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

Kenta Watanabe [1], Akira Shimizu[2,3,4], and Takashi Sugawara[5]

[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

Abstract

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

individuals. This is the first report of the polygamous breeding system not only in the genus *Psychotria*, but also in all heterostylous taxa.

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

Introduction

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

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

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

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

Materials & Methods

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

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

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

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

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

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

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

To determine the ability of pollen tubes to access the styles, 22 flowers with type-I 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 Na_2HPO_4 (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).

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

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

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

$G = (F + P/2) / (F + M + P)$, where F, P and M are the number of female, perfect and male flowers per plant, respectively. Since the perfect flowers have both female and male function, we assumed the perfect flower has the half of the femaleness of the female flower. We used the number of collected flowers for this calculation and calculated E per population considering 50 flowers per individual.

Measurements of floral traits–To understand the relationship between morphological traits and functional sexual mode, we measured the anther and stigma height of 20 flowers from six individuals (120 flowers in total) of different gender in the Sueyoshi population.

Self-compatibility and agamospermy test–To test self-compatibility, we self-pollinated 136 flowers (34 Ic, 63 IIc, and 31 IIIa) from 31 individuals in the Oppa population. Inflorescences were bagged 1 d before pollination, and open flowers in bags were pollinated with pollen grains from other flowers in a bagged inflorescence of the same plant. Then, the self-pollinated flowers were bagged again, and the fruit set was observed approximately 30 d after pollination. We performed Fisher’s exact test using R 3.3.2 (R Core Team, 2016) to test the differences between

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

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

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

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

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

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

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

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

Flower visitors–Flower visitors were observed in all the five populations over 8 h (between 0700 and 1800), and some individuals were collected for identification. The number of visits to a single inflorescence during the 8-h observation period was classified into three categories: +++, more than 8 times; ++, 3–8 times; and +, 1–2 times. 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.

Results

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

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

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

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

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

Self-pollination and agamospermy test–The fruit set after self-pollination was significantly lower (36.2%) than that after cross-pollination (65.0%; $P < 0.001$, Fisher's exact test). Of 18 studied individuals, 13 showed self-compatibility. Additionally, none of the emasculated flowers set any fruit, revealing that agamospermy did not occur.

Fruit set under open pollination and bagging experiments–The fruit set under open pollination varied among populations and years (1.8–21.9%; Fig. 7; Table S2), and the full model (model 4) showed the smallest AIC (binomial GLMM; Table S3). Some male individuals (only male flowers at the time of flower collection) also produced fruit, although femaleness (G) was significantly correlated with fruit set in all populations ($P < 0.05$, Pearson's correlation test; Fig. S1), except for the Katsuu population ($P = 0.35$). Some flowers set fruit after bagging experiments, although the fruit set was significantly lower than that under open pollination ($P < 0.0001$, the likelihood ratio test after binomial GLMM).

Flowering phenology and flower types within inflorescence through flowering period–The flowering period of 10 individuals observed in the Oppa population was approximately 42 d (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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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FIGURE 1. Flowers of *Psychotria manillensis* on Iriomote Island, Japan. Male-like flowers (black arrow) and female-like flowers (white arrow) in a single inflorescence. Scale bar = 10 mm.

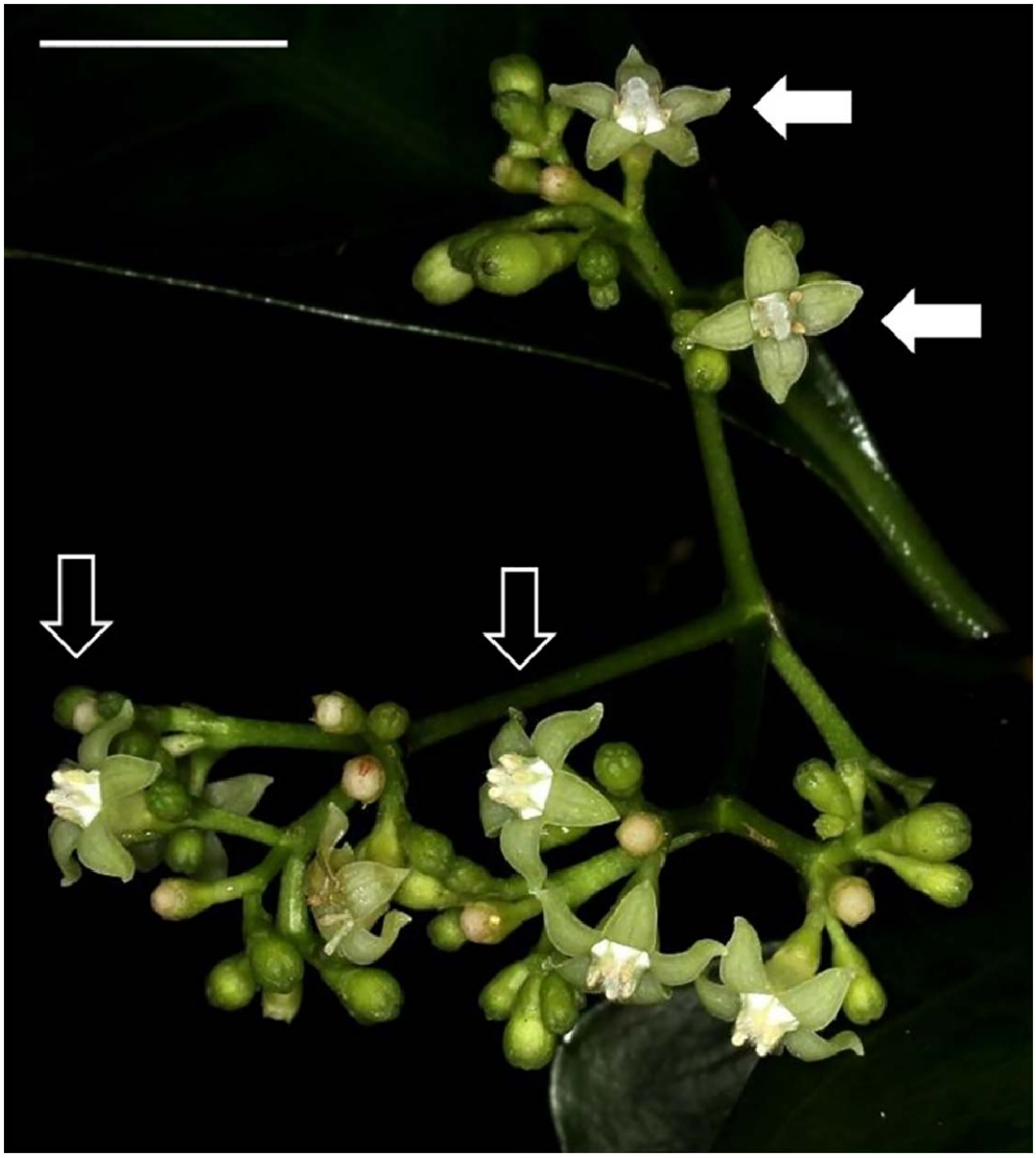

FIGURE 2. Three types of stigmas in *Psychotria manillensis*. Scanning electron microscopic images of stigmas: A, type-I stigma with well-developed stigmatic papillae; B and C, type-II stigma with moderately developed stigmatic papillae; D, type-III stigma with poorly developed stigmatic papillae. Scale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

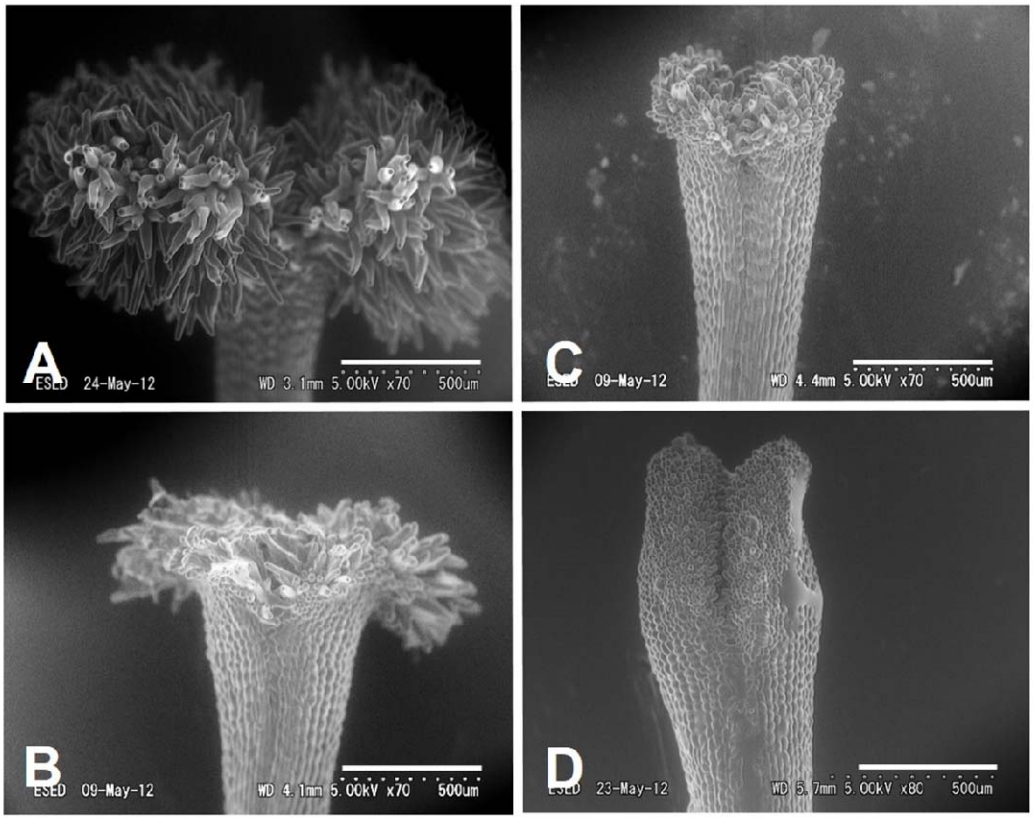

FIGURE 3. Three types of anthers in *Psychotria manillensis*: A, type-a anther with pollen sacs full of pollen grains; B, type-b anther with small pollen sacs half filled or less with pollen grains; and C, type-c anther with no pollen sac nor grain. Scale bar = 0.3 mm.

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.

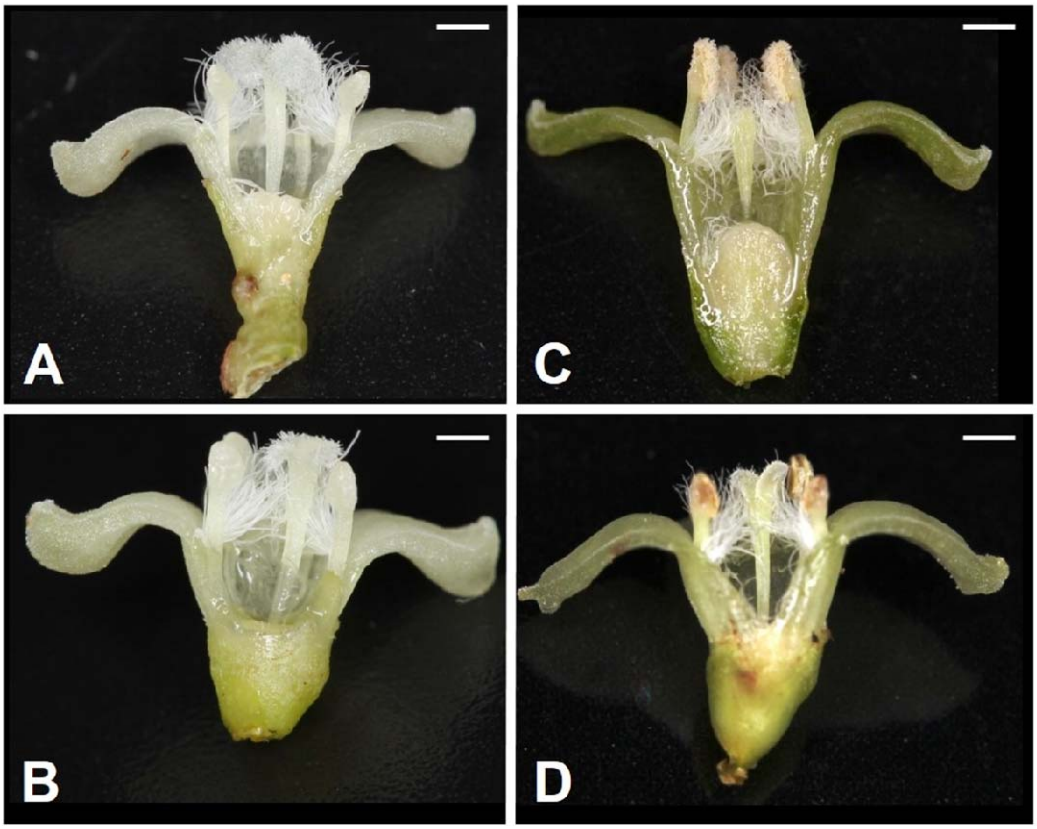

FIGURE 5. Relative abundance of female, male, perfect, and sterile flowers (A) and of female, male, monoecious, and hermaphroditic plants (B) in the five populations from Okinawa and Iriomote Islands, Japan.

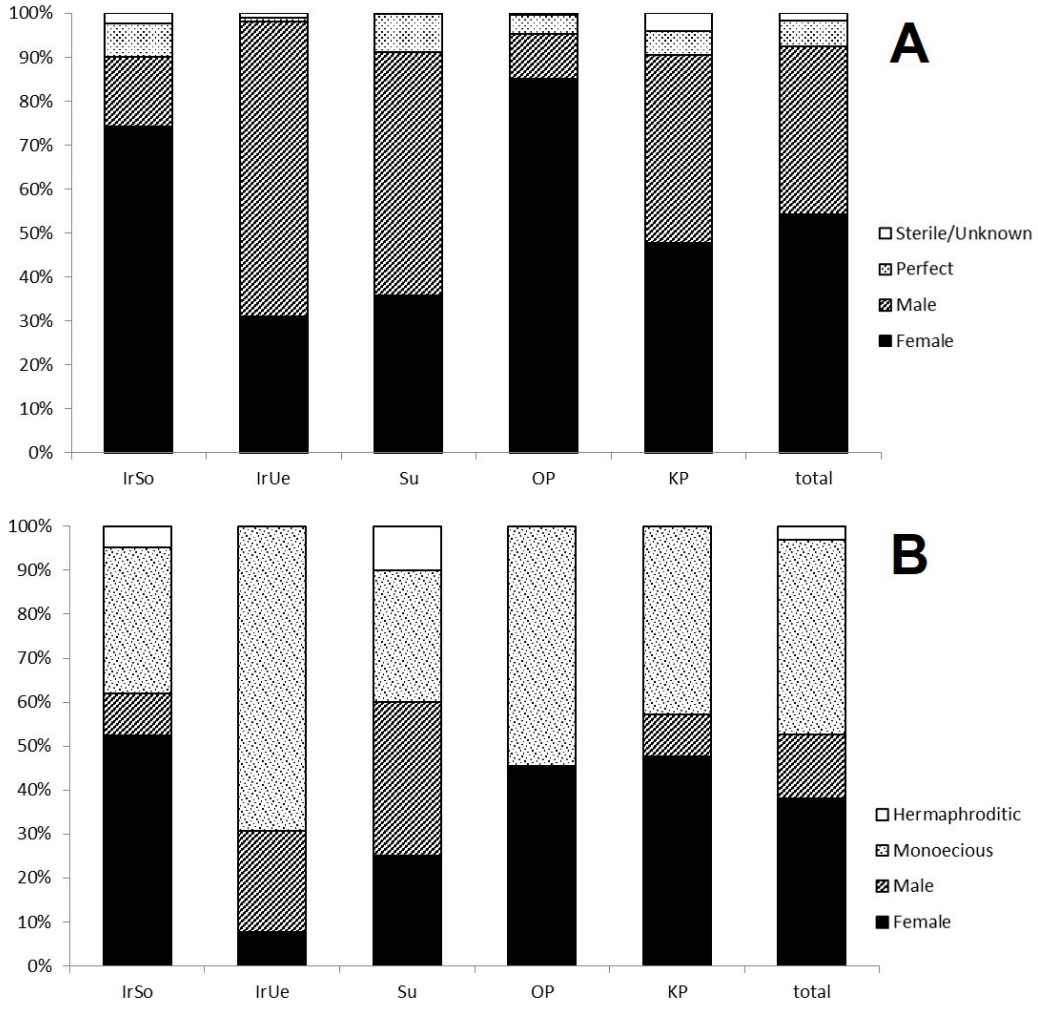

FIGURE 6. Anther/stigma height of female (closed circles), male (open circles), and perfect flowers (open triangles) in six plants of *Psychotria manillensis* in the Sueyoshi population from Okinawa Island, Japan.

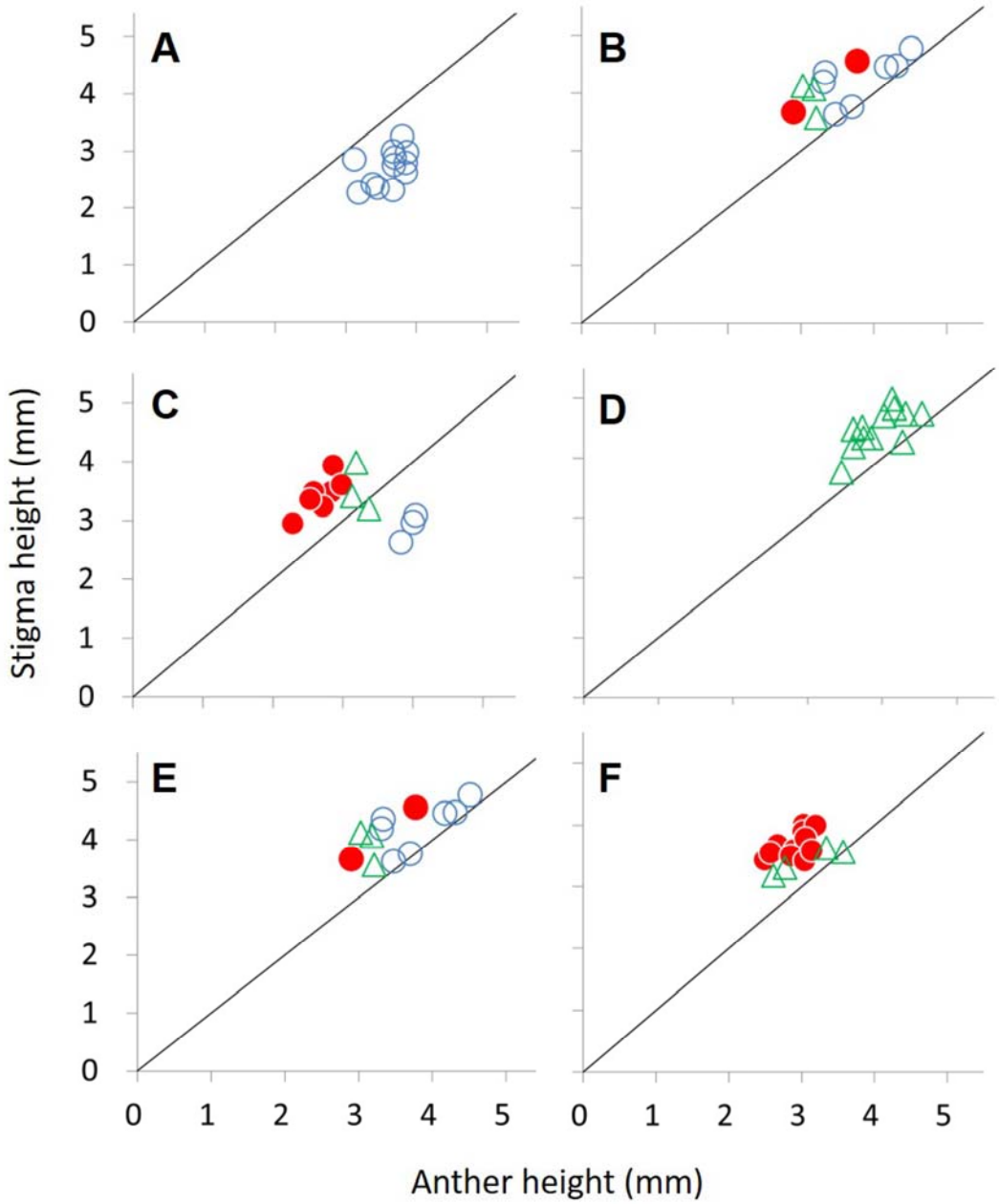

FIGURE 7. Fruit set under open pollination and bagging experiments in the five *Psychotria manillensis* populations from Okinawa and Iriomote Islands, Japan, in 2011–2013.

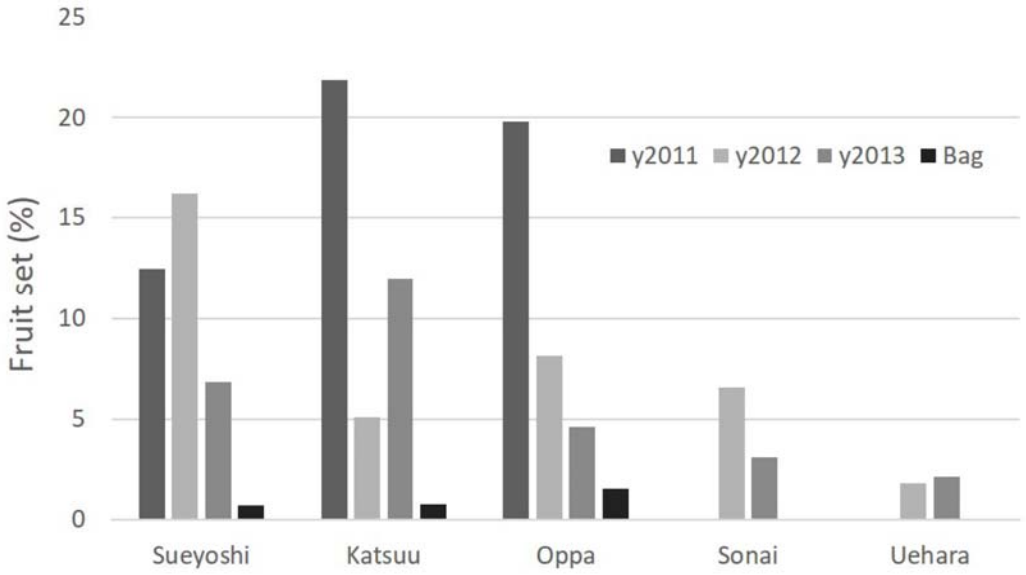

FIGURE 8. Flowering phenology (A) and relative femaleness (B) during the flowering period in 10 plants of *Psychotria manillensis* in the Oppa population from Okinawa Island, Japan. Relative femaleness (G) was calculated as follows: G = (F + P/2)/(F + M + P), where F, P and M are the number of female,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

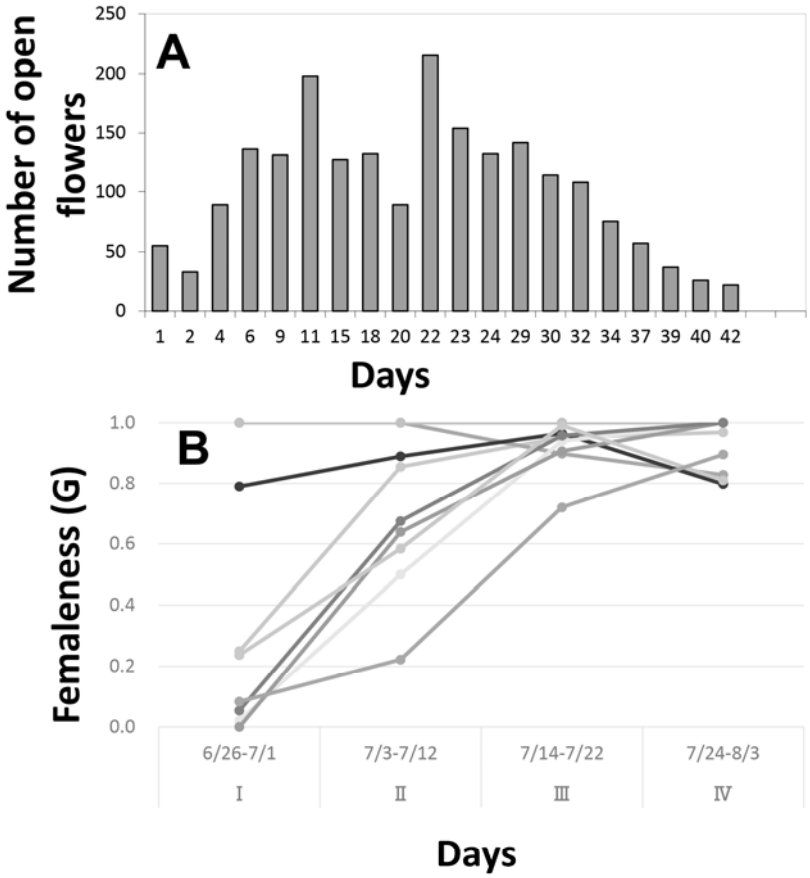

FIGURE 8.

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.

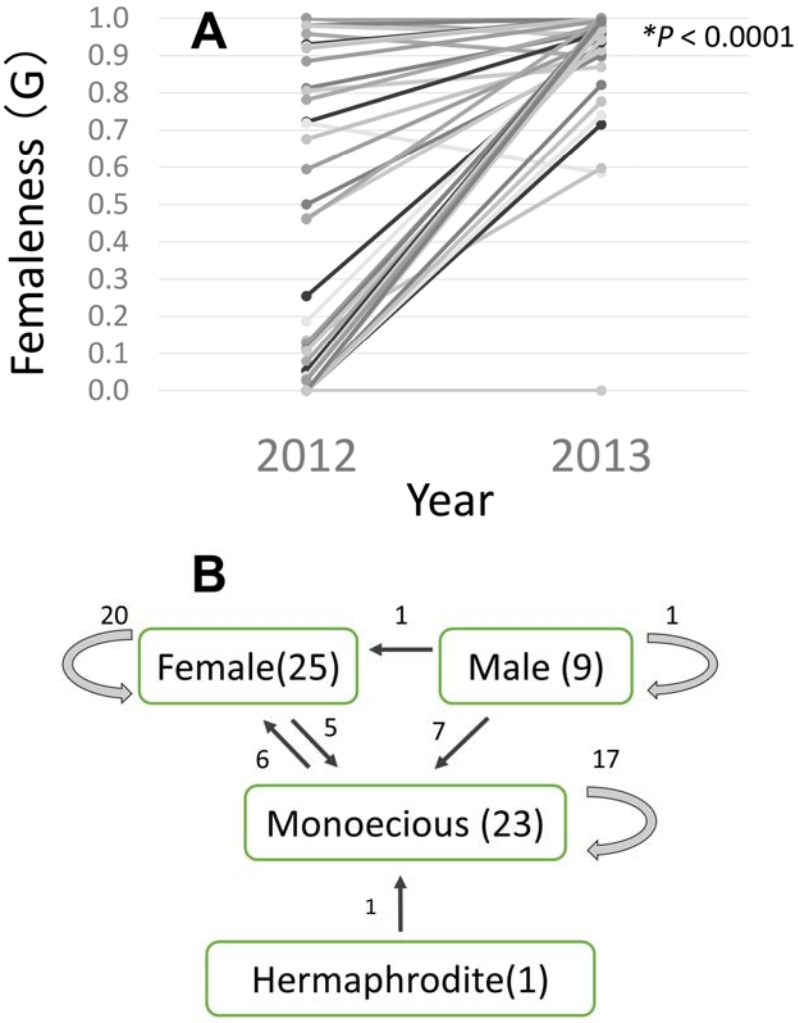

FIGURE 9.

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

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

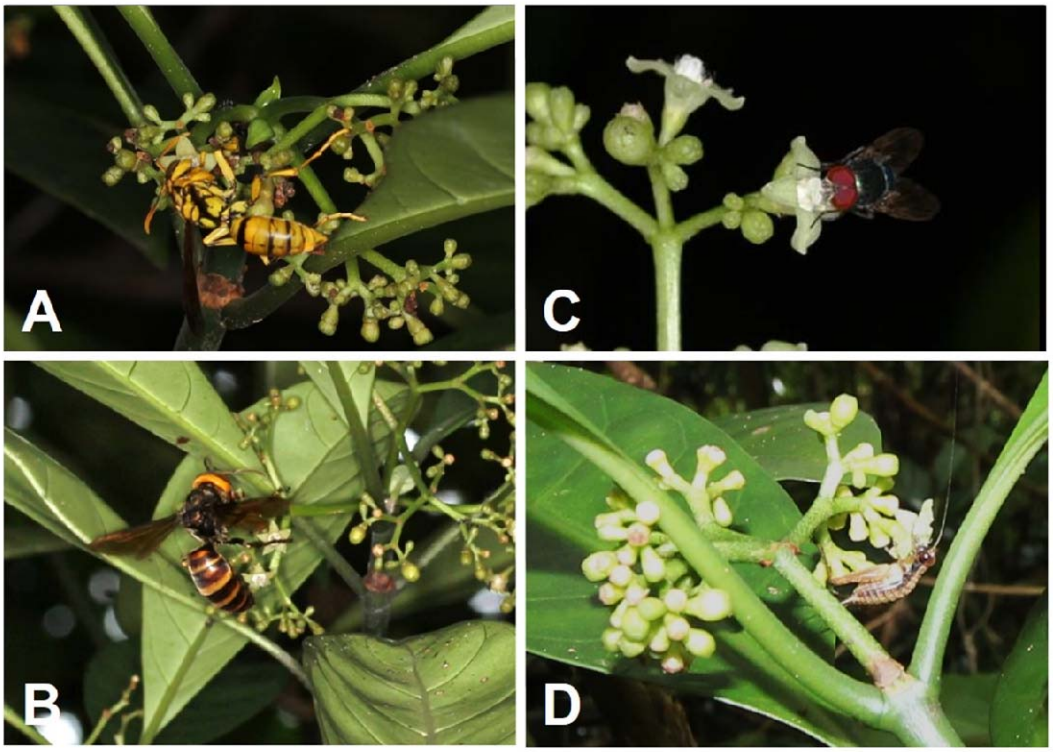

Table 1. Fruit set and pollen tube penetration after cross-pollination test for each stigma type of *Psychotria manillensis* on Okinawa Island, Japan.

Stigma type	A		B		C	
Fruit set		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.

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

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.