

Cryptic dioecy of *Symplocos wikstroemiifolia* Hayata (Symplocaceae) in Taiwan

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ABSTRACT. *Symplocos wikstroemiifolia* Hayata is one of the few morphologically androdioecious species in Symplocaceae. Although this species has been proposed as cryptically dioecious, little is known about its patterns of sexual expression in the field. We studied the breeding system and reproductive biology of *S. wikstroemiifolia* in Taiwan. Field investigations showed that anthers of most morphologically hermaphroditic flowers did not produce pollen grains; thus, this flower type should be considered female. Nearly all flowering individuals produced only male or female flowers. The sex ratios were slightly male-biased, but did not significantly deviate from 1:1, congruent with the proposed cryptic dioecy status. However, two individuals produced flowers that may have been functionally hermaphroditic, suggesting a variation of sexual expression in *S. wikstroemiifolia*. There were about 12 stamens per male flower but only five staminodes per female flower. No locules and ovules existed in the male flowers, while two locules within the ovary of the female flowers contained four ovules each (two large, two small). Male individuals produced cymes and thyrses, whereas female individuals only produced cymes. Some of these morphological characteristics differed from those previously described. Anthesis of most male and female flowers began before dawn and lasted for one to two days. Nevertheless, anther dehiscence, which only occurred under dry conditions, was not restricted to a specific time period during the day. The mean natural fruit set was 6.7%, which is relatively low compared to those in other *Symplocos* or dioecious species. In conclusion, *S. wikstroemiifolia* exhibited a nearly complete gender dimorphism, but with certain flexibility in sexual expression, and should thus be considered cryptically dioecious.

Keywords: Androdioecious; Breeding system; Cryptic dioecy; Gender dimorphism; Reproductive biology; *Symplocos wikstroemiifolia*.

INTRODUCTION

Dioecy, a plant breeding system in which a population consists of distinct male and female individuals (Sakai and Weller, 1999), is found in fewer than 7% of angiosperm species (Yampolsky and Yampolsky, 1922; Renner and Ricklefs, 1995; Richards, 1997). Dioecy however, is widespread among many plant families, and it has risen independently from hermaphroditism through various evolutionary pathways many times (Bawa, 1980; Charlesworth, 2002). The degree of opposite sex organs reduction on a dioecious plant also varies among different taxa (Mitchell and Diggle, 2005). If the rudiments cause unisexual flowers to have a hermaphroditic appearance and make the dioecious condition hard to recognize, the breeding system is defined as cryptic dioecy (Mayer and Charlesworth, 1991).

Cryptic dioecy can be classified into three types. In the first type (type I), the functionally female flowers release

sterile and usually inaperturate pollen grains (Mayer and Charlesworth, 1991; Cane, 1993; Furness, 2007), such as in some species of *Thalictrum* (Kaplan and Mulcahy, 1971; Penny and Steven, 2009), *Solanum* (Anderson, 1979; Anderson and Symon, 1989; Knapp et al., 1998) and *Vitis* (Kevan et al., 1985; Kevan et al., 1988; Caporali et al., 2003). In the second type (type II), the functionally female flowers do not release pollen grains during anthesis, such as in *Echinocereus coccineus* (Hoffman, 1992), *Withania aristata* (Anderson et al., 2006) and in some species of *Dombeya* (Humeau et al., 1999). In the third type (type III), the functionally female flowers release viable pollen grains that can sire seeds; however, seedlings sired by functional females tend not to survive in natural populations (Verdú et al., 2004). Thus, morphological form alone may not show its true reproductive function (Cruden and Lloyd, 1995; Sakai and Weller, 1999).

Androdioecy is a condition in which the male and hermaphroditic individuals coexist in a population, and it is extremely rare among plants (Darwin, 1877; Richards, 1997). Studies of many morphologically androdioecious plants often reveal that they are cryptically dioecious

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rather than truly androdioecious (Charlesworth, 1984; Pannell, 2002). However, such studies are very limited and require careful examination on the functionality of the flowers. One of the candidates showing androdioecy came from the genus *Symplocos* (Symplocaceae), found during our previous plant survey of Taiwan (Tseng et al., 2008). Symplocaceae, a family in the order Ericales, consists of about 300 tree or shrub species, occurring in humid tropical or subtropical montane forests in eastern and southern Asia, America and Australia (Heywood et al., 2007; APG III, 2009). The family has been classified into two genera (genus *Symplocos* and *Cordyloblaste*) and several taxa above the species level (two subgenera, three sections, and two series in *Symplocos*; Fritsch et al., 2008).

The breeding system of the genus *Symplocos* is predominately hermaphroditic (Heywood et al., 2007), but a few species show gender dimorphism (Aranha Filho et al., 2007, 2009a, b). Recently Aranha Filho et al. (2009a) documented prevalent type I cryptic dioecy in *Symplocos* section *Hopea*, which is mainly distributed in South America (Fritsch et al., 2008; Aranha Filho et al., 2010). Many of these are morphologically androdioecious and at least 10 species examined by Aranha Filho et al. (2009a) produced sterile and malformed pollen grains on morphologically hermaphroditic flowers.

The only Asian representative of *S.* section *Hopea* is *Symplocos wikstroemiifolia* Hayata (Fritsch et al., 2008; Aranha Filho et al., 2009a). This species has been considered “probably androdioecious” (Wu and Nootboom, 1996). Nagamasu (1998) described *S. wikstroemiifolia* as having “bisexual (female)” flowers in the 2nd edition of the *Flora of Taiwan*. Wang (2000) also stated that the stamens of morphologically hermaphroditic flowers are “undeveloped” in *S. wikstroemiifolia*. Although no detailed description exists in the literature, this species seems likely cryptically dioecious.

In this paper, we provide evidence for the functionality of the flowers in *S. wikstroemiifolia*, and also basic descriptions of the reproductive biology of this species in Taiwan. Sexual expressions of every flowering individual from two natural populations in Taiwan were recorded for at least two consecutive years. Flower morphology, phenology and natural fruit set were also investigated.

MATERIALS AND METHODS

Study species

Symplocos wikstroemiifolia Hayata is an evergreen shrub or tree with grayish or brownish bark. The leaves are simple, alternate, with an entire or sometimes a crenulate margin (Wang, 2000). This species is morphologically androdioecious (Wu and Nootboom, 1996; Nagamasu, 1998; Wang, 2000). The male flowers usually have more than 10 stamens, whereas the morphologically hermaphroditic flowers have only five stamens. Both flower types are white, small and inconspicuous. They both have a five-lobed corolla and a nectary disk on the superior part of the

ovary. There is one style on the morphologically hermaphroditic flower but none on the male flower, and only one seed per drupe when mature.

Symplocos wikstroemiifolia is distributed in southern China, Indochina, the Malay Peninsula, and Taiwan (Nagamasu, 1998). In Taiwan, the species is scattered across the middle and northern part of the island, from 700 m to 1,900 m altitude. They are not very common, and only locally abundant around Beichatian Mountain in Taiwan (Wang, 2000). Individuals start flowering from March to May, and set fruit from July to September (Wang, 2000).

Study sites

Field investigations were conducted at two sites where natural populations of *S. wikstroemiifolia* occur. One is located in the Chatianshan Nature Reserve, on the ridge around Beichatian Mountain (Figure 1). This area is one of the few places in Taiwan for *Fagus hayatae* (Fagaceae) (Liu and Su, 1972; Hsieh, 1989; Chiou et al., 1998; Lu et al., 1998). Beichatian Mountain is 1728 m high and *S. wikstroemiifolia* is particularly abundant at altitudes above 1,400 m on the surrounding ridge. The mean annual temperature is approximately 14.2°C (Chiou et al., 2004) and the mean annual precipitation, from 2005 to 2008, was 4,238 mm, as recorded by the Fushan Weather Station (about 5 km east of Beichatian Mountain and at 455 m altitude).

The other study site was the mountainous area of Pinglin District, New Taipei City (Figure 1). This site is along the roadside of Provincial Highway No. 9 near the “54.5 K” road sign located in a valley near the Sidu River at about 400 m altitude. The mean annual temperature, from

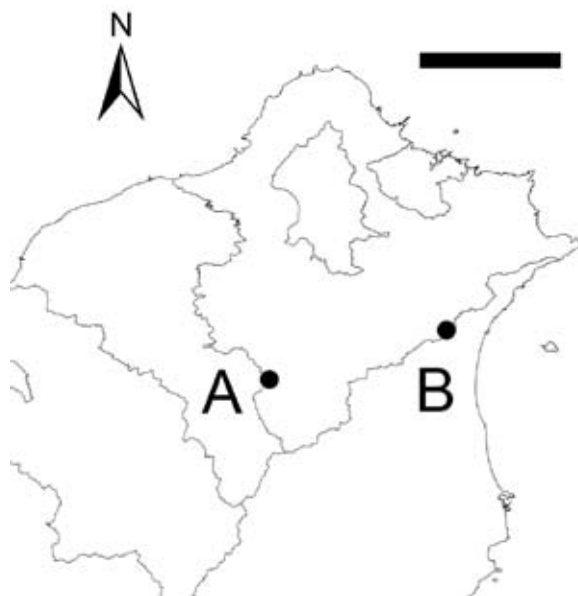


Figure 1. Study sites. Label A indicates the location of Beichatian Mountain. Label B indicates the second study site “Pinglin”. The image is a magnification of a region from northern Taiwan. Scale bar = 25 km.

2005 to 2008, was 19.6°C, and the mean precipitation was 4,108 mm, based on the records in the Sidu Weather Station.

In the following text, we use the term “Beichatian Mountain” for the mountain ridge around Beichatian Mountain, and “Pinglin” for the second site. The distance between the two study sites is about 33 km.

Patterns of sexual expression and sex ratio

From 2007 to 2009, a total of 190 *S. wikstroemiifolia* plants were labeled at Beichatian Mountain (188) and at Pinglin (2). During their 2007 and 2008 flowering seasons, individuals' floral morphologies were examined and recorded. We continued to monitor the flower production patterns in some individuals during 2009. Thirty or more flowers from each randomly selected individual's inflorescence were examined to verify the consistency of flower types. In small trees with fewer than 30 flowers, all flowers were observed. Flowers were inspected either in the field or in a laboratory under a stereomicroscope. The presence versus absence of pollen grains from the anthers of morphologically hermaphroditic flowers was carefully examined. The sex ratio, defined as the ratio of individuals with male flowers (male individuals or plants) to individuals with morphologically hermaphroditic flowers (morphologically hermaphroditic individuals or plants) in a population, was calculated with data from 2007 and 2008. Chi-square tests with Yates' correction were conducted to determine whether the sex ratios significantly deviated from 1:1.

Flower and inflorescence morphology

The lengths of floral organs, i.e. sepal, petal, stamen, style, and ovary, were measured with the software ImageJ, which can analyze the pixel distance on a digital photo (Abramoff et al., 2004) (Table 2). Flowers were randomly collected in the field and fixed in FAA (formalin: glacial acetic acid: 95% ethanol: water = 2:1:10:7 in volume), and digitally photographed. The length and width of each floral organ were then measured and calculated with ImageJ on a computer. We did not measure all the floral organs within a single flower because some could not be fully flattened and stretched when they were photographed, which would have caused inaccurate measurements using ImageJ. The number of stamens per flower was recorded, and the number of ovules per flower was observed from the dissected ovaries under a stereomicroscope.

To estimate pollen grain number per anther and flower, 10 flower buds near anthesis were collected from four male individuals and stored in 70% ethanol. From each flower bud, one to four indehiscent anthers were randomly chosen, and a total of 20 anthers were examined. An indehiscent anther was placed in a 0.2 mL PCR tube to which a droplet of 5 µL 70% ethanol was added, and the anther was smashed with tweezers. After the anther released pollen grains, 1 µL of the suspension solution containing pollen grains was transferred and placed on a microscopic

slide. All grains in the droplet were counted under a light microscope. This procedure was repeated until all remaining solution was examined. After that, another 5 µL 70% ethanol was added to the tube and all previous counting procedures were repeated. When no more pollen grains were found in the 1 µL ethanol droplet after three successive counts and either no or a negligible number of pollen grains were left in the PCR tube, the anther counts were completed. The sum of all numbers obtained from each counting procedure represented the total pollen grains per anther. The mean pollen grains per flower was calculated by multiplying mean pollen grains per anther by the mean stamens per flower.

To examine other flower characteristics, such as anther and ovary morphology, flower buds near anthesis were collected in the field and fixed in FAA. They were dehydrated through a serial ethanol concentration gradient and transferred to Histo-Clear II® (Electron Microscopy Science, Pennsylvania, USA), then embedded in paraplast. Serial cross- and longitudinal sections were cut at 6–10 µm thickness using a rotary microtome, then were mounted onto slides that were coated with Mayer's adhesive. The slides were then stained with Safranin O and Fast Green, mounted with coverslips using Permount® (Fisher Scientific, New Jersey, USA), and observed under a light microscope.

Inflorescence morphology was observed in the field. Numbers of flower per inflorescence were counted from 101 inflorescences of six male individuals and 93 inflorescences of eight morphologically hermaphroditic individuals.

To compare the means in each measurement of different flower categories, students' *t*-tests or one-way ANOVAs were conducted. Flower characteristics were compared with either Welch's ANOVAs (Day and Quinn, 1989) or standard ANOVAs. Duncan's multiple range tests were applied if ANOVAs showed a significant difference among different flower categories.

Flowering phenology and natural fruit set

Symplocos wikstroemiifolia flowering phenology was observed at three different levels: the duration of the flowering season in one population, the flowering sequence within one inflorescence, and the flowering course during one day.

The duration of flowering was recorded from five male and five morphologically hermaphroditic plants in Beichatian Mountain in 2008. If individuals had fewer than about 100 inflorescences, the number of all inflorescences was counted. Otherwise, only several shoots were selected and the numbers of inflorescences among these shoots were counted. During the flowering season, the number of inflorescence with open flowers was monitored weekly. The number recorded was divided by the initial number of inflorescences per plant and expressed as a percentage. In Pinglin, in 2008, the same method was also applied to one male (Plant No. 115) and one morphologically hermaph-

roditic plant (Plant No. 116). To investigate the flowering sequence within one inflorescence, flowering shoots that contained numerous inflorescences on Plant No. 115 and Plant No. 116 were observed for 12 consecutive days at Pinglin in 2009. The floral longevity and the flowering sequence were recorded with a digital camera. To understand the detailed flowering progression during one day, a continuous 24-hour observation was performed in Pinglin. During the observation periods, the flowering shoots on Plant No. 115 and 116 were photographed every one or two hours. The observation began at about 4:00 pm on March 22nd, 2009 and ended at about 6:00 pm on March 23rd, 2009.

The natural fruit set of *S. wikstroemiifolia* was recorded for 10 morphologically hermaphroditic plants in Beichatian Mountain in 2009. Numbers of flowers and flower buds near anthesis on each individual were counted during the flowering season. If the plants had fewer than 100

flowers, all the flowers and flower buds were counted. Otherwise, only part of the flowers and flower buds on selected flowering shoots and branches was counted. About two months later, the number of developing fruits was recorded, and the fruit set was calculated as the number of developing fruits divided by the number of flowers and flower buds.

RESULTS

Patterns of sexual expression and sex ratio

In the two study sites, from 2007 to 2009, 83 individuals of *S. wikstroemiifolia* produced flowers during at least one flowering season. Only two flowering individuals were found in Pinglin, and the other 81 individuals were all located in Beichatian Mountain. Visual inspection of flowers showed that the anthers of male flowers all contained pollen grains (Figure 2A), whereas those of most

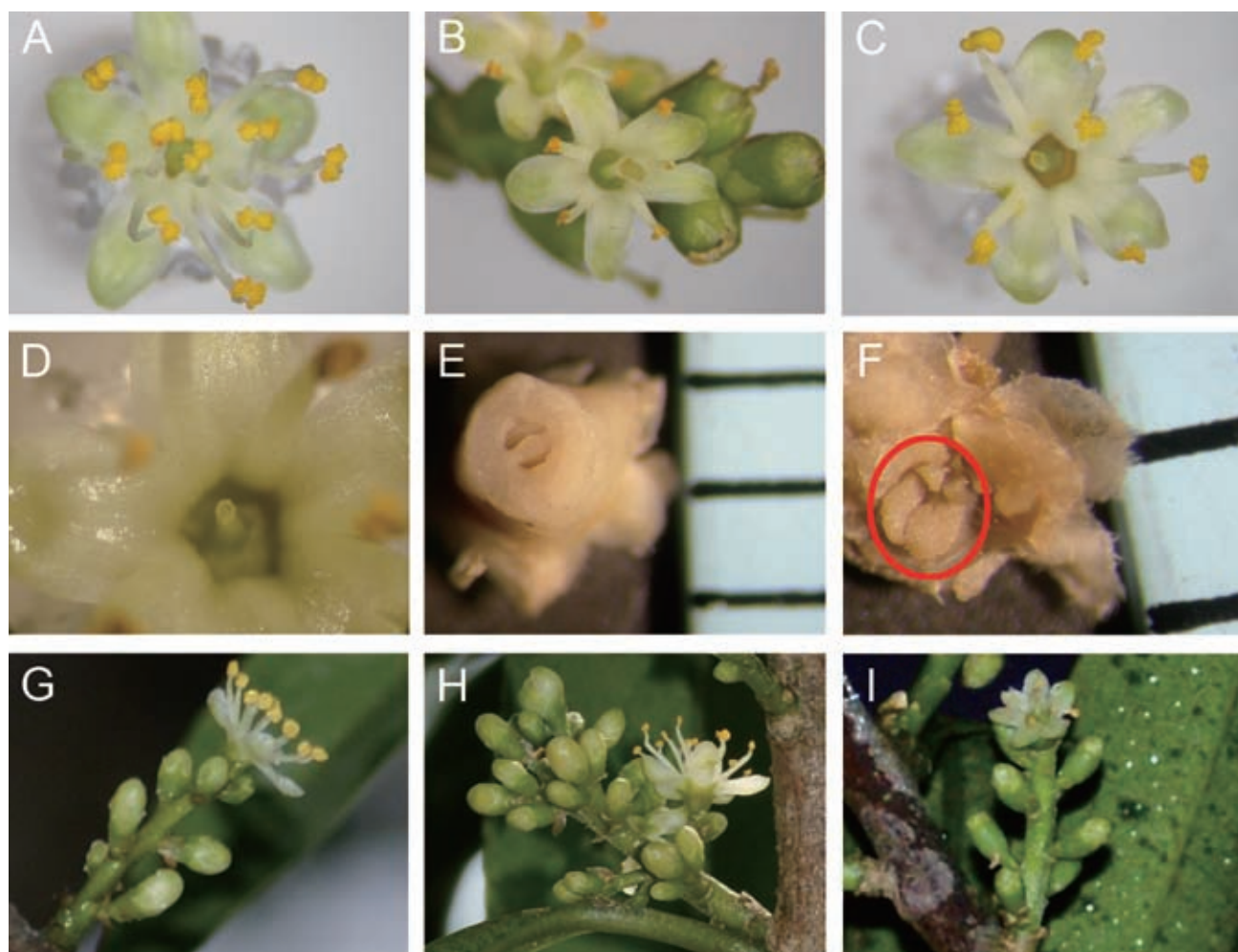


Figure 2. Flower and inflorescence morphology of *S. wikstroemiifolia*. (A) A male flower; (B) A female flower on one inflorescence; (C) A morphologically hermaphroditic flower from Plant No. 29. The anthers produced pollen grains; (D) A morphologically hermaphroditic flower from Plant No. 189 (note the short style in the flower); (E) A cross-section of the proximal region of one ovary from a female flower. There were two locules in one ovary separated by one septum; (F) The ovules in one of the two locules (see the red circle). Two small ovules were above two large ovules; (G) An unbranched male cymose inflorescence. The apical flower opened first; (H) A branched male thyrsoid inflorescence; (I) A female cymose inflorescence.

morphologically hermaphroditic flowers were empty (Figure 2B). Serial sections of flower buds revealed the same pattern (Figures 3A, 3B and 3C). Morphologically hermaphroditic flowers contain normally developed ovaries and ovules; therefore they can be considered having female-only function. To avoid confusion, we use the term “female flowers” to refer to the typical morphologically

hermaphroditic flowers that did not produce pollen grains. Thus, individuals with female flowers are described as “female individuals” or “female plants” in the following text. Two individuals (Plant No. 29 and 189) in Beichatian Mountain produced morphologically hermaphroditic flowers that bore pollen grains (Figures 2C and 2D). Plant No. 29 produced one inflorescence with a few female flowers in 2007, had no flower production in 2008, and produced one inflorescence with a few unusual hermaphroditic flowers in 2009. Anthers of the unusual hermaphroditic flower from Plant No. 29 in 2009 released pollen grains (Figure 2C). In contrast, Plant No. 189 produced unusual hermaphroditic flowers both in 2008 and 2009. Not all anthers of unusual hermaphroditic flowers from Plant No. 189 bore pollen grains. In many flowers, some anthers were empty thus resembling those found in female flowers. The style lengths were shorter than those in female flowers, and highly variable (Figure 2D, Table 2). The stigma surface in Plant No. 29 and 189 was reduced compared to those in female plants (Figures 2B, 2C and 2D). Pollen grains from Plant No. 29 and 189 were similar in morphology to those from ordinary male plants. All of these pollen grains

Table 1. Sex ratios of flowering individuals of *S. wikstroemiifolia* in Beichatian Mountain.

Year	M	F	Sex Ratio	Chi-square and <i>P</i> value
2007	35	30	1:0.86	$\chi^2 = 0.25, P = 0.62$
2008	24	18	1:0.75	$\chi^2 = 0.6, P = 0.44$
Total	44	35	1:0.8	$\chi^2 = 0.81, P = 0.37$

M= the number of male plants, F= the number of female plants. There are 17 male and 6 female plants that flowered in two of the three years; therefore, the figure in the bottom row represents the actual number of flowering individuals among the population during the study period.

Table 2. Flower morphology measurements and stamen number per flower of *S. wikstroemiifolia*.

	Male	Female	Plant No. 29	Plant No. 189	<i>F</i> and <i>P</i> value
Sepal length (mm)	0.77 ± 0.018 [1.122, 0.485] (A) (55, 15)	0.56 ± 0.013 [0.821, 0.382] (C) (69, 16)	0.72 ± 0.025 [0.963, 0.401] (A,B) (24, 5)	0.7 ± 0.021 [0.885, 0.454] (B) (24, 5)	<i>F</i> = 34.98 <i>P</i> *****
Petal length (mm) [†]	2.9 ± 0.05 [3.756, 2.101] (A) (65, 15)	1.81 ± 0.028 [2.289, 1.141] (D) (65, 16)	2.48 ± 0.046 [2.848, 2.017] (B) (25, 5)	2.1 ± 0.052 [2.748, 1.557] (C) (25, 5)	<i>F</i> = 136.37 <i>P</i> *****
Petal width (mm) [†]	1.69 ± 0.034 [2.337, 1.16] (A) (64, 15)	1.17 ± 0.029 [1.641, 0.646] (C) (65, 16)	1.61 ± 0.031 [1.935, 1.361] (A) (25, 5)	1.29 ± 0.041 [1.736, 0.853] (B) (25, 5)	<i>F</i> = 59.41 <i>P</i> *****
Stamen length (mm) [†]	2.31 ± 0.05 [4.099, 1.167] (A) (138, 15)	1.18 ± 0.037 [1.883, 0.637] (D) (63, 16)	2.07 ± 0.061 [2.905, 1.138] (B) (34, 5)	1.59 ± 0.05 [1.985, 0.998] (C) (29, 5)	<i>F</i> = 130.2 <i>P</i> *****
Ovary length (mm)	1.15 ± 0.048 [1.521, 0.826] (B) (15, 15)	1.47 ± 0.059 [1.766, 0.951] (A) (16, 16)	1.46 ± 0.117 [1.85, 1.159] (A) (5, 5)	1.22 ± 0.11 [1.504, 0.989] (B) (5, 5)	<i>F</i> = 6.41 <i>P</i> ****
Ovary width (mm) [†]	1.01 ± 0.038 [1.279, 0.712] (15, 15)	1.09 ± 0.024 [1.242, 0.909] (16, 16)	1.12 ± 0.03 [1.166, 1.015] (5, 5)	1.08 ± 0.092 [1.293, 0.808] (5, 5)	<i>F</i> = 1.49 <i>P</i> = 0.27
Style length (mm)	N.A.	1.8 ± 0.088 [2.38, 1.129] (B) (16, 16)	2.29 ± 0.049 [2.407, 2.134] (A) (5, 5)	0.87 ± 0.251 [1.666, 0.218] (C) (5, 5)	<i>F</i> = 19.58 <i>P</i> *****
Stamen number/flower [†]	12.3 ± 0.39 [18, 8] (A) (40, 40)	4.6 ± 0.18 [5, 3] (B) (16, 16)	6.6 ± 0.4 [8, 6] (B) (5, 5)	6.2 ± 0.49 [8, 5] (B) (5, 5)	<i>F</i> = 97.68 <i>P</i> *****

Flower characteristics compared with Welch's ANOVAs are noted with “†”. The *F* and *P* values resulting from ANOVAs or Welch's ANOVAs are presented in the last column. The *P* value is denoted as **** and ***** when less than 0.005 and 0.0001, respectively. The mean with standard error (± SE) for each measurement is listed on the first row of each cell. The maximum and the minimum values are given as [maximum, minimum] on the second row. Letters in the beginning of the third line represent significant differences between categories at the 0.05 level based on Duncan's multiple range test. The numbers of samples measured and flowers used in each measurement are noted in the third line as (samples, flowers). The styles in male flowers are absent; therefore, no length is indicated.

were triporate and spheroidal, with small granules on the surface.

All other recorded flowering individuals produced only one type of flowers, either male or female. None of the 81 individuals changed their sexes throughout the observation period (2007-2009). In Pinglin, one flowering individual was male (Plant No. 115) and the other was female (Plant No. 116). Both of them flowered each year from 2007 to 2009. In Beichatian Mountain, 44 male and 35 female plants were found. Among these plants, 17 male and six

female plants flowered two of the three years from 2007 to 2009, and six males and nine females flowered every year. There were more flowering male than flowering female individuals in Beichatian Mountain in 2007 and 2008, as counted either separately or cumulatively, but the sex ratios, which do not include Plant No. 29 and 189, did not deviate significantly from 1:1 (Table 1).

Flower and inflorescence morphology

We measured flower morphologies for male flowers,

Table 3. The locule number per ovary and description of the ovule morphology in flowers from female plants and plants No. 29 and 189. N, the number of flowers examined.

Types and numbers of the locule and ovule in one flower	Female (N = 16)	No. 29 (N = 5)	No. 189 (N = 5)
Two locules per ovary. Two large ovules and two small ovules in each locule	15	0	0
One locule per ovary. Two large ovules and two small ovules in each locule	1	5	2
One locule per ovary. One large ovule and one small ovule in each locule	0	0	1
No locule and ovule in the ovary	0	0	2

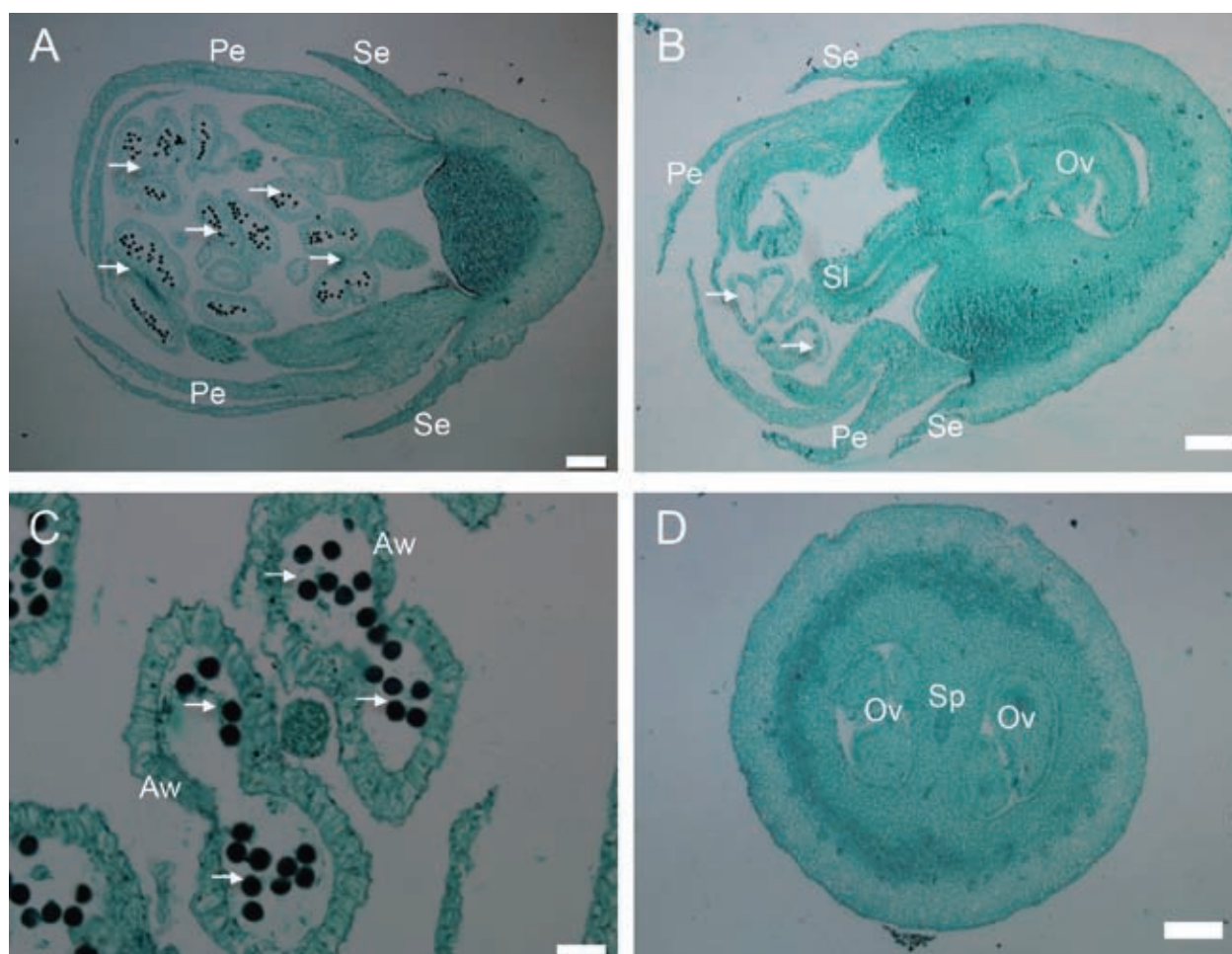


Figure 3. A male (A,C) and a female (B,D) flower bud of *S. wikstroemiifolia*. (A) A male flower bud in a longitudinal section; (B) A female flower bud in a longitudinal section. The anthers were empty; (C) A close-up of one anther of a male flower bud in cross-section; (D) An ovary of a female flower in cross-section. AW, anther wall. Ov, ovule. Pe, petal. Se, sepal. Sl, style. Sp, septum. Arrows indicate the anthers in (A,B) and pollen grains in (C). Scale bars = 200 μ m in (A, B, D); scale bar = 50 μ m in (C).

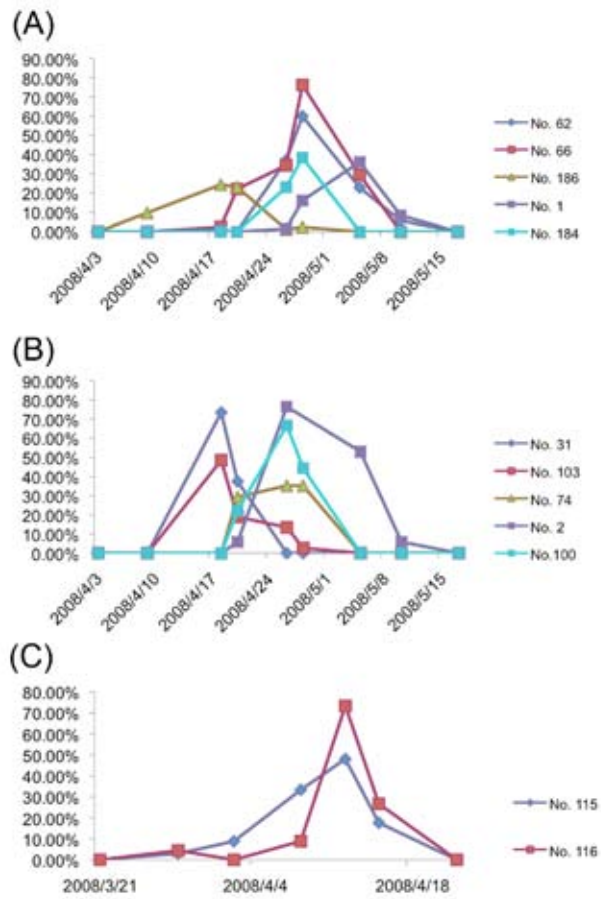


Figure 4. The flowering season duration of *S. wikstroemiifolia* in 2008. (A) Male plants in Beichatian Mountain. (B) Female plants in Beichatian Mountain. (C) Plants No. 115 and 116 in Pinglin. The y-axis indicates the percentage of inflorescences with open flowers.

female flowers, and morphologically hermaphroditic flowers from Plant No. 29 and 189. Table 2 shows the mean, standard error (SE), maximum, minimum, and sample size of each measurement in flower morphology and stamen number per flower. The morphological characteristics that were compared with Welch's ANOVAs are also presented in Table 2. Male flowers showed significantly longer and larger sepals, petals and stamens than female flowers or the two unusual plants (No. 29 and 189) ($P < 0.0001$), except that Plant No. 29 had a sepal length and petal width comparable to the males. Female flowers had significantly longer ovaries than the males and Plant No. 189 ($F = 6.41$, $P < 0.005$), but did not differ significantly from Plant No. 29. The width of the ovaries was not significantly different among the different flower types ($F = 1.49$, $P = 0.27$). The average style length was significantly different in female flowers, Plant No. 29 and 189 ($F = 19.58$, $P < 0.0001$). Morphologically hermaphroditic flowers from Plant No. 29 contained the longest mean style length compared to the others, whereas the mean style length was the shortest in flowers from Plant No. 189 (Table 2). The styles of some flowers from Plant No. 189 were highly reduced (Figure 2D).

The mean stamen number per flower significantly differed among flower categories (Welch's ANOVA, $F = 97.68$, $P < 0.0001$) (Table 2). The value in male flowers ranged from 8 to 18, with a mean of 12.3 (SE = 0.39), which was significantly higher than that in the other flower types. Most male flowers contained fewer than 15 stamens (80% of 40 examined male flowers). The stamen (staminode) number per female flower ranged from three to five, and most female flowers (69% of 16 examined female flowers) contained five stamens (staminodes). The range of stamen number per flower was six to eight and five to eight in Plant No. 29 and 189, respectively.

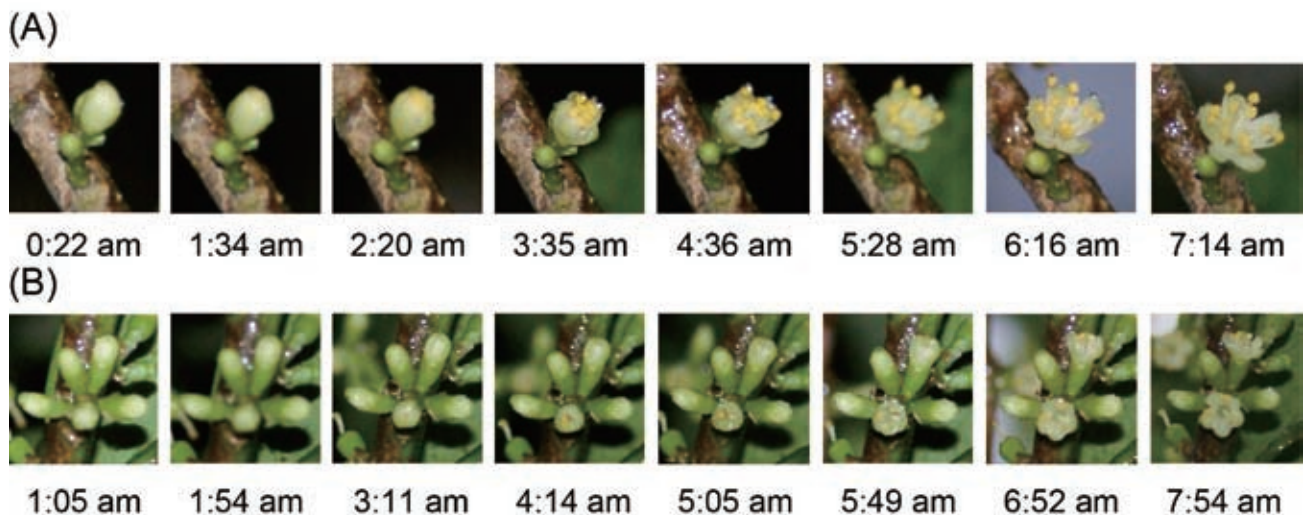


Figure 5. Anthesis of male and female flowers in *S. wikstroemiifolia*. Photos were taken in Pinglin on March 23rd 2009. The time is indicated below each photo. (A) A male flower from Plant No. 115. The petals opened slightly between 1:34 am to 2:20 am, and the flower completely opened about 7:14 am. (B) Female flowers from Plant No. 116. The petals opened slightly before 3:11 am, and the flowers completely opened about 7:54 am.

The number of pollen grains per anther in male flowers ranged from 181 to 1602, with a mean of 883 (SE = 101.12). The mean of pollen grains per male flower was estimated 10860.9 (SE = 1243.84).

In female flowers, we generally found two locules per ovary (Figures 2E and 3D), but occasionally a single locule was found (sample from Plant No. 33) (Table 3). Each locule contained four pendulous and curved ovules, two large and two small, per locule (Figure 2F). The size of the large ovules was almost 1 mm long, whereas that of the small ovules was about half or one-third this size. In Plant No. 29, each morphologically hermaphroditic flower had only one locule, whereas in Plant No. 189, the five flowers examined showed different kinds of degeneration in the ovary. Two flowers had only one locule containing two large and two small ovules. The other single flower had only one large and one small ovule in one locule, whereas the remaining two flowers lacked both ovules and locules (Table 3). No ovules or locules existed in the ovaries of male flowers.

Male plants produced both unbranched (Figure 2G) and branched inflorescences (Figure 2H), whereas female plants produced only unbranched inflorescences (Figure 2I). The average flower number per inflorescence in male plants was 15.2 (SE = 0.89, range from 2 to 47), whereas that in female plants was 4.1 (SE = 0.21, range from 1 to 11). The difference was significant between the two sexes ($t = 12.09$, $P < 0.0001$).

Flowering phenology and natural fruit set

In Beichatian Mountain, the flowering season began in early April and ended in middle May, whereas in Pinglin it began in late March and ended in middle April (Figures 4A and 4B). The duration of flowering in each individual lasted for two to three weeks. Among the observed flowering individuals in Beichatian Mountain, one male individual (Plant No. 186) began to flower earlier than the other male and female individuals, whereas in Pinglin, the male (Plant No. 115) and female (Plant No. 116) individual began to flower at approximately the same time (Figure 4C).

In most unbranched inflorescences from both male and female plants, the distal flower opened first, and so fit the definition of a cyme (Figures 2G and 2I). In most branched inflorescences from male plants, the lateral unit of inflorescences was cymose because the distal flower opened first, but the main axis of the inflorescence was racemose such that the apical flower buds were still small when the flowers on the lateral unit of inflorescences opened. Branched inflorescences, therefore, fit the definition of thyrses (Figure 2H). Despite the general trend, in a few unbranched inflorescences from both male and female plants the proximal flower opened first.

Anthesis of most male and female flowers began before dawn (Figure 5). In most male flowers, the tips of the petals opened slightly in the beginning, and part of the anthers were exposed. At this stage, the filaments were still covered by the petals and could not be seen from outside.

After 2:00 am, the petals opened slowly and gradually, lasting about four hours or more. By 6:00 or 7:00 am, the petals had opened completely.

The flowering progress was similar for most female flowers. Their petals also gradually opened before dawn, and fully opened after sunrise. Both male and female flowers of *S. wikstroemiifolia* generally opened for one to two days, but a few flowers lasted for three to four days. The anther dehiscence in male flowers was mainly dependent on weather conditions. Anthers only dehiscence under dry conditions; in humid weather, anthers would not dehiscence even when the petals had fully opened for several hours. When fully opened, both male and female flowers produced a small amount of nectar with their nectary disks.

The average natural fruit set was 6.7% (SE = 2.05%) and ranged from 0% to 17.8% among 10 female individuals. We tagged an additional 144 flowers and flower buds in Plant No. 189, and recorded the natural fruit set in 2009. During the fruiting season only one developing fruit was found, so the fruit set was extremely low in this unusual individual (0.69%). The natural fruit set of Plant No. 29 was not investigated due to the scarcity of flowers on this individual in 2009.

DISCUSSION

The breeding system of *Symplocos wikstroemiifolia*

Our study shows that *S. wikstroemiifolia* exhibits nearly complete gender dimorphism. Although female flowers display a hermaphroditic appearance, the anthers on these flowers do not produce any pollen grains. This result is compatible with Nagamasu's (1998) and Wang's (2000) descriptions, and reveals that this type of flower has only female reproductive function. During the period of field investigations, nearly all flowering individuals produced either only male or female flowers, and among these plants, none of them showed changes of sex. *Symplocos wikstroemiifolia* was thus confirmed as cryptically dioecious. However, two flowering individuals produced morphologically hermaphroditic flowers that produced pollen grains, styles and ovules; these may thus be functionally hermaphroditic (but in some flowers from Plant No. 189 the ovules were absent. See later discussion).

The sex ratios in the population were slightly male-biased, but did not significantly deviate from 1:1, which provided further evidence of cryptic dioecy (*sensu* Charlesworth, 1984; Verdú et al., 2004). In dioecious species, female plants usually invest more resources in reproduction due to the production of fruits and seeds, whereas male plants lack this investment (Delph, 1999; Obeso, 2002; Wheelwright and Logan, 2004). Therefore, female plants may reproduce less frequently or reach reproductive maturity later than male plants (Delph, 1999; Queenborough et al., 2007), which would result in a male-biased flowering sex ratio (Bullock and Bawa, 1981; García and Antor, 1995; Obeso et al., 1998).

In other cryptically dioecious species in *Symplocos* section *Hopea*, the functionally female flowers produce inaperturate and sterile pollen grains (Aranha Filho et al., 2009a). These sterile pollen grains might be maintained as pollinator rewards (Cane, 1993; Kawagoe and Suzuki, 2004). However, female flowers of *S. wikstroemiifolia* did not bear any pollen grains, and although the morphologically hermaphroditic flowers from Plant No. 29 and 189 produced pollen grains, the morphology of these grains was identical to those from the male flowers, triplicate and spheroidal (Wang and Ou, 2000), not inaperturate (Aranha Filho et al., 2009a). According to Fritsch et al. (2008), *S. wikstroemiifolia* is sister to *S. tinctoria*, and the next two related lineages are *S. longipes* and *S. guadeloupensis*. These species, except *S. wikstroemiifolia*, are all hermaphroditic. Other cryptically dioecious species within this section form a monophyletic group, which is sister to *S. guadeloupensis*. Therefore dioecy is probably an autapomorphy for *S. wikstroemiifolia*. This interpretation can explain why only *S. wikstroemiifolia* displays type II cryptic dioecy in *S.* section *Hopea*.

Flower and inflorescence morphology

Several flower and inflorescence characteristics of *S. wikstroemiifolia* examined in this study differed from those in previous descriptions. The first difference was the stamen number per male flower. *Symplocos wikstroemiifolia* was reported to have 15 to 20 stamens in a male flower (Wu and Nooteboom, 1996; Nagamasu, 1998; Wang, 2000), but in our measurements, the range was 8 to 18, with most male flowers (80%) having fewer than 15 stamens.

The second difference was the locule number per ovary in the female flowers. Although the ovaries of female flowers in *S. wikstroemiifolia* were described as having three locules (Nagamasu, 1998; Wang, 2000), i.e., the same number as the other species in *S.* section *Hopea* (Fritsch et al., 2008), we generally found only two locules and in a few female flowers, only one. The ovule number per locule varies from two to four in Symplocaceae (Heywood et al., 2007) and was previously described as four in *S. wikstroemiifolia* (Nagamasu, 1998; Wang, 2000). Although we indeed found four ovules per locule, two of the four ovules were significantly larger than the others. To our knowledge, the ovule dimorphism has not previously been reported in Symplocaceae. We suggest that the two smaller ovules represent a type of early abortion, and fertilization may only occur in the two larger ovules.

Finally, the inflorescence morphology was also incongruent with published descriptions. Nagamasu (1998) considered the inflorescences to be panicles in male plants and racemes in female plants, respectively. In contrast, Wang (2000) described the inflorescence as spikes. However, we describe the inflorescences as cymes or thyrses in male plants and cymes in female plants, based on the flowering sequence within one inflorescence. Although the flowers

at the base of the unbranched inflorescences sometimes opened prior to the others, the apical flower buds at that time were well-developed and near anthesis. We suggest that these unbranched inflorescences could be derived from thyrsoid inflorescences with some reductions of the lateral units. To verify this hypothesis, it will be necessary to observe the development of the inflorescence buds.

The thyrsoid inflorescences in male plants that allow a higher number of flowers per inflorescence, together with the larger petals size of male flowers (Table 2), provide a larger floral display that may attract more pollinators than do the female plants. Males tend to be limited by mating opportunities, whereas females tend to be limited by the resources necessary for producing offspring (Bateman, 1948; Bell, 1985; Arnold, 1994). Male plants may require a higher pollinator visitation rate compared to female plants. Some studies indicate that pollinators tend to visit plants with a larger number or size of flowers or inflorescences more frequently (Conner and Rush, 1996; Vaughton and Ramsey, 1998; Ohashi and Yahara, 2001). Male individuals may therefore be more advantageous when showing larger floral display (Bell, 1985). Another explanation for this sexual dimorphism is that because female individuals generally incur a higher cost of reproduction, they cannot produce as many flowers as males (Bullock and Bawa, 1981; Armstrong and Irvine, 1989; Carr, 1991).

Flowering phenology and natural fruit set

In most dioecious species, the beginning of the flowering season is earlier in male than in female plants (Lloyd and Webb, 1977). In Beichatian Mountain, one male individual of *S. wikstroemiifolia* began to flower prior to the others observed (Figures 4A and 4B). In the first week of April, 2008 we found only male but not female flowers in this site. This suggests that male plants of *S. wikstroemiifolia* tend to flower earlier than females in the population. The flowering season began earlier in Pinglin than in Beichatian Mountain. The lower mean annual temperature in Beichatian Mountain may have caused the delay of floral initiation in *S. wikstroemiifolia*.

The natural fruit set of *S. wikstroemiifolia* in Beichatian Mountain was quite low (6.7%) compared to the value for other dioecious species. Bawa and Opler (1975) described the mean fruit set in some tropical forest dioecious trees as about 26.3%, and Sutherland and Delph (1984) even reported an average of 73.8% fruit set in dioecious species. The fruit set of *S. wikstroemiifolia* was also relatively low when compared with other *Symplocos* species. Wesselingh et al. (1999) recorded a *Symplocos* species with an average of 26.9% fruit set in a tropical montane forest in Costa Rica. Devy and Davidar (2006) reported a mean fruit set of 46% for *S. wynadense* in a wet evergreen forest in India. These comparisons raise the question of what factors cause the low fruit set in *S. wikstroemiifolia*. Further pollinator activity and pollen supplementation studies are essential to explain these factors.

Unusual hermaphroditic flowers

Although *S. wikstroemiifolia* displayed nearly complete dioecy, the population still showed slight gender variability. Among all the flowering individuals, we found two plants that produced morphologically hermaphroditic flowers that contained pollen grains. Several morphological traits of these unusual hermaphroditic flowers, such as perianth size and stamen number per flower, were between the range of male and female flowers and thus did not simply contain the organ of the opposite sex in the flowers. It seems that the recovery of hermaphroditic reproductive function may alter the development of other flower characteristics, not only the fertile, but the sterile parts.

Furthermore, our observations suggest that the male and female reproductive abilities in these unusual hermaphroditic flowers may be limited, compared to ordinary male and female flowers. Fewer stamens were present in these than in male flowers. Moreover, not all anthers contained pollen grains in flowers from Plant No. 189. These flowers may produce fewer pollen grains. Significantly fewer ovules were also present in the morphologically hermaphroditic flowers, some had no ovules at all. Their style length is highly variable, and their stigmatic surface area is smaller than on those of female flowers. These factors may result in low female reproductive function, as revealed by the extremely low natural fruit set in Plant No. 189 (0.69%).

The origin and reproductive role of the unusual hermaphroditic individuals of *S. wikstroemiifolia* remain unclear. The sexual expression pattern in Plant No. 29 indicated that a few female individuals of *S. wikstroemiifolia* might recover partial male reproductive function, since in 2007, this plant produced relatively fewer female flowers. Other flowering individuals of *S. wikstroemiifolia* did not change their sex during the observation period, thus only a few individuals in the *S. wikstroemiifolia* population may have the potential to become hermaphroditic. The occasional existence of hermaphrodites has been documented in many dioecious plant species (Lloyd and Bawa, 1984). When pollen transfers are insufficient, the occasional hermaphrodites may produce seeds through selfing (Baker and Cox, 1984), and thus allow dioecious species to persist. However, no evidence suggests that these unusual hermaphroditic flowers of *S. wikstroemiifolia* are capable of self-fertilization. Future studies should investigate such male and female reproductive functions as pollen fertility and stigma receptivity on these flowers to examine their potential for self-fertilization. Sexual expression studies of longer duration will also help elucidate the reproductive roles in these plants.

Our research clearly demonstrates that *S. wikstroemiifolia* is cryptically dioecious, but a few individuals showed incomplete gender dimorphism differentiation. Several flower and inflorescence characteristics of *S. wikstroemiifolia* differ from those of previous descriptions. The level of natural fruit set for *S. wikstroemiifolia* was lower compared to those in other dioecious or *Symplocos* species

studies. More extensive research focusing on flower development and pollination ecology is necessary to understand the evolution of dioecy in Symplocaceae.

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台灣產蕘花葉灰木之隱性雌雄異株之研究

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蕘花葉灰木 (*Symplocos wikstroemiifolia* Hayata) 是灰木科 (Symplocaceae) 中少數外觀形態為雄花 / 兩性異株花 (androdioecy) 的物種。雖然過往的研究指出蕘花葉灰木可能是隱性雌雄異株 (cryptic dioecy)，但是對該物種在野外生育地的性別表現狀況並不清楚。我們針對台灣產的蕘花葉灰木進行生殖系統 (breeding system) 與繁殖生物學 (reproductive biology) 的研究。生殖系統的調查結果顯示，來自外觀兩性花上的雄蕊並不具花粉，因此這種型態的花朵應被視為雌花。幾乎所有的開花植株在調查期間只開雄花或雌花，族群中雖然記錄到較多的雄株，但是性別比例並沒有明顯偏離 1:1。這些結果符合預想中隱性雌雄異株的特徵。然而，有兩株個體產生了一些可能具有兩性生殖功能的花朵，這顯示少數植株在性別表現上仍具備可塑性。花部外形測量顯示雄花平均具有約 12 枚雄蕊但雌花只具有 5 枚假雄蕊。每朵雄花並不具有腔室或胚珠，而每朵雌花其子房具有兩室，而每室中具有 4 枚胚珠，且胚珠依照大小可以分成兩型。雄株會產生不分支的聚繖花序 (cymes) 與密錐花序 (thyrses)，而雌株只會產生不分支的聚繖花序。以上這些外形特徵有些與過往文獻中的敘述並不一致。開花物候觀察顯示大部分的雄花和雌花在凌晨開放花苞，且單花壽命大約是一至兩天，然而，雄蕊只在乾燥的環境下開裂，因此開裂的時間並不固定。族群中的天然結實率為 6.7%，比起其他灰木屬或是雌雄異株的物種，蕘花葉灰木具有較低的天然結實率。總而言之，蕘花葉灰木的性別表現幾乎完全分化為雄性與雌性兩種型態 (gender dimorphism)，但是還是有一些小變異存在於族群中，因此這個物種應被視為隱性雌雄異株。

關鍵詞：雄花 / 兩性異株花；生殖系統；隱性雌雄異株；性別表現分化；生殖生物學；蕘花葉灰木。

