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碩士論文

Institute of Ecology and Evolutionary Biology

College of Life Science

National Taiwan University

Master Thesis

台灣瑞香之性別系統研究

Sexual System of *Daphne arisanensis* Hayata (Thymelaeaceae)

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中華民國 106 年 7 月

July, 2017

誌謝



本論文的完成，首先要鄭重感謝胡哲明老師在研究與論文撰寫上給予的發想與指導，並且讓我有充分的發揮空間，感謝口試委員王震哲老師、王俊能老師及呂長澤老師給予的指正與建議。感謝于仁勇學長提供台灣瑞香的族群樣點與觀察經驗；林奐宇學長提供標本資料庫及軟體操作的協助；曾妤馨學姐與楊承瑞學長對試驗設計提供建議；馬弘文同學與陳凱修同學在論文主題上的討論；李鳳鳴老師熱心的提供試驗藥品；莊羚、王錦堯同學及洪培芳同學協助野外工作；鐘詩文老師、黃俊溢學長、翁明毅先生、蘇福欣先生、潘旻翰同學及張家昀同學分享野外觀察記錄。感謝 1227 研究室各位夥伴在研究上的各種協助，感謝我的家人們給予的支持。此外也感謝特生中心高海拔試驗站、救國團觀雲山莊及中油關原加油站同仁在野外調查行程中時常給予的熱心幫忙，感謝太魯閣國家公園管理處及陽明山國家公園管理處惠允試驗進行。

摘要



被子植物在性別系統上具有豐富的多樣性，除大多數種類只具單一型兩性花 (hermaphrodite) 外，單性花亦在許多類群獨立演化出現，各種性別系統的演化歷史及其適應意義是植物生殖生物學中的核心問題。台灣瑞香 (*Daphne arisanensis* Hayata) 為台灣本島產瑞香科 (Thymelaeaceae) 特有種灌木，過去文獻罕有關於其性別系統的紀錄，而據野外觀察，台灣瑞香族群中存在花藥發育不全的雄不孕型，與雌雄花器皆完整的兩性花兩種花型，就此形態而言，暗示台灣瑞香之性別系統可能為雌花兩性花異株 (gynodioecy)，然而欲確定其真實的性別系統，仍須進一步檢驗兩種花型個別的生殖潛力，以及花型在族群中的分佈模式。

本研究於 2014-2017 年沿省道臺 14 甲線，追蹤梅峰、鳶峰、合歡山、大禹嶺及關原地區台灣瑞香族群的開花型表現，並分別於中海拔關原族群及合歡山周圍高海拔族群進行授粉試驗。結果顯示三年之間沒有植株改變其開花型；雄不孕型花除了在自然授粉及人工授粉下皆正常結實外，於無授粉條件下仍有部分的花朵能夠結實，顯示雄不孕型為具無融合生殖能力 (apomixis) 的雌花；兩性花型則在所有授粉處理下幾乎都無結實，支持兩性花型為功能性的雄花。調查中另有兩株兩性花型植株 (即功能性雄株) 開出少量具不同程度雄不孕的花型，極端雄不孕的形態與雌花相似，且在自然授粉狀態下能夠結實，顯示雄株偶能重建部分的雌性生殖能力，而所有授粉條件下產生的種子皆具有萌芽的潛力。透過活體花粉萌發試驗則發現，雄花的柱頭對自體或異株花粉仍都具有接受性 (receptivity)。不同族群間在形態特徵、花型比例及開花物候上都有高度的變異。相較於關原族群，高海拔族群具有較大的花朵、較長的開花時間、較低的自然結實率以及較低的無融合生殖能力，而兩族群皆觀察到由多種非專一性夜行蛾類進行授粉。

此外，為探討台灣瑞香性別系統可能的演化背景，本研究另於 2017 年春季陽明山地區，針對同屬中另一兩性花物種白花瑞香 (*Daphne kiusiana* Miq. var. *atrocaulis* (Rehder) Maekawa) 進行套袋試驗。結果顯示白花瑞香在隔絕授粉者之下仍有 49% 的花朵能結實，支持瑞香屬植物中普遍具有自發性自花授粉的現象。

綜合上述結果，本研究確認台灣瑞香的性別系統為具滲漏性 (leakiness) 的隱性雌雄異株 (cryptic dioecy)，開花特性在不同族群間有高度變異，而此雌雄分化的生殖適應可能與瑞香屬中普遍的自花授粉特性有關。



關鍵字：隱性雌雄異株、花部多型性、授粉生物學、自花授粉、瑞香科

ABSTRACT



Angiosperms exhibit great diversity of sexual systems, as the majority of extant species are hermaphrodite, the evolution of unisexuality has drawn attention since Darwin's age. The reproductive biology of *Daphne arisanensis* Hayata (Thymelaeaceae), an endemic shrub in Taiwan, had rarely been studied before. Field observations implied that populations of *D. arisanensis* comprise morphologically bisexual and male-sterile individuals, hence this species may be gynodioecious. However, no unequivocal evidence had been presented yet. To clarify the true sexual system of *D. arisanensis*, I investigated the spatial and temporal expression of sexuality, and tested the reproductive potential of each floral morph.

In this study, phenology surveys were conducted in Meifeng, Yuanfeng, Hehuanshan, Dayuling and Guanyuan during 2014-2017. Pollination experiments were operated in Guanyuan and the Hehuanshan-nearby alpine populations respectively. No individual altered the flowering type in this three-year observation. Male-sterile flowers had the ability to set fruits either with pollination or without fertilization (i.e. apomixis). The gynoecia of morphologically bisexual flowers displayed receptivity to pollens from either itself or other plants, but they hardly set fruits among all pollination treatments. In addition, unusual flowers with different degrees of male-sterility were occasionally found on morphologically bisexual plants. The extremes were quite similar to the typical "female" flowers in male-sterile plants and indeed set fruit under open pollination. The seeds from all kinds of treatments showed germination potential. Great variations in morph ratio, floral traits and floral phenology were found among populations. Compared to the Guanyuan population, individuals in alpine areas showed larger flower size, longer floral lifespan and lower fruit-set rates under both open pollination and apomixis. Various

nonspecific nocturnal moths were observed pollinating in both of the populations.

To provide materials for inferring the possible evolutionary scenario of the gender divergence in *D. arisanensis*, I also conducted bagging experiments to another congeneric hermaphroditic species, *D. kiusiana* Miq. var. *atrocaulis* (Rehder) Maekawa, in Yangmingshan. The results show that 49% of the bagged flowers had set fruits via autonomous selfing, suggesting that self-pollination may be common in genus *Daphne*.

These findings indicate that the sexual system of *D. arisanensis* is cryptic and leaky dioecy. The flowering phenology and floral traits varied among populations, and the evolution toward dioecism would probably be correlated with the self-pollination pressure in genus *Daphne*.

Keywords: cryptic dioecy; floral polymorphism; pollination biology; self-pollination; Thymelaeaceae

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Chapter 1 Introduction



1.1 Diversity of sexual systems in angiosperms

Sexual system, referring to the sexual expression pattern (female and male) in populations, is a key factor in reproductive biology and evolutionary process (Bawa, 1980). Angiosperms exhibit astonishing diversity of sexual systems, with various combinations of male and female function at flower, inflorescence, and individual level. While the majority of extant species are monomorphic with all individuals containing single type of bisexual flowers (i.e. hermaphrodite) in the flowering plants, unisexual flowers had repeatedly evolved hundreds of times among different lineages (Renner, 2014). Besides hermaphrodite, monoecy is another monomorphic system characterized by the co-presence of female and male flowers on each individual. As for dimorphic sexual systems, populations comprised of two distinct morphs with differential sexual expression. The sexes of the two morphs could be entirely male or female, known as dioecy, or one morph is bisexual with the other being male (androdioecy) or female (gynodioecy) (Barrett, 2002a). More complicated cases also could be found, such as trimorphism in papaya (Deputy et al., 2002), temporal sex changing in *Arisaema* (Policansky, 1981) and polygamy with various flowering morphs and individuals in *Acer* (Sakai & Oden, 1983). Complex combinations of bisexual and unisexual flowers among individuals make the great sexual diversity in plants. Barrett (2002a) concluded that without recognizing the quantitative nature of sexual expression and the potential lability, one would fail to interpret the sexual systems appropriately. In addition, the morphological appearance of floral morphs sometimes is inequivalent to their actual function, thus identifying sexuality in plants should be done with functional criteria.

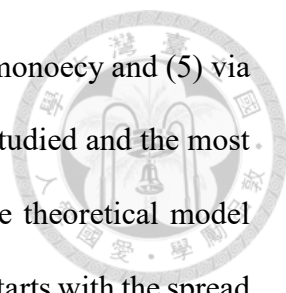
1.2 Evolution of dioecy and gynodioecy

Dioecy, characterized by separating female and male function into distinct individuals, is found in only about 6% of angiosperm species (Bawa, 1980; Renner, 2014). Despite the rareness at species level, Renner (2014) estimated that extant dioecism had 871-5000 independent origins in 43% of the angiosperm families, indicating frequent transitions from hermaphroditism to dioecy during the course of evolution, and thus fascinated and puzzled many botanists since Darwin's age (Barrett, 2010a; Darwin, 1876).

Having separate sexes could potentially bring disadvantages in several aspects for the flowering plants. First, pollination may be less efficient in the unisexual flower system, since single visit of pollinator in bisexual flowers could accomplish pollen dispersal and pollen receiving simultaneously. Second, the effective population size decreases as male plants lose their ability to sire seeds. Finally, because fertilization between unisexual plants totally relies on pollen vectors, dioecious plants would raise the risk of being unable to reproduce when pollinator service is scarce or the opposite sex is not present nearby (Bawa, 1980).

On the contrary, dioecism could be beneficial as it guarantees outcrossing rate and avoids inbreeding depression, and the gender optimization after sexual selection may lead to greater reproductive success in total (Barrett, 2002a). It is noteworthy that many predominantly dioecious systems are actually leaky or labile, with occasional presence of opposite sex or bisexual flowers in unisexual individuals, or individuals have the ability of changing sexual expressions (Humeau et al., 1999; Venkatasamy et al., 2007). The flexible reproductive strategies in these species could overcome the drawback of dioecy and still be able to produce seeds.

Bawa (1980) proposed five evolutionary pathways from hermaphrodite toward



dioecy: (1) directly, (2) via gynodioecy, (3) via androdioecy, (4) via monoecy and (5) via heterostyly. Among them, gynodioecy pathway is perhaps the most studied and the most frequent one toward the evolution of dioecy (Barrett, 2002a). In the theoretical model based on Charlesworth and Charlesworth (1978), gender divergence starts with the spread of male-sterile mutants (i.e. female) in the ancestral hermaphrodite population. The females should have some relative advantages in reproduction to compensate their loss of male function, which may result from higher quality of the outcrossed-offspring, higher fecundity of the female flowers and the greater floral display owing to resource reallocation. The required magnitude depends upon the underlying inheritance mechanism of male-sterility. In nuclear inheritance system of sexuality, the fecundity of females has to be at least twice over the bisexual plants, while no such criterion is required in nuclear-cytoplasmic inheritance system. Once the female frequency is maintained stably in the population, a gynodioecious interstate is met. The second step toward dioecy involves the transition of the other bisexual plants to males. The presence of females would further exert selective pressure of maleness to the bisexual plants. Mutant with allocating to better male function or even totally males would be favored, and they could gradually substitute the bisexual plants. At the end of this process, bisexuality is basically ruled out, and all individuals are either female or male.

According to this model, gynodioecy is usually thought to be correlated with dioecy, and the relative maleness in the bisexual flowers could be interpreted as the evolutionary status toward dioecy (Spigler & Ashman, 2012). Alternatively, gynodioecious systems may be actually stable and does not necessarily lead to dioecy. Dufay et al. (2014) suggested that if the relative female advantage is not strong enough in a gynodioecious system, the second step toward dioecy may not occur. This condition probably would be met in those self-incompatible lineages, in which the bisexual plants have no inbreeding

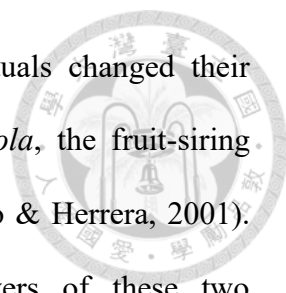
depression and the female advantage only stems from resource allocation (Karron et al., 2011).



1.3 Previous studies about sexual diversity in *Daphne*

Daphne (Thymelaeaceae) is a genus containing about 95 species mainly distributed in Asia, Europe and Northern Africa to Northern India (Herber, 2003). The diversity center may be in East Asia, since Flora of China alone lists 52 *Daphne* species and 41 of them are endemic to China (Wang et al., 2007). The delimitation between genus *Daphne* and *Wikstroemia* was thought to be ambiguous and difficult (Herber, 2003), and Halda (2001) further included several related genera, *Diplomorpha*, *Eriosolena*, *Rhamnoneuron* and *Wikstroemia*, into *Daphne sensu lato*, but only the traditionally recognized *Daphne* species are considered here. Most of the *Daphne* are regarded as hermaphrodites, while several species have been proposed to be dioecious or gynodioecious.

Four *Daphne* species in Japan had been documented being dioecious without empirical data or illustration: *Daphne kiusiana* Miq, *Daphne pseudomezereum* A. Gray, *Daphne miyabeana* Makino and *Daphne odora* Thunb. (Hamaya, 1955). The credibility of these descriptions had been doubted in Flora of Japan (Iwatsuki et al., 1999). Mathew (1989) also noted that some transplanted individuals of *D. kiusiana* were actually hermaphrodite, suggesting the sexual systems of *Daphne* in Japan should be reconsidered. To my knowledge, only two *Daphne* species were reported to be gynodioecious with unequivocal evidence. One is *Daphne jezoensis* Maxim. in Northern Japan (Kikuzawa, 1989; Sinclair et al., 2016) and the other is *Daphne laureola* L. in Europe (Alonso & Herrera, 2001; Alonso et al., 2007). These two species comprised of female flowers and bisexual flowers in their natural populations. In *D. jezoensis*, bisexual flowers had less

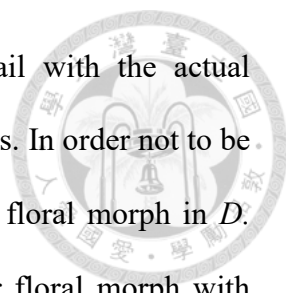


female fecundity compared to female flowers, and some individuals changed their sexuality among years (Sinclair et al., 2016). While in *D. laureola*, the fruit-siring potential of bisexual flowers was similar to female flowers (Alonso & Herrera, 2001). The differential allocations to maleness in the bisexual flowers of these two gynodioecious species were interpreted as their evolutionary status toward dioecy (Sinclair et al., 2016).

1.4 Floral polymorphism in *Daphne arisanensis*

Daphne arisanensis Hayata (Thymelaeaceae) is an endemic shrub to Taiwan. In the publication of this species, Hayata (1912) described two forms of individuals coexisted in Mt. Alishan (Arisan in original text, 阿里山), one with white flowers and yellow berries and the other with yellow flowers and red berries, but he did not mention any difference in sex organs or other floral morphology. The differential flower colors were regarded as variations within species since then by the followed taxonomists.

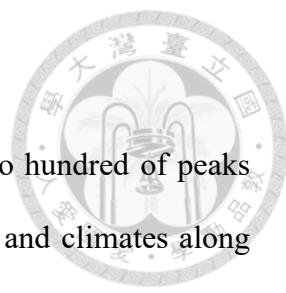
It was not until 2014 that the sexual polymorphism in *D. arisanensis* was first recorded in the taxonomic work by Yu (2014). Yu noted that certain populations have both female flowers and bisexual flowers. The female flowers were characterized by their smaller size of floral tube and stamens. Yu further stated that these two morphs could be presented on the same individual, and the ovaries in bisexual flowers seemed to be fertile (Yu, field observation), and he suggested *D. arisanensis* was likely to be gynomonoeious. However, later in August 2014, I visited one of the sampled populations by Yu (2014) in Guanyuan and found that the two morphs of flowers were mainly separated on different individuals, indicating a gynodioecious system instead. The preliminary observations provided hints about the consequence of gender divergence in *D. arisanensis*, but the



actual status of the sexual system have to be evaluated in detail with the actual reproductive function of each floral morph among natural populations. In order not to be confused between the morphology and the actual function of each floral morph in *D. arisanensis*, here I use the term “male-sterile” to infer the smaller floral morph with shriveled anthers, and “morphologically bisexual” (but abbreviated as “bisexual” hereafter) for the morph with larger flowers and complete anthers, both in the sense of morphology. Detailed description and figures of these two morphs can be found in Chapter 2. The functional states about these two floral morphs are discussed in Chapter 4.

Besides *D. arisanensis*, several *Daphne* species are known to Taiwan. Yu (2014) treated many of these names and relative species as synonyms of *D. arisanensis*, including: *D. morrisonensis* C.E. Chang, *D. chingshuishaniana* S.S. Ying, *D. nana* Tagawa, and *Stellera formosana* (Hayata) H.L. Li. The previously recognized *D. morrisonensis* was described as being similar to *D. arisanensis* and distinguished by narrowly linear leaf shape and obscure lateral veins (Chang, 1993). Yu (2014) found these characteristics were actually included in the continuous variation of *D. arisanensis*, and the samples of “*D. morrisonensis*” were indistinguishably clustered with typical *D. arisanensis* in the phylogenetic analysis. Here I followed this taxonomic treatment and included the previously identified *D. morrisonensis* in this study. The other two recognized species of *Daphne* in Taiwan by Yu (2014) were *Daphne kiusiana* Miq. var. *atrocaulis* (Rehder) Maekawa and *Daphne genkwa* Siebold & Zucc, both reported to be hermaphroditic.

1.5 Research aims



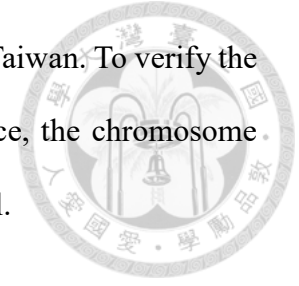
Taiwan is a mountainous subtropical island, with more than two hundred of peaks over 3000 m in height (Hsieh & Shen, 1994). Complex landscapes and climates along elevation gradient bring Taiwan an abundant flora, more than 4000 native plant species have been described and nearly 26% of them are endemic (Hsieh, 2003). In contrast to the relatively complete survey on species diversity, detailed information on reproductive biology of plants in Taiwan is extremely insufficient (Tseng et al., 2008). Most of the data about sexual systems rely on the morphological description in Flora of Taiwan (Tseng et al., 2008), which may be ambiguous and even inequivalent to their real function (Wang & Hu, 2011), and still some floral polymorphisms may have not been recognized yet, as in *D. arisanensis*. Thus, more intensive field observations and empirical evidences are fundamental to complete our knowledge of plant sexual diversity in Taiwan.

The primary aim of this research is to clarify the true sexual system of *D. arisanensis*, and it was achieved via three aspects: (1) Measurements of various floral traits to quantify the morphological difference between the two floral morphs. (2) Investigations of the spatial and temporal expression of floral morph in natural populations. (3) The reproductive potential of each floral morph, which was examined under pollination experiments, *in vivo* pollen germination, and seed germination tests.

Furthermore, great variations in floral biology were found among populations during the survey. To compare the reproductive strategy of *D. arisanensis* in different environments, I conducted another set of pollination experiments in the alpine populations and observed the pollinating fauna.

Finally, to sketch the possible evolutionary scenario of the sexual system in *D. arisanensis*, I also investigated the self-pollinating ability in the congeneric *Daphne*

kiusiana var. *atrocaulis*, another hermaphroditic *Daphne* species in Taiwan. To verify the possible interaction between polyploidization and gender divergence, the chromosome number of these two species was examined to infer their ploidy level.



Chapter 2 Materials and Methods



2.1 Study species and sites

Daphne arisanensis Hayata, is widely distributed on forest floor at the altitude from 1500 m to 3500 m in Taiwan. The individual heights range from 0.5 m to 3 m. The inflorescences are capitate and usually formed at terminals of branches. The flowers comprise of 4-lobed calyx tube without petals, the colors are white to greenish-yellow. The eight stamens arrange in two rows. In male-sterile morph, the anthers are brownish and shriveled at anthesis, with no pollen inside. The ovary is superior with insignificant style and single-loculed with only one ovule (Figure 2-1 and Figure 2-2). The flowering season differs among populations.

The flowering phenology observations were conducted along Provincial Highway 14A across central Taiwan during August 2014 to April 2017 (Figure 2-3). Main populations included Meifeng (梅峰, *ca.* 2100 m), Dayuling (大禹嶺, *ca.* 2700 m) and Guanyuan (關原, *ca.* 2400 m). Other small populations with less than ten individuals were Yuanfeng (鳶峰, *ca.* 2700 m), Xiaoqilai (小奇萊, *ca.* 3100 m) and Xiaofengko (小風口, *ca.* 3000 m).

Each flowering plant was labeled and the floral morph was recorded. Labeled plants were tracked for sexual expression at least once every three months. The pollination experiments and fresh flower collection were mainly conducted in the Guanyuan population. In addition, I also visited Taipingshan (太平山, Ilan County), Shaulaishan (稍來山, Taichung City) and Alishan (阿里山, Chiayi County) to examine the status of floral dimorphism among different mountain regions.

2.2 Floral traits measurement

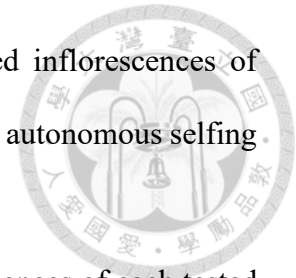
For each floral morph, 30 fresh flowers from 10 individuals were collected from Guanyuan population and stored at 4°C until measured. The flowers were dissected and pictured under stereo microscope (SteREO Lumar.V12, Carl Zeiss, Germany). The open diameter (diameter of the outer two lobes), diameter of tube throat, length of tube, length of ovary, diameter of ovary, length of ovule, and the distance between stigma and anthers were measured with imaging software AxioVision SE64 Rel. 4.9.1 (Carl Zeiss, Germany) (Figure 2-4).

The existence of pollens in anthers were examined under fluorescent staining of flower buds that near anthesis. The freshly collected buds of each floral morph were first softened with 8M NaOH solution for 60 minutes and washed in distilled water. Anthers were picked out and stained with 0.1% decolorized aniline blue for 20 minutes, then flattened with a coverslip on a microscope slide. The flattened anthers were observed under a fluorescence microscope (BX51, Olympus, Japan) to examine the existence of pollen grains, and the viable pollens would show bright florescence.

2.3 Pollination experiments

To examine the reproductive potential of each floral morph, pollination experiments were conducted in the Guanyuan population during the summers of 2015 and 2016. The treatments included: (1) open pollination of bisexual morph; (2) artificial crossing of bisexual morph; (3) autonomous selfing of bisexual morph; (4) open pollination of male-sterile morph; (5) artificial crossing of male-sterile morph; (6) bagging of male-sterile morph. The open-pollination treatments of each floral morph were employed to evaluate the natural pollination efficiency, and the artificial-crossing treatments provided


estimations of the maximum fecundity. The unmanipulated bagged inflorescences of bisexual morph and male-sterile morph were regarded as undergoing autonomous selfing and unpollinating respectively.



To conduct open pollination treatments, 1-4 unopened inflorescences of each tested individual were labeled on the branches and left unbagged. In the other treatments, bagged inflorescences were used to exclude flower visitors and herbivores before the experiments. Two-nine unopened inflorescences in each plant with medium to large size were bagged by nylon net, with the mesh size approximately 0.3 mm x 0.3 mm. To conduct artificial crossing, freshly opened flowers in the bagged inflorescences were chosen. I first picked up some fresh bisexual flowers at least 100 m away from the tested individual as pollen donors, and used fine brushes as pollinating tools. Pollens could be easily deposited on the stigmas of male-sterile morph but not of the bisexual morph. To reach the stigma of bisexual flowers, the upper floral tube and stamens were cut off in operation. After artificial pollination, the rest buds in the same inflorescence were removed to avoid confusion, and the branches were bagged again. In 2015, I used all flowers which looked fresh in the same inflorescence in artificial crossing, whereas in 2016, only those at the first day of opening were chosen. In bisexual flowers, because pollens easily fell on their own stigma after anther-dehiscence, the bagged inflorescences without pollination were regarded as undergoing autonomous selfing. The developmental status of ovaries was recorded every two weeks after treatments. Once the ovary swelled and broke up the floral tube, the flower was regarded as setting fruit.

Because of the influence of Typhoon Soudelor on August 8, 2015 in this area, the roads to Guanyuan were blocked for several days and many branches in test were broken. The fruit-set count in 2015 experiments was only traced to August 5, and the other unopened buds in the tested inflorescences were excluded.

2.4 Seed germination



To exam the seed viability among different pollination treatments, the ripe fruits in previous section were collected. The seeds were picked out and the aril-like membranes were removed with tweezer in laboratory (Figure 2-2G). After washing, the fresh seed weight was measured. The seeds with broken seed coat or unusually light weight were excluded from germination test. Since the appropriate germination condition for *D. arisanensis* seeds had not been elucidated yet, I tried various treatments to break seed dormancy in this study, including: cold stratification, scarification, immersion and ½MS medium. For cold stratification, seeds were stored at 4°C for 2-8 weeks. For scarification, the seed coat was scarred with scalpel at the bottom right before planting. For immersion, the seeds were dipped in distilled water for 24 hours. Half-MS media were tried together with cold stratification, the recipe was modified from Murashige and Skoog (1962) with half concentration in salt and additional 0.3% agarose. Before planted on the ½MS media, seeds were surface-sterilized in 70% ethanol for 60 seconds and in 1% bleach for 15 minutes subsequently. The other seeds were sown on wet paper towel, together incubated in a growth chamber at 22°C with a photoperiod of 12 hours. After planting, I examined the seeds twice a week and supplied water when necessary. Once the radicle emerged to a length of 0.5 cm, the seed was scored as ‘germinated’.

2.5 *In vivo* pollen germination

To test the stigma receptivity of each floral morph, the pistils were collected and fluorescent-stained after pollination. For male-sterile morph, nine of the artificial crossed flowers and two of the unpollinated flowers were collected. For bisexual morph, eight of the artificial crossed flowers and five of the aided-selfing flowers were collected. The

pollination procedure was the same as described in section 2.3 except for providing additional pollens in selfing bisexual flowers. The gynoecia were collected 24 hours after pollination and fixed in ethanol and glacial acetic acid mixture (3:1).

The staining procedure of pollen tubes was modified from Mori et al. (2006). Pistils were first picked out and longitudinally cut half under stereo microscope, the ovules were discarded. The half-pistils were washed two times in distilled water and softened in 8M NaOH solution for 60 minutes. Softened half-pistils were then carefully washed two times in distilled water and stained with 0.1% decolorized aniline blue for 20 minutes. The half-pistils were flattened with a coverslip. Pollen tubes were observed under a fluorescence microscope (BX51, Olympus).

2.6 Pollination experiments for alpine populations of *Daphne arisanensis*

To investigate different reproductive strategies among populations, I also performed pollination experiments to the alpine populations of *D. arisanensis* during the spring of 2017. The individuals distributing at the altitude from 2700 m to 3400 m in Hehuanshan (合歡山) and the neighboring areas usually have a linear leaf shape and bloom in spring, which were previously identified as *D. morrisonensis*, now a synonym of *D. arisanensis* according to Yu (2014). The studied populations comprised of Yuanfeng, Xiaoqilai, Xiaofengko and Dayuling. Each flowering plants was labeled, and the fresh flowers were collected for measuring floral traits as described in section 2.2, the subjects were 24 flowers from eight bisexual individuals and 21 flowers from seven male-sterile plants. Pollination experiments included (1) open pollination of bisexual morph; (2) autonomous selfing of bisexual morph; (3) open pollination of male-sterile morph; (4) bagging of

male-sterile morph. The detailed procedures were the same as described in sections 2.3 and 2.5. Due to the difficulty in handling the small-sized alpine populations, no artificial crossing was conducted in this experiment. Some of the falling flowers in the second treatment, autonomous selfing of bisexual morph, were collected and carried out fluorescent staining to examine the receptivity of their stigmas.

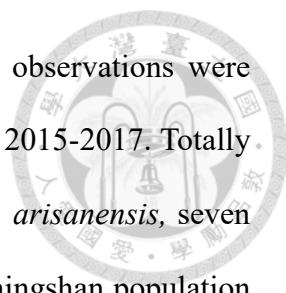
2.7 Pollination experiments for the hermaphroditic

Daphne kiusiana* var. *atrocaulis

To compare the reproductive strategies between *D. arisanensis* and other hermaphroditic *Daphne* species, I conducted another set of pollination experiments to *D. kiusiana* var. *atrocaulis* in Yangmingshan (陽明山, Taipei City) during the spring of 2017. To examine the floral monomorphism, 26 flowering individuals were field-observed in their floral morphology, and 24 fresh flowers from eight individuals were collected and dissected for floral traits measurements as described in section 2.2. The pollination treatments included (1) open pollination; (2) autonomous selfing; (3) emasculation without pollination; (4) emasculation with artificial pollination. The bagging procedure was the same as described in section 2.3. Emasculations were conducted when the buds were near open and the anthers were still indehiscent. Emasculations without pollination were employed to examine the existence and the relative contribution of apomixis in *D. kiusiana* var. *atrocaulis*. In the last treatment, supplementary pollens were hand-delivered on emasculated flowers to evaluate the influence of cutting damages to a flower.

2.8 Observation of flower visitors

The diurnal flower visitors were recorded while proceeding with other surveys and



pollination experiments in previous sections. Additional nocturnal observations were made with a two-hour period each time during flowering seasons of 2015-2017. Totally there were seven observation periods in Guanyuan population of *D. arisanensis*, seven times in the alpine populations of *D. arisanensis*, and four in Yangmingshan population of *D. kiusiana* var. *atrocaulis*. The detailed date and time of each observation is listed in Appendix Table A-1. In these observation periods, I wore a headlight and observed among the bloomed plants, the encountered flower visitors were pictured if accessible and their behavior was recorded.

2.9 Chromosome preparation

Actively growing root tips of the two *Daphne* species were collected to check the ploidy level. For *D. arisanensis*, two transplanted individuals from Meifeng and Guanyuan respectively, and four seedlings obtained in section 2.4 which had undergone apomixis were used. As for *D. kiusiana* var. *atrocaulis*, root tips were collected from a natural-growing plant in Yangmingshan. The root tips were cut off at noon and subsequently treated with 2 mM 8-hydroxyquinoline at 18°C for 6 hours, then fixed in ethanol and glacial acetic acid mixture (3:1) overnight. Before staining, the root tips were acid-hydrolyzed with 1N HCl at 60°C for 8 minutes then washed two times in distilled water. The hydrolyzed root tips were stained in leuco-basic fuchsin solution for one hour and soften with 1% pectinase solution for 30 min. Then the root tips were squashed in a drop of 45% acetic acid on a microscope slide and examined under a light microscope (BX51, Olympus).

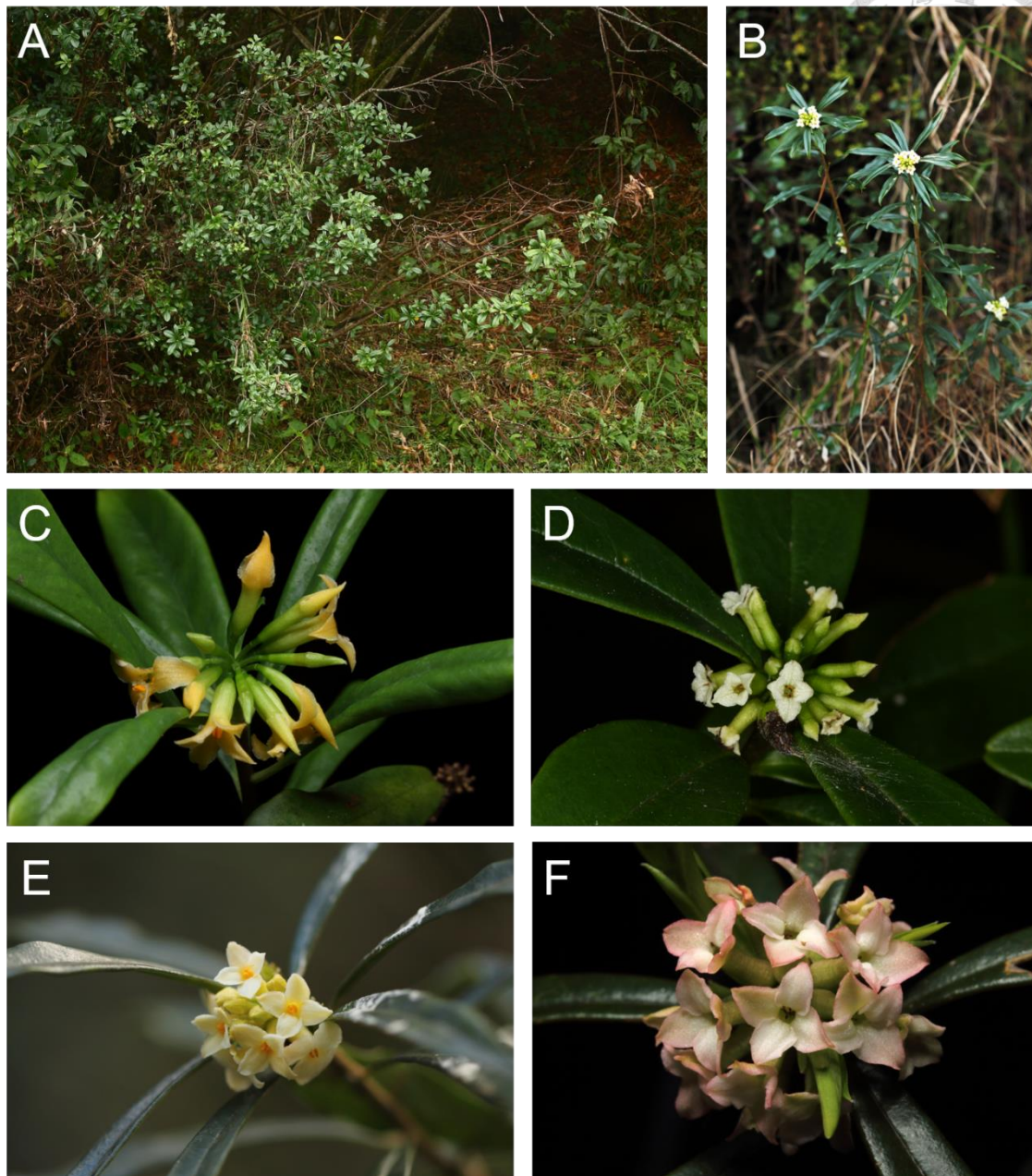


Figure 2-1. Morphology of *Daphne arisanensis*.

(A) A two meters high shrub in Guanyuan. (B) A 0.5 meters high flowering individual in Dayuling. (C) Bisexual inflorescence in Meifeng. (D) Male-sterile inflorescence in Guanyuan. (E) Bisexual inflorescence in Dayuling. (F) Male-sterile inflorescence in Dayuling.

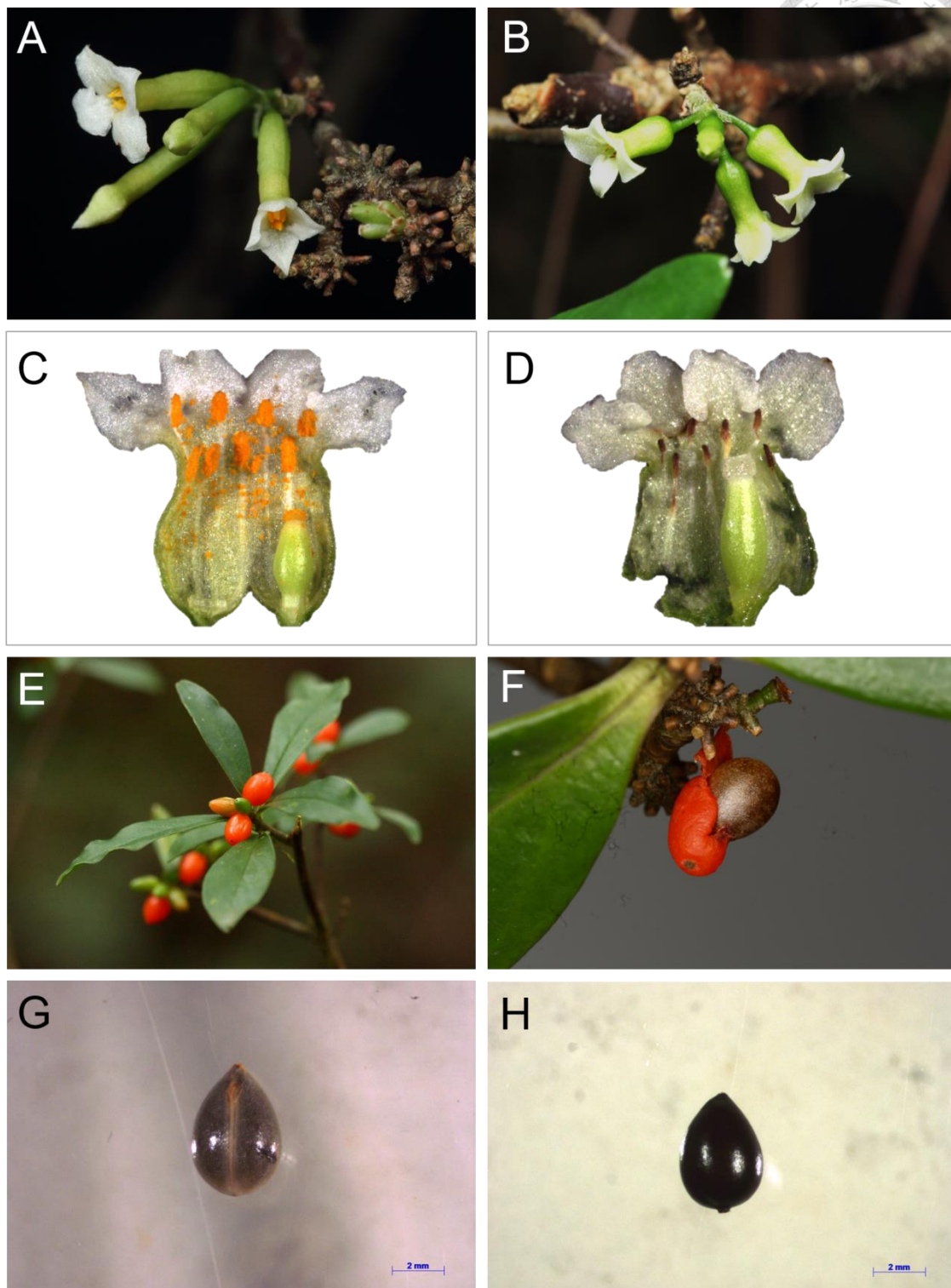


Figure 2-2. Flowers, fruits and seeds of *Daphne arisanensis*.

(A) Bisexual flowers. (B) Male-sterile flowers. (C) Dissection of a bisexual flower. (D) Dissection of a male-sterile flower. (E) Fruits. (F) Single-seeded fruit (G) Seed with aril-like membrane. (H) Washed seed.

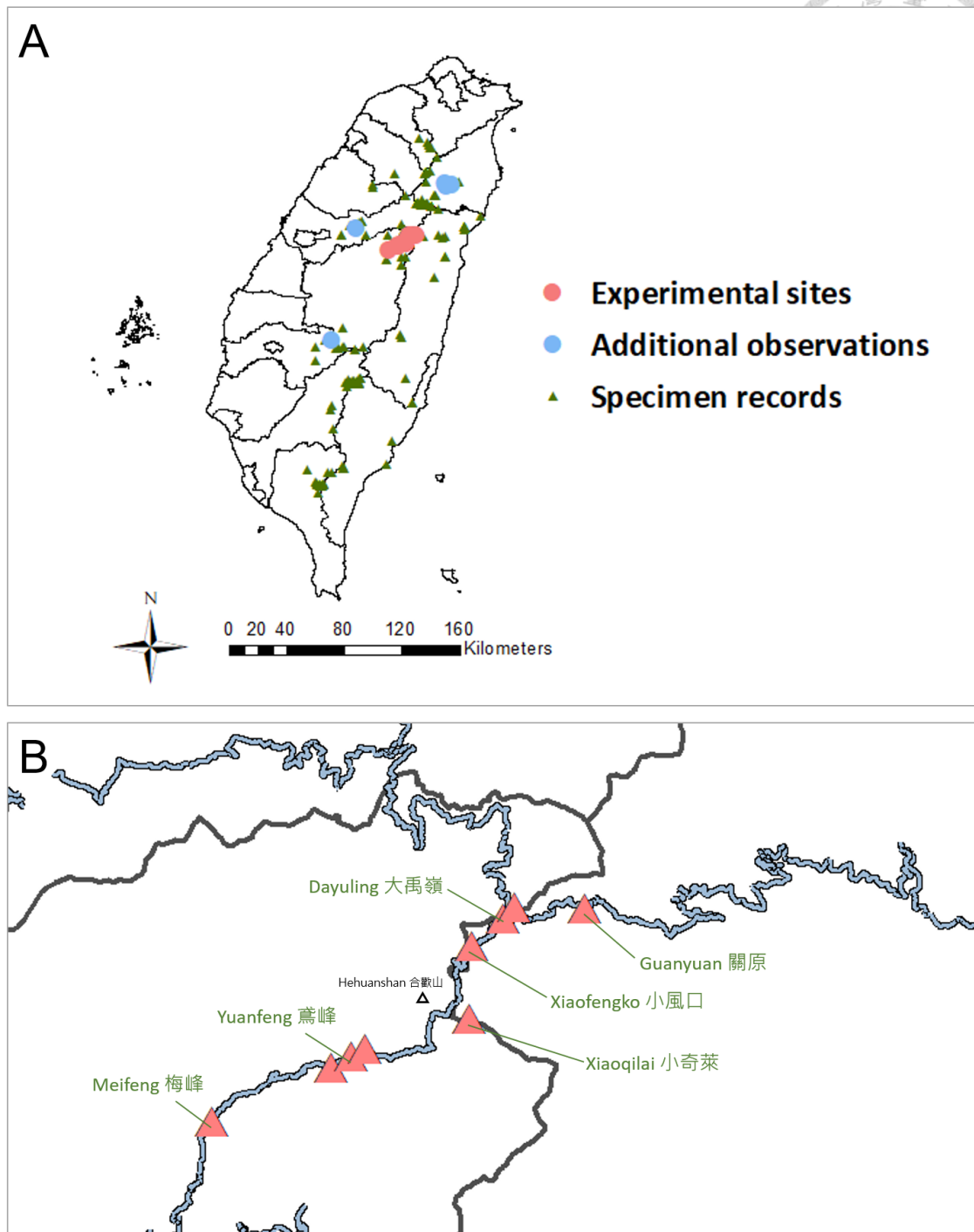


Figure 2-3. Map of study sites.

(A) Relative locations of studied areas in Taiwan. (B) Populations along Provincial Highway 14A, the blue line is road and the black line is county boundary. The “alpine populations” include individuals from Meifeng, Yuanfeng, Xiaoqilai, Xiaofengko and Dayuling.

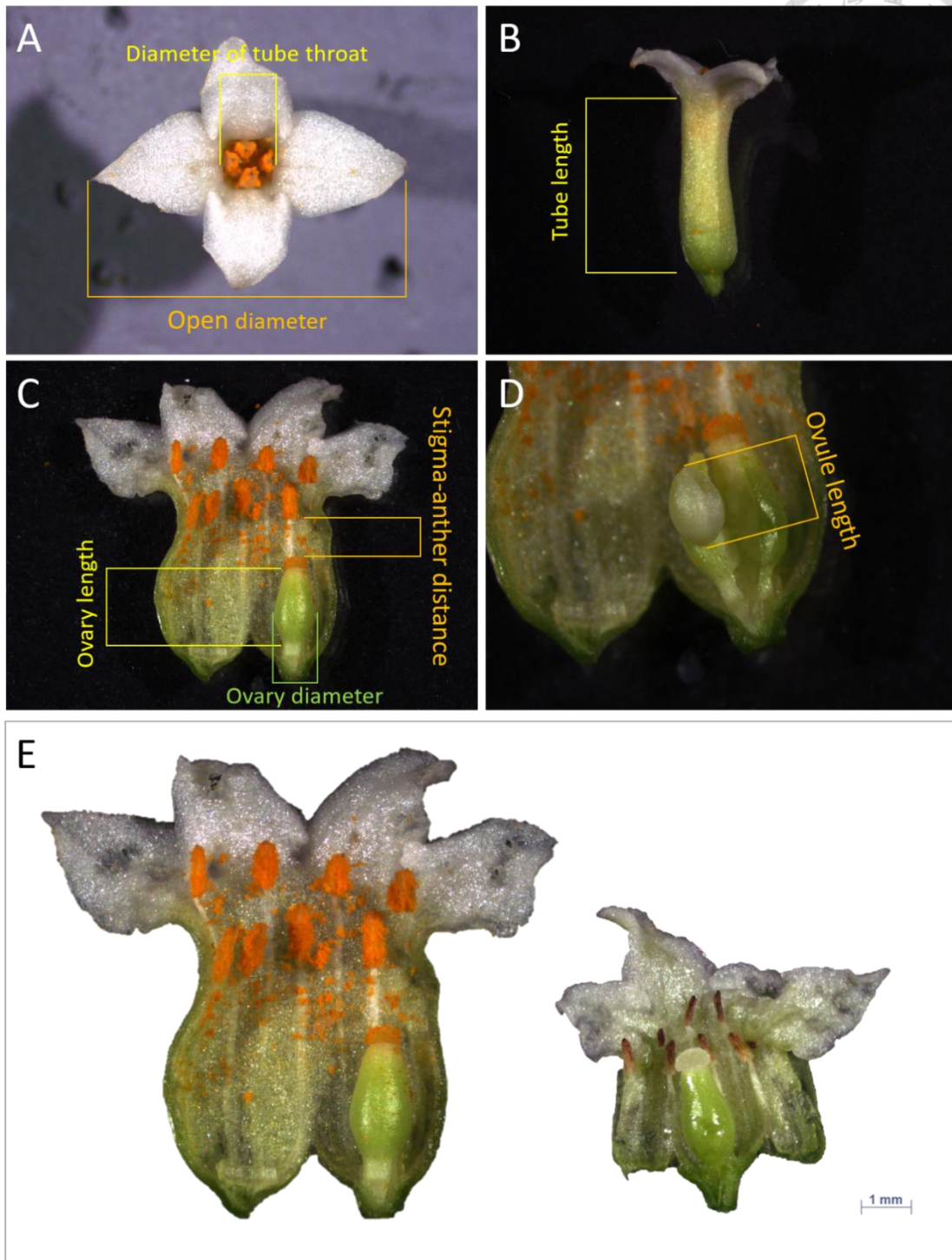


Figure 2-4. Floral traits measurements in *Daphne arisanensis*. (A) Top view. (B) Lateral view. (C) Longitudinal dissection. (D) Ovule. (E) Comparison of bisexual and male-sterile flower.

Chapter 3 Results



3.1 Flowering phenology and morph ratio

The floral dimorphism in *Daphne arisanensis* was widespread in Taiwan. Both male-sterile and bisexual morph were found in Taipingshan, Shaulaishan and Alishan.

During 2014-2017, a total of 142 flowering individuals along Provincial Highway 14A were labeled, and 81 of them were recorded for at least two continuous flowering seasons. Among the 142 flowering individuals, 72 plants produced purely male-sterile flowers and the other 70 plants bore almost all morphologically bisexual flowers (Table 3-1). No individual changed its major floral morph during the three-year observation. Some unusual flowers (less than 1%) with different degrees of male-sterility were occasionally found on the predominantly bisexual plants (Figure 3-1), the extremes were quite similar to those in a typical male-sterile plant. Fruits were commonly found on male-sterile plants while only ten of the 70 bisexual plants were recorded bearing a few fruits.

The morph composition and flowering season were quite different among populations (Table 3-1). Plants in Guanyuan usually bloomed during July-August and were biased to bisexual (deviated from 1:1 with a marginal significance, $P = 0.06$). The Dayuling population had a nearly 1:1 morph ratio and only flowered in January-April, together with other alpine populations. Individuals found in Meifeng were almost male-sterile and did not have a well-synchronized flowering time. The bisexual flowers in the Guanyuan population, which bloomed in summer, had a short lifespan, usually fell off in two days after anthesis, while the male-sterile flowers could remain fresh for around two weeks. In contrast, both bisexual and male-sterile morphs in the alpine populations which flowered in spring were relatively long-lived, and were able to hold on for 2-4 weeks.



3.2 Floral traits

Table 3-2 shows the floral traits measurements of each morph. Flowers in the alpine populations were larger than those of Guanyuan population (discussed later in section 3.6). Based on *t*-tests, the floral tubes of bisexual flowers were significantly larger than the male-sterile ones in both width and length. Ovaries and ovules were similar-sized for the two morphs. There was usually a spatial separation between the stigma and anther position in bisexual flowers. In comparison, stigmas of male-sterile flowers displayed a direct contact to the shriveled anthers, likely due to the shortening on floral tube. Despite the spatial separation, stigmas of bisexual flowers were usually found clogged by their own pollens. Fluorescent staining showed an average pollen size in bisexual flowers of 25 μm , whereas no such particle existed in the shriveled anthers of male-sterile flowers (Figure 3-2).

3.3 Pollination experiments

The results of pollination experiments conducted in Guanyuan population are shown in Table 3-3. Male-sterile flowers had a fairly high fruit-set rate (71.4% in 2015 and 62.3% in 2016) under natural pollination, while the morphologically bisexual flowers merely set one fruit among all treatments. There was a slight difference in operated subjects of open-pollination between the two years. In 2016, all flowers in tagged inflorescences were successfully monitored to the end, whereas in 2015, only those which had set fruit or fallen before the disturbance of Typhoon Soudelor were recorded. Compared to 2016, the fruit-set rate of artificially-crossed male-sterile flowers in 2015 was strangely lower (27.5%), this might be owing to the inappropriate operations described in section 2.3. The difference of fruit-set rate between artificially crossed flowers and the naturally pollinated

ones in 2016 could be interpreted as the pollen-limitation level in the Guanyuan population. Both in 2015 and 2016, some of the bagged male-sterile flowers showed the ability to set fruits even without pollination, i.e. apomixis, which gave a 13.8% and 14.7% fruit-set rate respectively. The only one bisexual flower which set fruit under bagging treatment was observed with partial male-sterility.

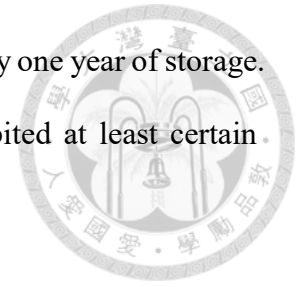
3.4 Seed viability

Due to the extremely low fruit-set rate of bisexual flowers, additional 13 naturally-pollinated fruits were also collected from nearby individuals for testing seed viability. The 13 seeds sired by bisexual plants were slightly smaller than those coming from male-sterile plants (Figure 3-3). There was no significant difference in fresh weight between the apomictic seeds and the pollinated ones.

The seeds of *D. arisanensis* had various lengths of dormancy, some of the unmanipulated seeds even retained their viabilities for more than 30 weeks and successfully germinated at last (Figure 3-4). The combination of cold stratification and scarification significantly boosted the breakdown of dormancy in my experiment. Nineteen of the 24 seeds germinated within eight weeks, whereas the cold stratification solely did not have such effect, which gave a minimum germination time of 65 days (data not plotted). Twenty-four hours of immersion seemed to have no notable influence on germination, the six seeds with this pretreatment germinated in 9-19 weeks (data not plotted). The apomictic seeds had a slightly slower germination curve than the pollinated ones (Figure 3-4). Unfortunately, seeds easily got moldy in the $\frac{1}{2}$ MS media as time passed, and 12 of the 13 bisexual-sired seeds rotted during the experiment, while the last one succeeded in germinating. It's noteworthy that besides this germination test, another

bisexual-sired seed also germinated in the 4°C refrigerator after nearly one year of storage.

In summary, seeds from different pollination treatments exhibited at least certain degree of fertility.



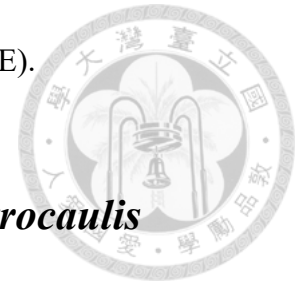
3.5 *In vivo* pollen germination

Pollens germinated vigorously in pollinated gynoecia of both male-sterile and bisexual flowers (Figure 3-5). There was no apparent difference in pollen germination between self-pollinated and artificial-crossed bisexual flowers, i.e. the stigma of bisexual flower displayed receptivity to pollens from either itself or other individuals. The pollen tubes could reach the position of micropyle within 24 hours after pollination.

3.6 The alpine populations of *D. arisanensis*

Compared with the Guanyuan population, the alpine *D. arisanensis* differed in leaf shape, flowering season, morph ratio and flower size (see section 3.1 and 3.2). Both bisexual and male-sterile morphs were larger than those of the Guanyuan population, but still with some overlap (Table 3-2 and Figure 3-6). The relatively long lifespan of flowers made a greater floral display throughout the blooming season (see Figure 2-1). Results of pollination experiments indicated a similar pattern but different frequencies in fruit-set (Table 3-4). The fruit-set rate of male-sterile flowers under natural pollination was 29.7%, quite lower than in the Guanyuan population (71.3% and 62.4%). Though apomixis in male-sterile flowers was also presented in the alpine populations, it turned out to be a rare event, with only 1.3% of the unpollinated flowers succeeding in setting fruit. Similar to the Guanyuan population, the bisexual flowers hardly set fruits among all treatments and the only one was also in a partially male-sterilized inflorescence. The gynoecia of

bisexual flowers exhibited receptivity to self-pollens too (Figure 3-5E).

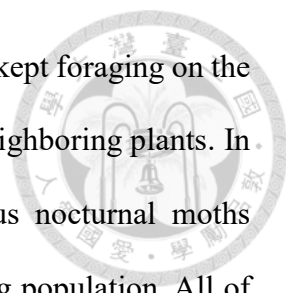


3.7 The hermaphroditic *D. kiusiana* var. *atrocaulis*

According to the field observations and floral traits measurements, there was no detectable floral polymorphism in *D. kiusiana* var. *atrocaulis* (Figure 3-7C). Among the 26 individuals observed in Yangmingshan, there was only one bearing with unusual petaloid flowers and the others were all bisexual (Figure 3-8). The petaloidy seemed to be an occasional sterile mutation, hence I concluded this species is certainly hermaphroditic. Table 3-5 shows the results of pollination experiments. All tested individuals exhibited a fairly high fruit-set rate under natural pollination. Nearly half of the bagged flowers succeeded in setting fruits, which were supposed to undergo self-pollination. The outcome of the two fruits in the emasculation-without-pollination treatment indicated a limited ability of apomixis. Compared with the open pollination treatment, emasculation followed by artificial pollination only brought about a limited fruit-set rate (22.9%), indicating emasculation itself might have a negative effect on fruit development, or the pollens had lost their viability before the stigmas reached maturity. Fluorescent staining showed the self-compatibility in pollen germination, as in the case of *D. arisanensis* (Figure 3-5F).

3.8 Flower visitors

Direct observations indicated biased recruitment to nocturnal visitors both in *D. arisanensis* and *D. kiusiana* var. *atrocaulis*. In this three-year study, only three diurnal visitors had been observed in the Guanyuan population, including a bee (Apidae) and two hoverflies (Syrphidae) (Figure 3-9). All these three insects were found on bisexual



flowers with their mouth parts contacting the anthers. The hoverflies kept foraging on the same individual for minutes, while the bee quickly moved among neighboring plants. In contrast to the poor pollinating fauna during the daytime, various nocturnal moths (Lepidoptera) had been recorded both in the Guanyuan and Dayuling population. All of the moths showed a similar foraging behavior on either bisexual or male-sterile plants, they crept on the branches and kept stretching their proboscises to find flowers. Once the proboscis sucked in a flower tube, the moth would successively visit all the other flowers in the same inflorescence. After visiting several inflorescences, they might take a rest for dozens of minutes and might stay on the same plant for hours. The headlight and the flashlights seemed to have no immediate influence on the moths' behavior. Not all of the visiting moths were successfully recorded, some of them were staying too high and others were disturbed when approached. Besides moths, many earwigs (Dermaptera) were also found feeding on pollens upon bisexual flowers in the Guanyuan population. A diurnal bumblebee (*Bombus sonani*, Figure 3-9I) was also found feeding on pollens at night in Dayuling, but it remained at the same position till the next day. As for Yangmingshan population of *D. kiusiana* var. *atrocaulis*, only four visiting moths had been recorded during the nocturnal observations, though the flowers were in full bloom. The complete list of the recorded nocturnal visitors is shown in Appendix Table A-1.

3.9 Ploidy level

For *D. arisanensis*, individuals from both Meifeng and Guanyuan population, together with the seedlings which had undergone apomictic fruiting, showed the mitotic chromosome count $2n = 18$ (Figure 3-10A-C). The Yangmingshan population of hermaphroditic *D. kiusiana* var. *atrocaulis* also gave the same chromosome number

(Figure 3-10D). Given the basis of $x = 9$ (Appendix Table A-2), *D. arisanensis* is apparently a diploid species.

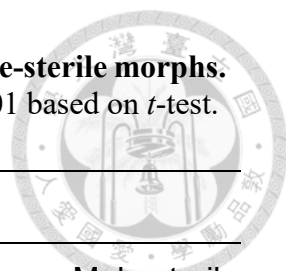


Table 3-1. Morph ratios of *Daphne arisanensis*.

Populations	♀-Male-sterile	♀-Bisexual	Total	♀:♂ Ratio	Chi-square and <i>P</i> value ^a
Overall	72	70	142	1.03	$\chi^2 = 0.028$ $P = 0.8667$
Guanyuan	25	40	65	0.63	$\chi^2 = 3.462$ $P = 0.0628$
Meifeng	20	4	24	5.00	$\chi^2 = 10.667$ $P = 0.0011$
Dayuling	20	22	42	0.91	$\chi^2 = 0.095$ $P = 0.7576$
Others	7	4	11	1.75	-

^a Chi-square tests of the deviation of morph-ratio from 1:1

Table 3-2. Comparison of floral traits between bisexual and male-sterile morphs.
Data shown are mean \pm SD. * indicates $P < 0.05$ and ** for $P < 0.01$ based on t -test.



Guanyuan population of *Daphne arisanensis*

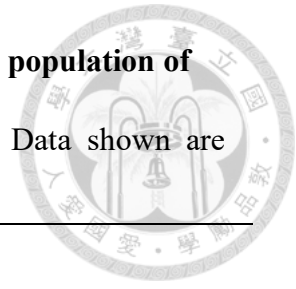
Floral traits	Bisexual	Male-sterile
Open diameter (mm)	7.50 \pm 0.79	5.63 \pm 0.54**
Tube throat diameter (mm)	1.32 \pm 0.20	0.97 \pm 0.13**
Tube length (mm)	6.84 \pm 0.61	3.73 \pm 0.36**
Ovary length (mm)	2.38 \pm 0.30	2.23 \pm 0.25*
Ovary-tube length ratio	0.35 \pm 0.06	0.60 \pm 0.06**
Ovule length (mm)	1.16 \pm 0.16	1.19 \pm 0.18
Stigma-anther distance (mm)	1.50 \pm 0.53	0**
	N = 30	N = 30

Alpine populations of *Daphne arisanensis*

Floral traits	Bisexual	Male-sterile
Open diameter (mm)	9.91 \pm 1.25	8.33 \pm 1.24**
Tube throat diameter (mm)	1.63 \pm 0.27	1.32 \pm 0.32**
Tube length (mm)	7.30 \pm 0.91	4.54 \pm 0.79**
Ovary length (mm)	3.02 \pm 0.47	2.88 \pm 0.40
Ovary-tube length ratio	0.41 \pm 0.07	0.64 \pm 0.10**
Ovule length (mm)	1.27 \pm 0.26	1.26 \pm 0.19
Stigma-anther distance (mm)	1.19 \pm 0.57	0**
	N = 24	N = 21

Table 3-3. Results of pollination experiments in the Guanyuan population of *Daphne arisanensis*.

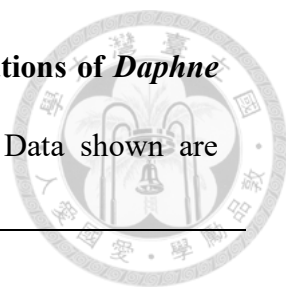
♀ means male-sterile and ♂ means morphologically bisexual. Data shown are fruiting-counts / tested number.



2015 Summer				
Treatments	Individuals	Inflorescences	Flowers	Fruit-set rate
♀ - Open pollination	4 / 4	15 / 15	102 / 143	71.3%
♀ - Artificial crossing	5 / 8	6 / 10	11 / 40	27.5%
♀ - Unpollinated	7 / 11	7 / 16	30 / 217	13.8%
♂ - Open pollination	--	--	--	--
♂ - Artificial crossing	0 / 6	0 / 7	0 / 22	0%
♂ - Autonomous selfing	0 / 4	0 / 6	0 / 64	0%
2016 Summer				
Treatments	Individuals	Inflorescences	Flowers	Fruit-set rate
♀ - Open pollination	10 / 10	22 / 24	171 / 274	62.4%
♀ - Artificial crossing	10 / 11	21 / 26	30 / 38	79.0%
♀ - Unpollinated	10 / 10	15 / 26	45 / 306	14.7%
♂ - Open pollination	0 / 11	0 / 27	0 / 302	0%
♂ - Artificial crossing	0 / 12	0 / 26	0 / 36	0%
♂ - Autonomous selfing	1 / 11	1 / 33	1 / 333	0.3%

Table 3-4. Results of pollination experiments in alpine populations of *Daphne arisanensis*.

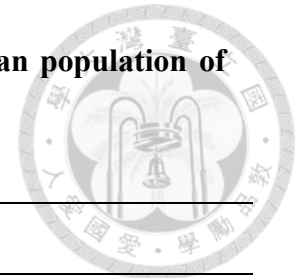
♀ means male-sterile and ♂ means morphologically bisexual. Data shown are fruiting-counts / tested number.



2017 Spring				
Treatments	Individuals	Inflorescences	Flowers	Fruit-set rate
♀ - Open pollination	10 / 13	25 / 39	130 / 437	29.7%
♀ - Unpollinated	4 / 12	5 / 37	5 / 376	1.3%
♂ - Open pollination	0 / 8	0 / 32	0 / 333	0%
♂ - Autonomous selfing	1 / 8	1 / 33	1 / 362	0.3%

Table 3-5. Results of pollination experiments in Yangmingshan population of *Daphne kiusiana* var. *atrocaulis*.

Data shown are fruiting-counts / tested number.



2017 Spring

Treatments	Individuals	Inflorescences	Flowers	Fruit-set rate
Open pollination	11 / 11	32 / 33	263 / 363	72.5%
Autonomous selfing	11 / 11	31 / 32	146 / 301	49%
Emas. unpollinated	2 / 7	2 / 14	2 / 77	2.6%
Emas. artificial pollinated	4 / 4	4 / 5	8 / 35	22.9%

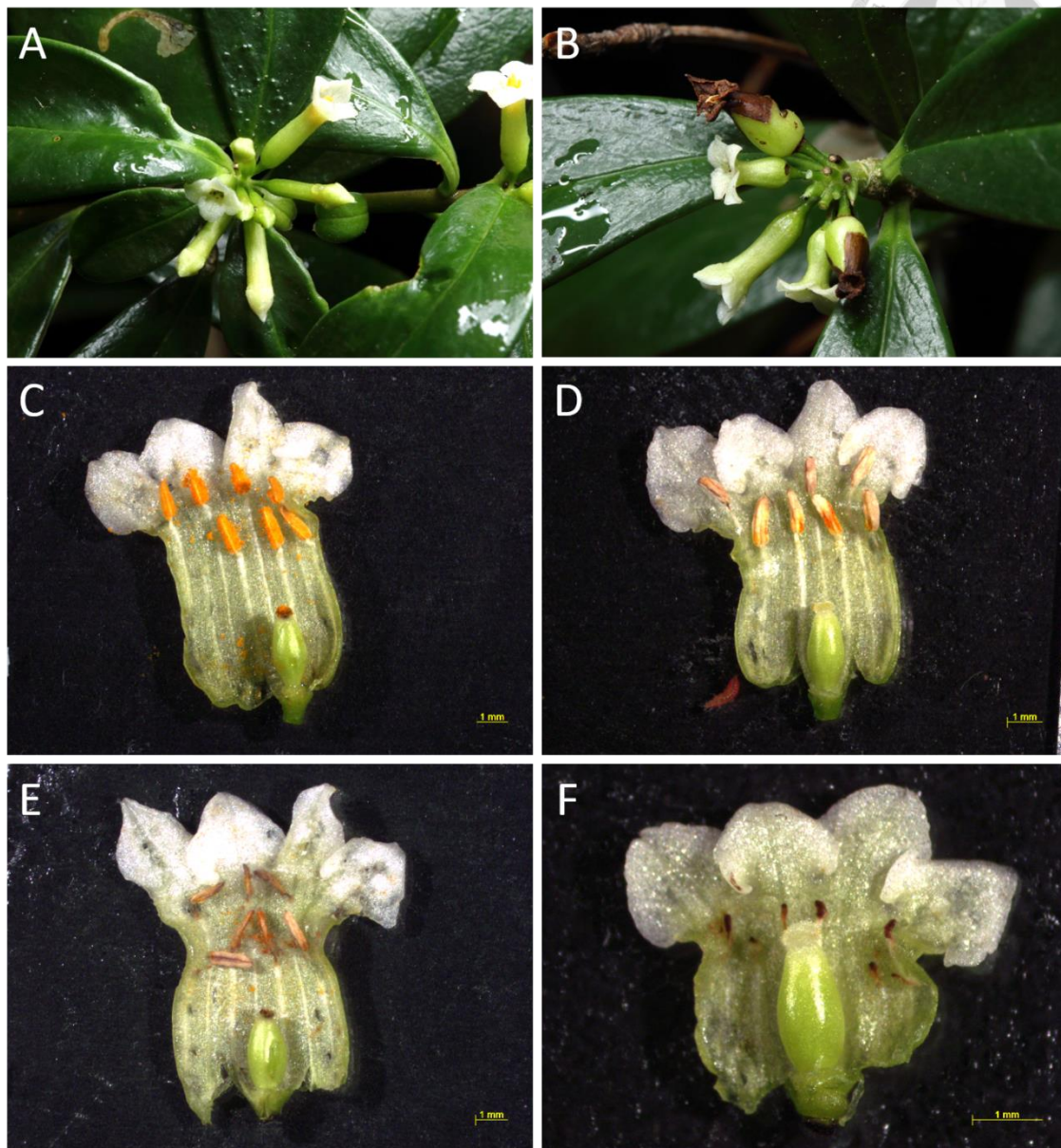


Figure 3-1. Occasional male-sterility in a bisexual individual of *Daphne arisanensis*.

(A) A male-sterile flower and other typical bisexual flowers borne on the same inflorescence. (B) Developing ovaries. (C) Typical bisexual flower. (D)—(F) Different degrees of male-sterility in bisexual flowers.

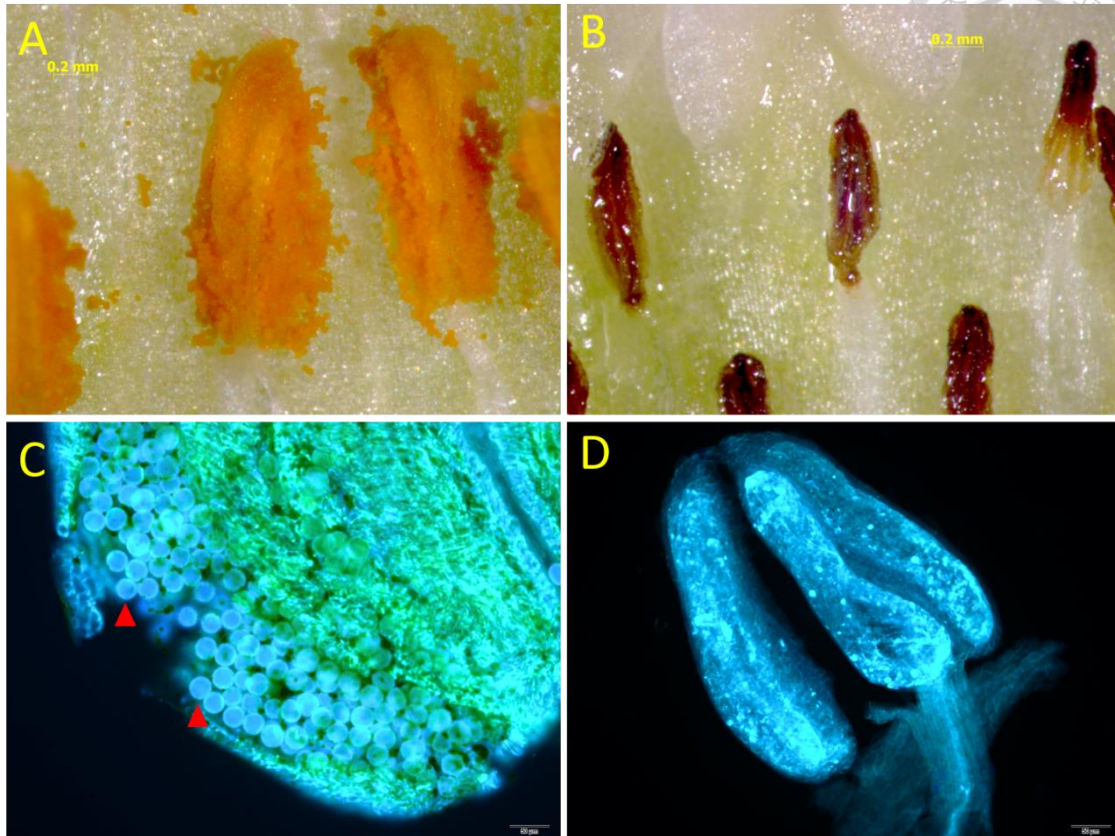


Figure 3-2. Comparison of anthers in bisexual and male-sterile flowers.
(A) Anthers in fresh bisexual flower. **(B)** Shriveled anthers in fresh male-sterile flower. **(C)—(D)** Fluorescent staining of anthers in bisexual **(C)**, and male-sterile **(D)** flower. Arrowheads indicate pollens. **(C)** and **(D)** are at same scale.

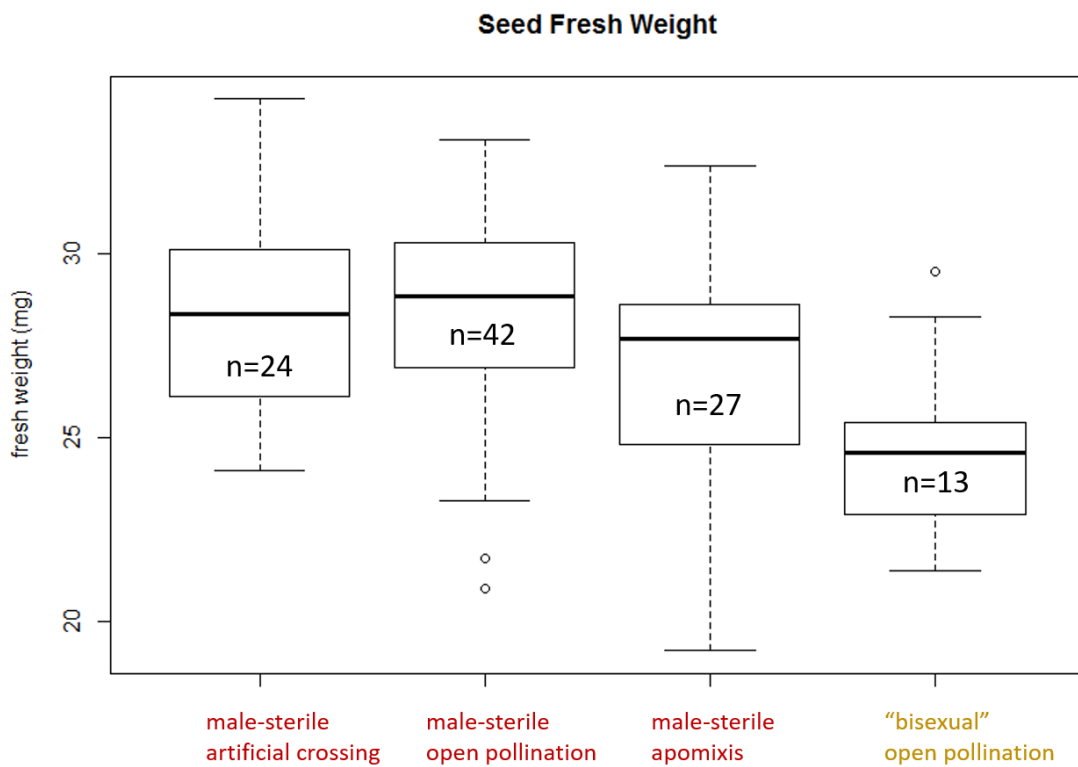


Figure 3-3. Fresh weight of seeds from different pollination treatments. The boxes represent first and third quartile, black lines are median, whiskers represent minimum and maximum, outliers are shown as circles.

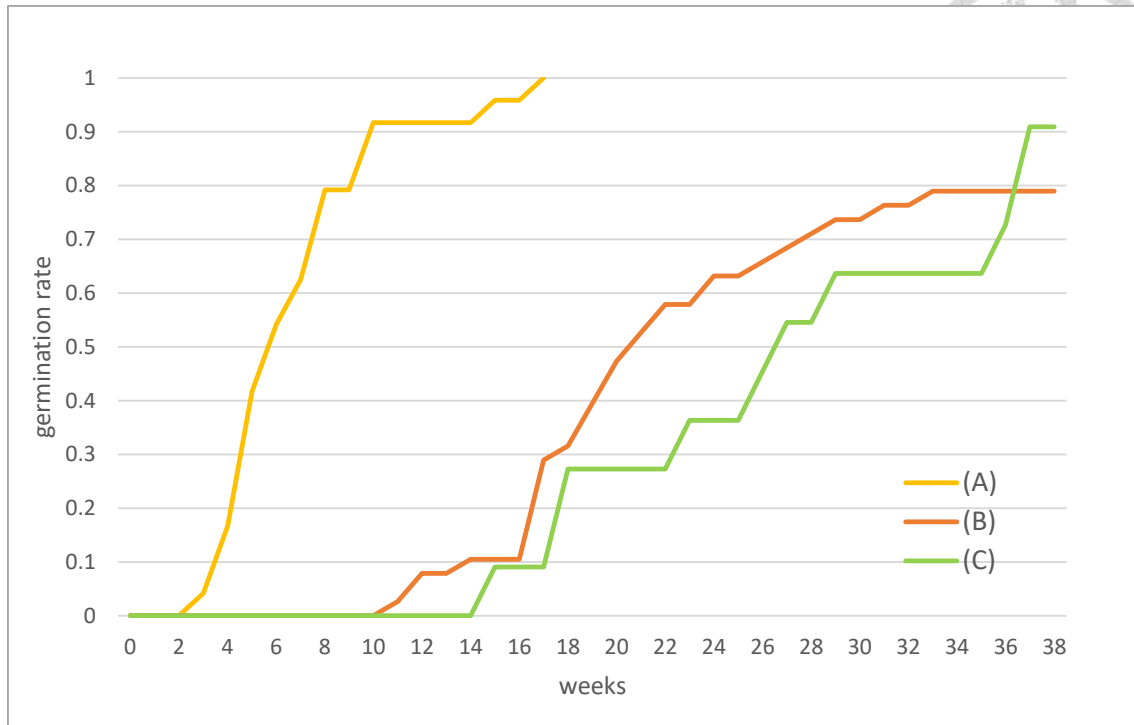


Figure 3-4. Cumulative seed germination rate.

(A) Pollinated seeds with scarification and 30 days of cold stratification, N = 24. **(B)** Pollinated seeds without manipulation, N = 38. **(C)** Apomictic seeds without manipulation, N = 11.

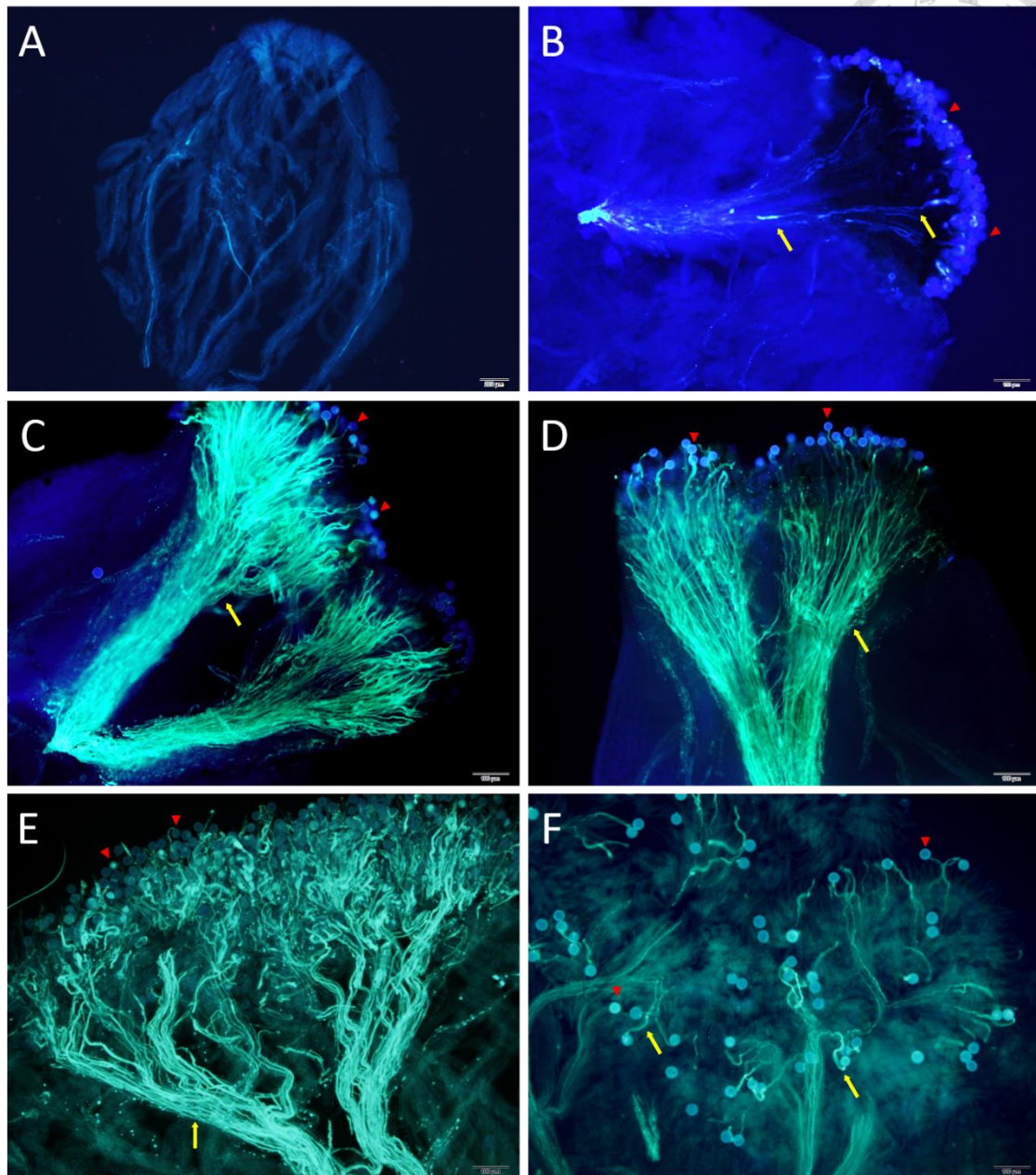


Figure 3-5. Fluorescent staining of pollen tubes in gynoecea.

(A)—(D) *Daphne arisanensis* from Guanyuan population. (A) Male-sterile flower without pollination. (B) Artificially crossed male-sterile flower. (C) Aided-sefling bisexual flower. (D) Artificially crossed bisexual flower. (E) Autonomous-selfing bisexual flower of *Daphne arisanensis* from Dayuling population. (F) Autonomous-selfing bisexual flower of *Daphne kiusiana* var. *atrocaulis* from Yangmingshan population. Arrowheads, pollens. Arrows, pollen tubes.

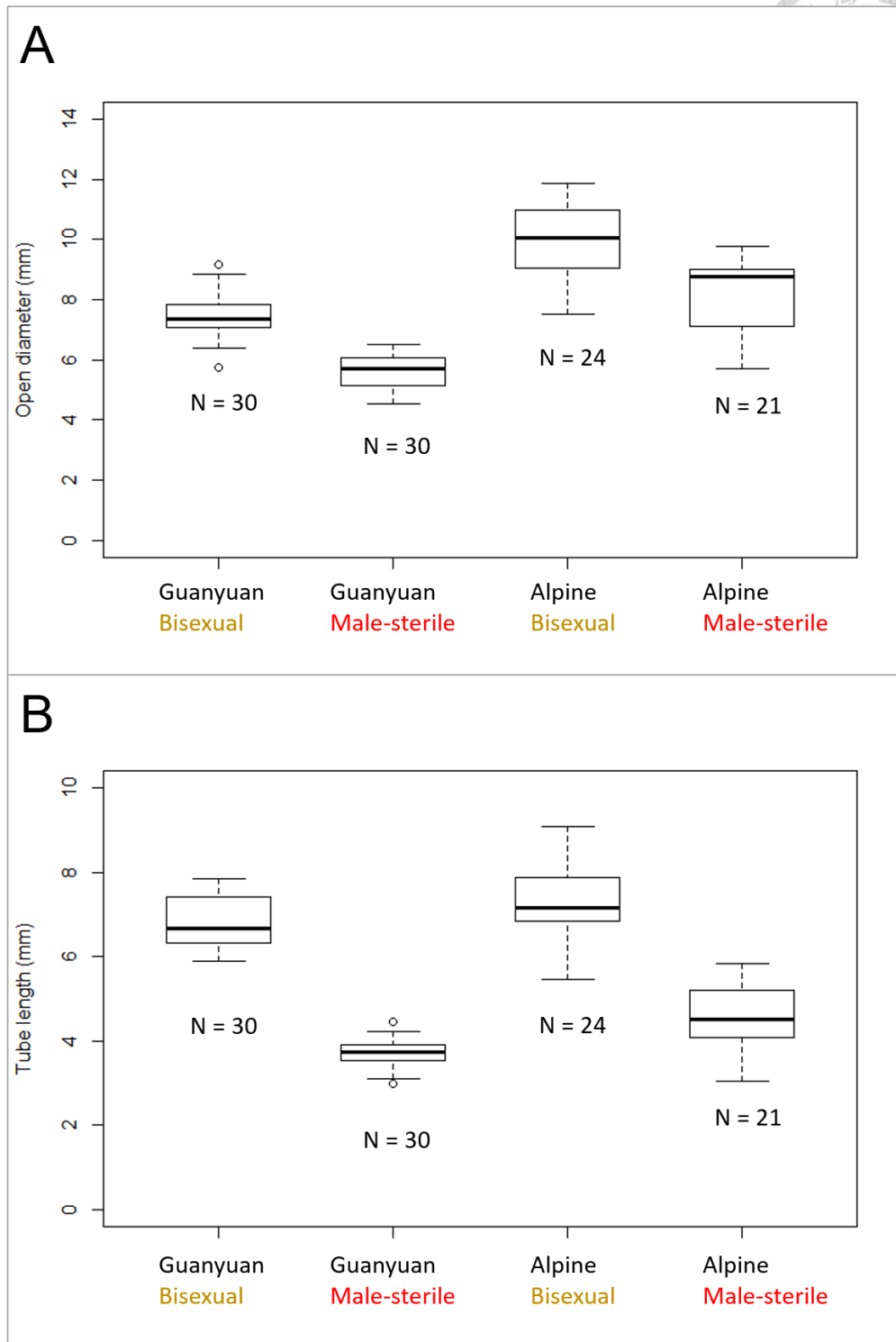


Figure 3-6. Comparison of flora traits between populations.

(A) Open diameter. **(B)** Tube length. The boxes represent first and third quartile, black lines are median, whiskers represent minimum and maximum, outliers are shown as circles

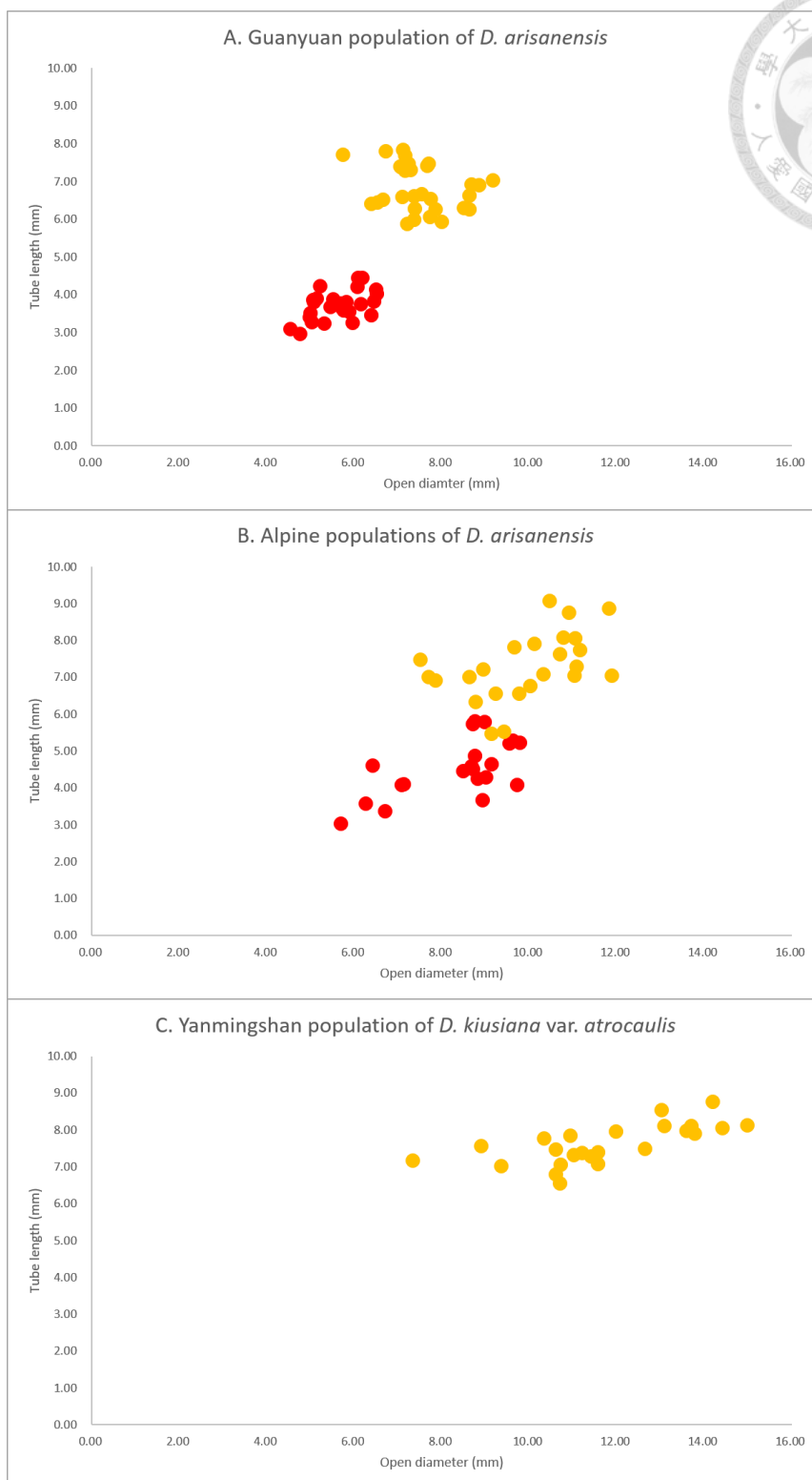


Figure 3-7. Distribution plot of tube length to open diameter. (A) Guanyuan population of *Daphne arisanensis*. (B) Alpine populations of *Daphne arisanensis*. (C) Yangmingshan population of *Daphne kiusiana* var. *atrocaulis*. Red dots represent male-sterile flowers. Yellow dots represent morphologically bisexual flowers.

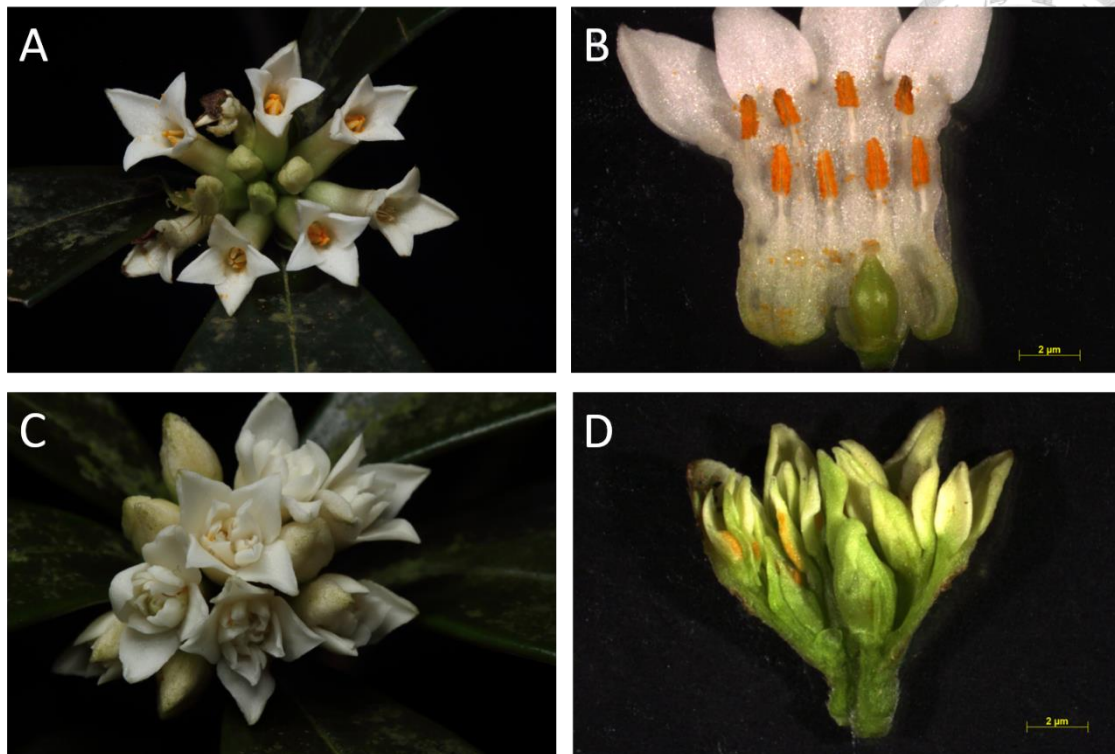


Figure 3-8. Morphology of *Daphne kiusiana* var. *atrocaulis*.

(A) Inflorescence. **(B)** Longitudinal section of a flower. **(C)** An occasionally formed petaloid mutant in Yangmingshan. **(D)** Longitudinal section of a petaloid flower.

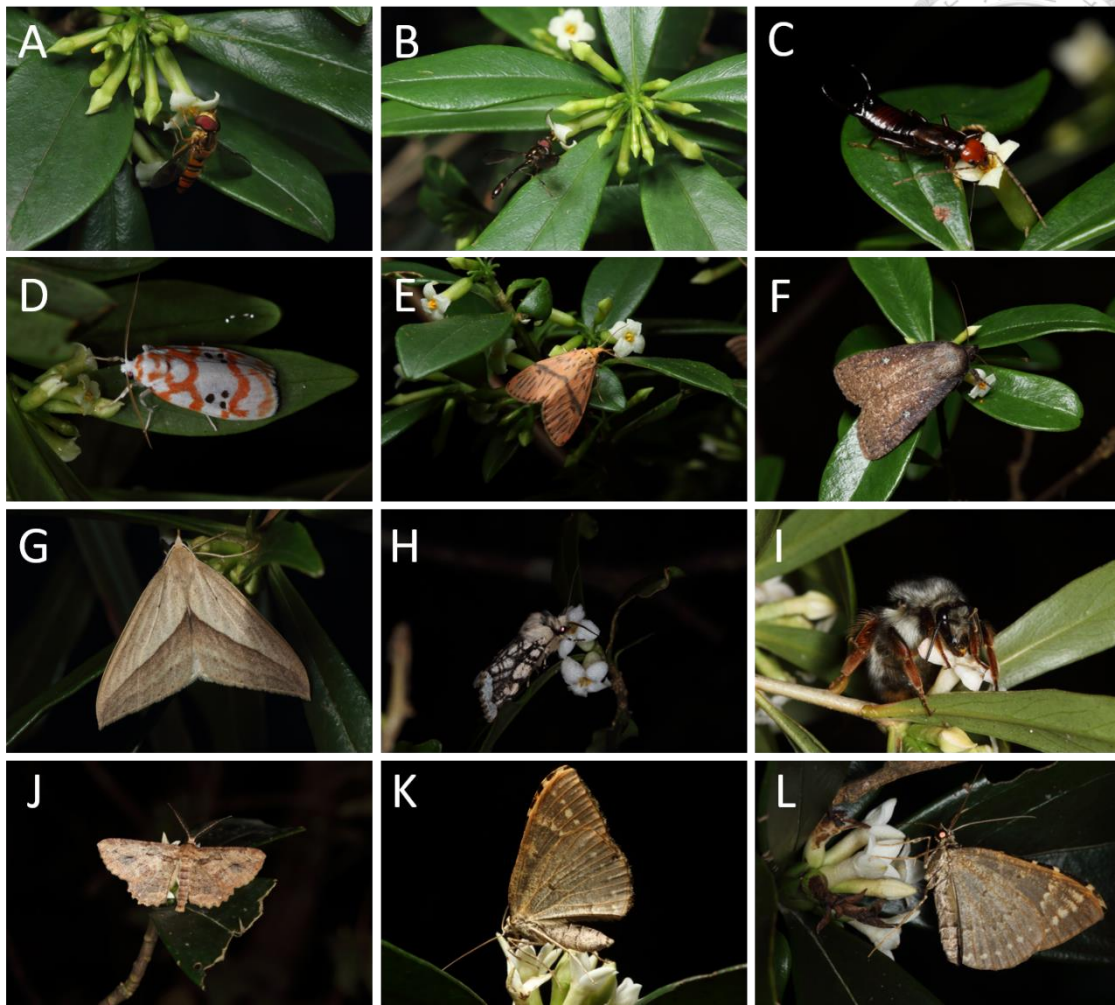


Figure 3-9. Selection of flower visitors.

(A)—(F) In Guanyuan population of *Daphne arisanensis*. (G)—(I) In Dayuling population of *Daphne arisanensis*. (J)—(L) In Yangmingshan population of *Daphne kiusiana* var. *atrocaulis*. (A) *Episyrphus* sp. (B) *Baccha* sp. (C) Earwig. (D) *Cyana formosana*. (E) *Lyclene wenchiyei*. (F) *Perinaenia mingchyrica*. (G) *Loxaspilates arrizanaria*. (H) *Orthosia reticulate*. (I) *Bombus sonani*. (J) Geometridae. (K) *Sibatania arizana*. (L) *Sibatania arizana*. Complete list of recorded moths is shown in Appendix Table A-1.

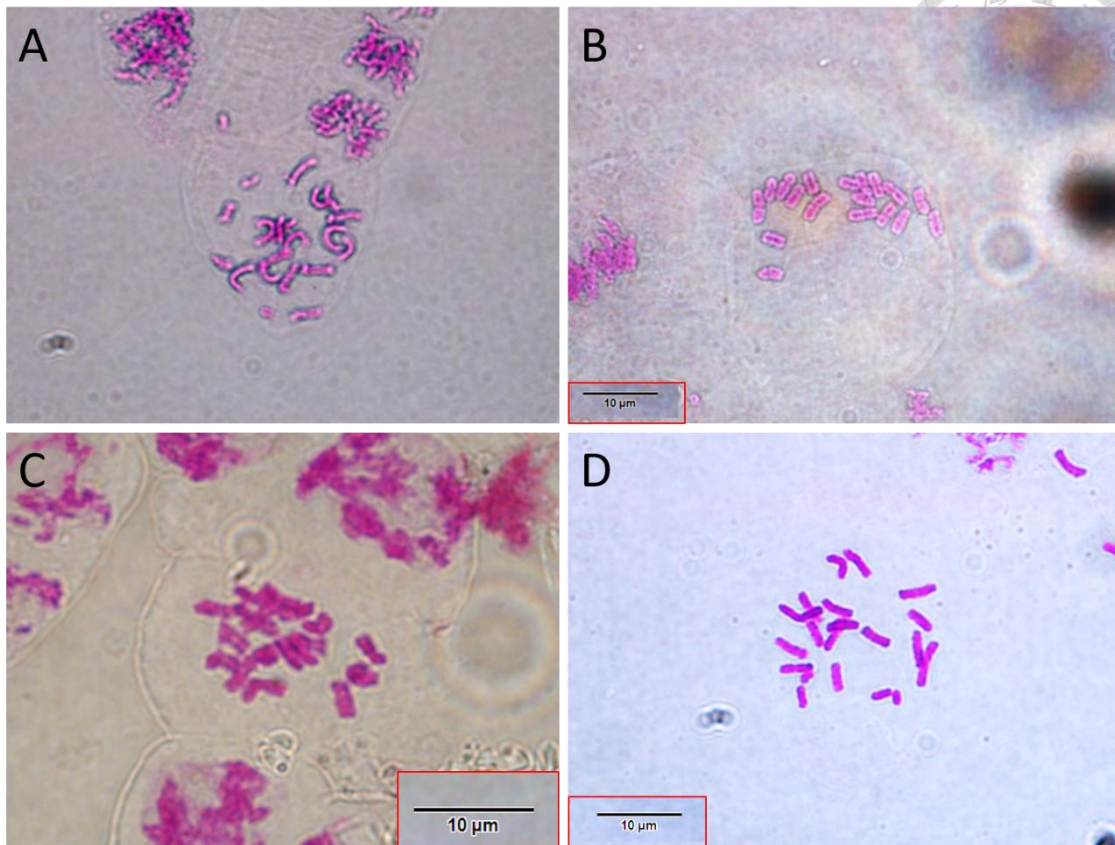


Figure 3-10. Chromosome examination.

(A) Guanyuan population of *Daphne arisanensis* (W. J. Huang 1520, TAI). (B) Meifeng population of *Daphne arisanensis* (W. J. Huang 1519, TAI). (C) Apomictic seedling of *Daphne arisanensis*. (D) Yangmingshan population of *Daphne kiusiana* var. *atrocaulis* (W. J. Huang 1518, TAI). All give the chromosome count $2n = 18$.

Chapter 4 Discussion

4.1 The sexual system of *D. arisanensis*



The results of pollination experiments had confirmed that *D. arisanensis* is indeed functionally dioecious. The male-sterile flowers had a complete ability in siring seeds, while the morphologically bisexual flowers served as almost purely pollen-donors in natural populations. Hence, these two morphs function complementarily as females and males. However, various degrees of male-sterility and a few fruits were occasionally found on male plants. I suspect the restoration of seed-siring ability in males was restricted to the male-sterilizing event, since the two fruits obtained in pollination experiments were either partially male-sterilized or in a such inflorescence. The typical male flowers (i.e., morphologically bisexual) without male-sterility might completely lose the ability to set fruits. For not confusing with the simply low fruit-set rate of bisexual flowers in other gynodioecious *Daphne* species (Sinclair et al., 2016), I suggest using the term “feminization” to describe this complex phenomenon. There were two seeds collected from “male individuals” succeeding in germinating (one in germination test and the other was stored at 4°C), suggesting occasional feminization in males was valid at least to a certain degree in reproduction. Instead of simply making discrete discrimination between “male” and “bisexual” in the morphologically bisexual flowers of a gynodioecious system, it is better to recognize the quantitative nature of varieties in expressing female function (Barrett, 2010a). The intensity of male-bias in bisexual flowers might imply their evolutionary stage toward dioecism (Sinclair et al., 2016; Spigler & Ashman, 2012). Considering the rareness of feminization in male plants and the consistency of sexual expression in this three-year observation, I regarded the little maternal output in male-plant reproduction is a leaky phenomenon.

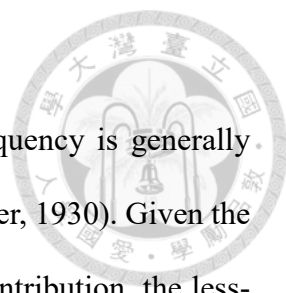
4.2 Variations among populations

Leaf shape and flowering phenology variation

Daphne arisanensis exhibited great variations in floral traits among populations, including flowering season, sex ratio, flower color, flower size, and floral lifespan. The individuals in Hehuanshan and the neighboring alpine areas (> 2700 m) usually showed linear leaf shape, a key character of the previously recognized *D. morrisonensis* (Chang, 1993), now a synonym of *D. arisanensis* (Yu, 2014). Yu regarded those extremes still as *D. arisanensis* based on the continuous variations in leaf shape of these two taxa and the result from phylogenetic analysis. According to my observation, the leaves in small individuals (0.5-1 m) of alpine populations were more or less linear shaped but typically oblanceolate in the larger plants (1-3 m). However, the alpine populations are distinct in their flowering phenology. The individuals in alpine areas produced flower buds during November-December and bloomed in January-April of the next year, the flowers were usually larger and long-lived up to 3-4 weeks. In contrast, the Guanyuan population had a peak bloom in summer, but additional buds and flowers could also be found all year round, and most of the male flowers were quite short-lived, falling within two days after anthesis. As for the Meifeng population, individuals were strongly female-biased and no well-synchronized flowering season had been found, and the flowers were more or less yellowish. According to herbarium specimens (Appendix Table A-3) flowering seasons and leaf shapes were also diverse among regions in Taiwan, and the flowers were not always large-sized and spring-blooming in other alpine areas (e.g. July in Yushan, *T. C. Huang 14243*, TAI; June in Hsiangyangshan, *S. T. Chiu 2934*, HAST).

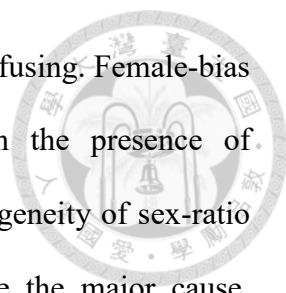


Sex ratio variation



Owing to the negative frequency-dependent selection, sex frequency is generally expected to be equal in dioecious species (Delph & Kelly, 2014; Fisher, 1930). Given the fact that each offspring needs both maternal and paternal genetic contribution, the less-abundant sex would enjoy a relative advantage in reproduction. Sex-biases may occur if sexual preference exists between their life-history traits, such as mortality, flowering frequency, and the minimum plant size required for flowering (Lloyd & Webb, 1977), and local segregation of specific sex may also result from small-scale niche partitioning (Bierzychudek & Eckhart, 1988). In this study, the overall sex-ratio of labeled *D. arisanensis* was close to 1:1 (72 females to 70 males), but great variations existed among populations (Table 3-1). The Guanyuan population was male-biased with marginal significance. Several studies had also obtained male-biased sex-ratios in different dioecious species (see Field et al., 2013). The most common explanation for male-biases is that males are usually less-burdened in reproduction, thus males could reproduce at earlier life-stage, and females would suffer a higher mortality when setting fruits.

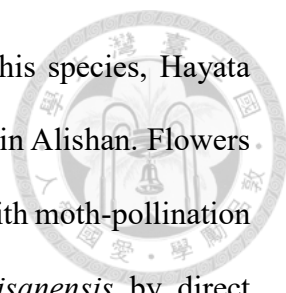
In Guanyuan, the majority of small individuals (shorter than 1 m) were indeed male, but flowering females at this size could be found among other populations, and the smallest was in Dayuling with flowers on a non-branching stem and merely ten leaves. It was not clear yet that whether female plants in Guanyuan preferred not to produce flowers until growing to a certain size, or a higher mortality existed among females. Nonetheless, plant size and age are difficult to estimate in *D. arisanensis* due to the irregular branching pattern, therefore a comprehensive analysis combining plant height, leaves number and stem thickness is needed. In addition, individuals sometimes formed dense patches, and it is difficult to identify them as vegetative propagations or from clustered seedlings unless the individuals are unearthed.



The strongly female-biased population in Meifeng was quite confusing. Female-bias in dioecious species had been proposed to be correlated with the presence of heteromorphic sex chromosomes (Field et al., 2013), but the heterogeneity of sex-ratio among populations indicated that site-specific incidents would be the major cause. Individuals in Meifeng were situated between the border of Highland Experimental Farm of NTU and the outlying farm road, and most of them were small to medium sized (0.5-1.5 m). Thus, the Meifeng population was supposed to be relatively newly-colonized and unstable. Considering the ability of apomictic reproduction in female plants, if the sexuality of unfertilized offspring was biased to female, a high female-frequency in new-formed population could be achieved under founder effect. However, it may take years to directly verify the existence of female-bias in those apomictic seedlings until they reach the size capable for flowering. Detailed genetic structure analysis might be able to provide evidence on the hypothesis. Alternatively, the female-bias could be actually exaggerated by the small sample size ($N = 24$) in Meifeng. More intensive field surveys in the neighboring areas are also needed.

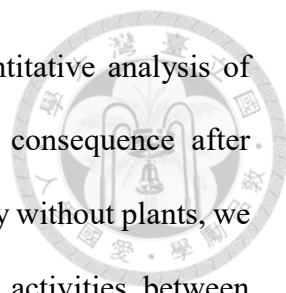
Floral trait variation

Since floral traits are directly correlated with reproductive success, the variations may reflect adaptations to local environments, especially in pollinating fauna (Cruden, 1976; Galen & Kevan, 1980). For example, Castilla et al. (2015) found that different pollinator-compositions might account for the geographic variations of flower length and flowering time in the gynodioecious *Daphne laureola*. Flower color and the color-pattern are important in visually attracting pollinators (Willmer, 2011). The *D. arisanensis* flowers were either whitish or yellowish, and the color-morphs were more or less consistent within population (Figure 2-1), though a white-flowered individual was found



in the yellow-based Meifeng population. As in the publication of this species, Hayata (1912) had already noted the coexistence of these two color-morphs in Alishan. Flowers with white color and the lack of nectar guides were often associated with moth-pollination syndrome (Fenster et al., 2004), which was confirmed in *D. arisanensis* by direct observations in Guanyuan and the alpine populations. Though no nocturnal observation had been conducted in the yellow-flowered Meifeng population, there was also no diurnal visitor found there. The yellow flowers were probably pollinated mainly by nocturnal insects, too. If so, the variation of flower color of *D. arisanensis* may have no direct effect to their pollination success. Correspondingly, Zhang et al. (2011) reported that the confamilial *Stellera chamaejasme* also comprised of yellow-flowered and white-flowered populations, but the variations of pollinator composition were not correlated with the flower color-morph.

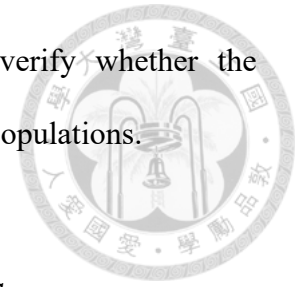
Flowers of *D. arisanensis* were significantly larger and long-lived in the alpine populations than those in Guanyuan, contributing a large floral display in early spring. In fact, *D. arisanensis* is one of the earliest flowering native plants in the alpine areas in Taiwan. During January-February, only some *Eurya* flowers were found contemporarily in Dayuling (2700 m, personal observation). Low temperature, occasional snowfalls and the unavailability of other flowers might together underlie an unreliable pollinator service in the alpine areas during winter and spring. The low pollinator activity in early spring had been addressed to account for the severe pollen limitation in the Mediterranean *D. laureola* (Alonso, 2004). Thus, the floral phenology and display of alpine populations might have been modified through pollinator-mediated selection. The relatively lower fruit-set rate (29.7%) under natural pollination suggested a stronger pollen limitation therein, though other ecological factors might also affect the female fecundity. While various non-specific nocturnal moths had been recorded pollinating alpine *D. arisanensis*



in the spring, the extant data still could not give an effective quantitative analysis of pollinator service. Since the presence of pollinators might be a consequence after adaptation, and the pollinator abundance could not be measured solely without plants, we should be careful to make reasonable comparisons of pollinator activities between populations and seasons. Alonso (2004) demonstrated a delicate method with periodic bagging to experimentally reduce flowering time in *D. laureola*, this would be potentially useful to evaluate the effect of floral lifespan and the relative importance of certain flowering-period. Also, directly counting pollens on female stigmas would be helpful to compare the pollination success between populations. As mentioned earlier, the flowering season was diverse among regions, and it was not always spring-opening in other alpiners. The whole matrix of floral biology and pollinators among populations could be very complicated.

Given that the fruit-set rate was lower in alpine populations under natural pollination, asexual reproduction (i.e. apomixis) was expected to be favored in the sense of reproductive assurance. However, the bagged female flowers in alpiners only gave a 1.3% apomictic fruiting rate, which was significantly lower than the 13.8% and 14.7% in Guanyuan. It might be caused by either a lower fitness of apomictic offspring or unsuitable ecological conditions for females to reproduce asexually in alpine areas. Harsh environments were suggested to favor higher genetic diversity (Barrett, 2010b; Dudash, 1990). The alpine environment might be stressful to *D. arisanensis*, and the advantages of apomixis might not be as beneficial as predicted. Both the energy level and metabolic efficiency could be lower in alpiners (Bliss & Mark, 1974). Without effective pollination, females might prefer to save their resources to invest in the future seasons for higher seed-quality. The extended floral lifespan of the alpine *D. arisanensis* could potentially increase pollination success, but could also be a constraint on the initiation of apomixis.

Additional experiments of artificial pollination are needed to verify whether the reproductive potential of female flowers is actually lower in alpine populations.

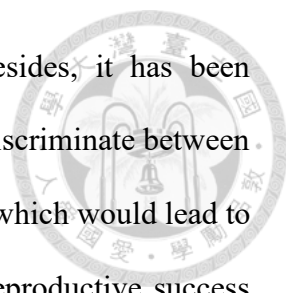


4.3 Reproductive features in *D. arisanensis*

Several ecological features found in *D. arisanensis*, including perennials, unspecialized pollinators, flesh fruits and island habitats, had been suggested to be correlated with dioecism (Bawa, 1980; Renner, 2014). Strictly dioecious species are thought to be obligate outbreeding, since one individual cannot reproduce sexually without its complementary gender. Due to this non-independence, the correlated ecological factors had been discussed principally from the aspect of promoting reproductive opportunities, together with gender optimization and effective colonization. The dioecy in *D. arisanensis* is not obligate but actually leaky, since either a female or a male could set fruits on its own through apomixis or partial feminization respectively. Nonetheless, these factors might still be influential in the predominantly outbreeding system, and the probable interactions are briefly discussed below.

Perennial life-form has a strong correlation with the occurrence of dioecy, and actually only several cases of dioecious annuals had been reported (Bram & Quinn, 2000; Decker & Pilson, 2000). For a newly-colonizing individual, it may take years to meet with the coincident arriving of opposite sex, thus the perennial-form would be more stable in dioecy than annual. Moreover, perennial trees with simultaneous large floral display would suffer greater pressure of self-pollination between neighboring flowers (i.e. geitonogamy), which could be a selective force driving gender divergence (discussed further in section 4.4).

Adaptations to various unspecialized pollinators may also benefit in colonizing new

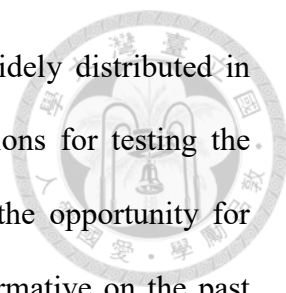


habitats, where pollination service could be more unreliable. Besides, it has been suggested that specialized pollinators might have a better ability to discriminate between the differential rewards of males and females in a dioecious species, which would lead to an unequal flower-visiting rate and have negative effect on total reproductive success (Charlesworth, 1993).

Daphne fruits are usually fleshy and berry-like (Wang et al., 2007), which is a character usually associated with zoochory in long distance dispersal. The maternal burden of setting such fruits had been suggested to associate with the evolution of differential resource allocation and the further gender divergence (Bawa, 1980).

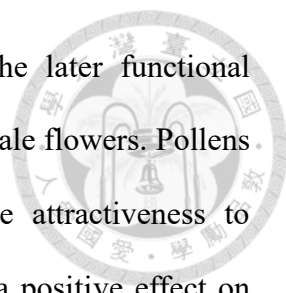
The occurrence of dioecy in islands seemed to be a controversy to Baker's law (Baker, 1955), which stated the relative difficulty of colonization after long-distance dispersal with a non-self-fruited individual (self-incompatible or unisexual). A complex integration among several factors may account for the necessary advantages, including the mentioned longevity, long-distance and multi-unit dispersal via zoochory, utilization of unspecialized pollinators, together with the probable strong selection for out-breeding in islands and the leakiness in dioecy (Baker & Cox, 1984; Emerson et al., 1996).

Leaky dioecy is characterized by the occasional presence of opposite sex or bisexual flowers within a unisexual individual (Baker & Cox, 1984). The leakiness could be either a remnant during gender divergence or a consequence of further selection with adaptive significance. As the majority of dioecious species are self-compatible (Charlesworth, 1985; Thomson & Barrett, 1981), leakiness could bring about small-scale reproductive assurance, and it would be especially beneficial during long-distance colonization. In *D. arisanensis*, male plants could produce several feminized flowers, which were capable of self-pollinating and siring viable seeds. Though no fertile male organ was found in the female plants, they could still reproduce via apomixis and contribute to a similar



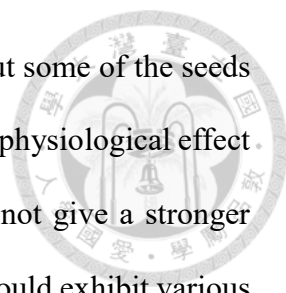
consequence of leakiness. Since *D. arisanensis* is endemic and widely distributed in Taiwan, it would be challenging to find suitable isolated populations for testing the dispersal model. Exploited areas like Meifeng might have given the opportunity for secondary colonization, and the population genetics would be informative on the past colonizing-pattern (see section 4.2). It is noteworthy that the occurrence of apomixis in dioecious species is relatively uncommon (Dupont, 2002; Richards, 1990). In this study, the hermaphroditic *D. kiusiana* var. *atrocaulis* also displayed apomictic reproduction in the emasculation-unpollination treatment (Table 3-5), but further examination in the ontogeny of embryos is needed to clarify the underlying mechanism and to exclude the possibility of contamination problem during manipulation. Rare fruit-set from unpollinated flowers were also reported in the congeneric gynodioecious *D. laureola* (Alonso & Herrera, 2001), both in bisexual flowers and female flowers, but the authors simply regarded the results as contaminations. If apomixis was common in *Daphne*, it could be a preadaptation to the gender divergence in *D. arisanensis*. However, given that the flowers of *Daphne* are easily self-pollinated (further discussed in section 4.4), the advantage of apomixis seem to be redundant in other hermaphroditic species. A broader-scale analysis is needed to clarify the existence of apomixis and the correlation with gender divergence in the whole genus.

The sterile but morphologically complete gynoecia in male flowers made them a bisexual appearance, thus the sexual system in *D. arisanensis* could be termed as cryptic dioecy (Mayer & Charlesworth, 1991). In cryptic dioecy, the rudimentary organ may represent an evolutionary remnant without adaptive significance, or have its specific function in pollination. In the former situation, a long time for selective pressure may be needed to promote degeneration of the sterile sex-organs, and the complicated genetic control of floral development may bring difficulties in reducing the non-functional floral



part without affecting others (Mayer & Charlesworth, 1991). The later functional explanation was mainly met with the presence of male organs in female flowers. Pollens and conspicuous anthers may be still important to maintain the attractiveness to pollinators in females, thus the retention of sterile androecia have a positive effect on pollination. While being adapted to nocturnal-moth-pollination, there was no need to produce sterile pollens as rewards in the female flowers of *D. arisanensis*. Generally, no obvious contribution to promoting pollen-dispersal could be found on the rudimentary gynoecia of male flowers, Mayer and Charlesworth (1991) suggested that reducing visual differences between the males and females would be helpful in maintaining equivalent visiting rate. In the case of *D. arisanensis*, many pollens would even be intercepted by the self-stigma, resulting a discount of potentially dispersible pollens (further discussed in section 4.4). Another possible functional perspective is that the retention of gynoecia could be essential in expressing leakiness (i.e. feminization in *D. arisanensis*), but the potential benefit seem to be inequivalent to the cost.

The fleshy fruits and the seeds of *Daphne* were supposed to be dispersed mainly via endozoochory of birds, which had been recorded in *D. gnidium* (Herrera, 1981) and *D. laureola* (Brickell & Mathew, 1976). The developmental and functional role of the aril-like membrane surrounding the seed coat was not clear yet. Due to its fragility, it probably would be destroyed when passing through the digestive tract of a disperser. It had been reported that pulp may inhibit seed germination in other endozoochorous species, and removing the pulp could be an indicator of successful dispersal and a suitable environment (Traveset et al., 2007). I was unable to test this hypothesis in *D. arisanensis* because the seeds easily got moldy with retaining the moist membranes for months. The seeds exhibited various and continuous lengths of dormancy. Low-temperature stratification seemed to have no synchronizing effect. Mechanical damage on the



crustaceous seed coat significantly boosted the germination curve, but some of the seeds still remained dormant for two-three months. Thus, both physical and physiological effect underlay the dormancy pattern. If dispersal by endozoochory does not give a stronger effect than manipulated-scarification, the naturally dispersed seeds would exhibit various lengths of dormancy in soil. Also considering the variable flowering seasons among populations, the emergence of seedlings may be irregular all year round. In summary, the germination strategy of *D. arisanensis* is likely a seed bank formation (Roberts, 1981).

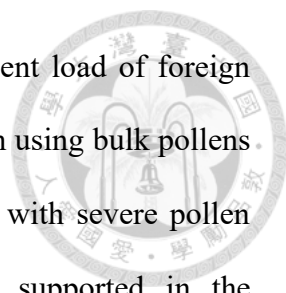
4.4 Self-pollination and gender divergence in *Daphne*

Considering the floral structure of *D. arisanensis*, I suspect the self-pollinating nature had played an influential role in sexual differentiation. The eight stamens are situated at upper part of the tube and block the opening, and the position of stigma is right beneath the anthers, with its receptive surface directly exposed (Figure 2-4). Despite a slight spatial separation, pollens would fall on stigma spontaneously after anther-dehiscence, which had also been reported in the congeneric *D. rodriguezii* (Rodríguez-Pérez & Traveset, 2011), *D. gnidium* (Roccatiello et al., 2009), *D. jezoensis* (Kikuzawa, 1989) and *D. kiusiana* var. *atrocaulis* (in this study), but not in *D. laureola* (Alonso & Herrera, 2001). This self-pollinating situation could be further enhanced by pollinator-mediation. Bulk self-pollens would be carried to the stigma when pollinators or their mouth parts enter the floral tube to collect nectar. Presence of tiny insects (e.g., thrips) within flowers would also bring about a similar consequence (Roccatiello et al., 2009). In *D. laureola*, Alonso and Herrera (2001) suggested that although selfing within flower was non-spontaneous, it could be simply achieved by the visiting of pollen vectors. Moreover, considering the simultaneous floral display on a single individual, and the

successively-foraging behavior of pollinators, self-pollination among flowers of same plant (i.e. geitonogamy) should be also frequent (Harder & Barrett, 1995).

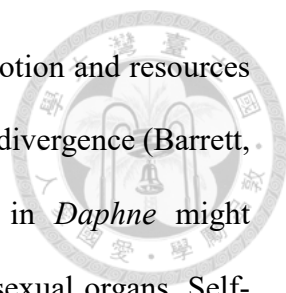
Despite the potential benefit from reproductive assurance, self-pollens might clog the stigma and have both physically and physiologically negative effects in sexual reproduction (Barrett, 2002b). Outcrossing pollens have to compete with the self-pollens in receptive surface and germinating resource. In self-compatible species, high inbreeding rate would lead to reduction in genetic diversity of offspring and even inbreeding depression (Charlesworth & Charlesworth, 1987). Though outcrossing rate is guaranteed under self-incompatibility system, presence of self-pollens could still bring about reduction in reproductive success through occupying the available stigmatic space for outcrossing pollens or interfering the pollen-tube growth (Barrett, 2002b). Studies in *Narcissus* (Barrett et al., 1996; Sage et al., 1999) also indicated that late-acting self-incompatibility system could operate through degenerating embryo sacs after self-pollination, resulting in discounting of potentially fertile ovules.

From the perspective of male-function, pollens intercepted by self-stigma are no longer available for dispersal, which is also a negative consequence of self-pollination termed pollen discounting (Barrett, 2002b). Thus, the fertility of clogged gynoecia is suspected to be lower in the self-incompatible *Daphne*, while a high inbreeding rate is expected in the self-compatible species. The former prediction had been verified in the gynodioecious Northern-Japanese *D. jezoensis*, Kikuzawa (1989) demonstrated that emasculation before anther-dehiscence significantly raised fruit-set in bisexual flowers, which had retrieved the female fecundity from pollen clogging. However, no such effect was detected in the hermaphroditic Mediterranean *D. rodriguezii* (Rodríguez-Pérez & Traveset, 2011), fruit-set did not differ between emasculated and non-emasculated flowers followed by hand-pollination. A possible explanation is that pollen-clogging in



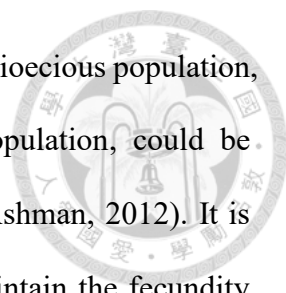
self-incompatible *Daphne* could be partially overcome by a sufficient load of foreign pollens, since each ovary contained only one ovule. Hand-pollination using bulk pollens might not directly reflect the natural condition, especially in areas with severe pollen limitation. The later prediction in self-compatible species was supported in the gynodioecious Mediterranean *D. laureola*, extremely low outcrossing rate and strong inbreeding depression were both reported in the bisexual flowers (Medrano et al., 2004).

In this study, *in vivo* pollen germination experiments had revealed the receptivity to self-pollens in male flowers of *D. arisanensis*, suggesting a probable self-compatible scenario in the ancestor. Polyploidization had been suggested to associate with breakdown of ancestral self-incompatibility and the promotion of further sexual divergence in *Lycium* and *Fragaria* (Ashman et al., 2013; Mable, 2004; Miller & Venable, 2000), while chromosome examination had certified the diploid *D. arisanensis* was not the case. Since direct evidence about ancestral state cannot be obtained, investigations in reproductive biology of the closely related species would be especially valuable (Igic et al., 2008). In Taiwan, the hermaphroditic *D. kiusiana* var. *atrocaulis* in Yangmingshan displayed high fecundity under autonomous selfing, nearly half of the bagged flowers set fruits, which might contribute a relative high inbreeding rate in the natural population. Another native hermaphroditic species, *D. genkwa*, was also supposed to be self-compatible in our preliminary observation. A transplanted individual of *D. genkwa* in laboratory was video-recorded setting fruits spontaneously without effective pollinators (Appendix Figure A-1), and those seeds also succeeded in germinating. As the most closely related species of *D. arisanensis* has not been identified yet, comprehensive studies in the vast diversity of *Daphne*, especially in China, in which up to 40 endemic species documented (Wang et al., 2007), are fundamental to give a whole picture about reproductive strategies and sexuality evolution in this genus.



Selective pressures for inbreeding avoidance, outcrossing promotion and resources allocation had been addressed for the principal forces driving gender divergence (Barrett, 2002a; Bawa, 1980). The easily self-pollinated floral structure in *Daphne* might contribute to high inbreeding rate and strong interference between sexual organs. Self-incompatibility would be preferred under this condition but only passively guarantee outcrossing. Spatial or temporal separation of male and female function would benefit more actively in promoting effective pollination and optimizing gender performance (Barrett, 2002a; Bawa, 1980). The coexistence of self-incompatibility and gender dimorphism in the gynodioecious *D. jezoensis* also supported this differential perspective (Kikuzawa, 1989), since inbreeding avoidance solely could not be interpreted as the main selective pressure driving gender divergence in a preexisted self-incompatible system (Barrett, 2002a, 2002b).

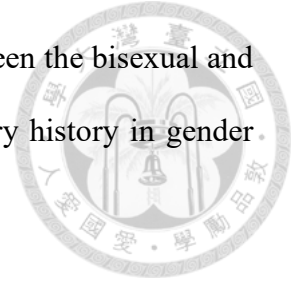
Sexual dimorphism in *Daphne* would evolve when the environment was no more suitable for the ancestral self-pollinating condition (either self-compatible or self-incompatible), or just substituting it with the more effective reproductive strategy. Considering the coexistence of morphologically bisexual and male-sterile plants in *D. arisanensis*, the evolution toward dioecism probably had undergone gynodioecy pathway. In the theoretical model, male-sterile mutant (i.e. female) could spread in the ancestral hermaphroditic population when the benefit of relieving from self-pollination and the increased seed fitness compensate the loss of paternal output (Charlesworth & Charlesworth, 1978). The increased frequency of females in population would further exert selective pressure for enhanced male function in other bisexual plants, thus different degrees of allocation to maleness in the bisexual flowers (gynodioecy) or even female-sterile individuals (subdioecy) could be presented at the same time (Spigler & Ashman, 2012). This intermediate stage could be temporally stable before transition to fully



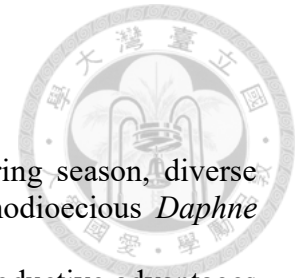
differentiation, and the relative maleness of bisexual flowers in gynodioecious population, or the relative fertility of purely male plants in subdioecious population, could be interpreted as the evolutionary position toward dioecy (Spigler & Ashman, 2012). It is noteworthy that pollinator service must be sufficient enough to maintain the fecundity advantage in females at the first step, because reproduction in the unisexual mostly rely on outcrossing. Severe pollen limitation might retard and even drive backwards the gender-diversifying progress (Alonso & Herrera, 2011; McCauley & Taylor, 1997). Therefore, geographical intraspecies variations in gender divergence might exist among populations and represent a complex hermaphrodite-gynodioecy-dioecy continuum, which had also been demonstrated in *D. laureola* (Alonso & Herrera, 2011; Cuevas et al., 2014).

Despite having morphologically bisexual flowers, the functional sexual system of extant *D. arisanensis* in studied populations had already evolved to predominant dioecy. The rarely restored female function in male plants (i.e. feminization) seemed to be restricted in feminization event, which was unlikely a simple remnant of bisexual flowers during the gynodioecy pathway. It would be controversial in directly referring the ecological factors of extant populations to the ancestral condition. However, considering the great variations in floral traits among populations, I'm also wondering whether the extant dioecy system could be actually diversified in Taiwan, specifically with existence of the relatively ancestral gynodioecy or subdioecy state. Indeed, I had examined a confusing specimen of *D. arisanensis* from Peitawushan (北大武山, Pingtung County), which seemed to have fruit-setting bisexual flowers (*C.H. Chen 567*, HAST). Although this could be a mistake of examining the floral morph in a dried specimen, and it had not been confirmed with field observations yet, there might still exist hermaphroditic individuals and even populations in Taiwan. If so, comparative investigations into the

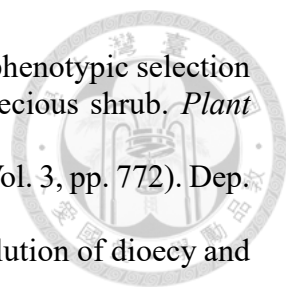
mating systems, and the post-pollination ontogeny of embryos between the bisexual and male flowers will be especially helpful to elucidate the evolutionary history in gender divergence of *D. arisanensis*.



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567-574.



APPENDIX



Table A-1. Record of nocturnal pollinator observations.

Each framed-cell represents a successfully recorded individual. “None” means no flower visitor was found during the period. “Unidentified” represents a moth individual without taxonomic identification.

Population	Date	Period	Flower visitors			
Guanyuan	2015/7/18	04:00-06:00	(unidentified)			
	2015/7/23	19:00-21:00	Geometridae	Geometridae	(unidentified)	(unidentified)
	2015/7/28	19:00-21:00	(unidentified)	(unidentified)	(unidentified)	
	2016/7/12	20:00-22:00	Geometridae	Geometridae	(unidentified)	(unidentified)
	2016/7/21	20:30-22:30	<i>Lyclene wenchiyehi</i>	Erebidae	<i>Rikiosatoa fucataria</i>	(unidentified)
	2016/7/22	20:00-22:00	(unidentified)	(unidentified)		
			<i>Paracymoriza cataclystalis</i>	Crambidae	<i>Perinaenia mingchyrica</i>	Erebidae
			<i>Paradarisa comparataria rantaizanensis</i>	Geometridae	Geometridae	(unidentified)
			(unidentified)			
	2016/8/3	20:30-22:30	<i>Menophra</i> sp. x2	<i>Paradarisa comparataria rantaizanensis</i> x2	<i>Cyana formosana</i>	<i>Menophra</i> sp.
		<i>Psyra conferta</i>	<i>Tanaoctenia haliaria</i>	<i>Menorpha</i> sp.	(unidentified)	
		(unidentified)	(unidentified)	(unidentified)		
Dayuling (alpine)	2016/1/26	19:30-21:30	(none)			
	2016/2/17	18:30-20:30	(none)			
	2016/3/7	19:30-22:00	<i>Loxaspilates arrizanaria</i>	<i>Orthosia alishana</i>	<i>Orthosia reticulata fuscovestita</i>	
	2016/4/1	19:30-22:00	(none)			
	2016/4/19	19:00-21:00	(none)			
	2017/2/7	19:00-21:30	<i>Harutalcis fumigata</i> x2	<i>Loxaspilates arrizanaria</i>	<i>Loxaspilates montuosa</i>	<i>Paradarisa comparataria rantaizanensis</i> x2
			Geometridae	Geometridae	Geometridae	
	2017/2/20	20:00-22:00	<i>Harutalcis fumigata</i>	(unidentified)	(unidentified)	(unidentified)
Yangmingshan	2017/2/14	18:30-21:00	(unidentified)			
	2017/2/17	21:30-23:30	<i>Sibatania arizana</i>	(unidentified)		
	2017/3/1	19:30-22:30	<i>Sibatania arizana</i>			
	2017/3/15	19:30-21:30	(none)			

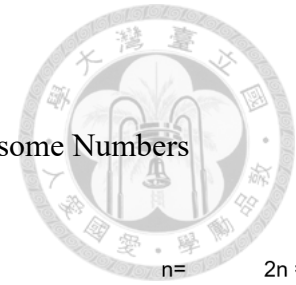


Table A-2. Chromosome numbers in genus *Daphne*.

Data are gathered from Flora of Japan and Index to Plant Chromosome Numbers (IPCN) website.

Species	Reference	n=	2n =
<i>Daphne</i> × <i>reichsteinii</i> Landolt & E. Hauser	Landolt, E. & E. Hauser. 1981. <i>Daphne reichsteinii</i> sp. nov., eine neue hybridogene <i>Daphne</i> -Art aus dem Gardasee-Gebiet. Ber. Geobot. Inst. ETH Stiftung Rübel 48: 36–47.		36
<i>Daphne arbuscula</i> Čelak.	Murín, A. 1990. Karyology of an endemic species <i>Daphne arbuscula</i> Celak. Acta Fac. Rerum Nat. Univ. Comeniana, Bot. 37: 35–40.		18
<i>Daphne arisanensis</i> Hayata	This study		18
<i>Daphne blagayana</i> Freyer	Van Loon, J. C. 1982. In IOPB chromosome number reports LXXVII. Taxon 31: 763–764.		18
<i>Daphne cannabina</i> Wall.	Roy, S. C., S. Ghosh & A. Chatterjee. 1988. A cytological survey of eastern Himalayan plants. II. Cell Chromosome Res. 11: 93–97.		18
<i>Daphne cneorum</i> L.	Krahulcová, A. 1991. Selected chromosome counts of the Czechoslovak flora III. Folia Geobot. Phytotax. 26(3): 225–368.	9	18
<i>Daphne gnidium</i> L.	Romano, S., P. Mazzola & F. M. Raimondo. 1986. Numeri cromosomici per la flora Italiana: 1070–1081. Inform. Bot. Ital. 18: 159–167.		18
<i>Daphne jasminea</i> Sibth. & Sm.	Montmollin, B. d. 1986. étude cytotaxonomique de la flore de la Crête. III. Nombres chromosomiques. Candollea 41: 431–439.	9	
<i>Daphne jezoensis</i> Maxim.	Ohba, H. 1999. Family Thymelaeaceae. Flora of Japan, IIc (Iwatsuki K, Boufford DE, Ohba H, eds). Kodan-sha, Tokyo, 146-151.		18
<i>Daphne kamtschatica</i> Maxim.	Nishikawa, T. 1990. Chromosome counts of flowering plants of Hokkaido (13). J. Hokkaido Univ. Educ., Sect. 2B 40: 19–30.		18
<i>Daphne kiusiana</i> Miq.	Ohba, H. 1999. Family Thymelaeaceae. Flora of Japan, IIc (Iwatsuki K, Boufford DE, Ohba H, eds). Kodan-sha, Tokyo, 146-151.		18
<i>Daphne kiusiana</i> Miq. var. <i>atrocaulis</i> (Rehder) Maekawa	This study		18
<i>Daphne laureola</i> L.	Peruzzi, L. & G. Cesca. 2003. Mediterranean chromosome number reports 13 (1348–1356). Fl. Medit. 13: 373–382.		18
<i>Daphne longilobata</i> (Lecomte) Turill	Kit Tan. 1980. Studies in the Thymelaeaceae I: Germination, seedlings, fruits and seeds. Notes Roy. Bot. Gard. Edinburgh 38: 149–164.		18
<i>Daphne mezereum</i> L.	Druskovic, B. & M. Lovka. 1995. IOPB chromosome data 9. Int. Organ. Pl. Biosyst. Newslett. (Zurich) 24: 15–19.		18
<i>Daphne miyabeana</i> Makino	Ohba, H. 1999. Family Thymelaeaceae. Flora of Japan, IIc (Iwatsuki K, Boufford DE, Ohba H, eds). Kodan-sha, Tokyo, 146-151.		18
<i>Daphne mucronata</i> Royle	Khaton, S. & S. I. Ali. 1993. Chromosome Atlas of the Angiosperms of Pakistan. Department of Botany, University of Karachi, Karachi.	9	
<i>Daphne odora</i> Thunb.	Ohba, H. 1999. Family Thymelaeaceae. Flora of Japan, IIc (Iwatsuki K, Boufford DE, Ohba H, eds). Kodan-sha, Tokyo, 146-151.		18, 24-28, 30
<i>Daphne oleoides</i> var. <i>oleoides</i>	Nikolov, N. A. 1991. Chromosome numbers of Bulgarian angiosperms from North Pirin Mountain: Reserve "Bajuv Dupki-D 3zind 3zirica". Fitologija 41: 70–75.		18
<i>Daphne pseudomezereum</i> A. Gray	Ohba, H. 1999. Family Thymelaeaceae. Flora of Japan, IIc (Iwatsuki K, Boufford DE, Ohba H, eds). Kodan-sha, Tokyo, 146-151.		18
<i>Daphne sericea</i> Vahl	De Montmollin, B. 1984. Etude cytotaxonomique de la flore de la Crête. II. Nombres chromosomiques. Bot. Helv. 94: 261–267.	9	

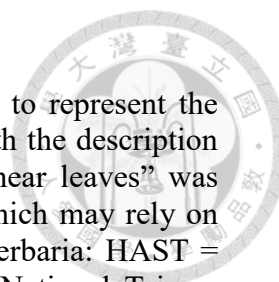


Table A-3. A selection of *Daphne arisanensis* specimens.

Some of the flowering *D. arisanensis* specimens are listed here to represent the variations in flowering time. Symbol “+” means specimen fits with the description and “-” means non-fitting. Presence of “large flowers” and “linear leaves” was judged on the basis of a typical size in Guanyuan population, which may rely on personal experiences. Ambiguous conditions are left unnoted. Herbaria: HAST = Biodiversity Research Center, Academia Sinica, Taipei; TAI = National Taiwan University, Taipei; TAIF = Taiwan Forestry Research Institute, Taipei; TNM = National Museum of Natural Science, Taichung.

Collecting Month	Collecting Date	Altitude > 2700 m	Large Flowers	Linear Leaves	Locality	Collection No.	Herbarium
JAN	1986/1/27	-	-	-	Pingtung	S. F. Huang 3360	TAI
JAN	1964/1/20	+	-	-	Ilan	C. C. Chuang 2514	HAST
JAN	1964/1/16	-	-	-	Ilan	C. C. Chuang 2476	TAI
FEB	1955/2/28	+	+	+	Nantou	(79120)	TAI
FEB	1990/2/27	-	+	-	Pingtung	C. I. Peng 13278	HAST
FEB	1993/2/14	-	+	-	Pingtung	S. Z. Yang 30241	HAST
MAR	1970/3/31	+	-	+	Hualien	(157500)	TAI
MAR	1989/3/19	+	+	+	Nantou	Y. K. Chen 959	HAST
MAR	1997/3/15	-	-	-	Taichung	T. C. Huang 16967	TAI
APR	2014/4/21	+	+	+	Nantou	(279987)	TAI
APR	1987/4/1	-	-	-	Kaohsiung	K. C. Yang 3541	TAI
APR	1994/4/2	-	+	-	Pingtung	C. H. Chen 567	HAST
MAY	1992/5/21	-	-	-	Kaohsiung	C. C. Wang 1131	HAST
MAY	1992/5/15	-	-	+	Ilan	S. F. Huang 4802	TAI
MAY	1974/5/28	+	-	-	Ilan	T. C. Huang 7768	TAI
JUN	1995/6/23	+	-	-	Kaohsiung	S. T. Chiu 2934	HAST
JUN	2010/6/28	+	-	-	Nantou	S. W. Chung 10038	TAIF
JUL	1989/7/11	+	-	-	Chiayi	T. C. Huang 14243	TAI
JUL	1995/7/14	-	-	-	Hualien	C. H. Chen 1165	HAST
JUL	1986/7/25	-	+	+	Nantou	C. I. Huang 2195	TAI
AUG	2006/8/3	-	-	-	Taichung	C. I. Huang 2777	HAST
AUG	2006/8/20	+	+	+	Nantou	P. F. Lu 12404	HAST
AUG	1969/8/10	-	-	-	Chiayi	Y. Ando 519	TAI
SEP	2009/9/4	-	-	-	Hualien	C. I. Huang 4159	HAST
SEP	1984/9/23	-	-	-	Hualien	C. I. Peng 7346	HAST
SEP	1993/9/15	-	-	-	Taichung	S. F. Huang 877	TAI
OCT	1977/10/13	+	-	-	Ilan	Y. C. Jeng 1955	TAI
OCT	1906/10/14	-	-	-	Chiayi	T. Kawakami 1708	TAI
OCT	2000/10/12	-	-	-	Nantou	J. J. Chen 424	HAST
NOV	2008/11/27	-	-	-	Taichung	C. I. Huang 3665	HAST
NOV	1997/11/16	-	-	-	Nantou	S. H. Wu 689	HAST
NOV	2007/11/18	-	-	-	Hualien	P. F. Lu 14998	HAST
DEC	1939/12/17	-	-	-	Chiayi	(79131)	TAI
DEC	2000/12/27	-	-	-	Ilan	Y. Y. Huang 190	TNM
DEC	2007/12/26	+	-	-	Taitung	(285493)	TAIF

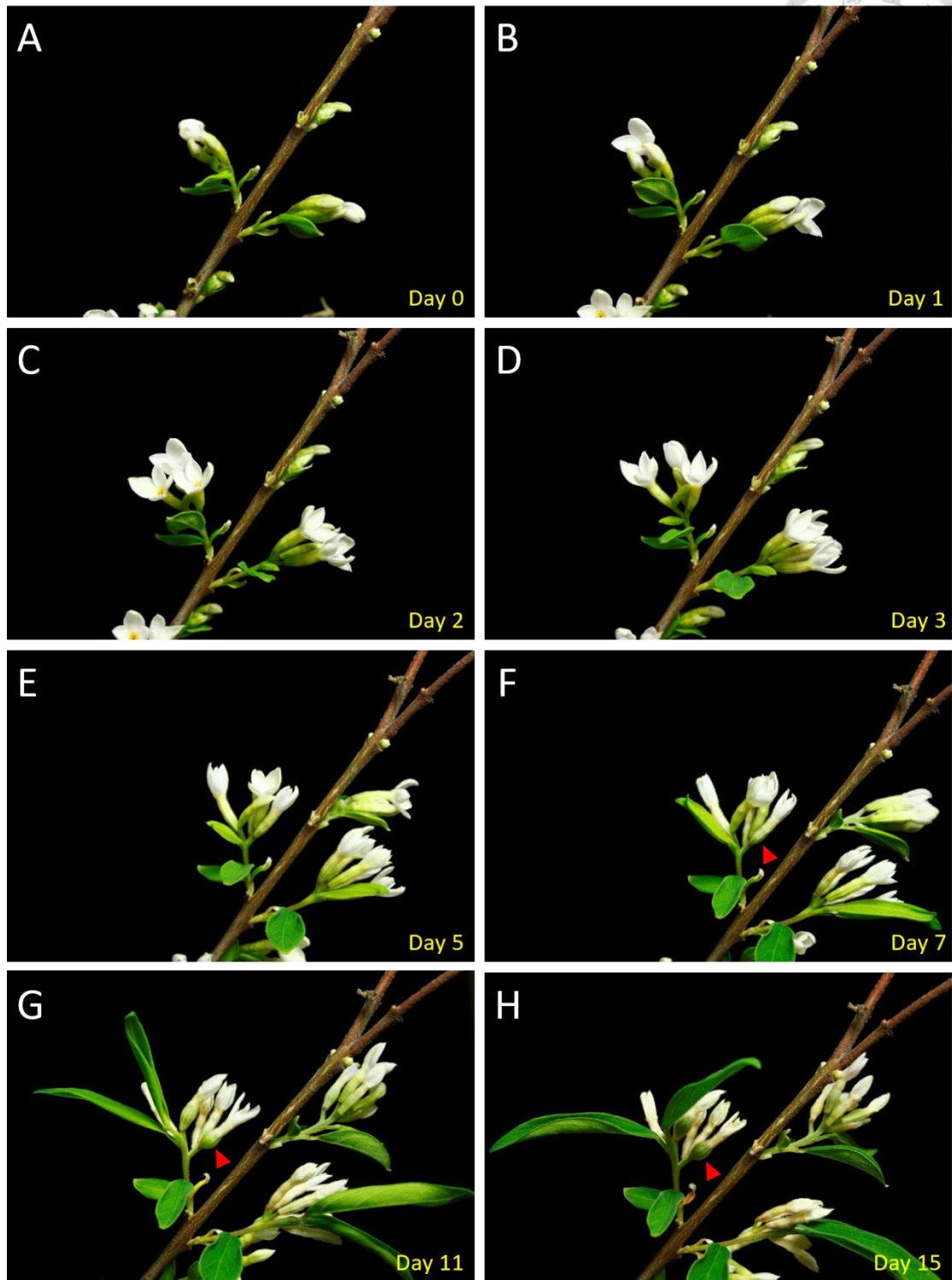


Figure A-1. Continuous video-recording of *Daphne genkwa* inflorescences. (A) Flower buds on recording-day 0. (B) Day 1. (C) Day 2. (D) Day 3. (E) Day 5. (F) Developing fruits on Day 7. (G) Day 11. (H) Day 15. Arrowheads indicate the swollen ovaries.