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Maintenance mechanism of gynodioecy in a summer-deciduous shrub, *Daphne jezoensis*

（夏期落葉性低木ナニワズにおける雌性両全性異株の維持機構）

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Contents

Summary

Sexual system of flowering plants is diverse and complex. Hermaphroditism is the most common sexual system in angiosperm (72%), while a separation of sex (dioecy) is found in only about 6% of angiosperm species. Dioecy is thought to have evolved from hermaphroditic ancestors in angiosperm. One of the postulated evolutionary pathways from hermaphroditism to dioecy is that through a gynodioecy stage (coexistence of female and hermaphroditic individuals), called a gynodioecy–dioecy pathway. That pathway is composed of the earlier stage (invasion of female phenotype into hermaphroditic population) and the later stage (replacement of hermaphroditic phenotype by male phenotype). However, there are controversial views on the credibility and generality of the transition from gynodioecy to dioecy. For a clarification of the possibility and generality of the gynodioecy–dioecy pathway, integrated studies on the mating properties and reproductive success of hermaphrodites in gynodioecious species are needed. A morphologically gynodioecious species *Daphne jezoensis* (Thymelaeaceae) is a summer-deciduous woody shrub inhabiting understory of deciduous forests. The female function (fruiting ability) of hermaphroditic plants is limited compared to female plants. Therefore, the sexual system of this species is expected to be in a later stage of the gynodioecy–dioecy pathway. In this study, I aim to clarify whether gynodioecy in *D. jezoensis* is a stable sexual system itself or on the transitional way to dioecy.

In Chapter 1, I evaluated the variations in sexual system and genetic differentiation across the geographic distribution range of *D. jezoensis* in Japan. I compared

reproductive traits among 14 populations and evaluated the selfing rate and genetic differentiation between populations using microsatellite markers. Although relatively high genetic separation was detected in southern most populations, there were no significant variations in the sexual system and fruiting ability of hermaphrodites among populations. Thus, gynodioecy with male-biased hermaphrodites may be a stable sexual system in this species throughout the geographic distribution range in Japan. Hermaphrodites were commonly self-compatible, but their seed-set ability was limited in every population even when enough pollen was supplied. Very low seed fertility of hermaphrodites suggests that the contribution of hermaphrodites as a seed producer is limited in the natural populations.

In Chapter 2, I searched the factors limiting seed production of *D. jezoensis* along the reproductive process; pollination, fertilization, and post-fertilization success. I conducted a hand-pollination treatment, observation of pollen germination on stigma and pollen-tube elongation toward ovules, and resource manipulation experiments (fertilizer addition treatment and shading treatment) in the field. Pollen quality significantly affected seed production of hermaphrodites. Seed-set rate of the self-pollinated inflorescences was lower than that of outcrossed ones. Pollen germination and pollen-tube growth were not restricted in hermaphroditic flowers. A series of the fertilizer addition and shading experiments revealed that the restricted fruiting ability of hermaphrodites was independent of the resource condition or size of maternal plants, indicating that the plasticity of fruit production in hermaphrodites was absent. Therefore, the low seed fertility in this species is supposed to be regulated by an internal physiological control.

In Chapter 3, I aimed to reveal the reason why gynodioecy with male-biased hermaphrodites can be stabilized. I measured the fitness gain through each sexual function and a trade-off between female and male functions under natural conditions. The degree of inbreeding depression was measured by a hand pollination experiment and germination test. For the estimation of male success under natural conditions, paternity analysis was conducted using microsatellite markers. In order to assess the reproductive performance over long periods, furthermore, I analyzed the long-term monitoring data of this species. A considerable inbreeding depression was detected in self-pollinated seeds in hermaphrodites. Considering the potentially low seed fertility and high selfing rate under natural conditions, fitness gain of hermaphrodites might be largely obtained through the male function as a pollen donor. Because donation success of individual plants increased with flower number, hermaphrodites need to keep large flower production. Individual with large fruit production decreased flower production next spring, indicating the cost of fruit production. Therefore, fruiting ability of hermaphrodites might be originally determined at low levels to mitigate the negative effect of fruit production.

The present study demonstrated that the gynodioecy with male-biased hermaphrodites is not necessarily a transitional stage to dioecy, but could be a stable sexual system. Because the seed productivity of male-biased hermaphrodites is remarkably small, its contribution as a seed producer is negligible in terms of the maintenance of existing population. However, the reproductive assurance in the colonization process may be advantageous to maintain gynodioecy in this species. Furthermore, the cost of fruit production was maintained at low levels, which enables to maintain the male-biased hermaphrodites. The conservative seed fertility, probably constrained by the summer-deciduous life-history, may be a proximate factor maintaining the gynodioecy as a stable sexual system.

General introduction

Sexual system of flowering plants is diverse and complex. Hermaphroditism, whose populations are composed of only hermaphroditic individuals, is the most common sexual system in angiosperm (72%). The separation of sex (dioecy) is thought to have evolved from hermaphroditic ancestors in flowering plants (Renner and Ricklefs, 1995). One of the postulated evolutionary pathways is that through gynodioecy, called as the gynodioecy–dioecy pathway (Charlesworth and Charlesworth, 1978; Spigler and Ashman, 2012). Gynodioecy is a sexual system in which hermaphroditic and female individuals coexist within a population. It occupies approximately 2–7% of angiosperm species (Richards, 1997; Dufay et al., 2014). In the earlier stage of the gynodioecy–dioecy pathway, female plants (male-sterile mutant) invade a hermaphrodite population. Hermaphrodites can gain fitness through both seed and pollen production, while females can gain fitness only through seed production. Therefore, females must have greater seed fertility (quantitatively or qualitatively superior seed production) than hermaphrodites (Charlesworth and Charlesworth, 1978; Bailey et al., 2003). As the frequency of females increases within a population, a selective pressure may act on hermaphrodites to invest more in male function, resulting in the decrease in seed fertility of hermaphrodites (Lloyd, 1976; Charlesworth, 1989; McCauley and Brock, 1998). In the later stage of the gynodioecy–dioecy pathway, the frequency-dependent selection may enable to establish pure male plants (female-sterile mutant) within a gynodioecious population: a stage of subdioecy in which hermaphroditic, female, and male individuals coexist. The transition from subdioecy to dioecy is accomplished by a replacement of hermaphrodites by males. However, there are controversial views on the credibility and generality of the transition from gynodioecy to dioecy (Spigler and Ashman, 2012). Several studies suggest that severe pollen limitation may help to maintain hermaphrodites within a gynodioecious population because some hermaphrodites can produce seeds by autonomous self-pollination as a reproductive assurance (Wolfe and Shmida, 1995; Fleming et al., 1998; Del Castillo and Argueta, 2009). This situation prevents the evolution of dioecy.

In the later stage of the gynodioecy–dioecy pathway, either males or male-biased hermaphrodites with low seed fertility are expected. There are many emperical studies supporting the existence of earlier stage of the gynodioecy–dioecy pathway, i.e., greater seed fertility of females than of hermaphrodites (Shykoff et al., 2003). In contrast, only a few studies have addressed the evolutionary significance of the later stage (Delph and Wolf, 2005; Ehlers and Bataillon, 2007; Spigler and Ashman, 2012). This is partly because the evaluation of male reproductive success, i.e., success as a pollen donor, is technically difficult in natural populations. Furthermore, effectiveness of ecological factors affecting the maintenance of hermaphrodite within a population, such as pollen limitation, selfing ability, and inbreeding depression, were insufficiently investigated in the previous studies. These ecological factors often vary among populations within a distributional range of a species. For a clarification of the possibility and generality of the gynodioecy–dioecy pathway, therefore, more integrated studies on the mating properties and reproductive success of hermaphrodites are needed across many populations in gynodioecious species.

It is crucial to select suitable plant species for a research of the gynodioecy–dioecy pathway in terms of the evolutionary status of the sexual system. *Daphne jezoensis* Maxim. (Thymelaeaceae) is reported as a morphologically gynodioecious species because each population is composed of hermaphroditic individuals having developed anthers and pistils and male-sterile individuals without pollen production (female plants). Furthermore, hermaphrodites produce small amount of fruits even when enough amount of pollen is received, indicating male-biased hermaphrodite (Kikuzawa, 1989; Sinclair et al., 2016). Therefore, the sexual system of this species is expected to be in the later stage of the gynodioecy–dioecy pathway. Because previous studies were conducted only in a few populations, geographic variations in the sexual system are unclear.

Environmental conditions and biotic features strongly affect reproductive success. Especially, pollinator availability, selfing ability, and inbreeding depression are important factors for the maintenance of hermaphrodites within a population (Wolfe and Shmida, 1997; Fleming et al., 1998). Pollinator availability, such as species composition and activity, may vary among populations across the wide distribution range. Site-specific selective forces might have changed the mating system and genetic diversity of local populations. At the marginal parts of the distribution range, higher selfing rate or evolution of self-compatibility is observed in several species (Busch, 2005; Michalski and Durka, 2007). These factors cause geographic variation in sexual system and genetic differentiation between populations. If the present sexual system of *D. jezoensis* is in a stable stage, male-biased gynodioecious populations will be observed constantly among local populations throughout the distribution range.

Although restricted seed production is commonly observed under natural conditions, multiple factors (both external and internal ones) may affect seed production differently. The process of seed production is composed of three stages; 1) pollination success, 2) pollen germination on stigma and pollen-tube elongation toward ovules (fertilization success), and 3) embryo survival and development after fertilization (post-fertilization success). The third process is often influenced by the resource conditions of maternal plants. Resource manipulation treatment and analysis of size dependency of fruit production are needed to test the resource limitation on fruit production.

According to the sex allocation theory, the fitness relationship between male and female function is a key issue to predict the evolutionary direction toward hermaphroditism or dioecy (Charnov, 1982). Based on the measurement of male and female fitness under natural conditions, I try to predict sexual selection acting on the sexual system in this species. In polycarpic plants, furthermore, large resource investment in the current reproduction often causes negative effects on future reproduction, growth, or survival, i.e., the cost of reproduction (Obeso, 2002). Therefore, fitness of individual plants should be evaluated based on the long-term reproductive events. I try to evaluate this using long-term monitoring data of this species.

In this study, I aim to clarify whether the male-biased gynodioecy of this species is a stable sexual system or a transitional stage to dioecy. The major objectives of this study are clarifications of 1) the geographic variation in sexual system across the distribution range, 2) factors restricting fruit production under natural conditions, and 3) reproductive success under natural conditions and a trade-off between female and male functions. Throughout these studies, I aim to clarify the evolutionary and ecological significance of the biased gynodioecious system (i.e., male biased hermaphrodites and females) in *D. jezoensis*. The integrated approach of this study should contribute to further understanding of the diversity of sexual system in plants and the evolutionary pathway from hermaphroditism to dioecy.

Study species

Daphne jezoensis (Thymelaeaceae) is a short (< 30 cm) summer-deciduous shrub inhabiting the understory of cool-temperate forests in central to northern Japan and Sakhalin (Fig. 0-1 a). Leaves emerged in autumn, overwinter, and several leaves are added in spring, then all leaves shed by early summer. Leaves were arranged as a whorl in each shoot. Flowering occurs in early spring, soon after snowmelt. Flowers persist until mid- to late May, and fruits mature in July. There are morphologically two distinct sex types of individuals within a population, female individuals (F) and hermaphroditic individuals (H); referred as sex morph (Fig. 0-1 b–d). Hermaphroditic flowers have developed anthers with reddish yellow color, while anthers of female flowers are highly reduced, whitish colored, and contain no pollen. Tubular flowers produce small amounts of nectar and are infrequently visited by moths, butterflies, and bumble bees. Small beetles and thysanopteran insects are also observed on the flowers for pollen foraging (personal observation). Individual fruits contain a single seed. Creeping and branching stems in this species sometimes make difficult to discriminate individual plants visually. In this study, separated stems at ground level were recognized as ramets, and a whole plant composed of genetically identical ramets was a single genet, F or H. Although sexually labile genets (yearly swinging between F and H) were occasionally observed (Sinclair et al., 2016), they were classified as either F or H depending on the dominant sex morph during the survey periods.

Genus *Daphne* is composed of approximately 70 species, and dominant sexual system is hermaphroditism including some gynodioecious species (Brickell and Marhew, 1976). Although comparative studies on the sexual system in *Daphne* species are limited, *D. rodriguezii* and *D. gnidium* are hermaphroditic, and *D. laureola* and *D. miyabeana* are reported as a gynodioecy of the early stage in the gynodioecy–dioecy pathway in which hermaphrodites have high seed-set ability and sometimes hermaphrodite populations exist (Alonso and Herrera, 2001; Alonso et al., 2007; Rodrıguez-Perez and Traveset, 2011; Sakata and Nakahama, 2018). In Japan, five gynodioecious *Daphne* species including *D. jezoensis* and *D. miyabeana* are distributed (Iwatsuki et al., 1999). Among *Dphne* species whose sexual system was studied in detail, the sexual system of *D. jezoensis* is likely to be nearest to dioecy. Thus, various stages of gynodioecy are existing in *Daphne* species but dioecious species are absent.

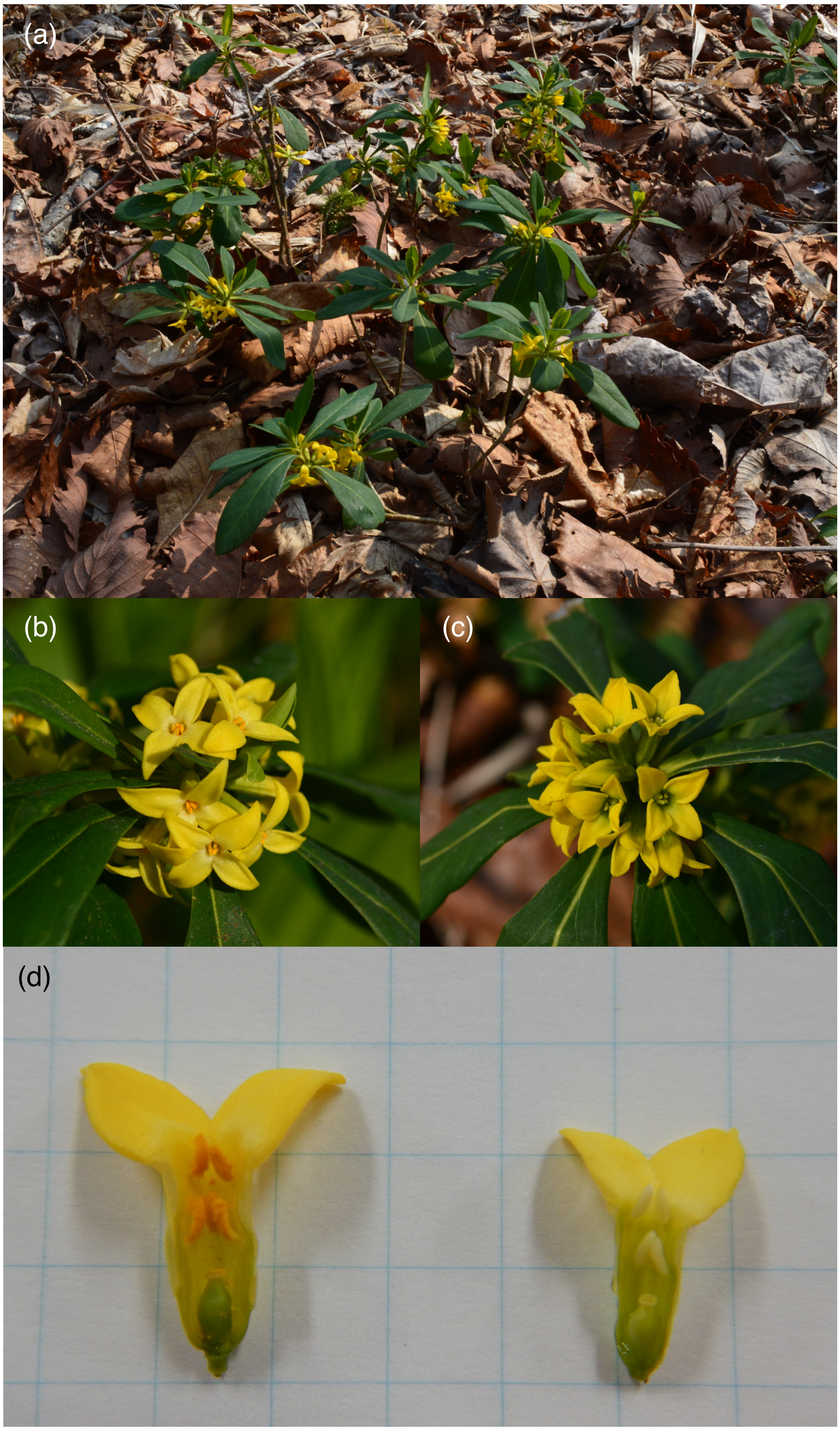

Fig. 0-1

Daphne jezoensis in flowering season (a), inflorescence of hermaphroditic individual (b), inflorescence of female individual (c), and the cross sections of hermaphroditic and female flowers (d). The grid size is 0.5 cm in (d).

Chapter 1: Geographic variation in sexual system and genetic differentiation

1.1 Introduction

In plant species whose sexual system is in a transitional stage from gynodioecy to dioecy, female function of hermaphroditic plants will be smaller, and seed fertility of hermaphrodites may vary among populations depending on the evolutionary stage. Understanding the patterns of geographic variation in sexual system provides us with possible selective factors affecting the evolution of sexual system (Barrett, 1992; Alonso et al., 2007; Caruso and Case, 2007). Previous studies reported that *Daphne jezoensis* is a morphologically gynodioecious species, but hermaphrodites rarely produced fruits, indicating a male-biased hermaphrodite (Kikuzawa, 1989; Sinclair et al., 2016). Therefore, sexual system of this species is expected to be in a later stage of the gynodioecy–dioecy pathway. Because these studies were conducted only in a few populations, however, geographic variations in the mating properties and sexual system are unclear. If the present sexual system is in a stable stage, gynodioecious populations with male-biased hermaphrodites will be consistently observed among populations throughout its distribution range. On the other hand, if the sexual system is in a transitional stage from gynodioecy to dioecy, female function of hermaphroditic plants may vary across the distribution range.

In the gynodioecy–dioecy pathway, sexual dimorphism of phenotypes is expected to be enforced toward dioecy. Sexual dimorphism in floral traits, such as flower size, shape, and display size of inflorescences, is widely reported in both dioecious and gynodioecious plants (Delph et al., 1996; Eckhart, 1999; Shykoff et al., 2003). In the later stage of the pathway, furthermore, female function of hermaphroditic phenotype is predicted to decrease and/or morphologically male phenotype exists within a population, i.e., a stage of subdioecy. In order to identify the evolutional stage, therefore, quantification of the degree of sexual dimorphism is a key issue.

Site-specific differences in the abiotic and biotic conditions may be related to the geographic variation in mating system of plants. In animal-pollinated species, pollinator availability largely affects reproductive success through female and male functions (Harder and Barrett, 1996). Severe pollen limitation often results in low seed-set success in which selfing ability without pollinators will be advantageous as a reproductive assurance (Wolfe and Shmida, 1997; Fleming et al., 1998). Local evolution of high selfing ability is frequently reported in the marginal populations of the distribution range in several plant species (Moeller, 2006; Michalski and Durka, 2007). Mating system, such as selfing, outcrossing, and mixture of them, affects genetic structure of populations. In species with high selfing ability, for instance, low genetic diversity within a population and/or large genetic differentiation among populations are expected (Charlesworth and Pannell, 2001; Charlesworth, 2003). Therefore, clarification of geographic variations in mating system and seed fertility of hermaphroditic phenotype are crucial to evaluate the evolutionary status of gynodioecious species.

In this chapter, I aim to evaluate the relationship between pollen limitation and the sexual and mating system among populations at a geographic scale. Here, I predict that if the sexual system of *D. jezoensis* is in a transitional stage, unstable sexual system ranging from gynodioey to dioecy may exist across the distribution range. In populations under severe pollen limitation, especially, hermaphroditic phenotype may have an autonomous selfing ability as a reproductive assurance. In such populations, low genetic diversity and/or genetic differentiation from other populations are expected. In contrast, in populations with sufficient pollinators, morphological or functional male phenotype lacking seed-set ability may exist. To test these predictions, I assessed geographic variations in floral morph, pollen limitation, sexual system, mating system, genetic diversity, and genetic differentiation among populations.

1.2 Methods

Study sites

Field survey was conducted in 14 populations across a whole distribution range of *Daphne jezoensis* in Japan: eight populations in Hokkaido and six populations in Honshu of Japan. The populations in Hokkaido were located at Notoro (44°06′N, 144°15′E), Tossyozan (43°51′N, 142°27′E), Arashiyama (43°48′N, 142°18′E), Nopporo (43°02′N, 141°31′E), Chitose (42°44′N, 141°44′E), Tomakomai (42°41′N, 141°34′E), Toyoura (42°36′N, 140°36′E), and Yokotsu (41°53′N, 140°45′E), hereafter referred as NOT, TOS, ARS, NOP, CTS, TOM, TOY, and YOK, respectively. The populations in Honshu were located at Asamushi (40°54′N, 140°52′E) and Shigabou (40°33′N, 140°38′E) in Aomori, Akita (39°54′N, 140°52′E), Niigata (37°41′N, 138°49′E), Ishikawa (37°01′N, 136°56′E), and Fukui (36°05′N, 136°28′E), hereafter referred as ASM, SGB, AKT, NGT, ISK, and FUK, respectively (Fig. 1-1). Permanent plots (50 m × 50 m) at NOP and CTS were established in 2009 and 2013, respectively (Sinclair et al., 2016). A permanent 30 m × 30 m plot was set up at TOM in April 2015. All ramets with floral buds were tagged and mapped, and observations were conducted every year.

For other five populations in Hokkaido and six populations in Honshu, field survey was conducted in 2017 and 2019, respectively.

Sex ratio and fruit production under natural conditions

At CTS, NOP, and TOM, all ramets producing floral buds were genotyped using genetic markers to determine genet individuality. In other 11 populations, about 100 flowering ramets with flowers were tagged in flowering season (Table 1-1). The flowering ramets were arbitrary selected at least 1-m apart from each other, in order to avoid sampling from same genets. Each ramet was marked with numbering tags on a stem in which about 15 flowers existed. For the measurements of sex ratio in each population and fruit production of individual ramets under natural conditions, sex morph, i.e., female (F) or hermaphrodite (H), and the numbers of flowers on tagged stems were recorded in flowering season, and the number of fruits was recorded in fruiting season.

Floral measurements

In order to evaluate the amount of sexual dimorphism in floral morphology within populations and the geographic variation in floral morphology, six floral parts were measured in 14 populations. A single flower was collected from each flowering ramet and stored in 70% ethanol until measurements. Corolla width, corolla tube length, corolla tube width, anther height, pistil width, and pistil length were measured using a digital caliper. A total of 60 flowers (F = 30, H = 30) from FUK, 85 flowers (F = 33, H = 52) from ISK, 98 flowers (F = 46, H = 52) from NGT, 58 flowers (F = 28, H = 30) from AKT, 50 flowers (F = 21, H = 29) from SGB, 52 flowers (F = 22, H = 30) from

ASM, 97 flowers (F = 28, H = 69) from YOK, 108 flowers (F = 52, H = 56) from TOY, 116 flowers (F = 49, H = 67) from TOM, 188 flowers (F = 68, H = 120) from CTS, 172 flowers (F = 57, H = 115) from NOP, 109 flowers (F = 39, H = 73) from ARS, 109 flowers (F = 68, H = 41) from TOS, and 110 flowers (F = 29, H = 81) from NOT were measured.

Hand pollination and bagging experiment

To evaluate seed fertility (potential fruiting ability) and pollen limitation under natural conditions, a hand pollination experiment was performed for female and hermaphroditic individuals in 13 populations (Table 1-2). The outcross-pollination was not performed for hermaphrodites at FUK and NGT because anthers of many hermaphroditic flowers had been dehisced and self-pollen grains had been deposited on the stigmas at the time of pollination experiment. The outcross-pollination treatment was performed on one or two inflorescences (composed of about 10 flowers) per ramet. Pollen for the outcross-pollination was collected from 5 to 10 hermaphroditic donors at least 10-m apart from the recipient genets and artificially deposited on the stigmas of flowers soon after opening. When fruits matured in mid-July, they were counted and fruit-set rate (identical to seed-set rate) was calculated. The degree of pollen limitation was calculated on the basis of mean fruit-set rate of females in each population as follows:

1 – (natural fruit-set rate) / (outcrossed fruit-set rate).

In order to test whether hermaphrodites have fruiting ability by automatic self-pollination, a bagging treatment was performed in hermaphrodites. All floral buds of selected hermaphroditic ramets were covered with fine-meshed nylon bags to prevent pollinator visits until fruit maturation. Then, the number of fruits was recorded, and fruit-set rate was calculated. This treatment was conducted at SGB, TOY, TOM, and ARS (Table 1-2). I did not conduct this treatment in other Honshu populations, because most of hermaphroditic flowers had opened at the time of survey.

Sampling and genotyping

One leaf was collected from each flowering ramet in each plot in the flowering season for ramet genotyping. In order to evaluate the selfing rate under natural conditions, matured fruits were harvested from tagged hermaphrodites during 2015 to 2019 depending on populations. Leaf samples were stored in a desiccator with silica gel at room temperature (20–25°C). Seed samples of the fruits were stored at –60°C until DNA extraction, which was performed using the cetyltrimethylammonium bromide (CTAB) protocol (Stewart and Via, 1993). Extracted DNA was amplified with a TaKaRa PCR Thermal Cycler Dice Gradient (Takara Bio Inc., Otsu, Shiga, Japan) using eight markers, DpJe010, DpJe074, DpJe044, DpJe076, DpJe020, DpJe082, DpJe059, and DpJe061. Two-step multiplex PCR was performed. In the first step, final volume of 5 µl containing 1 µl extracted DNA, 2.5 µl 2 × Type-it Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.5 µl F-tail mix (0.6 µM for each locus), 0.5 µl RS mix (2.4 µM for each locus), and 0.5 µl water with 5 min at 95°C, 23–32 cycles of 30 sec at 95°C, 90 sec at 60°C, and 40 sec at 72°C, followed by 8°C for holding. The number of cycles was changed depending on the quality of DNA, i.e., 23 cycles for leaf and 24–32 cycles for seed. Then, 0.5 µl 2 × Type-it Multiplex PCR Master Mix and 0.5 µl tail-Dye mix (1.2 µM for each color) were added for second step. Second step was performed with 3 cycles of 30 sec at 95°C, 90 sec at 60°C, and 40 sec at 72°C, followed by 30 min at 60°C. The DNA fragments were analyzed using an Applied Biosystems

3730 Genetic Analyzer with GeneScan 500 LIZ Size Standard (Thermo Fisher Scientific Inc., Waltham, MA, USA) and scored genotypes were analyzed using GeneMapper ver. 4.0 (Thermo Fisher Scientific Inc.).

Genetic structure and mating system

To evaluate the genetic diversity within populations, mean number of alleles (A), allelic richness (A_R), observed heterozygosity (H_O), and expected heterozygosity (H_E) were calculated with the diveRsity package (Keenan et al., 2013). R version 3.6.2 was used for all genetic and statistical analyses (R Core Team, 2019). Inbreeding coefficient (F_{IS}) as multilocus estimate was calculated, and deviations from Hardy-Weinberg equilibrium (HWE) was evaluated using Genepop version 4.7 (Rousset, 2008). The significance of the F_{IS} values was estimated using the Markov Chain algorithm with 1000 permutations, 100 batches and 1000 interactions per batch. Estimation of selfing rates was conducted for 13 of 14 populations because seed samples of hermaphrodites at ASM were limited. Multilocus outcrossing rates (t_m) were estimated and selfing rates ($s = 1 - t_\mathrm{m}$) were calculated for hermaphrodites in each population each year using MLTR ver. 3.2 (Ritland, 2002). The standard error of t_m was calculated based on 1000 bootstraps.

In order to estimate the genetic differentiation among populations, pairwise G'_{ST} (Hedrick, 2005) was calculated using the diveRsity package. The correlation between pairwise genetic distance (G'_{ST}) and pairwise geographic distance (km) was evaluated using Mantel test with 9999 permutations by the vegan package (Oksanen et al., 2019). For visualizing the patterns of population genetic structure, the dissimilarity of allele frequency was calculated based on the method of Kosman & Leonard (2005) with the

PopGenReport package (Adamack and Gruber, 2014), and was visualized using non-metric multidimensional scaling (NMDS) with the MASS package.

Statistical analyses

To test the deviation of sex ratio from 1:1 in each population, binomial test was performed. The correlation between female frequency and latitude or pollen limitation, and between the degree of pollen limitation and selfing rate of hermaphrodites, allelic richness, and F_{IS} were assessed by Pearson's test. R version 3.5.3 was used for all statistical analyses (R Core Team, 2019).

In order to compare floral morphology between females and hermaphrodites within a population, permutational multivariate analysis of variance (PERMANOVA) and generalized linear model (GLM) were performed for each population. PERMANOVAs were conducted using the Euclidean distances between six floral measurements with 999 permutations by the vegan package to test the significance of difference between sex morphs. GMLs postulating gaussian error distribution were conducted for four floral measurements, i.e., corolla width, corolla tube length, pistil length, and distance between anthers and stigma. Each floral measurement was a response variable, and sex morph was an explanatory variable. A best-fit model was selected based on Akaike's Information Criterion (AIC). In addition, in order to test the significance of difference in floral morphology between populations, PERMANOVAs were conducted using the Euclidean distances between six floral measurements with 999 permutations for each sex morph. To visualize the similarity in floral morphology, NMDS ordination was used. NMDS was performed based on the Euclidean distances between six floral measurements using the vegan package.

For the hand pollination experiment conducted for females, generalized linear mixed models (GLMMs) were performed to evaluate the degree of pollen limitation (compare fruit-set rate between natural and outcrossed-pollination) using the glmmTMB package, postulating a negative binomial error distribution with log-link function. Only the ramets used for the hand pollination experiment were included in this analysis. Female fruit-set rate was a response variable, i.e., fruit number was a response variable, flower number was set as an offset term, pollination treatment (natural and outcross-pollination) and population were explanatory variables with inclusion of their interaction, and ramet ID was set as a random factors. GLMM was run in each population if interaction was detected.

1.3 Results

Floral morphology and sex ratio

There was large sexual difference in floral morphology in every population (PERMANOVA, $P < 0.01$ in each population). Corolla was wider and longer in hermaphrodites than females in all populations ($P < 0.01$ in corolla width, $P < 0.01$ in corolla tube length; Fig. 1-2 a, b). Every hermaphroditic flower had a pistil, which was similar to female flowers at SGB (“sex morph” was excluded in the best-fit model), smaller than females at AKT ($P < 0.05$), and larger than females in other 12 populations ($P < 0.05$; Fig. 1-2 c). The distance between anthers and stigma was longer in hermaphrodites than females ($P < 0.05$; Fig. 1-2 d). Thus, morphological male phenotype without pistil was absent in any population. Floral morphology was different between populations in both of females and hermaphrodites ($F_{13,521} = 27.45$, $P < 0.001$

in female and $F_{13,777} = 36.17$, $P < 0.001$ in hermaphrodite; Fig. 1-3). In female flowers, variations in the length of corolla tube and the length and width of pistil (NMDS1) largely reflected the differences among populations. In hermaphroditic flowers, variations in length and width of corolla tube (NMDS1) largely reflected the differences among populations.

Sex ratio was not deviated from 1:1 in seven populations (FUK, NGT, AKT, TOY, TOM, CTS, NOP), while it was hermaphrodite-biased in five populations (ISK, ASM, YOK, ARS, NOT) and female-biased in two populations (SGB and TOS; Table 1-1). The trend in sex ratio was independent of geographic location and the degree of pollen limitation ($r = 0.01$, $P = 0.97$ for latitude; $r = –0.04$, $P = 0.90$ for pollen limitation; Fig. 1-4). All populations were composed of only females and hermaphrodites, indicating that the sexual system of *D. jezoensis* is morphologically gynodioecy throughout the distribution range.

Pollen limitation

A significant population × pollination treatment interaction was detected in fruit-set rate of females, indicating that the degree of pollen limitation was different between populations (Fig. 1-5). Outcrossed females showed significantly higher fruit-set rates than naturally pollinated females at TOY, CTS, NOP, ARS, TOS, and NOT ($Z > 2.65$, $P < 0.01$ in each population). The values of pollen limitation were positive in every population, indicating that pollen limitation was common (ranging 0.035–0.867; Table 1-2). However, pollen limitation was negligibly low at AKT and SGB (AKT: 0.044, SGB: 0.035). Even when outcross-pollination was conducted, fruit-set rates of females did not increase greatly: the maximum fruit-set rates under outcross-pollination was

53.9 at NGT. These results indicate that seed-set ability without pollen limitation of this species is not large even in females under natural conditions.

Fruiting and selfing abilities of hermaphrodites

In all populations surveyed, some hermaphrodites set fruits, but the fruit-set rates were very low compared to females (Table 1-2). Even when outcross-pollination was conducted, fruit-set rates remained at very low level (< 7.5%) in all populations, indicating that sexual function of hermaphrodites is male-biased.

Hermaphrodites produced seeds by selfing under natural conditions in all populations (Table 1-3). The estimated selfing rates of hermaphrodites at seed stage ranged from 32 to 87% across populations. The trend in selfing ability of hermaphrodites was independent of the degree of pollen limitation ($r = 0.25$, $P = 0.35$; Fig. 1-6 a). Hermaphrodites set fruits even when the bagging treatment was performed, indicating that self-pollination occurred automatically in both Honshu and Hokkaido populations (Table 1-2).

Genetic diversity

Mean number of alleles per locus (A) ranged from 3.9 to 11.1, and allelic richness (A_R) ranged from 3.6 to 9.5. These values were relatively low in the southernmost populations, FUK and ISK (A: 5.1 and 3.9, A_R: 4.7 and 3.6 at FUK and ISK, respectively, Table 1-4). The F_{IS} values were significantly higher than zero in four populations at ISK, NGT, CTS, and NOP. There were no significant relationships between the degree of pollen limitation and allelic richness ($r = 0.03$, $P = 0.93$; Fig. 1-6 b) and F_{IS} ($r = 0.16$, $P = 0.60$; Fig. 1-6 c).

Genetic differentiation among populations

Mean genetic distance values (G'_{ST}) was 0.58 (ranging from 0.11 to 0.91) between population pairs. G'_{ST} values were positively related to the geographic distance between populations ($r = 0.43$, $P < 0.01$, Fig. 1-7). The NMDS analysis of genetic similarity indicates that there is a moderate separation between Hokkaido populations and Honshu populations and that marginal populations, such as FUK (southernmost in Honshu), YOK (southernmost in Hokkaido), and NOT (northernmost in Hokkaido), were distributed at the marginal parts of the diagram (Fig. 8). Interestingly, ISK population was apparently isolated from other populations.

1.4 Discussion

Any significant difference in sexual system was not detected among populations across the distribution range of *D. jezoensis* in Japan. In every population, morphologically female and hermaphroditic phenotypes coexisted, and hermaphrodite had a male-biased sexual function, i.e., small seed-set ability even when enough pollen was supplied. Among populations studied, two southernmost populations were genetically isolated from other populations, and they had low genetic diversity. In this section, I discuss the effects of pollen limitation, selfing ability of hermaphroditic phenotype, and gene flow on the stability of gynodioecy in this species in view of geographic-scale variation.

Apparent sexual dimorphism was detected in corolla size in all populations. Large-sized hermaphroditic flowers may contribute to the improvement of male fitness. Presentation of conspicuous flowers and/or a large display size with many flowers often attracts more pollinators, resulting in larger success as a pollen donor (Bell, 1985;

Vaughton and Ramsey, 1998; Eckhart, 1999; Williams et al., 2000; Van Etten and Chang, 2014). Although the fruiting ability of hermaphrodites was significantly lower than that of females, pistil size of hermaphroditic flowers was generally larger than that of female flowers in most populations, indicating that there is no selective force reducing the pistil size of hermaphroditic flowers.

Morphological gynodioecy, composed of female and hermaphroditic phenotypes, was constantly observed in every population across the distribution range in Japan. Although sex ratio varied among populations to some extent (0.26–0.63), there were no significant relationships between sex ratio and geographic location or pollen limitation. Sex ratio of gynodioecious species is affected by the genetic mechanism of sex determination (Bailey and Delph, 2007). In species with a nuclear–cytoplasmic sex determination system, sex ratio often varied greatly among populations, whereas in species with a nuclear sex determination system, stable sex ratio is common in which female frequency is lower than 50%. A previous study suggested that gynodioecy in *D. jezoensis* might be determined by a nuclear inheritance system, because sex ratio showed small variation among seven populations in central Hokkaido over six years (Sinclair et al., 2016). Also in the present study, a nuclear inheritance system is expected because sex ratio was generally close to 1:1 in many populations. However, sex ratio in two populations (SGB and TOS) was significantly female biased. Several reasons may be considered for this deviation. Although I investigated sex ratio only once in many populations, observed trend might reflect actual sex ratio of each population. This is because stable sex ratio was confirmed in the long-term monitoring data at CTS, NOP, and TOM, indicating year-to-year variation in sex ratio is small in this species. However, female-biased sex ratio might be caused by mis-identification of individual genets because *D. jezoensis* sometimes creates large clonal patches of creeping stems and branches (Kudo et al., 2015). Furthermore, sex ratio of these populations might have not reached at a stable state yet.

There were some hermaphrodites having fruiting and selfing abilities in every population. A previous study reported that *D. jezoensis* might be self-incompatible, and stigma clogging with self-pollen might restrict the outcrossing fruit-set success of hermaphroditic flowers (Kikuzawa, 1989). In the present study, however, seed production was confirmed even on the bagged inflorescences of hermaphrodites in both Honshu and Hokkaido populations, indicating that hermaphrodites are physiologically self-compatible and self-pollination occurs automatically. Furthermore, genetic analysis showed that not a few hermaphrodite’s seeds were produced by selfing under natural conditions. Autonomous self-pollination can act as a reproductive assurance under the condition of severe pollen limitation (Wolfe and Shmida, 1997; Busch and Delph, 2012). In this study, pollen limitation was confirmed in most populations. This is because flowering of this species occurs early in the spring soon after snowmelt, when the activity of insects is low. If severe pollen limitation occurs every year, causing consistently small seed production, hermaphrodites could function as a reproductive assurance, under which selective force may act on hermaphrodites to have high selfing ability. However, there was no significant relationship between pollen limitation and selfing rate across populations. Furthermore, fruit-set rate of hermaphrodites was continuously low under natural conditions in every population (ranging 0–4.8%), resulting in negligibly low selfing rate at a population level. Therefore, autonomous self-pollination of hermaphrodites may not contribute as a reproductive assurance for the purpose of population maintenance.

When gene flow through pollination and/or seed dispersal occurs between geographically close populations, isolation by distance (IBD) is expected in genetic structure (Wright, 1943). In the present study, IBD was detected and the degree of genetic differentiation was relatively high (mean G'_{ST} was 0.58 and maximum was 0.91, and mean F_{ST} was 0.18 and maximum was 0.32), suggesting restricted gene flows between populations. This value was higher than other tree species using microsatellite markers, in which G'_{ST} values were 0.17 for 23 *Fagus crenata* populations (Hiraoka and Tomaru, 2009), 0.40 for 46 *Kalopanax septemlobus* populations (Sakaguchi et al., 2011), and 0.12 for 62 *Pinus densiflora* populations (Iwaizumi et al., 2013) in Japan. According to a review paper of allozyme data, long-lived woody species tend to exhibit little genetic differentiation among populations compared to short-lived woody species and herbaceous species (Hamrick et al., 1992). *D. jezoensis* seems to be a short-lived similar to other *Daphne* species (actual longevity was unknown but maximum was 40 years in a species) (Brickell and Marhew, 1976), whereas *F. crenata*, *K. septemlobus*, and *P. densiflora* are long-lived tree species. Furthermore, founder effects after long-distance seed dispersal often decrease genetic diversity within population and increase genetic differentiation from source populations (Le Corre and Kremer, 1998; Austerlitz and Garnier-Géré, 2003). Red ripen fruits of *D. jezoensis* seem to be dispersed by birds (fruits of a congener Japanese species *D. pseudomezereum* was reported to be dispersed by birds) (Suzuki, 2016). Therefore, a short lifespan and long-distance seed dispersal by birds may explain the unique genetic structure in this species.

NMDS result indicated that the southernmost populations, ISK and FUK, were genetically isolated. Furthermore, genetic diversity was low at ISK and FUK. Several studies reported that different mating system or sexual system was observed in isolated populations of gynodioecious species in which hermaphrodites with high selfing ability was recorded (Alonso and Herrera, 2011; Miller et al., 2019). In this study, however, mating system and sexual system of genetically isolated populations were not different from other populations. Although some geographic variations in the degree of pollen limitation, selfing rate, and genetic diversity were detected among populations, there were no significant relationships between these values. This might result in non-significant geographic variation in the sexual system of *D. jezoensis*. In conclusion, gynodioecy composed of male-biased hermaphrodites and females is a consistent sexual system in *D. jezoensis* throughout the distribution range in Japan.

Table 1-1. Numbers and frequencies of flowering ramets of female phenotype (F) and hermaphroditic phenotype (H) in each population.

Population	District	Ramet no. (F)	Ramet no. (H)	F frequency
FUK	Honshu	36	45	0.444
ISK	Honshu	39	63	**0.382**
NGT	Honshu	45	57	0.441
AKT	Honshu	41	60	0.406
SGB	Honshu	69	41	**0.627**
ASM	Honshu	30	60	**0.333**
YOK	Hokkaido	37	71	**0.343**
TOY	Hokkaido	52	56	0.481
TOM	Hokkaido	125	117	0.517
CTS	Hokkaido	187	202	0.481
NOP	Hokkaido	129	160	0.446
ARS	Hokkaido	36	73	**0.330**
TOS	Hokkaido	68	41	**0.624**
NOT	Hokkaido	29	81	**0.264**

* Bold character indicates sex ratio differed significantly from 1:1 ($P < 0.05$)

Table 1-2. Mean ± SE of fruit-set in females (F) and hermaphrodites (H) under natural-pollination, outcross-pollination, and bagging, and the degree of pollen limitation. In the parenthesis, the number of ramets setting any fruit (upper) and total number of ramets observed (lower) are shown.

Population	Natural (F)	Outcrossed (F)	Natural (H)	Outcrossed (H)	Bagging (H)	Pollen limitation
FUK	15.56 ± 3.56 (20/36)	22.25 ± 5.99 (9/13)	0.91 ± 0.46 (5/45)	-		0.301
ISK	20.48 ± 3.17 (30/39)	37.05 ± 5.93 (16/18)	1.86 ± 0.97 (6/63)	2.72 ± 1.25 (4/16)		0.447
NGT	18.64 ± 3.56 (27/45)	53.89 ± 9.09 (8/9)	1.01 ± 0.48 (5/57)	-		0.654
AKT	26.05 ± 4.83 (22/41)	27.24 ± 5.37 (16/20)	0.20 ± 0.19 (1/60)	4.15 ± 3.18 (3/17)		0.044
SGB	11.22 ± 1.75 (38/69)	11.63 ± 2.88 (11/22)	1.00 ± 0.58 (3/41)	0 ± 0 (0/21)	5.63 ± 3.00 (5/8)	0.035
ASM	14.22 ± 3.29 (14/30)	-	2.17 ± 1.29 (4/60)	-		-
YOK	20.15 ± 3.59 (28/50)	30.08 ± 7.66 (13/22)	0.64 ± 0.45 (4/94)	0 ± 0 (0/25)		0.330
TOY	22.19 ± 2.87 (38/52)	48.39 ± 3.89 (47/52)	3.25 ± 1.58 (10/56)	6.38 ± 2.11 (11/55)	0.65 ± 0.25 (7/28)	0.541
TOM	22.17 ± 2.08 (82/134)	44.32 ± 3.89 (57/74)	1.66 ± 0.50 (22/138)	6.41 ± 1.90 (14/81)	1.85 ± 1.17 (3/21)	0.500
CTS	22.83 ± 1.49 (197/293)	35.65 ± 3.43 (43/52)	4.79 ± 0.70 (86/340)	7.47 ± 2.23 (15/61)		0.360
NOP	32.17 ± 2.08 (136/188)	52.73 ± 4.72 (44/48)	2.29 ± 0.43 (66/306)	1.28 ± 0.48 (8/80)		0.390
ARS	6.46 ± 2.71 (6/27)	25.23 ± 5.31 (14/20)	2.62 ± 1.97 (3/31)	2.41 ± 1.05 (7/72)	1.59 ± 1.27 (2/15)	0.744
TOS	6.13 ± 1.69 (16/68)	46.05 ± 4.46 (36/40)	0 ± 0 (0/41)	1.60 ± 1.05 (3/41)		0.867
NOT	23.10 ± 4.02 (21/29)	45.93 ± 5.20 (25/29)	1.02 ± 0.38 (7/81)	4.38 ± 1.18 (15/77)		0.497

Table 1-3. Selfing rates at seed stage of hermaphrodites estimated by MLTR.

Population*	Sampling year	N (parent)	N (progeny)	Selfing rate
FUK	2019	11	33	0.35 ± 0.23
ISK	2019	16	61	0.75 ± 0.11
NGT	2019	9	17	0.87 ± 0.02
AKT	2019	4	7	0.83 ± 0.17
SGB	2019	4	18	0.32 ± 0.19
YOK	2017, 2018	17	67	0.68 ± 0.11
TOY	2017, 2018	23	90	0.47 ± 0.09
TOM	2016, 2017	24	48	0.32 ± 0.08
CTS	2016, 2017	14	36	0.60 ± 0.08
NOP	2015, 2016	39	54	0.43 ± 0.07
ARS	2017, 2018	4	10	0.70 ± 0.17
TOS	2017, 2018	6	9	0.69 ± 0.21
NOT	2017, 2018	10	19	0.86 ± 0.09

* Estimation of selfing rate was not conducted at ASM.

Table 1-4. Genetic diversity indices of *Daphne jezoensis* in 14 populations.

Population	N	A	A_R	H_O	H_E	F_{IS}*
FUK	30	5.13	4.66	0.53	0.57	0.0268
ISK	31	3.88	3.62	0.59	0.63	**0.0743**
NGT	30	11.13	9.54	0.76	0.79	**0.0554**
AKT	30	10.00	8.62	0.73	0.77	0.0633
SGB	32	7.13	6.27	0.72	0.71	–0.0023
ASM	30	7.63	7.01	0.81	0.78	–0.0289
YOK	29	7.88	6.72	0.70	0.66	–0.0523
TOY	39	7.38	6.30	0.75	0.71	–0.0397
TOM	38	7.00	6.10	0.71	0.73	0.0388
CTS	38	8.13	6.86	0.69	0.72	**0.0535**
NOP	75	10.00	7.39	0.66	0.71	**0.0877**
ARS	29	8.50	7.27	0.71	0.74	0.0291
TOS	27	6.88	6.22	0.69	0.69	0.0692
NOT	27	7.38	6.54	0.69	0.69	0.0225

N: Sample size, A: mean number of alleles per locus, A_R: allelic richness,

H_O: observed heterozygosity, H_E: expected heterozygosity, F_{IS}: inbreeding coefficient.

* Bold character indicates significance of heterozygote deficit ($P < 0.01$)

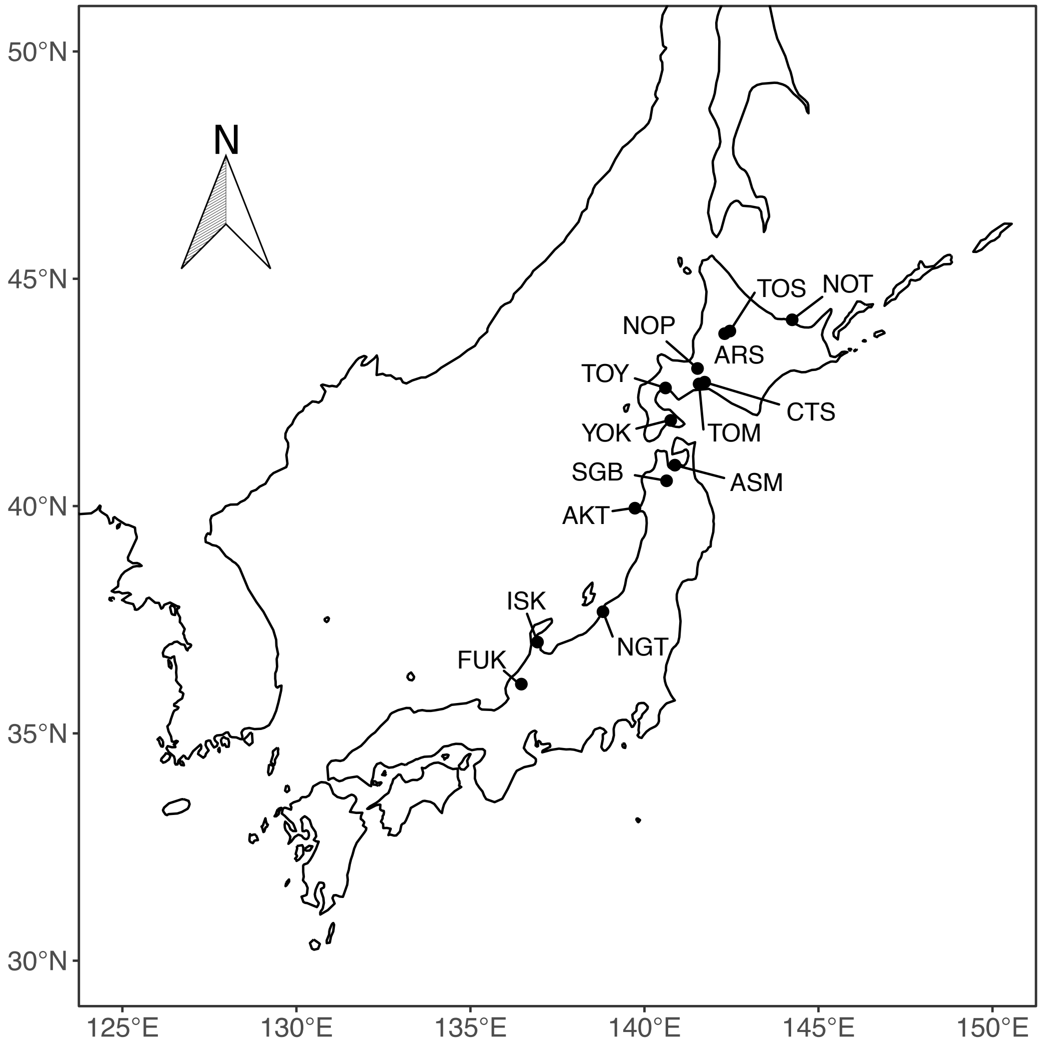

Fig. 1-1
Location of *Daphne jezoensis* populations surveyed in Japan.

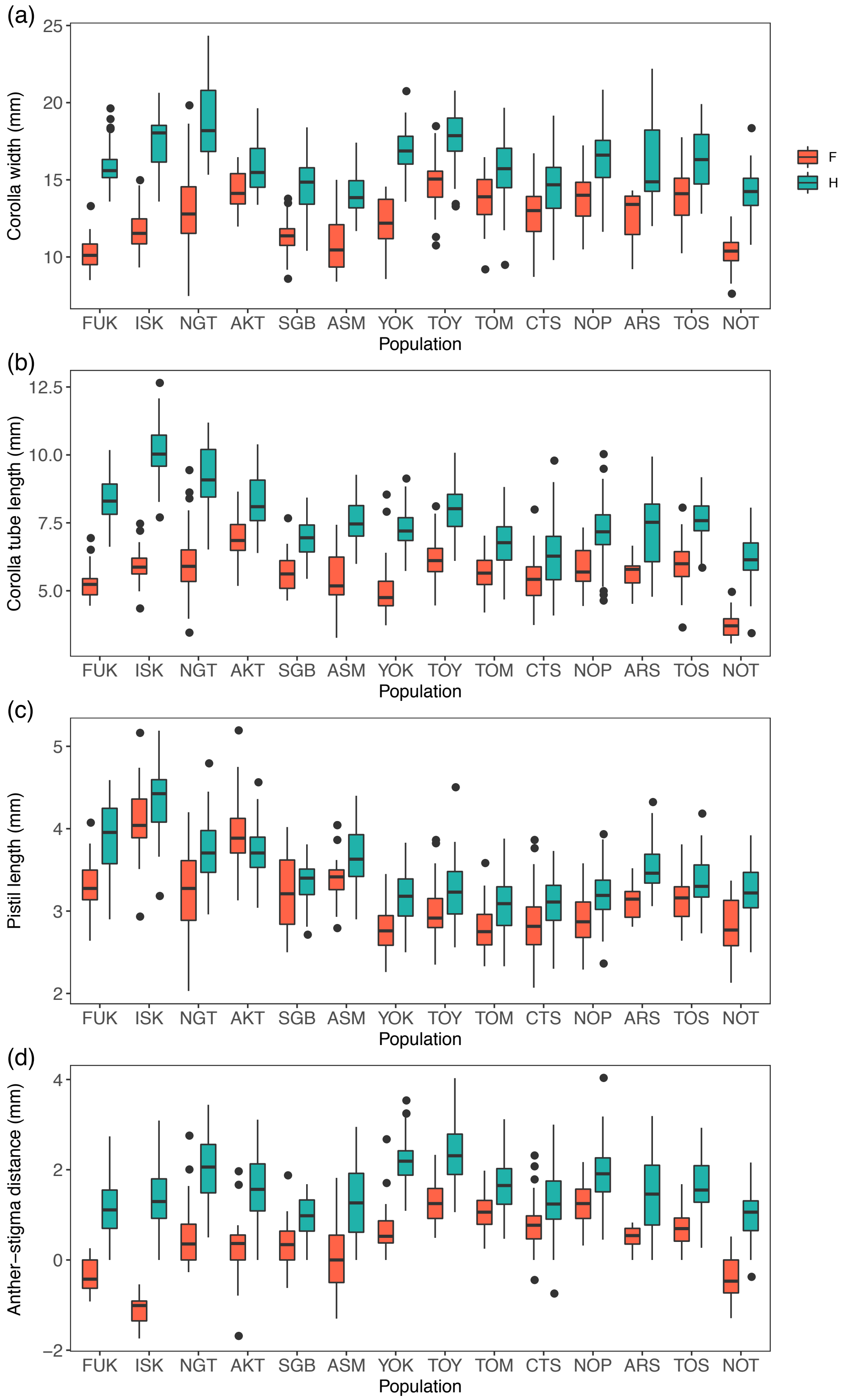

Fig. 1-2
Comparisons of floral morphologies; corolla width (a), corolla tube length (b), pistil length (c), distance between anther and stigma (d) between females and hermaphrodites.

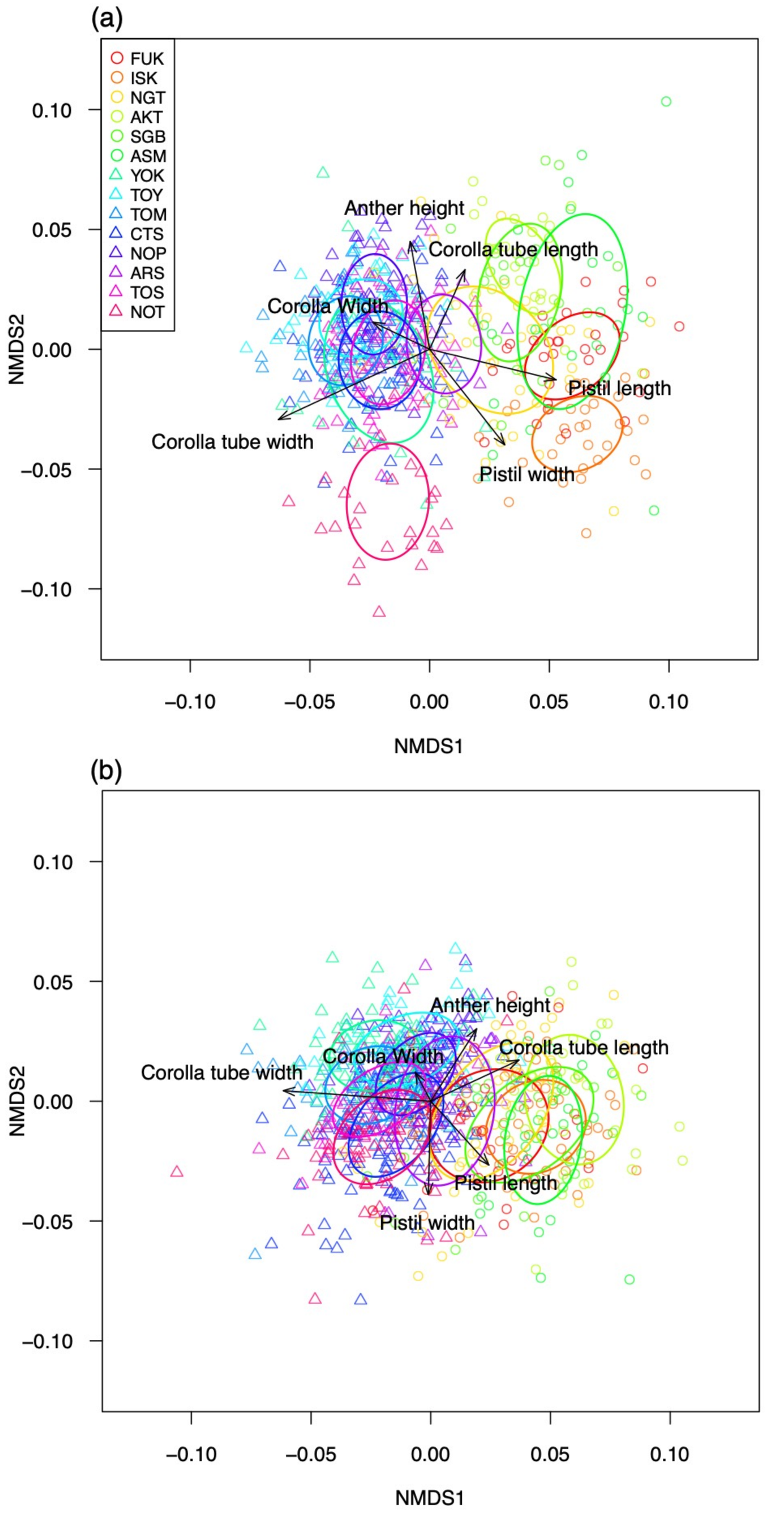

Fig. 1-3
Results of the non-metric multidimensional scaling (NMDS) ordination based on 6 floral measurements for females (a) and hermaphrodites (b).

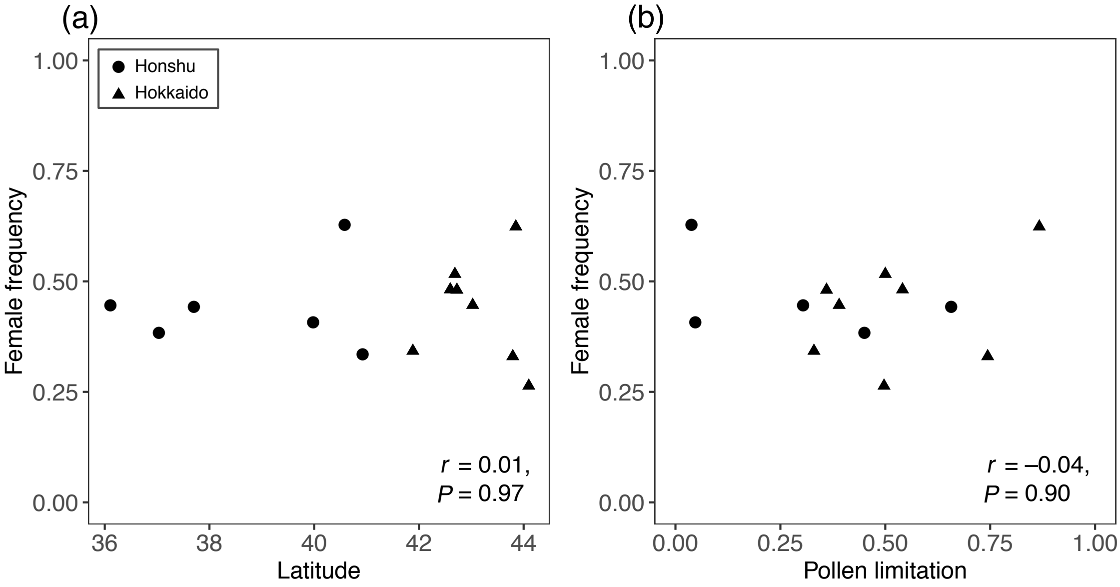

Fig. 1-4
Relationship between female frequency and latitude (a) or pollen limitation (b). Results of Pearson's test were indicated.

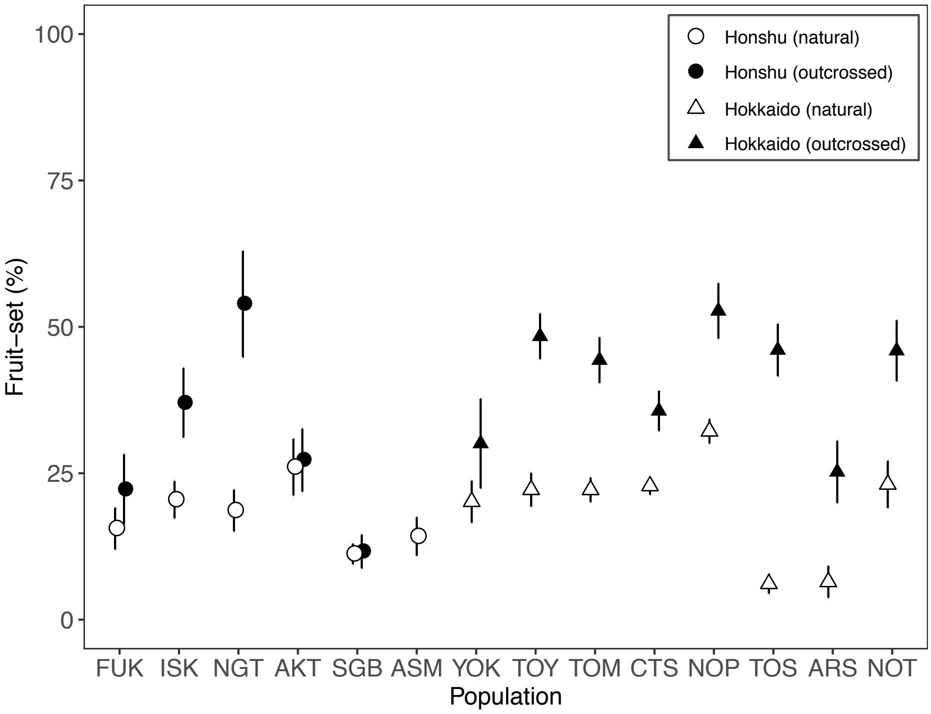

Fig. 1-5
Fruit-set (mean ± SE) of females under natural and outcross-pollination in 14 populations.

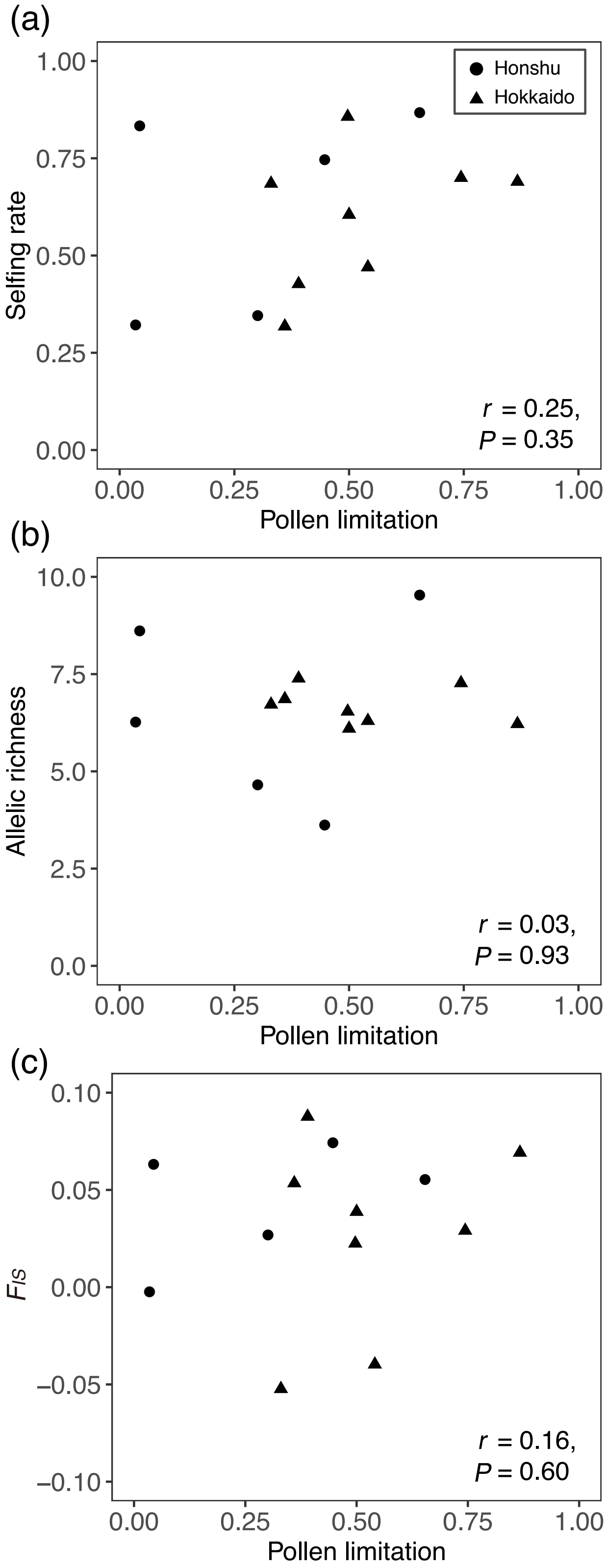

Fig. 1-6

Relationship between the degree of pollen limitation and selfing rate of hermaphrodites (a), allelic richness (b), and F_{IS} (c). Results of Pearson's test were indicated.

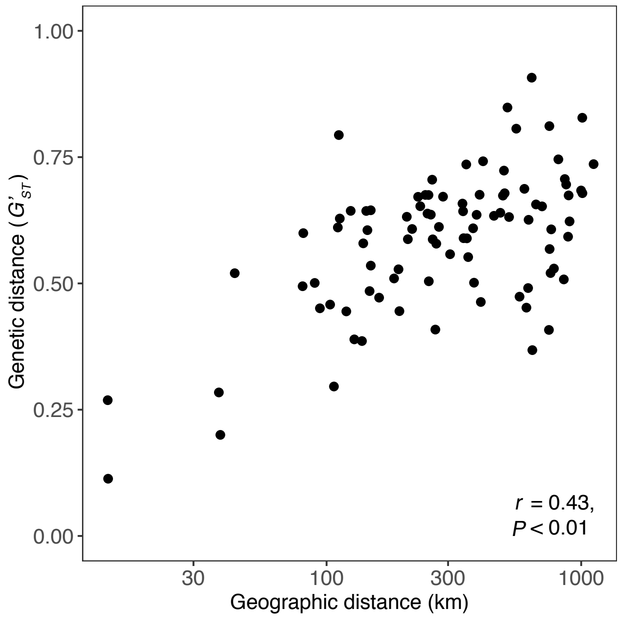

Fig. 1-7
Relationship between geographic distance (km) and genetic distance (pairwise G'_{ST}) for all population pairs. Result of mantel test was indicated.

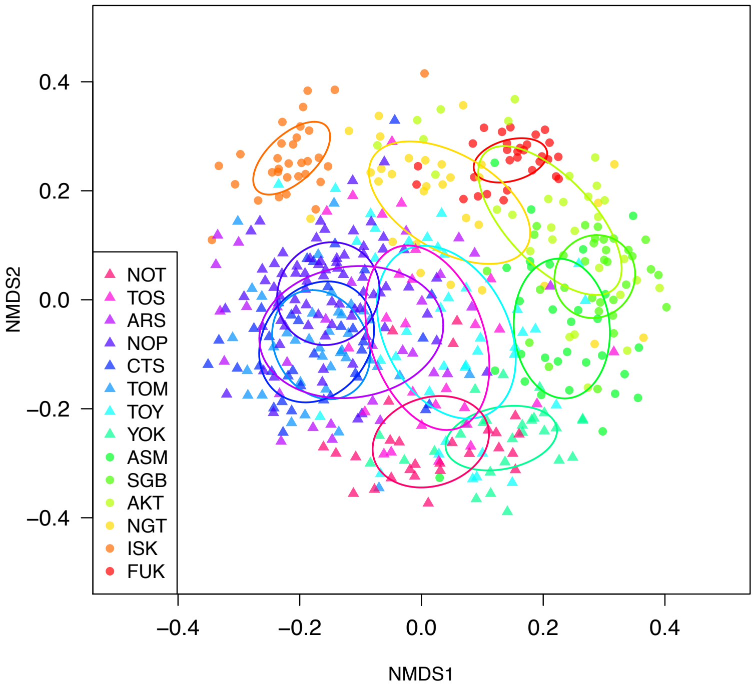

Fig. 1-8
Non-metric multidimensional scaling (NMDS) plot illustrating genetic differentiation among individual genotypes. Points represent individual genotypes, and circles indicate 95% inertia ellipses of populations.

Chapter 2: Factors affecting seed production of hermaphrodites and females

2.1 Introduction

A process of seed production is composed of three stages; 1) pollination success, 2) pollen germination on stigma and pollen-tube elongation toward ovules (fertilization success), and 3) embryo survival and development after fertilization (post-fertilization success) (Barrett and Harder, 2017). In Chapter 1, moderate and small seed-set success was observed under natural conditions in females (< 32.2%) and hermaphrodites (< 4.8%), respectively. Fruit-set ability of females was still moderate (< 53.9%) even when outcross-pollination was conducted. Furthermore, fruit-set ability of hermaphrodites was significantly lower than that of females. These results suggest the existence of other factors restricting fruit production than pollination success in each sex morph.

In hermaphrodites, seed production is restricted not only by pollen quantity but also by pollen quality (Harder et al., 2016). Under natural conditions, self-pollen is occasionally loaded on stigma automatically or by pollinators. In many self-incompatible species and some partially self-incompatible species, self-pollination reduces seed production by interference (Waser and Price, 1991; Ramsey and Vaughton, 2000; Barrett, 2002; Kawagoe and Suzuki, 2005). Therefore, in order to clarify the cause providing small seed production under natural conditions, the evaluations of the degrees of self-compatibility and interference of self-pollination are required.

Post-fertilization success is often influenced by the resource conditions of maternal plants (Ayre and Whelan, 1989). In gynodioecious species, resource limitation may affect seed production differently between females and hermaphrodites if each of sexual morphs has a different resource allocation strategy for reproduction (Ashman et al., 2001; Ashman, 2006). Females simply increase resource investment in fruit production as plant size (or resource availability) increases, while hermaphrodites may regulate resource allocation between flower and fruit production depending on size or resource availability (Delph, 1990; Ashman, 1999; Ashman et al., 2001). Size- or resource-dependent allocation to male and female functions has been observed in hermaphrodites of several gynodioecious and subdioecious species, where the male function of hermaphrodites tends to be enhanced in small-sized plants or under low resource conditions, indicating plastic sexual function of hermaphrodites (Delph, 1990; Ashman et al., 2001; Delph and Wolf, 2005).

In this chapter, I aim to clarify the factors regulating fruit production under natural conditions. Although hermaphrodites were self-compatible, the degree of self-compatibility was unknown in Chapter 1. Therefore, at first, I compared the fruiting ability under self- and outcross-pollination. Next, I assessed the physiological activity of stigmas and pistils of hermaphroditic flowers by observing pollen germination and pollen-tube elongation toward ovule within a pistil. Then, in order to assess the effects of resource availability of individual plants on fruit production, I conducted a resource manipulation experiment, and analyzed the size dependency of fruit production. The questions of this chapter are as follows: (1) Can sexual differences in seed fertility be explained by pollen receptive ability of female functions, i.e., stigma and ovary? (2) How extent do size and resource availability affect reproductive performance and seed fertility of females and hermaphrodites? (3) Does seed fertility of hermaphrodites change plastically?

2.2 Methods

Study sites

This study was conducted in three populations, CTS, NOP, and TOM (see Chapter 1). A permanent plot was established in 2009, 2013, and 2015, at CTS, NOP, and TOM, respectively (Sinclair et al., 2016), in which floral gender, flower production and fruit set have been recorded every year. Annual mean temperature is 7.1–7.2°C, ranging from –6. 3°C (January) to 20.8°C (August), and mean annual precipitation is 930–990 mm in three populations (Japan Meteorological Agency, www.jma.go.jp/jma/index.html). Snow usually covers the ground from mid-December to late March.

Hand pollination experiment

To evaluate the degree of selfing ability, a hand pollination experiment was performed in all populations in April 2016. Of all hermaphroditic plants producing floral buds, 39 genets at CTS, 33 genets at NOP, and 42 genets at TOM were randomly selected. One inflorescence per genet at TOM and two inflorescences per genet at CTS and NOP were covered with fine-meshed nylon bags to prevent pollinator visits. Outcross- and self-pollination treatments were performed for inflorescences within same hermaphroditic genets at CTS and NOP. Either outcross- or self-pollination was performed for single inflorescence of separate hermaphroditic genets at TOM (outcross = 22 ramets; self = 22 ramets). For the outcross-pollination treatment, pollen was collected from 5 to 10 hermaphroditic donors at least 10 m apart from the recipient genets and artificially deposited on the stigmas of all flowers of bagged female and hermaphroditic inflorescences soon after opening and before pollen dispersal for hermaphroditic flowers. For the self-pollination treatment conducted for hermaphroditic plants, pollen produced in the same genet was deposited on the stigmas. After pollination, the inflorescences were covered again with fine-meshed bags to prevent uncontrolled pollination and herbivore damage. When fruits matured in mid-July, they were counted and fruit-set rate (identical to seed-set rate) was calculated.

Observation of pollen germinability and pollen-tube growth

In order to test the physiological activity of stigmas and pistils of hermaphroditic flowers in comparison with female flowers, the situation of pollen germination on stigmas and pollen-tube elongation within pistils were observed on artificially outcrossed flowers at NOP. Anthers of hermaphroditic flowers were emasculated before dehiscent and covered with fine-meshed bags until hand pollination. When flowers opened completely, outcross-pollination was performed, and manipulated flowers were bagged again. After three days, pistils were collected and fixed in FAA solution (5% formalin, 5% acetic acid, 50% ethanol) during three days. Then, the samples were transferred into 70% ethanol solution for storage. Pistils were softened in 8-M NaOH for 30 min at 65°C, rinsed with distilled water, and stained for 30 min at 65°C in aniline blue (0.1%, dissolved in 0.1 M K_3PO_4). Each stained pistil was placed on a microscope slide with a drop of glycerol, carefully squashed with a coverslip, and observed under a fluorescence microscope (BX43, Olympus, Tokyo) using a filter cubes (340–390 nm excitation, 420– emission; U-FUW, Olympus). The number of pollen grains germinated on the stigma and pollen-tubes elongated toward ovule were counted with approximately 10 grains of error. In total, 66 pistils (F = 11 flowers from 11 genets, H = 55 flowers from 6 genets) were observed.

Resource manipulation

In order to evaluate the effects of resource availability on fruit production, a resource manipulation experiment composed of fertilization and leaf-cutting treatments was conducted from 2017 to 2019 at TOM. Approximately 5 g of fertilizer pellets (N: P: K = 8: 8: 8) was applied on the soil around the individual plants four times (November 2017, April and October 2018, and April 2019). In the leaf-cutting treatment, half number of leaves were cut off with scissors two times in April and October 2018. The fertilization treatment was conducted on plants growing in the unit of 5 m × 5 m subplots that were arranged within the 50 m × 50 m plots. Subplots of intact control were also set up. In total, 18 subplots (9 for fertilization and 9 for control) were set not to be adjacent between the fertilization and control subplots to avoid the contamination of fertilizer (Fig. 2-1). Leaf-cutting was conducted on randomly selected control plants. The number of control plants was 74 (F = 34, H = 40), that of fertilization plants was 58 (F = 25, H = 33), and that of leaf-cutting plants was 22 (F = 11, H = 11). Outcross hand-pollination was performed for all flowers of selected plants at flowering season in 2019. Pollen was collected from 5 to 10 hermaphroditic donors at least 10 m apart from the recipient genets. Anthers of hermaphroditic flowers were emasculated before dehiscent and covered with fine-meshed bags until the hand pollination. The number of fruits was recorded two months after the hand pollination.

Light availability manipulation and size dependency

In order to evaluate whether reproductive traits are influenced by light availability, seedlings that were cultivated from seeds were grown up at two sites under different light conditions (referred as bright and shaded site) at the experimental garden in Higashikawa (130 km northeast of NOP). The bright site was set at open place and the shaded site was set under the broad-leaved and coniferous mixed forest. Light intensity of the bright site was about four times higher than that of the shaded site (mean light intensity was 1494.0 and 381.5 μmol m^{-2} s^{-1} at the bright and shaded site, respectively). Seeds were sown in each of decomposable small pots (5 cm × 5 cm × 5 cm), these pots were then placed in larger planters and set at the bright site in August 2015. After germination, 1-yr-old seedlings were transplanted to the bright and shaded sites in late October 2016.

A total of 264 seedlings was transplanted (123 to the bright site; 141 to the shaded site), and 86 seedlings flowered in 2019 (F = 26 and H = 19 at the bright site; F = 22 and H = 19 at the shaded site). In order to test the size dependency of fruiting ability, plant size (expressed as basal stem diameter and total leaf number per stem) was measured in the spring of 2019. Then, outcross hand-pollination was performed for all flowers, whose anthers were emasculated before dehiscence and covered with fine-meshed bags until flowering starts in hermaphrodites. Pollen was collected from about 20 hermaphroditic donors from NOP and CTS populations outside the permanent plots one day before the treatment. The number of developing fruits was recorded two months after the hand pollination.

Size dependency in fruit-set rate in natural populations

To evaluate the relationship between plant size and fruiting ability in natural populations, outcross hand-pollination was performed in April 2017, and total leaf number was counted in early June 2017. For one or two inflorescences per genet, the outcross-pollination treatment was performed. The number of fruits was recorded two months after flowering. 39 genets at CTS (F = 23, H = 16), 46 genets at NOP (F = 18, H = 28), and 72 genets at TOM (F = 33, H = 39) were randomly selected. Pollen was collected from 5 to 10 hermaphroditic donors at least 10 m apart from the recipient genets.

Statistical analyses

R version 3.5.3 was used for all statistical analyses (R Core Team, 2019). For the hand pollination experiment, GLMM was conducted using the glmmTMB package postulating a zero-inflated negative binomial error distribution with log-link function. Genet ID was set as random factors. Fruit-set rate was a response variable and pollination treatment (outcross- and self-pollination) and population were explanatory variables with inclusion of their interaction. A best-fit model was selected based on Akaike's Information Criterion (AIC).

The effect of resource manipulation on reproduction was assessed by GLMMs postulating a negative binomial error distribution with log-link function. In the GLMMs of flower number and fruit number, resource treatment and sex morph were explanatory variables with inclusion of their interaction, and subplot ID was a random factor. In the GLMM of fruit-set rate, fruit number was set as a response variable and flower number was set as an offset term. When resource treatment was included in the best-fit model, Tukey’s post-hoc test was conducted to assess significance at *P* = 0.05 using multicomp and emmeans packages.

In order to assess the effects of light condition and plant size on reproduction, GLMMs were performed postulating a poisson error distribution for flower number and a negative binomial error distribution for fruit number and fruit-set rate with log-link function. In the GLMM, flower number, fruit number or fruit-set rate was a response variable, and site (light condition), log-transformed plant size (stem diameter or leaf number), and sex morph were explanatory variables with inclusion of their interaction. When the best-fit model included an interaction term, GLMM was performed separately for each sex morph. For fruit-set rate, fruit number was set as a response variable, flower number was set as an offset term, and genet ID of maternal plants was set as a random factor.

In order to assess the effects of plant size on fruiting ability in natural populations, GLMs were performed postulating a negative binomial error distribution for fruit-set rate with log-link function. In the GLM, fruit number was a response variable, flower number was set as an offset term, and population, log-transformed leaf number, and sex morph were explanatory variables with inclusion of their interaction. When the best-fit model included an interaction term, GLM was performed separately for each sex morph.

2.3 Results

Fruit production by self-pollination

Self-pollinated hermaphroditic flowers set fewer fruits than outcrossed flowers; mean values of fruit-set rates were 0.7–4.7% in self-pollination and 1.6–18.2% in outcross-pollination ($Z = -2.60$; $P < 0.01$; Fig. 2-2). Across populations, 60.0–91.2% of hermaphrodites failed to set any fruit by the outcrossing treatment, indicating a potentially low fruiting ability.

Pollen germinability and pollen-tube growth

Pollen germination on stigma and pollen-tube elongation toward ovule within a pistil were confirmed on most outcrossed flowers in both sex morphs (Fig. 2-3). The approximate number of germinated pollen grains ranged from 0 to 90 in hermaphroditic flowers and from 1 to 40 in female flowers (Fig. 2-4). The approximate number of elongated pollen-tubes toward ovule ranged from 0 to 60 in hermaphroditic flowers, and from 0 to 30 in female flowers. Therefore, hermaphroditic flowers had similar or even larger ability to activate pollen-tube growth compared to female flowers.

Effects of resource condition and plant size on reproduction

There were no significant effects of the fertilization or leaf-cutting treatment on flower and fruit production and fruiting success in the experiment at TOM (“treatment” was excluded in the best-fit model of flower production; Tukey’s $P > 0.09$ in the best-fit model of fruit production; Tukey’s $P > 0.12$ in the best-fit model of fruit-set rate; Fig. 2-5). Sexual difference was significant in fruit production and fruit-set rate ($Z = -4.89$, P

< 0.001 for fruit production; *Z* = –5.95, *P* < 0.001 for fruit-set rate), but not in flower production (“sex morph” was excluded in the best-fit model).

In the light availability experiment conducted in the experimental garden, light conditions (bright vs. shaded site) did not influence the flower and fruit production and fruiting success (“site” was excluded in the best-fit models; Fig. 2-6). Significant size effects were detected in the relationships between leaf number and some reproductive traits. Total flower number increased with leaf number without sexual difference (*Z* = 6.38, *P* < 0.001; Table 2-1). Fruit number and fruit-set rate were larger in females than in hermaphrodites (*P* < 0.001 in all models), size dependency was detected in the relationship between leaf number and fruit production (*Z* = 2.57, *P* < 0.05 in fruit number), but size effect was excluded in the best-fit model of fruit-set rate. There were no correlations between stem diameter and reproductive traits (*Z* = 1.67, *P* = 0.10 in flower production; “size” was excluded in the best-fit models of fruit production and fruit-set rate).

The significant relationship between leaf number and outcrossed fruit-set rate was also not detected in natural three populations (“size” was excluded in the best-fit models; Fig. 2-7). These results indicate that fruiting ability, i.e., fruit-set rate of outcrossed flowers, was independent of resource condition and plant size.

2.4 Discussion

A certain level of hermaphrodite’s seeds was produced by selfing in natural populations (32–87%; Chapter 1). However, self-pollinated flowers set fewer fruits than outcrossed flowers, indicating that *D. jezoensis* is partially self-incompatible. In hermaphroditic flowers, autogamous self-pollination occurs automatically because of the tubular-shaped upward floral morph in which anthers locate above the stigma. Therefore, autogamous self-pollination may cause an interference effect on seed production by outcrossing under natural conditions (Kikuzawa, 1989).

Hermaphroditic flowers showed similar or even larger ability to activate pollen-tube growth compared with female flowers. Since individual pistils contain only a single ovule, the number of pollen-tube reaching ovule should be large enough for fertilization. I observed that at least one pollen-tube reached ovule in > 90% pistils of both sex morphs in which outcross-pollination was conducted. This indicates that stigma receptivity and the activity of pollen-tube growth within a pistil cannot explain the limited fruit production in hermaphrodites and females. Also in the previous studies on gynodioecious species (including a congener species *Daphne laureola*), high receptivity of pollen-tube elongation in hermaphroditic flowers is reported when outcross-pollination was performed (Alonso, 2005; Molano-Flores and Faivre, 2015). However, these studies were conducted in the gynodioecious species, whose hermaphrodites had relatively high fruiting ability, different from *D. jezoensis*. Therefore, pre-fertilization process after pollination may be independent of the potential fruiting ability of female and hermaphroditic phenotypes. The fertilization treatment did not affect the reproductive performance, indicating that fruit production was independent of the nutrient condition in the natural population (TOM). Because *D. jezoensis* is commonly inhabiting the understory of broad-leaved deciduous forest with relatively fertile soil, reproductive performance and output might not be limited by soil nutrition at least in this experiment.

The leaf-cutting treatment and the manipulation of light availability did not influence any reproductive performance in this study. This indicates that carbon assimilation is not also a limiting factor of reproductive performance and output in the seedling of this species. In some gynodioecious species, however, resource availability affected fruit production or sex expression (Asikainen and Mutikainen, 2005; Varga and Kytöviita, 2016). *D. jezoensis* is a summer-deciduous shrub inhabiting understory of deciduous forests. They mainly gain carbon resources by photosynthesis during the spring before canopy closure of overstory vegetation and the autumn after defoliation of canopy (Lei and Koike, 1998), suggesting the importance of light availability for carbon assimilation. Because the experimental period of this study was two and half year (from autumn in 2016 to spring in 2019), a longer experiment may be needed to detect the impacts of carbon assimilation on reproductive performance in this species.

As another possibility, the effects of nutrient addition, leaf-cutting, and shading might be hindered by the negative effect of frost damage in the early spring of 2019. Because flowering starts soon after snowmelt in this species, freezing temperature in early spring sometimes causes frost damage on overwintering leaves and floral buds. Snowmelt at TOM usually occurs in late March around which nighttime temperature often decreases below freezing point. Actually, at TOM plot, there were 30 and 22 days when the temperature was below freezing point and the minimum temperature was –9.2ºC and –6.8ºC in March and April in 2019, respectively (personal observation). In addition, I observed withered leaves and damaged floral buds caused by frost in this population.

In contrast to the manipulation of resource availability, size-dependent flower and fruit productions were apparent in both sex morphs. The relationship between leaf and flower number was similar between sex morphs, whereas females showed stronger relationship between leaf and fruit number than hermaphrodites. This may reflect sex-specific strategy between female and hermaphroditic phenotypes (e.g., Dawson and Geber, 1999). Since females gain fitness only through seed production, they invest larger resources in fruit production. In contrast, male-biased hermaphrodites gain larger fitness as a pollen donor through pollen production. Both of females and hermaphrodites did not show any relationship between plant size and outcrossed fruit-set rate (fruiting ability) in the experimental garden and natural populations. These results imply that the degree of fruiting ability is potentially determined at low level by internal physiological control rather than resource dependent plasticity. Size-dependent sex allocation of hermaphrodites is reported in some gynodioecious species, indicating a plasticity in sex function of hermaphrodites (Delph, 1990; Ashman et al., 2001; Delph and Wolf, 2005). Delph and Wolf (2005) state that resource-mediated gender plasticity might help stabilize subdioecy, i.e., mixture of male phenotype in gynodioecious populations. In contrast, the present study demonstrated that sexual function of hermaphrodites in *D. jezoensis* is consistently male-biased independent of size or resource availability. This suggests that sexual system of this species may be in a stable stage.

Table 2-1. Effects of light condition, total leaf number, and sex morph (F or H) on reproductive traits. Results of GLMMs of best-fit models are indicated.

Variable	Coefficient	SE	Z value	*P* value
Flower number				
Intercept	0.15	0.30	0.49	0.63
Total leaf number	0.69	0.11	6.38	**< 0.001**
Fruit number				
Intercept (Sex = F)	0.02	0.61	0.03	0.98
Total leaf number	0.56	0.22	2.57	**< 0.05**
Sex (H)	−2.28	0.33	−6.92	**< 0.001**
Fruit-set rate				
Intercept (Sex = F)	−0.53	0.08	−6.23	**< 0.001**
Sex (H)	−2.20	0.30	−7.39	**< 0.001**

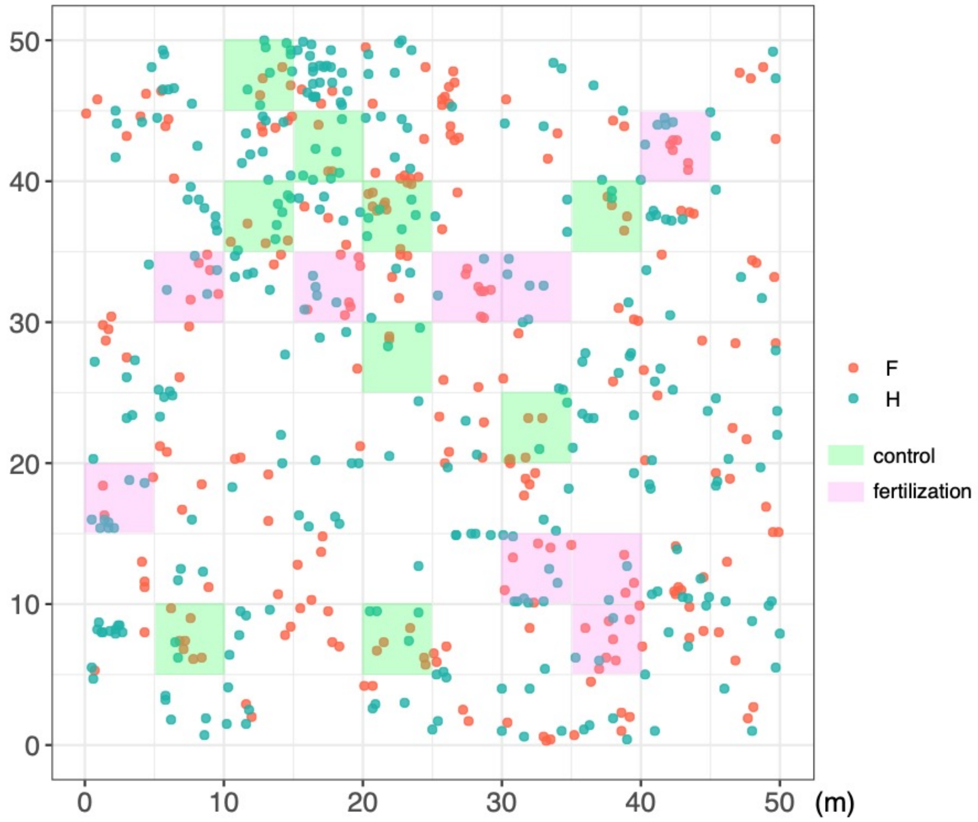

Fig. 2-1
Locations of individual plants and subplots used for the resource manipulation experiment in TOM population.

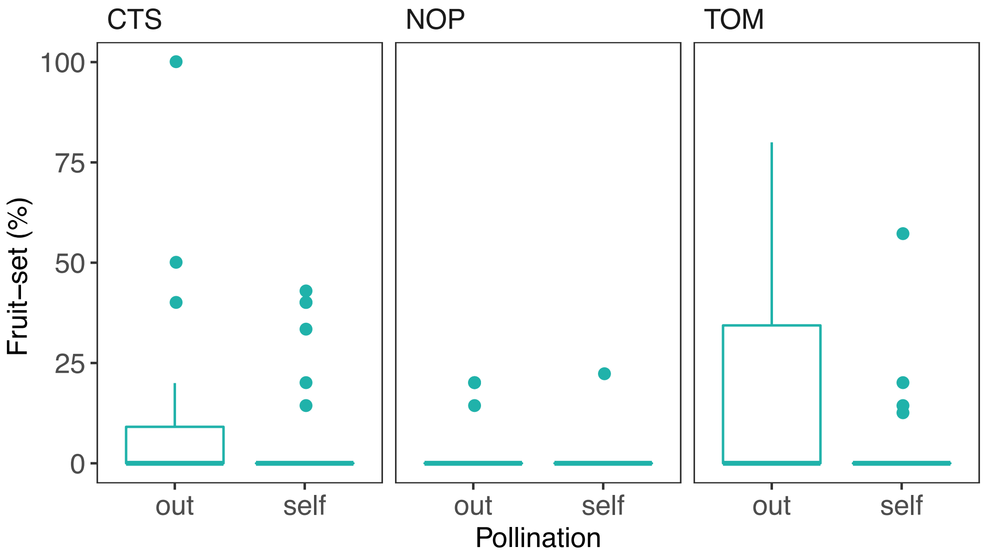

Fig. 2-2
Comparison of fruit-set of hermaphrodites between outcross- and self-pollination treatments in three populations.

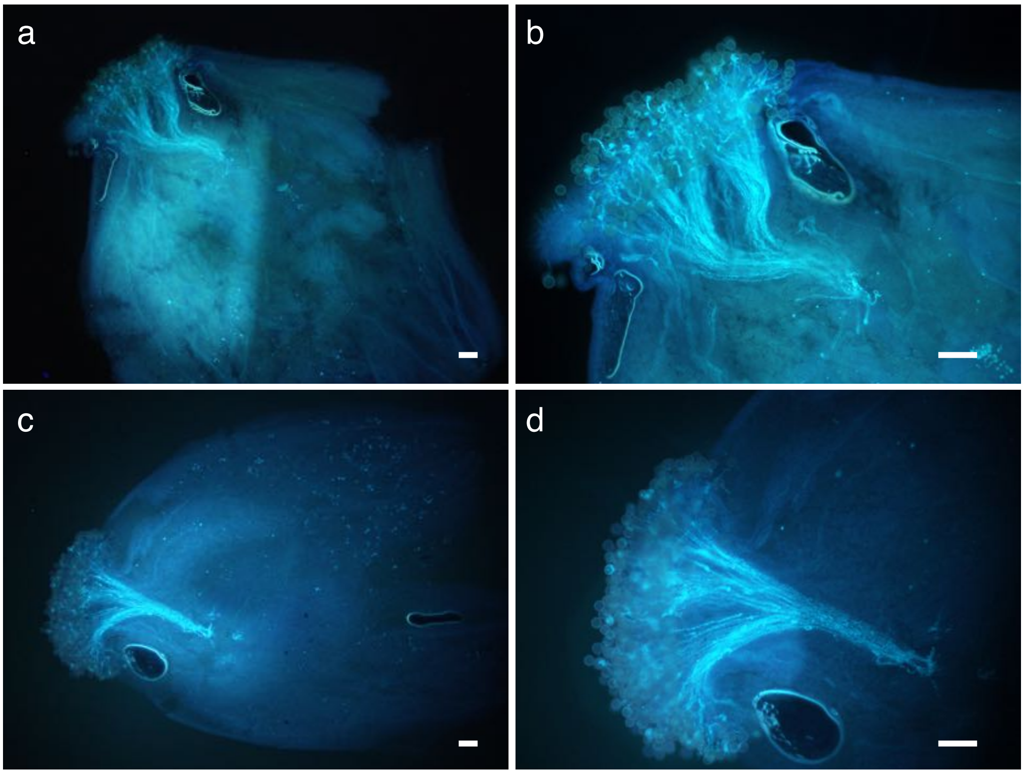

Fig. 2-3
Pollen germination on a stigma and pollen-tube elongation within a pistil of female (a, b) and hermaphroditic flowers (c, d). Scale bar indicates 100 µm.

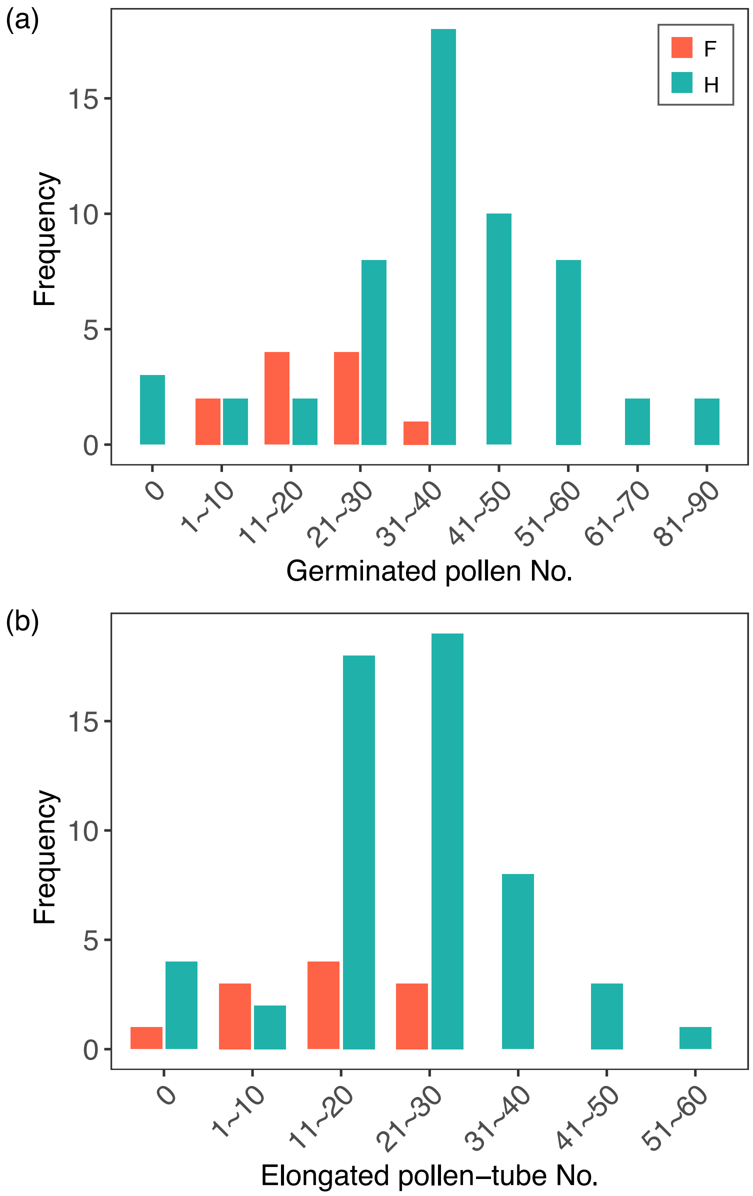

Fig. 2-4
The numbers of pollen grains germinated on a stigma (a) and pollen-tubes elongated within a pistil (b) in female (F) and hermaphroditic (H) flowers.

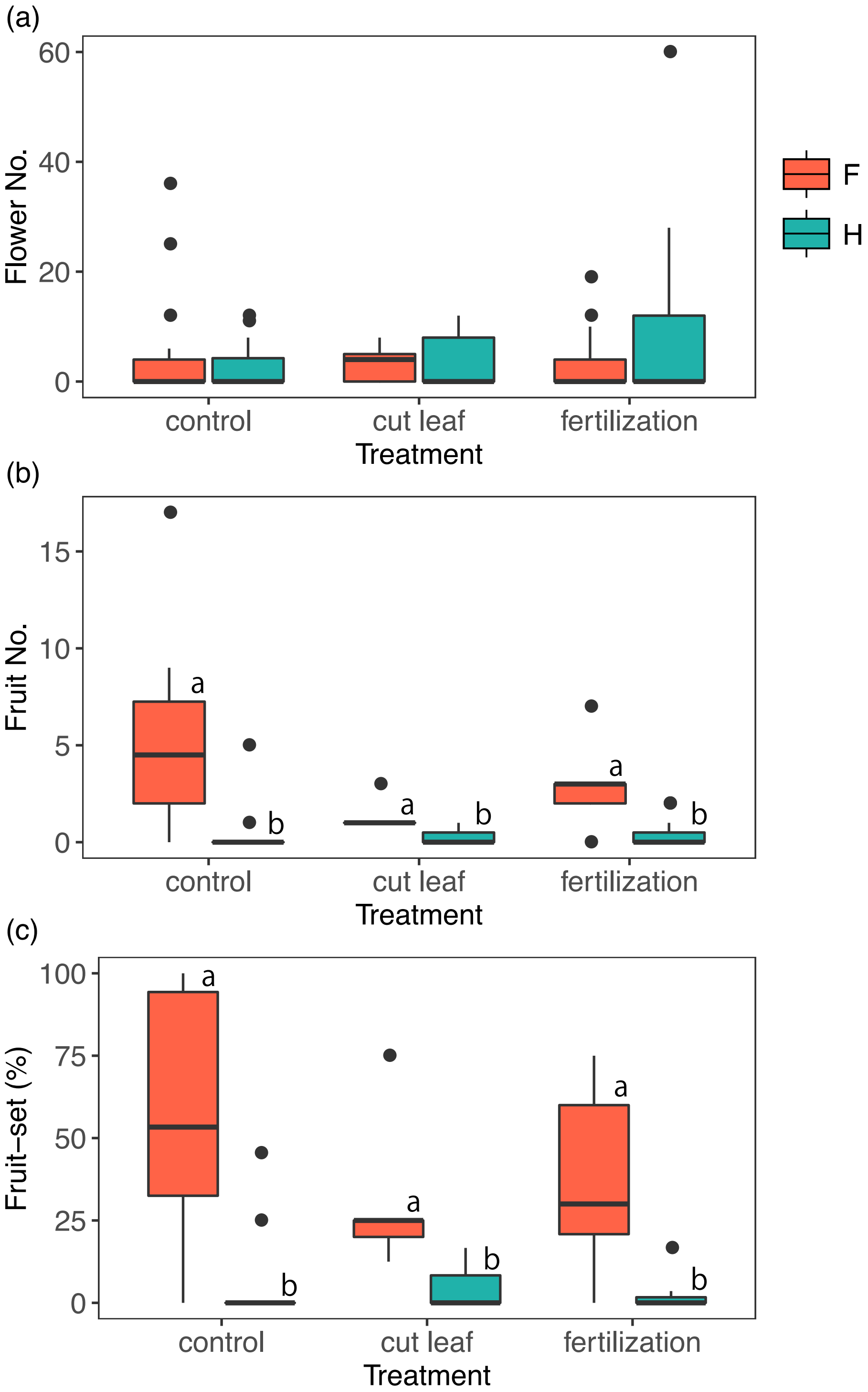

Fig. 2-5

Comparisons of flower number (a), fruit number (b), and fruit-set (c) among resource manipulation treatments in female (F) and hermaphroditic (H) plants. The letters above box plots denote significant difference between treatments and sex morphs ($P < 0.05$).

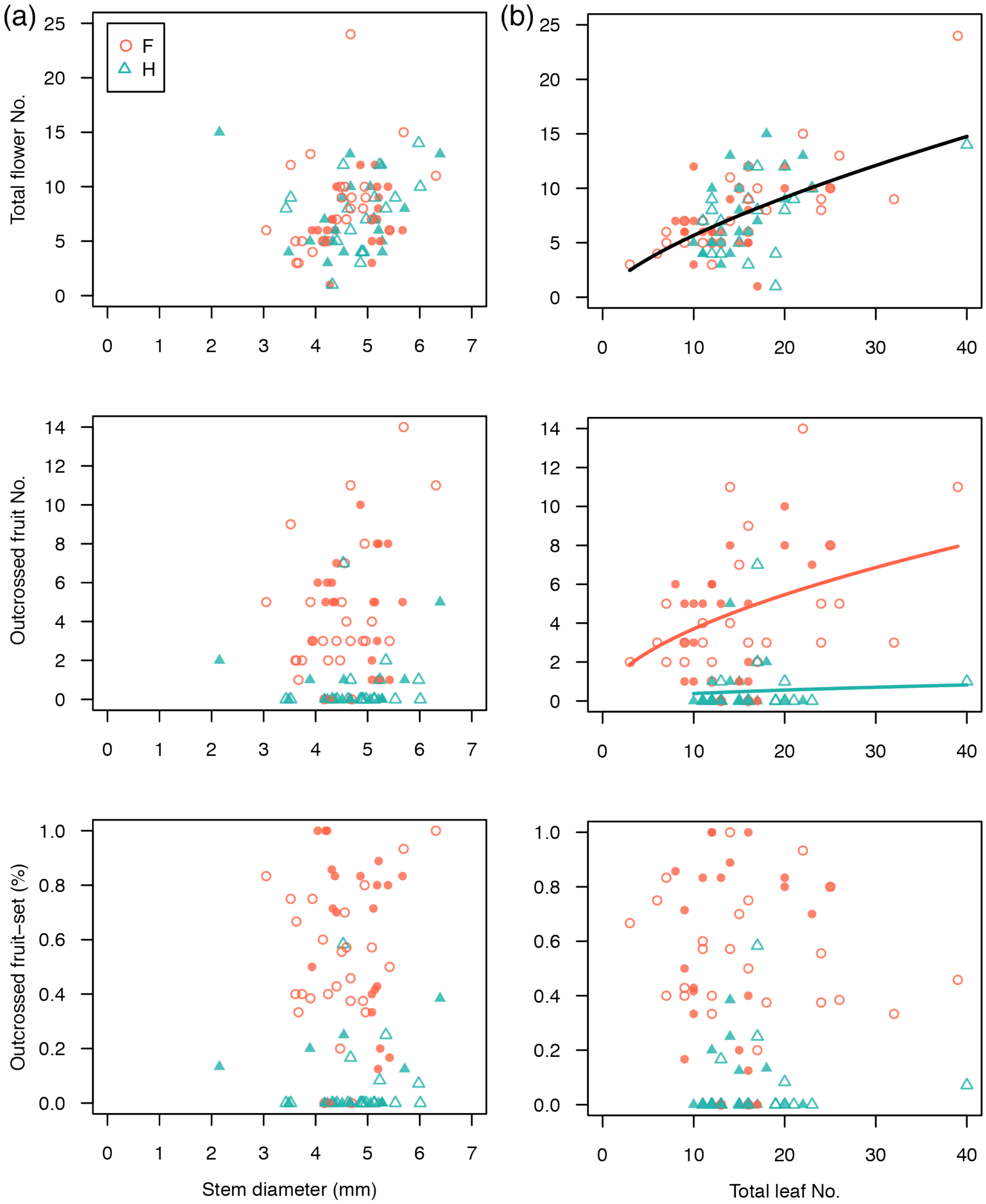

Fig. 2-6
The relationships between reproductive traits and plant size, i.e., stem diameter (a) and leaf number (b). Open and filled symbols represent the bright and shaded conditions, respectively. Lines indicate the predicted size-dependent responses by GLMMs.

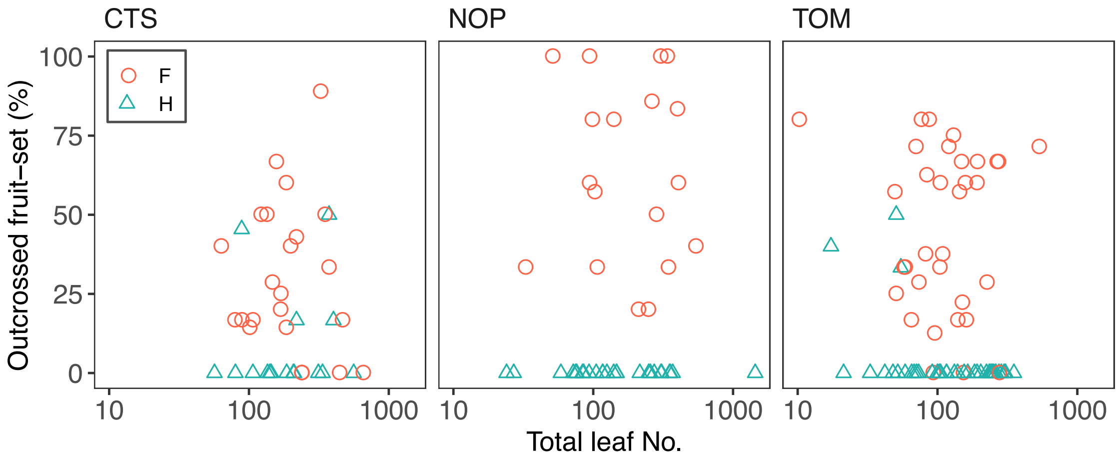

Fig. 2-7
The relationships between outcrossed fruit-set (fruiting ability) and leaf number of female (F) and hermaphroditic (H) plants in three populations.

Chapter 3: Reproductive strategies of female and hermaphroditic phenotypes with reference to the cost of reproduction and a trade-off between female and male functions

3.1 Introduction

The quantity and quality of seeds produced by hermaphrodites relative to females represent the functional role of hermaphrodites in gynodioecious populations. Fruit production generally requires more resources than flower production (Leigh et al., 2006; Vaughton and Ramsey, 2011), and selfed progeny often indicates smaller fitness than outcrossed progeny due to inbreeding depression (Sakai et al., 1997; Charlesworth and Willis, 2009). When there is a trade-off between current fruit production and future flower production, the cost of seed production in hermaphrodites may decrease the male fitness as a pollen donor. Furthermore, seed production by selfing may decrease the fitness through female function in hermaphrodites if inbreeding depression is large. To evaluate the contribution of hermaphrodites as a seed producer, it is necessary to evaluate the cost of fruit production and the degree of inbreeding depression under natural conditions.

When male-biased allocation is accelerated, seed fertility of hermaphrodites may decrease and eventually a female-sterile (functional male) phenotype may occur within a population (shift to subdioecy). These transitions are predicted in the later stage of the gynodioecy–dioecy pathway, but empirical data supporting this prediction is limited (Spigler and Ashman, 2012). Although the quantification of male and female fitness of hermaphrodites is crucial to evaluate the evolutionary stage of gynodioecious species, measurement of male fitness is often difficult in natural populations. In most previous studies, reproductive success as a pollen donor was estimated based on flower number, pollen production, frequency of pollinator visits and/or pollen removal by pollinators. However, these substitute values may often overestimate male fitness, and more precise estimation of male fitness using genetic markers is required (Spigler and Ashman, 2012).

A trade-off of resource allocation between male and female functions within individuals is a fundamental assumption in the sex allocation theory (Charlesworth and Charlesworth, 1978; Charnov, 1982). The sex allocation theory predicts that fitness gain through male and female functions is positively related to the resource investment in each sexual function. Hermaphroditism is expected when fitness gain through female function increases linearly with increasing resource investment but that through male function increases at a decelerating rate. In contrast, dioecy is expected when either male or female fitness increases at an accelerating rate with increasing resource investment. These patterns are expressed by the fitness-set approach that is the relationship between female fitness and male fitness. A convex-shaped relationship in fitness-set predicts that hermaphroditism is an evolutionary stable state of mating system, while a concave-shaped relationship predicts that dioecy is an evolutionary stable state (de Jong and Klinkhamer, 2005). Therefore, the measurement of fitness through male and female functions of hermaphrodites is important to evaluate the evolutionary stage of a gynodioecious species.

In polycarpic plants, large resource investment in current reproduction often causes negative effects on future reproduction, growth, and/or survival, i.e., the cost of reproduction (Obeso, 2002). Because fruit production commonly requires larger resources than flower production, continuous production of large amount of fruits is difficult not only for hermaphroditic individuals but also for female individuals. Reproductive strategy of polycarpic species should be interpreted based on a lifetime fitness rather than a success of single reproductive event. Even when the measurement of lifetime fitness is difficult, investigation of fruit and flower productions over multiple reproductive events is crucial to evaluate the reproductive strategy of hermaphrodites and females in gynodioecious populations.

In Chapters 1 and 2, it was revealed that the gynodioecy composed of male-biased hermaphrodites and females is a consistent sexual system in *D. jezoensis*. Hermaphrodites were partially self-compatible and their seed fertility was restricted at a small level even without pollen and resource limitations. Based on these results, I predict that the limited and moderate seed productivity is an intrinsic reproductive activity of hermaphroditic and female phenotype, respectively, reflecting the reproductive strategy to maximize the fitness. In this chapter, I aim to quantify the reproductive success of hermaphrodites through male and female functions using a paternity analysis under natural conditions. Furthermore, I test this prediction using the long-term monitoring data (7–11 year records in two populations) of reproductive output of this species. Specifically, I address the following questions: (1) How large is the inbreeding depression of hermaphroditic phenotype? (2) Is there a trade-off between female and male fitness in hermaphrodites? (3) What is the difference in reproductive performance over long periods between females and hermaphrodites?

3.2 Methods

Study sites

This study was conducted at three permanent plots in Hokkaido; CTS, NOP, and TOM (see Chapter 1). All ramets with floral buds were tagged, mapped, and genotyped using genetic markers to determine genet individuality (see below). Female genet frequency ranged 44.2–51.7% in these populations during the survey periods (Table 3-1).

Flower and fruit production over years

Total numbers of flowers and fruits produced during the survey periods were recorded for every tagged ramet in each plot (Table 3-1). Flowers were counted at the peak flowering season (late April to early May) and developing fruits were counted in mid-June before dispersal. Fruit-set rate (identical to seed-set rate) was calculated as a percentage of flowers that set fruits.

Inbreeding depression

Mature fruits produced in the hand-pollinated hermaphrodites in Chapter 2 were harvested in every plot in mid-July 2016. After removal of fruit pulp, exposed seeds were stored at room temperature (20–25°C). In August 2016, individual seeds were sown in each of small decomposable pots (5 cm × 5 cm × 5 cm), these pots were then placed in larger planters and set in the experimental garden in Higashikawa (130 km northeast of NOP). A total of 29 seeds (outcrossed 18, selfed 11), 5 seeds (outcrossed 3, selfed 2), and 25 seeds (outcrossed 19, selfed 6) originated from CTS, NOP, and TOM, respectively. Because of small sample size, all seeds were pooled for a comparison of germination rates between the pollination treatments. Germination rates were recorded after one year of sowing in October 2017. Survival rates of seedlings were measured after three years of sowing in October 2019.

Sampling and genotyping

One leaf was sampled from each flowering ramet at CTS, NOP, and TOM over the 2013–2016 flowering seasons to determine genet individuality by genotyping. For paternity analysis, progenies (seeds and leaves of seedlings) from females obtained under natural conditions at CTS and NOP in 2015 and 2016 were used (Table 3-2). Leaves were additionally sampled from all flowering hermaphroditic ramets outside the permanent plot at NOP (125 m × 125 m) in order to increase precision of paternity analysis. Leaf samples were stored in a desiccator with silica gel at room temperature (20–25°C). Seed samples were stored at –60°C until DNA extraction, which was performed using the cetyltrimethylammonium bromide (CTAB) protocol (Stewart and Via, 1993). Extracted DNA was amplified with a TaKaRa PCR Thermal Cycler Dice Gradient (Takara Bio Inc., Otsu, Shiga, Japan) using following microsatellite markers by Primer set unit. For identification of genet, eight markers (Primer set 1: Dp186, Dp238, Dp258, Dp419, Dp430, Dp499, Dp506, and Dp739) were used for TOM (Kameyama and Hirao, 2014), 16 markers (Primer set 2: DpJe010, DpJe074, DpJe044, DpJe076, DpJe020, DpJe082, DpJe059, and DpJe061; Primer set 3: DpJe054, DpJe003, DpJe012, DpJe077, DpJe018, DpJe024, DpJe028, and DpJe029) were used for CTS and NOP. Two-step multiplex PCR was performed. In a first step, final volume of 5 µl containing 1 µl extracted DNA, 2.5 µl 2 × Type-it Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.5 µl F-tail mix (0.6 µM for each locus), 0.5 µl RS mix

(2.4 µM for each locus), and 0.5 µl water with 5 min at 95°C, 23–32 cycles of 30 sec at 95°C, 90 sec at 60°C, and 40 sec at 72°C, followed by 8°C for holding. The number of cycles was changed depending on the quality of DNA, i.e., 23 cycles for leaf and 24 – 32 cycles for seed. Then, 0.5 µl 2 × Type-it Multiplex PCR Master Mix and 0.5 µl tail-Dye mix (1.2 µM for each color) were added for second step. Second step was performed with 3 cycles of 30 sec at 95°C, 90 sec at 60°C, and 40 sec at 72°C, followed by 30 min at 60°C. The DNA fragments were analyzed using an Applied Biosystems 3730 Genetic Analyzer with GeneScan 500 LIZ Size Standard (Thermo Fisher Scientific Inc., Waltham, MA, USA) and scored genotypes were analyzed using GeneMapper ver. 4.0 (Thermo Fisher Scientific Inc.).

Paternity analysis

To evaluate the reproductive success as a pollen donor (male reproductive success), paternity analysis was conducted at CTS and NOP based on 16 microsatellite markers using MasterBayes package version 2.55 in R (Hadfield et al., 2006). In the package, a probability of pollen donor is estimated for every hermaphroditic genet on the bases of genotypes of progenies from females, mothers, and all flowering pollen donors within a plot. The number of unsampled pollen donors and the genotyping error rate were also estimated jointly in the model. Paternity assignments with high probability (> 80%) were used for later analysis. Relative siring success of individual hermaphrodites (genets) was defined as follows: the number of seeds sired by a focal individual / total number of seeds on which specific pollen donors were assigned.

Reproductive success

Female reproductive success was identical to the number of seeds for females, whereas that for hermaphrodites was expressed as a combination of the number of fruits, selfing rate (s), and inbreeding depression (δ) as follows: seed number × $(1 - s \times \delta)$. The selfing rates obtained in Chapter 1 were used. Male reproductive success of hermaphrodites was calculated as follows: total number of seeds produced by females within the plot × relative siring success of a focal individual (genet).

Total reproductive success of hermaphrodites during the survey periods was predicted on the basis of the number of flowers of a focal individual (Fl_n), total number of female flowers within a 10-m radius of a focal individual (FFl_n), and the number of fruits (Fr_n) in each of CTS and NOP. Because the mean predicted distance between father and mother plants was around 10 m (see Results), total number of female flowers within a 10-m radius of a focal individual was used as a indicator of the amounts of mating chance for hermaphrodites. Female and male reproductive success of individual genets were estimated in each year, and sum of them were calculated as a total reproductive success. Male reproductive success (S_m) in each year was predicted using the following formulas that were obtained by GLMM (see Statistical analyses);

$$S_m = \exp\,(-0.54 + 0.16 \times \ln Fl_n + 0.39 \times \ln FFl_n) \text{ for CTS, and}$$

$$S_m = \exp\,(2.02 + 0.18 \times \ln Fl_n) \text{ for NOP.}$$

Then, total reproductive success during the survey periods was calculated as a sum of total success across years.

Cost of fruit production

A cost of fruit production, defined as a negative effect of current-year fruit production on the next-year's reproductive effort (total flower number), was examined for every genet at the permanent plots of CTS and NOP. All genets, which experienced at least one flowering event during the long-term survey, were used for this analysis (F = 182 and H = 196 during 7 years at CTS; F = 126 and H = 156 during 11 years at NOP).

As an index of fruiting ability, mean fruit-set rates under natural conditions over 7 and 11 years were calculated for individual genets at CTS and NOP, respectively. The genets, which were alive at least five years during the observation period, were used (F = 155 and H = 101 at CTS; F = 97 and H = 82 at NOP).

Statistical analyses

The relationship between flower number and fruit number was analyzed using a GLMM postulating a zero-inflated negative binomial error distribution with log-link function. Fruit number was a response variable, and flower number and sex morph (F or H) were explanatory variables with inclusion of their interaction, and genet ID was a random factor. A pooled data of flowering genets (2015–2019) was used for the analysis. GLMM was run in each sex morph if interactions were detected. Fisher's exact test was conducted to compare the germination rates between outcrossed and selfed seeds and survival rates after germination.

The relationships between flower number and male reproductive success in 2015 and 2016 were analyzed using a GLMM postulating a zero-inflated negative binomial error distribution with log-link function. Male reproductive success was a response variable, number of flowers (log-transformed) and total number of female flowers within a 10-m radius of focal genet were explanatory variables with inclusion of their interaction, and genet ID was a random factor.

For the analysis of the cost of fruit production, GLMM postulating a negative binomial error distribution with log-link function was fitted for each population. The total flower number in the next year (year *t* + 1) was a response variable, and current-year fruit number (year *t*) and sex morph were explanatory variables with inclusion of their interaction. Current-year flower number (year *t*) was set as an offset term, and genet ID was set as a random factor.

In order to estimate the relationships between fruiting ability and flower production, fruit production or total reproductive success over long periods, GLMs postulating a negative binomial error distribution with log-link function were fitted for each sex morph and population. Cumulative flower number, cumulative fruit number or total reproductive success for each genet over long periods was a response variable, and fruiting ability (expressed as mean fruit-set rate over long periods) was an explanatory variable with a quadratic term to test a unimodal relationship.

3.3 Results

Female reproductive success

During the survey periods, 28.2–58.7% of hermaphrodites set at least one fruit (Table 3-1). The number of flowers was larger in hermaphrodites than in females (Fig. 3-1 a). However, females showed 6.8–61.1 times higher fruit-set and produced 4.6–31.4 more fruits than hermaphrodites in three populations over five years (Fig. 3-1 b). The number of fruits significantly increased with the number of flowers in both sex morphs (Fig. 3-

2). However, this trend was much stronger in females ($Z = 16.79$, $P < 0.001$ at CTS; $Z = 22.31$, $P < 0.001$ at NOP; $Z = 11.00$, $P < 0.001$ at TOM) than in hermaphrodites ($Z = 4.61$, $P < 0.001$ at CTS; $Z = 7.12$, $P < 0.001$ at NOP; $Z = 2.20$, $P < 0.05$ at TOM) in every population. There were a few hermaphrodites having high fruit-set ability as much as females, while most hermaphrodites showed small fruit-set.

Germination rate (one year after sowing) of outcrossed seeds was significantly higher than that of selfed seeds produced in hermaphrodites (95.0% in outcrossed seeds and 47.4% in selfed seeds, $P < 0.001$). Survival rates of seedlings for three years after germination, by which many seedlings started to set floral buds, were also higher in outcrossed seeds than in selfed seeds although this difference was not significant statistically (81.6% in outcrossed seeds and 66.7% in selfed seeds, $P = 0.38$). Cumulative fitness until reproductive initiation was 0.775 (= 0.95 × 0.816) in outcrossed seeds and 0.316 (= 0.474 × 0.667) in selfed seeds. Cumulative inbreeding depression until reproductive initiation was calculated as 0.59 (= 1 – 0.316/0.775). Therefore, reproductive success through female function of hermaphrodites decreases with increasing selfing rate.

Male reproductive success

In the paternity analysis, pollen donors of about 60% of progenies were assigned (Table 3-2). Mean distance between pollen donor (father) and recipient (mother) genets was 6.9–11.4 m in both populations. Male reproductive success, i.e., siring success, significantly increased with the number of flowers produced in hermaphrodites in two populations ($Z = 3.18$, $P < 0.01$ at CTS; $Z = 2.80$, $P < 0.01$ at NOP; Fig. 3-3). A positive correlation between female flower number (recipient flower density around pollen donor) and siring success was detected at CTS but not at NOP ($Z = 3.27$, $P < 0.01$ at CTS).

Trade-off between male and female functions

Hermaphrodites produced offspring through both of female and male functions in natural populations (Fig. 3-4). However, most hermaphrodites had larger fitness through male function than through female function, indicating the male-biased gender function.

Reproductive effort, i.e., total number of flowers in the next year, decreased after large fruit production in both of hermaphrodites and females at CTS and NOP (Table 3-3; Fig. 3-5), indicating the cost of fruit production. A negative correlation was detected between fruiting ability (mean fruit-set rate over long periods) and total number of flowers in hermaphrodites at both CTS and NOP (Table 3-4 a; Fig. 3-6 a). This means that hermaphrodites having higher fruiting ability tend to produce smaller amount of flowers, i.e., potentially smaller male success over long periods. In females, however, unimodal relationship was detected between fruiting ability and total flower number at both populations. This indicates that high fruiting ability (> 35% at NOP and > 20% at CTS) resulted in smaller flower production over long periods due to the cost of fruit production. On the other hand, unimodal relationship was detected between fruiting ability and total fruit production in both sex morphs at CTS and NOP (Table 3-4 b; Fig. 3-6 b). This result indicates that moderate fruiting ability is better to maximize fruit production at a long time scale in both sex morphs. The optimal value of fruiting ability was lower in hermaphrodites (10–20%) than in females (30–50%). A negative relationship was detected between total reproductive success and fruiting ability over long periods in hermaphrodites at NOP, but not at CTS (Table 3-4 c; Fig. 3-6 c). This means that hermaphrodites with small fruiting ability tend to gain more fitness at NOP.

3.4 Discussion

Although hermaphrodites produced offspring through both of female and male functions in natural populations, most of them gained fitness as a pollen donor. Thus, the male-biased gender function of hermaphrodites is predominant in *D. jezoensis*. The cost of fruit production was detected in both females and hermaphrodites. Interestingly, too higher fruiting ability resulted in lower long-term reproductive output even in females. These results indicate that reproductive strategy should be evaluated over multiple reproductive events rather than a single reproductive event. Here, I discuss the reproductive strategies of females and hermaphrodites considering the cost of reproduction and a trade-off between female and male functions.

About a half to two-thirds of hermaphrodites (41–72%) did not produce any seed during the survey periods in three populations. Seed production per genet was much smaller in hermaphrodites (mean seed number was 0–1.8) than in females (1.9–12.3) in three populations over five years. Furthermore, germination rate of selfed seeds was about a half of the outcrossed seeds produced in hermaphrodites, and the survival rate of seedlings until young reproductive stage (for three years) tended to be in selfed progenies. These results indicate the existence of inbreeding depression. In many gynodioecious species, hermaphrodites produce seeds that are quantitatively and/or qualitatively inferior to seeds from females (Wolfe and Shmida, 1997; Shykoff et al., 2003; Dufay and Billard, 2012; Wang et al., 2014). In total, 33–63% seeds produced by hermaphrodites were self-pollinated ones in three natural populations (see Chapter 1), and about 60% of selfed seeds were supposed to be inactive, resulting in a waste of resources for hermaphrodites. Under such situation, sexual selection will act on resource allocation toward male function in hermaphrodites, i.e., more flower and pollen production at the expense of seed fertility (Spigler and Ashman, 2012).

Hermaphrodites produced more flowers than females, and flower number was positively related to the male reproductive success at CTS and NOP. These trends reflect a selective force acting on the male function of hermaphroditic phenotype. Production of many flowers may increase the chance of pollination success because larger floral display often attracts more insects (Bell, 1985; Vaughton and Ramsey, 1998). During the survey periods, I recorded visitations of bumble bees (*Bombus hypocrita sapporoensis* and *B. hypnorum koropokkrus*), bee flies (*Bombylus major*), butterflies (*Inachis io* and Hesperiidae sp.), and moths (*Illiberis psychina*), indicating that *D. jezoensis* is a generalist for pollinators. However, the frequency of insect visits to flowers was small due to cool weather conditions in early spring.

Paternity analyses revealed that the mean distances of father and mother individuals were 6.9–9.8 m at CTS and 10.7–11.4 m at NOP, which were shorter than the mean distances between each individual pair (26.6 ± 12.7 m at CTS and 38.7 ± 28.2 m at NOP). Thus, actual pollen dispersal occurs within much smaller distances than random pollination within the population (or plot). The maximum distances of father and mother individuals were 55.6 m at CTS and 91.9 m at NOP. In the previous study on the pollen dispersal of forest herb, *Primula elatior* (Van Rossum et al., 2011), pollen dispersal showed exponential decay distributions, with the most of the recipient occurring at < 50 m and a few longer distance dispersal events (up to 151 m). Although studies on the pollen dispersal distance of understory plants are limited, a large number of pollen in *D. jezoensis* was transported for a relatively short distance, with occasional long-distance dispersal.

Density of recipient flowers was positively related to the reproductive success as a pollen donor at CTS, but not at NOP. This difference might be caused by the difference in population size and sampling method. At NOP, where population size was relatively small, most flowering genets were genotyped and seeds were harvested from almost all plants within the population. Thus, accurate estimation of pollen donors was expected. At CTS, whose population size was larger than NOP, however, sampling and genotyping of ramets were conducted only within the 50 m × 50 m plot, but there were a lot of flowering individuals outside the plot. Therefore, estimation of male success was conducted under limited situation and the density effect of recipient flowers might be artifact.

Hermaphroditic plants having high fruiting ability produced smaller amount of flowers, indicating a trade-off between fruit production and flower production. A trade-off between female and male functions in hermaphrodites was reported in a few gynodioecious species, in which ovule or seed number was negatively related to pollen number within a season (Atlan et al., 1992; Ashman, 1999; but see Varga and Kytöviita, 2017). Furthermore, the fitness set obtained in this study (Fig. 3-4) showed concave-shaped curves. These results predict the evolution toward dioecy (Charnov, 1982). In hermaphrodites at NOP, a negative relationship was detected between fruiting ability and total reproductive success (Fig. 3-6 c), but the negative trend was moderate when fruiting ability was low (i.e., < 10% fruit-set rate). Therefore, the selective force acting on the fruiting ability may not be strong enough to lose it completely, resulting in the maintenance of low fruiting ability in hermaphrodites. In contrast, negative relationship was not detected at CTS, probably due to a low predictability of male success in this population as mentioned above.

Also for females, large fruit production resulted in the decrease in next-year's reproductive effort, and higher fruiting ability caused a negative effect on the long-term female success. Therefore, modest fruit production may be adaptive for females to maximize the lifetime fruit production. As shown in Chapter 1, fruit-set success was pollen limited in most populations, but seed-set rate retained at moderate level even when hand pollination was conducted. This indicates a potentially modest fruiting ability in females. The moderate reproductive performance may be related to the life-history traits of this species. Flowering of *D. jezoensis* occurs in early spring soon after snowmelt, when the risk of frost damage is high. Actually, frost damage on floral buds and overwintered leaves was sometimes observed as mentioned in Chapter 2. Furthermore, low pollinator activity in early spring makes pollination success uncertain (Schemske et al., 1978; Motten, 1986). Pollen limitation was detected across the wide distribution range (Chapter 1). Thus, photosynthetic carbon gain and pollination success are unpredictable in this species. Leaf habit of this species is summer-deciduous, in which leaves shed before the fruiting season (late spring) and new leaves and floral buds come up in early autumn (Lei and Koike, 1998). Therefore, fruits develop using a stored carbon resource that is also used for new shoot and floral bud productions. These trade-off relationships might cause the moderate seed fertility in order to sustain growth and/or flower production in both of females and hermaphrodites (Delph, 1999). Because of the woody growth form with creeping stems, lifespan of at least several decades is expected in this species. Monitoring long-term fitness is crucial to understand the reproductive strategy of such polycarpic plants.

Table 3-1. The number reproductive genets and the proportion of sex morphs during the survey years in each population.

Population	Survey year	Sex morph	Genet number (fertile H*)	Percentage (fertile H*)
CTS	7	Female	188	49.7
		Hermaphrodite	190 (104)	50.3 (54.7)
NOP	11	Female	119	44.2
		Hermaphrodite	150 (88)	55.8 (58.7)
TOM	5	Female	125	51.7
		Hermaphrodite	117 (33)	48.3 (28.2)

*The number of fertile hermaphroditic genets, which produced at least one fruit

Table 3-2. The number of progenies analyzed and assigned the donor plants by the paternity analysis, and mean distance between father and mother plants.

Population	Year	N (progeny analyzed)	N (progeny assigned)	Father–mother distance (m)
CTS	2015	222	133	6.9
	2016	214	124	9.8
NOP	2015	200	113	10.7
	2016	178	112	11.4

Table 3-3. Results of GLMMs for the effects of current-year fruit production on next-year flower production (reproductive effort). Current-year flower number (year *t*) was set as an offset term. Best-fit models by AIC are indicated.

Population	Variable	Coefficient	SE	*Z* value	*P* value
CTS	Flower no. (next year)				
	Intercept	−0.11	0.04	−3.00	**< 0.01**
	Fruit no. (current year)	−0.01	0.00	−3.05	**< 0.01**
NOP	Flower no. (next year)				
	Intercept	0.22	0.05	4.30	**< 0.001**
	Fruit no. (current year)	−0.02	0.00	−5.62	**< 0.001**

Table 3-4. Results of GLMs for the effects of fruiting ability on cumulative flower number (a), cumulative fruit number (b), and cumulative total reproductive success (c) in each sex morph over long periods.

Variable	Coefficient	SE	*Z* value	*P* value
(a) Cumulative flower no.				
CTS: Female				
Intercept	5.21	0.21	25.15	< **0.001**
Fruiting ability	0.05	0.02	2.57	< **0.05**
Fruiting ability^2	−0.001	0.0004	−3.73	< **0.001**
CTS: Hermaphrodite				
Intercept	6.40	0.12	53.90	< **0.001**
Fruiting ability	−0.06	0.01	−4.16	< **0.001**
NOP: Female				
Intercept	4.32	0.30	14.4	< **0.001**
Fruiting ability	0.07	0.002	3.94	< **0.001**
Fruiting ability^2	−0.001	0.0002	−4.93	< **0.001**
NOP: Hermaphrodite				
Intercept	6.40	0.11	55.91	< **0.001**
Fruiting ability^2	−0.003	0	−3.50	< **0.001**
(b) Cumulative fruit no.				
CTS: Female				
Intercept	1.68	0.31	5.38	< **0.001**
Fruiting ability	0.13	0.02	7.35	< **0.001**
Fruiting ability^2	−0.001	0.0002	−6.69	< **0.001**
CTS: Hermaphrodite				
Intercept	1.29	0.18	7.17	< **0.001**
Fruiting ability	0.45	0.06	7.70	< **0.001**
Fruiting ability^2	−0.02	0.002	−6.24	< **0.001**
NOP: Female				
Intercept	1.75	0.21	8.22	< **0.001**
Fruiting ability	0.17	0.02	8.83	< **0.001**
Fruiting ability^2	−0.002	0.0003	−7.48	< **0.001**
NOP: Hermaphrodite				
Intercept	1.63	0.16	10.32	< **0.001**
Fruiting ability	0.24	0.04	6.15	< **0.001**
Fruiting ability^2	−0.006	0.002	−3.85	< **0.001**

Continued

(c) Cumulative total reproductive success				
CTS: Female				
Intercept	1.75	0.21	8.22	**< 0.001**
Fruiting ability	0.17	0.02	8.83	**< 0.001**
Fruiting ability^2	−0.002	0.0003	−7.48	**< 0.001**
CTS: Hermaphrodite				
Intercept	4.45	0.03	127.1	**< 0.001**
NOP: Female				
Intercept	1.68	0.31	5.38	**< 0.001**
Fruiting ability	0.13	0.02	7.35	**< 0.001**
Fruiting ability^2	−0.001	0.0002	−6.69	**< 0.001**
NOP: Hermaphrodite				
Intercept	4.97	0.06	82.99	**< 0.001**
Fruiting ability^2	−0.001	0.0005	−2.32	**< 0.05**

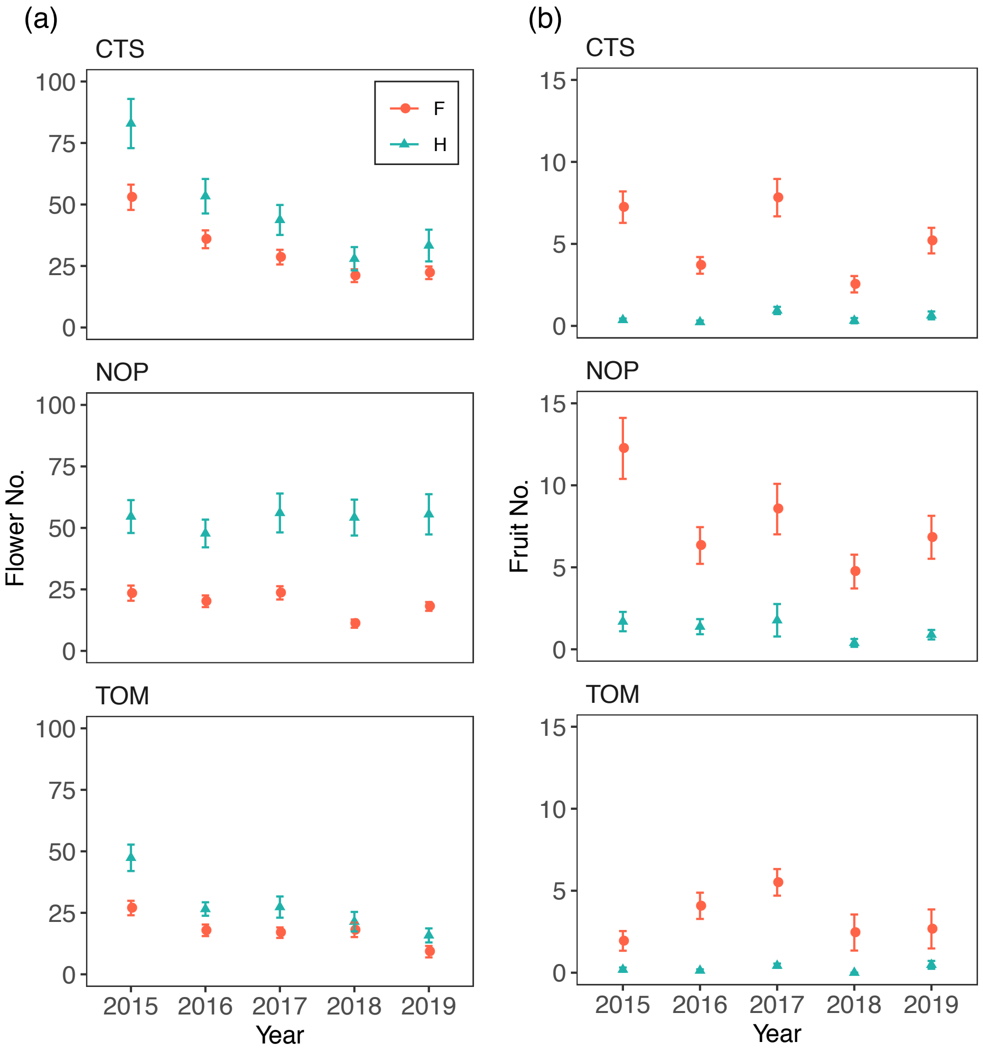

Fig. 3-1
Yearly variations in flower production and fruit production of female (F) and hermaphroditic (H) genets under natural conditions from 2015 to 2019 in three populations. Mean ± SE of genet was indicated.

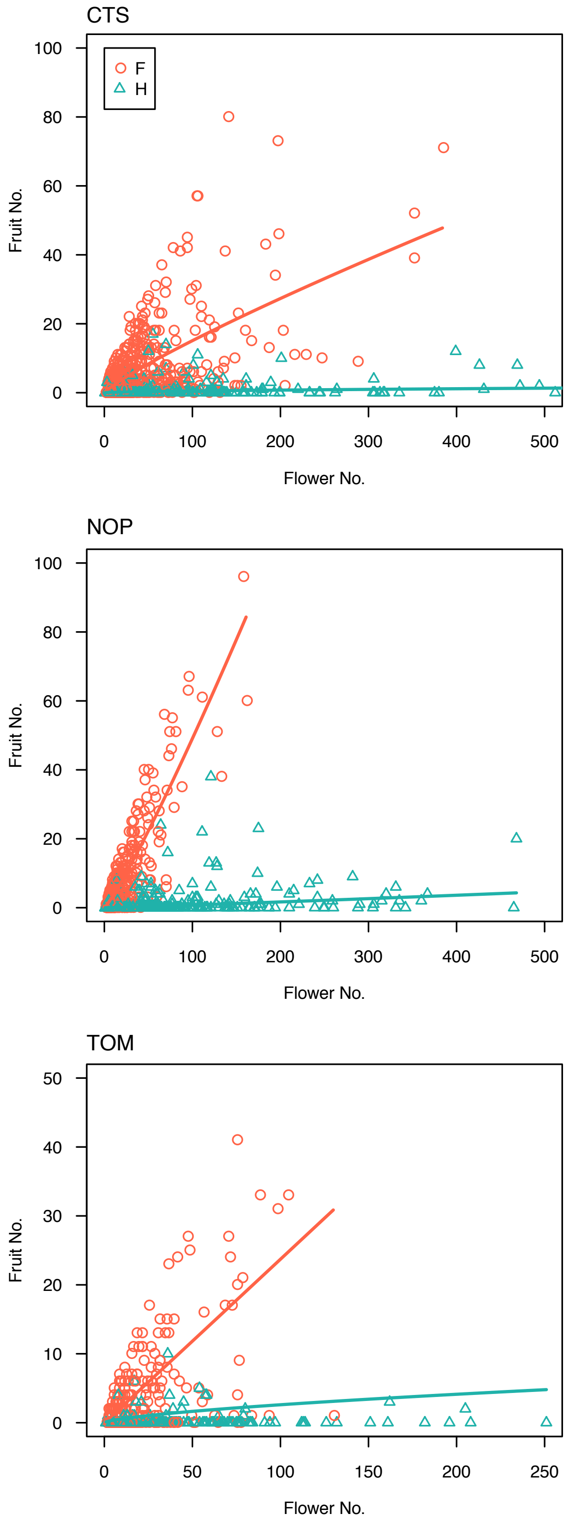

Fig. 3-2
The relationships between flower number and fruit number of female (F) and hermaphroditic (H) genets under natural conditions in three populations over five years.

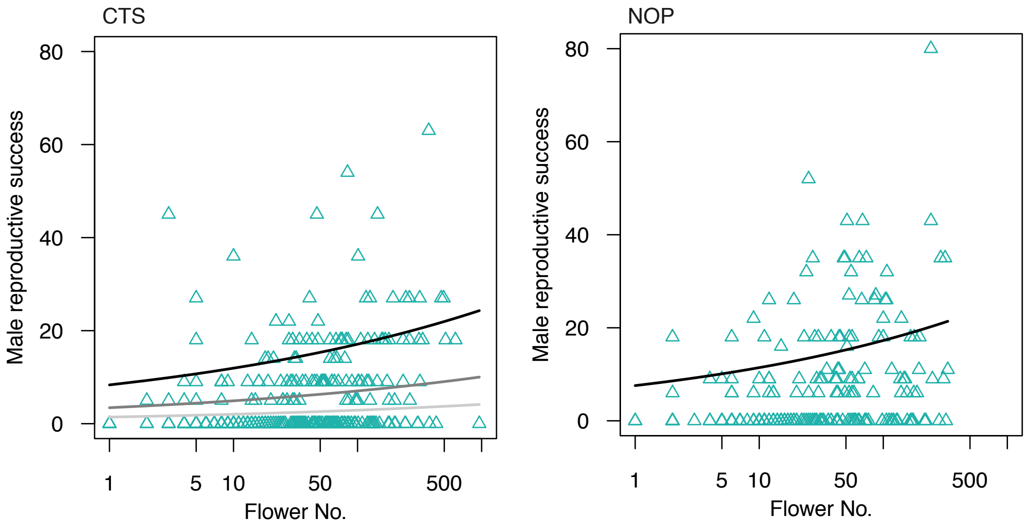

Fig. 3-3
The relationships between flower number and male reproductive success (number of sired seeds) in hermaphrodites at CTS and NOP. Lines indicate the predicted relationship by GLMMs. At CTS, the color depth of lines represents total female flower number within a 10-m radius of a focal pollen donor, i.e., density of potential recipient flowers (black: 1000, dark grey: 100, light grey: 10).

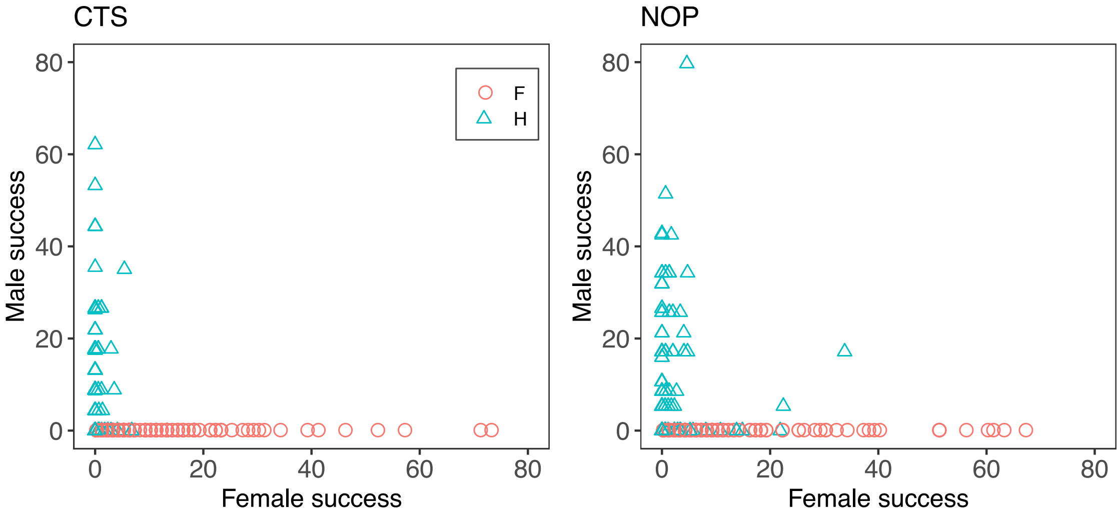

Fig. 3-4
The relationships between reproductive success through female function (seed production) and male function (pollen donation) of female (F) and hermaphroditic (H) genets at CTS and NOP.

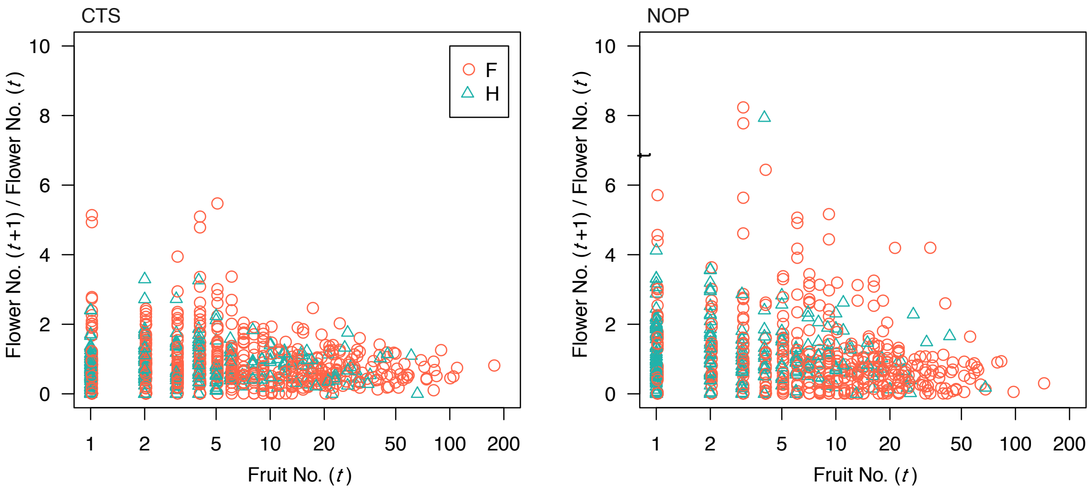

Fig. 3-5
The relationship between fruit number in current year (*t*) and relative flower production (a ratio of flower no. in next year to flower no. in current year) in the next year (*t*+1) of female (F) and hermaphroditic (H) genets at CTS and NOP.

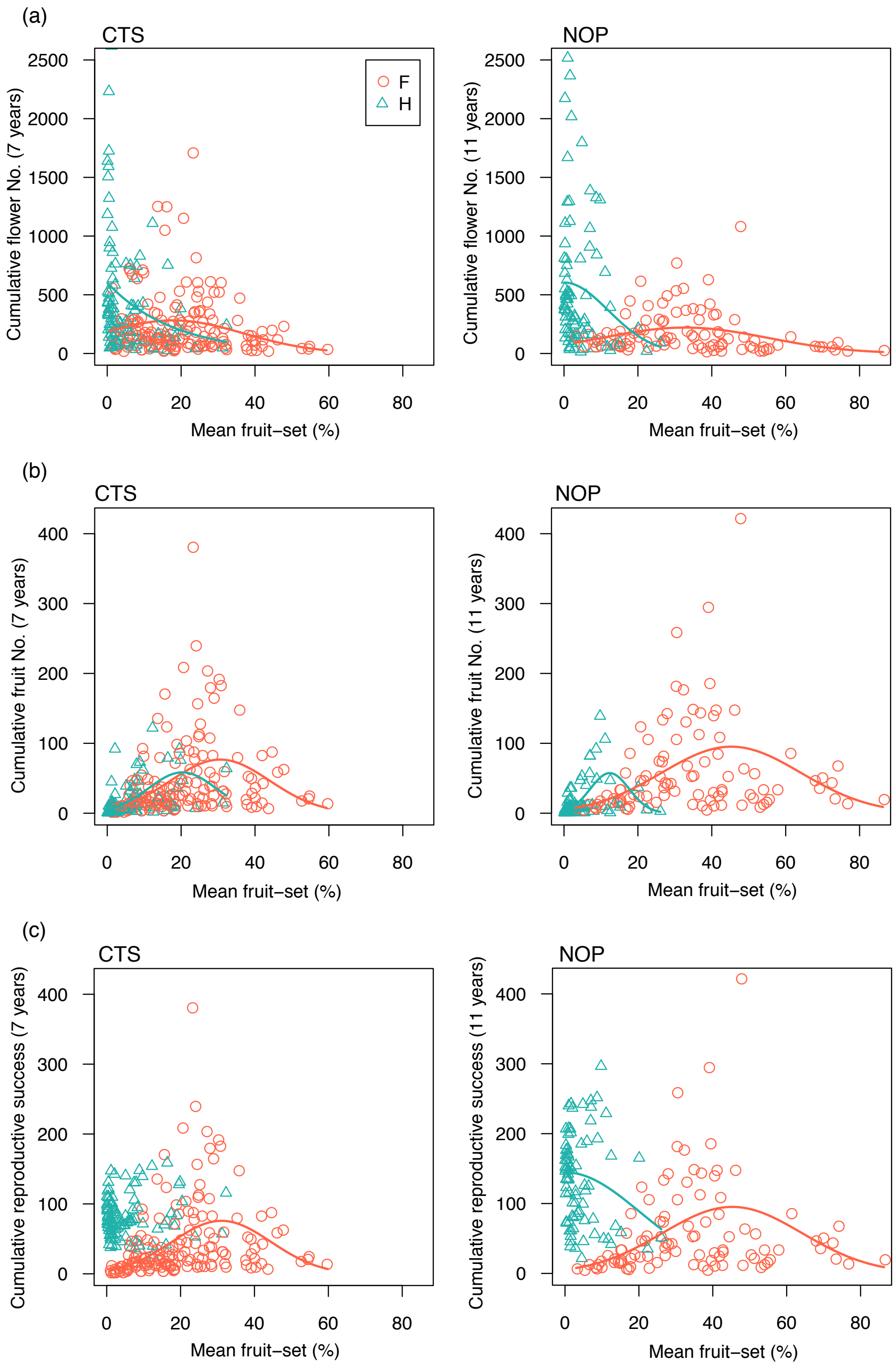

Fig. 3-6

The relationships between mean fruit-set (fruiting ability) and cumulative flower number (a), cumulative fruit number (b), and cumulative reproductive success (sum of seed number produced and sired) (c) of female (F) and hermaphroditic (H) genets over long periods at CTS and NOP.

General discussion

Throughout the study, following aspects were revealed in *Daphne jezoensis*. First, the gynodioecy composed of female and male-biased hermaphroditic individuals was a consistent sexual system across the distribution range in Japan (Chapter 1). Second, low seed fertility of hermaphrodites was a fixed trait independent of size and resource condition of individuals (Chapter 2). Third, however, the fitness-set showed a concave-shaped relationship, suggesting that dioecy could be a stable sexual system on the basis of the sex allocation theory (Chapter 3). In this section, I discuss the reason why male-biased hermaphroditic phenotype is maintained by focusing on the ecological advantages of the female function in hermaphrodites and other possibilities contributing to the maintenance of gynodioecy. Finally, I overview the evolutionary perspectives on the gynodioecy–dioecy pathway in angiosperm.

Ecological factors affecting the transition from gynodioecy to dioecy

In the review of the later stage in the gynodioecy–dioecy pathway, Spigler and Ashman (2012) stated that three ecological factors could be key issues interpreting the absence or presence of male phenotype in gynodioecious populations; that are (1) advantage of seed production by selfing in hermaphrodites, (2) sex allocation plasticity in hermaphrodites, and (3) interactions between plants and enemies, i.e., herbivores, pathogens, and nectar robbers). Among these factors, the third factor, i.e., sexual differences in herbivore damage, was not tested in this study. This is because herbivore damage on flowers and fruits was basically small in this species across the populations (personal observation). Furthermore, this factor can be important in the transition stage from subdioecy to dioecy in which male-biased attack by enemies may interfere the evolution of dioecy (Ashman, 2002, 2006). In *D. jezoensis*, there was no evidence of the existence of subdioecious populations across the distribution range. Therefore, interactions between plants and enemies may be not important for the maintenance of the sexual system in *D. jezoensis*.

For the second factor, i.e., resource-dependent plasticity in sex allocation, it is predicted that gender plasticity in hermaphrodites should delay the transition to dioecy (Delph and Wolf, 2005; Ashman, 2006). The present study revealed that reproductive performance (flower and fruit production) was size dependent but the seed fertility of hermaphrodites was independent of size and resource conditions (Chapter 2). This implies that the plasticity in sexual function of hermaphrodites is limited in this species. In contrast, the possibility of the first factor needs to be assessed carefully because hermaphrodites were self-compatible and significant inbreeding depression was detected. Thus, I discuss the ecological significance of seed production in hermaphrodites as a reproductive assurance function.

Reproductive assurance function in hermaphrodites

Several studies reported that severe pollen limitation may help to maintain hermaphrodites within a gynodioecious population, because some hermaphrodites can produce seeds by autonomous self-pollination as a reproductive assurance (Wolfe and Shmida, 1995; Fleming et al., 1998; Del Castillo and Argueta, 2009). For a reproductive assurance, plant species needs to have self-compatibility and the ability of autonomous self-pollination (Lloyd, 1992). Actually, every *D. jezoensis* population showed both of these features across the wide distribution range (Chapter 1). Furthermore, the quality and quantity of selfed progenies are important for the consideration of function as a reproductive assurance. In the populations observed in this study, selfing rate of hermaphrodites ranged between 32–87% (Chapter 1), but the contribution of hermaphrodites as a seed producer was very small, corresponding to only 7–17% of total seed production (Chapter 3). Furthermore, considerable inbreeding depression ($\delta = 0.59$) existed in selfed progenies due to low seed germination ability and relatively low survival rate of seedlings (Chapter 3). Therefore, the reproductive assurance function of selfing in hermaphrodites was negligibly small in terms of the maintenance of existing populations.

On the other hand, occasional establishment of seedlings from selfed seeds may have advantages at a colonization stage, such as the foundation of new population after long-distance seed dispersal. This colonization advantages of self-compatible was proposed by Baker (1955), and was also suggested in several gynodioecious species (Taylor et al., 1999; Alonso and Herrera, 2011). Because fruits of *D. jezoensis* are single seeded and dispersed by birds, mating partners may be limited or even absent in early stage of the colonization because of small number of individuals and/or deviated sex ratio in the early stage of population establishment (Kudo et al., 2015). Under such situation, hermaphrodites producing seeds by autonomous self-pollination may be advantageous. Therefore, I present a new hypothesis that the maintenance of male-biased hermaphrodites in this species is advantageous in colonization process. Hermaphrodites having selfing ability would be positively selected in recently colonized populations due to reproductive assurance advantages (Pannell, 1997). To test this hypothesis, genetic researches of meta-populations including recently established small populations are required.

A trade-off between female and male functions

A large resource investment in the current reproduction often causes negative effects on future reproduction, growth and/or survival due to the cost of reproduction (Obeso, 2002). In *D. jezoensis*, the cost of fruit production, i.e., a negative correlation between current-year fruit production and next-year reproductive effort (flower number) was detected (Chapter 3). This indicates that a large fruit production in one year results in a decrease in flower production next year, resulting in lower reproductive success as a pollen donor (Chapter 3). Furthermore, high fruiting ability caused smaller amounts of flowers in a long period. These relationships indicate a trade-off between male and female functions in hermaphrodites.

The sex allocation theory postulates the existence of a trade-off in resource allocation between male and female functions, and a positive correlation between resource investment in each sexual function and fitness gain through the sexual function (Charnov, 1982). A concave-shaped fitness-set predicts that dioecy is a stable sexual system in *D. jezoensis* (Chapter 3). Nevertheless, gynodioecy with male-biased hermaphrodites was a consistent sexual system across the geographic distribution rage. As possible reasons of the discrepancy between actual pattern and theoretical prediction, at least two explanations are possible. First explanation is the colonization advantage hypothesis after long-distance seed dispersal as mentioned above. Second explanation is that the trade-off in resource allocation between male and female functions is small in the hermaphroditic phenotype.

In contrast to the existence of the cost of fruit production, both of the resource-dependent and size-dependent fruiting ability, i.e., plasticity in sexual function, were absent in hermaphrodites (Chapter 2). The fruiting ability of hermaphrodites might be originally determined at a low level, resulting in small fruit production even when resource conditions of individual plants are improved. The conservative fruit production in hermaphrodites without the plasticity of seed fertility may result from a selective force toward decreasing the cost of fruit production for a long period. Because of the conservative resource investment in fruit production, flower production of hermaphrodites may be maintained at higher level. Under such regulation of resource allocation to reproductive functions, the selective force at the present moment may be not strong enough to establish pure male phenotype within a population and replace hermaphrodites with male phenotype, leading to dioecy. Interestingly, a conservative fruit production was evident also in females (Chapter 1). Furthermore, females with modest fruiting ability were predicted to be adaptive for the maximization of long-term fitness in this species (Chapter 3). The conservative fruit production seemed to be related to the summer-deciduous leaf habit of this species as discussed in Chapter 3. If so, the life-history strategy of carbon assimilation may determine the resource allocation between male and female functions in hermaphrodites, resulting in the maintenance of small fruiting ability.

Gynodioecy as a stable sexual system

Previous angiosperm-wide analysis revealed that gynodioecious species were found in 81 families (> 18% of all angiosperm families), of which 29 families did not include any dioecious species (Dufay et al., 2014). This trend suggests that gynodioecy is not necessarily the transitional sexual system toward dioecy. There are approximately 70 species in the genus *Daphne* including many gynodioecious species, but there is no dioecious species (Brickell and Marhew, 1976). One congeneric species, *D. laureola*, is composed of hermaphroditic and gynodioecious populations in the Iberian Peninsula, and recent phylogenetic study revealed that different sexual systems have been repeatedly evolved in the region, but purely dioecious populations are absent (Alonso and Herrera, 2011). The evolutionary stage of *D. laureola*, therefore, is on the way to hermaphroditism–gynodioecy evolutionary pathway, i.e., early stage in gynodioecy. Existence of females in the hermaphroditic populations has been explained by the combination of high selfing rate and inbreeding depression (Medrano et al., 2005). In contrast, the present study revealed that the gynodioecy with male-biased hermaphrodites in *D. jezoensis* could be a stable sexual system, i.e., late stage in gynodioecy. The selective force leading to a dioecy was supposed to be not strong in this species. In male-biased hermaphrodites, a trade-off relationship between female and male functions can be mitigated by a small fruit production in this species.

In conclusion, the conservative seed fertility could maintain the gynodioecy as a stable sexual system in this species. As an ultimate explanation of the low seed fertility in hermaphrodites, reproductive assurance function in colonization process is predictable. For a generalization of the maintenance mechanism of gynodioecy in angiosperm, interspecific comparisons of genetic structure at the meta-population scale and physiological control of reproductive allocation are required.

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