.333*
P-4				1.000	0.561***	0.367*	-0.206	0.036	0.064	-0.061	0.061	0.082
P-5					1.000	0.922***	-0.753***	-0.535***	-0.486**	-0.561***	-0.438**	-0.144
P-6						1.000	-0.678***	-0.517***	-0.476**	-0.526***	-0.452**	-0.184
P-7							1.000	0.906***	0.877***	0.886***	0.791***	0.443**
P-8								1.000	0.937***	0.905***	0.902***	0.584***
P-9									1.000	0.928***	0.915***	0.630***
P-10										1.000	0.892***	0.645***
P-11											1.000	0.806***
P-12												1.000

***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$.

Table 3. Summary of the twelve major biomolecular peaks for *S. invicta*, *S. richteri*, and various hybrid groups based on Ward's minimum variance hierarchical cluster analysis.

		Mean Percent from Maximum							
								F Test†	
Peak	Retention Time (min)	*S. invicta* ($n = 11$)	*S. richteri* ($n = 6$)	Hybrid (core) ($n = 9$)	Hybrid (out) ($n = 3$)	Hybrid (*S.i.*) ($n = 5$)	Hybrid (*S.r.*) ($n = 5$)	*F* value	$P > F$
1	20.01 - 20.20	6.17 ± 4.50 c	39.30 ± 6.79 a	54.35 ± 16.79 a	36.61 ± 24.56 ab	11.83 ± 5.11 bc	13.55 ± 10.61 bc	21.85	<0.0001
2	22.55 - 22.65	0.39 ± 0.68 c	5.15 ± 0.58 ab	6.99 ± 2.66 a	2.97 ± 2.58 bc	2.87 ± 0.66 bc	2.35 ± 0.92 bc	19.58	<0.0001
3	23.20 - 23.44	55.57 ± 8.79 b	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a	94.86 ± 6.42 a	100.00 ± 0.00 a	106.10	<0.0001
4	23.68 - 23.88	34.37 ± 3.24 bc	17.17 ± 3.08 d	55.02 ± 10.34 a	40.71 ± 18.37 ab	41.82 ± 10.42 ab	21.91 ± 7.77 cd	18.57	<0.0001
5	26.75 - 27.01	100.00 ± 0.00 a	2.23 ± 0.56 d	64.96 ± 12.09 b	46.09 ± 40.32 bc	90.21 ± 13.44 a	30.65 ± 6.92 c	58.17	<0.0001
6	27.19 - 27.37	38.61 ± 5.87 a	1.91 ± 0.46 d	16.03 ± 4.47 b	14.59 ± 6.91 bc	33.29 ± 8.64 a	5.07 ± 2.32 cd	57.39	<0.0001
7	28.46 - 28.56	0.24 ± 0.27 c	5.92 ± 1.29 a	2.79 ± 0.96 b	7.39 ± 3.04 a	1.02 ± 0.29 c	1.76 ± 0.48 bc	38.77	<0.0001
8	29.01 - 29.11	0.04 ± 0.09 c	1.82 ± 0.52 b	1.40 ± 0.56 b	3.91 ± 1.15 a	0.54 ± 0.29 c	0.48 ± 0.29 c	39.41	<0.0001
9	31.10 - 31.16	0.05 ± 0.09 c	3.80 ± 1.39 b	3.13 ± 1.33 b	10.31 ± 3.11 a	0.63 ± 0.74 c	1.50 ± 1.47 bc	34.82	<0.0001
10	31.85 - 31.97	0.04 ± 0.07 d	2.14 ± 0.52 b	1.61 ± 0.79 bc	5.29 ± 2.74 a	0.54 ± 0.23 cd	1.19 ± 0.83 bcd	19.80	<0.0001
11	32.52 - 32.62	0.04 ± 0.08 d	1.98 ± 0.58 b	1.98 ± 0.62 b	7.25 ± 0.80 a	0.83 ± 0.62 cd	1.34 ± 0.79 bc	83.55	<0.0001
12	33.10 - 33.18	0.28 ± 0.40 b	0.10 ± 0.21 b	0.94 ± 0.35 b	4.10 ± 1.79 a	0.91 ± 0.69 b	1.07 ± 1.06 b	16.37	<0.0001

† values from ANOVA to test for overall cluster group difference for each peak.

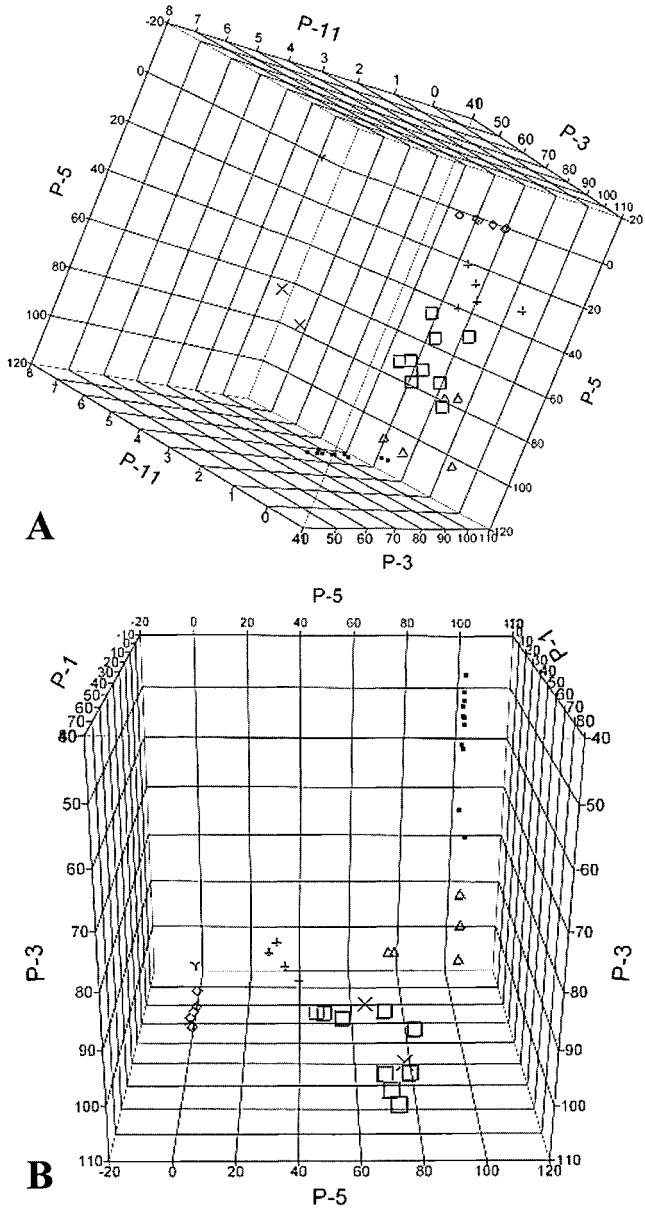

Fig. 4. (A) Three-dimensional scatterplot of *S. invicta*, *S. richteri*, and various hybrid groups using peaks 3, 5, and 11. (B) Scatterplot of *S. invicta*, *S. richteri*, and various hybrid groups using peaks 3, 5, and 1. Graph axes have been rotated to optimize cluster resolution. Symbols used to distinguish clusters are the same as those used in the dendrogram.

some of the 'core' hybrids (i.e., ant colonies 4 and 25). Alternatively, a three-dimensional scatterplot using the alkenylpiperidines represented by peaks 3, 5, and 1 (Fig. 4B) associated 2 out of the 3 'outlier' hybrids (specifically ant colonies 26 and 35, denoted by small "x" symbols) with the 'core' hybrid cluster.

Comparison of imported fire ant classification based on the Ward model of hierarchical clustering (12 peaks) with the predicted group outcomes from linear discriminant analysis (Table 4) indicated that 2 ant colony samples (4 and 30) were misclassified (5% of the total). In the first case (ant colony 4), the sample was categorized as a 'core' hybrid (HH); its discriminant score returned a probability (0.70) that it was a hybrid close to *S. invicta* (HR). The second sample (ant colony 30), originally classified as a 'core' hybrid was most likely (0.83) better characterized as a hybrid close to *S. richteri* (HB).

Discussion

Brill and colleagues (1985a, b) first used computerized pattern recognition algorithms in the differentiation of *S. invicta* and *S. richteri* based on cuticular hydrocarbon assemblages extracted from individual ants. Brill et al. (1985a) concluded that the optimum number of chromatogram features (i.e., GC peaks) to distinguish between these 2 species was around 3 or 4; 'noise level' increased if more features were included. Vander Meer et al. (1989) used principal component analysis to resolve inter and intracolony relationships among *S. invicta* GC profiles of cuticular hydrocarbons and other variables such as geographic source (Florida and Mississippi) and field collected versus laboratory-reared. In this investigation, we expanded on these prior fundamental works and used forms of nonsupervised classification (i.e., hierarchical cluster and principal component analyses) as well as normal mixtures ('supervised' by the specification of cluster number) to examine hybrid relationships to the parental species based on a 12-peak assemblage of GC-MS features including venom alkaloids and hydrocarbons.

Hybridization in imported fire ants was first reported by Vander Meer et al. (1985) from colonies collected in the vicinity of Starkville, MS. These hybrid workers were

Table 4. Comparison of classification based on hierarchical clustering with predicted group outcomes from linear discriminant analysis.

	Predicted group					
	S. invicta	Hybrid (*S.i.*)	Hybrid (out)	Hybrid (core)	Hybrid (*S.r.*)	*S. richteri*
S. invicta	11	0	0	0	0	0
Hybrid (*S.i.*)	0	5	0	0	0	0
Hybrid (out)	0	0	3	0	0	0
Hybrid (core)	0	1 (0.70)†	0	7	1 (0.83)‡	0
Hybrid (*S.r.*)	0	0	0	0	5	0
S. richteri	0	0	0	0	0	6

† ant colony 4; ‡ ant colony 30.

morphologically characterized as *S. richteri*; however, their GC profiles (from ants soaked in hexane 2 + d) indicated admixtures of venom alkaloids as well as cuticular hydrocarbons. Thus, the authors concluded hybridization was expressed biochemically rather than morphologically. Earlier to this pivotal work, Vander Meer et al. (1982) had recognized that soaking times in excess of several hours resulted in increased hydrocarbon content most likely leached from the postpharyngeal gland. Later, Ross et al. (1987) reported on the development of a 'combined index' (*I*) that served to quantify the direction of hybridization with parental forms *S. richteri* ranging from 0.00 - 0.06 and *S. invicta* greater than or equal to 0.85. However, the authors cautioned that values obtained for the alkaloids did not necessarily correspond to those for hydrocarbons (Ross et al. 1987) "indicating the independence of the two biochemical characters" (Vander Meer and Lofgren 1988).

In the current study, we decided to include all of the chromatogram peaks resolved (that had some of the ant colony samples with responses ≥0.2% percent from maximum); thus, preserving biomolecular details that had been merged by Ross et al. (1987) to formulate correction factors for *S. invicta* and *S. richteri* (spanning retention times from 17 - 24 min using their GC-MS procedure). In addition, we have analyzed the biochemical data for alkaloids and hydrocarbons of whole ant bodies in an attempt to conserve the integrity of the biomolecular 'signature' of each ant colony. The relative proportions of 12 peaks served to differentiate imported fire ant hybrids into 4 assemblages – hybrids closely allied with *S. invicta*, hybrids closely related with *S. richteri*, a 'core' hybrid grouping, and an 'outlier' hybrid group. Four 'key' peaks of chemotaxonomic utility were resolved using discriminant analysis, namely peaks 3 (alkenylpiperidine, C19H37N), 11 (3,11-diMeC25), 5 (2-methyl-6-pentadecenyl-6-piperideine, C21H39N), and 1 (alkenylpiperidine, C17H35N).

Closer inspection of the ant colonies identified as 'outliers' (by nonsupervised classification methods as well as jackknifed distances) revealed that ant sample numbers 26, 29, and 35 had very uneven/skewed distributions of worker sizes with the samples from colonies 26 and 35 dominated by major workers; whereas, colony 29 consisted largely of minor workers. Lok et al. (1975) noted that hydrocarbon composition varied both qualitatively and quantitatively (based on 10 min hexane soaks) for imported fire ants, detailing differences by developmental stage for workers and reproductives. Thus, in the current study, inclusion of an alkane peak (in this case peak 11, 3,11-dimethylpentacosane (3,11 diMeC25), appears to facilitate the recognition of colony subsamples that may be unusual in terms of worker size distribution.

Our colony sampling strategy was season-specific and more inclusive with the broad spectrum of workers represented (reserves, foragers, and nurses - though brood were carefully extracted from the latter group) to gain a more comprehensive perspective of each colony's profile. In addition, the soak time used in this investigation was standardized at 1 h, and by the use of the chloroform:methanol solution, leaching was greatly reduced. Because the social interactions of colony workers promote the rapid transference of cuticular chemicals throughout the colony (e.g., Sorensen et al. 1985) and more recent studies have demonstrated that the cuticular colony profile is strongly concordant with postpharyngeal gland (PPG) contents such as tricosanes (e.g., Bagneres and Morgan 1991, Akino et al. 2004), the extraction method that we used may represent a potential avenue for integrating environmental influences into the colony profile (e.g., Hefetz 2007).

In the current study, 2-methyl-6-pentadecenyl-6-piperideine (C21H39N) was linked to peak 5 and associated with the *S. invicta* ant colony samples. Our findings have

also indicated that this venom alkaloid (peak 5) is a key compound serving as one of the 'central organizers' in the hierarchical clustering of these summer-season, nonnative *Solenopsis* colonies with significant separation for ants assigned to the '*S. invicta*-to-hybrids-close-to *S. invicta*' aggregation from all of the other 4 groups as well as significant distinctions between *S. richteri* and the various hybrid clusters. Peak 7, 3-methyltricosane (3-MeC23), is present in *S. invicta*, *S. richteri*, and hybrid samples; but peak 8, 3,11 dimethyltricosane (3,11–diMeC23), is absent in polygyne *S. invicta* samples; in addition, peak 11, 3,11 dimethylpentacosane (3,11-diMeC25) is absent from 5 out of the 6 polygyne *S. invicta* ant colonies included in this study. These findings may indicate that the ants are selectively using tricosanes and 3,11-dimethylpentacosane in the nestmate recognition process. Brandstaetter et al. (2008) demonstrated that ants of the genus *Camponotus* can discriminate multicomponent nestmate recognition cues from a distance of at least 1 cm via olfactory sensilla. These investigators used *cis*-9-tricosene (C9T – which is not a component of the *C. floridanus* cuticular hydrocarbon profile) in their behavioral experiments and concluded that this long-chain hydrocarbon alone was likewise detected by ant workers at close range and that C9T not only interfered with nestmate recognition when added to nestmate PPG extracts, but that workers' response changed over time (with the seasons). In the natural environment, *cis*-9-tricosene may serve as a cue in detecting the presence of other insects (e.g., Bagneres et al. 1991).

Chromatograms based on our 'surface' extraction method and GC-MS protocols showed additional peaks which represented hydrocarbons of shorter chain length that may potentially have higher volatility than the hydrocarbon constituents of the colony profiles resulting from methodologies used by Lok et al. (1975) or Ross et al. (1987). Vander Meer and Morel (1998) have previously commented that cuticular surface extracts oftentimes contain short-chain exocrine gland products, regurgitates and/or excretory products in addition to the long-chain cuticular lipids. With this cautionary note in mind, another potentially suitable compound for separating black imported fire ant colony samples from the others was Peak 6 which was linked to nonadecane (n-C19, C19H40) for *S. richteri* (at retention times ranging from 27.19 - 27.37 min); whereas, it was identified as 2-methyl-6-pentadecyl-6-piperideine, C21H41N (at retention times ranging from 27.40 - 27.37 min) for *S. invicta* and hybrids.

Ant colony samples identified using Ward's minimum variance model when compared with predicted group outcomes from linear discriminant analysis produced the fewest misclassification errors. Use of the 3 compounds represented by peaks 3, 11, and 5 (i.e., alkenylpiperidine (C19H37N), 3,11-diMeC25, and 2-methyl-6-pentadecenyl-6-piperideine, C21H39N, respectively) resulted in misclassification of 5% of the ant colonies; whereas, use of 4 compounds represented by peaks 3, 11, 5, and 1 (i.e., alkenylpiperidine (C19H37), 3,11-diMeC25, 2-methyl-6-pentadecenyl-6-piperideine (C21H39N), and alkenylpiperidine (C17H35), respectively) resulted in the misclassification of 2.5%. Ward's minimum variance model has been characterized as sensitive to outliers (Milligan 1980); however, we were able to use this 'feature' to our advantage, namely in the identification of ant colony samples that may exhibit an unusual (skewed) distribution of workers.

This alternative GC-MS method used in tandem with a multivariate approach in the assessment of biochemical data obtained from red, black and hybrid imported fire ant colonies may facilitate the finer-scale distinction of hybrid colonies in terms of their surficial semiochemical complexity and 'alliance' with parental species. However, the biological significance of our findings, based on ant colonies acquired during the summer,

needs to be expanded upon to gain a better understanding of seasonal variations in these biochemical signatures especially among hybrid populations. These techniques are complementary to a widely-used method by Ross et al. (1987) based on calculating a combined cuticular hydrocarbon and venom alkaloid index to identify hybrids. Over the past few decades, much effort has been directed at species-specific control methods such as toxic bait formulations incorporating pheromones, the release of pathogens and/or parasites, or some combination of these. Glancey et al. (1989) were the first to question if these species-specific approaches would be effective for control of the hybrid. Application of the techniques presented in this paper would be especially useful in refining regionally-based biological control strategies such as the release of phorid flies (*Pseudacteon* spp.) or other species that use semiochemicals to detect appropriate hosts. An alternative potential use would be in the refinement of ant repellant and bait efficacy evaluations as different populations may vary substantially in their response to treatment based on degree of introgression.

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