Research Article

Variation in floral characters, particularly floral scent, in sapromyophilous *Stemona* species

Running title: Variation in floral scent in *Stemona* species

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Edited by: Alice Y. Cheung, University of Massachusetts, Amherst, USA

Received: July 12 2017; Accepted: August 22, 2017

Abstract

Flowers or inflorescences often deploy various signals, including visual, olfactory, and gustatory cues, that can be detected by their pollinators. In many plants, these cues and their functions are poorly understood. Deciphering the interactions between floral cues and pollinators is crucial for analyzing the reproductive success of flowering plants. In this study, we examined the composition of the fetid floral scents produced by several *Stemona* species, including nine *S. tuberosa* populations from across China, using dynamic headspace adsorption, gas chromatography, and mass spectrometry techniques. We compared variations in floral phenotype, including floral longevity, nectar rewards, pollinator behavior, and flower length and color among the *Stemona* species. Of the 54 scent compounds identified, the major compounds include fetid dimethyl disulfide, dimethyl trisulfide, 1-pyrroline, butyric acid, *p*-cresol, isoamyl alcohol, and indole. We detected striking differentiation in floral scent at both the species and population level, and even within a population of plants with different colored flowers. Floral characteristics related to sapromyophily and deceptive pollination, including flower color mimicking livor mortis and a lack of nectar, were found in five *Stemona* species, indicating that *Stemona* is a typical sapromyophilous taxon. Species of this monocot genus might employ evolutionary tactics to exploit saprophilous flies for pollination.

Keywords: Dimethyl disulfide; Floral scents; Indole; Oviposition site mimicry; *p*-Cresol; Pollination; 1-Pyrroline; Sapromyophily

INTRODUCTION

Sapromyophily, i.e., pollination by carrion- and dung-breeding flies, has evolved several times during angiosperm radiation (Faegri and van der Pijl 1979; Vereecken and McNeil 2010; Jürgens et al. 2013). To attract saprophilous flies that pollinate their flowers or inflorescences, sapromyophilous plants mimic the fetid odor signals that the flies use to locate their oviposition sites and/or food resources on animal dung or carrion (Stensmyr et al. 2002; Jürgens et al. 2006, 2013; Vereecken and McNeil 2010). Fetid volatiles emitted by the flowers/inflorescences of sapromyophilous plants include oligosulfides (dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS)); 1-pyrroline, which has a semen-like odor associated with carrion; aliphatic acids, which are generally associated with rotting fruits; pyrazines, which are associated with urine or late-stage carcasses; and *p*-cresol and indole, which are associated with dung (Kite et al. 1998; Johnson and Jürgens 2010; van der Niet et al. 2011; Jürgens et al. 2013; Chen et al. 2015a, 2015b). The function of these fetid compounds as pollinator attractants has been demonstrated in physiological experiments and field or laboratory bioassays (Stensmyr et al. 2002; Shuttleworth and Johnson 2010; van der Niet et al. 2011; Chen et al. 2015a, 2015b). To date, detailed studies of the floral profiles of sapromyophilous plants have focused on Araceae, Apocynaceae, Orchidaceae, and Rafflesiaceae, only a subset of the more than 20 angiosperm families that include sapromyophilous taxa (Jürgens et al. 2013).

In addition to olfactory cues, the syndrome of sapromyophily also likely involves visual signals, because flower/inflorescences in many sapromyophilous arums, stapeliads, orchids, and birthworts are often red or purple with patterns reminiscent of "livor mortis" (Proctor et al. 1996; Raguso 2004; Jürgens et al. 2013; Chen et al. 2015a). A recent study revealed that necrophagous flies cannot discriminate between the color of an *Amorphophallus konjac* inflorescence, livor mortis, and floral pigments (Chen et al. 2015a). The authors suggested that mimicking livor mortis might represent a common tactic for attracting pollinators by sapromyophilous plants. Some saprophilous flies can perceive different colors, suggesting that flower colors is important for attracting these pollinators (Fukushi 1989; Aak and Knudsen 2011; Jersáková et al. 2012; Chen et al. 2015a). For example, visual cues are important when a female blowfly selects a final landing site for oviposition and/or food (Wall and Fisher 2001; Gomes et al. 2007). In addition, flowers or inflorescences of sapromyophilous plants often deploy tactile or thermal cues for saprophilous pollinators (Seymour et al. 2003; Raguso 2004). Floral structures, rewards, and flowering period may also affect the floral visitor's behavior (Dafni 1984; Raguso 2004; Davis et al. 2008; Jürgens et al. 2013). However, the relationship between these floral characteristics and saprophilous flies is poorly understood with respect to sapromyophily.

Stemonaceae is a small, ethnobotanically important monocotyledonous family comprised of four genera and 36 species (Ji and Duyfjes 2000). The largest genus, *Stemona*, consists of 23 species, many of which are traditionally used for medicinal purposes in Southeast Asia (Ji and Duyfjes 2000). Flowers of these species emit foul odors. The fetid floral scent and dull-red coloration of these flowers led to the hypothesis that these species are

sapromyophilous plants (Duyfjes 1991). Some *Stemona* species, e.g., *S. tuberosa* and *S. javanica*, produce a strong putrid smell reminiscent of carrion or cheese (Duyfjes 1991). However, except for *S. japonica* (Chen et al. 2015b), floral scent composition in Stemonaceae has not yet been investigated. Our field experience suggested that most *Stemona* species emit a fetid odor from their flowers and are pollinated by saprophilous flies (Chen et al. 2015b; Figure 1). Therefore, in the current study, we selected six Chinese *Stemona* species and investigated their floral scent composition and relative emission rates, flowering period, floral rewards, and morphological profiles (e.g., flower size, color, and chamber morphology), because these floral traits may be altered in response to selection. We specifically address the following questions: 1) Is *Stemona* a typical taxon exhibiting sapromyophily; 2) Are the floral morphological profiles in *Stemona* related to sapromyophily; and 3) What are the ecological functions of fetid floral scents in *Stemona*? We also investigated whether floral volatiles emitted by *Stemona* spp. are similar to those emitted by other known sapromyophilous plants.

RESULTS

Floral scent characteristics of *Stemona* species

We tentatively calculated the approximate amounts of compounds in a sample of extract via the internal standard method, because we were unable to run external standards due to a lack of standard compounds. The emission rate of floral compounds per flower per hour from *S. japonica* was ~1,527.36 ± 42.42 ng/flower/h (n = 5). The emission rates of *S. sessilifolia* and *S. shandongensis* were similar, at ~23.76 ± 11.88 and 30.54 ± 1.53 ng/flower/h, respectively. The emission rate of *S. parviflora* was 736.48 ± 24.55 ng/flower/h (n = 10; Table 2). The intraspecific emission rate variation of *S. tuberosa* was often high, ranging from 1,107.4 ± 126.3 to 7,079.7 ± 222.1 ng/h/per flower (n = 58). More details about these values (mean ±*SE*) are given in Tables 2 and S1.

A total of 54 different volatile compounds were identified from the flowers of *S. japonica*, *S. parviflora*, *S. sessilifolia*, *S. shandongensis*, and *S. tuberosa*. Chemical analysis and smelling tests using human subjects did not detect floral scents from *S. mairei* flowers during the day-time (Tables 2 and S1). The number of scent compounds identified ranged from four in *S. sessilifolia* and *S. shandongensis* to 46 in *S. tuberosa*. The identified compounds were divided into six chemical classes (Table 2): fatty acid derivatives (28), benzenoids (10), C5-branched compounds (five), nitrogen-containing compounds (five), sulfur-containing compounds (four), and terpenoids (two). Each species emitted diverse profiles of scent compounds. For example, *S. sessilifolia* and *S. shandongensis* mainly emitted sulfur-containing compounds, comprising 88.06 ± 2.58% and 64.48 ± 4.67% of the total floral scent profile, respectively. *S. japonica* primarily emitted C5-branched compounds (35.71 ± 2.75%) and nitrogen-containing compounds (64.29 ± 2.66%), while *S. parviflora* mainly emitted fatty acid derivatives (42.03 ± 6.48%), sulfur-containing compounds (20.56 ± 4.63%), nitrogen-containing compounds (10.33 ± 4.82%), and C5-branched compounds (24.29 ± 3.33%). *S. tuberosa* primarily emitted fatty acid derivatives (46.48 ± 2.97%) and benzenoids (38.12 ± 2.58%). These five *Stemona* species did not emit (or emitted low levels of) terpenoid

compounds (Tables 2, S1). Moreover, the scent profiles of each species were dominated by only a few compounds (two to three), including the following: *S. japonica*, 1-pyrroline (64.29 ± 2.66%) and 2-methyl-1-butanol (25.72 ± 2.97%); *S. parviflora*, 3-hydroxy-2-butanone (34.74 ± 9.51%) and isoamyl alcohol (23.7 ± 6.3%); *S. sessilifolia*, dimethyl disulfide (78.63 ± 3.61%) and isoamyl alcohol (10.06 ± 2.63%); *S. shandongensis*, dimethyl disulfide (36.45 ± 2.84%), isoamyl alcohol (29.13 ± 5.94%), and dimethyl trisulfide (28.04 ± 3.64%); and *S. tuberosa*, butyric acid (24.49 ± 2.97%), *p*-cresol (18.37 ± 2.26%), and phenol (12.85 ± 1.86%) (Tables 2, S1).

PERMANOVA tests suggested that the floral scent profiles differed significantly among species ($F_{4, 62} = 26.16$, $P < 0.001$). The results of ANOSIM analysis, which are in accordance with the results of PERMANOVA, also indicated a significant difference among *Stemona* species ($R = 0.8862$, $P < 0.001$). Moreover, we detected inter-specific differences in floral scent compounds among species in the non-metric multi-dimensional scaling (NMDS) ordination, with high linear and non-metric fits ($R^2 = 0.59$ and 0.25, respectively) and a low stress value (0.141). There was no overlap between species (Figure 2A).

Analysis of intra-specific levels of variance dispersion using Bray-Curtis distances among individuals or populations revealed significant inter-specific differences (Figure 2B). Each species showed different levels of intra-specific variation; for example, *S. japonica* showed the highest intra-specific similarity, followed by *S. sessilifolia* and *S. shandongensis*. *Stemona parviflora* and *S. tuberosa* exhibited the largest intra-specific dissimilarity. Significant population differences were found in *S. tuberosa* (one-way ANOSIM; $R = 0.8685$, $P < 0.001$).

NMDS ordination based on scent data from *S. tuberosa* produced high linear and non-metric fits ($R^2 = 0.75$ and 0.18, respectively) and a low stress value (0.114; Figure S1). The NMDS biplot indicates that most *S. tuberosa* populations form discrete clusters, but there are also some overlaps between populations, such as populations Mz, Mlp, and Cx, as well as populations Hn-p and Yc (Figure S1). We also evaluated the intra-specific contribution of each floral scent based on relative levels among the examined *S. tuberosa* populations through principal component analysis (PCA). The first two PCs explain most of the total variance (47.1% and 25.0%, respectively). Butyric acid mainly contributes to populations Bn, Lc, and Mz, while *p*-cresol mainly contributes to populations Cx, Mlp, and Yc. Phenol mainly contributes to the four remaining populations: Jx, Hn-w, Hn-p, and Gx (Fig. S2).

Independent component analysis (ICA) detected 16 diagnostic scent compounds, which were significantly associated with certain species. These scent compounds, which are considered to be good “indicator” or “predictor” compounds (Table S2), include the following: 1-pyrroline, isoamyl alcohol, 2-methyl-1-butanol, 3-methyl butanal, and 2-methyl butanal for *S. japonica*; 3-hydroxy-2-butanone, 2,5-dimethylpyrazine, and S-methyl butanethioate for *S. parviflora*; dimethyl disulfide for *S. sessilifolia*; dimethyl trisulfide for *S. shandongensis*; and *p*-cresol, butyric acid, indole, phenol, n-tetradecane, and 2-methyl butyric acid for *S. tuberosa*. The ICA values for *S. sessilifolia* and *S. shandongensis* were lower (0.64 and 0.63, respectively) than those of the three other species examined. Additionally, we detected strong specificity in floral scent in *S. japonica* (5/5), *S.*

sessilifolia (1/4), and *S. shandongensis* (1/4), while more shared scent compounds were detected in *S. parviflora* (17/20) and *S. tuberosa* (48/54).

Canonical variate analysis (CVA) ordination illustrated the five species for which floral volatiles could be detected (MANOVA; Wilks $\lambda_{252,110.7}$ = 8.915e-11, $P < 0.001$). All individuals of each group were classified according to their *a-priori* designations, implying that floral scents are an important component for each taxon examined. The first two canonical axes account for 69.5% and 25.4% of the total variation, respectively (Figure 2C). The key volatile compound of this taxon on the x-axis is 1-pyrroline, which clearly distinguishes *S. japonica* from the other four species. Seven other scent compounds contribute greatly to the y-axis, including butyric acid, *p*-cresol, phenol, 3-hydroxy-2-butanone, dimethyl disulfide, isoamyl alcohol, and dimethyl trisulfide. In *S. tuberosa*, butyric acid, *p*-cresol, and phenol contribute positively to the y-axis. On the other hand, 3-hydroxy-2-butanone, dimethyl disulfide, isoamyl alcohol, and dimethyl trisulfide contribute negatively to the y-axis, with different loadings on the x-axis. For example, 3-hydroxy-2-butanone contributes greatly to *S. parviflora*, whereas dimethyl disulfide, isoamyl alcohol, and dimethyl trisulfide contribute greatly to *S. sessilifolia* and *S. shandongensis* (Figure 2C). Moreover, one-way SIMPER revealed 54.8% scent similarity between *S. sessilifolia* and *S. shandongensis* (Table S3).

Floral profiles not including scent

All species examined have chamber flowers with a single flowering period of 1 d, except for *S. mairei*, which has a flowering period of 2-3 d (Figure 1; Table 1). Flowers in these species range from 8 to 79 mm in length, with tepal color ranging from light red to dark purple. Nectar secretion at the base of the flowers could not be detected in any of these species and, indeed, previous studies have also indicated that *Stemona* species lack nectaries (Rudall et al. 2005). The flowers typically opened at ~8:30-9:30 in the morning and closed at ~18:30-19:30 in the evening. Interestingly, the nine staff members at Kunming Botanical Garden (KBG) detected fetid floral odor from five species, which emanated from the large stamens and appendages of the flowers (Figure 1M), due to the putrid smells of 2-methyl-1-butanol, isoamyl alcohol, dimethyl disulfide, dimethyl trisulfide, and 1-pyrroline. Bagging experiments suggested that spontaneous self-pollination does not occur in these *Stemona* species, since bagged flowers did not develop fruits. However, manual cross-pollination of different individuals and species at KBG yielded high fruit set in six species, ranging from 63.3% to 86.7% (Tables 2, S1). These results imply that fruit set in *Stemona* is related to pollinator and/or pollen availability at the study location. Several floral pollinators were observed to visit the flowers of different *Stemona* species (Figure 1). *S. japonica* flowers were pollinated by *Atherigona* flies (Chen et al. 2015b). *S. sessilifolia* and *S. shandongensis* might have been successfully pollinated by flies of the genus *Paregle* (Fig. 1N). Among *S. tuberosa* fly pollinators, more than 87% (692 individuals) were *Aldrichina graham*, *Chrysomya pinguis*, *Lucilia sericata*, or *Ravinia striata* (Chen et al., unpublished data; Figure 1A-J). The behavior of flies visiting the flowers and apparently searching for a reward or oviposition site included crawling around the base of the flower, the tepals, and the stamens. The flies acquired

pollen accidentally on their bodies when they moved within the flowers. No active grooming/pollen gathering behavior was observed. When the flies left the flowers, their bodies were coated in pollen. During our preliminary investigation, no obvious oviposition behavior was observed, and no eggs were detected in the flowers. Previous studies suggest that tactile and thermogenesis cues might play roles in sapromyophily (Urru et al. 2011; Jürgens et al. 2013); these cues should be investigated in future studies.

DISCUSSION

Stemona is a new taxon exhibiting Sapromyophily

Sapromyophily has evolved several times in unrelated angiosperm families (Vereecken and McNeil 2010; Jürgens et al. 2013; Chen et al. 2015a, 2015b). The flowers/inflorescences of these plants mimic carrion, dung, or the fruiting bodies of fungi by emitting putrid smells. Saprophagous insects are unable to distinguish between these odors and those from their natural oviposition sites (Vereecken and McNeil 2010; Jürgens et al. 2013). Floral odor plays a particularly important role in sapromyophilous mimicry systems, with the dominant volatiles being oligosulfides, *p*-cresol, phenol, isoamyl alcohol, and indole (Urru et al. 2011; Jürgens et al. 2013). These volatile compounds appear to act as attractants to floral visitors, as supported by several behavioral studies (Shuttleworth and Johnson 2010; Moré et al. 2013; Chen et al. 2015a, 2015b). In the current study, we identified volatile compounds emitted by *Stemona* species that are rarely present in the floral scent volatiles of angiosperms. These volatiles represent a wide range of different degradation products of plant and animal material, e.g., dimethyl disulfide, dimethyl trisulfide, 1-pyrroline, butyric acid, and *p*-cresol (Knudsen et al. 2006; Jürgens et al. 2013; Tables 2, S1). Based on previous findings (Jürgens et al. 2006, 2013; Chen et al. 2015) and the current study, we propose that *Stemona* species that emit scents with a semen-like odor, which have a high 1-pyrroline content, display a unique type of sapromyophily involving carcass mimicry. Other types of mimicry displayed by *Stemona* species, such as herbivore feces mimicry, carnivore/omnivore feces mimicry, and urine mimicry, were described previously in other angiosperm families (Jürgens et al. 2006). Our analysis of fetid floral scents from *Stemona* lays the foundation for further investigations of the evolution of floral scents involved in sapromyophily.

Floral scent variation among *Stemona* species

Statistical analyses suggested that the floral scent profiles differed among the six *Stemona* species examined (Figure 2; Tables 2, S1, S2). Strong divergence in floral scents among closely related species is most commonly reported for species pollinated by different genera, families, or orders of pollinators (Stuurman et al. 2004; Shuttleworth and Johnson 2010). Indeed, extreme divergence in floral scents at the population level is a common phenomenon in various species (Azuma et al. 2001; Dötterl et al. 2005; Chen et al. 2014). In this study, the floral scents markedly diverged in nine populations of *S. tuberosa* (Figures S1, S2; Table S1), although all were planted in a common garden. Therefore, the genetic constitution rather than the environmental basis of *S. tuberosa* appears to influence floral scent compositions and emission in these populations (Table S1). Furthermore, local

pollinator adaptation, the biosynthetic capacity to produce volatiles, balancing selection via herbivory, genetic drift, and phenotypic plasticity might affect intraspecific floral scent variation in wild populations of *S. tuberosa*. In addition to the population differences observed in *S. tuberosa*, we also detected differences within the Hunan population (Hn), where two flower color morphs were observed and sampled (Figure 1A, B). Floral scent composition significantly differed between the two floral morphs (Figures S1, S2; Table S1), suggesting that a change in floral color might affect the floral scent emission level or composition. Similar results were reported in other studies (Salzmann and Schiestl 2007; Majetic et al. 2008; Chen et al. 2014, 2015c). We suggest that the difference in fitness between the two floral morphs of *S. tuberosa* represents an interesting ecological question; further studies are needed to explore genetic structure across the different populations of *S. tuberosa* that may be linked to differences in floral volatiles.

Ecological significance of floral scents

Although carrion and dung odors from various flowers or inflorescences have traditionally been considered to represent an adaptation for attracting flies and beetles for pollination (Vereecken and McNeil 2010; Jürgens et al. 2006, 2013), Lev-Yadun et al. (2009) proposed that fetid odors from flowers may also have another, previously overlooked function as anti-herbivore agents. Such odors might deter mammalian herbivores, especially during the critical period of flowering. In the current study, *Stemona* species were pollinated by several types of saprophilous flies (Figure 1). We also found that *Apis cerana* could carry *S. tuberosa* pollen, but these bees seldom pollinated these fetid flowers (Figure 1L). Perhaps the fetid odor from these flowers can repel some visitors, even though they have a suitable body size, or perhaps some compounds mediate a shift between fly and bee pollination systems, as was recently reported (Shuttleworth and Johnson 2010; Peter and Johnson 2014). Our preliminary observation also revealed that earwigs consume pollen and stamens of *S. tuberosa* (Figure 1K); at KBG, approximately 20.0% of flowers were consumed by earwigs. The attractive and defensive functions of fetid flowers need to be clarified in future studies.

Notably, several uncommon compounds of significance were detected in this study. For example, we detected 1-pyrroline, an attractive pheromone component produced by the male Mediterranean fruit fly *Ceratitis capitata* (Baker et al. 1985). This compound, along with other floral volatiles, plays a role in attracting *Atherigona* flies (Chen et al. 2015b). Furthermore, pyrazines, species-specific volatile compounds for *S. parviflora*, are the predominant volatile compounds emitted from urine (Jürgens et al. 2006). A recent study showed that pyrazines act as sex pheromones in a sexually deceptive orchid (Bohman et al. 2014). A comparative study by Jürgens et al. (2013) showed that the emission of sulfur-containing compounds has evolved independently in at least five different plant families of flowering plants. In this study, DMDS and DMTS are reported as floral volatiles in Stemonaceae. Interestingly, the similar chemicals dimethyl disulfide and dimethyl trisulfide often resulted in different biological responses in saprophilous flies (Zito et al. 2014).Various other compounds, such as *p*-cresol and indole, are potent lures for saprophilous coprophagous flies (Kite et al. 1998; Jeanbourquin and Guerin 2007;

Jürgens et al. 2006, 2013; Shuttleworth and Johnson 2010; Zito et al. 2015). We propose that the functions of the fetid floral scents emitted by *Stemona* species identified in our study be tested in the field in future studies.

Floral morphological profiles of *Stemona* related to Sapromyophily

Floral volatiles are complex, multi-functional signals that are often used by pollinators in combination with other signals, such as color (Schiestl 2015). In this study, we found a strong divergence in scent profiles among six *Stemona* species with similar pollinator guilds (Figure 1). Nevertheless, the flowers of the five scented *Stemona* species have similar textures and are all dull green with reddish markings (Figure 1). Perhaps flower color is limited by strong natural selection; indeed, sapromyophilous flowers often present dull-red color signals to their potential pollinators (Raguso 2004; Urru et al. 2011; Chen et al. 2015a). Similarly, flower color may be highly important for attracting fly pollinators (Wall and Fisher 2001; Gomes et al. 2007; Aak and Knudsen 2011; Chen et al. 2015a). Color changes in a carcass, including dull-red livor mortis and greenish discoloration, are caused by various physical and chemical processes in blood (Nashelksy and McFelley 2003; Goff 2009). We recently demonstrated that mimicking livor mortis is a substantiated color profile in sapromyophily (Chen et al. 2015a). Therefore, we hypothesize that *Stemona* flowers mimic different decay phases to attract a wider range of saprophagous insects. In this study, we did not detect fly eggs in the floral chambers of *Stemona* spp. This result suggests that although fly pollinators are lured to flowers, they may discriminate between the model and the mimic at a later stage and avoid laying eggs in flowers. In addition, there was a high diversity in the genera and species of flies pollinating the *Stemona* species examined (Figure 1), indicating functional specialization (nearly all fly-pollinated) but ecological generalization (quite a number of different fly genera and species can act as pollinators) among *Stemona* species.

MATERIALS AND METHODS

Study species and locations

Species of *Stemona* are well-known traditional medicinal plants in Southeast Asia. Various *Stemona* species were collected from wild populations in China and transplanted in Kunming Botanical Garden (KBG: 25.127 N and 102.743 E, 1788 m.a.s.l.), Kunming Institute of Botany, Chinese Academy of Sciences in 2004–2015. Collection permits were required for these species in China, as most are threatened species. The six selected *Stemona* species were *S. japonica*, *S. mairei*, *S. parviflora*, *S. sessilifolia*, *S. shandongensis*, and *S. tuberosa*. *Stemona parviflora* was obtained with permission from Mr. Ming-Xing Fu's private forest. To compare floral scent variation within the widely distributed *S. tuberosa* species, floral scents from nine populations were investigated in this study (Table 1). Moreover, during our field investigation, two flower color morphs (white and purple) were found in the *S. tuberosa* population in Hunan (Hn) (Figure 1A, B). Voucher specimens for each species were deposited at the Herbarium of Kunming Institute of Botany (KUN), Chinese Academy of Sciences. The geographic origins of the investigated species are listed in Table 1. Floral scents from *S. parviflora* were collected from a wild population in

Nansha, Hainan province, China. Floral scents from the five other species were collected from different individuals at the KBG location.

Collection and analysis of floral scents

Based on our observations in KBG, the single flowering period of most *Stemona* species is approximately 1 d. Therefore, floral scents were collected using the dynamic headspace adsorption method during the sunniest time of the day, between 11:30 and 16:30, which coincided with the time when the flower was fully open (usually during midday and early afternoon) and the time when flies visit flowers. Due to differences in floral size, one sample included either a single flower from *S. tuberosa*, three from *S. sessilifolia* and *S. shandongensis*, two from *S. mairei*, or five from *S. japonica* or *S. parviflora* collected from a single individual. Replicates for each species are given in Table 2 and Table S1. Flowers from the different *Stemona* species were cut from various individuals and the flower stalks were wrapped with wet cotton. The material was placed in a culture dish and enclosed in a Tedlar bag (Dupont, USA). The scent was drawn from the bag into a tube containing the adsorbent Porapak Q (150 mg, mesh 60/80, Waters Associates, Inc.) using a pump with an inlet flow rate of 300 mL min^{-1} for 4 h. To identify background contamination, the scent from stems with floral buds was collected as a control. Floral scents from *S. japonica* were collected according to Chen et al. 2015b. Trapped volatile organic compounds (VOCs) were eluted with 300 μL dichloromethane (99.5%) and concentrated to one-fifth of the original volume under a gentle stream of nitrogen (200 mL min^{-1}). Before concentration, 1000 ng *n*-nonane was added to all samples as an internal standard. The extracts were stored at –20°C in a freezer until subsequent analysis.

Samples from flowers and control tissue were analyzed using an Agilent Technologies HP 6890 gas chromatograph (GC) equipped with an HP-5MS column (5% phenylmethylpolysiloxane; 60 m long, 0.32 mm inner diameter, 0.25 mm film thickness) and linked to an HP 5973 mass spectrometer (MS). Helium was used as a carrier gas, at a flow rate of 1 mL min^{-1}. The split inlet and MS were held at 250°C. Column temperature was programmed to rise from 40°C (5 min. hold) to 250°C (20 min. hold) at 3°C/min. The mass spectra were taken at 70 eV (in EI mode) with scanning from m/z 35 to 500. Compounds were tentatively identified by comparing mass spectra and relative retention times with those of standard compounds purchased from Sigma-Aldrich, USA and with the Wiley 7n.1 mass spectral library. The average relative amounts (%) of compounds in a sample were determined based on peak area measurements. The approximate amounts of compounds in a sample extract were tentatively calculated by the internal standard method (Chen et al. 2015b), because performing GC-MS without running external standards might have reduced the precision of quantification in this study.

Statistical analysis of floral scents

Because the "individual × compound" matrix of floral scents did not meet the assumptions of multivariate normality of variances (Shapiro-Wilk normality test, W = 0.7418, $P < 1.46e\text{-}11$) and of multivariate homogeneity of group dispersions (Variances, ANOVA $F_{4, 62} = 9.75$, $P = 3.68e\text{-}222$), non-parametric tests were performed to characterize the floral scent differences among samples and *Stemona* species. PERMANOVA tests using the

average Bray-Curtis distances among samples of floral scents (relative amounts in %) were conducted with 10,000 random permutations. ANOSIM similarity analyses using the average Bray-Curtis distance among samples with 10,000 random permutations were also conducted as an alternative way to test statistically whether there were significant differences in the composition of floral scents among *Stemona* species. All of these tests were performed using PAST (Version 2.08; Hammer et al. 2001).

To characterize the dissimilarities in floral scent among samples associated with different species or populations within a species, a non-metric multi-dimensional scaling (NMDS) ordination based on a matrix of Bray-Curtis distances was calculated using the relative amounts of volatile compounds (in % of the total blend). The appropriateness of the NMDS results was determined by comparing (in a Shepard diagram) the distances among samples in the ordination plot with the original distances; the stress value generated by NMDS analysis reflects how well the ordination summarizes the observed distances among samples. NMDS analyses were performed using PAST. The similarity and distance indices between samples within a species were also calculated with PAST and plotted using Origin 8.5 (Origin Lab, USA).

To visually compare the contribution of each scent compound between different populations within *S. tuberosa*, principal components analysis (PCA) was performed with PAST. A variance-covariance matrix of the floral scents (relative amount, in %) was used, and the Jolliffe cut-off value obtained provided an informal indication of how many principal components should be considered significant (Jolliffe 1986). Components with eigenvalues smaller than the Jolliffe cut-off were considered to be insignificant. The "Biplot" option was used to show a projection of the original axes (variables) on the scatter plot and to visualize PCA loading, which gives an indication of the contributions of each compound among populations within a species.

To detect floral scent compounds whose presence is statistically associated with certain species, an indicator compound analysis (ICA) with 999 random permutations was performed. The calculated indicator value of each compound reflects both its relative abundance (specificity, "A": the probability that the volatile compound belongs to the target species) and relative frequency (fidelity, "B": the probability of finding the volatile compound in other species). The associated *p*-values indicate whether specific compounds are significant indicators of certain species (Dufrene and Legendre 1997; Cáceres and Legendre 2009). ICA was performed using PC-ORD (Version 5.0).

To visualize the homogeneity among *a-priori* groups (species), canonical variate analysis (CVA) was performed using PAST. A within-group covariance matrix of the floral scents (relative amount, in %) polled over all groups participating in the MANOVA was used. There were five *a-priori* groups in CVA of all *Stemona* taxa investigated, with all *S. tuberosa* individuals from nine populations taken as a whole. CVA was performed to reveal the floral scent compounds into which CVA axes partition *Stemona* species, provide an idea of group distinctiveness, and identify the characters most strongly associated with each partition. The "Biplot" option was used to show a projection of the original axes (variables) on the scatter plot, providing a visual representation of

the CV loading used to infer the contributions of CVs within a group. The inter-specific similarities in floral scents were also calculated by one-way SIMPER using PAST.

Floral profiles not including scent

To determine the longevity of a single flower, 10 flowers from five individuals for each *Stemona* species (two flowers per individual) were observed at KBG. The opening and closing times of the perianths were recorded. To measure floral size, 15 flowers from five individuals for each *Stemona* species were measured (three flowers per individual). The length and width of mature flowers were recorded. When measuring these characteristics, the flowers were carefully inspected and the presence of chamber flowers and floral color were recorded. During the flowering period for each single flower, the presence of nectar at the base of the flower was measured with a 10 μL capillary (N = five individuals, four flowers per individual, at the KBG location) in May–July in both 2013 and 2014. For each species, 30 flower buds from six individuals (five buds per individual) were tagged with thin, inconspicuous green threads and monitored. Hand-pollination was also performed at the KBG location. For each species, 30 flower buds on five individual plants were randomly selected, labeled, and enclosed in fine nylon nets (one flower per net) to exclude flower visitors and allow only spontaneous autogamous self-pollination. Thirty flowers from five individuals were cross-pollinated by hand to examine whether the plants could produce fruits. The reproductive success (fruit set) was then examined using the individuals planted at KBG in 2010–2015. Floral pollinators of six *Stemona* species were observed in their natural habitats. Floral pollinators of the investigated *Stemona* species were observed continuously during flower opening (09:30–18:30) for 4 d per species in 2010–2015, totaling 36 h. During the observation, the behaviors of floral pollinators were observed, such as movements on the flower, contact with floral organs, and the potential oviposition behavior of the flies. Furthermore, whether the floral pollinators collected pollen as foragers or acquired the pollen incidentally on their bodies was also investigated. Some of the representative floral pollinators were caught using a handheld net and preserved for later identification by insect taxonomists. Voucher specimens (Table 1) have been deposited at KBG. Sapromyophilous flowers often emit odors that appear fetid to the human sensory system; the olfactory descriptions of floral smell and the origin of fetid floral odors were evaluated by nine staff members at KBG.

ACKNOWLEDGEMENTS

We thank Z. Yu for analyses of floral scents from different *Stemona* species. Support for this study was provided by grants from the NSFC-Yunnan joint fund to support key projects to G. Chen (U1602266), the National Natural Science Foundation of China (31670322), and the Young Academic and Technical Leader Raising Foundation of Yunnan Province (2015HB091) to G. Chen. We thank local administration departments of natural protection area for their permission for *Stemona* species collection.

AUTHOR CONTRIBUTIONS

G. Chen and W.C. Gong designed the experiment. J. Ge and B. Wang carried out the experiments. J. Schinnerl performed the data analysis. W.B. Sun and G. Chen contributed to writing the paper.

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Table 1. The flower characteristics of different *Stemona* species and their floral visitors

Species	Origin	Longitude	Latitude	Elevation (m)	Floral length (mm)	Floral smell	Flower colour	Voucher
Stemona tuberosa								
Hn-p	Chengzhou	E112°56′	N25°35′	796	55-65	Fetid, dung, carrion	Purple	CG-2012-05-06
Hn-w	Chengzhou	E112°56′	N25°35′	796	55-65	Fetid, dung, carrion	White	CG-2012-05-06
Mlp	Malipo	E104°43′	N23°4′	1150	58-79	Fetid, dung	Dark purple	CG-2010-06-19
Lc	Lincang	E99°26′	N23°40′	1847	60-75	Fetid, dung, ink	Dark red	CG-2007-05-19
Gx	Guilin	E110°19′	N25°4′	164	58-67	Fetid, dung	Dark red	CG-2011-04-26
Mz	Mengzi	E104°14′	N24°37′	1284	60-70	Fetid, dung	Dark purple	CG-2008-05-26
Jx	Jingxi	E106°32′	N23°5′	822	50-65	Fetid, dung, carrion	Dark red	CG-2013-06-03
Cx	Chuxiong	E101°47′	N24°28′	1492	60-70	Fetid, dung	Dark purple	CG-2013-11-03
Yc	Yichang	E111°24′	N30°28′	67	65-75	Fetid, dung, carrion	Dark purple	CG-2014-07-23
Bn	Banna	E101°1′	N22°5′	1113	65-75	Fetid, dung	Light red	CG-2009-10-17
S. shandongensis	Taian	E117°7′	N36°13′	310	10-15	Rotting flesh	Purple	CG-2013-07-28
S. japonica	Hangzhou	E120°6′	N0°13′	120	10-15	Semen-like odour	Light red	CG-2012-06-21
S. mairei	Lijiang	E100°13′	N27°18′	1640	18-25	No smell to human?	White	CG-2010-09-12
S.parviflora	Baisha	E109°33′	N19°2′	880	8-11	Smelly feet	Dark red	CG-2011-07-26
S. sessilifolia	Nanjing	E118°51′	N32°4′	169	10-15	Rotting flesh	Dark red	CG-2013-05-11

Table 2. Average relative amounts (%) of floral scent volatiles in five *Stemona* species (Mean ± *S.E.*)

Compounds Samples of floral scents from different individuals	*S. japonica* (*n* = 5)	*S. parviflora* (*n* = 10)	*S. sessilifolia* (*n* = 12)	*S. shandongensis* (*n* = 9)	*S. tuberosa* (*n* = 58)
Number of compounds	4	17	4	4	46
Fruit set (%, *n* = 30)	66.7	63.3	83.3	86.7	66.7
Emission rate (ng/flower/h)	1527.36±42.42	736.48±24.55	23.76±11.88	30.54±1.53	3527.4±171.54
Fatty acid derivatives	—	42.03±6.48	1.88±0.69	2.61±0.85	46.48±2.97
C5-branched compounds	35.71±2.75	24.29±3.33	10.06±2.63	29.13±5.94	6.02±0.47
Benzenoids	—	1.19±1.19	—	—	38.12±2.58
Sulfur-containing compounds	—	20.56±4.63	88.06±2.58	64.48±4.67	1.97±0.54
Nitrogen-containing compounds	64.29±2.66	10.33±4.82	—	—	7.65±0.87
Terpenoids	—	—	—	—	0.22±0.14
Fatty acid derivatives					
Propionic acid*	—	—	—	—	2.22±0.45
3-Hydroxy-2-butanone	—	34.74±9.51	—	—	0.34±0.14
Isobutyric acid	—	—	—	—	2.76±0.54
Butane-2,3-diol	—	—	—	—	0.13±0.05
1,3-Butanediol	—	—	—	—	0.56±0.24
Butyric acid*	—	—	—	—	24.49±2.97
n-Propyl acetate	—	—	—	—	1.56±0.62
n-Pentanoic acid*	—	—	—	—	1.52±0.38
Caproaldehyde	—	—	—	—	0.03±0.02
4-Methyl-2-pentanol	—	—	—	—	0.45±0.14
Caproic acid	—	0.20±0.15	—	—	0.34±0.15
n-Butyl acetate*	—	0.47±0.27	1.88±0.69	2.62±0.85	1.39±0.42
Methyl-2-methylvalerate	—	2.08±1.11	—	—	—
4-Methylvaleric acid	—	—	—	—	3.75±1.10
4-Heptanol	—	—	—	—	0.09±0.06
4-Methyl-1-hexanol	—	—	—	—	0.08±0.04
Heptanoic acid	—	—	—	—	0.07±0.07
Octanal	—	—	—	—	0.57±0.13
Octanoic acid	—	—	—	—	0.03±0.03
Nonanoic acid	—	—	—	—	0.19±0.09
1-Octyl alcohol	—	—	—	—	0.01±0.01

4-Methylhexanoic acid	—	—	—	—	0.70±0.18
Decanoic acid	—	0.58±0.58	—	—	0.18±0.07
n-Dodecane*	—	1.54±0.26	—	—	0.56±0.11
n-Tridecane*	—	—	—	—	0.14±0.08
n-Tetradecane*	—	—	—	—	0.78±0.13
n-Pentadecane*	—	0.94±0.94	—	—	1.31±0.19
Isobutyric acid propyl ester	—	—	—	—	1.05±0.48
Benzenoids					
Benzaldehyde*	—	—	—	—	0.41±0.12
Phenol*	—	0.33±0.33	—	—	12.85±1.86
Benzyl alcohol*	—	—	—	—	4.52±1.48
p-Cresol*	—	—	—	—	18.37±2.26
Methyl benzoate*	—	—	—	—	0.18±0.11
Phenethyl alcohol*	—	—	—	—	0.93±0.22
2-Methoxy-4-methylphenol	—	—	—	—	0.03±0.03
Ethyl benzoate	—	—	—	—	0.52±0.43
Benzoic acid-1-methylethyl ester	—	—	—	—	0.10±0.07
4-Ethoxybenzoic acid ethyl ester	—	0.86±0.86	—	—	0.18±0.07
C5-branched compounds					
3-Methyl butanal*	0.78±0.11	-	-	-	-
2-Methyl butanal*	2.34±0.18	-	-	-	-
2-Methyl-1-butanol*	25.72±2.97	—	—	—	0.67±0.23
Isoamyl alcohol*	—	23.70±6.3	10.06±2.63	29.13±5.94	2.47±0.21
2-Methyl butanoic acid*	—	0.59±0.43	—	—	2.81±0.46
Sulfur-containing compounds					
Dimethyl disulfide*	—	8.14±2..25	78.63±3.61	36.45±2.84	0.44±0.16
Dimethyl trisulfide *	—	5.26±2.15	9.43±3.57	28.04±3.64	1.46±0.38
Dimethyl tetrasulfide*	—	—	—	—	0.06±0.03
S-methyl butanethioate	—	7.16±2.93	—	—	—
Nitrogen-containing compounds					
1-Pyrroline*	64.29±2.96	—	—	—	—
2,5-Dimethylpyrazine	—	8.20±4.05	—	—	—
Trimethyl pyrazine	—	0.34±0.25	—	—	—
Tetramethyl pyrazine	—	0.98±0.41	—	—	—
Indole*	—	—	—	—	7.65±0.87
Terpenoids					
a-Pinene	—	—	—	—	0.08±0.06

Isolongifolene	—	—	—	—	0.14±0.08

Note: Compounds marked with asterisks (*) were identified on the basis of similarities of GC retention times and mass spectra to those of standard compounds purchased from Sigma-Aldrich, USA. The other compounds were identified according to their mass spectral data in Wiley 7n.1 mass spectral library. "-", not detected compounds in this study.

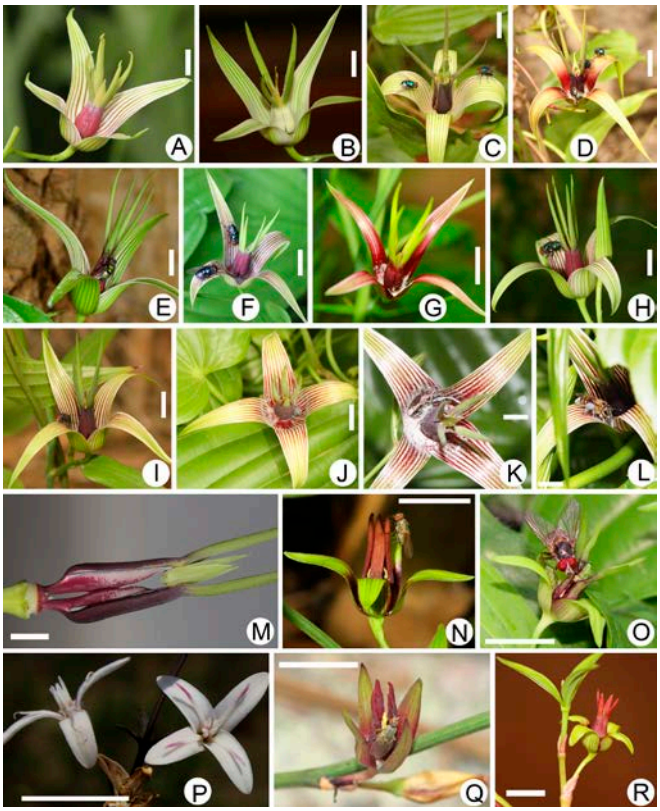

Figure 1. Flowers of the investigated *Stemona* species

(**A**–**M**) *S. tuberose*. (**A**) Sthn-p (Hunan population with purple flowers). (**B**) Sthn-w (Hunan population with white flowers). (**C**) Stmlp (Malipo population). (**D**) Stlc (Lincang population). (**E**) Stgx (Guangxi population). (**F**) Stmz (Mengzi population). (**G**) Stjx (Jingxi population). (**H**) Stcx (Chuxiong population). (**I**) Styc (Yichang population). (**J**) Stbn (Xishuangbanna population). (**K**) An earwig eating *S. tuberosa* pollen. (L) *Apis cerana* collecting pollen from *S. tuberose*. (**M**) Huge stamens and appendages of *S. tuberose*. (**N**) *S. shandongensis*. (**O**) *S. japonica*. (**P**) *S. mairei*. (Q) *S. parviflora* (photograph by Feng Gu). (**R**) *S. sessilifolia.* Scale bars: 1 cm.

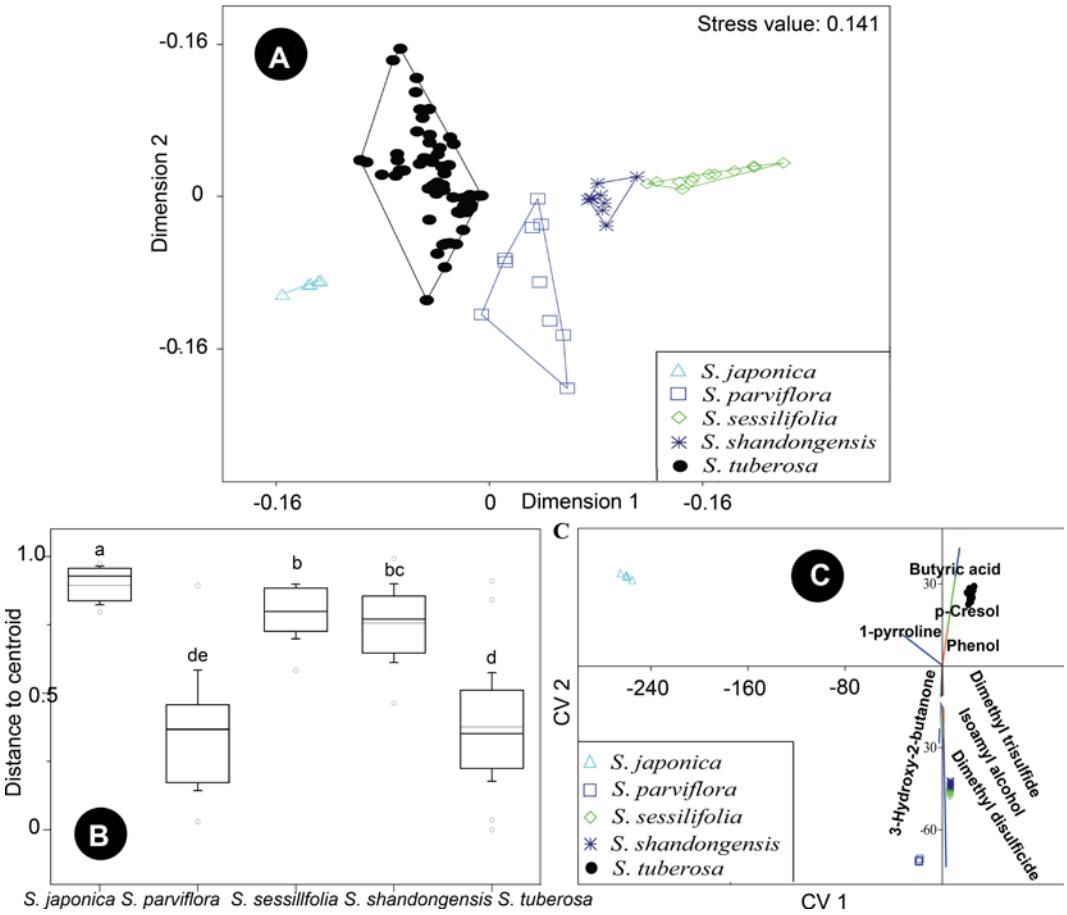

Figure 2. Floral scent differentiation among five *Stemona* species

(A) Non-metric multi-dimensional scaling (NMDS) biplot of floral scent differentiation based on a matrix of Bray-Curtis similarity distance calculated using the relative amounts of odor compounds (in % of the total blend). (**B**) Boxplot of intra-specific levels of floral scent dispersion using Bray-Curtis similarity distances among samples (different letters above the boxplots indicate significant differences based on Mann-Whitney *U*-test, $P < 0.05$). (**C**) Canonical variate analysis (CVA) of the identified floral scent compounds from five *Stemona* species.