Similarity in Venom Alkaloid Chemistry of Alate Queens of Imported Fire Ants: Implication for Hybridization between *Solenopsis richteri* and *S. invicta* in the Southern United States

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Both *cis*- and *trans*-2-methyl-6-undecylpiperidines, MC11P, have been previously reported as the major components of the venom of alate queens of the imported fire ants, *Solenopsis richteri* (black) and *S. invicta* (red). To identify the minor components of venom alkaloids from alate queens and compare the venom alkaloid chemistry of alate queen of their hybrid (*S. richteri* × *S. invicta*) with that of the two parental fire ant species (*S. richteri* and *S. invicta*), silica-gel short-column chromatography was utilized for separating *cis*-stereoisomers of venom alkaloids from *trans*-stereoisomers. GC/MS Analyses of venom-alkaloid chemistry of alate queens demonstrated that fewer alkaloid peaks were detected in the chromatograms of the alate queens compared to those of workers. Three new compounds, **7**, **12**, and **13**, were detected as minor components in the venom of alate queens of all three fire ant species. Alate queens of hybrid fire ants showed *cis*- and *trans*-alkaloid patterns similar to those of the parental species. Similarity in venom-alkaloid chemistry of alate queens of *S. richteri* and *S. invicta*, and their hybrid may indicate their reproductive compatibility in the hybrid zone in southern United States, where all three species occur sympatrically.

Introduction. – The imported fire ants, *Solenopsis richteri* Forel (black) and *S. invicta* Buren (red), were first detected in the United States (USA) in the early 20th century and have since spread across the southern USA [1]. Both species had previously been treated as subspecific variants, until *Buren* [2] separated them into two species. One support for such separation was that no hybrid between these two species was found at that time in the areas where they occurred sympatrically in their native South America. However, *Wilson* [3] first reported the occurrence of hybrids in the Mobile (Alabama) and Meridian (Mississippi) areas where *S. richteri* and *S. invicta* overlapped. *Vander Meer et al.* [4] reported that ants collected near the contact zone of the two species in Mississippi exhibited intermediate profiles of piperidine alkaloids and cuticular hydrocarbons, confirming the occurrence of hybrid fire ants between the two species. Hybrid fire ants are morphologically similar to *S. richteri* and *S. invicta*, and chemical analysis of ant venom and cuticular hydrocarbons or genetic characterization are required to distinguish hybrid ants from the parent species [4][5]. The method developed by *Vander Meer et al.* [4] to distinguish the hybrid from its parent species based on piperidine alkaloids and cuticular hydrocarbon profiles has been used for identifying hybrid imported fire ants for decades [6–11].

Hybridization between the two imported fire ant species in the USA occurs over an extensive area from western Georgia through Alabama to central Mississippi [5][8]. Populations in northwestern Georgia and northern Alabama are mostly hybrid, whereas populations in northern Mississippi are a mixture of hybrid and *S. richteri* with a predominance of *S. richteri* [6][8][9]. *S. invicta* was almost not found in northern areas of Georgia, Alabama, Mississippi, and entire state of Tennessee [8][9][11]. The *S. richteri* populations have been pushed by hybrid and *S. invicta* from northwestern Alabama to southwestern Tennessee, and the hybrid fire ant zone has expanded northward to southeastern Tennessee [11]. In contrast to the extensive hybridization of these species in the United States [4][5][12], hybridization is rare between *S. invicta* and *S. richteri* where their native ranges overlap in central Argentina and south Brazil [13].

Brand et al. [14] investigated the chemistry of the venom alkaloids in fire ant workers and alate queens of *S. xyloni*, *S. geminata*, *S. richteri*, and *S. invicta*. The first two species, which are indigenous to the southern USA, produce primarily 2,6-dialkylpiperidine alkaloids with short side C-chain, *i.e.*, (2*R*,6*S*)-2-methyl-6-undecylpiperidine (*cis*-MC11P) and (2*R*,6*R*)-2-methyl-6-undecylpiperidine (*trans*-MC11P), in both workers and alate queens. Workers of the two introduced (imported) fire ant species (*S. richteri* and *S. invicta*) produce qualitatively and quantitatively different venom alkaloids. The venom of alate queens of these four species contains essentially only MC11P components, and *cis*-MC11P is at least twice as abundant as *trans*-MC11P [15]. In previous studies [16][17], we have developed a method to isolate and purify venom alkaloids from workers of imported fire ants. By conventional silica-gel column chromatography, *cis*- and *trans*-alkaloids were separated, and a series of new 2,6-dialkyl-1,2-didehydropiperidines and 2,6-dialkyl-1,6-didehydropiperidines were identified [16][17]. Recently [18], we used this methodology to investigate new alkaloids from workers of hybrid fire ant. Hybrid workers showed a GC pattern of alkaloids intermediate to that of both parental species. Several unidentified compounds were detected in the *cis*-alkaloid fraction. Given the similarity in major components of venom alkaloids of alate queens of the two imported fire ant species, it is of interest to determine whether or not alate queens from hybrid *S. richteri* × *S. invicta* share similar venom-alkaloid profile with the two parental species.

In the present study, we conducted a comparative analyses of the venom alkaloid chemistry of alate queens of *S. richteri*, *S. invicta*, and their hybrid, *S. richteri* × *S. invicta*, *1*) to identify alkaloids produced by hybrid alate queens; *2*) to identify minor peaks other than two previously reported major components *cis*-MC11P and *trans*-MC11P; and *3*) to compare *cis*- and *trans*-alkaloid profiles in the venom of alate queens of the three imported fire ant species. Comparison of the venom alkaloid chemistry of alate queens from these three fire ant species (here, we consider reproductively active hybrid *S. richteri* × *S. invicta* as a species) may provide evidence to support current hybridization status occurring in the United States.

Results and Discussion. – *GC Patterns of the* cis- *and* trans-*Alkaloid Fractions of Fire Ant Alate Queens.* As previously reported [16–18], silica-gel chromatography allowed separation of the two alkaloid fractions (*cis*- and *trans*-alkaloid fractions) from whole body extracts of alate queens, with the *cis*-stereoisomers always being eluted before the corresponding *trans*-stereoisomers. The GC profiles of the *cis*-alkaloid fraction and the *trans*-alkaloid fraction were very similar among *S. richteri* and *S. invicta*, and their hybrid. Only one major peak was detected in each alkaloid fraction (*Figs. 1* and *2*). No key qualitative or quantitative differences were recorded among the three species, with the exception of peaks **1** and **1′** which were not detected in the extract of *S. invicta* alate queens.

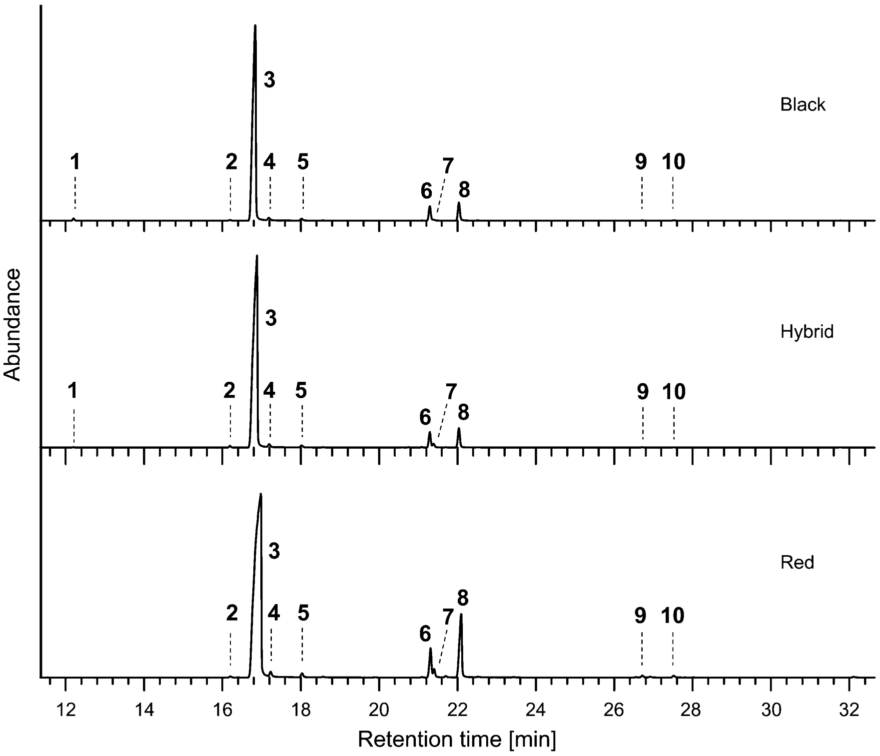


Fig. 1. *Typical GC traces of* cis-*alkaloid fraction*. Black, *S. richteri*; Hybrid, *S. richteri* × *S. invicta*; Red, *S. invicta*.

Identification of Alkaloids from Fire Ant Alate Queens. The chemical identities of major peaks in these two alkaloid fractions were easily determined by comparing with published characteristic peaks of fire ant venom alkaloids. The chemical identities of the minor peaks were determined by comparing their mass spectra and retention times (t_R) with those present in worker extracts of *S. richteri* and *S. invicta*, and their hybrid (*Figs. 1* and *2*, and *Table*).

In the *cis*-alkaloid fraction, peaks **1**, **2**, **3**, **6**, **8**, **9**, and **10** are *cis*-MC9P, *cis*-MC11 : 1P, *cis*-MC11P, *cis*-MC13 : 1P, *cis*-MC13P, *cis*-MC15 : 1P, *cis*-MC15P, respectively, with the characteristic base peak at *m/z* 98. The mass spectra of minor peaks **4** and **5** were absolutely identical to those reported by *Brand et al.* [19] for 2,6-dialkylpiperidines. The

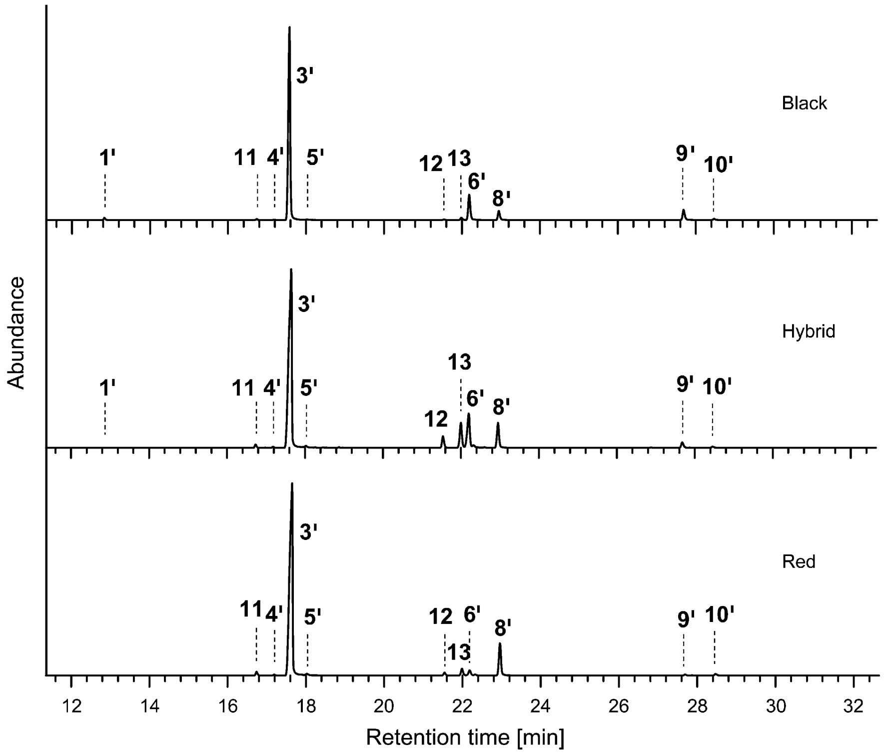
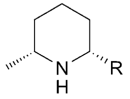


Fig. 2. *Typical GC traces of* trans-*alkaloid fraction*. Black, *S. richteri*; Hybrid, *S. richteri* × *S. invicta*; Red, *S. invicta*.

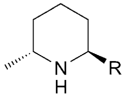
important peaks at *m/z* 96 and 111 indicate an N=C(6) bond, whereas the peaks at *m/z* 96, 97, 110 evidence an N=C(2) bond on the piperidine ring. Therefore, **4** and **5** were identified as 1,6-didehydro-2-methyl-6-undecylpiperidine and 1,2-didehydro-2-methyl-6-undecylpiperidine (structures shown in the *Table*), respectively. Peak **7** was eluted right after peak **6** in the GC (*Fig. 1*) with very similar mass spectrum to that of peak **6** (*Fig. 3*). We tentatively identified peak **7** as *cis*-MC13 : 1P with the position of the C=C bond on the side chain different from that in peak **6** (*Fig. 3*).

GC/MS Analyses of the *trans*-alkaloid fraction showed that the alate queens of the three fire ant species have corresponding *trans*-stereoisomers of major *cis*-alkaloids (*Figs. 1* and *2*, and *Table*). Peaks **1'**, **3'**, **6'**, **8'**, **9'**, **10'** were *trans*-MC9P, *trans*-MC11P, *trans*-MC13 : 1P, *trans*-MC13P, *trans*-MC15 : 1P, *trans*-MC15P, respectively. The mass spectrum and t_R of **4'** were identical to those of **4** in the *cis*-alkaloid fraction, suggesting that **4'** and **4** have the same structure. In the same manner, **5'** and **5** were identified as the same compound. The phenomenon occurred here was due to slow elution of didehydropiperidines on silica-gel column. Peak **11** had identical mass spectrum to that of peak **3** and same t_R as peak **3**. Therefore, peak **11** could be major venom alkaloid *cis*-MC9P (peak **3**) retained on the silica-gel column which was eluted with hexane containing increased amount of acetone along with *trans*-alkaloids.

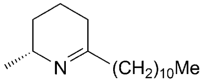
Table. *Chemical Identity of Known Alkaloids from* Solenopsis *Fire Ant Alate Queens*



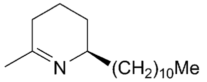
cis-Compound



trans-Compound



4 (=**4'**)



5 (=**5'**)

cis-Alkaloids				*trans*-Alkaloids			
Peak	Trivial name	Configuration	R	Peak	Trivial name	Configuration	R
1	*cis*-MC9P	(2*R*,6*S*)	$Me(CH_2)_8$	**1′**	*trans*-MC9P	(2*R*,6*R*)	$Me(CH_2)_8$
2	*cis*-MC11 : 1P	(2*R*,6*S*)	$Me(CH_2)_7CH{=}CHCH_2$				
3	*cis*-MC11P	(2*R*,6*S*)	$Me(CH_2)_{10}$	**3′**	*trans*-MC11P	(2*R*,6*R*)	$Me(CH_2)_{10}$
4		(2*R*)		**4′**		(2*R*)	
5		(6*R*)		**5′**		(6*R*)	
6	*cis*-MC13 : 1P	(2*R*,6*S*)	$Me(CH_2)_7CH{=}CH(CH_2)_3$	**6′**	*trans*-MC13 : 1P	(2*R*,6*R*)	$Me(CH_2)_7CH{=}CH(CH_2)_3$
8	*cis*-MC13P	(2*R*,6*S*)	$Me(CH_2)_{12}$	**8′**	*trans*-MC13P	(2*R*,6*R*)	$Me(CH_2)_{12}$
9	*cis*-MC15 : 1P	(2*R*,6*S*)	$Me(CH_2)_7CH{=}CH(CH_2)_5$	**9′**	*trans*-MC15 : 1P	(2*R*,6*R*)	$Me(CH_2)_7CH{=}CH(CH_2)_5$
10	*cis*-MC15P	(2*R*,6*S*)	$Me(CH_2)_{14}$	**10′**	*trans*-MC15P	(2*R*,6*R*)	$Me(CH_2)_{14}$

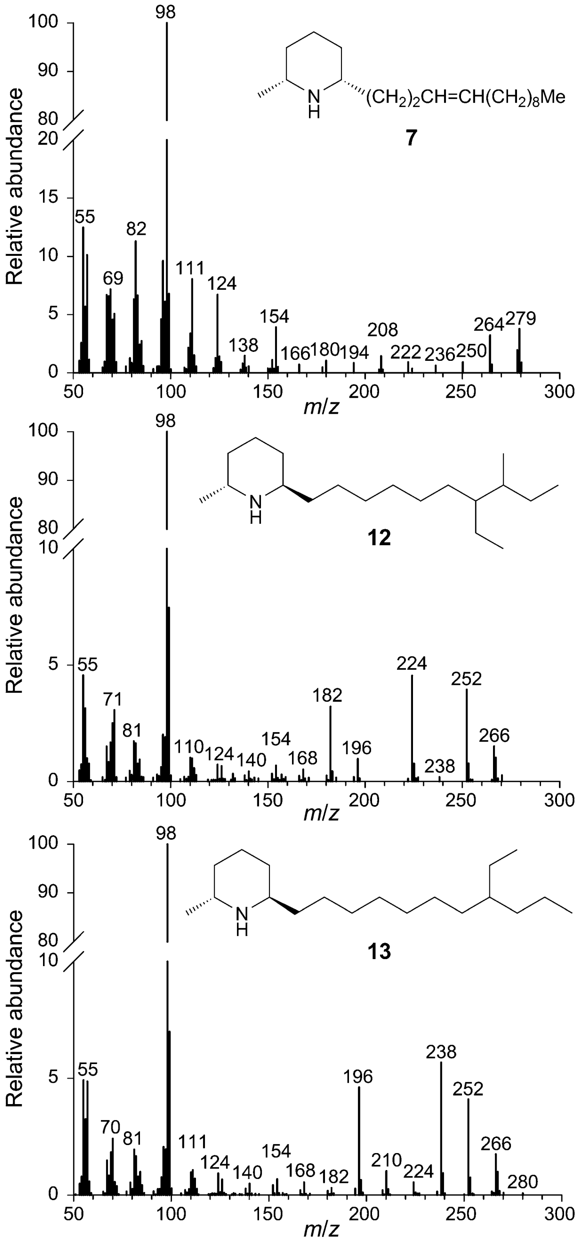
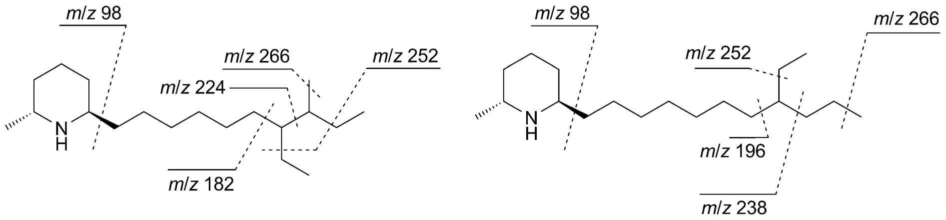


Fig. 3. *Mass spectra of new alkaloids identified from alate queens*

According to *Leclercq et al.* [20], the absolute configuration of the *trans*-alkaloids present in fire ant workers is always (2*R*,6*R*), while that of the *cis*-alkaloids is (2*R*,6*S*). Therefore, we assume that the alkaloidal components in fire ant queens have the same configuration as those in fire ant workers. As didehydropiperidines have been proposed to function as precursors for the syntheses of fire ant alkaloids [16][21], the absolute configuration of didehydropiperidines can be inferred to (2*R*)-Me for 1,2-didehydropiperidine **4** and (6*R*)-$C_{11}H_{23}$ for 1,6-didehydropiperidine (**5**).

The base peak at *m/z* 98 in the spectra of **12** and **13** indicated a characteristic piperidine ring (*Fig. 3*). The ion peaks at *m/z* 266 and 280 suggested that the mass of **13** was 281 as that of **8′** (*trans*-MC13P). Although a parent-ion peak for **12** was not visible, mass spectrum of **12** similar to those of **13** and **8′** indicated a mass of 281. The shorter retention times of **12** and **13** as compared to that of **8′** suggested the presence of branch(es) on side C-chain. *Fig. 3* depicts the mass spectra of **12** and **13**, and *Fig. 4* shows the molecular fragments that could be assigned to the corresponding peaks. As they were eluted in the *trans*-alkaloid fraction, we considered the absolute configuration of **12** and **13** as *trans*-(2*R*,6*R*).



12 **13**

Fig. 4. *Suggested interpretation of the MS fragmentation of* **12** *and* **13**

Both *cis*-MC9P (**1**) and *trans*-MC9P (**1′**) were detected as minor GC peaks of alate queens of *S. richteri*, and of the hybrid *S. richteri* × *S. invicta*, but not of *S. invicta*. *MacConnell et al.* [22] have previously reported the detection of both *cis*-MC9P and *trans*-MC9P in alate queens of *S. richteri*. Both *cis*- and *trans*-MC9P were also found in the venom of workers of *Solenopsis* (*Diplohoptrum*) species collected from Puerto Rico [23–25] and of *S. conjurata* collected from Costa Rica [26]. Except for *cis*-MC11:1P (**2**), all corresponding *trans*-stereoisomers of *cis*-MC11P (**3**), *cis*-MC13:1P (**6**), *cis*-MC13P (**8**), *cis*-MC15:1P (**9**), and *cis*-MC15P (**10**) were present in the *trans*-alkaloid fractions across the three fire ant species. Two possible biosynthetic precursors of fire ant venom alkaloids, didehydropiperidines **4** (=**4′**) and **5** (=**5′**), were found in both alkaloid fractions of alate queens, as in workers [16][17]. All of the alkaloidal components found in alate queens of the three fire ant species but the two new compounds **12** and **13** were also present in various amounts in conspecific workers. Although there were notable differences between the venoms of workers *vs.* alate queens of each fire ant species, with respect to the major alkaloidal components and the configurations of these components [14][15], we can still conclude that both castes have qualitatively similar alkaloid profiles, with the alate queen containing fewer varieties of venom alkaloids. The new compound **7** could be an unidentified minor component detected in the workers in our previous studies [16–18]. The two new compounds **12** and **13** from alate queens absent in the worker caste may play a special role in fire ant social organization. Further synthesis and behavioral tests are needed to determine the function of the latter two new alkaloids in fire ant queen's communication system.

Implication for Hybridization between S. richteri *and* S. invicta *in the Southern United States.* Both *S. richteri* and *S. invicta* likely entered the USA near the Port of Mobile, Alabama, in shipping dunnage during late 1910s and early 1930s, respectively [27][28]. The subsequent range expansion of both imported fire ants across the southern USA was facilitated by their rapid natural dispersal mechanism (*i.e.*, mating flights) and human assisted transport. By chemically analyzing alcohol-preserved fire ant specimens collected six decades ago, *Vander Meer* and *Lofgren* [29] determined that hybridization occurred as early as 1949 in southern Alabama. Hybridization might have occurred soon after the invasion of these ant species wherever the two species came into contact, and the hybrid zone has moved northward over the past several decades to occupy its present position in Mississippi, Alabama, and Georgia [5][8]. The distribution of *S. richteri* in the native range is restricted to the southernmost part of Brazil, Uruguay, and east Argentina [30][31], whereas the native range of *S. invicta* is relatively broader, extending through central and southern Brazil, Uruguay, northern Argentina, Paraguay, Bolivia, and southern Peru [32]. The range of *S. invicta* apparently overlaps with that of *S. richteri* only marginally at its extreme southern limits, *i.e.*, from central Argentina to south Brazil. Both species are reproductively isolated in their native South America even where their ranges overlap [33], but they readily hybridize in their expanded ranges in North America [4].

The width of the hybrid zone in the southern USA varies considerably from Georgia to Mississippi. In northwestern Mississippi, the ranges of the two parental species may come into contact during mating flight, whereas, in northern Georgia, *S. invicta* is separated from *S. richteri* by the hybrid zone extending over more than 100 km [34]. Normally, mating takes place between 90 to 300 meters high, and female alates may fly several kilometers or more under proper wind conditions [35]. Therefore, newly fertilized females cannot disperse far away from their original colony by natural mating flight. It is not likely that migration of the magnitude required by their current distribution of hybrid colonies could occur by natural mating flights of the sexuals from the two parental species. The finding that *S. richteri* and *S. invicta* produce reproductively viable hybrid might be responsible for the current distribution of hybrid fire ants. We propose several mating types occurring in hybrid zone: *1*) mating may occur between female and male alates from the two parental species in close ranges; *2*) sexuals from hybrid colonies may cross or backcross with opposite-sex sexuals from a parental species in the hybrid zone mixed with parental species, and in hybrid zone edges; *3*) sexuals from different hybrid colonies may mate with each other in large pure hybrid zones. Sexuals of *S. invicta* are nearly indistinguishable in shape from those of *S. richteri* [2], and we did not observe any evident morphological difference between sexuals of all three imported fire ant species. The above mating types imply that sexuals of *S. richteri*, *S. invicta*, and their hybrid can mate freely without any natural barriers. The observation that males of *S. richteri* and *S. invicta* have virtually identical structures of genitalia [14][32] may support this assertion. During mating flights, sex pheromones are most likely utilized to initiate activity and to assure that contact is made between the males and females in the air.

Although nothing is known about sex pheromone in *Solenopsis* fire ants, the queen pheromone system of *S. invicta* has been particularly well-studied, and three types of queen pheromones have been reported from poison sac of *S. invicta*. The queen poison sac was a source of a releaser (recognition) pheromone that is responsible for initiation and maintenance of retinue, eliciting attraction and queen tending activities by workers [36][37]. *Rocca et al.* [38][39] identified three compounds (queen recognition pheromones), which, when tested in combination, were reported to elicit a behavioral response from workers [40]. The poison sac also was the source of a queen primer pheromone that inhibits virgin queens from shedding their wings (dealating) and developing their ovaries [41][42]. Moreover, poison sac is the probable storage location of a water-soluble queen pheromone that induces workers to kill sexual larvae in the colonies of *S. invicta* [43].

The alkaloids in fire ant queens may not function necessarily as defensive compounds as in fire ant workers [44][45], conceivably as queen pheromones. It has recently been shown that some myrmicine ant species produce sex pheromones in the female's poison glands [46–48]. We suspect poison gland is the source of sex pheromone, if there is in *S. invicta* virgin queens. The nearly identical chemistry of venom alkaloids from alate queens of the three fire ant species suggests that other poison gland secretions, *e.g.*, queen pheromones and/or sex pheromones, are qualitatively and quantitatively congruent across the three fire species. Therefore, the males of a fire ant species (either one of parental species or hybrid) may not be able to differentiate their own female counterparts through sex pheromonal cues during mating flights so that hybridization can extensively occur where ranges of the two introduced species and their hybrids overlap. Thus, the incapability of species-specific sexual differentiation increases possibility for the two introduced species to interbreed during mating flight, and sexuals from hybrid colonies may be involved in mating flights to backcross with parental species as well. As fire ant populations were ten times more abundant in the USA than they were in Brazil [49], the strong interaction between the two introduced fire ant species may facilitate the interbreeding process. Eventually, the premating barriers which normally act to block genetic exchange between two species were compromised once the fire ants were introduced into the new environment [13][50]. Thus, it is reasonable to conclude that the similarity of poison gland secretions of *S. richteri*, *S. invicta*, and hybrid queens contributes to the hybridization success of these ants in their introduced range.

Closely related moth species that overlap in time and space usually share common sex pheromone components with different component ratios, *e.g.*, *Helicoverpa armigera* and *H. assulta* [51]. Although sex pheromone chemistry of *Solenopsis* fire ants is not yet known, we speculate that *S. richteri* and *S. invicta* likely have same sex-pheromone components probably with slightly different component ratio, and that sex pheromones in hybrid fire ants are similar to those of the two parental species with quantitatively intermediate ratio between the two parental species. Future studies on identification of sex-pheromone chemistry of the imported fire ants, and behavioral response of ant sexuals to conspecific and heterospecific sex pheromones may unveil the interbreeding mechanisms among the three fire ant species.

Conclusions. – Alate queens of the three fire ant species show very similar GC patterns of both *cis*- and *trans*-alkaloids. Two new compounds, **12** and **13**, absent in fire ant worker caste, were found to be present in alate queens from all three fire ant species tested. The alate queens of hybrid share the same venom alkaloids with the two parental species. Similarity in venom alkaloid chemistry of females, along with identical structures of genitalia in males, may facilitate hybridization between *S. richteri* and *S. invicta* in their introduced range in the southern USA. The poison gland, which is the source of a releaser pheromone, a primer pheromone, and an inducer pheromone, could also be the source of female sex pheromones in fire ant alate queens. The similarity in venom alkaloid chemistry in alate queens across the three fire ant species may imply the existence of very similar sex pheromones stored in the poison sac, as evidenced by the production of sex pheromones in the female's poison glands in some myrmicine ant species [46–48].

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China). GC Analysis: *Shimadzu GC17A* equipped with an *Rtx®-1MS* column (30 m × 0.25 mm i.d., 0.25 μm thickness, *Restek*, Bellefonte, PA). GC/MS: *Agilent 7890A* GC coupled to a *5975C* mass-selective detector, with a *HP-5ms* cap. column (30 m × 0.25 mm i.d., 0.25-mm film thickness).

Source of Colonies. Hybrid (*S. richteri* × *S. invicta*) fire ant colonies were collected from northern Alabama and northeastern Mississippi where hybrids are known to occur almost exclusively. The collection trip was made in June 2008 which yielded 25 hybrid colonies. At the same time, colonies of the black imported fire ant *S. richteri* (6 colonies) and the red imported fire ant *S. invicta* (12 colonies) fire ants were collected from Tennessee and Alabama (Auburn University campus), resp. An ant colony was collected by transferring *ca.* 1 l of soil (containing workers, alates, broods, and the queen(s)) from each mound into 1-gallon *Rubbermaid* plastic jars coated with *Fluon®* (*ICI*, Wilmington, DE) to prevent escape. All colonies were maintained in the laboratory and were fed 10% sugar-water and crickets.

Confirmation of Identity of Fire Ant Colonies. About 50 randomly selected worker ants from each colony were soaked in 1 ml of hexane for 2 h. One μl of hexane extract was directly injected into a *Shimadzu GC17A* equipped with an *Rtx®-1MS* column. GC Oven was programmed at 15°/min from 90° to 270°, with 2 min initial time and 16 min final holding time. Each colony was confirmed to be *S. richteri*, *S. invicta*, or hybrid by using both alkaloid and hydrocarbon characters [4][52].

Isolation and Identification of Alkaloids. A colony of each fire ant species (*S. richteri*, *S. invicta*, or hybrid) with enough alate queens was randomly selected for venom chemistry study. The ants were separated from the soil by flooding [53] and then transferred to a plastic box in which a *Petri* dish nest was used for them to settle down. Sugar soln. and crickets were provided as food sources. The ants were killed by freezing, and alate queens were removed from the colony using soft forceps. Alkaloids were extracted and isolated from alate queen samples as previously described for fire ant workers [16][17]. Briefly, *ca.* 1 g of alate queens was soaked in hexane (enough hexane to cover ant bodies) under laboratory condition for 24 h. The extract (0.4 ml) was loaded onto a silica-gel (0.75 g in *Pasteur* glass pipette) column and eluted with hexane containing increasing amounts of acetone to separate the extract into different fractions and thus to isolate the *cis*- and *trans*-alkaloids. GC/MS Analyses of alkaloid fractions were performed on an *Agilent 7890A-5975C* GC/MS. The GC oven temp. was programmed from 90° (isothermal for 1 min) to 160° at 10°/min, then to 250° at 3°/min, and held for 2 min. The injection temp.

and the transfer line temp. were both set at 250°. Alkaloids were identified by analysis of their mass spectra produced by EI (70 eV), as well as by comparison of characteristic peaks of the alkaloids in *Solenopsis* fire ants [16–18].

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