Polygamous breeding system identified in the distylous genus *Psychotria*: *P. manillensis* in the Ryukyu archipelago, Japan

Kenta Watanabe¹, Akira Shimizu^{2,3,4}, and Takashi Sugawara⁵

¹ National Institute of Technology, Okinawa College, 905 Henoko, Nago, Okinawa 905-2192, Japan.

² Department of Biological Sciences, Graduate School of Science and Engineering, Tokyo Metropolitan University, 1-1 Minami-Ohsawa, Hachioji, Tokyo 192-0397, Japan.

³ Research Institute of Evolutionary Biology, Inc., Setagaya-ku, Tokyo, 158-0098 Japan

⁴ University Museum, the University of Tokyo, Bunkyo-ku, Tokyo, 113-0033 Japan

⁵ Department of Botany, National Museum of Nature and Science, 4-1-1, Amakubo, Tsukuba, 305-0005 Japan.

Corresponding Author:

Kenta Watanabe

National Institute of Technology, Okinawa College, 905 Henoko, Nago, Okinawa 905-2192, Japan

Email address: watake98n@gmail.com

23 **Abstract**

24 The evolutionary breakdown of distyly has been reported in many taxa, and mainly involves a
25 shift toward monomorphism or dioecism. However, a shift toward monoecism has not been
26 reported in distylous species. *Psychotria* (Rubiaceae), one of the world largest genera, consists
27 of distylous species and their derivatives. In our preliminary study, however, we identified some
28 monoecious individuals in a population of *Psychotria manillensis*. To understand the breeding
29 system and reproductive biology of *P. manillensis*, we investigated floral traits, open fruit set,
30 and flower visitors, and performed hand pollination and bagging experiments in five
31 populations of Okinawa and Iriomote islands, Ryukyu Islands, Japan. The populations of *P.*
32 *manillensis* were composed mainly of monoecious individuals (44%), followed by female
33 (38%), male (14%), and hermaphroditic (3%) individuals at the time of flower collection. Of the
34 collected flowers, 94% were functionally unisexual (male or female), whereas only 6% were
35 perfect (hermaphroditic). However, some individuals changed sex mainly towards increasing
36 femaleness during the flowering period. Moreover, 35% of the studied plants changed their
37 sexual expression over the years. *P. manillensis* showed self-compatibility and no agamospermy.
38 The fruit set under open pollination varied among populations and years (1.8–21.9%), but it was
39 significantly higher than that of auto-selfing (0.68–1.56%). Wasps and flies were the main
40 flower visitors and probably the main pollinators of the species. In conclusion, *P. manillensis*
41 was revealed to be polygamous, composed of monoecious, female, male, and hermaphroditic

42 individuals. This is the first report of the polygamous breeding system not only in the genus

43 *Psychotria*, but also in all heterostylous taxa.

44

45 **KEY WORDS:** breeding system; distyly; monoecism; polygamous; *Psychotria manillensis*;

46 Rubiaceae

47

48 **Introduction**

49 Heterostyly is a genetic polymorphism composed of two (distyly) or three (tristyly) distinct
 50 floral morphs (Darwin 1877; Barrett 1992; Barrett 2019). Distyly is composed of a “long-styled
 51 morph” with the stigma above the anthers and a “short-styled morph” with anthers above the
 52 stigma (Lloyd and Webb 1992; Naiki 2012). The anthers and stigmas are usually reciprocal
 53 between the two morphs, and the species usually have self- and intramorph incompatibilities
 54 (Ganders 1979; Barrett et al. 2000; Barrett et al. 2002). These features are thought to be a
 55 mechanism for promoting outbreeding, and distyly has been reported for more than 28
 56 flowering plant families (Naiki 2012). The genus *Psychotria* L. (Rubiaceae) is one of the largest
 57 distylous genera in the world with more than 1,800 species (Davis et al. 2001 2009; Nepokroeff
 58 et al. 2003; Razafimandimbison et al. 2014), and most of the species are either distylous or have
 59 breeding systems derived from distyly (Hamilton 1990; Watanabe and Sugawara 2015).

60 The evolutionary breakdown or modification of distyly has occurred in many taxa, and
 61 several different patterns have been documented (Ganders 1979; Naiki 2012). Of these,
 62 monomorphism (homostyly or pin/thrum monomorphy), which is the most frequently reported
 63 evolutionary transition (Vuilleumier 1967; Naiki and Nagamasu 2004; Nakamura et al. 2007;
 64 Naiki 2012), has been identified in *Psychotria* (Hamilton 1990; Sakai and Wright 2008;
 65 Consolaro et al. 2011). The evolution of dioecy from distyly has also been reported for several
 66 species (Watanabe and Sugawara 2015), including the Hawaiian *Psychotria* (Beach and Bawa

1980; Muenchow and Grebus 1989; Sakai et al. 1995; Wagner et al. 1999) and *Psychotria*
asiatica in Japan (Watanabe et al. 2014b). However, there is no report of the evolution of
monoecism in any distylous taxa.

Tracking the evolutionary transitions from distyly is important to understand the
diversity of the breeding systems in the flowering plants. The evolution of distyly is extremely
difficult compared to its breakdown because of the complicated genetic system (Barrett 2013),
and hence, diversification is usually initiated from the breakdown of distyly. In highly
diversified plant taxa, such as the genus *Psychotria*, the diversification of the breeding system is
one of the key factors that cause speciation and adaptive radiation.

There are five *Psychotria* species in Japanese subtropical islands: *P. homalosperma*,
(Watanabe et al. 2014a; Watanabe et al. 2018), *P. boninensis* (Kondo et al. 2007; Sugawara et al.
2014), and *P. serpens* (Sugawara et al. 2013, 2016) that are both morphologically and
functionally distylous; *P. asiatica* (Watanabe et al. 2014b; this species in Japan, Taiwan and
Vietnam had been recognized as *P. rubra*, but now it is regarded as synonym of *P. asiatica*) that
is functionally dioecious with distylous morphology; and *P. manillensis* that has an unknown
breeding system. Our preliminary observations revealed that several individuals of *P.*
manillensis have both male and female flowers in a single inflorescence, and consequently, this
species may be monoecious. Although most individuals of *P. manillensis* have male- or female-

85 like flowers, some have perfect flowers. Additionally, the sexual forms are often variable among
86 the individuals. Since the sexual forms of each flower and/or individual in *P. manillensis* are
87 irregular and complex, more research is required to clarify the breeding system and reproductive
88 mechanism.

89 The objectives of this study were to investigate the breeding system of *P. manillensis*
90 and its reproductive mechanism in natural populations of the Ryukyu Islands, Japan. To reveal
91 the breeding system of *P. manillensis* and the possible occurrence of monoecy in a species likely
92 derived from distyly, we investigated the traits of flower organs and classified them into several
93 categories. We also performed hand-pollination experiments to test the function of each stigma
94 type. Based on the collected data, we classified the flowers and individuals into different flower
95 types and plant genders. We also studied the flowering phenology and gender changes during
96 the flowering period. Moreover, we checked the sex changes over the years in 2012 and 2013.
97 Self-pollination and bagging experiments were performed to test for self-compatibility and
98 possibility of auto-selfing. Finally, fruit set under open pollination was measured and flower
99 visitors were observed to understand the pollination biology of the species.

100

101 **Materials & Methods**

102 *Study plants and sites*—*P. manillensis* (Bartl. ex DC.) is a woody shrub, 2–3 m in height,
103 commonly found in limestone evergreen forests with alkali soil and widely distributed from the

104 Ryukyu Islands to the Lanyu and Ludao Islands of Taiwan and the Philippines (Yamazaki 1993).
 105 Inflorescences are terminal and each possesses 20–150 flowers (Fig. 1). Corollas are white to
 106 white green and have a short funnel shape, ca. 2–3 mm in length, with white hairs inside.
 107 Flowers bloom for a single day; they open in the morning and fade in the evening. Normally,
 108 one to five flowers in a single inflorescence bloom each day, and one inflorescence blooms for
 109 several weeks. The flowering season is from June to August, and fruits mature from November
 110 to January on Okinawa Island.

111 In the present study, we examined three populations located on Okinawa Island: the
 112 Katsuu population at Mt. Katsuu-dake (26.63°N, 127.94°E, 310 m above sea level [a.s.l.]), the
 113 Oppa population at Mt. Oppa-dake (26.67°N, 127°96'E, 250 m a.s.l.) and the Sueyoshi
 114 population at Sueyoshi Park (26.23°N, 127.72°E, 65 m a.s.l.) and two populations located on
 115 Iriomote Island: the Sonai population at the Sonai beach forest (24.37°N, 123.75°E, 5 m a.s.l.)
 116 and the Uehara population at the Uehara forest (24.39°N, 123.81°E, 40 m a.s.l.). All field
 117 studies were performed in 2011–2013.

118

119 ***Classification of stigmas and anthers of flowers***—We collected 294 flowers from 20 individuals
 120 in the Sueyoshi population in June–August 2011. We observed the sexual organs of all the
 121 collected flowers under a light microscope (SMZ800; Nikon, Tokyo, Japan) at a magnification

122 of 10–63× and the unfixed stigmas under a scanning electron microscope (SEM, S-3000N;
123 Hitachi, Tokyo, Japan) to examine their surface structure.

124 Based on the degree of development of the stigmatic papillae, we classified the stigmas
125 of *P. manillensis* into three types: I, stigmas with well-developed stigmatic papillae; II, stigmas
126 with moderately developed stigmatic papillae; and III, stigmas with poorly developed stigmatic
127 papillae (Fig. 2). We also classified the anthers of *P. manillensis* into three types based on the
128 amount of pollen: a, anthers with pollen sacs full of pollen grains; b, anthers with small pollen
129 sacs with half or less of the total capacity of pollen grains; and c, anthers with no pollen sac nor
130 grains (Fig. 3). Thus, there were nine possible combinations of stigma and anther types.

131

132 **Function of stigmas**—To evaluate the function of each stigma type, we cross-pollinated 103
133 flowers with type-I stigma (N: Number of individuals = 21), 111 flowers with type-II stigma (N
134 = 20), and 96 flowers with type-III stigma (N = 21) at the Oppa and Katsuu populations
135 transferring pollen from other individuals. Because the styles of some flowers were hidden
136 within the corolla tubes, we partially cut the floral tube for pollination. Approximately 50 d after
137 pollination, we counted the number of immature fruits to evaluate the fruit set percentage.

138 To determine the ability of pollen tubes to access the styles, 22 flowers with type-I
139 stigma (N = 10), 17 flowers with type-II stigma (N = 10), and 18 flowers with type-III stigma

(N = 10) were cross-pollinated in the laboratory. After 24 h, the pollen tube growth was observed as described by Kearns and Inouye (1993). Briefly, the pollinated styles were fixed in 5: 5: 90 formalin: glacial acetic acid: 70% ethanol and then, transferred to 70% ethanol for storage. The fixed styles were softened in 8 N NaOH at 4°C for 24 h, washed with water, transferred to 0.005% aniline blue in Na₂HPO₄ (pH 11), and stored at 4°C for at least 24 h. The styles were mounted on a slide in glycerol beneath a cover glass and gently pressed to spread the tissue. Pollen grains on the stigmas and pollen tubes in the styles were observed under a fluorescence microscope (Axio Imager Z2; Zeiss, Oberkochen, Germany).

148

Flower collection and counts of flowers by type—We collected 50–100 flowers from 20 individuals from each of the five populations in 2012 and from the three populations on Okinawa Island in 2013 and classified them into the nine flower types as defined above. Then, we classified the individuals by gender based on the flower sexual type(s) at the time of collection: female individuals (only with female flowers), male individuals (only with male flowers), monoecious individuals (with both male and female flowers and often with some perfect flowers), and hermaphroditic individuals (only with perfect flowers). To calculate the relative femaleness (G) of each plant, we simply used flower numbers of each types. Although the flower numbers of female, male and perfect flowers cannot represent the

158 “functional femaleness” of the individuals (Lloyd 1980), they can be the indicator of the

159 “relative femaleness”.

160 We used the following formula to calculate the relative femaleness (G):

161 $G = (F + P/2) / (F + M + P)$, where F, P and M are the number of female, perfect and male

162 flowers per plant, respectively. Since the perfect flowers have both female and male function,

163 we assumed the perfect flower has the half of the femaleness of the female flower. We used the

164 number of collected flowers for this calculation and calculated E per population considering 50

165 flowers per individual.

166

167 ***Measurements of floral traits***—To understand the relationship between morphological traits and

168 functional sexual mode, we measured the anther and stigma height of 20 flowers from six

169 individuals (120 flowers in total) of different gender in the Sueyoshi population.

170

171 ***Self-compatibility and agamospermy test***—To test self-compatibility, we self-pollinated 136

172 flowers (34 Ic, 63 IIc, and 31 IIIa) from 31 individuals in the Oppa population. Inflorescences

173 were bagged 1 d before pollination, and open flowers in bags were pollinated with pollen grains

174 from other flowers in a bagged inflorescence of the same plant. Then, the self-pollinated flowers

175 were bagged again, and the fruit set was observed approximately 30 d after pollination. We

176 performed Fisher’s exact test using R 3.3.2 (R Core Team, 2016) to test the differences between

177 self- and cross-pollination. To test agamospermy (apomixis), we emasculated 10 flowers from
178 nine individuals (90 flowers in total) before anthesis and observed the fruit set approximately 50
179 d after the treatment.

180

181 ***Fruit set under open pollination and bagging experiments***—Fruit set under open pollination
182 was surveyed for three to five inflorescences of 9–31 individuals (average of 19.8 individuals
183 per site per year) from the three populations on Okinawa Island in 2011 and all the five
184 populations on Okinawa and Iriomote Islands in 2012–2013. We counted the number of flower
185 buds in inflorescences and of immature fruits on the marked inflorescences approximately 50 d
186 after the flowering peak.

187 A bagging experiment was carried out to test for spontaneous self-pollination/fertilization in
188 2012. One inflorescence each of 10, 11, and 18 individuals from the Sueyoshi, Katsuu, and
189 Oppa populations, respectively, was bagged using a mesh bag before blooming, and the fruit set
190 ratio (proportion of flowers that produced fruit) was calculated after all flowers faded.

191 We analyzed the effect of population and year on fruit set under open pollination using
192 generalized linear mixed models (GLMM; binomial error distribution with log-link function)
193 with the “lme4” package of R 3.3.2. The models included the number of fruit as the response
194 variable, year and population as the explanatory variables and individual plant as a random

195 factor. We compared five models with different combinations of the explanatory variables:
196 model 1 (population), model 2 (year), model 3 (population and year), model 4 (population, year
197 and their interaction), model 0 (null). The model fit was evaluated with the Akaike information
198 criterion (AIC), and the model with the smallest AIC was regarded as the best fit for our data.

199 We also analyzed the effect of bagging compared with open pollination on fruit set using the
200 GLMM (binomial error distribution with log-link function) and performed the likelihood ratio
201 test for evaluating the statistical significance of the effect using R. In this analysis, models
202 included the number of fruit as the response variable, pollination treatment (bagging or open) as
203 the explanatory variable, and individual plant as a random factor. We compared the models with
204 (model 1) and without (model 0) the explanatory variable. We also performed Pearson's
205 correlation test to evaluate the association between femaleness (G) and fruit set under open
206 pollination in R 3.3.2.

207

208 ***Flower phenology***—We marked 13 inflorescences on 10 individuals in the Oppa population (130
209 inflorescences in total) and recorded the number and type of open flowers every 2 d from June
210 26 to August 3, 2012. We divided the flowering period into four spans and calculated the
211 relative femaleness (G) as described above for each span and plant.

212

213 ***Gender shift over the years***—We tagged 21, 18, 19 plants from Katsuu, Oppa and Sueyoshi
 214 populations on Okinawa Island respectively for multiple year tracking of the relative femaleness
 215 (G) and the gender of each individual.

216

217 ***Flower visitors***—Flower visitors were observed in all the five populations over 8 h (between
 218 0700 and 1800), and some individuals were collected for identification. The number of visits to
 219 a single inflorescence during the 8-h observation period was classified into three categories:
 220 +++, more than 8 times; ++, 3–8 times; and +, 1–2 times. Observations were performed on July
 221 17 and 18, 2011 and on July 14 and 20, 2012 in the Katsuu population; from July 6 to
 222 September 18, 2011 and on July 14, 2012 in the Oppa population; on July 9 and 27, 2011, on
 223 June 19 and July 1, 2012 in the Sueyoshi population; and on June 2, 3, 15, 16, and 17, 2011 and
 224 on June 13, 2013 in the Sonai and Uehara populations.

225

226 **Results**

227 ***Function of stigmas and anthers by type***—Of the nine possible combinations of stigma and
 228 anther types (Ia, Ib, Ic, IIa, IIb, IIc, IIIa, IIIb, and IIIc), we hypothesized that the type-III stigma
 229 and type-c anther were functionally sterile. Hence, there were four different sexual flower types:
 230 perfect flower (Ia, Ib, IIa, and IIb), male or staminate flower (IIIa and IIIb), female or pistillate
 231 flower (Ic and IIc), and sterile flower (IIIc). After cross-pollination, pollen tube growth was

232 observed in 100% of type-I stigmas and 82% of type-II stigmas, and fruit set was observed in
233 more than 60% of flowers with type-I and type-II stigmas (Table 1). No pollen tube growth or
234 fruit set was observed in flowers with type-III stigma. These results indicated that the stigmas of
235 type-I and type-II, but not type-III showed a female function. These results confirmed our
236 hypothesis regarding the distribution of the four sexual flower types.

237 All flowers had anthers and a stigma, but more than 94% of the flowers differentiated to
238 either male (IIIa and IIIb, 39%) or female (Ic and IIc, 55%). Only 5.9% of the flowers were
239 perfect (Ib, IIa, and IIb) and only 0.3% were sterile (IIc); no Ia type flowers were observed
240 (Fig. 4; Table S1, see Supplemental information of this article). The ratio of type-I and type-II
241 stigmas was 92:8 for the female flowers and 8:92 for the perfect flowers. The proportion of
242 flower types was almost consistent in all the five populations, although the number of perfect
243 flowers was relatively low in the Uehara population.

244 Monoecious individuals were the most common (44.3%) in the three populations,
245 followed by female and male individuals (38.1% and 14.4%, respectively; Fig. 5B), as well as
246 hermaphroditic individuals (3.1%). Female individuals usually consisted of only Ic (female)
247 flowers that had a high stigma with low anthers (Fig. 6F), whereas male individuals consisted of
248 IIIa (male) flowers that had a low stigma and high anthers (Fig. 6A). Of the monoecious
249 individuals, most of the flowers were Ic (female) and IIIa (male), but those types were not

250 distinct when mixed with perfect flowers (Fig. 6B–E).

251

252 ***Self-pollination and agamospermy test***—The fruit set after self-pollination was significantly

253 lower (36.2%) than that after cross-pollination (65.0%; $P < 0.001$, Fisher’s exact test). Of 18

254 studied individuals, 13 showed self-compatibility. Additionally, none of the emasculated flowers

255 set any fruit, revealing that agamospermy did not occur.

256

257 ***Fruit set under open pollination and bagging experiments***—The fruit set under open pollination

258 varied among populations and years (1.8–21.9%; Fig. 7; Table S2), and the full model (model 4)

259 showed the smallest AIC (binomial GLMM; Table S3). Some male individuals (only male

260 flowers at the time of flower collection) also produced fruit, although femaleness (G) was

261 significantly correlated with fruit set in all populations ($P < 0.05$, Pearson’s correlation test; Fig.

262 S1), except for the Katsuu population ($P = 0.35$). Some flowers set fruit after bagging

263 experiments, although the fruit set was significantly lower than that under open pollination ($P <$

264 0.0001, the likelihood ratio test after binomial GLMM).

265

266 ***Flowering phenology and flower types within inflorescence through flowering period***—The

267 flowering period of 10 individuals observed in the Oppa population was approximately 42 d

268 (Fig. 8A). Femaleness (G) increased in seven individuals and decreased only in one plant during

the flowering period, whereas two individuals produced only female flowers (Fig. 8B). Of 127 inflorescences, 44 (35%) had only female flowers, 44 (35%) had male, female, and perfect flowers, 23 (18%) had male and female flowers, 14 (11%) had male and perfect flowers, and two (1.6%) had only male flowers (Fig. S2).

Gender shift over the years—of the 58 checked plants, relative femaleness increased in 24 plants (41%) and decreased in three plants (5%, Fig. 9A). Although, majority of female and monoecious plants kept the same gender in 2012-2013 season, 20 plants (35%) changed their sex (Fig. 9B). Five female plants changed to monoecious, six monoecious plants to female, one hermaphroditic plants to monoecious, seven male plants to monoecious, and one male plant to female.

Flower visitors—A total of 39 insect species of 20 families belonging to six orders visited the flowers of *P. manillensis* in the five populations (Table 2, Fig. 10). Of these insects, wasps of the family Vespidae (*Vespa* and *Polistes*) and flies of the families Calliphoridae, Syrphidae, and others were the most frequent visitors. Wasps visited the flowers, sucked nectar, and left quickly for other flowers, whereas flies stayed relatively longer after licking the nectar. Bees (*Apis mellifera*, *Amegilla* spp., Apidae and *Lasioglossum*, Halictidae) also visited the flowers, but

287 much less frequently than wasps and flies. Butterflies (*Papilio*, *Lampides*, and *Athyma perius*)
 288 and moths (Crambidae) also visited flowers on Okinawa Island. Hawkmoths (*Macroglossum*),
 289 beetles, stink bugs, and crickets were occasional visitors. Only syrphid flies (*Allobaccha*
 290 *nubilipennis* and *Sphaerophoria* sp.) fed on pollen, whereas all others fed on nectar.

291

292 Discussion

293 This study aimed to investigate the breeding and floral biology of *P. manillensis* in the natural
 294 populations of the Ryukyu Islands. The results showed that *P. manillensis* was polygamous,
 295 including monoecious, female, male, and hermaphroditic individuals, self-compatible, and
 296 flowers were visited by wasps and flies. To our knowledge, this is the first report of polygamous
 297 plants not only in the genus *Psychotria* but also in all heterostylous taxa worldwide.

298 Individuals observed in the five populations were monoecious plants with male, female,
 299 and often some perfect flowers (44%), female plants with only female flowers (38%), and male
 300 plants with only male flowers (14.4%), whereas hermaphroditic plants with only perfect flowers
 301 (3.1%) were found in three populations (Fig. 5B). Based on our data, the breeding system of *P.*
 302 *manillensis* could be characterized as “polygamous” (Darwin 1877; Sakai and Weller 1999),
 303 since no other better terms can be applied to describe the co-existence of monoecious, female,
 304 male, and hermaphroditic individuals in a single population (Sakai and Weller 1999).

305 In the present study, most of the flowers (94%) were unisexual (either male or female),
 306 and 5% were bisexual (hermaphroditic). Sexually intermediate anthers with a small number of
 307 pollen grains (type-b anther) and sexually intermediate stigmas with half-way developed
 308 stigmatic papillae (type-II stigma) might represent incomplete sexual differentiation, resulting in
 309 either perfect or sterile flowers. Some male individuals (as characterized at the time of flower
 310 collection) set fruits, showing that they might change gender during the flowering period. These
 311 results were supported by the increase in the relative femaleness during the flowering period in
 312 the Oppa population in 2012 (Fig. 8B). However, the increase in the relative femaleness with
 313 time could not be considered universal across the populations, since gender changes were not
 314 studied in all populations due to time limitation. Similarly, we observed the increase of the
 315 relative femaleness in 24 plants (41%, Fig. 9A), and sex changes in 20 individuals (35%, Fig.
 316 9B) over 2012-2013. These results indicate that this species has plasticity in sexual expression
 317 along with genetically-based differences.

318 Self-pollination experiments showed that *P. manillensis* is self-compatible, unlike other
 319 distylous species of the same genus, whereas emasculation experiments indicated that no
 320 agamospermy occurs in *P. manillensis*. Overall, at least self- or auto-self-pollination is required
 321 for fruit set. The fruit set after bagging experiments indicated that auto-self-pollination occurred
 322 without any pollinators; however, the fruit set was significantly lower than that under open

323 pollination, revealing that pollinators probably enhance the reproductive success of *P.*
324 *manillensis*. The main flower visitors were wasps (family Vespidae) and flies (family
325 Calliphoridae and others) in all the five populations (Table 2), and usually pollen grains were
326 attached to their heads. Although both wasps and flies licked the nectar, wasps moved more
327 actively among flowers, probably having a higher contribution to the pollination of *P.*
328 *manillensis* than flies. Although bees (family Apidae and Halictidae) were observed less
329 frequently than wasps and flies, they might also contribute to the reproductive success of the
330 species, because they touched both the stigmas and anthers (usually pollen attached to their
331 heads or bodies) and moved quickly from one flower to another. Butterflies and moths
332 (Lepidoptera) were infrequent visitors, and only occasionally their proboscises touched the
333 anthers or stigmas. Hence, wasps and flies were probably the most important pollinators of *P.*
334 *manillensis* in the Ryukyu Islands, similarly as occurs in the closely related species *P. asiatica*
335 (Watanabe et al. 2014b). The flowers of *P. manillensis* and *P. asiatica* are inconspicuous in the
336 dark forest understory, but the shallow and small flower forms allow insects with short mouth
337 parts to access the nectar secreted at the bottom of the flowers.

338 Distyly is the ancestral breeding system in the genus *Psychotria* (Hamilton 1989;
339 Watanabe and Sugawara 2015). Both short-styled (thrum) and long-styled (pin) monomorphy
340 derived from distyly have been reported for many *Psychotria* species in the neotropics

341 (Hamilton 1990; Sakai and Wright 2008; Consolaro et al. 2011). Dioecism probably derived
342 from distyly was reported in Hawaiian *Psychotria* (Sakai et al. 1995) and *P. asiatica* in Japan
343 (Watanabe et al. 2014b). This is the first report of partial monoecism in the genus *Psychotria*.

344 The adaptive consequences of the polygamous breeding system in *P. manillensis* remain
345 unclear. The imperfect shapes and functions of the reproductive organs indicate the unstableness
346 and imperfect adaptation of the breeding system. Sex changes in the flowering season and over
347 the years indicate the plasticity of the sexual expression. The polygamous system in *P.*
348 *manillensis* may be transitional and shift to different breeding systems. Further studies on the
349 polygamous system of *P. manillensis* may provide new insights into the diversification of the
350 breeding systems derived from distyly.

351 The phylogenetic background is important for identifying the origin of polygamous
352 breeding system in *P. manillensis*. DNA sequencing showed that *P. manillensis* is most closely
353 related to *P. asiatica*, which occurs in the Ryukyu Islands, Taiwan, South East Asia and China
354 (this species in Japan, Taiwan and Vietnam was known as *P. rubra*, but currently it is regarded
355 as synonym of *P. asiatica*). Distribution areas of these two species are overlapped only in the
356 Ryukyu Islands, and the detailed phylogenetic relationship of the two species remains unclear
357 due to lack of data from the Philippines (Watanabe et al. unpublished data). *P. manillensis* and *P.*
358 *asiatica* share many similar morphological traits such as a cymose inflorescence, cupuliform

359 corolla, elliptic-oblong leaves, caducous and triangular to broadly triangular stipules, and
 360 shrubby growth forms (Chao 1978; Yamazaki 1993; Tao and Taylor 2011). However, *P. asiatica*
 361 is functionally dioecious with distylous morphology (Watanabe et al. 2014b) and tetraploid ($2n$
 362 = 42) in the Ryukyu Islands, whereas *P. manillensis* is polygamous and octaploid ($2n = 84$;
 363 Nakamura et al. 2003). F_1 interspecific hybrids between the two species (hexaploid, $2n = 63$)
 364 have been also reported in the Ryukyu Islands (Nakamura et al. 2003).

365 It is possible that *P. manillensis* derived from an autopolyploid *P. asiatica* or an
 366 allopolyploid *P. asiatica* and another species (polyploid hybrid). It might be easier to explain the
 367 evolution of the polygamous system with partial monoecism in *P. manillensis* from dioecism
 368 through polyploidization than directly from distyly. Polyploidization and the evolution of
 369 monoecism from dioecism have also been reported to co-occur in *Mercurialis annua* (Pannell et
 370 al. 2004). The evolution of the polygamous breeding system from distyly is theoretically hard to
 371 be explained, because the sex expression is determined by diallelic genetic systems (Weller
 372 2009; Li et al. 2016). In *M. annua*, the occurrence of male and female flowers within a single
 373 plant could be explained by the heterogeneous expression of genes responsible for the sexual
 374 determination of flowers that doubled through polyploidization (Russell and Pannell 2015).
 375 Theoretically, a similar mechanism may be also operated in *P. manillensis*. Previous studies
 376 have explained the breakdown of distyly into homostyly or monomorphy by the recombination

377 and mutation of the distylous genes (Charlesworth and Charlesworth 1979; Ganders 1979;
378 Barrett and Shore 2008) and the evolution of dioecism from distyly by the plant-pollinator
379 interaction (Beach and Bawa 1980; Naiki and Kato 1999; Watanabe et al. 2014b). However,
380 these hypotheses failed to explain the different sex expressions within a single plant. It is also
381 unlikely that the pollinator shift drove the evolution of monoecism in *P. manillensis* from the
382 dioecious congener *P. asiatica*, because these two species have similar pollinator fauna (flies
383 and wasps, Watanabe et al. 2014b) and flower shapes.

384 Overall, this is the first report revealing the polygamous breeding system in the genus
385 *Psychotria* as well as in all heterostylous taxa worldwide. Understanding the evolution of the
386 breeding system in *P. manillensis* might help to better understand the diversification of the
387 breeding systems derived from distyly. Polyploidization could be related to the evolution of the
388 polygamous breeding system, although detailed information is still limited. Further research is
389 required to understand the evolutionary pathway, adaptive consequences, and origin of the
390 polygamous breeding system. Future studies should focus on the phylogenetic background and
391 genetic mechanisms for sex determination in *P. manillensis* as well as its relationship with *P.*
392 *asiatica*.

393

394 **Acknowledgements**

395 We thank Dr. S. Shinonaga and Mr. N. Kikuchi for their assistance in insect identification; Dr.

396 A. Iguchi for his advice on statistical analysis; Drs. M. Yokota, T. Denda, K. Nakamura, T.
397 Takaso, and T. Konjo for providing useful information. We are also grateful to Okinawa
398 Prefecture for allowing the collection of plant samples. This study was supported by the JSPS
399 KAKENHI (Grant number JP26840130 and 18K14782 provided to KW), and partly by the
400 Collaborative Research of Tropical Biosphere Research Center, University of the Ryukyu
401 provided to TS.

402

403 **References**

404 Barrett, S. C. H. 1992. Heterostylous genetic polymorphisms: model systems for evolutionary
405 analysis. *In* S. C. H. Barrett [ed.], *Evolution and function of heterostyly*, 1–29. Springer.
406 Berlin, Germany.

407 Barrett, S. C. H. 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* 3: 274–
408 284.

409 Barrett, S. C. H. 2013. The evolution of plant reproductive systems: how often are transitions
410 irreversible? *Proceedings. Biological Sciences/The Royal Society* 280: 20130913.

411 Barrett, S. C. H. 2019. ‘A most complex marriage arrangement’: recent advances on heterostyly
412 and unresolved questions. *New Phytologist*, 224(3), 1051–1067.

413 Barrett, S. C. H., and J. S. Shore. 2008. New insights on heterostyly: comparative biology,
414 ecology and genetics. *In* V. E. Franklin-Tong [ed.], *Self-incompatibility in flowering*

- 415 plants, 3–32. Springer, Berlin, Germany.
- 416 Barrett, S. C. H., L. Jesson, and A. Baker. 2000. The evolution and function of stylar
- 417 polymorphisms in flowering plants. *Annals of Botany* 85: 253–265.
- 418 Beach, J. H., and K. S. S. Bawa. 1980. Role of pollinators in the evolution of dioecy from
- 419 distyly. *Evolution* 34: 1138–1142.
- 420 Chao, J. M. 1998. *Psychotria*. L. In Editorial Committee of the Flora of Taiwan [ed.], Flora of
- 421 Taiwan vol. 4, 326–329. Epoch, Taipei, Taiwan.
- 422 Charlesworth, B., and D. Charlesworth. 1979. The maintenance and breakdown of distyly. *The*
- 423 *American Naturalist* 114: 499–513.
- 424 Consolaro, H., S. C. C. Silva, and P. E. Oliveira. 2011. Breakdown of distyly and pin-
- 425 monomorphism in *Psychotria carthagenensis* Jacq. (Rubiaceae). *Plant Species Biology*
- 426 26: 24–32.
- 427 Darwin, C. 1877. The different forms of flowers on plants of the same species. John Murray,
- 428 London, UK.
- 429 Davis, A. P., D. Bridson, C. Jarvis, and R. Govaerts. 2001. The typification and
- 430 characterization of the genus *Psychotria* L. (Rubiaceae). *Botanical Journal of the*
- 431 *Linnean Society* 135: 35–42.
- 432 Davis, A. P., R. Govaerts, D. M. Bridson, M. Ruhsam, J. Moat, and N. A. Brummitt. 2009. A

433 global assessment of distribution, diversity, endemism, and taxonomic effort in the

434 Rubiaceae 1. *Annals of the Missouri Botanical Garden* 96: 68–78.

435 Ganders, F. R. 1979. The biology of heterostyly. *New Zealand Journal of Botany* 17: 607–635.

436 Hamilton, C. 1990. Variations on a distylous theme in Mesoamerican *Psychotria* subgenus

437 *Psychotria* (Rubiaceae). *Memoirs of the New York Botanical Gardens* 55: 62–75.

438 Hamilton, C. W. 1989. A revision of mesoamerican *Psychotria* subgenus *Psychotria*

439 (Rubiaceae), part I: introduction and species 1–16. *Annals of the Missouri Botanical*

440 *Garden* 76: 67–111.

441 Kearns, C. A., and D. W. Inouye. 1993. Techniques for pollination biologists. University Press

442 of Colorado, USA.

443 Kondo, Y., M. Nishide, K. Watanabe, and T. Sugawara. 2007. Floral dimorphism in *Psychotria*

444 *boninensis* Nakai (Rubiaceae) endemic to the Bonin (Ogasawara) Islands. *The Journal*

445 *of Japanese Botany* 82: 251–258.

446 Li, J., J. M. Cocker, J. Wright, M. A. Webster, M. McMullan, S. Dyer, et al. 2016. Genetic

447 architecture and evolution of the S locus supergene in *Primula vulgaris*. *Nature Plants*

448 2: 16188.

449 Lloyd, D. G. 1980. Sexual strategies in plants III. A quantitative method for describing the

450 gender of plants. *New Zealand Journal of Botany*, 18: 103–108.

- 451 Lloyd, D., and C. Webb 1992. The selection of heterostyly. *In* S. C. H. Barrett [ed.], Evolution
452 and function of heterostyly, 179–207. Springer. Berlin, Germany.
- 453 Muenchow, G., and M. Grebus. 1989. The evolution of dioecy from distyly: reevaluation of the
454 hypothesis of the loss of long-tongued pollinators. *The American Naturalist* 133: 149–
455 156.
- 456 Naiki, A. 2012. Heterostyly and the possibility of its breakdown by polyploidization. *Plant*
457 *Species Biology* 27: 3–29.
- 458 Naiki, A., and M. Kato. 1999. Pollination system and evolution of dioecy from distyly in
459 *Mussaenda parviflora* (Rubiaceae). *Plant Species Biology* 14: 217–227.
- 460 Naiki, A., and H. Nagamasu. 2004. Correlation between distyly and ploidy level in
461 *Damnacanthus* (Rubiaceae). *American Journal of Botany* 91: 664–71.
- 462 Nakamura, K., T. Denda, K. Kameshima, M. Uehara, and M. Yokota. 2003. Chromosome
463 numbers of *Ophiorrhiza* and *Psychotria* (Rubiaceae) in the Ryukyus. *Biological*
464 *Magazine Okinawa* 41: 15–24.
- 465 Nakamura, K., T. Denda, O. Kameshima, and M. Yokota. 2007. Breakdown of distyly
466 *Ophiorrhiza* in a tetraploid variety of *Ophiorrhiza japonica* (Rubiaceae) and its
467 phylogenetic analysis. *Journal of Plant* 120: 501–509.
- 468 Nepokroeff, M., K. J. Sytsma, W. L. Wagner, and E. A. Zimmer. 2003. Reconstructing ancestral

469 patterns of colonization and dispersal in the Hawaiian understory tree genus *Psychotria*
470 (Rubiaceae): A comparison of parsimony and likelihood approaches. *Systematic*
471 *Biology* 52: 820–838.

472 Pannell, J. R., D. J. Obbard, and R. J. A. Buggs. 2004. Biological relevance of polyploidy:
473 ecology to genomics polyploidy and the sexual system: what can we learn from
474 *Mercurialis annua*? *Biological Journal of the Linnean Society* 82: 547–560.

475 R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for
476 Statistical Computing, Vienna, Austria. Website <http://www.R-project.org/>

477 Razafimandimbison, S. G., C. M. Taylor, N. Wikström, T. Pailler, A. Khodabandeh, and B.
478 Bremer. 2014. Phylogeny and generic limits in the sister tribes Psychotrieae and
479 Palicoureeae (Rubiaceae): Evolution of schizocarps in *Psychotria* and origins of
480 bacterial leaf nodules of the *Malagasy* species. *American Journal of Botany* 101: 1102–
481 1126.

482 Russell, J. R. W., and J. R. Pannell. 2015. Sex determination in dioecious *Mercurialis annua*
483 and its close diploid and polyploid relatives. *Heredity* 114: 262–271.

484 Sakai, A. K., W. L. Wagner, D. M. Ferguson, and D. R. Herbst. 1995. Origins of dioecy in the
485 Hawaiian flora. *Ecology* 76: 2517–2529.

486 Sakai, A., and S. Weller. 1999. Gender and sexual dimorphism in flowering plants: a review of

487 terminology, biogeographic patterns, ecological correlates, and phylogenetic
 488 approaches. In M. Geber, T. E. Dawson, and L. Delph [eds], Gender and sexual
 489 dimorphism in flowering plants, 1–31. Springer, Berlin, Germany.

490 Sakai, S. and J. Wright. 2008. Reproductive ecology of 21 coexisting *Psychotria* species
 491 (Rubiaceae): when is heterostyly lost? *Biological Journal of the Linnean Society* 93:
 492 125–134.

493 Sugawara, T., K. Watanabe, and M. Tabata. 2013. Distyly in *Psychotria serpens* (Rubiaceae) in
 494 the Ryukyu Islands, Japan. *Acta Phytotaxonomica et Geobotanica* 64: 113–122.

495 Sugawara, T., K. Watanabe, T. Takaso, M. Tabata, and A. Shimizu. 2016. Incompatibility and
 496 pollination of distylous *Psychotria serpens* (Rubiaceae) in the Ryukyu Islands, Japan.
 497 *Acta Phytotaxonomica et Geobotanica* 67: 37–45.

498 Sugawara, T., M. Yumoto, S. Tsuneki, and K. Watanabe. 2014. Incompatibility and
 499 reproductive output in distylous *Psychotria boninensis* (Rubiaceae), endemic to the
 500 Bonin (Ogasawara) Islands, Japan. *The Journal of Japanese Botany* 89: 22–26.

501 Saumitou-Laprade, P., Vernet, P., Dowkiw, A., Bertrand, S., Billiard, S., Albert, B., ... Dufay,
 502 M. 2018. Polygamy or subdioecy? The impact of diallelic self-incompatibility on the
 503 sexual system in *Fraxinus excelsior* (Oleaceae). *Proceedings of the Royal Society B:*
 504 *Biological Sciences*, 285(1873).

505 Saumitou-Laprade, P., Vernet, P., Vassiliadis, C., Hoareau, Y., De Magny, G., Dommée, B., &
506 Lepart, J. 2010. A self-incompatibility system explains high male frequencies in an
507 androdioecious plant. *Science*, 327(5973), 1648–1650.

508 Vernet, P., Lepercq, P., Billiard, S., Bourceaux, A., Lepart, J., Dommée, B., & Saumitou-
509 Laprade, P. 2016. Evidence for the long-term maintenance of a rare self-incompatibility
510 system in Oleaceae. *New Phytologist*, 210(4), 1408–1417. Tao, C., and C. M. Taylor.
511 2011. *Psychotria*. L. In T. Chen, C. M. Taylor, and C. Swartz C [eds], *Flora of China*,
512 294–301. Missouri Botanical Garden Press, St. Louis, MO, USA.

513 Vuilleumier, B. S. 1967. The origin and evolutionary development of heterostyly in the
514 angiosperms. *Evolution* 21: 210–226.

515 Wagner, W. L., D. R. Herbst, and S. H. Sohmer. 1999. *Manual of the Flowering Plants of*
516 *Hawai'i*, Vols. 1 and 2. University of Hawai'i and Bishop Museum Press, USA.

517 Watanabe, K., Kato, H., Kuraya, E., & Sugawara, T. 2018. Pollination and reproduction of
518 *Psychotria homalosperma*, an endangered distylous tree endemic to the oceanic Bonin
519 (Ogasawara) Islands, Japan. *Plant Species Biology*, 33(1), 16–27.

520 Watanabe, K., H. Kato, and T. Sugawara. 2014a. Distyly and incompatibility in *Psychotria*
521 *homalosperma* (Rubiaceae), an endemic plant of the oceanic Bonin (Ogasawara)
522 Islands. *Flora-Morphology, Distribution, Functional Ecology of Plants* 209: 641–648.

- 523 Watanabe, K., A. Shimizu, and T. Sugawara. 2014b. Dioecy derived from distyly and
524 pollination in *Psychotria rubra* (Rubiaceae) occurring in the Ryukyu Islands, Japan.
525 *Plant Species Biology* 29: 181–191.
- 526 Watanabe, K., and T. Sugawara. 2015. Is heterostyly rare on oceanic islands? *AoB Plants* 7:
527 plv087.
- 528 Weller, S. G. 2009. The different forms of flowers-what have we learned since Darwin?
529 *Botanical Journal of the Linnean Society* 160: 249–261.
- 530 Yamazaki, T. 1993. *Psychotria* L. In K. Iwatsuki, T. Yamazaki, D. E. Bufford, & H. Ohba (Eds.),
531 Flora of Japan IIIa (pp. 225–227). Tokyo: Kodansha.
- 532

533 **FIGURE 1.** Flowers of *Psychotria manillensis* on Iriomote Island, Japan. Male-like flowers (black arrow)

534 and female-like flowers (white arrow) in a single inflorescence. Scale bar = 10 mm.

535

536

537 **FIGURE 2.** Three types of stigmas in *Psychotria manillensis*. Scanning electron microscopic images of
538 stigmas: A, type-I stigma with well-developed stigmatic papillae; B and C, type-II stigma with
539 moderately developed stigmatic papillae; D, type-III stigma with poorly developed stigmatic papillae.
540 Scale bar = 500 μ m.

541

542

543 **FIGURE 3.** Three types of anthers in *Psychotria manillensis*: A, type-a anther with pollen sacs full of
544 pollen grains; B, type-b anther with small pollen sacs half filled or less with pollen grains; and C, type-c
545 anther with no pollen sac nor grain. Scale bar = 0.3 mm.

FIGURE 4. Various flower types in combination with different stigma and anther types of *Psychotria*
manillensis on Okinawa and Iriomote Islands, Japan. A, flower with type-I stigma and type-c anthers (Ic;
38%); B, flower with type-II stigma and type-c anthers (IIc; 11%); C, flower with type-III stigma and
type-a anthers (IIIa; 32%); D, flower with type-II stigma and type-b anthers (IIb; 9%). Ic and IIc flowers
function as female, IIIa as male and IIb as hermaphroditic. Scale bar = 1 mm.

FIGURE 5. Relative abundance of female, male, perfect, and sterile flowers (A) and of female, male, monoecious, and hermaphroditic plants (B) in the five populations from Okinawa and Iriomote Islands, Japan.

560 **FIGURE 6.** Anther/stigma height of female (closed circles), male (open circles), and perfect flowers
561 (open triangles) in six plants of *Psychotria manillensis* in the Sueyoshi population from Okinawa Island,
562 Japan.

563

564

565 **FIGURE 7.** Fruit set under open pollination and bagging experiments in the five *Psychotria manillensis*

566 populations from Okinawa and Iriomote Islands, Japan, in 2011–2013.

567

568

569 **FIGURE 8.** Flowering phenology (A) and relative femaleness (B) during the flowering period in 10
570 plants of *Psychotria manillensis* in the Oppa population from Okinawa Island, Japan. Relative femaleness
571 (G) was calculated as follows: $G = (F + P/2)/(F + M + P)$, where F, P and M are the number of female,
572 perfect and male flowers per plant, respectively.

FIGURE 8.

573

574

FIGURE 9. Relative femaleness G (A) and sex changes (B) of 58 plants in three populations on Okinawa Island in 2012-2013. Numbers in parenthesis are the numbers of plants in 2012, numbers with arrows indicate plants changed the sex. Relative femaleness was compared with a Wilcoxon test.

FIGURE 9.

580 **FIGURE 10.** Flower visitors of *Psychotria manillensis* on Okinawa and Iriomote Islands, Japan.

581 A, *Polistes rothneyi* ; B, *Vespa analis* ; C, *Lucilia porphyryna* ; D, *Cardiodactylus guttulus*.

582

Table 1. Fruit set and pollen tube penetration after cross-pollination test for each stigma type of *Psychotria manillensis* on Okinawa Island, Japan.

Fruit set	Stigma type	A		B		C	
		n (N)		n (N)		n (N)	
Cross pollination (%)		64.1	103 (21)	65.8	111 (20)	2.1	96 (21)
Pollen tube penetration (%)		100	22 (10)	82	17 (10)	0	18 (10)

N, number of plants

n, number of flowers

Table 2. Flower visitors of *Psychotria manillensis* on Okinawa and Iriomote Islands, the Ryukyu Islands, Japan, in 2011 – 2013.

Family	Species	Population				
		Okinawa			Iriomote	
		Kt	Op	Su	Ue	So
HYMENOPTERA						
Apidae	<i>Amegilla dulcifera</i>		+	+		
	<i>A. florea</i>					++
	<i>Apis mellifera</i>	+	+			
Halictidae	<i>Lasioglossum</i> sp.	+	+	++		
Icheumonidae	sp.		+			
Scoliidae	<i>Campsomeris prismatica</i>			+		
Vespidae	<i>Anterhynchium flavomarginatum</i>	+				
	<i>Pararrhynchium ishigakiense</i>					+
	<i>Polistes formosanus</i>				+++	+++
	<i>P. rothneyi</i>	+++	+++	+++	+++	+++
	<i>Vespa affinis</i>				++	++
	<i>V. analis</i>	+++	+++	+	+++	+++
	<i>V. ducalis</i>				++	++
DIPTERA						
Ephydriidae	sp.	+				
Calliphoridae	<i>Hemipyrellia</i> sp.	+			+	
	<i>Lucilia papuensis</i>	+			+	
	<i>L. porphyrina</i>	+++	+++	+++	+++	++
Muscidae	<i>Dichaetomyia flavipolis</i>	++	++	++		
	<i>D. watasei</i>	++	+			
	<i>Stomoxys calcitrans</i>	+				
Sarcophagidae	<i>Sarcophaga antilope</i>		+			
	<i>S. kanakovi</i>		+			
	sp.	+				
Syrphidae	<i>Allobaccha nubilipennis</i>	+++	++	+	+	
	sp.		+			
Tachinidae	sp.		+			
LEPIDOPTERA						
Crambidae	sp. 1		+			
	sp. 2		+			
	sp. 3		+			
Lycaenidae	<i>Lampides boeticus</i>	+				
Nymphalidae	<i>Athyma perius</i>		+			
Papilionidae	<i>Papilio memnon</i>	+				
	<i>Papilio okinawaensis</i>	+				
	<i>Papilio polytes</i>	++	+			
Sphingidae	<i>Macroglossum pyrrhosticta</i>		+			
COLEOPTERA						
Chrysomelidae	<i>Altica cyanea</i>	+				
HEMIPTERA						
Largidae	<i>Physopelta cincticollis</i>		+			
ORTHOPTERA						
Gryllidae	<i>Cardiodactylus guttulus</i>	+	+		+	+
	<i>Grylodes sigillatus</i>					+

Table 2. Continued. Number of visits to an inflorescence during an 8-h observation are classified in the following categories: +++, more than 8 times; ++, 3–8 times; +, 1–2 times observed in 8 hours observation period. Kt, Katsuu population; Op, Oppa population; Su, Sueyoshi population; Ue, Uehara population; So, Sonai population. Observations were performed on July 17 and 18, 2011 and on July 14 and 20, 2012 in the Katsuu population; from July 6 to September 18, 2011 and on July 14, 2012 in the Oppa population; on July 9 and 27, 2011, on June 19 and July 1, 2012 in the Sueyoshi population; and on June 2, 3, 15, 16, and 17, 2011 and on June 13, 2013 in the Sonai and Uehara populations.