Secretion Profiles of Venom Alkaloids in *Solenopsis geminata* (Hymenoptera: Formicidae) in Taiwan

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ABSTRACT *Solenopsis geminata* (F.) was introduced into southern Taiwan decades ago and has continued to threaten the residents. Although the venom compositions of various fire ant species have been studied, the effects of environmental cues on the secretion pattern have received relatively little attention in an area with subtropical climate and high humidity, such as Taiwan. This study characterizes the effects of temperature and season on the venom compositions of *S. geminata* in Taiwan. Pure venom was sampled by using a microcapillary pipette and immersing the whole ant in hexane and subjected to gas chromatography-mass spectrometry analysis. The results showed that the ratio of *cis* C_{11} to *trans* C_{11} alkaloids in major workers was significantly higher than that in minor workers. No significant differences could be found in either the relative alkaloids content or the ratio of *cis* C_{11} to *trans* C_{11} alkaloids in venom of minor workers while rearing at four temperature conditions. Nevertheless, the ratio of *cis* C_{11} to *trans* C_{11} alkaloids in the venom of minor workers was the highest in spring and the lowest in winter. The results also showed that the body length, abdomen length, head length, head width, and venom volume differed significantly between major workers and minor workers of *S. geminata*. The venom volumes of these two castes were positively correlated with their body sizes.

KEY WORDS *Solenopsis geminata*, venom volume, alkaloids, body length, abdomen length

The tropical fire ant, *Solenopsis geminata* (F.), a native of the southern United States and northern South America, was introduced into southern Taiwan (estimated as sometime before 1980; Nambu and Tano 1983). This ant species attracted public concern recently because of the introduction of the red imported fire ant, S. *invicta*, into Taiwan (Huang et al. 2004) and because its nests are usually found near populated areas. The symptoms elicited by venom include pain, wheals, and itching (Caro et al. 1957, deShazo et al. 1984), but it seldom causes a pustule reaction (Hoffman 1995). Lind (1982) also reported that the venom of *S. geminata* caused certain rat cells to release histamine.

The venom of *Solenopsis* species contains ≈95% water-insoluble alkaloids and a small amount of proteinaceous material (Baer et al. 1979). Fire ant alkaloids are *cis*- and *trans*-2-methyl-6-alkyl or -alkenyl piperidines (Brand et al. 1972, MacConnell et al. 1976), for which the name "solenopsins" has been coined (MacConnell et al. 1970, 1971). Gas chromatography (GC) has been used to analyze alkaloids in venom from *S. geminata* and has shown that the venom contains two main alkaloids: *cis*-2-methyl-6-*n*-undecyl piperidines (*cis* C_{11}) and *trans*-2-methyl-6-*n*-undecyl piperidines (*trans* C_{11}) (Brand et al. 1972, 1973a; MacConnell et al. 1976). Brand et al. (1973a) further found that the ratio of *cis* C_{11} to *trans* C_{11} and venom volume were higher for soldiers (major workers) than for workers (minor workers).

Our previous study showed that venom alkaloids of *S. invicta* vary with the social forms (monogyne versus polygyne workers) and the sampling methods (microcapillary tube versus whole body soaking) (Lai et al. 2008). We hypothesized that the venom composition of major workers and minor workers of *S. geminata* may also differ because of different sampling methods used. Furthermore, compared with that of *S. invicta*, the venom of *S. geminata* has not yet been characterized in areas with subtropical climate and high humidity, such as Taiwan. In this study, the effects of environmental factors on venom composition in *S. geminata* were investigated.

Materials and Methods

Collection of Colonies. Colonies of S. *geminata* were excavated from Tainan City (southern Taiwan), Taichung City, and Changhua County (central Taiwan), and transported in plastic containers that had been coated with Fluon (NP115; Northern Products Inc., Woonsocket, RI) to prevent the ants from es-

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caping. *S. geminata* colonies were kept in soil (mixed alluvial soils) and maintained at room temperature in the laboratory. Workers were fed mealworms (*Tenebrio molitor* L. larvae) and 10% (wt:wt) sugar solution in cotton-plugged test tubes (15 cm long by 1.5 cm diameter). A test tube half-filled with water and stoppered with a cotton ball was also provided.

Determination of Colony Social Organization. Because no allele variation (*B*- or *b*-like allele) at the *Gp-9* locus could account for the expression of social form in *S. geminata* (Ross et al. 2003), the social organization (polygyne or monogyne) was determined by inspecting genotype distributions from six individuals per nest at seven microsatellite loci (*Sol-11*, *Sol-20*, *Sol-42*, *Sol-49*, and *Sol-55*, Krieger and Keller 1997; *SolM-III*, and *SolM-V*, Chen et al. 2003). The methods for polymerase chain reaction (PCR) and allele sorting were similar those in Gotzek et al. (2007) and Yang et al. (2008). Because Ross et al. (1988) indicated that queens of *S. geminata* mate normally once with single haploid male, one could expect that more than three alleles at one locus may result from multiple matrilines mediated by several queens (Ross et al. 2003, Gotzek et al. 2007). Therefore, the presence of a maximum of two different genotypes at one given locus among nestmates indicates that the examined nest is monogyne.

Collection of Venom and Chemical Analyses

To study whether sampling method affects venom secretion, two sampling methods were used.

Method I: Milking. Pure venom was collected in a 5-μl microcapillary pipette (71900; Kimble, Toledo, OH) from minor workers and major workers of *S. geminata* (Blum et al. 1958). In brief, the fire ant gaster was pulled apart from the thorax with forceps and was stimulated to release venom droplets from the sting by microcapillary pipette. To obtain the greatest quantity of venom from an individual ant, we stimulated the gaster again after 5 min and repeated the procedure three to four times until the venom was exhausted. We pooled venom from three workers per colony and kept it in n-hexane (104367; Merck KGaA, Darmstadt, Germany) at −20°C for GC-mass spectrometry (MS) analysis. Nine colonies were sampled independently to study whether the sampling method affects venom secretion.

Method II: Soaking. Twenty-five minor workers and 25 major workers from each colony were respectively placed in a glass vial containing 1 ml n-hexane, and stored at −20°C. After 7 d, the fire ants were removed and the solvent was subjected to GC-MS analysis (Ross et al. 1987). Three colonies were sampled independently to study whether sampling method affects venom secretion.

A Finnigan Focus GC was coupled to a Finnigan Focus DSQ mass selective detector (Thermo Fisher, Waltham, MA) to separate and identify the venom alkaloids of *S. geminata* according to our previous publication (Lai et al. 2008). The mass spectral base peak at m/z = 98 corresponded to the fragment $C_6H_{12}N^+$ (MacConnell et al. 1971).

Venom Volume and Ant Size. Ants were divided into major and minor workers, the former defined as those with a body length >5.5 mm and the latter with a body length of <4.1 mm. Venom was collected from 20 major workers and 20 minor workers from examined colonies using 5-μl microcapillary pipettes. The venom from 10 ants was pooled in one microcapillary pipette to calculate the average volume of venom according to the diameter of the pipette. The sizes of abdomen length (the length of gaster, excluding petiole), head width, head length, and body length were determined for 300 major workers and 300 minor workers (Trager 1991, Lin and Wu 1996).

Effect of Temperature on Venom Secretion. Three colonies of *S. geminata* collected from Tainan were separated from soil by slowly flooding the collection bucket (Jouvenaz et al. 1977); minor workers from each colony were divided into four groups and reared in a Fluon-coated plastic container (21 cm long by 14.5 cm wide by 4.5 cm deep) with a lid at 15, 25, 35, and 27 ± 3°C (room temperature), respectively. Venom of three minor workers was collected with a microcapillary pipette from each group weekly until ants died and was pooled in n-hexane for GC-MS analysis.

Effect of Season on Venom Secretion. To study whether season affects venom components, three independent *S. geminata* colonies were excavated in summer (August), fall (October), and winter (December) 2006 and in spring (March and May) and summer (July) 2007 (18 colonies in total). The venom of three workers from each colony was studied using method I (milking) immediately after they were transported to the laboratory. According to the Central Weather Bureau (CWB) of Taiwan (http://www.cwb.gov.tw/), the monthly mean temperature in Tainan, Taiwan, was 29.3°C in August, 27.2°C in October, and 20.0°C in December 2006. In March 2007, the monthly mean temperature was 22.4°C; it was 28.0°C in May and 30.5°C in July.

Statistical Analyses. The major two peak areas were determined, and the proportion of each peak area to the sum of peak areas was calculated. The ratio of *cis* C_{11} to *trans* C_{11} in major workers and minor workers was estimated by comparing the two peak areas and analyzed by two-sample *t*-test (Microsoft Excel 2002), and the data of season and temperature were analyzed by one-way analysis of variance (ANOVA) (SAS Institute 2006), using a significance level of $\alpha = 0.05$. Venom volume and ant size data were analyzed by regression analysis.

Results

Determination of Colony Social Organization. All examined nests of *S. geminata* collected from central (Taichung City and Changhua County) and southern Taiwan (Tainan City) represented, at maximum, two genotypes, consistent with a monogyne nest form inhabited by a single queen mated by a single haplod male.

Venom Study: Collection of Venom. Venom was collected from *S. geminata* using the milking and soak-

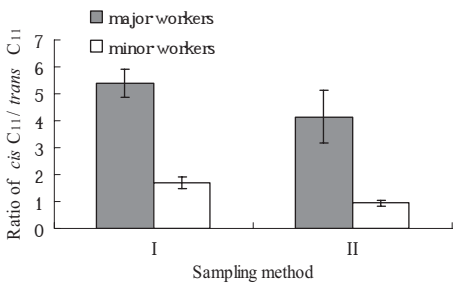

Fig. 1. Ratios of *cis* C_{11} to *trans* C_{11} piperidine alkaloid in venom from major workers and minor workers of *S. geminata.* Venom was sampled using milking (method I) or soaking (method II) methods and subjected to GC-MS analysis as described in the Materials and Methods section. Statistical difference between *cis* C_{11} to *trans* C_{11} ratios of major workers and minor workers was noted (df = 16, $P < 0.05$ for method I and df = 4, $P < 0.05$ for method II).

ing methods to compare venom composition. The GC-MS results included two major peaks corresponding to *cis*-2-methyl-6-*n*-undecyl-piperidine (*cis* C_{11}) and *trans*-2-methyl-6-*n*-undecylpiperidine (*trans* C_{11}). Several minor peaks in the GC-MS profiles of venom collected by the soaking method could represent C_{13} alkaloids and cuticular hydrocarbons.

Figure 1 shows that the ratio of *cis* C_{11} to *trans* C_{11} piperidine alkaloids in major workers was significantly greater than that for minor workers. The ratios of *cis* C_{11} to *trans* C_{11} piperidine alkaloids in venom collected using the microcapillary pipette were 5.39 ± 0.53 (major workers) and 1.70 ± 0.21 (minor workers) (mean ± SE; $t = 6.483$; df = 16; $P < 0.0001$). Venom collected by soaking yielded similar results: the ratio of *cis* C_{11} to *trans* C_{11} piperidine alkaloids in major workers (4.15 ± 0.97) also exceeded that of minor workers (0.94 ± 0.12; $t = 3.267$; df = 4; $P = 0.031$). It is notable that the C_{11} ratio in both castes of workers was not significantly different in the venom collected by milking or soaking methods ($P = 0.273$ for major workers; $P = 0.069$ for minor workers).

Venom Study: Venom Volume and Ant Size. Our study showed that both mean body size and venom volume of major workers were generally greater than those of minor workers (Table 1). The two-sample *t*-test indicated that the mean body length, the mean abdomen length, and the mean venom volume of major workers differed significantly from that of minor workers ($n = 15$; $t = 12.75$; df = 14; $P < 0.0001$ for mean body length, $t = 12.65$; df = 14; $P < 0.0001$ for mean abdomen length, and $t = 5.378$; df = 14; $P < 0.0001$ for mean venom volume, respectively). The mean venom doses per sting was 0.727 ± 0.099 and 0.368 ± 0.038 nl for major workers and minor workers (df = 14; $P = 0.0004$), respectively.

Table 1. Comparisons of body length, abdomen length, and venom volume between major workers and minor workers of *S. geminata*

	Major workers	Minor workers	*t*	df	*P*
Body length (mm)	5.98 ± 0.30	3.95 ± 0.20	5.602	28	<0.0001
Abdomen length (mm)	1.75 ± 0.08	1.17 ± 0.06	5.802	28	<0.0001
Venom vol (nl)	47.02 ± 7.07	15.56 ± 1.78	4.316	16	<0.001

All means were calculated from 15 colonies and presented as the mean ± SE.

Table 2 shows a significant correlation between body size and mean venom volume in both castes of *S. geminata.* The results indicated that the mean head length, mean head width, mean abdomen length, and mean body length were positively correlated with mean venom volume in major workers and minor workers of *S. geminata* ($n = 15$). Moreover, the mean body length was also positively correlated with the mean abdomen length regardless of castes in the colony ($n = 15$; major worker: $r = 0.985$; minor worker: $r = 0.975$).

Effect of Temperature on Venom Secretion. Table 3 indicates that the content of *cis* C_{11} alkaloid was generally higher than that of *trans* C_{11} alkaloid in venom of *S. geminata.* The ratio of *cis* C_{11} to *trans* C_{11} piperidine alkaloids was the lowest when *S. geminata* were reared at 15°C compared with those at other temperatures. However, the growth temperature did not significantly affect the alkaloid content and the ratio of *cis* C_{11} to *trans* C_{11} piperidine alkaloids ($F = 0.59$; df = 3,28; $P = 0.6254$).

Effect of Season on Venom Secretion. Colonies of *S. geminata* were collected from the field, and venom alkaloid of minor workers was analyzed to determine the seasonal variation of the secreted venom. The results show that the ratio of *cis* C_{11} to *trans* C_{11} piperidine alkaloids was the highest in colonies collected in spring and the lowest in those collected in winter, although it was not significantly associated with season ($F = 2.53$; df = 3,14; $P = 0.099$, ANOVA; Fig. 2).

The correlation of mean body length was significantly different among ants collected from southern (in summer), central (in summer), and southern Taiwan (in fall) ($F = 17.83$; df = 2,12; $P = 0.0003$ for major workers; $F = 56.35$; df = 2,12; $P < 0.0001$ for minor workers, ANOVA). The results of the ANOVA also showed a significant difference in venom volume between ants collected in summer (southern Taiwan and central Taiwan) and in fall (southern Taiwan) ($F = 25.69$; df = 2,12; $P < 0.0001$ for major workers; $F = 9.56$; df = 2,12; $P = 0.0033$ for minor workers, ANOVA; Fig. 3).

Discussion

This is the first study that describes the social organization and the relationship of venom secretion of *S. geminata* in the Asia-Pacific area. In this study, we found that *S. geminata* from Changhua County appears smaller in body size, raising the possibility that these nests may be polygyne considering the fact that workers of this ant from polygyne nests were considerably smaller than those from monogyne nsets in Mexico (MacKay et al. 1990). However, single family structure registered by the examined colonies showed that all of

Table 2. Linear regression analyses of venom vol (*Y*) versus measurements (*X*) of major workers and minor workers of *S. geminata*

Measurement	Caste	Regression equation	r^2	$F_{1,13}$	*P*
Head length	S	$Y = 65.079X - 51.17$	0.5513	15.97	0.0015
	W	$Y = 26.489X - 8.396$	0.4627	11.19	0.0053
Head width	S	$Y = 71.501X - 58.38$	0.6672	26.06	0.0002
	W	$Y = 35X - 12.901$	0.5611	16.62	0.0013
Abdomen length	S	$Y = 69.821X - 74.968$	0.6621	25.47	0.0002
	W	$Y = 21.057X - 9.053$	0.4395	10.19	0.0071
Body length	S	$Y = 19.752X - 71.061$	0.7059	31.20	<0.0001
	W	$Y = 6.006X - 8.172$	0.4567	10.93	0.0057

S, major workers; W, minor workers.

them are monogyne regardless where these colonies were collected.

The distribution of 2,6-dialkyl (and alkenyl-) piperidine alkaloids in the venom of the different castes of four *Solenopsis* species has been described in various studies (Brand et al. 1972, 1973a, b). Generally, *S. geminata* soldiers (referred to as "major workers" in this study) produced three to four times more *cis* C_{11} than *trans* C_{11}, whereas workers (referred to as "minor workers" in this study) produced these two alkaloids in approximately equal amounts. However, this study showed that the ratio of *cis* C_{11} to *trans* C_{11} piperidine alkaloids was in the range of three to six for major workers and one to two for minor workers. Surprisingly, the ratio of *cis* C_{11} to *trans* C_{11} in venom sampled by milking is always higher than that by soaking. This is probably caused by the lower passive ejection of venom by directly immersing ants in hexane.

The secretion pattern of venom alkaloids in *S. geminata* may shed light on the evolutionary status of this species. Two presumed steps have been suggested for the evolution of *Solenopsis* species with respect to venom alkaloids: (1) the venom in recently derived species is characterized by a major shift from *cis* C_{11} to *trans* C_{11} and abundant levels of *trans* C_{13}; and (2) the addition of *trans* C_{15} and *trans* C_{17} was accompanied by a reduction in the production of *trans* C_{11}. From this point of view, *S. geminata* would seem to be a more ancestral species (Brand et al. 1973b; Chen and Fadamiro 2009a, b). The study conducted by Jouvenaz et al. (1972) showed that the growth of gram-positive bacteria was inhibited by synthetic fire ant venom with the toxic order of solenopsin A (*trans* C_{11}) > B (*trans* C_{13}) > C (*trans* C_{15}). Our current studies showed that the venom of major workers contained more abundant *cis* C_{11} than that of minor worker (74.92 ± 1.08% for major workers and 59.96 ± 2.70% for minor workers). The result indicated that the quantity of *cis* C_{11} in venom of *S. geminata* might play an important role in ant social behavior or venom toxicity.

To further explore the effect of temperature and seasonal variation on venom composition, we maintained colonies in various temperatures. The results indicated that temperatures did not significantly affect the alkaloid content or the ratio of *cis* C_{11} to *trans* C_{11} (Table 3). However, the ratio of *cis* C_{11} to *trans* C_{11} was the lowest when minor workers were maintained at 15°C, which is consistent with venom obtained from minor workers in winter.

This study also found that the body size of ants collected from central and southern Taiwan in summer was smaller than that collected from southern Taiwan in fall (df = 10; $P = 0.0003$ for major workers; df = 9; $P = 0.0001$ for minor workers). This finding provides an explanation to the contention of the variation in body size between colonies (df = 14,285; $P < 0.0001$ for major workers; df = 14,285; $P < 0.0001$ for minor workers). Moreover, this study also showed that the venom volumes of major workers and minor workers of *S. geminata* were positively correlated with their body size.

This study also showed that the ratio of *cis* C_{11} to *trans* C_{11} in the venom of minor workers was three

Table 3. Effect of rearing temperature on venom alkaloids in minor workers of *S. geminata*

Temperature	*cis* C_{11} (% ± SE)[a]	*trans* C_{11} (% ± SE)[a]	*cis* C_{11}/*trans* C_{11} (± SE)[a]
15°C	59.81 ± 2.59	40.05 ± 2.57	1.62 ± 0.17
25°C	63.82 ± 3.10	35.43 ± 2.96	1.96 ± 0.24
27 ± 3°C (room temperature)	63.53 ± 2.54	36.47 ± 2.54	1.84 ± 0.20
35°C	65.66 ± 0.45	34.13 ± 0.24	1.92 ± 0.27

[a] ANOVA analysis ($F = 0.60$; df = 3, 28; $P = 0.6177$ for *cis* C_{11}; $F = 0.71$; df = 3, 28; $P = 0.5524$ for *trans* C_{11}, and $F = 0.59$; df = 3, 28; $P = 0.6254$ for *cis* C_{11}/*trans* C_{11}).

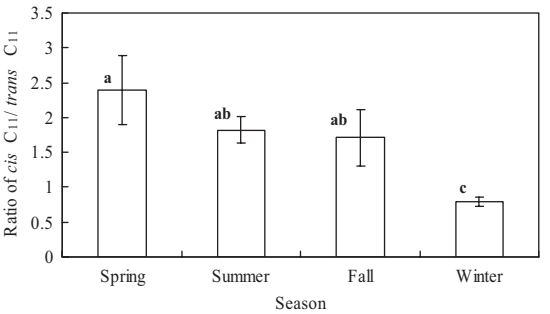

Fig. 2. Seasonal variation of *cis* C_{11} to *trans* C_{11} ratio in minor workers of *S. geminata*. Field samples of *S. geminata* were collected in different seasons in Tainan and subjected to venom alkaloid analysis. Letters above each bar indicate significant difference ($F = 2.53$; df = 3,14; $P = 0.0994$, ANOVA followed by Duncan's new multiple range test).

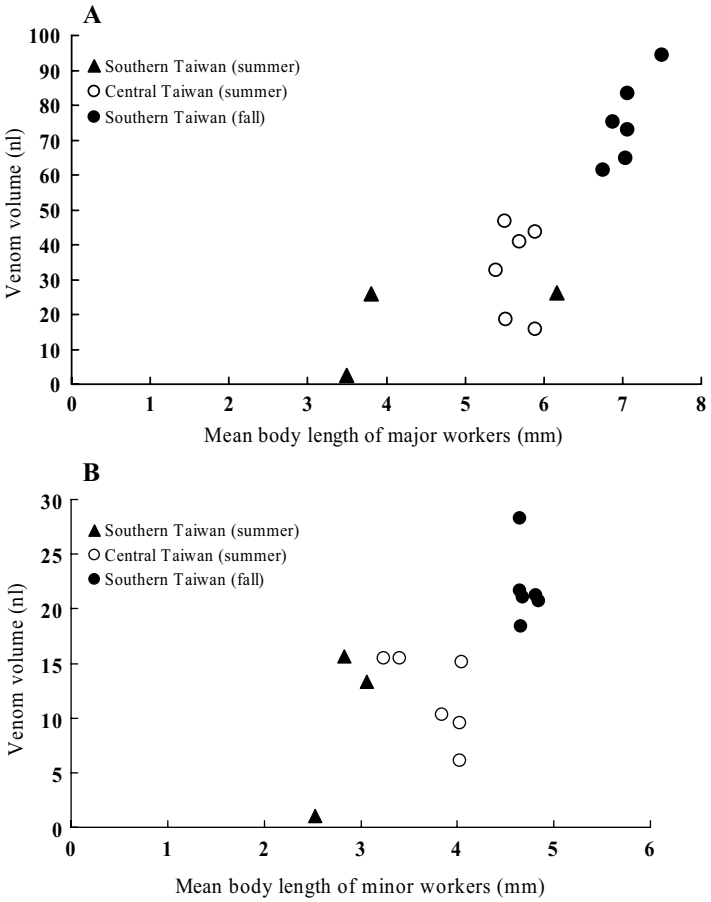

Fig. 3. Mean body length and venom volume among ants collected from different geographic locations of Taiwan. The mean body length for these three groups (southern Taiwan, in summer; central Taiwan, in summer; southern Taiwan, in fall) were strikingly different ($F = 17.83$; df = 2,12; $P = 0.0003$ for major workers [A]; $F = 56.35$; df = 2, 12; $P < 0.0001$ for minor workers [B]). The results of the ANOVA also show a significant difference in venom volume between ants collected in summer (southern Taiwan and central Taiwan) and in fall (southern Taiwan) ($F = 25.69$; df = 2,12; $P < 0.0001$ for major workers; $F = 9.56$; df = 2,12; $P = 0.0033$ for minor workers, ANOVA).

times greater in spring than in winter. The proportion of *cis* C_{11} alkaloid is higher than that of *trans* C_{11} in all seasons except in winter, where both alkaloids represent approximately equal amounts. According to historical data from the Central Weather Bureau (www.cwb.gov.tw), the mean temperature and precipitation in winter season were apparently lower than in other seasons (19.49°C and 28.4 mm, respectively). Additionally, March is typically spring season in Taiwan with frequent and heavy rainfall accompanied by thunderstorms that may increase the humidity in nest or force the fire ants to move their nests to other place. Thus, the variation in the ratio of *cis* C_{11} to *trans* C_{11} may be explained by climatic factors such as temperature and precipitation in Taiwan.

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