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Neotropical *Piper* species: Are they all hermaphroditic?

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ABSTRACT

Sexual expression has been used as a distinctive character among the clades of the genus *Piper*. Unisexual flowers are described for Paleotropical species, which are usually dioecious, whereas bisexual flowers are associated with Neotropical species, considered as hermaphroditic. However, the registration of an andromonoecious species in the Neotropics, associated with the morphological similarity of *Piper* flowers, suggests that the presence of functionally unisexual flowers may be more common than the one recorded so far. To investigate this possibility, we determined the sexual expression of 17 Neotropical *Piper* species, focusing on the functionality of the flowers along the anthesis in individuals of a semideciduous seasonal forest. We found 13 hermaphroditic species, one andromonoecious and three with variable combinations of hermaphroditic, andromonoecious and male individuals. The staminate flowers presented cryptic unisexuality. Species with more than one floral type presented sexual dimorphism, differing in length of their inflorescences and pistils of flowers. Although hermaphroditism is the predominant sexual expression among Neotropical *Piper* species, we observed variations of this expression in almost 25% of the species studied here, indicating that the unisexual flowers may have evolved independently in different clades of Neotropical and Paleotropical species.

1. Introduction

Most of the angiosperms species are hermaphroditic (Yampolsky and Yampolsky, 1922; Barrett and Hough, 2013), but there are other types of sexual expressions due to the presence of different floral types in one or more individuals of a species (Sakai and Weller, 1999). The determination of sexual expression is based on floral morphology, however, it is also necessary to analyze the functionality of the flowers because morphologically hermaphroditic flowers may present nonfunctional androecium or gynoecium (Mayer and Charlesworth, 1991). Thus, unusual sexual expressions can be detected as cryptic dioecy (Mayer and Charlesworth, 1991) and functional androdioecy (Liston et al., 1990; Zhou et al., 2016).

Piper L. has about 2000 species with Pantropical distribution (Quijano-Abril et al., 2006) and probably originated in the Neotropics (Smith et al., 2008), the region with the greatest species richness (Jaramillo and Manos, 2001). In Brazil, it is represented by 290 species (Guimarães et al., 2015) and the Tropical Atlantic Forest is one of the centers of endemism, with about 150 species (Jaramillo and Manos, 2001; Quijano-Abril et al., 2006).

The tiny flowers of *Piper* species are, at first sight, morphologically similar: perianthless, protected by a bract, with 1–10 stamens and 3–4 carpels (Yuncker, 1972, 1973; Jaramillo and Manos, 2001). Despite this, these species present a great diversity related to the floral bract morphology, the floral development and the dynamics of stigmatic exposure and pollen release (Tucker, 1982; Jaramillo et al., 2008; Valentin-Silva et al., 2018). Phylogenetic studies have shown that the genus is composed of three clades that differ in the geographic distribution and sexual expression of the species (Jaramillo and Manos, 2001; Jaramillo et al., 2008). Bisexual flowers and, therefore, hermaphroditic species have been described for the Neotropics, whereas, in the Paleotropics (Asia and South Pacific), unisexual flowers and dioecious species predominate (Wanke et al., 2007; Jaramillo et al., 2008). Despite this difference, it is not clear which sexual expression is a synapomorphy of these clades (Jaramillo and Manos, 2001).

Although hermaphroditism is considered the only sexual expression of Neotropical species, Figueiredo and Sazima (2000) showed that there may be variation, such as the andromonoecy observed in *Piper arboreum* Aubl. This species has bisexual flowers and functionally staminate flowers in the same plant, but the occurrence of sexual dimorphism, a common feature in species with more than one floral type (Eckhart, 1999), was not described for this species.

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The evaluation of floral characters in *Piper* herbarium specimens is difficult (Jaramillo and Manos, 2001), since it can mask small morphological variations in flowers and presents limitations in relation to the temporal sequence of anthesis events. Consequently, the presence of functionally unisexual flowers may be more common than the one recorded so far. Thus, we aimed to analyze sexual expression in Neotropical *Piper* species, focusing on the sexuality of flowers and plants, to test the occurrence of sexual expressions distinct from hermaphroditism. In addition, we tested whether the non-hermaphroditic species had sexual dimorphism between different floral types.

2. Methods

2.1. Study area and species

We carried out this study from April 2014 to March 2016 at the 'Station of Research, Environmental Training and Education Mata do Paraíso' (hereafter, Mata do Paraíso), located in Viçosa (20°47′-48′S, 42°50′-52′W), Minas Gerais State, southeastern Brazil. This reserve has 194 ha of seasonal semideciduous montane forest (Veloso et al., 1991) and altitudes varying from 690 to 870 m.

At the study area, we recorded 17 *Piper* species, which belong to different clades of the genus (Table 1). To sample the plants, we used 5 km of previously established transects to mark individuals to be studied. As it was necessary to have adult individuals (at the reproductive stage), we used as inclusion criterion the presence of reproductive structures (inflorescences with floral buds, flowers or fruits) or marks (scars) on the branches that indicate previous reproductive episodes (Valentin-Silva and Vieira, 2015). We deposited voucher specimens in the BOTU Herbarium (Table 1).

2.2. Sexual expression

We evaluated the floral morphology of all species to test whether there were variations in the androecium and gynoecium that could indicate the presence of functionally unisexual flowers. So, we collected inflorescences, which were analyzed either fresh or fixed (stored in 70% ethanol), with a hand lens (60X increase) or under a stereomicroscope. Additionally, we observed the functionality of flowers throughout the anthesis. We randomly sampled three individuals per species, from the ones initially marked (Table 1) and followed, in all inflorescences, the events occurring during flower anthesis until their senescence to assign the sex of the flowers. Then, we evaluated the distribution of the floral types observed in the individuals of each species to assign the sex of the plants.

In species in which we identified in field the presence of more than one floral type, we analyzed vouchers to observe the presence of these floral types in other populations. The presence of more than one floral type in populations other than the studied in Viçosa, indicates that this floral dimorphism is a characteristic of the species. The presence of staminate flowers in vouchers was corroborated when we observed inflorescences with flowers at anthesis, which were releasing pollen, but whose gynoecium was atrophied, with stigmas below the anthers (all bisexual flowers of studied species showed incomplete protogyny; Valentin-Silva, 2017).

To exemplify the floral types observed, we collected samples of inflorescences, fixed in FAA (formaldehyde, acetic acid and ethanol 50%, 1: 1: 18 v / v / v) for 48 h and stored in 70% ethanol (Johansen, 1940). Thereafter, we dehydrated samples in an ethanol series, subjected to critical-point drying using CO_2 and metal deposition with gold (Robards, 1978). We examined and captured images in a scanning electron microscope (model Quanta 200, Fei Company, Hillsboro, OR, USA).

In species with more than one floral type, we analyzed the length of the inflorescences composed of each floral type, the length of the pistil, the number of pollen grains produced by anther and the pollen viability in each floral type to test whether there was sexual dimorphism. We measured the length of inflorescences with a digital caliper or tape measure in 10 inflorescences (n = 10 individuals/floral type/species). To measure the length of the pistil, we photographed it in a stereomicroscopic coupled with a digital camera. We processed images in the program LAS 3.8, in which we did measurements in 10 flowers (n = 10 individuals/floral type/species).

We counted the number of pollen grains per anther, using a method adapted from Roulston (2005). In a tube, we put an indehiscent anther, macerated in 0.5 mL of distilled water and homogenized the sample in a vortex shaker for 30 s. We removed an aliquot of 0.01 mL and counted the number of pollen grains in a sedgwick rafter, under a light microscope. We extrapolated the value found in the aliquot for the initial sample. We counted the number of pollen grains in 10 anthers (n = 10 individuals/floral type/species).

We tested pollen viability with acetic carmine (Radford et al., 1974) in 10 inflorescences (n = 10 individuals/floral type/species). To do this, we macerated five anthers of different flowers of the same inflorescence in a drop of acetic carmine. In each slide, we counted 200 pollen grains under a light microscope, separating them into viable and

Table 1
Number of *Piper* individuals analyzed, type of inflorescence, number of stamens and stigmas by floral type and number of material collected in semideciduous seasonal forest, Viçosa, southeastern Brazil.

Species	Clade	Individuals	Inflorescence	Bisexual flowers		Unisexual flowers	Voucher
				Stamens	Stigmas	Stamens	(BOTU)
Piper aduncum L.	Radula	13	spike	4	3		32,180; 32,198
P. amplum Kunth	Radula	11	spike	6	3		32,187; 32,194
P. anisum (Spreng.) Angely	Ottonia	12	raceme	4	4		32,188
P. arboreum Aubl.	Macrostachys	10	spike	4	3–4	4	32,181
P. caldense C. DC.	Peltobryon	15	spike	4	3	4	32,195; 32,201
P. cernuum Vell.	Macrostachys	14	spike	4	3–4	4	32,172; 32,200
P. chimonanthifolium Kunth	Radula	15	spike	4	3	4	32,196; 32,199
P. corcovadensis (Miq.) C. DC.	Ottonia	15	raceme	4	4		32,191
P. crassinervium Kunth	Radula	11	spike	4	3		32,175; 32,177
P. gaudichaudianum Kunth	Radula	12	spike	4	3		32,173; 32,183
P. hispidum Sw.	Radula	15	spike	4	3		32,174; 32,184
P. lucaeanum Kunth	Schilleria	15	spike	3	3		32,182; 32,193
P. malacophyllum (C. Presl) C. DC.	Radula	10	spike	4	3		32,178; 32,185
P. mollicomum Kunth	Radula	10	spike	4	3		32,179; 32,190
P. pubisubmarginalum Yunck.	Schilleria	15	spike	3	3		32,192
P. umbellatum L.	Pothomorphe	15	umbel of spikes	2	3		32,176
P. vicosanum Yunck.	Radula	15	spike	4	3		32,189; 32,197