Ecological Study on Plant Reproduction and Pollination for Establishing Appropriate Management of Ex-situ Conservation in Botanical Garden Setting

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Chapter 1 General Introduction

1.1 Importance of reproductive and pollination ecology in conservation

Human impacts are a main threat to biodiversity worldwide and this critical situation has not been resolved (Godet & Devictor, 2018). Conservation of plant diversity is an urgent situation; however, there are several challenges to achieving this (Corlett, 2016). In this context, an integrated approach of in-situ and *ex-situ* conservation methods (Volis & Blecher, 2010) is a recommended.

Determinant of extinction of species are considered to be caused by factors such as habitat fragmentation, habitat deterioration, destruction, changes in land use. In conservation strategy, artificial conservation measures using physical protection or environmental modification could lead to successes of conservation or recovery program depending on the situations. However, reproductive and pollination process are one of the uncertain and uncontrollable factors in conservation. Therefore, they could have the difficulty in controlling the conservation compared to other factors (Ye et al., 2006; Primack et al., 2008). More than 80% of land plants depend on animal pollinator for their pollination process (Ollerton et al., 2011), and it is an essential component for the regeneration. Pollination systems are under increasing threat from anthropogenic factors, including fragmentation of habitat, changes in land use, modern agricultural practices, use of chemicals such as pesticides and herbicides, and invasions of non-native plants and animals (Kearns *et al.* 1998). Especially, fragmentation of habitat caused by human impacts could directly affect plant pollination and pollinator abundance. In plant conservation, decrease of reproductive success and output due to pollen limitation (i.e., fewer mating partners) in self-incompatible plant species, and increase of inbreeding in

self-compatible plant species are the major concern for endangered species (Ghazoul 2005). In general, outcross-pollination could be a driving force for genetic diversity and an essential element for a persistence of plant population against stochastic factors. Thus, outcross-pollination is considered to have a positive effect on viability of individual and population (Law et al. 2010; Castro et al. 2015). On the other hand, inbreeding depression is known to increase the risk of extinction through the loss of genetic diversity and viability of individual and population in the long term (Frankham 2008). Pollination process is the drivers for the reduction in reproductive outputs (reproductive quantity) and genetic diversity (reproductive quality), hence, it is a factor that must be given importance in conservation.

Reproductive and pollination process also affect the plant evolution. For instance, evolutionary trend to self-mating system under the pollinator scarce condition (Ushimaru *et al.* 2014), evolution of floral traits such as floral shape and scent due to the pollinator change (Gervasi and Schiestl 2017) are case studies. Same as pollination, florivory is also ecological and evolutionary process caused by plant-animal interaction and it could change the ecological and evolutionary consequence (McCall and Irwin 2006). Reproductive systems, including flowering phenology, flower structure, and pollination, are key determinants for population persistence through genetic effects (Charlesworth 2006; Eckert et al. 2010; Castro et al. 2015). In other words, pollination process could also affect the persistence of population in indirect way. Distortion of the evolutionary trajectory by human-induced impact could prevent the value of plant conservation.

Although reproductive biology of endangered plants is crucial for implementing appropriate conservation measures (Schemske et al. 1994), the lack of knowledge about breeding systems or pollination is a major problem for modern conservation efforts (Zych

and Jakubiec 2008; Bhattacharya 2010). In plant conservation, conservation measures based on plant-animal interactions including pollinators and florivore should be considered (Bhattacharya 2010; Morellato et al. 2016). However, applications of conservation based on plant-animal interactions remains few cases.

1.2 Application of reproductive and pollination ecology in *ex-situ* conservation

Successful in-situ conservation, including restoration and reintroduction, is one of the main goals of conservation ecology. *Ex-situ* conservation plays a complementary role (Volis and Blecher 2010; Schwartz et al. 2017). The Global Strategy for Plant Conservation (Secretariat of the Convention on Biological Diversity 2010) aims to conserve 75% of globally threatened plant taxa in *ex-situ* collections (e.g., seed banks, cryopreserved, tissue cultures, and living collections) and prepare 20% of these taxa for conservation programs in 2020. Establishing a conservation system of *ex-situ* living collections is crucial in developing a conservation strategy. Botanical gardens play an important role in *ex-situ* conservation (Heywood 2017; Mounce et al. 2017, Primack & Miller-Rushing 2009) and are suitable places for investigations into pollination ecology (Chen and Sun, 2018) or researching and finding new aspects of plant behavior (Faraji et al., 2020). The importance of reproduction and pollination is mostly mentioned under in-situ conservation and is rarely mentioned under *ex-situ* conservation contrary to its importance. Therefore, such issues should be considered in order to improve *ex-situ* conservation measures (Bhattacharya, 2010). However, empirical studies are limited to few cases (e.g., pollen limitation in *ex-situ* conservation; Tang et al., 2020 or pollen-mediated gene flow in an *ex situ* population; Wang et al., 2008) and the available information is scarce.

Small and isolated conserved populations specific to *ex-situ* setting (Chen and Sun 2018) could be are subject to the ecological process such as Allee effect accompanying inbreeding which directly deteriorate genetic diversity. For instance, isolated populations and small populations are subject to pollen limitation due to fewer mating partners, reduced seed production and increased self-pollination due to lack of pollinators (Kearns and Inouye 1997; Ghazoul 2005; Berec et al. 2007). These problems are connected with the pollination environments surrounding conserved plant species. This ecological process could also be simulated in *ex-situ* environment and result in deterioration of *ex-situ* living collections due to improper management practices, including 1) adaptation to the *ex-situ* cultivation (Frankham, 2008; Ensslin et al., 2015), 2) genetic erosion and decline of genetic diversity of *ex-situ* collections with the duration of cultivation (Enßlin, Sandner, and Matthies, 2011; Lauterbach, Burkart, and Gemeinholzer, 2012; Rucińska and Puchalski, 2011), and 3) fitness decline of *ex-situ* collections due to genetic drift and inbreeding depression (Hedrick and Kalinowski, 2000). Therefore, the exploration of appropriate conditions for promoting the outcross-pollination against the degradation of *ex-situ* living materials is essential to develop the conservation strategy in *ex-situ* conservation.

Botanical gardens serve as bases for the *ex situ* protection and have unique landscapes wherein many plant taxa are cultivated together. Under this environment, heterospecific pollen transfer could be a potential concern for conservation, which could cause the hybridization among sympathetically cultivated species in *ex-situ* (Zhang et al., 2010). Heterospecific pollen transfer is considered as an ecological process affecting plant reproduction potentially and they may indirectly cause pollen limitation(Celaya et al. 2015; Tur et al. 2016). As *ex-situ* environment are different between from wild

condition and may simulate new artificial selection or unintentional ecological process derived from environment (Ashton 1988), adaptation to the cultivation condition (Frankham 2008; Ensslin et al. 2015) could occur and reduce the quality of *ex-situ* living materials.

1.3 Objectives of this study

The aim of this thesis were to specify the pollination interactions focusing on pollination network level and reproductive ecology focusing on intra-species reproduction for efficient conservation management and strategies. Especially, I focused on the main two topics: I) the heterospecific pollen transfer both in plant and generalist pollinator, II) reproduction including pollination and floral biology of *Eriocaulon heleocharioides,* extinct in the wild, to develop the evidence-based conservation measures. First, I evaluated the heterospecific pollen transfer between the cultivated plant species in botanical garden setting to clarify the distance of plant-plant interference (Chapter 2). Second, I investigated the heterospecific pollen transfer using generalist pollinator hoverflies to specify the garden scale pollination network (Chapter 3). Third, I investigated the pollination system and reproductive performance of *Eriocaulon heleocharioides* previously ignored reproductive characteristics to clarify the magnitude of reproductive success by the pollination types and inbreeding depression (Chapter 4). Forth, I surveyed the floral and pollination ecology of *Eriocaulon heleocharioides* to specify the optimal reproductive condition to develop conservation management (Chapter 5). Finally, I conclude with a discussion on how I could use findings of these study for appropriate management of *ex-situ* conservation in botanical garden setting.

Chapter 2 Detection of heterospecific pollen transfer under *ex-situ* condition for cultivation management

Summary

Ex-situ setting including botanical garden have unique environment where wide variety of plant species together in one place unlike original habitats. This situation could cause unintentional pollination interaction that could not occur under natural conditions. As pollination process could be ecological process driving an evolutionary force on plant reproductive system, pollination process that cannot be simulated in situ may cause possible adaptation to *ex-situ* environment. In botanical garden setting where many plant species are cultivated in many density condition, heterospecific pollen transfer (HPT) through pollinators sharing is possible factors affecting quality and quantity of pollination. However, the patterns of heterospecific pollen transfer remains unclear. In this study, to reveal the factors affecting HPT under artificial conservation situation, I addressed: (i) Does heterospecific pollens inhibit the deposition of conspecific pollens (ii) Does spatial status of multiple species condition affect HPT.

In this study, heterospecific pollens in the total number of deposited pollen grains on stigma increased, the percentage of conspecific pollens decreased in used 5 plant model plant species. In all species, extracted cluster from pollination network contained the neighboring cultivated plant species or weed plant species with high eigenvector centralities were and these species were confirmed around the model plant within the range of approx. 0-50 m.

Heterospecific pollen transfer may potentially reduce the reproductive success,

however, direct evidence that characteristics of pollen grains (the size or stickiness of pollen grains) or pollen tube growth affect the reproductive output should be clear. In addition, when evaluating the pollination network for conservation purpose, effects of neighboring plant species should be considered to determine how neighboring plant species might drive the pollination process and consequent results.

Chapter 3 Potential role of generalist hoverflies as indicators for pollination networks based on pollen DNA barcoding in a botanical garden setting

Summary

To conserve the wild pollinator species for maintaining ecological services such as pollination of plants including agricultural crops, establishment of pollinator-friendly gardens is considered to be effective method using green spaces. Aside from general pollinator gardens, botanical gardens could also function as secondary pollinator gardens due to its high floral resource abundances of cultivated plants. However, the role of botanical garden as a pollinator garden is not evaluated even though botanical garden is a suitable location for investigating pollination ecology. Under botanical garden setting, process of pollination could be easily revealed because botanical garden have the regular arrangement in plant cultivation. Therefore, in this study, combination of hoverflies and pollen DNA barcoding of deposited pollen grains on hoverflies were used for monitoring pollination status in garden scale. Bipartite pollination networks by hoverflies were generalized whereas invasive plant species were dominant. Pollen transfer distances and process of pollination through hoverflies behavior were significantly affected by environmental condition. Invasive plant taxa were incorporated into hoverfly-mediated networks and might behave as unintentional super-generalists, which may cause the disruption of pollen transfer dynamics. Utilization of hoverflies for detecting the possible distance of pollen transfer and the invasive pollen transfer in a garden setting could have the potentials monitoring the pollination status.

Chapter 4 Biological and ecological constraints to the reintroduction of Eriocaulon heleocharioides (Eriocaulaceae): A species extinct in the wild

Summary

Reintroduction is one of the more practical methods of in-situ conservation, however, there are many unsuccessful examples of relocation and reintroduction programs. Consistent with this, inappropriate *ex-situ* conservation causes changes in genetic features and traits, have been recognized as factors obstructing reintroduction of *ex-situ* conserved populations. In *Eriocaulon heleocharioides* Satake (Eriocaulaceae), which is extinct in the wild, reintroduction attempt has been conducted in its last natural habitat from 2008, however, reintroduction project failed after 2013. Especially, reproductive and environmental effects related to long-term *ex-situ* cultivation are suspected to be the main constraints in the reintroduction of *E. heleocharioides*. Therefore, in order to evaluate current status of *ex-situ* conserved population of *E. heleocharioides* and to establish appropriate conservation methods, the reproductive ecology of *E. heleocharioides* should be examined. To this end, this study addressed the following questions: (i) patterns of reproductive success of *E. heleocharioides* between self-pollination and outcross-pollination (ii) offspring performance between pollination methods, (iii) interplay effect between offspring performance (inbreeding depression) and different environmental conditions (underwater and wet-soil).

First, we performed open outcross-pollination and self-pollination to verify the levels of reproductive success along the capitulum position (1st to 6th inflorescences). Subsequently, growth experiments (number of leave, number of inflorescence and

maximum leaf length) incorporating the maternal effect were conducted using inbred and outbred offspring. Evaluation of survival rate were carried out under the combination of pollination treatment and environmental conditions (underwater and wet-soil).

Compared to self-pollination, reproductive output of outcross-pollination significantly decreased and negative effects were more pronounced in the first and in the last capitulum position. The growth of the outbred offspring was superior to that of the inbred offspring in approximately 30–40% of the mother plants. The positive effects derived from outcross-pollination could only be detected in part. Regarding the number of inflorescences and maximum leaf length, the overall negative effects of outcross-pollination increased with time. Pattern of inbreeding depression in survival rate was significantly greater in the underwater condition whereas inbreeding depression was not detected in wet-soil conditions.

E. heleocharioides has a preference for self-pollination based on the seed set under *ex-situ* cultivation setting. Increase of negative effects of outcross-pollination in growth traits with time may suggest the genetic unhealthiness of *E. heleocharioides*. High survival rate of inbred individuals also indicates the ability of inbred offspring to prevail under wet-soil conditions. Additionally, the preference for self-pollination in *E. heleocharioides* could accelerate the frequency of inbred individuals in the conserved population. However, underwater condition could limit the spread of inbred individuals in the conserved population, also contributing to reduce the adaptation to *ex situ* conditions. In terms of sustaining the viability of *E. heleocharioides*, reduction of the frequency of inbred offspring in the conserved population using underwater cultivation is effective, and could also function in reducing the adaptation to the *ex-situ* environment

(wet-soils) because pollination (e.g., avoidance of geitonogamy) cannot be completely regulated. To ensure the success of in-situ conservation, the use of appropriate environmental condition and lineages management in which the mother plants exhibit low extent of inbreeding depression is required to avoid losing the few valuable genetic resources based on the current status of the conserved population.

Chapter 5 Floral and pollination characteristics of Eriocaulon heleocharioides, an extinct species in the wild, for evidence-based conservation management

Summary

Reproductive biology of endangered plants is crucial for implementing appropriate evidence-based conservation measures, dearth of knowledge about breeding systems or pollination in endangered species is major problem even in modern conservation. For extinct species in the wild under conservation, there are few available references regarding reproductive ecology to inform conservation management, and the information that exists is often quite basic. To maximize potential reproductive output for conservation purposes, floral or inflorescence characteristics could provide essential information regarding not only pollination but also florivore. Comprehension of the role of floral ecology in *Eriocaulon heleocharioides* Satake (Eriocaulaceae), which is extinct in the wild, could help fill the gaps in both reproductive ecology in Eriocaulaceae and conservation management plan incorporating previously ignored reproductive characteristics in this species. In the present study, we investigated the basic reproductive characters focusing on the sexual function of inflorescence and pollination mechanisms to develop the conservation strategies for *E. heleocharioides*. We evaluated 1) the functionality of capitula depending on capitulum position, 2) the changes in reproductive success and florivory depending on capitulum positioin, and 3) the effect of plant density on pollinator behaviors and pollen transfer in *E. heleocharioides*.

First, the functional characteristics of capitula, pollen-ovule ratio, and reproductive status (maximum pollination success/florivory damage by larva) were

investigated along 6 flowering sequences of capitulum. Second, to clarify the relationship between plant density and pollination efficiency, we established two density plots (high density: 195 flowering individuals/m^2, low density: 30 flowering individuals/m^2) and total deposited pollen on stigma, insect visitation, and visit duration per capitulum were observed.

Pollen-ovule ratio of first capitulum position was significantly lowest Among 6 flowering capitula. The highest pollination success was found in the second–fourth capitula, whereas florivory increased along the terminal fifth-sixth capitulum position. High plant density affected the pollen deposited on stigmas via insect visitation and low pollinator visit duration.

The capitulum characteristics of E. *heleocharioides* have different functional effects: different sexual functions among the capitulum position, enhancement of the quality and quantity of reproductive output through active pollen transfer, and avoidance of herbivory in the middle flowering capitula position. High plant density could facilitate pollen transfer in *E. heleocharioides*. For example, higher density reduces the duration of pollinator visits and the size of capitula and may also alleviate intra-plant pollination (geitonogamous pollination). This basic information from multiple perspectives can help to determine the potential reproductive success in *ex-situ* conservation setting. Moderate plant density management promoting pollen transfer while utilizing the capitula' functionality could be effective in enhancing conservation program efficiency such as reductions in the required time, money, and labor) for *E. heleocharioides*.

Chapter 6 General Discussion

6.1 Comprehension of interaction between abiotic/biotic factors and reproductive factors for developing the conservation measures

In recent years, conservation management practices taking into account the plant ecology and evolution become important based on the results of adaptation to *ex-situ* conservation condition or consequential conservation failures in in-situ condition. However, there are few cases that effect of ecological processes and factors are clearly identified even in *ex-situ* conservation (Ye *et al.* 2006; Wang *et al.* 2008). Ecological processes in reproduction and the quality and quantity of reproductive success could be key factors influencing conservation strategies under both in-situ and *ex-situ* condition. Especially, these process is considered to be strongly implicated in the processes of reduction in genetic diversity and adaptation. Thus, reproduction process is an important factor that should be addressed under conservation settings.

In this thesis, I firstly focused on pollination as an ecological process in plant-plant interaction through a pollinator (chapter 2), potential plant-pollinator interaction through a generalist pollinator (chapter 3). Result of pollination network mediated by hoverflies in chapter 3 showed that the invasive plant taxa were incorporated into hoverfly-mediated networks and could possibly behave as unintentional super-generalists, which may cause harmful heterospecific pollen transfer. This suggested the potential selection on other plants species flowering simultaneously and sympatrically via heterospecific pollen transfer by generalist pollinator such as the hoverflies. In general, the reproduction process is known to exert selection pressure as a strong driver and when invasive alien species is incorporated into the pollination network, it can

disrupt the pollination network and interrupt the reproduction of other species. Heterospecific pollen transfer is considered to be a kind of ecological process in reproduction (Tur *et al.* 2016). Especially in conservation context, reproductive interference interrupting the seed formation or healthy offspring through the physical and physiological reaction by pollen grains of closely related species (Matsumoto *et al.* 2010) become concern. In *ex-situ* environment where many plant species are cultivated together, the risk for heterospecific pollen transfer become more serious concern. Based on the results of plant-plant interaction (chapter 2), model plants species under the *ex-situ* condition could receive the heterospecific pollens from the distance of approx. 0-50 m in maximum. However, the most important pollination network in each model species contained the heterospecific pollen transfer from more neighboring plant species. Therefore, not only evaluating the indirect and potential effects of heterospecific pollen transfer (chapter 3), but also the direct heterospecific pollen transfer in pollen-pistil interaction (chapter 2) is crucial for specifying and developing the appropriate management measures regardless of the presence or absence of reproductive interference between cultivated plant species. As heterospecific pollen transfer is considered to be affected by plant density, behavior of pollinator or landscape factors (Evans *et al.* 2017), the environment around the conservation site as a reproductive environment should be considered on developing the conservation strategy. This is because neighboring plants may not necessarily have a direct, but indirect, effect on the pollination process (Fig. 3). In particular, for maintaining the rare and small *ex-situ* conserved population, ecological and evolutionary loads on pollination might be monitored based on in this study. Because unexpected pollination process and consequence might be occur even in *Eriocaulon heleocharioides* which is an

inconspicuous plant species under botanical garden setting. Heterospecific pollen transfer distance is also suitable index for both heterospecific pollen transfer and conspecific pollen transfer to promote the outcross pollination. The information on the range of pollen transfer could strengthen the pollination-focused cultivation plan.

On the other hand, for reducing conservation costs and preventing unintended conservation consequences (Ashton 1988; Schemske *et al.* 1994), more species-focused management is also essential for ensuring appropriate conservation management. In this thesis, using the case of *Eriocaulon heleocharioides,* extinct species in the wild*,* interaction of abiotic factors and reproductive performance (Chapter 4), plant-pollinator and plant-florivore interactions (Chapter 5) were evaluated. The interaction between reproductive performance such as inbreeding depression and the environment in has become an important factor (Frankham, 2005) because inbreeding depression is considered to be stronger in harsher environmental conditions (Cheptou et al., 2000; Gargano et al., 2011; Waller et al., 2008). Especially in *E. heleocharioides*, reduction of the frequency of inbred offspring in the conserved population using underwater cultivation is effective in terms of sustaining the viability of this species, and could also function in reducing the adaptation to the *ex-situ* environment (wet-soils condition). In addition, to prevent lower reproductive performance in terms of long-term conservation management, the potential and basic mechanisms should be revealed. In chapter 5, functionality of capitulum, direct and indirect process of reproductive success were evaluated. Moderate plant density management promoting outcross-pollination while utilizing the inflorescences' functionality could be effective in enhancing conservation program efficiency for *E. heleocharioides*. For developing the robust conservation plan, multiple perspectives are important for determining potential reproductive success

(Berec *et al.* 2007; Ruane *et al.* 2014) especially in *ex-situ* conservation.

6.2 Implications for the evidence-based *ex-situ* conservation management

In wild condition, plants are immobile and always be exposed to various environmental factors. However, in *ex-situ* condition, plant could be exposed to more unintentional environmental factors. For instance, focusing on reproduction-oriented conservation measures, there are complex factors involving such as heterospecific pollen transfer (chapter 2), proximity of cultivated plants (chapter 2), characteristics and behavior of pollinator (chapter 3), interaction with inbreeding depression and abiotic factors (chapter 4), interaction effect of pollinator or florivore (chapter 5). Broad and detailed description of conservation action, consequences of action including problems should be fully used for developing the conservation strategy and practice.

On focusing on the functionality of botanical garden and pollinators as pollination environment, the unintended effects of heterospecific pollen transfer by pollinator-sharing could be concerns in plant conservation. Artificial environmental change, causing the unintentional behavior of generalist plant-pollinator interaction, could disrupt the structure of the native plant–pollinator networks and pollen transfer dynamics. Thus, hoverflies as potential indicators monitoring unintentional pollen transfer could be important for plant conservation management. To prevent unintentional ecological consequences, the surrounding environment should be considered in botanical garden management where highly mobile pollinators, such as hoverflies, are concerned although this could not evaluate direct chapter pollen-pistil interaction.

Complex factors involving pollination in botanical garden condition (e.g.,

landscape, cultivation plant density) could mediate the patterns of pollinator behavior and might result in positive effects (e.g., promotion of outcrossing in chapter 5) and negative effects (e.g., heterospecific pollen transfer in chapter 2 and 3). The risk for heterospecific pollen transfer due to generalist pollinator should be carefully studied especially in dipteran flies (e.g., hoverflies in chapter 3) in high abundance often underestimated its contribution to pollination. On the other hands, conservation of pollination mutualisms is important challenge. In chapter 5, the underestimated contribution to pollination in *Eriocaulon heleocharioides* by small flies missing in the observation was suggested based on statistical models. Ramos *et al.* (2005) reported that a decrease in potential pollinators would affect the maintenance of populations of the Eriocaulaceae. Therefore, pollinator-friendly management will be required to maintain the contribution of potential pollinators during conservation procedures considering the balance between positive and negative effects caused by pollinator.

When conducting the maintenance of *ex-situ* conserved populations, management approach to prevent or reduce the negative effects of genetic problems under controlled breeding, pedigree selection design is needed. In chapter 4, the importance of outcross-pollination was confirmed for some traits (i.e., growth traits , survival rate in under-water condition). However, *E. heleocharioides* is an annual plant species with short-lived seeds, surviving for 1-4 years in *ex situ* condition (Miyamoto, 1995). Thus, postponement of regeneration by seed (Ensslin *et al.*, 2015; Frankham, 2008) is not applicable. This indicates that the risk of adaptation or genetic degradation increases every year. Cross-bred lineage in cultivation is essential to avoid losing the few genetically healthy lineages/individuals or valuable genetic resources in the *ex-situ* conserved population. Therefore, efficient reproductive management plans should aim

at reproductive output through active pollen transfer, and avoidance and escape from florivory in the middle flowering capitulum. High plant density could facilitate pollen transfer and reduce the duration of pollinator visits alleviate intra-plant pollination (geitonogamy) in *E. heleocharioides*.