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SEX EXPRESSION, BREEDING SYSTEM AND POLLINATORS OF *Piper caldense* (PIPERACEAE) IN THE BRAZILIAN ATLANTIC FOREST

Expresión sexual, sistema reproductivo y polinizadores de *Piper caldense* (Piperaceae) en un bosque Atlántico

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ABSTRACT

Neotropical *Piper* species have bisexual flowers. Such reproductive trait is considered basal in this pantropical genus. However, neotropical species having unisexual (staminate) flowers along with bisexual ones have also been reported. Dichogamy is common in the genus, associated with either self-compatibility or -incompatibility, as well as with entomophily. We analyzed a natural population of *Piper caldense* in a Atlantic Forest area (Viçosa municipality, Minas Gerais state, southeastern Brazil). Preliminary observations indicated that the species produces two flower types. We analyzed flower sex in spikes of 50 plants. We obtained additional information through morphological and anatomical studies and scanning electron microscopy analyses. The longevity and exposure dynamics of stigmatic papillae and the pollen release sequence of all four stamens were investigated to verify the degree of dichogamy. Pollination tests were performed, pollinators were identified and their visitation frequency was recorded. *Piper caldense* has both bisexual and staminate flowers, each flower type being located on separate spikes. These spikes occurred on the same plant, thus indicating andromonoecy; furthermore, plants having only spikes with staminate flowers were also observed. This gender had not yet been reported to the genus. Gradual and sequential exposure of stigmatic papillae associated with asynchronous pollen release (one stamen a day) resulted in incomplete protogyny. Hand pollination tests showed that the species is self-incompatible. Social bees, mainly *Apis mellifera* and *Melipona* spp., were the major pollinator group. Our study reinforces the need to associate morphological analysis with floral biology and indicates future changes in studies addressing reproductive traits associated with the phylogeny of the *Piper* genus.

Keywords: andromonoecy, entomophily, incomplete protogyny, self-incompatibility, social bees.

RESUMEN

Las especies neotropicales de *Piper* presentan flores bisexuales, condición considerada basal al interior de este género pantropical. Sin embargo, fueron observadas especies neotropicales con flores unisexuales (estaminadas), además de las bisexuales. La dicogamia es común en el género y se encuentra asociada a la autocompatibilidad o incompatibilidad, al igual que la entomofilia. Fue analizada una población natural de *Piper caldense* en un área de bosque Atlántico (municipio de Viçosa, Minas Gerais, sureste de Brasil). Las observaciones preliminares indicaron que esta especie produce dos tipos florales, para lo cual fue analizada la sexualidad de las flores en 50 espigas a través de estudios morfológicos, anatómicos y análisis con microscopia electrónica de barrido. La longevidad y dinámica de exposición de las papilas estigmáticas y la secuencia de liberación de los granos de polen en los cuatro estambres fueron observados para verificar el grado de dicogamia. El sistema reproductivo se evaluó mediante el test de autopolinización espontánea y polinización abierta. Los visitantes florales fueron identificados y se registró su frecuencia de visita. *Piper caldense* presenta flores bisexuales y estaminadas, cada tipo floral se encuentra en espigas separadas. Estas espigas se encuentran en la misma planta, lo que indica que es andromonoica; adicionalmente, fueron observadas plantas que solo presentaban espigas con flores estaminadas, lo que se constituye en una combinación sexual inédita para el género. La exposición gradual y secuencial de las papilas estigmáticas asociada con la liberación asincrónica de los granos de polen (un estambre por día) indica una protoginia incompleta. Los test

de polinización mostraron que la especie es autoincompatible. Las abejas sociales, principalmente *Apis mellifera* y *Melipona* spp., fueron los polinizadores principales. Este estudio refuerza la necesidad de asociar los analisis morfológicos con la funcionalidad de las estructuras florales, además indica los cambios futuros en estudios que abordan características reproductivas asociadas a la filogenia de las especies de *Piper*.

Palabras clave: abejas sociales, andromonoica, autoincompatibilidad, entomofilia, protoginia incompleta.

INTRODUCTION

Piper is a pantropical genus with around 2000 species, being one of the most important components of the understory in tropical forests (Quijano-Abril *et al.*, 2006), including the Brazilian one (Rossetto and Vieira, 2013). The genus is represented in Brazil by 290 species, 179 of which are endemic, like *P. caldense* C. DC. (Guimarães *et al.,* 2015). Despite the species richness of the genus and its ecological importance, information on the reproductive biology of its representatives is available for only 6 % of the species occurring in the country (A. Valentin-Silva, pers. comm.).

Floral morphology is similar among *Piper* representatives. Flowers in the genus are small, usually arranged in spikes, bisexual or unisexual, perianthless, and fully protected by a bract at the flower bud stage (Yuncker, 1972; Yuncker, 1973; Jaramillo and Manos, 2001). In view of such similarity in flower morphology among *Piper* representatives, sexual expressions were considered important reproductive traits to distinguish paleotropical from neotropical species: those from Asia and the South Pacific are dioecious, while those from Africa are monoecious or dioecious and those from the neotropics are hermaphrodite (Jaramillo and Manos, 2001). However, in the neotropical *P. arboreum* Aubl. (Figueiredo and Sazima, 2000) and *P. gaudichaudianum* Kunth (Valentin-Silva and Vieira, 2015), unisexual (staminate) flowers were observed along with bisexual ones in andromonoecious populations from southeastern Brazil. Additionally, in preliminary observations made on a *P. caldense* population from the same area of the present study, both flower types have also been observed in the analyzed individuals. These findings show that, conversely to what was described above, sex expression may not be a diagnostic feature for neotropical species.

Dichogamy is common among species with bisexual flowers (Figueiredo and Sazima, 2000; Valentin-Silva *et al.,* 2015), being associated with either self-compatibility (Marquis, 1988; Sasikumar *et al.,* 1992; Figueiredo and Sazima, 2000, Valentin-Silva *et al.,* 2015) or self-incompatibility (Figueiredo and Sazima, 2000; Kikuchi *et al.,* 2007). In the latter case, studies have shown that insects, especially bees and flies, as well as wind, are pollinators (Martin and Gregory, 1962; Semple, 1974; Fleming, 1985; Figueiredo and Sazima, 2000; Thomazini and Thomazini, 2002; Kikuchi *et al.,* 2007; Valentin-Silva *et al.,* 2015). On species having unisexual flowers, Merrett *et al.* (2007) concluded that pollination is anemophilous in *Macropiper excelsum* Miq. (= *Piper excelsum* G. Forst.), a dioecious species, due to the absence of biotic pollinators and to the high pollen:ovule ratio. On species having both bisexual and staminate flowers, *P. arboreum* was considered self-incompatible and ambophilous (Figueiredo and Sazima, 2000).

The purpose of this study was to analyze the sexuality, morphology and biology of *Piper caldense* flowers, aiming to clarify the species sex expression and degree of floral dichogamy. We also investigated the species breeding system and identified flower visitors, including pollinators.

MATERIALS AND METHODS

Study area and species

The studies were conducted from July 2013 to September 2014 at the Station of Research, Environmental Training and Education Mata do Paraíso, (hereafter, Mata do Paraíso; 20°48' S and 42°51' W, 690 m.a.s.l.), the largest forest fragment in Viçosa municipality, Minas Gerais state, southeastern Brazil. The fragment has Atlantic Forest vegetation (Oliveira-Filho and Fontes, 2000), being classified as montane seasonal semideciduous forest (Veloso *et al.,* 1991).

According to Köppen's classification, climate in Viçosa is type Cwa, i.e., mesothermal with hot wet summers and cold dry winters. Annual means of rainfall, relative humidity and temperature are, respectively, 1221.4 mm, 81 %, and 19.4 °C. The dry cold season extends from April to September while the warm rainy season begins in October and lasts until March.

Piper caldense is a 2-3 m high shrub. The hanging spikes are 3 to 5 cm long. Flowers are protected by a triangular subpeltate bract with fringed margins; the androecium consists of four stamens; the gynoecium has a superior ovary, short style and three stigmas; and the fruit is a drupe (Carvalho-Okano and Alves, 1998). A voucher specimen was deposited in the VIC Herbarium (A Valentin-Silva 82, DL Vargas-Rojas).

At Mata do Paraíso, *P. caldense* plants occur in moist and partially shaded areas of the understory. All analyzed plants had spikes with flower buds throughout the study period. These spikes remained latent for up to 12 months and were approximately 0.5 cm long. Spikes with flowers at anthesis were observed in the dry season, between August and September 2013 and 2014.

Sex expression and floral morphology and biology

To define the sex expression of plants and flowers, we analyzed flowers on spikes of 50 plants with a hand lens (30 x). Additionally, spike length and diameter (central

portion) were measured in 49 spikes (14 with bisexual flowers and 35 with unisexual ones) having flowers at late anthesis. To assess the average number of flowers per spike, the number of flowers per inflorescence was counted in ten spikes for each flower type.

Fresh spikes were stored in 70 % ethanol and examined under a stereomicroscope (model EZ4D, Leica Microsystems, Deerfield, Illinois, USA) to verify the presence of androecium and/or gynoecium. In addition, stigma receptivity, which was characterized by the exposure of turgid stigmatic papillae (Valentin-Silva *et al.,* 2015), was monitored in bisexual flowers of 15 spikes from different plants in the field, for 10 consecutive days, with a hand lens. The dynamics of pollen release from the four stamens of both bisexual and unisexual flowers was observed. Pollen grain viability was tested with acetic carmine (Radford *et al.,* 1974). To this end, 40 spikes (20 per floral type) were fixed in FAA_{50} (formaldehyde, acetic acid, and 50 % ethanol, 1:1:18 v/v/v) for 48 h and then stored in 70 % ethanol (Johansen, 1940). Completely exposed stamens (not protected by the floral bract) were used. On each slide, five anthers of flowers from the same spike were squashed in a drop of acetic carmine. A total 200 pollen grains were counted per slide, being then separated into viable and non-viable (Kearns and Inouye, 1993).

To obtain further information on floral morphology and biology, morphoanatomical studies were performed at the Laboratory of Plant Anatomy of Federal University of Viçosa, and scanning electron microscope analyses were conducted at the Center for Microscopy and Microanalysis at the same institution. We used samples of spikes having bisexual and staminate flowers at different stages, previously fixed in FAA_{50} and stored in 70 % ethanol (Johansen, 1940). The adopted techniques were similar to those described by Valentin-Silva *et al.* (2015).

Breeding system and flower visitors

Following the methods proposed by Dafni *et al.* (2005), two tests were conducted in spikes with bisexual flowers: spontaneous selfing–15 spikes were bagged at pre-anthesis until fruit formation or inflorescence abortion; and open pollination–15 spikes were labelled and left exposed to pollinators. Spikes were isolated with cloth (fully closed mesh) bags. The percentage of fruiting in these tests was computed, considering the mean number of flowers per spike.

We recorded the visitation frequency on five non-consecutive days in August 2014, from 7:00 AM to 5:00 PM, totalizing a sampling effort of 40 h. Floral visitors were captured to localize the pollen deposition sites on their bodies. Voucher specimens were deposited in the Entomology Museum of Federal University of Viçosa.

Statistical analysis

The above-mentioned measurements, as well as those of pollen grain viability, were subjected to analysis of variance (ANOVA) with software R, version 3.0.1 (R Development Core Team, 2010). We assessed the number of flowers with a means test (t test), using the same software.

RESULTS

Sex expression and floral morphology

Piper caldense has bisexual and unisexual (staminate) flowers, each flower type being located on separate spikes. In 15 of the 50 analyzed plants, all spikes having flowers still at the bud stage were aborted, which rendered flower sex identification impossible. Of the other 35 plants, 14 had spikes with staminate flowers as well as spikes with bisexual flowers, while the other 21 plants had only spikes with staminate flowers. Therefore, the number of plants having only staminate flowers was 1.5 times higher than that of plants having both flower types. There were size differences between spikes with bisexual flowers and spikes with staminate flowers (Table 1), with statistical differences being detected in both spike length ($F = 16.7$, $p = 3.6\ e^{-6}$) and diameter ($F = 33.2$, $p = 1.18\ e^{-9}$). Moreover, spikes with bisexual flowers had on average more flowers than spikes with staminate flowers: 252.8 ± 27.2 and 198.3 ± 27.5, respectively. These averages differed from each other ($t = -4.5$, $p = 0.003$).

Bisexual flowers have a floral bract which, at the bud stage, fully protects all four stamens; white, dithecous anthers (Fig. 1A); a pistil with trifid stigma; and a superior, unilocular, uniovular ovary with basal placentation. Staminate flowers also have a floral bract that fully protects all four stamens, having the same characteristics of the androecium of bisexual flowers and also a pistillode (non-functional, reduced pistil; Fig. 1B).

Table 1. Size (mean ± standard deviation) of *Piper caldense* spikes having both bisexual and staminate flowers and viability (mean ± standard deviation) of pollen grains from all four stamens. Values in a same column followed by the same letter do not differ statistically.

Plants	Flowers	Spike size (mm)		Pollen grain viability (%)			
		Length	Diameter	Stamen 1	Stamen 2	Stamen 3	Stamen 4
Andromonoecious	Bissexual	36.3 ± 4.8 a	4.6 ± 0.5 A	96.9 ± 1.2	97.4 ± 1.8	97.0 ± 1.7	97.2 ± 2.0
	Staminate	30.5 ± 4.3 b	3.6 ± 0.4 B	97.8 ± 1.6	97.6 ± 1.5	96.1 ± 1.8	97.5 ± 2.3
Male	Staminate	27.5 ± 4.2 b	3.5 ± 0.3 B	-	-	-	-

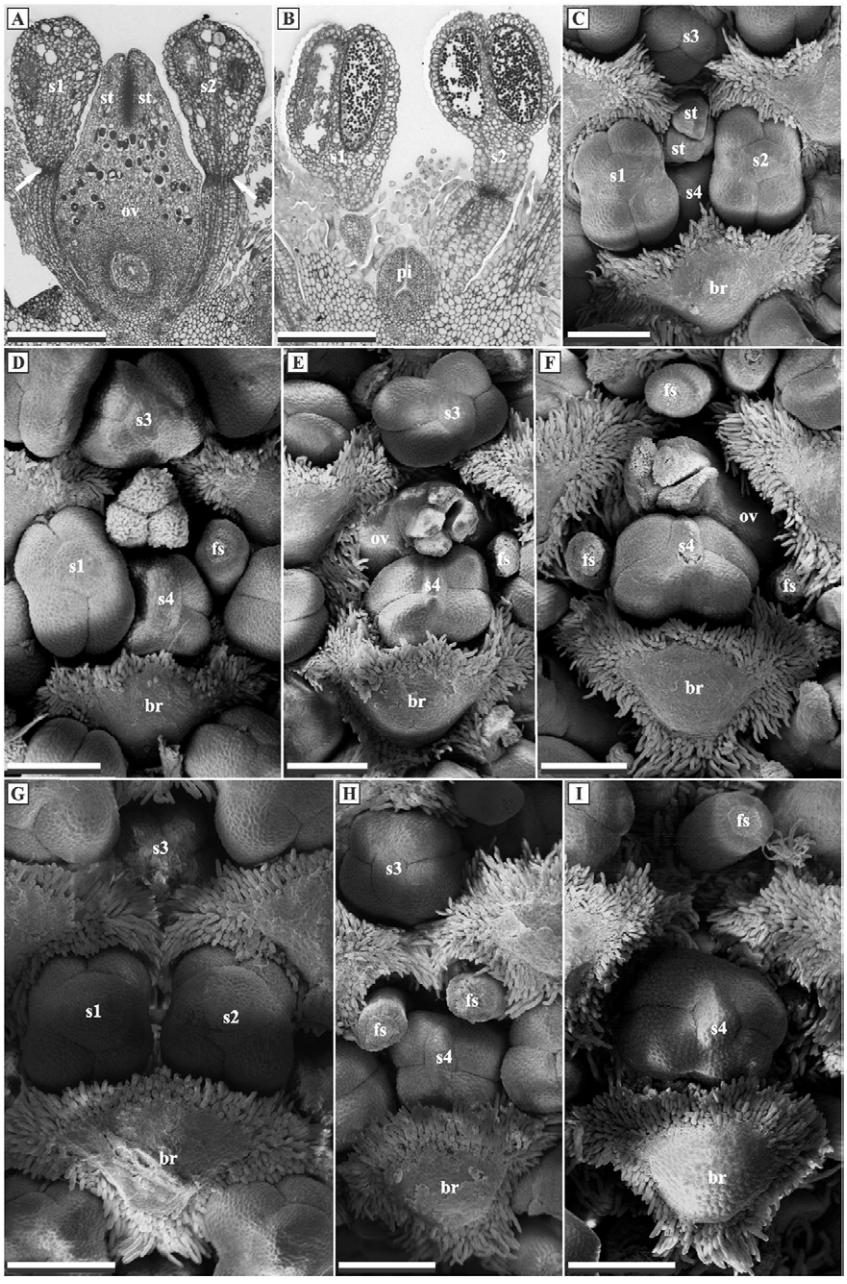

Figure 1. *Piper caldense* flowers. A) bisexual flower, with two of the four stamens and the pistil; note the abscission tissue in the filament (arrow). B) staminate flower, with two of the four stamens and the pistillode. C) bisexual flower with partially exposed anthers of stamens 1, 2 and 3 and upright, juxtaposed stigmas. D) bisexual flower with three stamens; note the filament scar of the first stamen that released pollen. E) bisexual flower with stamens 3 and 4. F) bisexual flower with stamen 4. G) staminate flower with partially exposed anthers of three stamens (1, 2 and 3). H) staminate flower with stamens 3 and 4; note the filament scar of stamens 1 and 2. I) staminate flower with exposed anther of stamen 4. The bract apex is directed toward the distal region of the spike. br = bract, fs = filament scar, ov = ovary, pi = pistillode, st = stigma, s1 = stamen 1, s2 = stamen 2, s3= stamen 3, s4 = stamen 4. Scale bars: 400 mm.

Biology of bisexual flowers

In bisexual flowers, there was a partial opening of the bract and exposure of the anthers of three stamens (stamens 1-3, Fig. 1C) for two to three days (stamens were numbered according to the position of the floral bract; Fig. 1C). Subsequently, for up to three additional days, the upright, juxtaposed stigmas were visible among those three stamens, as was the apical portion of the anther of stamen 4 in some flowers (Fig. 1C), due to the continuous bract opening. Thus, these changes occurred in the flowers for three to six days before anthesis.

Anthesis of bisexual flowers began with stigma receptivity, being characterized by the exposure of turgid papillae at the distal portion; stamens remained as shown in Fig. 1C, with indehiscent anthers. The occurrence of stigma receptivity in the period prior to pollen release indicates protogynous dichogamy. The pistillate flower phase lasted an average two days.

The bisexual phase began with pollen being released from flowers while stigmas were still receptive, a characteristic of incomplete protogyny. Either stamen 1 or 2 was the first one to release pollen. One of these stamens was higher than the other, due to complete exposure (filament and anther), and released pollen first, beginning in late morning but occurring mainly in the afternoon, at around 2:00 PM (same time of release of the other stamens). Thereat, the distance among stigmas increased, almost completely exposing the still-turgid stigmatic papillae.

On the second day of the bisexual phase, the second stamen to undergo anther dehiscence (either stamen 1 or 2; Fig. 1D) released pollen. By then, the stamen that had released pollen the day before may either still have residual pollen, in which case such stamen has characteristics that indicate senescence, such as anther darkening and wilting; or it may have already been aborted (note the filament scar of the stamen that first released pollen, in Fig. 1D). At this stage, the bract is fully open, further exposing the anther of stamen 4. Furthermore, in some flowers, the distal portion of stigmas was darkened and had plasmolyzed papillae, indicating the onset of cell senescence.

On the third day, stamen 3 was completely exposed and pollen was released from the anther (Fig. 1E). The stamen that had released pollen the day before may either have already been aborted or still have a senescent anther with residual pollen. In that occasion, the anther of stamen 4 remained indehiscent. Stigmas then were totally exposed (Fig. 1E); from this stage, the darkened portion of stigmatic surface was quite variable, but was thereat observed on the middle portion of the stigma towards its base.

On the fourth day, senescence- or abortion-related characteristics were observed on the anther of stamen 3 (Fig. 1F). Stamen 4 was completely exposed and its anther released pollen. Most flowers had completely darkened stigmas with collapsed papillae, indicating the end of stigma receptivity; however, thereat some flowers may have turgid papillae in the proximal third of stigmas.

Anthesis of bisexual flowers thus lasted six days. Thereafter, ovary size increased due to fruit formation in fertilized flowers.

Biology of staminate flowers

In staminate flowers, with the beginning of bract opening for two to three days, the anthers of three stamens (stamens 1-3; Fig. 1G) were partially exposed. At the end of this period, stamens 1 and 2 were more exposed than stamen 3. Flowers may remain at this stage for up to five additional days. Therefore, these changes occurred in the flower at three to eight days before anthesis.

Anthesis began with pollen release (see the filament scar of stamens that had already released pollen, in Fig. 1H-I), with similar dynamics to the one of bisexual flowers, for four consecutive days. In stamen 4, we observed that some flowers might have residual pollen for up to two days. Thereafter, spikes dried out and were aborted.

Pollen viability, breeding system and flower visitors

Pollen grain viability was similar between stamens of bisexual and staminate flowers, as well as among stamens of each flower (Table 1). Means did not differ statistically ($F = 0.5$, $p = 0.861$).

No fruiting was observed in the spontaneous self-pollination test, while a 63.3 % fruit set was observed in the open-pollination test, thus indicating the occurrence of self-incompatibility in *P. caldense*. The fact that the number of fruits/spike ($\overline{X} = 161.5 \pm 93.1$) was smaller than the one of flowers/spike ($\overline{X} = 252.8 \pm 27.2$) indicates that not all flowers were pollinated and/or fertilized.

Spikes were visited by insects of two orders, each being represented by one family: Hymenoptera, Apidae (bees, Fig. 2) and Diptera, Syrphidae (flies) (Table 2). These insects collected pollen, the only floral resource available.

Table 2. Visitation frequency (number and percentage of visits per insect) of flower visitors on *Piper caldense* flowers at Mata do Paraíso, Viçosa municipality, Minas Gerais state, southeastern Brazil.

Order/Family/Species	Visits	
	No.	%
Hymenoptera/Apidae		
Apis mellifera Linnaeus 1758	127	30.5
Melipona bicolor Lepeletier 1836	45	10.8
Melipona mondury Smith 1863	36	8.7
Melipona quadrifasciata Lepeletier 1836	50	12.0
Schwarziana quadripunctata Lepeletier 1836	34	8.2
Tetragona elongata (Lepeletier & Serville 1828)	9	2.2
Tetragonisca angustula (Latreille 1825)	8	1.9
Trigona spinipes (Fabricius 1793)	40	9.7
Subtotal	349	84.0
Diptera/Syrphidae		
Argentinomyia sp.1	1	0.2
Ocyptamus sp.1	43	10.3
Ocyptamus sp.2	11	2.6
Ocyptamus sp.3	7	1.7
Ocyptamus sp.4	1	0.2
Salpingogaster sp.1	2	0.5
Toxomerus sp.1	2	0.5
Subtotal	67	16.0
Total	416	100.0

Among bees, we observed eight species grouped into six genera (Table 2). These insects made 349 flower visits. *Apis mellifera* accounted for the highest visitation frequency, followed by *Melipona quadrifasciata*, *M. bicolor*, *Trigona spinipes*, *M. mondury* (Fig. 2A), and *Swarziana quadripunctata* (Fig. 2B); these bees altogether performed 79.9 % of all visits (Table 2). As for flies, we observed seven species grouped into four genera (Table 2). These insects made 67 flower visits. The highest visitation frequencies were of three *Ocyptamus* species; these flies altogether accounted for 14.6 % of all visits (Table 2).

Bees began visiting at around 8:00 AM, which lasted until 4:00 PM. Visits peaked between 9:00 and 11:00 AM, with another minor peak at 2:00 PM (Fig. 3). The timetables and visitation peaks of flies and bees were similar, differing only in number of visits (Fig. 4).

The insect visitation frequencies and deposition sites of pollen grains on insect bodies (in the ventral region and legs) both suggest that the two groups of flower visitors, especially bees, are effective pollinators of *P. caldense*.

Figure 2. Bees on spikes of *Piper caldense*. A). *Melipona mondury*. B). *Schwarziana quadripunctata*.

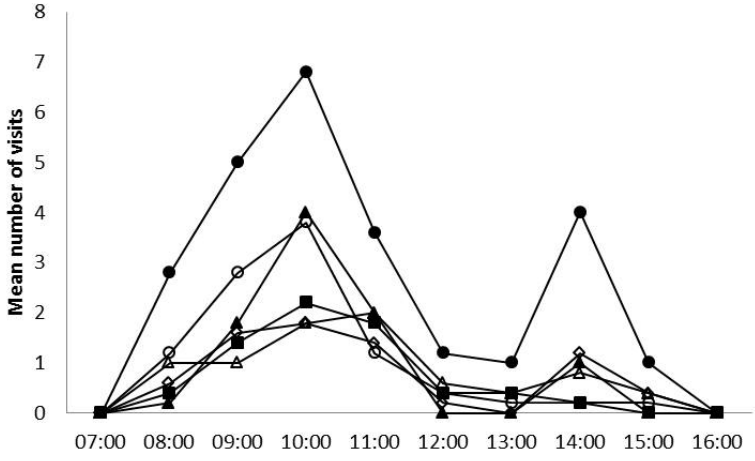

Figure 3. Number of visits of the most frequent bees on *Piper caldense* flowers, from 7:00 AM to 4:00 PM, at Mata do Paraíso, Viçosa municipality, Minas Gerais state, southeastern Brazil. ● = *Apis mellifera*, ▲ = *Melipona bicolor*, ◊ = *Melipona mondury*, ○ = *Melipona quadrifasciata*, ■ = *Schwarziana quadripunctata*, Δ = *Trigona spinipes*.

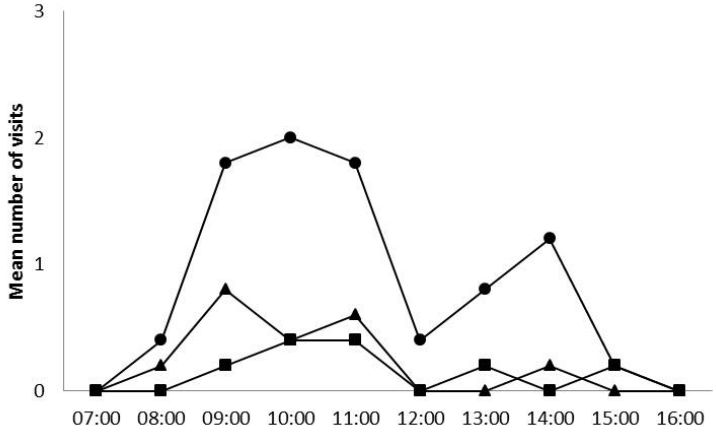

Figure 4. Number of visits of the most frequent flies on *Piper caldense* flowers, from 7:00 AM to 4:00 PM, at Mata do Paraíso, Viçosa municipality, Minas Gerais state, southeastern Brazil. ● = *Ocyptamus* sp.1, ▲ = *Ocyptamus* sp.2, ■ = *Ocyptamus* sp.3.

DISCUSSION

Bisexual flowers, which were observed in *P. caldense*, are considered basal in Piperaceae (Tebbs, 1993) and represent one of the characteristics that define the clade of neotropical *Piper* species (Jaramillo *et al.,* 2008). However, besides bisexual flowers, this species has also staminate flowers. This result contradicts the report by Jaramillo *et al.* (2008), who claimed that all neotropical *Piper* species have only bisexual flowers. The similarity in floral morphology among species in this genus must have motivated that statement, along with an almost complete lack of information on the sexual reproduction characteristics of those species. Our study reinforces the need to associate floral morphology analysis with data on flower biology, and indicates future changes in studies involving reproductive traits associated with the phylogeny of the genus.

According to Richards (1997), the presence of spikes with bisexual flowers and spikes with staminate flowers on a same plant characterizes andromonoecy, a gender which has been observed in two other neotropical *Piper* species (Figueiredo and Sazima, 2000, Valentin-Silva and Vieira, 2015). However, the presence of plants that produce only spikes with staminate flowers, in addition to the presence of andromonoecious plants, is reported here for the first time to the genus. There is no term that defines this combination between andromonoecious plants (a monomorphic population *sensu* Sakai and Weller, 1999) and male plants. Therefore, according to the terminology of Sakai and Weller (1999), the studied *P. caldense* population is dimorphic, i.e., it has two kinds of individuals, each one having different gender. This sex expression of the plants might be a derived character, bearing in mind that most neotropical *Piper* species are hermaphrodite (Jaramillo and Manos, 2001; Jaramillo *et al.,* 2008).

Incomplete protogyny, as observed in *P. caldense*, has also been described for other species of the genus (Figueiredo and Sazima, 2000, Valentin-Silva *et al.,* 2015). Temporal separation between the functions of the gynoecium and androecium is considered a strategy that favors cross-pollination (Lloyd and Webb, 1986). Nevertheless, incomplete protogyny does not prevent self-pollination due to the occurrence of the bisexual flower phase. In this case, self-incompatibility, as observed here, is a strategy that prevents autogamy. Figueiredo and Sazima (2000) found this combination of incomplete protogyny with self-incompatibility in six *Piper* species from southeastern Brazil.

The long-lived stigmas (at least four days) of *P. caldense* seem to favor incomplete protogyny, as reported to *P. vicosanum* Yunck. by Valentin-Silva *et al.* (2015). Another similarity between these two species is the gradual and sequential exposure and senescence of the stigmatic papillae, in a basipetal direction. According to these authors, this mode of stigmatic exposure is the mechanism that explains stigma longevity, which is related to the long flower duration. In addition, the darkening of *P. caldense* stigmas associated with senescence of stigmatic papillae should be the result of accumulation of phenolic compounds, as found in *P. vicosanum* (Valentin-Silva *et al.*, 2015).

The asynchronous sequence of pollen release in *P. caldense* is a trait that has also been described by Valentin-Silva *et al.* (2015) for *P. vicosanum*. The pattern (see Valentin-Silva *et al.*, 2015) involving the species studied here determines that pollen from stamens 1 and 2 must be released on subsequent days, separated by an interval of at least 24 h. Stamens 3 and 4 also released pollen on subsequent days, yet we observed no such one-day interruption, as observed in *P. vicosanum* (Valentin-Silva *et al.,* 2015). Pollen release from each stamen on different days should be advantageous to the species, since thereat the period of offering of this resource in the flower is extended, and the chances of cross-pollination are consequently increased. In addition, asynchronous pollen release is also associated with long flower duration (Valentin-Silva *et al.,* 2015).

Spikes with staminate flowers in *P. caldense* might be related to the attraction of pollinators, as suggested by Primack and Lloyd (1980) and O'Brien (1994), since pollen is the only floral resource in the species. Diurnal flower anthesis, especially regarding pollen release time, combined with insect visitation frequency, favor entomophily in *P. caldense*. Most species of *Piper* are entomophilous (Martin and Gregory, 1962; Semple, 1974; Fleming, 1985; Figueiredo and Sazima, 2000; Thomazini and Thomazini, 2002; Kikuchi *et al.,* 2007).

Among the insect pollinators, bees stand out in species richness, behavior and visitation frequency. *Apis mellifera* and *Melipona quadrifasciata* were the most frequently observed bees on spikes. These two species are social and polyletic, and therefore exploit floral resources of several plant species (Minussi and Alves-Santos, 2007, Nogueira and Augusto, 2007), including the one studied herein.

On the other hand, the lower visitation frequency by flies indicates that these insects are secondary pollinators. Flies commonly feed on pollen (Gilbert, 1981, Larson *et al.,* 2001), and the flowering of *P. caldense* in a time of low availability of this resource (dry season) might have stimulated their visit to flowers of this species. According to Gilbert (1981), the activity of flies in flowers is more related to the availability of floral resources.

Although intensely visited, the spikes with bisexual flowers of *P. caldense* produced less fruits than flowers. This reproductive feature seems to be common among andromonoecious species (Primack and Lloyd, 1980; O'Brien, 1994). However, the prevalence of spikes with staminate flowers over those with bisexual flowers apparently rules out pollen limitation in the studied population. A likely explanation for these results may be the visitation behavior. Bees and flies tend to visit several flowers per spike and more than one spike per plant, thus promoting geitonogamy in

self-incompatible species. Another explanation could be a limitation on plant nutrients, as suggested by O'Brien (1994), which also might have contributed to a decreased fruit set. Additional studies are required to clarify this issue.

CONCLUSIONS

In *P. caldense*, the observed sexual traits – two flower types, incomplete protogyny, gradual and sequential exposure of stigmatic papillae, asynchronous pollen release, self-incompatibility, and entomophily – seem to favor the maintenance of the studied population. The combination of spikes having bisexual flowers and spikes having staminate flowers in one plant with spikes having only staminate flowers in another is a new observation in the *Piper* genus. Staminate spikes seem to extend pollen availability, pollen being the only floral resource available to pollinators, which are mainly social bees.

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CONFLICT OF INTEREST

The authors declare that they have not conflict of interest.

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