



## Evolution of distyly breakdown in Palicoureeae Robbr. & Manen and Psychotrieae Cham. & Schldl. (Rubiaceae)

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### ABSTRACT

Distyly is a floral polymorphism with reciprocal placement of male and female structures, heteromorphic self-incompatibility, and other ancillary traits. However, breeding system breakdowns and loss of polymorphism are common. Here we traced the diversification of breeding strategies in the type genera of tribes Palicoureeae and Psychotrieae and discussed the evolution of distyly in a phylogenetic framework. We used literature and field information for breeding systems transitions in 46 species of *Palicourea* and *Psychotria*. Beyond distyly, we found four additional breeding systems, including monomorphism with herkogamy, homostyly (without herkogamy), monoecy and dioecy. Breeding transitions arose independently and were mostly derived from distyly. Only two species presented monomorphism as an intermediate state into gender specialization. It was not possible to evaluate the origin and evolutionary pathways for distyly in *Psychotria* and *Palicourea* as a whole, since distyly seems to be ancestral to their diversification. Breeding transitions in *Psychotria* and *Palicourea* appeared to be phylogenetically and biogeographically independent and occurred mostly in islands or isolated forest fragments, with distinct divergence times. Breeding transitions were not related to changes in ploidy. We propose that evolution of breeding transitions in *Psychotria* and *Palicourea* represent phylogenetically independent strategies to reproductive assurance in isolated or disturbed habitats.

**Keywords:** breeding system evolution, heterostyly, homostyly, monomorphism, plant reproduction

### Introduction

Distyly is a genetically controlled floral polymorphism with two morphs which differ in the expression of herkogamy (Ganders 1979; Barrett 1992). Distylous species present flowers with a long-styled morph (pin), with anthers

below the stigma (approach herkogamy), and a short-styled morph (thrum), where anthers are placed above the stigma (reverse herkogamy) (Cardoso *et al.* 2018). In truly distylous species, female (pistil) and male (stamens) sexual organs of opposite floral morphs are placed at reciprocal height (Barrett 2019). In addition to the morphological floral syndrome, many distylous plants usually present a

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diallelic self-incompatibility system, in a way that ovule fertilization occurs only when a flower receives pollen from the opposite morph. Altogether, the morphological variation and incompatibility system are interpreted as mechanisms to promote seed production through cross-pollinations (Ganders 1979; Barrett 1992; Barrett 2019).

Distyly is, however, an unstable breeding strategy (Barrett 2013; Jiang *et al.* 2018). The breakdown of distyly may result in loss of the polymorphism and acquisition of self-compatibility (Taylor 1989; Mast *et al.* 2006; Sakai & Wright 2008; Ferrero *et al.* 2009; Consolaro *et al.* 2011; Yuan *et al.* 2017). The breakdown of distyly commonly results into homostyly, with loss of intrafloral herkogamy and reproductive whorls placed at the same height within flowers (Ganders 1979; Yuan *et al.* 2017). Alternatively, the loss of the polymorphism can lead to the occurrence of populations with only one flower morph that resembles either the long-styled or short-styled morph (Ganders 1979; Barrett 1992; Yuan *et al.* 2017). These are referred also as homostylous (*e.g.* Yuan *et al.* 2017), but since they retain herkogamy, they have also been referred to as pin- or thrum monomorphism (Cardoso *et al.* 2018). These atypical morphologies in distylous groups may occur in distinct ecological contexts, appearing in flowers of the same individual or different individuals (Sakai & Wright 2008), kept only in isolated populations (Consolaro *et al.* 2011), or be spread to the whole geographic distribution of the species (Rodrigues & Consolaro 2013).

Several authors have proposed hypotheses for the evolution of distyly. Ernst (1936), Mather & Winton (1941) and Baker (1966) framed the evolution of heterostyly under a strong genetic perspective, although these hypotheses differ in the temporal sequence of assumptions. Later, and particularly for the family Rubiaceae, Anderson (1973) proposed the “Morphological hypothesis” for the origin of distyly. In this model, the ancestral condition to distyly is a protandrous and self-compatible flower with delayed maturation and elongation of the style. The short-styled morph appears as a result of a mutation making the stigma matures below the anthers. The establishment of mutants occurs as the morphology enhances self-pollination and seed output. In Anderson’s (1973) scenario, the short-styled flowers of distylous Rubiaceae self-pollinate while the long-styled flowers experience outcross pollination. Therefore, according to this hypothesis, the sex polymorphism evolved prior to the establishment of an incompatibility system. In contrast, the model proposed by Charlesworth & Charlesworth (1979) predicted the evolution of the incompatibility system before the morphological polymorphism. Specifically, under a context of inbreeding depression, the appearance of self-incompatibility would be rapidly selected in an homostylous and self-compatible morphology; then, mutations occurred, and reciprocal placement of anthers and stigma (long-styled and short-styled morphs) would be favored in the population by

avoiding self-interference, leading to the promotion of cross-pollination and fixation of distyly. Another model was proposed later by Lloyd & Webb (1992a; b), based on an ecological perspective by comparing the variation of flower morphology in distylous species and their close relatives. They predicted the evolution of distyly from an ancestral flower with approach herkogamy and partially outcrossing. Then, a dominant mutation for short style length would lead to a morph with reverse herkogamy (short-styled morph), which would spread in the population favored by pollinators promoting pollen flow between the two floral morphs. After that, ancillary traits (system of incompatibility and other floral polymorphisms) would evolve in this reciprocal herkogamous population and distyly with self-incompatibility would be finally established. Lloyd & Webb (1992a) considered the morphology of homostylous flowers as derived from distyly, caused by linkage breakdown of the supergene that controls the full heterostyly syndrome expression (Lloyd & Webb 1992b). The most widely invoked selective pressure to explain distyly breakdown has been reproductive assurance (Yuan *et al.* 2017), although the breeding strategy transition would depend on genetic breakdown processes.

Aside from the wide occurrence of heterostyly across plant families, the ancestral state reconstruction has not been well documented outside the Amaryllidaceae (Graham & Barrett 2004; Santos-Gally *et al.* 2012), Boraginaceae (Schoen *et al.* 1997; Ferrero *et al.* 2009), Passifloraceae (Truyens *et al.* 2005), Primulaceae (Mast *et al.* 2006) and Plumbaginaceae (Costa *et al.* 2019). These studies allow us to comprehend if heterostyly evolved more than once in those families and which theoretical models for evolution can be inferred (Barrett & Shore 2008; Barrett 2019). Interestingly, these studies revealed different underlying mechanisms for the changes in the breeding system. For example, in Passifloraceae (Truyens *et al.* 2005) and in Primulaceae (Mast *et al.* 2006) breeding system transitions were attributed to changes in ploidy level and recombination in a supergene. In *Narcissus* L. (Amaryllidaceae) changes in the breeding system correlated with changes in functional pollinators (Pérez-Barrales *et al.* 2006; Santos-Gally *et al.* 2012). Again, variation in ploidy level and inefficiency in pollination service in marginal habitats in Boraginaceae seemed to have driven evolutionary shifts in distylous breeding system (Schoen *et al.* 1997; Ferrero *et al.* 2009). Another interesting shift in the breeding system of distylous species involves gender specialized flowers, as explained by Beach & Bawa (1980). In their model, the origin of dioecy from distyly has been caused by a gradual process, triggered by a disruption in the disassortative pollen flow among the distylous morphs and a shift in pollinator fauna (*e.g.* long-tongued to short-tongued bees), followed by unidirectional pollen flow and ultimately the selection of unisexual flowers, the female flower from the pin morph and the male flower from the thrum morph.





Rubiaceae is one of the plant families with the largest number of distylous species, in which distyly seems to have evolved and been lost multiple times (Barrett & Shore 2008; Barrett 2019). Monomorphism (either pin or thrum) and homostyly are hypothesized to be alternative reproductive strategies derived from distyly in the Rubiaceae (Ganders 1979; Hamilton 1990), although the proper genetic mechanisms are yet to be detailed. The tribe Psychotrieae, holding more than 2,000 species, is traditionally considered to be monophyletic (e.g. Taylor 1996; Bremer & Eriksson 2009) and, possibly, presents the largest number of distylous species in the Angiosperms (Naiki 2012). Among the genera considered in Psychotrieae, *Psychotria* and *Palicourea* present complex relationships. Recent studies supported the division of the genera into two sister tribes (Psychotrieae and Palicoureeae) based on molecular, morphological and chemical differences (Robbrecht & Manen 2006; Razafimandimbison *et al.* 2014; 2017). Despite the large number of distylous species, few studies have attempted to understand breeding system evolution for the groups. Sakai & Wright (2008) studied genetic relationships and breeding system transitions in 21 species of *Psychotria* in the Barro Colorado Island, Panama. They detected a repeated and independent evolution pattern of breeding system transitions derived from distylous ancestors. In the subfamily Rubioideae, Ferrero *et al.* (2012) pointed that distyly is ancestral to the Psychotrieae and Spermaceae Alliances, suggesting that more detailed studies would be important to understand breeding system evolution in the Rubiaceae. These breeding transitions in Rubiaceae are sometimes linked to changes in ploidy, but a consistent pattern has not been detected for the family (Naiki, 2012).

One decade after the evolutionary studies of Sakai & Wright (2008) in the genus *Psychotria*, here we conducted a literature review and interpreted the breeding system evolution for a larger and worldwide sample of species of Psychotrieae and Palicoureeae, with a focus on their type genera. We particularly aimed to address the following questions: i) what breeding systems do occur in the species and whether are they derived from distyly?; ii) are the shifts in breeding systems associated with ecological conditions (e.g. isolated habitats) or polyploidy? iii) how many times has distyly evolved and been lost in these taxa and whether is there a phylogenetic signal for evolution of these traits?; iv) when, in a paleobotanical context, have breeding system shifts occurred?; v) which of the theoretical models for evolution and breakdown of distyly can be supported for these genera?

## Material and methods

### *Selected species and breeding system data*

We selected 46 species of *Psychotria* and *Palicourea* species for which breeding system information was known

from field and/or herbarium observations and effectively published. The studied species (names and authority in Tab. 1; whenever necessary, *Palicourea* was abbreviated to *P.* and *Psychotria* to *Psy.*) belong to five biogeographic regions: Neotropical, Panamanian, Sino-Japanese, Oriental and Hawaiian (Holt *et al.* 2012; Razafimandimbison *et al.* 2014). We used their up-to-date accepted names based on IPNI and World Flora Online ([www.ipni.org](http://www.ipni.org) and <http://www.wfo.org>) and provided synonyms due to recent combinations (e.g. Delprete & Kirkbride 2016). We focused on the type genera (*Palicourea* and *Psychotria*) since they include most of the accepted species, and to avoid the ongoing taxonomic reorganization within the sister genera in each tribe.

We classified the breeding system of the species based on Ganders (1979), considering homostyly when flowers presented no herkogamy and monomorphism when all flowers had a morphology like one of the distylous floral morphs. We also included habitat information for the species, whether they occurred in insular, isolated or disturbed areas, or continental and pristine continuous habitats. Both breeding systems and ecological information were retrieved from literature and direct field observations. We added chromosome number information whenever possible based on CCDB (<http://ccdb.tau.ac.il>), Correa *et al.* (2010), and Kiehn & Berger (2020), and compared the data with chromosome numbers and ploidy levels observed for the genera and the Rubiaceae as a whole (Naiki 2012; Kiehn & Berger 2020). We tested if there was a relationship between ploidy levels and breeding system by contingency analysis and chi-square independence test (Sokal & Rohlf 1994).

### *Molecular data and analyses*

We downloaded all DNA sequences available for species of the genera *Psychotria* and *Palicourea* in GenBank ([www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)) and maximized the number of species with the same sequences available. As a result, we focused on complete 45S ribosomal DNA (composed by 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 26S ribosomal RNA). DNA sequences were aligned and edited in Geneious version 11.0 (<http://www.geneious.com>, Kearse *et al.* 2012) using the MAFFT v. 7 algorithms (Katoh & Standley 2013). The nucleotides substitution model was estimated in Mega 7.0 (Kumar *et al.* 2016) and the best model was selected using the AIC values. The phylogenetic relationship of the species was estimated through Maximum Likelihood analysis based on the GTR model in Mega 7.0 (Kumar *et al.* 2016). Node supports were estimated by performing 1,000 bootstrap replications (Felsenstein 1985). Branch lengths and divergence times were estimated rooting and calibrating the tree with *Faramea multiflora* A. Rich. as an outgroup and the estimated age for fossil records of the genus *Faramea* Aubl. as the Oligocene (Graham 2009). The divergence times of species with shifts in their breeding system were estimated using the fossil-calibrated phylogenetic tree (using ~34 mya for *Faramea*).



**Table 1.** Studied species of *Palicourea* (*P.*) and *Psychotria* (*Psy.*). The accepted names used through the text is followed by the GeneBank reference; recent synonyms due to new combinations; breeding systems; habitat of breeding system studies, either insular, fragmented or continental and relatively pristine; references for breeding system information; and level of ploidy (chromosome number). Pin-mono = Pin-monomorphism, Thrum-mono = Thrum-monomorphism.

Accepted name (World Flora)	Genbank reference	Recent Synonyms	Breeding system	Habitat	Breeding system reference	Plody 2n
<i>Palicourea alpina</i> (Sw.) DC.	KJ804878.1		<b>Homostyly</b>	Island	Tanner 1982	
<i>P. calophlebia</i> Standl	AF149321.1		Distyly	Fragment	Taylor 1997	
<i>P. corymbifera</i> (Müll.Arg.) Standl.	AF149320.1		Distyly	Fragment	Santos 2016	
<i>P. crocea</i> (Sw.) Schult.	AF149322.1		Distyly	Fragment	Costa & Machado 2017	
<i>P. semirasa</i> Standl.	AF149324.1		Distyly	Fragment	Lau & Bosque 2003	
<i>P. guianensis</i> Aubl.	AF072010.1		<b>Pin-mono</b>	Continental	Taylor 1997	
<i>P. lasiorrhachis</i> Oerst.	AF072009.1		Distyly	Continental	Feinsinger & Busby 1987	44
<i>P. macrobotrys</i> (Ruiz & Pav.) Schult.	AF149335.1		<b>Homostyly</b>	Fragment	Coelho & Barbosa 2003	
<i>P. macrocalyx</i> Standl.	KC480539.1		Distyly	Fragment	Taylor 1989	
<i>P. montivaga</i> Standl.	KC480540.1		<b>Thrum-mono</b>	Fragment	Taylor 1989	
<i>P. padifolia</i> (Willd. ex Schult.) Taylor & Lorence	AF072008.1		Distyly	Fragment	Ree 1997	44
<i>P. petiolaris</i> Kunth	AF149337.1		Distyly	Continental	Sobrevilla <i>et al.</i> 1983	
<i>P. pittieri</i> Standl.	AF149338.1		Distyly	Island	Sakai & Wright 2008	
<i>P. rigida</i> Kunth	AF149342.1		Distyly	Continental	Machado <i>et al.</i> 2010	22
<i>Psychotria cuspidata</i> Bredem. ex Schult.	EF667969.1	<i>P. acuminata</i> (Benth.) Borhidi	Distyly	Fragment	Bawa & Beach 1983	
<i>Psy. brachiata</i> Sw.	AF072001.1	<i>P. brachiata</i> (Sw.) Borhidi	<b>Pin-mono</b>	Continental	Faivre & McDade 2001	
<i>Psy. gracilentia</i> Müll. Arg.	AF072004.1	<i>P. gracilentia</i> (Müll. Arg.) Delprete & Kirkbr.	<b>Pin-mono</b>	Island	Sakai & Wright 2008	44
<i>Psy. nuda</i> (Cham. & Schltld.) Wawra	AF072053.1		Distyly	Fragment	Silva & Vieira 2015	22
<i>Psychotria capitata</i> Ruiz & Pav.	AF072005.1	<i>P. violacea</i> (Aubl.) A. Rich	Distyly	Island	Sakai & Wright 2008	22/44
<i>Psy. carthagenensis</i> Jacq.	KC480533.1		<b>Pin-mono</b>	Fragment	Consolaro <i>et al.</i> 2011	44
<i>Psy. cephalophora</i> Merr.	KJ804900.1		Distyly	Island	Watanabe <i>et al.</i> 2015	
<i>Psy. chagrensis</i> Standl.	AF072051.1		Distyly	Island	Sakai & Wright 2008	
<i>Psy. deflexa</i> DC.	AF072006.1	<i>P. deflexa</i> (DC) Borhidi	Distyly	Continental	Sá <i>et al.</i> 2016	32
<i>Psy. elata</i> (Sw.) Hammel	AF072011.1	<i>P. elata</i> (Sw.) Borhidi	Distyly	Fragment	Silva & Vieira 2015	44
<i>Psy. grandiflora</i> H.Mann	AY350670.1		Distyly	Island	Sohmer 1978	
<i>Psy. grandis</i> Sw.	KJ804909.1		Distyly	Island	Sakai & Wright 2008	
<i>Psy. hathewayi</i> Fosberg	AY350664.1		Distyly	Island	Sohmer 1978	
<i>Psy. hexandra</i> H. Mann.	AF034907.1		Distyly	Island	Sohmer 1977	



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Table 1. Cont.

Accepted name (World Flora)	Genbank reference	Recent Synonyms	Breeding system	Habitat	Breeding system reference	Plody 2n
<i>Psy. hobbayi</i> Sohmer	AF034906.1		Distyly	Island	Sohmer 1977	88
<i>Psy. hoffmannseggiana</i> (Willd. ex Schult.) Müll.Arg.	EF667970.1	<i>P. hoffmanseggiana</i> (Willd. ex Schult.) Borhidi	<b>Pin-mono</b>	Island	Sakai & Wright 2008	22
<i>Psy. horizontalis</i> Sw.	AF072047.1		Distyly	Island	Sakai & Wright 2008	
<i>Psy. limonensis</i> K. Krause	AF072052.1		Distyly	Island	Sakai & Wright 2008	
<i>Psy. manillensis</i> Bartl. ex DC.	AF072025.1		<b>Monoecy</b>	Island	Watanabe <i>et al.</i> 2015	84
<i>Psy. mapourioides</i> DC.	AF072040.1		<b>Homostyly</b>	Fragment	Pers. obs.	40
<i>Psy. marginata</i> Sw.	AF072049.1		Distyly	Island	Sakai & Wright 2008	22/24
<i>Psy. micrantha</i> Kunth	AF072048.1		<b>Pin-mono</b>	Island	Sakai & Wright 2008	
<i>Psy. nervosa</i> Sw.	AF072046.1		Distyly	Continental	Hernández-Ramírez 2012	22
<i>Psy. cyanococca</i> Seem. ex Dombrain	AF071998.1	<i>P. cyanococca</i> (Dombrain) Borhidi	Distyly	Island	Sakai & Wright 2008	22
<i>Psy. poeppigiana</i> Müll.Arg.	AF071993.1	<i>P. tomentosa</i> (Aubl.) Borhidi	Distyly	Continental	Coelho & Barbosa 2004	44
<i>Psy. pubescens</i> Sw.	AF071997.1	<i>P. berteroa</i> (DC.) Borhidi	Distyly	Island	Sakai & Wright 2008	
<i>Psy. racemosa</i> Rich.	AF071995.1	<i>P. racemosa</i> (Aubl.) Borhidi	<b>Pin-mono</b>	Island	Sakai & Wright 2008	
<i>Psy. asiatica</i> L.	AF072035.1		<b>Dioecy</b>	Island	Watanabe <i>et al.</i> 2013	22/44
<i>Psy. serpens</i> L.	AF072036.1		Distyly	Island	Sugawara <i>et al.</i> 2014	22
<i>Psy. tenuifolia</i> Sw.	AF072050.1		<b>Pin-mono</b>	Island	Sakai & Wright 2008	
<i>Psy. trichophora</i> Mull.Arg.	AF149407.1	<i>P. trichophora</i> (Müll. Arg.) Delprete & Kirkb.	Distyly	Fragment	Sá <i>et al.</i> 2016	22
<i>Psy. viridis</i> Ruiz & Pav.	FJ208620.1		Distyly	Fragment	Pers. obs.	44
<i>Coussarea hydrangeifolia</i> (Benth.) Benth. & Hook. f. ex Müll. Arg.	EU145360.1		Distyly		Pereira 2007	22
<i>Faramea multiflora</i> A.Rich. ex DC.	EU145363.1		Distyly		Consolaro 2008	22
<i>Hedyotis acutangula</i> Champ. ex Benth.	HQ148754.1		Distyly		Wu <i>et al.</i> 2010	
<i>Oldenlandia pulcherrima</i> (Dunn) Chun.	JF976500.1		Distyly		Liu <i>et al.</i> 2012	18
<i>Mussaenda lancipetala</i> X.F.Deng & D.X.Zhang	KM005547.1		<b>Pin-Mono</b>		Duan <i>et al.</i> 2018	
<i>Mussaenda macrophyla</i> Wall.	KM005544.1		Distyly		Duan <i>et al.</i> 2018	22
<i>Mussaenda shikokiana</i> Makino	KC339498.1		<b>Dioecy</b>		Duan <i>et al.</i> 2018	
<i>Rudgea hostmanniana</i> Benth.	AF072014.1		Distyly		Zappi 2003	
<i>Rudgea stipulacea</i> (DC.) Steyerf.	JX155099.1		Distyly		Zappi 2003	
<i>Rudgea virburnoides</i> (Cham.) Benth.	KJ804983.1		Distyly		Zappi 2003	



Besides *Faramea multiflora*, we included other species as outgroups to allow a better representation and support to the ancestral state inference (see Tab. 1 for names and authority): a species of another Rubioideae tribe, Coussareae; three species in the sister Spermaceae Alliance; two less related species inside the Psychotriace Alliance itself; and three *Rudgea* Salisb., a sister genus to *Palicourea* and *Psychotria* inside the Psychotriace Alliance. The availability of DNA sequences and breeding system information was also considered for the selection of the outgroups.

We traced the ancestral states for the breeding system in species of *Psychotria* and *Palicourea* (and whenever possible to the outgroups) based on the consensus phylogenetic tree. Breeding system character state reconstruction was built-up using maximum likelihood in Mesquite 2.5 (Maddison & Maddison 2008). We also used trace characters over trees with maximum likelihood. Breeding system information was categorized as 0 – monomorphism, 1 – distyly, 2 homostyly, 3 – dioecy and 4 – monoecy. We used stochastic mapping character reconstruction using continuous Markov's chain model, which allows trait changes in all possible evolutionary pathways (Nielsen 2002). Independent evolution of the breeding systems of the studied species was calculated using Pagel's Lambda considering free homoplasy of characters, which calculates likelihoods using a speciation/extinction model reduced from the BiSSE model. This index ranges from zero, which means no phylogenetic signal in the trait, to one meaning strong phylogenetic signal in the trait. Low likelihood values (e.g. closer to zero) indicate independent trait evolution (Maddison *et al.* 2007).

## Results

### Taxa breeding system and distribution

Out of the 46 studied species (Tab. 1), 31 were truly distylous and 15 presented other breeding systems. We recorded ten species with monomorphism, mostly pin-monomorphism with stigmas above the anthers (except *Palicourea montivaga*, which is thrum-monomorphic), three homostylous species, one dioecious species and one monoecious species. Among the outgroups, most were typically distylous. However, *Mussaenda lancipetala* is thrum-monomorphic and *Mussaenda shikokiana* is dioecious. Dioecy is reported for *Coussarea*, although the studied species is truly distylous. Breeding system transitions were commonly associated with island populations and isolated forest fragments, 11 in 15 transition cases, although those habitats were also more common among the studied species (see Tab. 1).

The chromosome number data obtained was still inconclusive to define ploidy trends. Studied species were either diploid ( $2n = 2x = 22/24$ ), tetraploid ( $2n = 4x = 44$ ) or octoploid ( $2n = 8x = 88/84$ ) with a few aneuploidy cases. For the 21 species which we got chromosome number estimates

(Tab. 1), only six showed anomalous distyly and only one was diploid. Among the truly distylous, seven species were diploid and eight species polyploid. A chi-square test for the contingency analysis showed no significant dependence between breeding system and ploidy ( $p=0.098$ ). Limited data preclude finer analyses for chromosome data.

### Phylogenetic inference

The phylogenetic relationships between *Psychotria* and *Palicourea* species was inferred using 716bp following the GTR nucleotides substitution model (Fig. 1). We found two groups of species of the two genera which were clearly separated from *Rudgea* species (84 bootstrap) and from the other outgroups. The first group included exclusively Neotropical species of *Psychotria* (*Heteropsychotria*) and *Palicourea*, all in the tribe Palicoureeae (26 species). The second group was distinct from the first with a support of 49 bootstrap, and included species of heterogeneous origin (Fig. 1) but all from the tribe Psychotriace (20 species). Although some recent synonyms may indicate otherwise, the accepted names in The World Flora were all congruent with phylogenetic placement.

### Ancestral state inference and breakdown of distyly

The mating system transitions were mostly derived from distyly and were present across the species phylogeny, both in tribe Palicoureeae (eight transitions) and Psychotriace (six transitions). The divergence time analyses indicated tribe divergence ca. 25 mya (Fig. 1). Dioecy and monoecy appeared only in species of Psychotriace of the Sino-Japanese regions, and the divergence time analyses indicated they arose up to ca. 20 mya. in *Psy. manillensis*. Homostyly and monomorphism shifts were recorded in 12 species, mostly in the Neotropics, and occurred mostly in the Pliocene and Pleistocene (less than five mya). However, *Psy. racemosa*, *Psy. brachiata* (Palicoureeae) and *Psy. mapourioides* (Psychotriace) may have diverged earlier, up to ca. 20 mya. The 14 shifts in breeding system seemed to have evolved independently of the phylogenetic similarities between the studied species (Pagel's lambda = 0.21.)

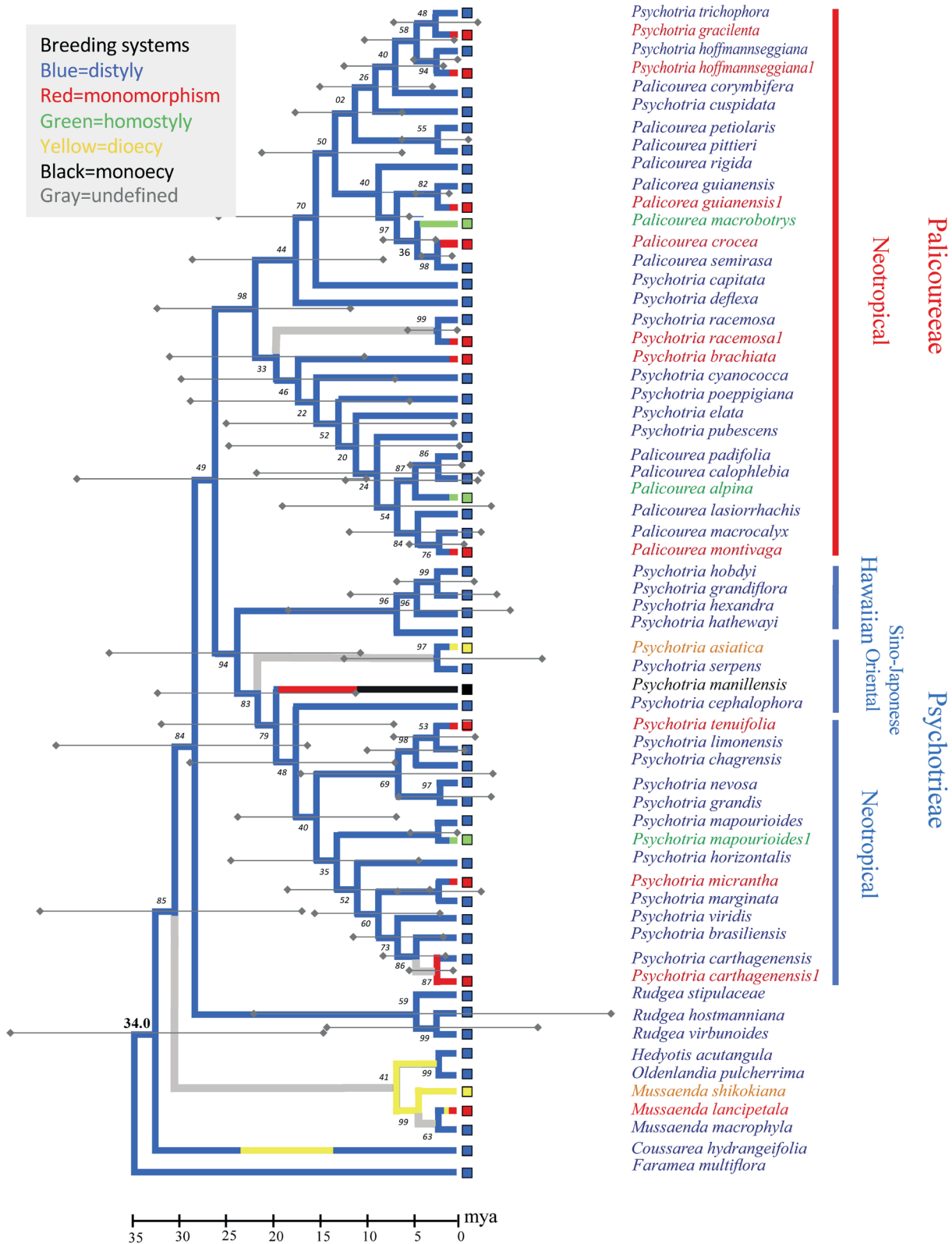
## Discussion

Our results showed evolutionary transitions in breeding systems in species of *Psychotria* and *Palicourea* as derived from distyly. Shifts in the breeding system and anomalous distyly appeared independently multiple times across the phylogenetic analyses, with different types of breeding systems and with at least one possible reversion to a distylous stage. Monoecy and dioecy appeared only in Asian taxa and were of Miocene origin, while homostyly and herkogamous monomorphism occurred mostly in Neotropical taxa and appeared mostly later in the ancestral state reconstruction. These trends are discussed in detail below.





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**Figure 1.** Phylogenetic consensus tree of *Psychotria* and *Palicourea* species (Rubiaceae) inferred by maximum likelihood of rRNA sequences. Numbers at branches indicate bootstrap values. Taxonomic classifications and Biogeographical regions followed Holt *et al.* (2012) and Razafimandimbison *et al.* (2014). Trace over tree breeding system information and estimated transitions are presented in different colors (gray branches indicated undefined ancestral breeding system). Tree was rooted and calibrated with *Fareamea* fossil record (~34 mya) to infer divergence times. Different colors in species names represent different breeding system strategies. Bars represent standard deviation of divergence times.



## Breeding systems

Most species analyzed here were truly distylous with different flower morphs in the studied populations. We did not finer analyses of distylous species for changes in mating systems or isoplethy, which may precede distyly breakdown (Sá *et al.* 2016), but most distylous species here are self-incompatible and distyly is functional. Despite our limited sample (possibly less than 10 % of the species of either group) we found in *Palicourea* and *Psychotria* species almost all the kinds of breeding transitions described in literature, including classical homostyly without herkogamy, pin- and thrum-monomorphism, monoecy and dioecy (Barrett 2019). But monomorphism was by far the most common kind of breeding deviation in the species.

The occurrence of monomorphism in species of *Psychotria* and *Palicourea* may be due to the variation in self-incompatibility expression in Rubiaceae (Bawa & Beach 1983). The weakening or breakdown of physiological incompatibility can lead to unbalanced population morph ratio (unisoplethy) and even to the establishment of monomorphic populations (Sá *et al.* 2016; Barrett *et al.* 1989). Among the species of our study, only *P. montivaga* showed short-styled, thrum-monomorphism (reverse herkogamy; Taylor 1989), all the other transitions were pin-monomorphic with approach herkogamy. The exposure of pin stigma and possibly incompatibility breakdown may explain why, most of the time, the long-styled morph is the one fixed in monomorphic populations and species (Arroyo *et al.* 2002; Sakai & Wright 2008; Barrett 2015; Balogh & Barrett 2016). It is hypothesized that the approach herkogamy in pin flowers has a better performance in founding populations since they are more likely to receive pollen grains than the stigma in thrum flowers (Baker *et al.* 2000). Moreover, in genetic models of distyly expression in other angiosperms, the long-styled morph genotype is commonly recessive (*ss*), and the short-styled morph is heterozygote (*Ss*) (Dulberger 1964; Barrett 2019). Thus, the allelic frequency (*s/S*) in distylous population under negative frequency-dependent selection is respectively 3:1, and the odds of losing the *S* allele is much greater than the odds of losing the *s* allele. If this model holds for Rubiaceae, this can be the reason why pin-monomorphic populations of *Psychotria* and *Palicourea* are more common than thrum-monomorphic populations.

The monomorphism of *Psy. hoffmansegianna*, *Psy. racemosa*, *Psy. brachiata*, *Psy. tenuifolia*, *Psy. micrantha* (Sakai & Wright 2008), *Palicourea guianensis* (Taylor 1997) and *P. montivaga* (Taylor 1989) were reported in island populations studies in Central America. In *Psy. carthagenensis* the monomorphism was also associated with populations somewhat isolated or disturbed, or in the edge of species distribution range (Consolaro *et al.* 2011, E. Rodrigues unpublished studies). All monomorphic populations of *Palicourea* and *Psychotria* studied so far are

self-fertile and are viewed as examples of the advantage of selfing and reproductive assurance as strategies in colonizing islands or marginal distribution habitats, as predict by Baker's law (Baker 1967; Pannel *et al.* 2015). Evolutionary studies on distyly showed that breeding system variation across populations (distyly and monomorphism with approach herkogamy) were associated with differences in pollinators morphology (short-tongued pollinivorous and long-tongued nectivorous), as in *Narcissus papyraceus* (Amaryllidaceae) (Pérez-Barrales & Arroyo 2010). In contrast, the monomorphism in populations of *Luculia pinceana* (Rubiaceae) seems to be linked to founder effect events and differences in the self-incompatibility of the floral morphs (Zhou *et al.* 2012). Founder effect may also be responsible for the fixation of monomorphism in species and populations of *Palicourea* and *Psychotria*. Among the studied species, there are records of truly distylous populations for *Psy. hoffmansegianna*, *Psy. racemosa* and *P. guianensis* in Brazilian forests (Sá *et al.* 2016, E. Rodrigues unpublished studies), while the same species are monomorphic in island habitats (Sakai & Wright 2008). Homostyly without herkogamy was reported in *P. macrobotrys*, *P. alpina* and *Psy. mapourioides*. In *P. macrobotrys* homostyly occurred in marginal habitats of the species distribution (Coelho & Barbosa 2003) and in *P. alpina* in an island population in Jamaica (Tanner 1982). In these species, homostyly seems to be fixed at the species level, since there are no records of distyly in either species elsewhere (Taylor 1997). In *Psy. mapourioides*, homostyly occurred in a population in the Brazilian Northeastern region (Parque Estadual Mata do Pau-Ferro, E. Rodrigues, pers. obs.) in a rain forest fragment isolated amid the dry Caatinga vegetation (Veloso *et al.* 1991). Contrastingly, in forests of the Cerrado region, the species appears to be truly distylous (Tangará da Serra, Mato Grosso; Parque Nacional de Brasília, Distrito Federal Brazil, E. Rodrigues, pers. obs.).

Classic homostyly has been considered a result of recombination in the distylous supergene, as in *Primula* (Conti *et al.* 2000; Mast *et al.* 2006), in *Turnera* (Barrett & Shore 1987) and in *Villarsia albiflora* (Menyanthaceae) (Ornduff 1988). Despite the lack of similar genetic studies with the distylous *Psychotria* and *Palicourea* species, homostyly is probably analogous to the accepted for other distylous plants groups (Barrett & Shore 2008). Recent data has shown that the structure of the purported supergene is more complex (Barrett 2019) and recombination may be rarer (Cocker *et al.* 2018). However, homostyly occurred independently at least in 45 species of *Primula* (Mast *et al.* 2006; Barrett 2019). Breakdown events and homostyly in *Psychotria* and *Palicourea* may represent similar events and offer a great model for the study of the evolution of the distyly expression and regulation.

Regardless of the genetic process which leads to distyly breakdown and homostyly, transitions will putatively depend on ecological pressures to be established in populations





(Richards 1998). Pollinator-mediated selection processes, as in *Exochaenium* Griseb. (Gentianaceae; Kissing & Barrett 2013), or founder effect, as in Plumbaginaceae (Costa *et al.* 2019) and *Amsinckia* (Boraginaceae; Schoen *et al.* 1997), may explain homostyly establishment in insular or isolated Rubiaceae populations. Thereby, ecological and biogeographic factors seem to influence the breeding system transitions for both *Psychotria* and *Palicourea* species, probably leading to a uniparental colonization or loss of one of the morphs when species colonize islands (Sakai & Wright 2008) or when populations are isolated by ecological factors such as disturbance, habitat fragmentation, or reduced pollination services (Consolaro *et al.* 2011; Zhou *et al.* 2012; Costa & Machado 2017). But other transitions, such as gender distinction, may be more complex and require a sequence of events.

Dioecy and monoecy were the breeding system in *Psy. asiatica* (*Psy. rubra*) and *Psy. manillensis*, respectively. The breeding system transition of both species occurred in the Japanese archipelago (Watanabe *et al.* 2013). Beach & Bawa (1980) predicted the evolution of dioecy from distyly by disruption of disassortative pollen flow between the distylous morphs under a context of shifts in pollinator fauna. Thomson & Barrett (1981) pointed out the importance of self-incompatibility ancestor in the evolution and selection of dioecy. However, for *Psy. asiatica* it is unknown if the species ancestors had self-incompatibility or whether there are distylous populations outside the Japanese island, which hinders the evaluation of possible pathways for the evolution of dioecy from distyly. The monoecious *Psy. manillensis* also occurs in a Japanese island habitat. Putatively the closest related species of *Psy. asiatica*, *Psy. manillensis* is polyploid, suggesting that chromosome doubling might be responsible for the origin of male and female flowers in this species (Watanabe & Sugawara 2015). However, our phylogenetic reconstruction (see below) did not support this inference since the species appeared apart and possibly required monomorphism as intermediate stages from a hermaphrodite ancestor (Beach & Bawa 1980). A complex pathway to dioecy would also require disruptive selection in male and female sex allocation (Barrett 2002), usually associated with some degree of male sterility and selection of unisexual flowers. Actually, recent studies indicate polygamous populations of *Psy. manillensis*, with different flower morphologies and breeding behaviors (Watanabe *et al.* 2020). Thus, intermediate stages and self-interference may have played important roles on the evolution of unisexual flowers, as previously proposed (Casper & Charnov 1982; Charlesworth 1989; Charlesworth & Morgan 1991). In insular habitats, like the Japanese Islands, these evolutionary transitions to gender isolation may have ensured cross-pollination and eliminated the risks of interference between sexual functions.

Heterostyly has been postulated as rare or absent in islands (Pailler *et al.* 1998) and the breakdown of distyly has been observed during species colonization in oceanic

islands (Barrett *et al.* 1989; Sakai & Wright, 2008; Barrett & Shore, 2008; Watanabe & Sugawara, 2015). However, this mating system transition seems to be species specific, since there are also truly distylous species in islands, such as *Psy. cephalophora* and *Psy. boninensis* (Hayata) Nakai and *Psy. serpens* in the Japanese archipelago (Sugawara *et al.* 2014; Watanabe *et al.* 2015; Watanabe *et al.* 2013), and *Psy. deflexa*, *Psy. chagrensis*, *Psy. marginata* and other *Psychotria* species in Barro Colorado Island, Panama (Sakai & Wright 2008). So, the breakdown of distyly and breeding transitions seems to not be more frequent in those habitats, at least in tribes Psychotrieae and Palicoureeae.

Different ploidy levels were present in typical and anomalous distylous species. No clear relationship among breeding systems and polyploidy was found. This relationship is not clear either for species of *Amsinckia* (Boraginaceae) (Schoen *et al.* 1997) and Turneraceae (Shore *et al.* 2006). There is also evidence for Rubiaceae species that polyploidization has no clear link with the breakdown of heterostyly (Naiki 2012). Although wider sampling may show otherwise, the breeding systems transitions of *Palicourea* and *Psychotria* do not seem to be related to chromosome number or polyploidy.

### Phylogenetic insights

The phylogenetic reconstruction attempted here was limited to the species of *Psychotria* and *Palicourea* with both rRNA sequences and breeding system information, so that it is limited in scope, should be used cautiously, and has no taxonomic intent. However, it resulted in a topology that broadly agrees with recent phylogenetic studies in the Rubiaceae (Razafimandimbison *et al.* 2008; 2014; 2017; Wikström *et al.* 2020). The clear exception was the position of *Rudgea*, placed in Palicoureeae Alliance in Razafimandimbison *et al.* (2014) and here as a sister group Palicoureeae and Psychotrieae, probably a result of our limited sampling. In any case, the phylogenetic framework for the studied group is an ongoing discussion and may result in further reorganization.

Despite being limited by available breeding system information and rRNA sequences, our sample included species from the two tribes in similar proportions and from different biogeographical regions worldwide; and breeding system transitions appeared in both groups and regions. The multiple and apparently independent shifts were similar to the observed by Sakai & Wright (2008) for Barro Colorado *Psychotria*. Due to this ample distribution, we expect the shifting events will be even more numerous and independent whenever a wider sample of species of the tribes are put together. Numerous breeding systems shifts and distyly breakdown events have been described for Rubiaceae (Ferrero *et al.* 2012) and distylous angiosperms as a whole (Barrett 2019), and seem to be an homoplasious trait (*e.g.* Zhong *et al.* 2019).



Distyly appeared as ancestral to the diversification of the genera *Psychotria* and *Palicourea* as previously proposed for the Psychotriaceae Alliance (Ferrero *et al.* 2012). The breeding system ancestral state reconstruction for *Psychotria* and *Palicourea* species did not corroborate the model of evolution of distyly proposed by Anderson (1973). As far as we could see, there was no protandry as ancestral state or as breeding system deviation in the species of our study. The results of the phylogenetic reconstruction did not agree with Charlesworth & Charlesworth (1979) predictions either. Homostyly was mostly derived from distyly and there was little evidence of homostyly as ancestral breeding system or reversion to distyly from homostyly. Our results corroborate Hamilton (1990) and Lloyd and Webb (1992a; b) predictions about derived floral morphology from distyly. As they both proposed, homostyly and monomorphism were basically derived from distyly. However, the breeding system phylogenetic reconstruction of our study does not allow inferences for the evolution of distyly using Lloyd and Webb (1992a) model. Distyly seems to have been already well established before the diversification of the Psychotriaceae Alliance (Ferrero *et al.* 2012). Further studies and addition of more species of *Psychotria* and *Palicourea*, or even at the family level, will be required to elucidate the evolution of the floral polymorphism, making clear what is the ancestral condition of distylous Rubiaceae.

The breeding system transitions in *Psychotria* and *Palicourea* seemed to have occurred in distinct geological times. The Sino-Japanese species, which distyly breakdown events involved gender specialization, had their estimated diversification before the Miocene (more than 10-13 mya), while the Neotropical species, which distyly breakdown events led to monomorphism, had their estimated diversification mostly less than five mya, in the Pliocene and in the Pleistocene (nine out of 12 species). In the Miocene, under a warmer climate, the Sino-Japanese flora presented extensive humid forests (Tanai 1972; Hsu 1983). In the Neotropics, dry and humid forests had multiple expansion cycles during the Pleistocene and even earlier than that (Ratter 1992; Oliveira-Filho and Ratter 1995; Werneck *et al.* 2012). The species with breakdowns in distyly evolved under climate shifts of the Miocene and Pleistocene that have been seen as trigger mechanisms for species diversification (Tanai 1972; Antonelli & Sanmartín 2011). In addition, in these new colonizing areas scenario, distyly breakdowns may have been strategies for reproductive assurance (Yuan *et al.* 2017).

The diverse breeding systems observed for the studied *Psychotria* and *Palicourea* derived from distyly and evolved independently across the species of this study. They were not associated with the tribe phylogenetic divisions either, evolving independently in *Psychotria* and *Palicourea* of different origins and possibly at different geological times. Despite limited sampling, transitions did not appear to be linked to ploidy changes either. Nevertheless, breeding system transitions in *Psychotria* and *Palicourea* were reported

in populations and species that occurred in islands or relatively isolated forest habitats, ecological scenarios where founder effect may have played an important role in establishment of species and populations with breeding systems derived from distyly. Our results indicate that, although distyly is widespread in *Psychotria* and *Palicourea* across their Pan-tropical distribution, these plants repeatedly evolved alternative breeding strategies, possibly to ensure reproductive success in their diversity of habitats.

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