

# Workers and alate queens of *Solenopsis geminata* share qualitatively similar but quantitatively different venom alkaloid chemistry

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*Solenopsis geminata* group (Hymenoptera: Formicidae) encompasses ant species commonly called fire ants because of their painful sting. The many physiological effects of the venom are caused by 2-methyl-6-alkyl and/or alkenylpiperidine alkaloids. The variation in piperidine alkaloid structures has useful taxonomic characters. The most well studied *Solenopsis* species is *Solenopsis invicta*, which was accidentally imported into the USA in the 1930s from South America. It quickly spread throughout the southern USA and is now a major invasive pest ant in the USA and in other parts of the world. Interestingly, the invasive *S. invicta* has largely displaced a native USA fire ant, *S. geminata*, from the southern USA. We explore the possibility that differences in venom chemistry could be correlated with this displacement. The *cis* and *trans* alkaloids from body extracts of workers and alate queens of *S. geminata* were separated by silica gel chromatography, identified, and quantitated by GC-MS analysis. Both workers and alate queens produce primarily *cis*- and *trans*-2-methyl-6-*n*-undecylpiperidines, as well as other minor alkaloid components. Imported fire ant, *S. invicta*, alate queens produce the same alkaloids as *S. geminata* alate queens, but in contrast *S. invicta* workers produce piperidine alkaloids with longer side chains, which are purported to be physiologically more effective. These results are discussed in relation to the evolutionary progression of fire ant venom alkaloids and displacement of *S. geminata* by *S. invicta* in the USA.

**Keywords:** *Solenopsis geminata*, fire ant, piperidine, GC-MS, isomer

## Introduction

Ants of the genus *Solenopsis* (Hymenoptera: Formicidae) are known as fire ants because of their painful stings (Vinson and Greenberg, 1986; Blum, 1992). The sting is used for colony defense, prey capture, and antimicrobial action (Blum, 1985). *Solenopsis* venom is characterized by the presence of up to 95% water-insoluble alkaloids and a small amount of protein (Baer et al., 1979; Jones et al., 1982). The venom sac is the source of large amounts of venom alkaloids (MacConnell et al., 1971). The venoms are composed mainly of 2-methyl-6-alkyl or alkenyl piperidines known as the solenopsins which are responsible for the toxic properties of the venom (MacConnell et al., 1970, 1971). These piperidine alkaloids found in *Solenopsis* fire ants differ in the lengths

of their alkyl/alkenyl chains, which can have 9, 11, 13, 15, or 17 carbon atoms (Brand et al., 1972; MacConnell et al., 1974; Blum et al., 1992). The two alkyl substituent groups can be either on same side of the piperidine ring as *cis*-solenopsin or on different side of the piperidine ring as *trans*-solenopsin. Carbon numbers have been widely used to represent these solenopsins (Brand et al., 1972). For example, *cis* C11 denotes the alkaloid with an 11-carbon side chain *cis* to the ring methyl group; *cis* C11:1 denotes the alkaloid with one double bond in the 11-carbon side chain *cis* to the ring methyl group. The absolute configurations of the *cis*- and *trans*-solenopsins were determined to be (2*R*,6*S*) and (2*R*,6*R*), respectively (Leclercq et al., 1994).

The mix of solenopsins, their *cis* or *trans* configuration, and relative proportions, vary both among castes and within *Solenopsis* species (Brand et al., 1972, 1973a; MacConnell et al., 1976). The venoms of workers of *Solenopsis richteri* Forel and *Solenopsis invicta* Buren contain mainly *trans* solenopsins with C13 and C15 side chains whereas these compounds in the venoms of alate queens of these species are essentially lacking (Brand et al., 1973b). In the venoms of workers of *Solenopsis xyloni* McCook and *S. geminata* (F.), only *cis* and *trans* C11 components are abundant (Brand et al., 1973b). The difficulty of classical taxonomy in *S. geminata* and *Solenopsis saevissima* complexes has been recognized for a long time because of the morphological similarity of many *Solenopsis* species in these complexes (Creighton, 1930; Buren, 1972; Trager, 1991). The species-specific pattern of venom alkaloids, a biochemical taxonomic character, may be useful in sorting out this very complex taxonomic problem (MacConnell et al., 1976). Indeed, venom alkaloid and cuticular hydrocarbon chemistry has been reported as useful taxonomic tool for differentiating *Solenopsis* species (Vander Meer, 1986; Vander Meer and Lofgren, 1988; Dall'Aglio-Holvorcema et al., 2009).

The tropical fire ant, *S. geminata*, is native to coastal areas from the southern United States to Central America and northern South America, and has been introduced into both tropical Asia and west Africa (Trager, 1991; Wetterer, 2011). This species co-occurs extensively with the distantly related fire ant *S. saevissima* along Brazil's Atlantic coast (Ross et al., 2010), but has been displaced from much of its range in the United States by the introduced congener, *S. invicta* (Porter et al., 1988; Porter, 1992; Wojcik, 1994). Compared with *S. invicta*, the chemistry of *S. geminata* has not been extensively studied. Venom alkaloids of *S. geminata* workers and alate queens were first determined by Brand et al. (1972, 1973b), and confirmed by Cruz-Lopez et al. (2001), as primarily (2*R*,6*S*)-2-methyl-6-*n*-undecylpiperidine (*cis*-C11) and (2*R*,6*R*)-2-methyl-6-*n*-undecylpiperidine (*trans*-C11). Furthermore, *cis*-2-methyl-6-*n*-nonylpiperidine (*cis*-C9) was found to be a minor component in the venom of alate queens of *S. geminata* (MacConnell et al., 1974). In a series of previous studies (Chen and Fadamiro, 2009a,b; Chen et al., 2010b, 2012; Yu et al., 2014), we developed a method to purify venom alkaloids from the imported fire ants using conventional silica gel column chromatography. Two new alkaloid series, 2,6-dialkyl- $\Delta^{1,2}$ -piperideines and 2,6-dialkyl- $\Delta^{1,6}$ -piperideines were identified. Qualitative analysis demonstrated that alate queens

from *S. richteri*, *S. invicta*, and hybrid *S. richteri*  $\times$  *S. invicta* share similar piperideine venom alkaloid profiles. It was thus of interest to investigate whether the *S. geminata* populations would have similar or different piperideine compositions or produce other minor alkaloid components. In the present study, we conducted both qualitative and quantitative analyses of the venom alkaloid chemistry of workers and alate queens in pooled samples of *S. geminata*.

## Materials and Methods

### Insects

Five colonies of the tropical fire ant, *S. geminata*, were collected in late March, 2014 in Alachua County, Florida, USA where *S. geminata* continued to persist with the red imported fire ant *S. invicta* (Tschinkel, 1988; Porter, 1992). Two of these colonies were a red form of *S. geminata* collected from the west side of Gainesville along Parker Road while other three colonies were a dark form obtained east of Gainesville near Boulware Springs. The alate queens of the dark form had distinctly smaller heads than those of the red form. Each colony was separated from the soil and reared in the laboratory with 1.5M sugar water and domestic crickets (*Acheta domesticus* L.) (Gavilanez-Slone and Porter, 2013). All colonies that had been in the lab for 3 months prior to alkaloid extraction contained both workers and alate queens. There were 1200–1600 workers per gram of worker ants from both red and dark forms. The mean weight of alate queens from red and dark forms were  $13.40 \pm 4.51$  mg and  $6.92 \pm 0.24$  mg, respectively.

### Chemicals

HPLC grade *n*-hexane (CNW Technologies GmbH, Düsseldorf, Germany) was used for extraction and purification of the venom alkaloids. Analytical reagent grade acetone and triethylamine (Beijing Chemical Reagent, Beijing, China) were redistilled before use. Silica gel (300–400 mesh, Qingdao Marine Chemical Factory, Qingdao, China) was used for column chromatography. Racemic *cis*-2-methyl-6-undecylpiperidine (*cis*-C11) was synthesized by reduction of corresponding pyridine with hydrogen and palladium/carbon catalyst (Pianaro et al., 2012), and used as external standard for quantitative analysis.

### Sample Preparation

Alkaloids were extracted and isolated from fire ant samples as previously reported (Chen et al., 2012; Yu et al., 2014). For each colony, ant workers, or alate queens (1g) were killed by freezing and extracted with hexane (enough hexane to cover ant bodies, ~3.5 mL) for 24 h. The whole body extraction proved to adequately represent the gland extractions (data not shown) and have been widely used for fire ant venom alkaloid analysis (For example, Deslippe and Guo, 2000; Eliyahu et al., 2011). The extract was dried over anhydrous sodium sulfate and concentrated to 0.5 mL under a mild stream of N<sub>2</sub>, and then loaded onto a 17 mm (o.d.) chromatography column (20 g silica gel). The column was eluted with hexane containing 2% triethylamine and stepwise increasing amounts of acetone

(hexane/acetone ratio ranging from 50:1 to 10:1) to obtain the alkaloids. The chemistry of each collection (*ca.* 2 mL) was analyzed by gas chromatography (GC) on an Agilent 7890A GC equipped with a flame ionization detector (FID) and a 30 m × 0.25 mm i.d., 0.25 μm, HP-5ms capillary column (Agilent Technologies). Nitrogen was used as carrier gas at a flow rate of 2 mL/min. The injection port and detector temperatures were set at 270 and 280°C, respectively. The GC oven was programmed at a rate of 15°C/min from 90 to 270°C, with 2 min initial time and 16 min final holding time. The collections were pooled based on changes observed in the GC chromatograms of each collection to obtain two major alkaloid fractions corresponding to *cis* and *trans* alkaloids. The two alkaloid fractions for each colony were concentrated to a volume of 4 mL for GC-MS analyses.

### GC-MS Analysis

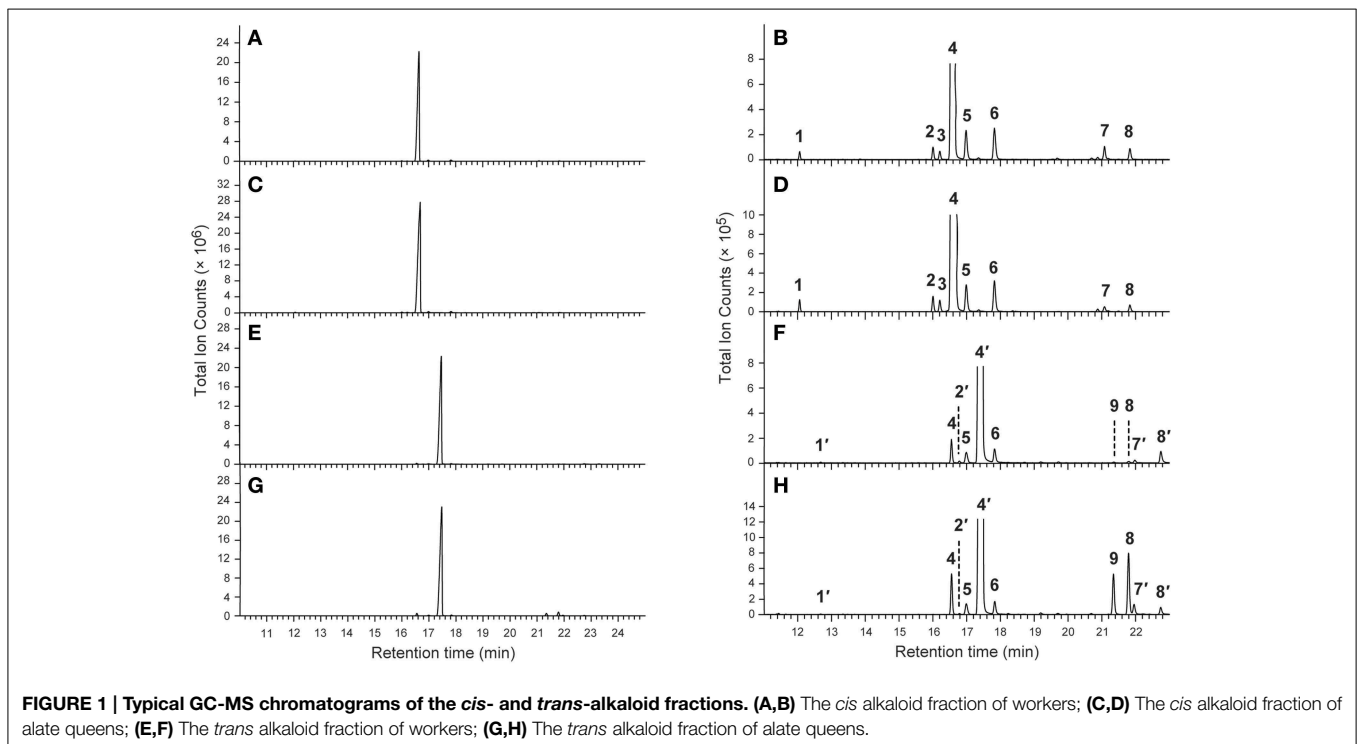
GC-MS analyses of alkaloid fractions were performed on an Agilent 7890A GC coupled to a 5975C mass selective detector, with an HP-5ms capillary column as described above. Helium was used as carrier gas at a flow rate of 1 mL/min. Injections (1 μL) were made in the splitless mode at an injector temperature of 230°C. The GC oven temperature was programmed from 90 (isothermal for 1 min) to 160°C at 10°C/min, then to 250°C at 3°C/min, and held for 2 min. Total run time was 40 min. The transfer line temperature was set at 250°C. Mass spectra were obtained using electron impact (EI, 70 eV). The chemical identities of alkaloids were determined by analysis of their mass spectra and by comparison of diagnostic ion fragments with published results (Chen and Fadamiro, 2009a,b; Chen et al., 2010b, 2012).

A stock solution of standard compound, synthetic racemic *cis*-C11 (2000 ng/μL), was prepared and then diluted to a series of concentrations ranging from 1.95 to 1000 ng/μL. All dilutions were transferred to the GC autosampler, and 1 μL of each dilution was used for GC-MS analysis under same conditions as described above. A standard curve was calculated by linear regression analysis. The concentrations of the identified venom alkaloids in the *cis* and *trans* alkaloid fractions were calculated against the standard curve. The obtained concentration for a given alkaloid component was multiplied by the final volume of the alkaloid fraction, 4 mL, and then divided by the weight of ants used for extraction to give exact amount of the alkaloid component per 1 g of ants. The absolute abundances of the alkaloid components were analyzed using PROC TTEST procedure ( $P < 0.05$ ) to establish significant difference between workers and alate queens (SAS Institute, 2004).

## Results

### GC Patterns of the *cis* and *Trans* Alkaloid Fractions of Workers and Alate Queens

As previously reported (Chen and Fadamiro, 2009a,b; Chen et al., 2010b, 2012), the *cis* and *trans* alkaloid fractions from whole body extracts of workers and alate queens were readily separated through silica gel chromatography. The GC profiles of the *cis* alkaloid fraction and the *trans* alkaloid fraction from workers and alate queens were very similar. Only one major peak was detected in each alkaloid fraction (Figure 1). No key qualitative differences were observed between workers and alate queens.



## Identification of Alkaloids from Workers and Alate Queens

The chemical identities of major peaks in these two alkaloid fractions were readily determined by comparison with published characteristic peaks of fire ant venom alkaloids. Major peak **4** in the *cis* alkaloid fraction is *cis*-C11 and peak **4'** in the *trans* alkaloid fraction is *trans*-C11, having exactly same mass spectra with the characteristic base peak ion  $m/z$  98 and parent ion  $[M^+]$  252. The chemical identities of the minor peaks (**Figure 1** and **Table 1**) were determined by comparing mass spectra and retention times with those already characterized from extracts of workers and alate queens of *S. richteri*, *S. invicta* and their hybrid (Chen and Fadamiro, 2009a,b; Chen et al., 2010b, 2012).

In the *cis* alkaloid fractions, minor peaks **1**, **2**, **7**, **8** were *cis*-C9, *cis*-C11:1, *cis*-C13:1, *cis*-C13, respectively, having the characteristic base peak ion  $m/z$  98. The configuration of the side chain double bond in the piperidines (dehydrosolenopsins A, B, C, D) is always *Z* (*cis*) (MacConnell et al., 1971); therefore, we assign the *cis* configuration to the double bond in the *cis*-C11:1 (**2**) and *cis*-C13:1 (**7**) side chains. The mass spectrum of peak **3** was almost identical to that of peak **2**. Considering that this peak eluted right after peak **2**, we tentatively identified **3** (*cis*-C11:1') as a stereoisomer of **2** (*cis*-C11:1) with a possible *trans* double bond on the alkyl side chain. We also proposed that the absolute configuration of **3** was the same as **2** (2*R*,6*S*) (**Table 1**). The mass spectra of minor peaks **5** and **6** were identical to 2,6-dialkylpiperideines found generally in *Solenopsis* fire ants. The important mass peaks at  $m/z$  96, 111 in **5** indicated an N-C<sub>6</sub> double bond, whereas ions at  $m/z$  96, 97, 110 in **6** indicated an N-C<sub>2</sub> double bond on the piperideine ring. Therefore, **5** and **6** were identified as 2-methyl-6-*n*-undecyl- $\Delta^{1,6}$ -piperideine and 2-methyl-6-*n*-undecyl- $\Delta^{1,2}$ -piperideine, respectively. Because piperideines were proposed to function as precursors for the syntheses of fire ant alkaloids (Leclercq et al., 1996; Chen and Fadamiro, 2009a) and the chirality of 2-CH<sub>3</sub> was always found to be *R* in *Solenopsis* fire ants (Pianaro et al., 2012), we inferred absolute configuration of  $\Delta^{1,6}$ -piperideine (**5**) to be (2*R*)-CH<sub>3</sub>. However, both (6*R*)- and (6*S*)-C<sub>11</sub>H<sub>23</sub> configurations are possible for  $\Delta^{1,2}$ -piperideine (**6**).

In the *trans* alkaloid fraction, minor peaks **1'**, **2'**, **7'**, **8'** were *trans*-C9, *trans*-C11:1, *trans*-C13:1, *trans*-C13, respectively. The mass spectrum and retention time of peak **4** in the *trans* alkaloid fraction were identical to those of peak **4** in the *cis* alkaloid fraction, suggesting that they were same compound. In the same manner, peaks **5**, **6**, **8** in the *trans* alkaloid fraction were identical to those in the *cis* alkaloid fraction. These *cis* alkaloids and piperideines retained in silica gel column due to strong absorption by silica gel were eluted with hexane containing increased amount of acetone along with *trans* alkaloids. The mass spectrum of peak **9** in the *trans* alkaloid fraction was almost identical to that of peak **7** in the *cis* alkaloid fraction. We tentatively identified **9** (*cis*-C13:1') as stereoisomer of **7** (*cis*-C13:1) with a *trans* double bond on the alkyl side chain.

## Quantitative Analysis of Alkaloids

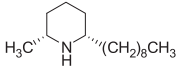
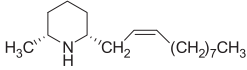
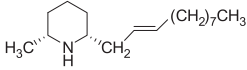
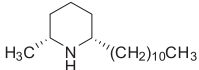
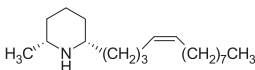
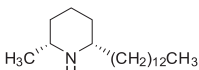
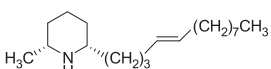
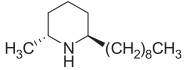
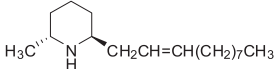
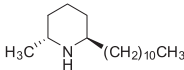
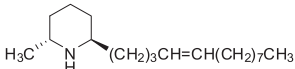
The external standard method was used to quantitate the separated piperidine and piperideine alkaloids. A calibration curve was constructed with log-transformed data,  $\log(A) = 1.1681\log(C) + 5.3575$  ( $r^2 = 0.9797$ ,  $P < 0.001$ ), where A is the peak area, C is the concentration of the standard compound (ng/ $\mu$ L). The obtained calibration curve was used to quantitate alkaloidal components in all ant samples (**Table 1**).

Over 90% of the venom alkaloids from workers and alate queens are composed of *cis*-C11 and *trans*-C11 (**Table 1**). In red and dark form workers, the total percentages of these two major components were 94.00 and 94.80%, respectively. Similarly, in red and dark form alate queens, the total percentages of these two major components were 93.79 and 92.93%, respectively. Apparently, *cis*-C11 was the only dominant component in the *cis* alkaloid fraction, while *trans*-C11 was the only major component in the *trans* alkaloid fraction, irrespective of ant form. Furthermore, the amount of *cis*-C11 from alate queens ( $1219.24 \pm 123.03$   $\mu$ g/g ant in red form;  $1467.05 \pm 49.54$   $\mu$ g/g ant in dark form) was significantly greater than that from workers ( $442.41 \pm 156.30$   $\mu$ g/g ant in red form;  $528.78 \pm 103.75$   $\mu$ g/g ant in dark form). On the contrary, although the amount of *trans*-C11 from alate queens ( $582.82 \pm 97.60$   $\mu$ g/g ant in red form;  $267.40 \pm 23.32$   $\mu$ g/g ant in dark form) was lower than that from workers ( $873.57 \pm 99.76$   $\mu$ g/g ant in red form;  $850.61 \pm 108.47$   $\mu$ g/g ant in dark form), significant difference in the amount of *trans*-C11 was only detected for dark form ( $t = 2.08$ ,  $P = 0.1726$  for red form;  $t = 5.26$ ,  $P = 0.0063$  for dark form). The amounts of two piperideines,  $\Delta^{1,6}$ -C11 and  $\Delta^{1,2}$ -C11, were quite low ranging from  $17.87 \pm 3.16$  to  $31.23 \pm 2.57$   $\mu$ g/g ant in workers and alate queens. The amounts of other minor components, *cis*-C9, *cis*-C11:1, *cis*-C13:1, *cis*-C13, *trans*-C9, *trans*-C11:1, *trans*-C13:1, and *trans*-C13, were all under 24  $\mu$ g per 1 g of ant. In general, the alate queens had more *cis* alkaloid components and less *trans* alkaloid components than workers, with only one exception, *trans*-C13:1, even though the differences were not always statistically significant.

We further calculated the total amount of alkaloids depending on structure of piperidine ring (**Table 2**). The trend of difference in total amount of alkaloids of different alkaloid type between workers and alate queens was similar to the trend concluded from data in **Table 1**. The alate queens had higher amount of *cis* alkaloids and lower amount of *trans* alkaloids than workers. There was no significant difference in total amount of piperideines between workers and alate queens ( $df = 2$ ,  $t = 0.77$ ,  $P = 0.5226$  for red form;  $df = 4$ ,  $t = 2.34$ ,  $P = 0.0795$  for dark form). Furthermore, the total amount of alkaloid components in alate queens was relatively higher, but not significantly, than that in workers. The total amount of alkaloids produced by 1 g of ant workers was about 1.4 mg, and the total amount of alkaloids produced by 1 g of ant queens was about 1.9 mg.

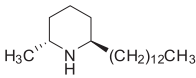
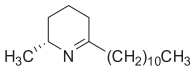
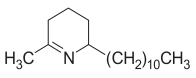
Moreover, we calculated the ratio of *cis*-C11 to *trans*-C11 for workers and alate queens (**Table 3**). To obtain more accurate values of the *cis*-C11 to *trans*-C11 ratio, 1  $\mu$ L of diluted ant body extracts was subjected to GC-MS analysis prior to silica gel chromatography. Areas of GC peaks were used to calculate the

TABLE 1 | Quantitation of alkaloids from *S. geminata* fire ants ( $\mu\text{g/g} \pm \text{SE}$ ).

Peak	Structure	Name	Red form		Dark form	
			Workers	Alate queens	Workers	Alate queens
<b>cis ALKALOIDS</b>						
1		<i>cis</i> -C9	1.36 $\pm$ 0.59 (0.10)	5.02 $\pm$ 1.37 (0.26)	2.44 $\pm$ 0.39 (0.17)	9.52 $\pm$ 2.74* (0.51)
2		<i>cis</i> -C11:1	2.42 $\pm$ 1.02 (0.17)	9.13 $\pm$ 0.32* (0.48)	2.55 $\pm$ 0.60 (0.18)	10.77 $\pm$ 1.58* (0.58)
3		<i>cis</i> -C11:1'	1.81 $\pm$ 0.77 (0.13)	7.41 $\pm$ 0.33* (0.39)	1.60 $\pm$ 0.29 (0.11)	10.50 $\pm$ 1.96* (0.56)
4		<i>cis</i> -C11	442.41 $\pm$ 156.30 (31.60)	1219.24 $\pm$ 123.03* (63.46)	528.78 $\pm$ 103.75 (36.34)	1467.05 $\pm$ 49.54* (78.60)
7		<i>cis</i> -C13:1	2.81 $\pm$ 1.13 (0.20)	5.79 $\pm$ 1.63 (0.30)	4.25 $\pm$ 1.21 (0.29)	5.32 $\pm$ 1.07 (0.28)
8		<i>cis</i> -C13	4.76 $\pm$ 2.35 (0.34)	15.64 $\pm$ 2.29 (0.81)	7.85 $\pm$ 1.83 (0.54)	23.71 $\pm$ 7.57 (1.27)
9		<i>cis</i> -C13:1'	0.59 $\pm$ 0.43 (0.04)	10.81 $\pm$ 0.39* (0.56)	1.12 $\pm$ 0.52 (0.08)	7.02 $\pm$ 3.74 (0.38)
<b>trans ALKALOIDS</b>						
1'		<i>trans</i> -C9	0.53 $\pm$ 0.17 (0.04)	0.21 $\pm$ 0.10 (0.01)	0.43 $\pm$ 0.13 (0.03)	0.13 $\pm$ 0.03 (0.01)
2'		<i>trans</i> -C11:1	3.91 $\pm$ 0.92 (0.28)	0.78 $\pm$ 0.07 (0.04)	2.03 $\pm$ 0.31 (0.14)	0.24 $\pm$ 0.05* (0.01)
4'		<i>trans</i> -C11	873.57 $\pm$ 99.76 (62.40)	582.82 $\pm$ 97.60 (30.33)	850.61 $\pm$ 108.47 (58.46)	267.40 $\pm$ 23.32* (14.33)
7'		<i>trans</i> -C13:1	3.69 $\pm$ 0.32 (0.26)	5.34 $\pm$ 0.31 (0.28)	3.26 $\pm$ 0.53 (0.22)	3.58 $\pm$ 0.95 (0.19)

(Continued)

TABLE 1 | Continued

Peak	Structure	Name	Red form		Dark form	
			Workers	Alate queens	Workers	Alate queens
8'		<i>trans</i> -C13	8.25 ± 2.13 (0.59)	2.53 ± 0.37 (0.13)	10.75 ± 1.61 (0.74)	1.80 ± 0.26* (0.10)
<b>PIPERIDEINES</b>						
5		Δ <sup>1,6</sup> -C11	25.49 ± 2.25 (1.82)	26.97 ± 0.62 (1.40)	17.87 ± 3.16 (1.23)	28.32 ± 2.10* (1.52)
6		Δ <sup>1,2</sup> -C11	28.34 ± 0.70 (2.02)	29.74 ± 0.35 (1.55)	21.53 ± 4.10 (1.48)	31.23 ± 2.57 (1.67)

Data in parentheses are percentage of each component (%). An \* indicates a statistical difference between workers and alate queens from same form ( $P < 0.05$ , *t*-Test).

TABLE 2 | Total amounts of alkaloids (μg/g ± SE).

Alkaloids	Red form		Dark form	
	Workers	Alate queens	Workers	Alate queens
<i>cis</i> Alkaloids	456.16 ± 162.59	1273.03 ± 121.53*	548.58 ± 107.70	1533.88 ± 63.08*
<i>trans</i> Alkaloids	889.95 ± 103.30	591.68 ± 97.71	867.08 ± 110.85	273.15 ± 24.56*
Piperideines	72.32 ± 3.45	75.21 ± 1.47	39.40 ± 7.25	59.56 ± 4.66
Total alkaloids <sup>a</sup>	1436.93 ± 269.85	1958.42 ± 217.26	1455.06 ± 225.44	1866.59 ± 83.02

<sup>a</sup>Total alkaloids mean sum of the *cis*, *trans* alkaloids, and piperideines.

An \* indicates a statistical difference between workers and alate queens from same form ( $P < 0.05$ , *t*-Test).

*cis*-C11 to *trans*-C11 ratio. The ratios of *cis*-C11 to *trans*-C11 in alate queens were significantly greater than in workers for both ant forms. A similar difference between alate queens and workers was found in the ratios of *cis*-C11 to *trans*-C11 obtained from quantitation data. We also found that there was no significant difference in the ratios of *cis*-C11 to *trans*-C11 of workers between red form and dark form ( $df = 3$ ,  $t = 1.60$ ,  $P = 0.2069$  for “direct injection,”  $df = 3$ ,  $t = 1.01$ ,  $P = 0.3850$  for “quantitation,” *t*-test). However, the ratio of *cis*-C11 to *trans*-C11 in alate queens of dark form was significantly greater than that of red form ( $df = 3$ ,  $t = 4.74$ ,  $P = 0.0178$  for “direct injection,”  $df = 3$ ,  $t = 5.50$ ,  $P = 0.0118$  for “quantitation,” *t*-test).

## Discussion

The results of this study confirm that both workers and alate queens of *S. geminata* produce prominently *cis*-C11 and *trans*-C11 piperidine alkaloids as previously reported (Brand et al., 1972, 1973b; MacConnell et al., 1976). We also identified some minor components as *cis*-C9, *cis*-C11:1, *cis*-C13:1, *cis*-C13, *trans*-C9, *trans*-C11:1, *trans*-C13:1, *trans*-C13, Δ<sup>1,6</sup>-C11, and Δ<sup>1,2</sup>-C11. An earlier report (Brand et al., 1972) only showed minor components, *cis*-C13:1 and *cis*-C13 in the venom of *S. geminata*

workers. With better analytical instrumentation and methods, we clearly showed the presence of both *cis* and *trans* stereoisomers of C9, C11:1, C13:1, and C13 in workers and alate queens.

In the present study, we report for the first time the presence of *cis*- and *trans*-C9 as minor components in *S. geminata* workers. Both *cis*- and *trans*-C9 have been reported from the venom of alate queens of *S. geminata*, *S. richteri* and hybrid *S. richteri* × *S. invicta* (MacConnell et al., 1974; Chen et al., 2012), from the venom of workers of *Solenopsis* (*Diplohoptrum*) species collected from Puerto Rico (Jones et al., 1982, 1996; Blum et al., 1985), and of *S. conjurata* Wheeler collected from Costa Rica (Jones et al., 1984). Chen and Fadamiro (2009a) also reported the detection of *cis*-C9 in the venom of *S. richteri* workers. *cis*-C11:1, but not *trans*-C11:1, has been previously reported in alate queens of *S. richteri*, *S. invicta*, and their hybrid (Chen et al., 2012). The *cis* and *trans* stereoisomers of C13:1 and C13 are major components in worker venom of *S. richteri*, *S. invicta*, and their hybrid (Chen and Fadamiro, 2009a,b; Chen et al., 2010b).

Brand et al. (1972) first reported the identification of piperideine Δ<sup>1,2</sup>-C11 from *S. xyloni* venom. Recently, a number of studies have confirmed the presence of two series of piperideines including Δ<sup>1,6</sup>-C11 and Δ<sup>1,2</sup>-C11 in workers and alate queens of *S. richteri*, *S. invicta*, and their hybrid (Chen and

TABLE 3 | Ratio of *cis*-C11 to *trans*-C11.

Caste	Red form			Dark form			
	Col. 1	Col. 2	Mean ± SE	Col. 3	Col. 4	Col. 5	Mean ± SE
<b>WORKERS</b>							
Direct injection <sup>a</sup>	0.36	0.43	0.40 ± 0.03	0.64	0.67	0.41	0.57 ± 0.08
Quantitation <sup>b</sup>	0.37	0.62	0.49 ± 0.12	0.66	0.66	0.51	0.61 ± 0.05
<b>ALATE QUEENS</b>							
Direct injection	2.09	2.36	2.22 ± 0.14*	7.34	7.12	5.14	6.53 ± 0.70*
Quantitation	1.97	2.26	2.12 ± 0.14*	6.46	5.42	4.82	5.57 ± 0.48*

<sup>a</sup>Direct injection means that ant body extract was directly injected into GC-MS for calculation of peak ratio.

<sup>b</sup>Quantitation means that peak ratios were calculated with quantitation data obtained from Table 1.

An \* indicates a statistical difference between workers and alate queens from same form ( $P < 0.05$ , *t*-Test).

Fadamiro, 2009a,b; Chen et al., 2009, 2010a,b, 2012). Piperidine  $\Delta^{1,2}$ -C11 has been hypothesized to act as an intermediate in the interconversion of *cis* and *trans* ring isomers (Brand et al., 1972). Later, the piperidines,  $\Delta^{1,6}$ -C11 and  $\Delta^{1,2}$ -C11, were proposed to function as precursors for the biosynthesis of fire ant alkaloids, *cis*- and *trans*-C11 (Leclercq et al., 1996; Chen and Fadamiro, 2009a). The detected piperidines could be the result of the extraction process, which would halt the normal biosynthetic progression and trap piperidine biosynthesis intermediates. As both *cis*- and *trans*-C11 are major components in the venoms of workers and alate queens of *S. geminata*, it is likely that enantioselective enzymes are present in this species which can reduce  $\Delta^{1,6}$ -C11 into both (2*R*,6*S*)-*cis*-C11 and (2*R*,6*R*)-*trans*-C11, and  $\Delta^{1,2}$ -C11 mainly into (2*R*,6*S*)-*cis*-C11 and partially into (2*R*,6*R*)-*trans*-C11. The presence of both  $\Delta^{1,6}$ - and  $\Delta^{1,2}$ -piperidines further supports the hypothesis that these piperidines are biosynthetic precursors for fire ant alkaloids. However, it is unlikely that already produced piperidines would undergo *cis* and *trans* ring interconversion through piperidine intermediates.

The total amount of alkaloids produced by 1 g of *S. geminata* workers was about 1.4 mg. However, 1 g of the imported *Solenopsis* ant workers produce 8–10 mg alkaloids (Yu et al., 2014), meaning that the imported fire ant workers can produce 5–6 times more alkaloids than *S. geminata* workers. It follows that the significantly higher production of total alkaloids in *S. invicta* would increase their fitness and competitive capability. Therefore, the much lower production of alkaloids in *S. geminata* workers may be a factor in the displacement of this species in its native range by introduced *S. invicta*. However, a previous study has demonstrated that the amounts of major venom alkaloid components in laboratory-maintained *S. invicta* colonies decreased 3–6 times in 3 months (Liu et al., 2015). Because the *S. geminata* colonies used for alkaloid extraction in the present study were maintained in the laboratory for several months, it is possible that total amounts of alkaloids produced by freshly collected *S. geminata* colonies are quantitatively similar to those produced by freshly collected *S. invicta* colonies.

The ratios of *cis*-C11 to *trans*-C11 in workers of two forms of *S. geminata* ranged from 0.36 to 0.67, indicating that *trans*-C11 was more dominant than *cis*-C11 in workers. In this study,

we presented data calculated from 1 g of pooled ant samples. Brand et al. (1973a) analyzed venoms milked from 10 individuals with 1  $\mu$ L capillary tube and estimated the ratio of *cis*-C11 to *trans*-C11 by a comparison of the two peak heights. For workers the ratio ranged from 0.71 to 1.98, for major workers 2.89–7.21, and for alate queens from 2.53 to 3.90. The venoms of two other *Solenopsis* species native to the United States, *Solenopsis aurea* Wheeler, and *S. xyloni*, consisted mainly of *cis*-C11 and *trans*-C11 as well. The ratio of *cis*-C11 to *trans*-C11 in workers of these two species was about 4:1 (Blum et al., 1973; Brand et al., 1973b; MacConnell et al., 1976). This ratio matched that of the equilibrium mixture formed during their chemical synthesis through  $\Delta^{1,6}$ -C11 or  $\Delta^{1,2}$ -C11 piperidines in the laboratory (Hill and Yuri, 1977; Jefford and Wang, 1993). While this concurrence is interesting and supports piperidine involvement in piperidine biosynthesis, we realize that a ratio based on thermodynamic equilibrium in a chemical reaction can be readily modified by enzymatic reactions during biosynthesis, thus resulting in the variety of *cis/trans* C11 ratios reported for *Solenopsis* species.

Chemical characteristics of *Solenopsis* piperidine alkaloids have been used as an indicator of fire ant evolution. The primitive venom alkaloid profile would consist of primarily C11 with the *cis* isomer dominating and the *trans* isomer at a much lower level (Brand et al., 1973b). This relatively simple venom was followed by a biosynthetic change in the *cis*- and *trans*-C11 ratio to a thermodynamically highly unfavorable decrease in *cis* and an increase in *trans* isomers, and then by further addition of abundant *trans* homologs with longer saturated and unsaturated side chains. Thus, the venoms of *S. richteri* and *S. invicta* (Brand et al., 1973b; Brand, 1978; Chen and Fadamiro, 2009b) are composed of primarily *trans* isomers and side chains greater than C11. On this basis, *S. aurea*, *S. xyloni*, and *S. geminata* would be classified as the more primitive species. The venom of *Solenopsis eduardi* Forel (a close relative of *S. geminata*) collected from Colombia consisted of more than 98% *trans*-C11, and about 1% of *cis*-C11 (MacConnell et al., 1976), which might represent a major shift from *cis*-C11 to *trans*-C11 alkaloids. It is likely that  $\Delta^{1,6}$ - and  $\Delta^{1,2}$ -piperidines, as biosynthetic precursors, play an important role in the evolutionary progression.

Although alate queens produced slightly more alkaloids than workers, there were no qualitative differences between the venoms of workers and of alate queens. The alate queens of *S. geminata* were found to have a significantly higher ratio of *cis*-C11 to *trans*-C11. The reported ratio of *cis*-C11 to *trans*-C11 in alate queens of *S. geminata*, 2.53–3.90 (Brand et al., 1973a), was slightly higher than our reported ratio for the red form and lower than that of the dark form. Even though peak height, instead of peak area, was used for calculation of the ratio in the previous report (Brand et al., 1973a), the actual ratio of *cis*-C11 to *trans*-C11 in alate queens may not be much different. Therefore, it can be suspected that the dark form of *S. geminata* has not been studied previously. The *cis*-C11 to *trans*-C11 ratio in alate queens of *S. xyloni* ranged from 12.37 to 20.75 (Brand et al., 1973a,b), which was much higher than that in alate queens of *S. geminata*, suggesting that *S. xyloni* is evolutionarily more primitive than *S. geminata*. The alate queens produce significantly higher amount of *cis*-C11, but much lower amount of *trans*-C11 than workers, suggesting that these same alkaloids may play distinct roles in alate queens and workers. Workers inject venom directly into other animals for defense and predation, or spray it throughout the nest environment, presumably for protection against microbial pathogens (Chen, 2007). The proportion of *cis* piperidines was found to gradually increase in the venom of queens as they became fully reproductive (Eliyahu et al., 2011). Queens apply their venoms over eggs as they are laid, presumably for protecting eggs from entomopathogenic fungi (Vander Meer

and Morel, 1995; Tschinkel, 2006). These alkaloids in queens may not necessarily function as defensive compounds as in workers (Blum et al., 1958; Javors et al., 1993), but conceivably as queen pheromones. Venom alkaloids on the surface of eggs may play a role in advertising the presence and fertility status of queens (Vander Meer and Morel, 1995; Eliyahu et al., 2011). The workers are aggressive defenders against nest disturbance; however, queens move away from disturbances and seldom attempt to sting. The difference in venom alkaloid chemistry could account for these different behavior patterns of alate queens and workers. With this behavioral difference there would be a strong positive selection pressure for the production of more effective venom in the workers, but probably little selection pressure for more potent venom in the queens (Brand et al., 1973b). Therefore, the workers and queens in the more primitive species as *S. xyloni* and *S. geminata* have roughly the same venom composition, and the increasing differences in venom chemistry between workers and queens in newer species as *S. richteri* and *S. invicta* indicate evolutionary progression.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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