Multidisciplinary analyses on the intraspecific variation of pollination system in *Lycoris sanguinea* Maxim.

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General introduction

Angiosperms are the most diversified group among land plants (e.g. Wikström *et al.* 2001). Since Darwin (1877), plant researchers have been believed that diversifications of flowering plants were promoted by plant-pollinator interactions (Van der Niet & Johnson 2012; Willmer 2011). Basically plants cannot move from their birth places to other points by themselves but use other mobile factors for their reproduction and propagule dispersal, such as wind, water, and a variety of animal species (Ackerman 2000; Culley *et al.* 2002; Stebbins 1970; Tussenbroek *et al.* 2016). In approximately 87.5% of extant flowering plants, their reproductive successes are thought to be dependent on animal pollination (Ollerton *et al.* 2011). Animals visit to flowers for floral rewards such as nectar or pollen, and they also carry conspecific pollen grains to stigma. Many studies showed that flowers were adapted to the most efficient pollinators and their traits changed for more fruit and seed production (e.g. Stebbins 1970). These floral adaptations could cause reproductive isolation between populations and promote plant speciation (Kay 2006; Ramsey *et al.* 2003). Therefore, close relationships between plants and their pollinators have been one of important research subjects of evolutionary biology and ecology.

Reproductive success of flowers is determined by the quality and quantity of carried pollen. Insufficient visitation frequencies of pollinators or mismatches between floral parts and pollinators' body sizes cause lower production of fruits and seeds (Aizen & Harder 2007). These conditions are named as pollen limitation, and it is considered to be one of the main factors which could cause ecological and evolutionary consequences such as evolving the mechanisms of reproductive assurance or promoting adaptation to

inefficient pollinators to flowering plants (Ashman *et al.* 2004). Compared to ecological impacts, evolutionary changes caused by pollen limitation have been widely studied (e.g. Harder & Aizen 2010). Plants evolved their flowers to reduce the influences of pollen limitation. In the condition of quantitative pollen limitation, plants may acquire the mechanisms of reproductive assurances such as autonomous self-pollination, or adapt to most effective pollinators by attracting or fitting floral shapes to them. Previous study suggested the commonness of pollen limitation (e.g. Knight *et al.* 2005), and it might indicate that pollen limitation promoted plant diversifications.

One of the representative examples of the results of floral adaptation is pollination syndrome. Plants with specific pollinator species receive similar selective pressures to floral traits, and they are changed to have similar floral colors and/or shapes. In Schiestl & Johnson (2013) and cited therein, clear examples of pollination syndrome across unrelated three taxa were shown. For example, flowers with hummingbirds as pollinators tend to have reddish color and large volume of dilute nectar; on the other hand, hawkmoth-pollinated flowers have white floral color and floral scent at night (e.g. Raguso *et al.* 2003; Wilson *et al.* 2004). Recently genetic backgrounds of pollination syndromes have been gradually revealed (Hermann *et al.* 2013 and references therein; Wessinger *et al.* 2014; Sheehan *et al.* 2016), and quantitative evaluations of the predictability of pollination syndromes have also been performed (Rosas-Guerrero *et al.* 2014). Although the pollination syndrome concepts have been received widely in researchers, inconsistent cases have been frequently reported in some taxa and reliabilities of this concept has been still controversial (e.g. Faegri & van der Pijl 1979; Fenster *et al.* 2004; Ollerton *et al.* 2009; Rosas-Guerrero *et al.* 2014).

In general, flowers have various types of animal visitors including bees,

butterflies, and hummingbirds (e.g. Waser et al. 1996; Sahli & Conner 2007; Gomez *et al.* 2014). However, some of them do not act as effective pollinators but collect floral rewards with few or no pollination. These ineffective visitors are categorized as nectar/pollen thieves or robbers. Nectar thieves (nectar stealers without damage to flowers) or robbers (with damage) have been well studied mainly about their ecological influences (Inouye 1980), perhaps because one of the famous nectar thieves were bumblebees, most popular floral visitors in pollination biology. Nectar thieves or robbers indirectly reduce plant fitness through affecting behaviors of other visitors (Irwin *et al.* 2010). Compared to nectar stealers, the study cases of pollen thieves or robbers have been limited (Hargreaves *et al.* 2009). Pollen grains are unique because they are not only floral reward for visitors but also floral gametes (Hargreaves *et al.* 2009; Muth *et al.* 2016). Unnecessary pollen consumptions reduced seed productions directly, and pollen thieves or robbers would create the conditions of pollen limitation. Pollen thieves are predicted to affect plant fitness more strongly than nectar thieves; however, the numbers of studies including theoretical or empirical ones are insufficient to understand the comprehensive impacts of pollen theft.

Against to pollen theft, plants could respond through the floral adaptation. Hargreaves *et al.* (2009) reviewed the patterns of floral adaptation to pollen thieves: tolerance, resistance and converting thieves into pollinators. Plants can endure the thieves by increasing pollen production or resist them by morphological or chemical defenses. For example, *Alcea rosea* has spinose pollen grains and this mechanical structure protected pollen to collection by corbiculate bees (Lunau *et al.* 2015). Furthermore, the larval developments of specialized bees, which depended on the resource of pollen of a plant species, were impeded from non-host pollen grains (Praz *et*

al. 2008). Similarly, floral traits which mimic or conceal additional pollen have been recognized as adaptations to pollen thieves (e.g. Lunau 2000). Finally, the utilizations of pollen thieves by changing their function to pollinators can occur by decreasing the space or time separations of reproductive organs, although these adaptations could increase self-pollination. However, no study has been directly investigated whether pollen theft promoted floral adaptation.

Pollinator-mediated evolution of flowering plants has been mainly studied from two perspectives; adaptation of floral traits to pollinators (microevolution) and speciation with shifts of pollination systems (macroevolution). Pollinator-mediated floral adaptation has been examined through the inter- or intraspecific comparison of floral traits and their pollinators between populations (e.g. Anderson & Johnson 2008; Anderson *et al.* 2014; Newman *et al.* 2015; Pauw *et al.* 2008; Shutterworth & Johnson 2010; Sun *et al.* 2014). Recently Grant-Stebbins model, firstly suggested by Johnson (2006), has been accepted widely. In Grant & Grant (1965), they hypothesized pollinator distributions were not equal throughout the plant distribution. Stebbins (1970) also suggested that the pollination efficiencies of each pollinator would change, depending on the frequencies of each pollinator, structure of visited flowers, and their surrounding environments. Grant-Stebbins model is the combined model of them, and it suggests that geographic mosaic of pollinator distribution give different selective forces and promotes divergent selections (Johnson 2006; 2010).

The degrees of pollen limitations caused by pollen theft would also be different between populations. Floral visitors can change their behaviors smoothly and they act as pollen thieves in some plants but also do as effective pollinators for others (e.g. Hargreaves *et al.* 2012). Their behaviors could be changed by their surrounded

environments such as the abundances of visitors or other flowering plants. These differences would cause various intensities of pollen limitation between populations, promoting geographic differences of the patterns of floral adaptation. Although inter- or intraspecific variations of the degrees of pollen theft have been examined (Hargreaves *et al.* 2012; Solís-Montero *et al.* 2015), the question whether these differences could cause various patterns of floral adaptation has remained to be answered. This may be due to the lack of model cases for investigating the evolutionary influences of pollen theft. Especially, although the phenomenon of converting thieves into pollinators by floral adaptations could be common in the evolutionary interaction between plants and flower visitors, specific cases that can be clearly attributed to the phenomenon have not been known.

Differences of floral visitor faunas could not only promote morphological variations but also generate genetic differences at neutral genetic loci. This is because that pollinator shifts can reduce gene flow by separating pollen vectors and thus act as a prezygotic isolation barrier. Although this issue has been still controversial, plant speciation by pollinator shifts have been widely accepted (Van der Niet et al. 2014). Even for the plant species with generalist pollinators, geographical mosaic of floral visitor assemblages including both effective pollinators and pollen/nectar stealers may impose floral trait divergence among populations and also affect population genetic structure of the species.

In order to answer these questions, multiple approaches including field observations and experiments, phylogenetic analyses and population genetics would be needed. Recently image quality of low-cost digital video cameras has been dramatically improved, enabling the burden for field researchers of pollination biology to decrease

(e.g. Phillips *et al.* 2014). The advent of next generation sequencing (NGS) technology has also enabled us to determine numerous amounts of DNA sequences easily and cheaply, and shed light on genetic and genomic mechanisms of ecological and evolutionary perspectives even in non-model organisms. One of the NGS methods, restriction site-associated DNA sequencing (RADseq), has largely contributed to these studies (Andrews *et al.* 2016). This method can collect genome-wide single nucleotide polymorphism (SNP) flanking to cutting sites of restriction enzymes without any information about genomes of target or related species.

The genus *Lycoris* is distributed in Eastern Asia, mainly in China, Korea, and Japan. Approximately 20 species are included in the genus, and they have several interesting features that attract researchers: variation of floral traits, frequent hybridization in nature, separation of vegetative and reproductive phases, and polymorphisms of chromosome numbers. Floral shapes of *Lycoris* species are divided into mainly two patterns; funnel shapes or radiated ones. For example, Higan-bana (*Lycoris radiata* var. *radiata*) that is familiar to Japanese as a beautiful autumn flower, has radiated flowers with bright reddish color. Floral colors in the genus *Lycoris* are varied: red, orange, yellow, peach, white, and mixed ones of them (Hsu *et al.* 1994). In *L. longituba* var. *longituba*, intraspecific polymorphisms of floral colors were observed (He *et al.* 2011). Most of the *Lycoris* species show vegetative period after or before flowering season. Leaves appear above the ground and expand rapidly. After that, the leaves disappear before the appearances of scape. The periods of vegetative and reproductive phases are different among species. Chromosome polymorphisms have been well studied for taxonomical aspects. As for fertile diploid taxa, chromosome numbers varies from 2n=14 to 2n=22 in *Lycoris* (Hsu *et al*. 1994). The chromosomes of

Lycoris can be classified into three types: acrocentric (A), metacentric (M) and telocentric (T) chromosomes. The 2n=22 taxa have only A-type chromosomes (2n=22A) and include *L. radiata* var. *pumila*, *L. sprengeri* and *L. sanguinea*. The choromosomes of the taxa of 2n=12, 14 and 16 were composed of M and T chromosomes (2n=10M+2T, 8M+6T and 6M+10T, respectively).

Lycoris sanguinea Maxim is a perennial herb with bulb and distributed in Japan and Korea. There are three varieties with different floral traits; *L. s.* var. *sanguinea*, *L. s.* var. *kiushiana* T.Koyama, and *L. s.* var. *koreana* (Nakai) T.Koyama (Kurita 1988). *Lycoris s.* var. *sanguinea* is distributed mainly in wide ranges of central Japan, *L. s.* var. *kiushiana* from Kyusyu to western parts of Honsyu, and *L. s.* var. *koreana* in limited area of southern Korea and Nagasaki and Miyazaki Pref. (Kawano 2009; Hsu *et al.* 1994). *Lycoris s.* var. *koreana* has been considered extinct in the wild (EW) but the individuals were recently observed in the Tsushima Island (Ministry of the Environment Japan 2015). The sizes of floral parts are different among the three varieties; for example, the anthers of *L. s.* var. *sanguinea* are not exserted from corollas, but the other two have longer and exserted anthers (Hsu *et al.* 1994). Their funnel-shaped and reddish-orange flowers were partly consistent with the characters common to butterfly-pollinated flowers based on the pollination syndrome (Faegri & van der Pijl 1979). Limited informations about floral visitors have been available for *L. sanguinea*. Kawano (2009) and Chung *et al.* (1999) listed the species of floral visitors. However, visitation and pollination frequencies of each visitor have not been reported. In some cases, frequent visitors were not always effective pollinators (i.e. the visitation and pollination frequencies by each floral visitor were not positively correlated) (King *et al.* 2013 and references therein). Therefore, I need to evaluate each floral visitor in terms of

the effectiveness for reproductive success of *L. sanguinea*.

In this thesis, I focused on a new observation in pollination ecology of *L. sanguinea* var. *sanguinea*: breaking-bud pollination by small bees. I observed that small bees visited to partially opened flowers of *L. s.* var. *sanguinea*. I called this stage of flowers as breaking buds, which had just started to open. Previous studies reported similar insect behavior. In X*yris tenneseensis*, *Lasioglossum zephyrum* visited to premature flowers and removed floral sheath to collect pollens (Wall *et al.* 2002). Another study suggested that the dichogamous (protogynous) flowers could be pollinated by bees before they fully opened (Thomson & Plowright 1980). However, in our knowledge, there has been no study about the insect-mediated pollination process at partially opened stage.

This thesis is composed by four chapters. In the first chapter, I reported pollinator frequencies of *Lycoris sanguinea* var. *sanguinea* in multiple sites and examined whether visitation of small bees at the breaking-bud stage was effective for fruit and seed set. Pollinator observations in five populations of the Kanto region showed that most frequent visitors were small bees, *Lasioglossum japonicum*. They also visited to breaking buds at five populations. Bagging experiments showed that the small bees can pollinate flowers even at the breaking-bud stage because stigmas were receptive even 1 or 2 days before anthesis. Comparison among experimental manipulations in the field suggested that breaking-bud pollination would contribute considerably to the reproduction of *L. s.* var. *sanguinea*.

In the second chapter, I collected the data of pollinator frequencies and floral morphologies of 13 populations of the three varieties of *L. sanguinea*. Pollinator observations showed that only three populations had breaking-bud pollination. Cluster

analysis based on eight floral morphological characters suggested that the 13 populations were divided into three groups, which were partly inconsistent with the three varieties. One of the three groups consisted of the three populations in which breaking-bud pollination was observed, suggesting the evolutional association between floral traits and this novel pollination process. Statistical analyses showed significant correlations between frequencies of breaking-bud pollination and anther-stigma length at breaking-bud stage.

In the third chapter, I examined whether any floral adaptation could be detected in the populations in which breaking-bud pollination was observed, by transplantation experiments. I transplanted individuals from one breaking-bud pollination (Aichi) and two non-breaking-bud pollination (Ehime and Hiroshima and Ehime) to Chiba, where breaking-bud pollination is frequently observed. Bagging experiments showed that Ehime and Hiroshima populations were not adapted to breaking-bud pollination because their stigma was not receptive at the breaking-bud stage. Furthermore, I manipulated flowers to be visited only by small bees at different flowering stages. The results showed that pollination efficiency of small bees at breaking-bud stage was higher than that at fully-opening stage for the samples of Chiba.

In the fourth chapter, I collected huge amount of genetic data by RAD sequencing. Estimations of population genetic structures showed clear pattern of isolation by distance (IBD). Neighbor-net tree of populations showed that populations were not grouped according to either traditional taxonomic classification or the clusters on floral morphologies, suggesting that similar set of morphological characters were generated independently. I hypothesized that differences of pollinator assemblages can promote the formation of these morphological patterns, although other factors can also

do.

Finally, I discussed about the pollinator functions for the evolution of *Lycoris sanguinea* varieties. Pollinator differences between populations could promote to divergences of floral traits, but geographical factors would also associate to population divergences. I also discussed about the taxonomic identification of these varieties. It could be difficult to identify these varieties based on the genetic and morphological information. Morphological variation in this species would be clinal, just as indicated by the IBD pattern, and partly caused by regional selective pressure of pollinators.

References

Ackerman J.D. (2000) Abiotic pollen and pollination: ecological, functional, and evolutionary perspectives. *Plant Systematics and Evolution*. 222, 167-185.

Aizen M.A. & Harder L.D. (2007) Expanding the limits of the pollen-limitation concept: Effects of pollen quantity and quality. *Ecology*. 88, 271-281.

Anderson B., Johnson S.D. (2008) The geographical mosaic of coevolution in a plant-pollinator mutualism. *Evolution*. 62, 220-225.

Anderson B., Ros P., Wiese T.J., Ellis A.G. (2014) Intraspecific divergence and convergence of floral tube length in specialized pollination interactions. *Proceedings of the Royal Society B*. 281, 20141420.

Andrews K.R., Good J.M., Miller M.R., Luikart G. Hohenlohe P.A. (2016) Harnessing the power of RADseq for ecological and evolutionary genomics. *Nature Review Genetics*. 17, 81-92.

Ashman, T.-L., Knight T.M., Steets J.A., Amarasekare P., Burd M., Campbell D.R., Dudash M.R., Johnston M.O., Mazer S.J., Mitchell R.J., Morgan M.T. & Wilson W.G. (2004) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology*. 85, 2408–2421.

Bradshaw H.D., Schemske D.W. (2003) Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature*. 426, 176-178.

Byers K.J.R.P., Vela J.P., Peng F., Riffell J.A., Bradshaw H.D. (2014) Floral volatile alleles can contribute to pollinator-mediated reproductive isolation in monkeyflowers (*Mimulus*). *The Plant Journal*. 80, 1031-1042.

Chung, M.Y., Chun, C.P., Chung, M.G. (1999) Clonal and spatial genetic structure in a

population of the endangered herb *Lycoris sanguinea* var. *koreana* (Amaryllidaceae). *Genes & Genetics Systems*, 74, 61–66.

Culley T.M., Weller S.G., Sakai A.K. (2002) The evolution of wind pollination in angiosperms. *Trends in Ecology and Evolution*. 17, 361-369.

Darwin C. (1877) *The different forms of flowers on plants of the same species*. John Murray, London

Dodd M.E., Silvertown J., Chase M.W. (1999) Phylogenetic analysis of trait evolution and species diversity variation among angiosperm families. *Evolution*. 53, 732-744.

Faegri K, van der Pijl L (1979) *The principles of pollination ecology*. Pergamon Press, Oxford

Fenster C.B., Armbruster W.S., Wilson P., Dudash M.R., Thomson J.D. (2004) Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution and Systematics*. 35, 375–403.

Gómez J.M., Muñoz-Pajares A.J., Abdelaziz M., Lorite J., Perfecutti F. (2014) Evolution of pollination niches and floral divergence in the generalist plant *Erysimum mediohispanicum*. *Annals of Botany*. 113, 237-249.

Grant V., Grant K.A. (1965) *Flower pollination in the Phlox family.* New York, NY: Columbia University Press.

Harder L.D. & Aizen M.A. (2010) Floral adaptation and diversification under pollen limitation. *Philosophical Transactions of the Royal Society B.* 365, 529-543.

Hargreaves A.L., Harder L.D. & Johnson S.D. (2009) Consumptive emasculation: The ecological and evolutionary consequences of pollen theft. *Biological Reviews*. 84, 259-276.

Hargreaves A.L., Harder L.D. & Johnson S.D. (2012) Floral traits mediate the

vulnerability of aloes to pollen theft and inefficient pollination by bees. *Annals of Botany*. 109, 761-772.

He Q., Shen Y., Wang M., Huang M., Yang R., Zhu S., Wang L., Xu Y., Wu R. (2011) Natural variation in petal color in *Lycoris longituba* revealed by anthocyanin components. *PLoS One*. 6, e22098.

Hermann K., Klahre U., Moser M., Sheehan H., Mandel T. & Kuhlemeier C. (2013) Tight genetic linkage of prezygotic barrier loci creates a multifunctional speciation island in petunia. *Current Biology*. 23, 873–877.

Hsu P.S., Kurita S., Yu Z.Z., Lin J.Z. (1994) Synopsis of the genus *Lycoris* (Amaryllidaceae). *Sida*. 16, 301–331.

Inouye D.W. (1980) The terminology of floral larceny. *Ecology*. 61, 1251-1253.

Irwin R.E., Bronstein J.L., Manson J.S. & Richardson L. (2010) Nectar robbing: ecological and evolutionary perspectives. *Annual Review of Ecology, Evolution, and Systematics* 41, 271–292

Johnson S.D. (2006) Pollinator-driven speciation in plants. In *The ecology and evolution of flowers* (eds L. D. Harder & S. C. H. Barrett), 295–310. Oxford, UK: Oxford University Press.

Johnson S.D. (2010) The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philosophical Transactions of the Royal Society London B, Biological Sciences*. 365, 499–516.

Kawano S. (2009) Life-history monographs of Japanese plants. 13: *Lycoris sanguinea* Maxim. (Amaryllidaceae). *Plant Species Biology.* 24, 139–144.

Kay K.M. (2006) Reproductive isolation between two closely related hummingbird-pollinated neotropical gingers. *Evolution*, 60, 538–552.

King C., Ballantyne G., Willmer P.G. (2013) Why flower visitation is a poor proxy for pollination: measuring single-visit pollen deposition, with implications for pollination networks and conservation. *Methods in Ecology and Evolution*, **4**, 811–818.

Knight T.M., Steets J.A., Vamosi J.C., Mazer S.J., Burd M., Campbell D.R., Dudash M.R., Johnston M.O., Mitchell R.J., Ashman T.-L. (2005) Pollen limitation of plant reproduction: Patterns and process. *Annual Review of Ecology, Evolution and Systematics*. 36, 467-497.

Lunau K. (2000) The ecology and evolution of visual pollen signals. *Plant Systematics and Evolution*. 222, 89–111.

Lunau K., Piorek V., Krohn O. & Pacini E. (2015) Just-spines—mechanical defense of malvaceous pollen against collection by corbiculate bees. *Apidologie*. 46, 144-149.Ministry of the Environment Japan. (2015) *Red Data Book 2014–Threatened wildlife of Japan, Vol. 8: Vascular Plants.* Gyousei, Tokyo, Japan.

Muth F., Francis J.S. & Leonard A.S. (2016) Bees use the taste of pollen to determine which flowers to visit. *Biology Letters*. 12, 20160356.

Newman E., Manning J., Anderson B. (2015) Local adaptation: mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities. *Evolution*, 69, 2262–2275.

Ollerton J., Alarcon R., Waser N.M., Price M.V., Watts S., Cranmer L., Hingston A., Peter C.I., Rotenberry J. (2009) A global test of pollination syndrome hypothesis. *Annals of Botany*. 103, 1471-1480.

Ollerton J., Winfree R., Tarrant S. (2011) How many flowering plants are pollinated by animals? *Oikos*, 120, 321–326.

Pauw A., Stofberg J., Waterman R.J. (2008) Flies and flowers in Darwin's race.

Evolution. 63, 268-279.

Phillips R.D., Scaccabarozzi D., Retter B.A., Hayes C., Brown G.R., Dixon K.W., Peakall R. (2014) Caught in the act: pollination of sexually deceptive trap-flowers by fungus gnats in *Pterostylis*. *Annals of Botany*. 113, 629-641.

Praz C.J., Müller A. & Dorn S. (2008) Specialized bees fail to develop on non-host pollen: Do plants chemically protect their pollen? *Ecology*. 89, 795-804.

Raguso R.A., Levin R.A., Foose S.E., Holmberg M.W., McDade L.A. (2003) Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. *Phytochemistry*. 63, 265-284.

Ramsey J., Bradshaw H.D., Schemske D.W. (2003) Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution*. 57, 1520-1534.

Rosas-Guerrero V., Aguilar R., Marten-Rodriguez S., Ashworth L., Lopezaraiza-Mikel M., Bastida J.M., Quesada M. (2014) A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecology Letters*. 17, 388–400.

Sahli H.F., Conner J.K. (2007) Visitation, effectiveness and efficiency of 15 genera of visitors to wild radish, *Raphanus raphanistrum* (Brassicaceae). *American Journal of Botany*. 94, 203-209.

Schemske D.W., Bradshaw H.D. (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America*. 96, 11910-11915.

Schiestl F.P., Johnson S.D. (2013) Pollinator-mediated evolution of floral signals. *Trends in Ecology and Evolution*. 28, 307-315.

Sheehan H., Moser M., Klahre U., Esfeld K., Dell'Olivo A., Mandel T., Metzger S.,

Vandenbussche M., Freitas L. & Kuhlemeier C. (2016) *MYB-FL* controls gain and loss of floral UV absorbance, a key trait affecting pollinator preference and reproductive isolation. *Nature Genetics*. 48, 159-166.

Shutterworth A., Johnson S.D. (2010) The missing stink: sulphur compounds can mediate a shift between fly and wasp pollination systems. *Proceedings of the Royal Society B*. 277, 2811-2819.

Solís-Montero L., Vergara C.H. & Vallejo-Marín M. (2015) High incidence of pollen theft in natural populations of a buzz-pollinated plant. *Arthropod-Plant Interactions*. 9, 599–611.

Stathos A., Fishman L. (2014) Chromosomal rearrangements directly cause underdominant F1 pollen sterility in *Mimulus lewisii-Mimulus cardinalis* hybrids. *Evolution*. 68, 3109-3119.

Stebbins G.L. (1970) Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. *Annual Review of Ecology and Systematics 1*, 307–326.

Sun M., Gross K., Schiestl F.P. (2014) Floral adaptation to local pollinator guilds in a terrestrial orchid. *Annals of Botany*. 113, 289-300.

Thomson J.D., Plowright R.C. (1980) Pollen carryover, nectar rewards, and pollinator behavior with special reference to *Diervilla lonicera*. *Oecologia*. 46, 68-74.

Van Der Niet T., Johnson S.D. (2012) Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology & Evolution*, 27, 353–361.

Van der Niet T., Johnson S.D. (2012) Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology & Evolution*. 27, 353-361.

Van der Niet T., Peakall R., Johnson S.D. (2014) Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Annals of Botany*, 113, 199–211.

van Tussenbroek B.I., Villamil N., Márquez-Guzmán J., Wong R., Monroy-Velázquez L.V., Solis-Weiss V. (2016) Experimental evidence of pollination in marine flowers by invertebrate fauna. *Nature Communication*. 7, 1-6.

Wall M.A., Teem A.P., Boyd R.S. (2002) Floral manipulation by *Lasioglossum zephyrum* (Hymenoptera: Halictidae) ensures first access to floral rewards by initiating premature anthesis of *Xyris tennesseensis* (Xyridaceae) flowers. *Florida Entomologist*. 85, 290–291.

Waser N.M., Chittka L., Price M.V., Williams N.M., Ollerton J. (1996) Generalization in pollination systems, and why it matters. *Ecology*, 77, 1043–1060.

Wessinger C.A., Hileman L.C. & Rausher M.D. (2014) Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*. *Philosophical Transactions of the Royal Society B.* 369, 20130349.

Whittall J.B., Hodges S.A. (2007) Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature*. 447, 706-709.

Wikström N., Savolainen V., Chase M.W. (2001) Evolution of the angiosperms: calibrating the family tree. *Philosophical Transactions of the Royal Society London B, Biological Sciences*. 268, 2211-2220.

Willmer P. (2011) *Pollination and floral ecology*. Princeton: Princeton University Press.

Wilson P., Castellanos M.C., Hogue J.N., Thomson J.D., Armbruster W.S. (2004) A multivariate search for pollination syndromes among penstemons. *Oikos*. 104, 345-361.

Wu C.A., Lowry D.B., Cooley A.M., Wright K.M., Lee Y.W., Willis J.H. (2008) *Mimulus* is an emerging model system for the integration of ecological and genomic studies. *Heredity*. 100, 220-230.

Yuan Y.W., Sagawa J.M., Young R.C., Christensen B.J., Bradshaw H.D. (2013) Genetic

dissection of a major anthocyanin QTL contributing to pollinator-mediated reproductive isolation between sister species of *Mimulus*. *Genetics*. 194, 255-263.

Chapter 1: Breaking-bud pollination: a new pollination process in partially opened flowers by small bees.

[The content of this chapter has been published in Yamaji and Ohsawa (2015).]

Introduction

Some plants and animals interact closely to perform functions such as plant defense, pollination and seed dispersal (Agrawal 2011; Fontaine *et al.* 2006; Willson and Traveset 2000) and mutualistic interactions between plants and pollinators have attracted the attention of naturalists for more than a hundred years (e.g., Darwin 1877). Previous studies suggest that various plant lineages have undergone convergent evolution to produce the same flower characteristics that are adapted to specific pollinators, and these results generated the concept of pollination syndromes (Faegri and van der Pijl 1979; Fenster *et al*. 2004). For example, plants pollinated by butterflies tend to have erect, radiating flowers; long, narrow corolla tubes; and vivid, pure red or pink coloration (Faegri and van der Pijl 1979; Proctor *et al.* 1996). Recently, some studies found genetic backgrounds (Hermann *et al.* 2013; Wright and Bomblies 2013) and quantitative evidence of pollination syndromes (Rosas-Guerrero *et al.* 2014) indicating the presence of convergent relationships between plants and animals. However, other studies have argued that there are mismatches between floral characteristics and effective pollinator types (Ollerton *et al.* 2009; Waser *et al.* 1996), and the reliability of this concept is still debated.

Although plant–pollinator interactions have been studied from many viewpoints, no research has been conducted on flowers before full flowering, or on their

way to opening. Flowers in such opening stages have been studied mainly on cleistogamous plants. About 700 plant species produce cleistogamous flowers, which pollinate by themselves, without opening, for resource conservation or reproductive assurance (Culley and Klooster 2007; Redbo-Torstensson and Berg 1995; Waller 1984). In chasmogamous plants, which have opening flowers, the breeding technique called bud pollination has been used at the bud stage to resolve the self-incompatibility problem (e.g., Nasrallah 1974). A previous study showed that, in natural conditions, a sweat bee *Lasioglossum zephyrum* manipulates the premature flowers of *Xyris tennesseensis* (Xyridaceae) to ensure floral rewards (Wall *et al.* 2002); however, no research has been done to determine whether insect pollination is occurring at these flowering stages (see also Boyd *et al.* 2011). During the flowering season, the flower buds of an individual plant can be in various stages of maturation and opening at any given time. Pollinators can visit the flowers at the 'breaking-bud stage,' when there are small gaps between the petals wide enough for small pollinators to enter. Insects might visit some flowering plants before their opening stages, like *X. tennesseensis*, and such unpredictable visits might also be linked to the pollination of plants.

In this chapter, I carried out pollinator observations, bagging experiments, and counts of pollen grains on *Lycoris sanguinea* Maxim. var. *sanguinea* (Amaryllidaceae) at multiple sites. In other genera of this family, some studies have found interesting examples of plant–pollinator interactions, such as the relationships between style polymorphism and the tongue length of main visitors to flowers (e.g., Arroyo and Dafni 1995). However, the genus *Lycoris*, which includes our target species, has been scarcely studied in terms of the relationships between plants and floral visitors, although the plants of this genus have more diversified characteristics than other well-known flowers

of Amaryllidaceae (Hsu *et al.* 1994; Wang *et al.* 2013). *L. sanguinea* var. *sanguinea* has showy reddish-orange flowers to which various types of insect visit (Kawano 2009), yet details of the activities of these insects as pollinators (e.g., the visitation frequency and the effects on plant reproduction) has not been described. Our results show (1) the list of pollinators of *L. sanguinea* var. *sanguinea*, (2) the frequency of flower visitation by several types of insect, (3) the effects of these pollinators on fruit and seed set in this plant, and (4) the pollen grain numbers of *L. sanguinea* var. *sanguinea* on the body of main floral visitors and on the anthers of randomly-selected and manipulated flowers. I then report for the first time on a new pollination process, breaking-bud pollination, which occurs in the breaking buds of *L. sanguinea* var. *sanguinea*.

Materials and methods

Plant species

The genus *Lycoris* has approximately 20 species, and these plants occur mainly in East Asia, including China, Korea and Japan. Five species of this genus are found in Japan, including *L. albiflora* Koidz, *L. aurea* (L'Herit.) Herb, *L. radiata* (L'Herit.) Herb, *L. sanguinea* Maxim., and *L. squamigera* Maxim (Kawano 2009). Our studied species, *L. sanguinea*, has varieties including *L. sanguinea* var. *sanguinea*, *L. sanguinea* var. *kiushiana* Makino, and *L. sanguinea* var. *koreana* (Nakai) Koyama. *Lycoris sanguinea* var. *sanguinea* grows on deciduous forest floors from central Honshu to Shikoku in Japan, on the Korean Peninsula, and in China (Kawano 2009). As in other *Lycoris*, the vegetative growth and reproductive phases of *L. sanguinea* var. *sanguinea* are seasonally separated. Leaves emerge in late March to April, but die back to the ground by early summer. Two to six flowers borne on a 30- to 50-cm-tall, leafless stalk appear in late July to August. *Lycoris sanguinea* var. *sanguinea* has showy, reddish-orange, funnel-shaped flowers that lack odor, which partly correspond to the features of butterfly-pollinated flowers based on pollination syndrome concepts (Faegri and van der Pijl 1979). The stamens of this flower are shorter than the perianths and the pistil is approximately as long as the perianths. A bud on the stalk finishes opening approximately 5 h after the beginning of the opening of the perianths, and the buds of a given stalk open over a period of approximately 5 days (personal observation). Flowers are visited by various insect species, such as *Amegilla florea* (Hymenoptera: Apidae), *Thymelicus sylvaticus* (Lepidoptera: Hesperiidae), and unidentified small bees. We have limited information on the reproductive ecology of this species, particularly concerning

the breeding system (Kawano 2009).

Observations of pollinators

Our studies were conducted at five sites (Sites 1–5), mainly in Izumi Nature Park, a natural park in Chiba Prefecture, central Japan (Table 1-1). At each study site, *L. sanguinea* var. *sanguinea* grew on the floor of a forest of deciduous trees, such as *Quercus acutissima* Carruth (Fagaceae) and *Quercus serrata* Murray (Fagaceae). Pollinator observations at Site 1 were carried out over the entire flowering season of *L. sanguinea* var. *sanguinea* in 2011 and 2012, whereas the other four sites were studied over part of the 2013 flowering season (Table 1-2). At a given site, I selected several target flowers of *L. sanguinea* var. *sanguinea* (Table 1-2) and logged the species of each insect visitor. I only counted the insects which landed on flowers as floral visitors. These data allowed the visitation frequencies of species to be calculated. I also recorded insect behavior, both by direct observation and by using a GZ-E220 video camera recorder (JVC Kenwood, Japan) to obtain video clips of pollinators visiting the flowers of *L. sanguinea* var. *sanguinea*. In 2011, I carried out night-time observations for a few nights and verified that no nocturnal pollinators visited. Therefore, our observations were conducted primarily between 05:00 and 13:00 h, with the observation time varying depending on weather conditions and pollinator activity. Dates of pollinator observations are given in Table 1-2. At the conclusion of the pollinator observations, I compared visit frequencies of each insect among sites and years with two-way ANOVAs using R version 2.15.2. For the main and following visitors, I also performed post hoc tests with Tukey's Honest Significant Difference (HSD) test. In 2011, I observed that only small bees entered the breaking buds. Therefore, I captured 10 such

bees using a pooter (aspirator) and sent the specimens to Professor Osamu Tadauchi, a research professor at Kyushu University, for identification. Other insects were captured with insect nets and identified by the authors.

Effect of pollinator visitation

To estimate the relative contributions of insect visitors, I conducted bagging experiments for two types of opening stages: partially opened flowers classified as 'breaking buds', and fully opened flowers classified as 'flowering'; both types were of interest because many small bees visited and entered breaking buds through small gaps between the tepals to collect pollen. The anthers and stigma were more closely apposed in breaking buds than at the flowering stage, and the stigma of flowers at the breaking-bud phase was near the point at which the small bees entered the buds. Hence, I hypothesized that these bee species could carry pollen to the stigma of breaking buds.

In 2011 and 2012, I carried out bagging experiments at Site 1 to calculate the fruit set ratios and seed numbers per fruit of each flower pollinated by different insect visitors. I was particularly interested to test whether small bees can pollinate the plants at the breaking-bud stage. The following seven treatments were applied: (1) Control: flowers were freely exposed to insect visitors. (2) Breaking-bud: just after small bees left flowers at the breaking-bud stage, the flowers were emasculated and then enclosed in bags to block subsequent insect visits. (3) Flowering: insect visits during the breaking-bud stage were prevented by bagging then the bags were removed after flower opening. (4) Large-insect exclusion: some plants that did not have opening flowers but did set some buds were covered with a 1-cm mesh wire cage until the end of anthesis. This cage excluded insects, except for small bees (approximately 5-mm body length),

and could evaluate the relative contribution of the bees for fruit and seed production throughout the entire flowering period. (5) Hand-self: buds were enclosed in bags for the whole duration of flowering and the flowers were artificially self-pollinated at the flowering stage. (6) Hand-bud pollination: buds were emasculated 1 or 2 days before anthesis, pollinated with pollen grains from other individuals and then enclosed in bags. (7) Auto-self: buds were enclosed in bags for the duration of flowering to test whether autonomous selfing occurred. Draining bags made of non-woven fabric were used for the bagging treatments. These bags were sufficient to prevent insect flower visitation, including those by the small bees, even though the bees were only 5 mm in size. All treated flowers were tagged, and the fruit-set and seed-set ratios were examined 3–4 weeks after the treatments. All fruit samples were collected to count seed numbers. I also counted the ovule number in the ovary of flowers in Site 1, and this value (10) was utilized for calculating the seed-set ratios. To compare the fruit-set and seed-set ratios between each treatment, I applied Fisher's exact test using R version 2.15.2.

Count of pollen grains on the body of small bees

In 2013 I captured the visiting small bees at Site 1 to examine the amounts of pollen adhering to their bodies. I caught small bees on the flowers as soon as they visited using a pooter (aspirator), they were then killed quickly in the tube with ethyl acetate. I separated the pollen grains from the bodies of the small bees by washing in 70 % ethanol. The collected bees were placed in a 2-mL tube with 200 μL of 70 % ethanol and were washed by vortexing. The insects were washed repeatedly until no pollen grains were visible on the bodies when viewed under the microscope. The number of pollen grains in the washings was estimated using a haemocytometer. Ten pollen counts

were performed per sample. Finally, I classified the counted samples into seven categories: 0, 1–10, 11–50, 51–100, 101–500, 501–1000, 1001–5000, 5001–10000, and > 10001.

Count of pollen grains on anthers

In 2012, I counted pollen grains remaining on the anthers of the flowers to assess the ability of small bees to collect pollen grains. I randomly selected some flowers that were fully opened (‘control’) and some breaking buds that did not reveal whether small bees had visited them (‘breaking-bud’). I also bagged some buds until they had fully opened (‘pollinator rejection’). I then collected the anthers of the selected and bagged flowers. Anthers of bagged flowers were collected just after I had removed the bag. The collected anthers were stored in 1000 μL of 70 % ethanol. After collection, I counted the number of pollen grains using a haemocytometer and performed statistical analysis with one-way ANOVA. When a statistically significant difference was detected, Tukey’s test was used for comparison of the treatments. Results were reported on a per-flower basis.

Results

Observations of pollinators

Total numbers and frequencies of insect visits to the target flowers of *L. sanguinea* var. *sanguinea* are listed in Table 1-3. Total visit frequencies of all insect visitors differed significantly by study site (two-way ANOVA, $df = 4$, $F = 4.6691$, $P < 0.01$) and by year (two-way ANOVA, $df = 1$, $F = 15.1058$, $P < 0.001$). Six insect families visited the target flowers: Apidae, Halictidae, Hesperiidae, Lycaenidae, Papilionidae and Syrphidae (Table 1-3). At all study sites, the small bee *Lasioglossum japonicum* (Hymenoptera: Halictidae) was the most frequent visitor, but the visit frequencies of the bees were different among sites (two-way ANOVA, $df = 4$, $F = 5.473$, $P < 0.001$) and years (two-way ANOVA, $df = 1$, $F = 18.271$, $P < 0.001$). *Amegilla florea* (Hymenoptera: Apidae) and *Episyrphus balteatus* (Diptera: Syrphidae) were the next most frequent visitors. Visitation frequencies of *A. florea* were different among sites ($df = 4$, $F = 4.4522$, $P < 0.01$) and years ($df = 1$, $F = 7.4866$, $P < 0.01$), but those of *E. balteatus* were not (site: $df = 4$, $F = 1.6507$, $P = 0.18$; year: $df = 1$, $F = 0.6202$, $P = 0.44$). Additionally, Tukey's tests detected statistically significant differences in the visit frequencies of both *L. japonicum* and *A. florea* by study site, between Site 1 and 3, and Site 1 and 5 (*L. japonicum*: $P < 0.05$ in Site 1–Site 3, $P < 0.05$ in Site 1–Site 5, respectively; *A. florea*: $P < 0.05$ in Site 1–Site 3, $P < 0.05$ in Site 1–Site 5, respectively), and by year, between 2011 and 2012, and 2011 and 2013 (*L. japonicum*: $P < 0.001$ in 2011–2012, $P < 0.01$ in 2011–2013, respectively; *A. florea*: $P < 0.05$ in 2011–2012, $P < 0.05$ in 2011–2013, respectively).

Lasioglossum japonicum visited breaking buds to collect pollen (Fig. 1-1a–e), and these

visits were observed at every site (Table 1-4: two-way ANOVA, site: $df = 4$, $F = 0.8642$, $P = 0.50$; year: $df = 1$, $F = 10.9034$, $P < 0.01$). At the flowering stage, most of the small bees landed on the indehiscent anthers and collected pollen using their mandibles (Fig. 1-1f), but some subsequently travelled down to the base of the perianth. No other insect species visited breaking buds. *Amegilla florea* would land on a flower, obtain nectar, and then leave immediately for other flowers. This species touched the anthers when collecting nectar, and pollen grains could have become attached to their bodies and be transferred to the same or other flowers. *Episyrphus balteatus* visited flowers to collect pollen grains and did not appear to touch the stigma. *Papilio macilentus* (Lepidoptera: Papilionidae) and *Thymelicus sylvaticus* (Lepidoptera: Hesperiidae), which were anticipated to be the main pollinators based on pollination-syndrome reasoning, rarely visited the experimental flowers. They inserted their proboscis to the bottom of flowers to suck nectar, thus they might have incidentally carried some pollen.

Effect of pollinator visitation

The results of the bagging experiments are given in Table 1-5. The breaking-bud treatment showed that flower visits at the breaking-bud stage by the small bee *L. japonicum* resulted in effective pollination. In 2011, I bagged the breaking buds after *L. japonicum* visits, and thirty percent of the bagged flowers set fruit with seeds despite protection against subsequent insect visits (Table 1-5). In 2012, I observed small bees entering flowers through a wire cage with a 1-cm diameter mesh (large-insect exclusion treatment), and 43 % of these cage-enclosed flowers produced fruit and seeds (Table 1-5). These fruit-set ratios were significantly higher than that of the auto-self treatment, which prevented visitations by all insects (Fisher's exact test, $P < 0.05$ in 2011, $P < 0.05$

in 2012, respectively).

Further, I compared fruit-set and seed-set ratios of each treatment, particularly between the breaking-bud and other treatments. The control treatments had significantly higher fruit-set ratio than the 'breaking-bud' treatment (Fisher's exact test, $P < 0.05$ in 2011 and 2012, respectively), although this was not the case for seed-set ratio (Fisher's exact test, $P = 0.58$ in 2011 and $P = 0.08$ in 2012, respectively). The fruit-set ratio of the breaking-bud treatment was not significantly different from that of the flowering treatments, in which insect visits were permitted only at the flowering stage, in 2011 and 2012 (Fisher's exact test, $P = 0.13$ in 2011, $P = 0.61$ in 2012, respectively). On the other hand, the seed-set ratio of the breaking-bud treatment was significantly lower than that of the flowering treatment in 2012 (Fisher's exact test, $P < 0.05$), but not in 2011 ($P = 0.29$). Additionally, there were no significant differences in the fruit-set and seed-set ratios between the breaking-bud treatment and the large-insect exclusion treatment (Fisher's exact test, $P = 0.32$ in fruit-set ratio, $P = 0.13$ in seed-set ratio, respectively).

The other treatments revealed additional reproductive features of *L. sanguinea* var. *sanguinea*. The results of the hand-self treatment indicated self-compatibility of *L. sanguinea* var. *sanguinea*. A low fruit-set ratio from the auto-self treatment suggested the rarity of automatic self-pollination in *L. sanguinea* var. *sanguinea*. The hand-bud pollination treatment showed that stigmas were receptive even 1 or 2 days before anthesis.

Counts of pollen on the bodies of small bees

I collected samples from 94 *L. japonicum* visiting the flowers. I confirmed that some pollen grains of our target plants were attached to the bodies of all of them (Fig. 1-2).

The highest number of pollen grains on a small bee was 10496.0, and average pollen numbers were 1018.5 (±201.0). Captured bees mainly had pollen on the proximal parts of their legs and abdomen. Under the microscope, I found pollen grains of other species in some samples, but those of *L. sanguinea* var. *sanguinea* were predominant in all samples I did not include any pollen grains of other species in the count.

Counts of pollen grains on anthers

Anthers of 'control', 'breaking-bud', and 'pollinator rejection' treatments were collected from 28, 10, and 8 flowers, respectively. There were significant differences (one-way ANOVA, $df = 2$, $F = 169.37$, $P < 0.001$) among treatments. The very low number of pollen grains of 'breaking-bud', [mean pollen number = 5400.0 (±2304.4)] relative to those of 'pollinator rejection' [mean pollen number = 118,812.5 (±13522.4)] treatments, showed that the small bees collected most of the pollen produced by flowers during the breaking-bud stage (Fig. 1-3). Pollen grain numbers were not significantly different between 'control' and 'breaking bud' treatments [mean pollen numbers = 3732.1 (± 640.5) vs. 5400.0 (± 2304.4), respectively; Tukey's test, $P = 0.96$]. A few pollen grains were shed onto the perianths of some flowers, but these were not included in the analysis.

Discussion

This is the first report of breaking-bud pollination, a pollination process mediated by insect species at the breaking-bud stage. Unlike the case of *Xyris tennesseensis*, which visits premature flowers of *Lasioglossum zephyrum* by removing the floral sheath (Wall *et al.* 2002), *Lasioglossum japonicum* entered the breaking buds of *Lycoris sanguinea* var. *sanguinea* through tiny spaces between tepals with no damage to the flower. In this study, I conducted bagging experiments in a research site in Chiba prefecture. However, our target plant *L. sanguinea* var. *sanguinea* is found from central Honshu to Shikoku in Japan (Kawano 2009), and the only breaking-bud visitor, *L. japonicum*, is widely distributed from Honshu to Yakushima Island (Image Database HANABACHI: Tadauchi *et al.* 2001). This overlap of distribution areas indicates that breaking buds of *L. sanguinea* var. *sanguinea* may be visited and pollinated by *L. japonicum* in other populations, and I in fact observed the visitation of the bees to breaking buds in other study sites (Table 1-3). Furthermore, this visitation method may occur in other plant species, because most flowering plants open gradually and have an opening stage similar to breaking-buds. I perceive that there are at least two essential requirements for breaking-bud pollination: (1) small visitors that can touch the stigma of the breaking buds, and (2) a stigma that is receptive to pollination at the breaking-bud stage. Consequently, flowers of other species (e.g., slowly-flowering protogynic hermaphrodites) may set fruit and seed via a pollination process as observed in this study. Compared to cleistogamy, i.e., autonomous self-pollination within closed flowers (Lord 1981), breaking-bud pollination by small bees is unique because an insect participates in the pollination process, and outcrossing can occur if the insect has

already visited other individual plants. If breaking-bud pollination provided the highest fitness for plants, the opening of flowers would no longer be important. Thus flowers might be able to omit the opening stage, creating a pollination process like cleistogamy, and further promoting the floral adaptation of these plants. Although the fruit-set and seed-set ratios in the breaking-bud treatment did not have significant differences in comparison with the flowering treatment in 2011 or the large-insect exclusion treatment (Table 1-5), and although I cannot conclude whether breaking-bud pollination had a significantly different contribution to the reproduction of *L. sanguinea* var. *sanguinea* than the other pollination methods, the discovery of breaking-bud pollination may lead to novel insights into pollination biology. In particular, most plant scientists observe fully open flowers when studying floral visitors, but this approach may miss important pollination events that occurred at the breaking-bud stage.

Previous research has shown that the first visitor to a flower will probably obtain the largest floral reward (Galen and Stanton 1989; Harder 1990; Harder and Thomson 1989), thus it should be advantageous for small bees to visit breaking buds. Except for *L. japonicum*, I did not observe any other insects visiting breaking buds. Consequently, breaking buds would have a good supply of pollen grains, which are consumed by the larvae of most bee species (Roulston and Cane 2000). In pollinator observations, the visitation frequencies of *L. japonicum* on breaking buds were higher than those on fully-open flowers, except for Site 3 (Table 1-4). In addition, the dominant pollen resource of *L. japonicum* was *L. sanguinea* var. *sanguinea* at the blooming season of this plant species (Fig. 1-2), and most of the pollen was removed at the breaking-bud stage (Fig. 1-3). Therefore, I hypothesize that it is the most profitable strategy for them. In addition, the fruit set ratios of the flowers that were visited only by

small bees were not significantly different between experimental years, while those of the flowers visited by all kinds of visitors were significantly different (Table 1-5). These results suggest that *L. japonicum* is a stable pollinator of *L. sanguinea* var. *sanguinea*, thus the *L. japonicum* pollination strategy might be the best for the plant also.

However, it is not clear that breaking-bud pollination is adaptive for *L. sanguinea* var. *sanguinea*. The fruit-set ratios of the breaking-bud and large-insect exclusion treatments were significantly lower than those of the control treatment in both experimental years (Table 1-5; Fisher's exact test, $P < 0.05$ in 2011, $P < 0.05$ in 2012, respectively), and the fruit-set and seed-set ratios of the flowering treatment were comparable to those of the breaking-bud and large-insect exclusion treatments (Table 1-5). Our results indicate that pollination by insects other than small bees, such as other larger bees and butterflies, at the flowering stage, is also effective. Moreover, pollination by small bees might reduce the genetic diversity of *L. sanguinea* var. *sanguinea*. I observed that *L. japonicum* collected pollen grains into pollen masses at the base of the legs, and this pollen was not used for pollination (Thorp 1979, 2000). Instead, the small bees might promote self-pollination, as they move around in the breaking-bud and some pollen grains could easily be transferred to the stigma of the same flower. Additionally, the involvement of small insects such as *L. japonicum* may lead to shorter pollen-dispersal distances than those of larger insects (Gathmann and Tscharntke 2002; Greenleaf *et al.* 2007; Zurbuchen *et al.* 2010), which could cause increased inbreeding (Kettle *et al.* 2010). In the bagging experiments, I showed that *L. sanguinea* var. *sanguinea* is self-compatible (Table 1-5; see also Ma *et al.* 2000, 2001). However, it is unknown whether the progeny of breaking-bud-pollinated plants suffer from inbreeding depression. Many seeds resulting from self-pollination in *L. sanguinea*

var. *sanguinea* germinated through embryo rescue (Ma *et al.* 2000, 2001), but it is unknown whether these seeds can germinate under natural conditions. Future studies should be undertaken to examine seed qualities (e.g., germination rate) vs. level of selfing by means of various bagging experiments.

In addition to the discovery of breaking-bud pollination, our study shows that *L. japonicum* is the most frequent floral visitor of *L. sanguinea* var. *sanguinea*, whereas the predicted butterfly species rarely visited (Table 1-3). In recent years, several biologists have questioned the predictive power of the pollination syndrome theory (Faegri and van der Pijl 1979; Johnson and Steiner 2000; Ollerton *et al.* 2009; Waser *et al.* 1996). Our results also suggest a mismatch between the predictions of pollination-syndrome theory and the actual pollinator faunas, and this might be the result of floral adaptations to other pollinators. At two sites in Kyushu, I observed insects visiting a different variety of *L. sanguinea*, *L. sanguinea* var. *kiushiana*, which has larger flowers than var. *sanguinea*. I established that *Papilio bianor* and *P. helenus* (Lepidoptera: Papilionidae) were the most frequent visitors, small bees were unfrequent visitors and they did not enter the breaking buds of this plant at all (unpublished data). It can be conjectured that the flower of *L. sanguinea* var. *kiushiana* is adapted to butterfly pollinators, whereas *L. sanguinea* var. *sanguinea* has floral features altered by selection by smaller insects, such as *L. japonicum*.

References

Agrawal A.A. (2011) Current trends in the evolutionary ecology of plant defence. *Functional Ecology*. 25, 420–432.

Arroyo J., Dafni A. (1995) Variations in habitat, season, flower traits and pollinators in dimorphic *Narcissus tazetta* L. (Amaryllidaceae) in Israel. *New Phytologist*. 129, 135–145.

Boyd R.S., Teem A., Wall M.A. (2011) Floral biology of an Alabama population of the federally endangered plant, *Xyris tennesseensis* Kral (Xyridaceae). *Castanea.* 76, 255–265

Culley T.M., Klooster M.R. (2007) The cleistogamous breeding system: a review of its frequency, evolution, and ecology in angiosperms. The Botanical Reviews. 73, 1–30.

Darwin C. (1877) *The different forms of flowers on plants of the same species*. John Murray, London

Faegri K., van der Pijl L. (1979) *The principles of pollination ecology*. Pergamon Press, Oxford

Fenster C.B., Armbruster W.S., Wilson P., Dudash M.R., Thomson J.D. (2004) Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution and Systematics*. 35, 375–403.

Fontaine C., Dajoz I., Meriguet J., Loreau M. (2006) Functional diversity of plant-pollinator interaction webs enhances the persistence of plant communities. *PLoS Biology*. 4, 129–135.

Galen C., Stanton M.L. (1989) Bumble bee pollination and floral morphology: factors influencing pollen dispersal in the alpine sky pilot, *Polemonium viscosum*

(Polemoniaceae). *American Journal of Botany*. 76, 419–426.

Gathmann A., Tscharntke T. (2002) Foraging ranges of solitary bees. *Journal of Animal Ecology*. 71, 757–764.

Greenleaf S.S., Williams N.M., Winfree R., Kremen C. (2007) Bee foraging ranges and their relationship to body size. *Oecologia*. 153, 589–596.

Harder L.D. (1990) Pollen removal by bumble bees and its implications for pollen dispersal. *Ecology*. 71, 1110–1125.

Harder L.D., Thomson J.D. (1989) Evolutionary options for maximizing pollen dispersal of animal-pollinated plants. *American Naturalist*. 133, 323–344.

Hermann K., Klahre U., Moser M., Sheehan H., Mandel T., Kuhlemeier C. (2013) Tight genetic linkage of prezygotic barrier loci creates a multifunctional speciation island in petunia. *Current Biology*. 23, 873–877.

Hsu P.S., Kurita S., Yu Z.Z., Lin J.Z. (1994) Synopsis of the genus *Lycoris* (Amaryllidaceae). *SIDA* 16, 301–331

Johnson S.D., Steiner K.E. (2000) Generalization vs. specialization in plant pollination systems. *Trends in Ecology & Evolution*. 15, 140–143.

Kawano S. (2009) Life-history monographs of Japanese plants. 13: *Lycoris sanguinea* Maxim. (Amaryllidaceae). Plant Species Biology. 24, 139–144.

Kettle C.J., Maycock C.R., Ghazoul J., Hollingsworth P.M., Khoo E., Sukri R.S.H., Burslem D.F.R.P. (2010) Ecological implications of a flower size/number trade-off in tropical forest trees. *PLoS One*. 6, e16111.

Lord E.M. (1981) Cleistogamy: a tool for the study of floral morphogenesis, function and evolution. The Botanical Review. 47, 421–449.

Ma B., Tarumoto I., Morikawa T. (2000) Cytological studies on selfed plants and

interspecific crosses produced in four species of genus *Lycoris* (Amaryllidaceae). *Scientific Report of the Colleage of Agriculture, Osaka Prefecture University*. 52, 13–18.

Ma B., Tarumoto I., Nakamura N., Kunitake H. (2001) Production of interspecific hybrids between *Lycoris incarnata* and four other *Lycoris* species through embryo culture. *Journal of the Japan Society for Horticultural Science*. 70, 697–703

Nasrallah M.E. (1974) Genetic control of quantitative variation in selfincompatibility proteins detected by immunodiffusion. *Genetics*. 76, 45–50

Ollerton J., Alarcon R., Waser N.W., Price M.V., Watts S., Cranmer L., Hingston A., Peter C.I., Rottenberry J. (2009) A global test of the pollination syndrome hypothesis. *Annals of Botany*. 103, 1471–1480.

Proctor M., Yeo P., Lack A. (1996) *The natural history of pollination*. Harper Collins, London

Redbo-Torstensson P., Berg H. (1995) Seasonal cleistogamy: a conditional strategy to provide reproductive assurance. *Plant Biology*. 44, 247–256.

Rosas-Guerrero V., Aguilar R., Marten-Rodriguez S., Ashworth L., Lopezaraiza-Mikel M., Bastida J.M., Quesada M. (2014) A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecology Letters*. 17, 388–400.

Roulston T.H., Cane J.H. (2000) Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution*. 222, 187–209.

Tadauchi O., Dawut A., Inoue H. (2001) On image database file HANABACHI based on the Japanese bees. *ESAKIA*. 41, 149–154.

Thorp R.W. (1979) Structural, behavioral, and physiological adaptations of bees (Apoidea) for collecting pollen. *Annals of Missouri Botanical Garden*. 66:788–812.

Thorp R.W. (2000) The collection of pollen by bees. *Plant Systematics and Evolution*. 222, 211–223.

Wall M.A., Teem A.P., Boyd R.S. (2002) Floral manipulation by *Lasioglossum zephyrum* (Hymenoptera: Halictidae) ensures first access to floral rewards by initiating premature anthesis of *Xyris tennesseensis* (Xyridaceae) flowers. *Florida Entomologist*. 85, 290–291.

Waller D.M. (1984) Differences in fitness between seedlings derived from cleistogamous and chasmogamous flowers in *Impatiens capensis*. *Evolution*. 38, 427–440.

Wang R., Xu S., Jiang Y., Jiang J., Li X., Liang L., He J., Peng F., Xia B. (2013) *De novo* sequence assembly and characterization of *Lycoris aurea* transcriptome using GS FLX Titanium platform of 454 Pyrosequencing. *PLoS One.* 8, e60449.

Waser N.M., Chittka L., Price M.V., Williams N.M., Ollerton J. (1996) Generalization in pollination systems, and why it matters. *Ecology*. 77, 1043–1060

Willson M.F., Traveset A. (2000) The ecology of seed dispersal. In: Fenner M (ed) *Seeds: the ecology of regeneration in plant communities*. CAB Int, Wallingford, 85–110.

Wright K.M., Bomblies K. (2013) Evolutionary genetics: inheritance of a complex pollination syndrome. *Current Biology.* 23, R525–R527.

Yamaji F., Ohsawa A.T. (2015) Breaking-bud pollination: a new pollination process in partially opened flowers by small bees. *Journal of plant research*, **128**, 803–811.

Zurbuchen A., Landert L., Klaiber J., Muller A., Hein S., Dorn S. (2010) Maximum foraging ranges in solitary bees: only few individuals have the capability to cover long distances. *Biological Conservation.* 143, 669–676.

Table 1-1. Locations of the five study sites

	Site name	Latitude	Longitude
Site 1	Izumi Nature Park, Noro-cho, Chiba Pref.	35°34′38″N	140°13′50″E
Site 2	Sonnou no Mori, Inage-ku, Chiba Pref	35°38′49″N	140°06′52″E
Site 3	Sugawara Shrine, Kamiozuki, Kanagawa Pref	35°21′50″N	139°14′23″E
Site 4	Mannyou Nature Park, Iwafune-cho, Tochigi Pref	36°19′26″N	139°37′53″E
Site 5	Kogushi Katakuri no Sato, Yoshii-machi, Gunma Pref	36°15′12″N	139°00′56″E

Table 1-2. Target flower number (sample size, n), and experimental dates and times in pollinator observations

	Year	Flower n			Total time of observations	Dates of pollinator observations
		Breaking bud	Flowering	Total		
Site 1	2011	26	125	151	20 h	3–6, 8, 10, 19, 24, 25 August
	2012	9	66	75	144 h	1–24 August
Site 2	2013	2	21	23	24 h	3–6 August
Site 3	2013	3	19	22	30 h	30, 31 July; 1 August
Site 4	2013	1	13	14	24 h	8–10 August
Site 5	2013	1	20	21	18 h	11–13 August

Table 1-3. Pollinator visit numbers by insect species

Family	Species	Site 1		Site 2	Site 3	Site 4	Site 5
		2011	2012				
Halictidae	*Lasioglossum japonicum*	773 (1.89)	6348 (15.26)	380 (4.24)	196 (0.95)	236 (2.46)	52 (0.68)
Apidae	*Amegilla florea*	5 (0.04)	255 (0.8)	89 (0.64)	11 (0.18)	6 (0.19)	34 (0.36)
Apidae	*Apis mellifera*	-	-	-	-	2 (0.13)	-
Syrphidae	*Episyrphus balteatus*	-	24 (0.17)	1 (0.04)	6 (0.1)	13 (0.21)	-
Syrphidae	*Baccha maculata*	-	-	2 (0.08)	1 (0.02)	-	2 (0.17)
Syrphidae	sp.	9 (0.21)	18 (0.13)	-	-	-	-
Papilionidae	*Papilio macilentus*	-	2 (0.25)	-	-	-	-
Hesperiidae	*Thymelicus sylvaticus sylvaticus*	3 (0.09)	6 (0.38)	-	-	-	-
Lycaenidae	*Pseudozizeeria maha*	-	-	-	1 (0.1)	-	-
Lycaenidae	sp.	-	-	2 (0.06)	-	-	-
Megachilidae	sp.	-	-	6 (0.18)	-	-	1 (0.17)

The figures in parentheses show frequencies in visits h−1. Dashes indicate no visitation

Table 1-4. Pollinator visit numbers by *Lasioglossum japonicum* at the breaking-bud and flowering stages

	Site 1		Site 2	Site 3	Site 4	Site 5
	2011	2012				
Breaking bud	442 (2.7)	1067 (33.82)	39 (8.25)	15 (0.68)	33 (4.71)	1 (1.0)
Flowering	331 (1.17)	5281 (12.73)	341 (3.71)	181 (0.99)	203 (2.27)	51 (0.65)
Total	773 (1.89)	6348 (15.26)	380 (4.24)	196 (0.95)	236 (2.46)	52 (0.68)

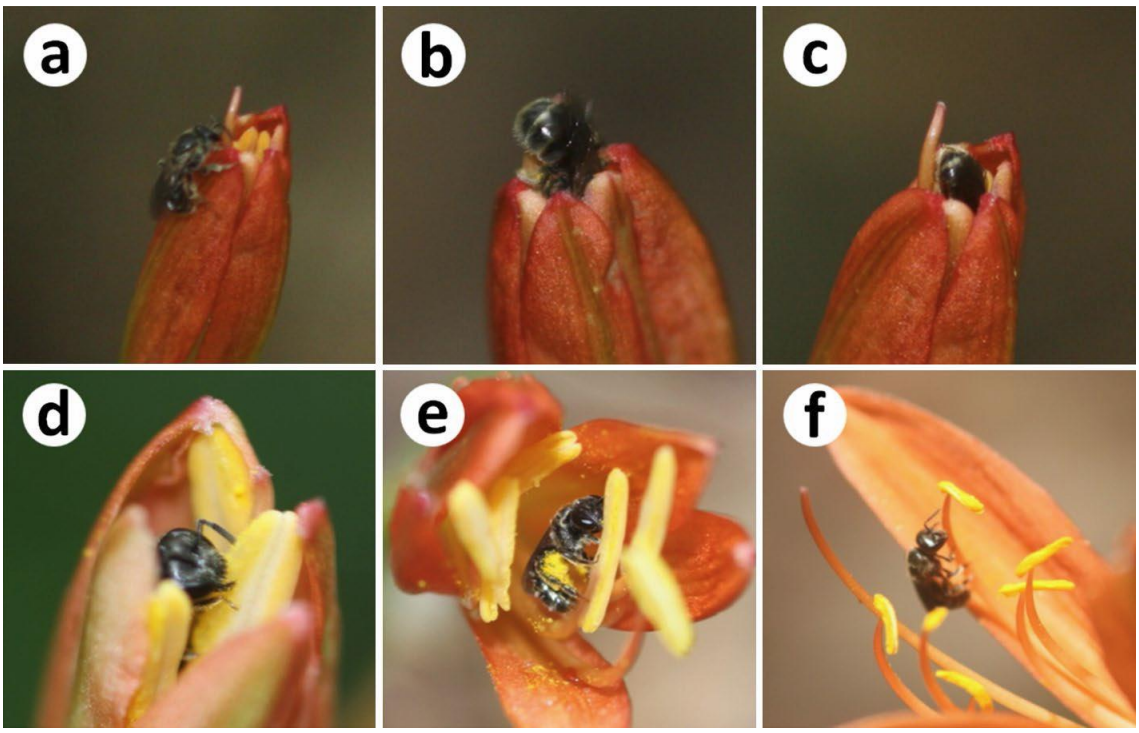
The figures in parentheses show frequencies in visits h^{-1}

Table 1-5. Results of bagging experiments at Site 1

Treatment type	2011			2012		
	n	FS	SS	n	FS	SS
Control	81	56.8*	17.0	124	57.3*	23.9
Breaking bud	20	30.0	13.3	-	-	-
Flowering	21	9.5	25.0	94	38.3	26.9*
Large-insect exclusion	-	-	-	86	43.0	23.0
Auto-self	38	5.3*	45.0	-	-	-
Hand-self	-	-	-	47	63.8	17.3
Hand-bud pollination	-	-	-	19	63.2	20.0

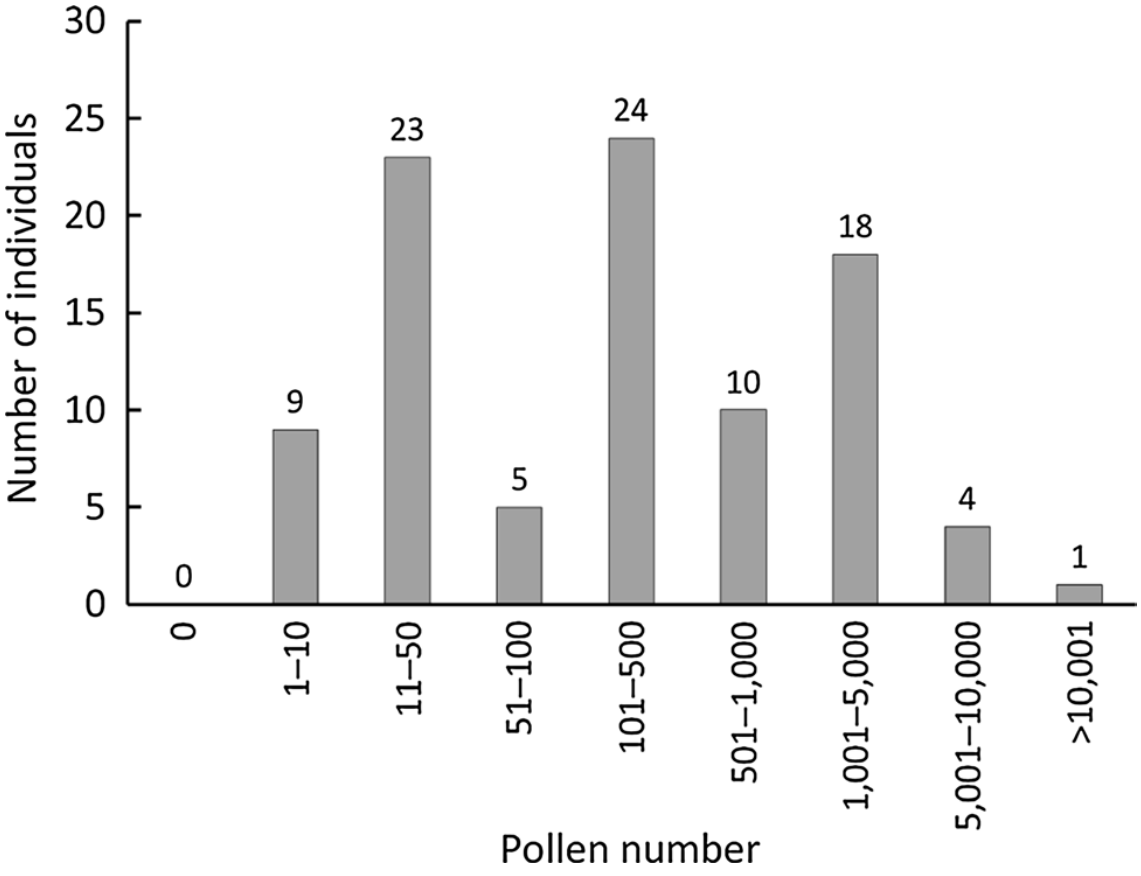
The values marked with an asterisk indicate a significant difference ($P < 0.05$) compared to the breaking-bud treatment. Dashes indicate no data. *n* flower number for each experiment, *FS* fruit-set ratio (percentage), *SS* seed-set ratio (percentage)

Figure 1-1. The process of breaking-bud pollination.



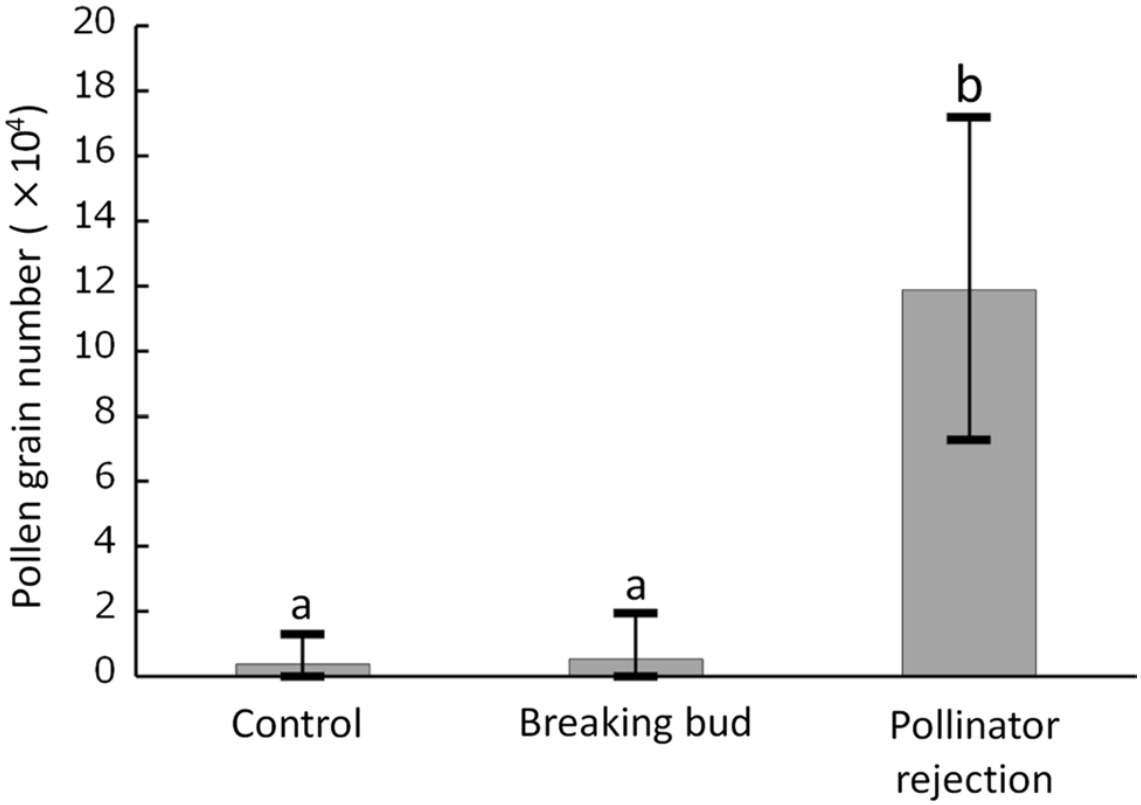
A small bee visits a breaking bud (**a**) and enters (**b**, **c**). The bee uses its mandibles to open the anthers and collect pollen (**d**, **e**). Small bees have difficulty carrying pollen to the stigmas of fully opened flowers because the anthers and stigma are too far apart (**f**)

Figure 1-2. Histogram of pollen grain numbers on the bodies of small bees.



Individual numbers of each category are shown on the *bars*

Figure 1-3. Pollen grain numbers remaining on anthers after three types of treatment.



Grey bars show the mean grain numbers per flower after each treatment. Limits are the maximum and minimum values of pollen grain number. 'Pollinator rejection', all pollinators excluded; 'Control', no pollinators excluded; 'Breaking bud', anthers harvested just after small bees left the flowers at the breaking-bud stage. *Different letters* indicate that pollen grain numbers were significantly different by Tukey's test ($P < 0.001$)

Chapter 2: Relationships between pollinator fauna and floral morphology in *Lycoris sanguinea*: effects of a novel pollination mechanism.

Introduction

Studies of plant-pollinator interactions have shown strong evidence of the ecological and evolutionary connections between plants and pollinators (Kay & Sargent 2009; Johnson 2010; Willmer 2011). Approximately 87.5% of flowering plants are dependent on pollinators for their reproductive success (Ollerton *et al*. 2011). Pollinators select floral traits, and floral diversification has been strongly promoted by relationship changes between flowers and pollinator fauna (Fenster *et al*. 2004; Van der Niet & Johnson 2012), although other environmental factors are also important (Herrera et al. 2006; Perez-Balares *et al*. 2007; Anderson & Johnson 2008; Cosacov *et al*. 2013).

In generalist flowers, the strength and direction of pollinator-mediated selection vary between populations because the type and number of pollinator species differ between these populations (e.g., Waser *et al.* 1996). The geographical mosaics of pollinator fauna can exert different selective forces on individual plants and may lead to different pollinator ecotypes in intraspecific plant populations (Johnson 2010; Van der Niet *et al*. 2014). Pollinator-selected floral traits possess two different functions (Gómez & Zamora 2006). Attractant traits attract pollinators to flowers by floral scent or colour (e.g., Byers *et al.* 2014; Schestl 2015), while mechanical traits select pollinators by structural fit (e.g., Kay 2006; Zhang & Li 2014; Paudel *et al.* 2016). Divergent selection caused by different frequencies of the same pollinators promotes variance only of mechanical traits (e.g., Newman *et al*. 2015). In generalist plants, the relationships

between pollinator fauna and floral morphology have been investigated by several groups (e.g., Cooley *et al*. 2008; Gomez *et al*. 2014; Medel *et al*. 2007); however, few studies regarding mechanical adaptations within the geographic mosaic of pollinators have been conducted.

The genus *Lycoris* (Amaryllidaceae), which comprises approximately 20 species, has been introduced as ornamental and medicinal plants in eastern Asia (Hsu *et al.* 1994; Chang *et al.* 2009). Compared to those of other genera in the Amaryllidaceae family, such as *Narcissus*, the plant-pollinator interactions of *Lycoris* have received relatively little study (e.g., Marques *et al.* 2016; Simón-Porcar *et al.* 2013; Pérez-Barrales *et al.* 2007). *Lycoris sanguinea* Maxim., which have been previously studied about the pollinator relationships (Yamaji & Ohsawa 2015, 2016), is a perennial bulb in Japan. This plant exhibits floral size variations and is divided into three described varieties: *L. sanguinea* var. *sanguinea*, *L. sanguinea* var. *kiushiana* Makino, and *L. sanguinea* var. *koreana* (Nakai) Koyama (Hsu *et al.* 1994; Kurita 1988). The flowers of these varieties attract several taxonomic groups of insects (Chung *et al.* 1999; Kawano 2009; Yamaji & Ohsawa 2015). In *L. sanguinea* var. *sanguinea*, the most frequent visitors are small bees of the species *Lasioglossum japonicum* (Yamaji & Ohsawa 2015), although its floral characteristics have been thought to attract butterfly species (Faegri & van der Pijl 1979; Johnson & Steiner 2000). In contrast, my preliminary research revealed that large butterfly (*Papilio*) species frequently visited *L. sanguinea* var. *kiushiana*, which has the largest flowers of the three varieties. Based on these observations, I predicted that the differences of flower morphology in *L. sanguinea* were strongly influenced by pollinators. However, no comprehensive research on the floral morphology of *L. sanguinea* and its pollinators has been

conducted.

Furthermore, I recently found a novel pollination mechanism in *Lycoris sanguinea* var. *sanguinea* (Yamaji & Ohsawa 2015). *Lasioglossum japonicum* visited and collected the immature pollen of partially opened flowers (breaking buds) using their mandibles, and they touched the stigma while foraging and collecting pollen. This mechanism, which I named breaking-bud pollination, has been observed in all five populations across a limited region of the distribution area (Yamaji & Ohsawa 2015). In my observations, the bees did not actively pollinate in breaking buds but instead accidentally attached pollen to the stigma. My previous study suggested that breaking-bud pollination increased fruit and seed set compared to that of pollination only at the fully open stage (Yamaji & Ohsawa 2015). Additionally, *L. sanguinea* var. *sanguinea* is self-compatible, and the stigma of this plant is receptive at the breaking-bud stage (Yamaji & Ohsawa 2015). Therefore, the main condition for breaking-bud pollination is morphological, dependent on whether small bees can touch the stigma. I hypothesized that populations pollinated at the breaking-bud stage could adapt to small bees and change their floral morphology compared to that of populations without breaking-bud pollination.

The goal of this study was to investigate the relationships between the floral morphology of *L. sanguinea* and its pollinators. I hypothesized that different pollinator fauna in intraspecific populations of *L. sanguinea* promote the morphological variation between them. I specifically focused on the interaction of breaking-bud pollination with floral morphology. However, my previous study did not clarify whether breaking-bud pollination occurs widely in *L. sanguinea*. Therefore, I conducted pollinator observations and morphological measurements of floral traits in multiple populations,

followed by statistical analysis to compare these values between populations and test significant correlations between floral traits and pollinator frequencies.

Materials and methods

Study species and sites

Lycoris sanguinea, a perennial bulb in the Amaryllidaceae family, is mainly found growing on deciduous forest floors in Japan. Its flowering season occurs from mid-July to August. Each individual has two to six flowers, which are reddish-orange and funnel-shaped without a floral scent (Kawano 2009). Three varieties of *L. sanguinea* have been described based on taxonomic and ecological characteristics: *L. sanguinea.* var. *sanguinea*, *L. sanguinea.* var. *kiushiana*, and *L. sanguinea.* var. *koreana* (Kurita 1988). *L. sanguinea* var. *sanguinea* is distributed mainly in central Honshu. The stamens of this variety are shorter than its petals. *L. sanguinea* var. *kiushiana* is distributed mainly from Shikoku to Kyusyu, and its stamens are exserted beyond its corollas. *L. sanguinea* var. *koreana* is narrowly distributed in Tsushima Island and southern Korea. This variety can be distinguished by its flower size, smaller than that of *L. sanguinea* var. *kiushiana*, and by its stamens, longer than those of *L. sanguinea* var. *sanguinea*. Previous genetic analyses using allozyme loci have suggested that *L. sanguinea* var. *koreana* has a limited dispersal range (Chung *et al*. 1999).

I examined 13 populations in this study (Figure 2-1; Table 2-1). These sites covered the distribution regions of the three varieties from central Honshu to Kyusyu and Tsushima Island. Most of the populations were located in humid subtropical climates, except for population 11, which was located in a humid continental region. Populations 3 and 4 were located in higher altitudes, while the other 11 study sites were in lowlands. In each population, I identified flowering individuals by *L. sanguinea* variety according to floral morphology, flowering period, and location, based on the

work of Hsu *et al*. (1994). At the Hiroshima site (populations 7 and 8), I identified two varieties, *L. sanguinea* var. *sanguinea* and *L. sanguinea* var. *kiushiana*, based on morphological traits. Therefore, I added these two populations to my study. Each population covered approximately 1 km and grew under similar conditions. However, the population of *L. sanguinea* var. *kiushiana* occurred in a slightly darker area than that of *L. sanguinea* var. *sanguinea*.

Floral morphology measurements

In 2015, I randomly selected 30 *L. sanguinea* individuals in each population for floral morphology measurements. I measured only one freshly opened flower per individual to avoid the effects of floral degradation or herbivore damage. After selection, eight floral traits were measured using a digital calliper: stamen length (STL), pistil length (PIL), anther-stigma length (ASL), petal length (PEL), petal width (PEW), petal-petal length (PPL), pedicel length (PED), and corolla tube length (CTL) (Figure 2-2A). PPL represents the average distance of petal tips between diagonally-located petals, and ASL indicates the average distance between the tips of the stamens and pistil. PPL and ASL were measured in situ, and the other six traits were measured using collected samples preserved in 70% ethanol.

Observations of floral visitors

To investigate the pollinator assemblages of each population, I observed opened flowers and breaking buds. I first searched for and selected freshly opened or breaking buds as target flowers at each site, then set digital video cameras (GZ-E220 and GZ-E265, JVC Kenwood, Japan) with tripods in front of the selected flowers. I recorded floral visitors

for an average of 8 hours a day. I then checked the videos and categorized the floral visitors into functional groups based on the work of Fenster *et al.* (2004). Furthermore, I identified "potential pollinators", which were defined as floral visitors that contacted the stigma with conspecific pollen. To estimate the contact frequencies of the visitors (touches per observation time of each functional group), I carefully checked the pistil movements. I established the following criteria for potential pollinators: the figures of the visitors and stigma in the videos overlapped, and the pistil moved after the visitors left in windless conditions. I identified potential pollinators in accordance with these criteria and recorded their pollination events. I conducted the above pollinator research for 11 populations in 2015; research was conducted in populations 3 and 4 in 2013.

Visitor pollen counts

To reveal the potential pollinators in visitor assemblages, I observed the body surfaces of floral visitors and checked the attachment of *L. sanguinea* pollen grains. At each site, I caught floral visitors as soon they visited the flower using insect nets or a pooter (aspirator). Small and large bees were killed using ethyl acetate, and large butterflies were quickly killed by applying finger pressure to the thorax. These specimens were preserved in 100% ethanol or soft plastic bags, and their body surfaces were microscopically observed in my laboratory. The positions of pollen grains on the insect body surfaces were recorded in for five partitions (head, thorax, abdomen, legs, and wings). I also counted the attached *L. sanguinea* pollen numbers on each partition.

Data collection for abiotic factors

To investigate the influence of environmental factors, I extracted 19 bioclimatic factors,

mean temperature, and precipitation for the flowering season of *L. sanguinea* (i.e., Jul.–Aug.) These data were extracted from the WorldClim database (Hijmans *et al.* 2005) for the longitude and latitude coordinates of each population. These environmental data were interpolations of observed data from 1950 to 2000 at a spatial resolution of 5 km^2.

Statistical analyses

Comparisons of floral morphology between populations

Each floral trait was statistically compared between populations using generalized linear models (GLMs) with gamma errors (identity link), and these models were compared to null hypotheses for likelihood ratio tests. To determine which populations were statistically different, I also conducted a post-hoc test, Tukey's honestly significant differences (TukeyHSD) test, using the glht function in the multcomp library of the R package (Hothorn *et al*. 2008).

I divided the populations based on the measured morphological trait data using hierarchical clustering methods. The mean morphological data of the eight traits in each population were converted to Bray-Curtis similarities using the vegdist function in the vegan package, and the populations were clustered based on the converted scores obtained by the unweighted pair group method with arithmetic mean (UPGMA) in the hclust function. I estimated the actual number of clusters based on Beale's index using the NbClust function (Charrad *et al*. 2014). Furthermore, I performed an analysis of similarity (ANOSIM) for the statistical test of clustering results. The contributions of each floral trait to the clusters were also statistically tested by similarity percentage (SIMPER). Both statistical analyses were performed in the vegan R package.

Comparisons of floral visitors and potential pollinators between populations

I calculated the visitation frequencies of each visitor and functional group as the total number of visitors per observation time. I also measured the pollination frequencies of each functional group. This value was calculated as number of insects with stigma contact per flower per hour in potential pollinators with conspecific pollen.

Using these frequency values, I performed clustering analyses to compare the 13 populations. I used the mean pollination frequencies of each floral visitor for UPGMA clustering in hclust and determined the best number of clusters in NbClust using Beale's index. I also performed ANOSIM to investigate the significance of these clustering results and SIMPER to select the factors with the greatest contribution to clustering. I selected visitors and pollinators using the criterion of cumulative contributions in SIMPER until 80%. These representative groups were used in the following statistical analyses.

Relationships between floral morphologies and pollinators

To study the relationships between floral morphologies and pollinator species, I selected representative parts of floral morphologies and pollinator functional groups based on the SIMPER analyses. I also used generalized linear mixed models (GLMMs) using glmer functions in R package lme4. Each value of floral morphologies was used as response values and pollination frequencies of each pollinator group were as explanatory values. Population sites were also used as random effects for GLMMs. Furthermore, I estimated significant correlated factors for each floral morphology by multiple comparisons with Holm corrections in glht function of the R package multcomp.

Relationships between floral morphology and biotic/abiotic factors

I first translated each value of the morphological and environmental factors into principal component analysis (PCA) axis scores because many of the factors in each variable were correlated. Twenty-one environmental variables were standardized prior to PCA, and eight morphological variables were used with no standardization. To investigate the relationships between morphological axis scores and other factors (pollination frequencies, environmental axis scores), GLMs with Gaussian errors (identity link) were performed. Models were prepared for each morphological data axis score as a response variable and the pollination frequencies of representative visitors and environmental axis scores as explanatory variables. Statistical tests were also performed by multiple comparison tests with Holm corrections using the glht function of the package multcomp.

Test of breaking-bud-pollination effects on floral morphology

I tested two hypotheses about the effect of breaking-bud pollination on floral morphology. Under the stigma-anther hypothesis (SA hypothesis), breaking-bud pollination occurs due to the timing of pollen collection by small bees, and this process is encouraged by decreasing the anther-pistil gaps at the breaking-bud stage. Under the stigma-petal hypothesis (SP hypothesis), breaking-bud pollination does not happen under the conditions of the SA hypothesis but instead occurs at the entrances of breaking buds. Pollination is thus promoted by placement of the stigma closer to the petal tips (Figure 2-3).

To test these hypotheses, I performed generalized linear mixed models

(GLMMs) using the glmer function in the package lme4. I first calculated the distances between the stigma and anthers and between the stigma and petal tips by calculating PIL – STL and PIL – PEL, respectively. These values were used as response variables for the SA and SP hypotheses. The pollination numbers of representative functional groups and of small bees for breaking-buds were used as fixed effects, and each population was a random effect. For the SA and SP hypotheses, GLMMs with gamma errors (log links) were performed. GLMMs were also performed with the offset variable of log observation hours per day. I investigated the influences of each variable by multiple comparison tests with Holm corrections using the glht function.

Results

Comparisons of floral morphology between populations

Of my study populations, six were identified as *Lycoris sanguinea* var. *sanguinea*, five as *L. sanguinea* var. *kiushiana*, and two as *L. sanguinea* var. *koreana* (Table 2-1). The eight floral morphology traits significantly differed between populations (GLMs with gamma errors: $P < 0.001$), and statistically different populations determined using TukeyHSD are shown in Figure 2-2 as different symbols. In the Hiroshima prefecture, two sympatric populations (populations 7 and 8) significantly differed in five traits (Figure 2-2), indicating the weak relationship between floral morphology and geography.

Clustering analyses divided the 13 study populations into three groups (Figure 2-4). These groups were not consistent with the varieties based on my identifications. Group1 consisted of four populations with larger flowers than those of the other groups. Group2 included six populations with intermediate flower size. Populations 9, 10, and 13 belonged to Group3, which had the smallest flowers in the study population. ANOSIM showed the clustering analysis to be statistically significant (ANOSIM: $P < 0.001$), and SIMPER showed that four traits contributed to clustering with a percent cumulative contribution (STL, PEL, PPL, and PIL).

Observations and comparisons of floral visitors and potential pollinators

In total, I recorded 1854 floral visitors across all populations and categorized them into six functional groups: 938 small bees, 358 large bees, 35 small butterflies, 283 large butterflies, 229 hoverflies, and 11 hawkmoths (Figure 2-5). The pollination

frequencies of eastern populations tended to be higher for small and large bees, and those of western populations were higher for large butterflies (Figure 2-6). In population 5, I could not identify contact with the stigma by any floral visitor, and I removed it from the following analyses of pollination frequencies.

Based on the visitation frequencies, the 13 populations were divided into two groups (Figure 2-7A; ANOSIM: P = 0.003). The similarity percentage (SIMPER) results revealed that small bees and large butterflies represented 61.3% of cumulative contributions. Cluster analysis using pollination frequencies yielded statistically similar results (ANOSIM: P = 0.005) to those of analysis based on visitation frequencies (Figure 2-7B). Only populations 7 and 11 belonged to different clusters in the results of the two analyses. No contact by smaller insects was observed in population 7, and population 11 had the lowest visitation and pollination frequencies among the study sites, which may have caused the differences in clustering. SIMPER analysis showed high contributions of large butterflies and large bees (66.7% of cumulative contributions). By SIMPER analysis, three functional groups with an 87.9% cumulative contribution were selected as representative visitor groups (small bees, large bees, and large butterflies).

Visitor pollen counts

In total, I captured 119 floral visitors (Table S2-1). Most visitors had pollen of *L. sanguinea* varieties, suggesting that they could be pollinators if they touched the stigma. Small bees had pollen grains on their whole bodies, but they tended to have larger pollen amounts on their abdomens and legs. Large bees also had large amounts of pollen on their abdomens and legs, but few individuals had pollen on their thoraxes. All

captured large butterflies had pollen grains on their legs and wings. Although I did not observe visitations of small bees in populations 3 and 11 (Figure 2-3), I observed their visitation to flowers and confirmed that captured small bees in the former population also had *L. sanguinea* pollen.

Relationships between floral morphologies and pollinators

In SIMPER analyses, four and three factors were selected in floral morphologies and pollinator groups respectively; STL, PEL, PPL and PIL in floral morphologies, small bees, large bees and large butterflies. All four parts of floral traits had significant relationships to small and large bees (Table 2-2; multiple comparisons with Holm correction, $P<0.01$).

Relationships between floral morphology and biotic/abiotic factors

The results are shown in Table 2-3. GLMs with Gaussian errors showed that the scores of PC1 based on morphological data were significantly related with the contact frequency of small bees (GLM with Gaussian error: t value = 3.681, P = 0.008; multiple comparison tests with Holm adjustments: $P < 0.01$). Two abiotic factors were not significantly related with the PC1 score (GLM with Gaussian error of abiotic factor 1: t value = -0.247, P = 0.812; GLM with Gaussian error of abiotic factor 2; t value = -1.275, P = 0.243). The PC2 score was not supported significantly by any explanatory variables (multiple comparison tests with Holm adjustments: P = 1).

Test of breaking-bud-pollination effects for floral morphologies

The results of the hypothesis testing are shown in Table 2-4. The estimated pollination

frequencies of breaking-bud pollination were significantly related to the stigma-anther length with other three explanatory variables according to the GLMM with gamma error (GLMM with gamma error: t value = 2.254, P = 0.024; multiple comparison tests with Holm adjustments: P = 0.048). Conversely, stigma-petal length showed a weak but nonsignificant relationship with breaking-bud-pollination frequency (GLMM with gamma error: t value = 2.232, P = 0.026; multiple comparison tests of stigma-petal length: P = 0.051).

Discussion

In this study, I compared floral morphology and pollinator faunas between populations in the genus *Lycoris*. Clustering analyses by floral morphology divided the 13 study populations of *L. sanguinea* into 3 groups. These clusters were better described by pollinators groups than by abiotic factors. Although I could not identify potential pollinators in population 5, the results suggested that the floral morphology of *L. sanguinea* was strongly influenced by pollinator fauna. More importantly, I observed the presence of breaking-bud pollination in three populations that were morphologically clustered together. My analysis showed a significant relationship between anther-stigma separation and breaking-bud-pollination frequency. Although the pollination frequencies of small and large bees were also significantly related to floral morphology, the result of the generalized linear models suggested that breaking-bud pollination was related to these traits, especially to gap length between the tips of the male and female reproductive organs.

In my previous study, small bees collected approximately 95% of pollen grains produced by *L. sanguinea* var. *sanguinea* at the breaking-bud stage (Yamaji & Ohsawa 2015). This excessive pollen collection would decrease the available pollen for reproduction, causing pollen limitation (Ashman *et al.* 2004). This condition could promote floral adaptation against small bees and cause variation in floral morphology. The cluster analysis based on floral morphology grouped the populations with breaking-bud pollination, suggesting relationships between the measured traits and the pollination system. However, my data showed a positive relationship between the pollination frequency of small bees and the anther-stigma distance at the breaking-bud

stage (Table 2-4). This result is unusual because the separation of the anthers and stigma should decrease to allow small bees to contact the stigma more easily. To reduce the ratio of self-pollination, some plants have separated their reproductive organs in time or space (e.g., Lloyd & Webb 1986; Webb & Lloyd 1986). My data may show a similar pattern for preventing self-pollination, although the varieties of *L. sanguinea* are self-compatible (Table S2-2; Yamaji & Ohsawa 2015). Therefore, my results may suggest that seedlings or growing plants produced by self-pollination have lower fitness.

Although the floral morphology of *L. sanguinea* varieties varied significantly and was related to pollination frequency, I found that the pollinator fauna between populations of *L. sanguinea* var. *sanguinea* slightly differed. This plant-pollinator interaction was similar to adaptive wandering, proposed by Wilson & Thomson (1996). This theory suggests that slight differences of pollinator communities do not cause the evolution of mechanisms that could exclude other pollinators, so floral evolution occurs without specialization of the pollination system in generalist flowers. For example, I focused on the pollinator community differences between populations 6 and 9. Both populations have two main pollinators, *Lasioglossum japonicum* and *Amegilla florea*, with *L. japonicum* as the most frequent pollinator. These two populations were located together according to clustering analysis based on pollination frequencies, but their flowers were divided into two clusters based on floral morphology. These results suggest that the floral morphology of *L. sanguinea* var. *sanguinea* could be produced by floral adaptation mediated by local pollinator fauna. However, I found considerable pollinator overlap between populations. The dispersal distance of *L. sanguinea* is thought to be limited (Chung *et al.* 1999), and it is easier to treat geographic isolation rather than pollinator difference as the cause of reproductive isolation.

Based on the results of the present study, I suggest that pollinator-mediated adaptation occurred in *L. sanguinea* for a rare pollination process. Most of the observed pollinators had intraspecific pollen (Table S2-1), but the actual number of pollinators that touched the stigma varied. To investigate the relationships between the lineage's split history and its pollinator changes, phylogenetic analyses with molecular data have been used (e.g., Ng & Smith 2016; Van der Niet & Johnson 2012). The floral traits of *L. sanguinea* are partly consistent with those of butterfly-pollinated flowers (Faegri & van der Pijl 1979), and the shift from butterflies to bees may have promoted the variation of floral morphologies. Previous results and my unpublished data suggest that the three varieties of *L. sanguinea* are positioned nearest to each other in the genus (Shi *et al.* 2006), and further analyses of the divergence history between *L. sanguinea* populations could reveal plant-pollinator interactions more precisely.

References

Anderson B., Johnson S.D. (2008) The geographical mosaic of coevolution in a plant–pollinator mutualism. *Evolution*, **62**, 220–225.

Ashman T.L., Knight T.M., Steets J.A., Amarasekare P., Burd M., Campbell D.R., Dudash M.R., Johnston M.O., Mazer S.J., Mitchell R.J., Morgan M.T., Wilson W.G. (2004) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology*, **85**, 2408–2421.

Byers K.J.R.P., Bradshaw H.D.Jr., Riffell J.A. (2014) Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). *Journal of Experimental Biology*, **217**, 614–623.

Charrad M., Ghazzali N., Boiteau V., Niknafs A. (2014) NbClust package: finding the relevant number of clusters in a dataset. *Journal of Statistical Software*, **61**, 1–36.

Chang Y.C., Shi C.T., Chung M.C. (2009) Variations in ribosomal RNA gene loci in spider lily (*Lycoris* spp.). *Journal of the American Society for Horticultural Science*, **134**, 567–573.

Chung, M.Y., Chun, C.P., Chung, M.G. (1999) Clonal and spatial genetic structure in a population of the endangered herb *Lycoris sanguinea* var. *koreana* (Amaryllidaceae). *Genes & Genetics Systems*, **74**, 61–66.

Cooley A.M., Carvallo G., Willis J.H. (2008) Is floral diversification associated with pollinator divergence? Flower shape, flower colour and pollinator preference in Chilean *Mimulus*. *Annals of Botany*, **101**, 641–650.

Cosacov A., Cocucci A.A., Sersic A.N. (2013) Geographical differentiation in floral traits across the distribution range of the Patagonian oil-secreting *Calceolaria polyrhiza*:

do pollinators matter? *Annals of Botany*, **113**, 251–266.

Faegri K., van der Pijl L. (1979) *The principles of pollination ecology*. Pergamon Press, London, UK, pp 244.

Fenster C.B., Armbruster W.S., Wilson P., Dudash M.R., Thomson J.D. (2004) Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution and Systematics*, **35**, 375–403.

Gómez J.M., Zamora R. (2006) Ecological factors that promote evolution of generalization in pollination systems. *In:* Waser N., Ollerton J. *(Eds), Plant–pollinator interactions: from specialization to generalization. University of Chicago Press;* Chicago, IL, USA*:* 145–166.

Gómez J.M., Muños-Pajares A.J., Abdelaziz M., Lorite J., Perfectti F. (2014) Evolution of pollination niches and floral divergence in the generalist plant *Erysimum mediohispanicum*. *Annals of Botany*, **113**, 237–249.

Herrera C.M., Castellanos M.C., Medrano M. (2006) Geographical context of floral evolution: towards an improved research programme in floral diversification. In: Harder L.D., Barrett S.C.H. (Eds), *Ecology and evolution of flowers.* Oxford University Press; Oxford, UK: 278–294.

Hijmans R.J., Cameron S.E., Parra J.L., Jones P.G., Jarvis A. (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, **25**, 1965–1978.

Hothorn T., Bretz F., Westfall P. (2008) Simultaneous Inference in General Parametric Models. *Biometrical Journal*, **50**, 346–363.

Hsu P.S., Kurita S., Yu Z.Z., Lin J.Z. (1994) Synopsis of the genus *Lycoris* (Amaryllidaceae). *Sida*, **16**, 301–331.

Johnson S.D., Steiner K.E. (2000) Generalization versus specialization in plant pollination systems. *Trends in Ecology and Evolution*, **15**, 140–143.

Johnson S.D. (2010) The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philosophical Transactions of the Royal Society London B, Biological Sciences*, **365**, 499–516.

Kawano S. (2009) Life-history monographs of Japanese plants. 13: *Lycoris sanguinea* Maxim. (Amaryllidaceae). *Plant Species Biology*, **24**, 139–144.

Kay K.M. (2006) Reproductive isolation between two closely related hummingbird-pollinated neotropical gingers. *Evolution*, **60**, 538–552.

Kay K.M., Sargent R.D. (2009) The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annual Review of Ecology Evolution and Systematics*, **40**, 637–656.

King C., Ballantyne G., Willmer P.G. (2013) Why flower visitation is a poor proxy for pollination: measuring single-visit pollen deposition, with implications for pollination networks and conservation. *Methods in Ecology and Evolution*, **4**, 811–818.

Kurita S. (1988) Variation and evolution in the karyotype of *Lycoris*, Amaryllidaceae VI. Intrapopulational and/or intraspecific variation in the karyotype of *L. sanguinea* Max. var. *kiushiana* and *L. sanguinea* Max. var. *koreana* (Nakai) Koyama. *Cytologia*, **53**, 307–321.

Lloyd D.G., Webb C.J. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *New Zealand Journal of Botany*, **24**, 135–162.

Marques I., Jürgens A., Aguilar J.F., Feliner G.N. (2016) Convergent recruitment of new pollinators is triggered by independent hybridization events in *Narcissus*. *New*

Phytologist, **210**, 731–742.

Medel R., Valiente A., Botto-Mahan C., Carvallo G., Pérez F., Pohl N., Navarro L. (2007) The influence of insects and hummingbirds on the geographical variation of the flower phenotype in *Mimulus luteus*. *Ecography*, **30**, 812–818.

Newman E., Manning J., Anderson B. (2015) Local adaptation: mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities. *Evolution*, **69**, 2262–2275.

Ng, J., Smith, S.D. (2016) Widespread flower color convergence in Solanaceae via alternate biochemical pathways. *New Phytologist*, **209**, 407–417.

Ollerton J., Winfree R., Tarrant S. (2011) How many flowering plants are pollinated by animals? *Oikos*, **120**, 321–326.

Paudel B.R., Shrestha M., Burd M., Adhikari S., Sun Y.S., Li Q.J. (2016) Coevolutionary elaboration of pollination-related traits in an alpine ginger (*Roscoea purpurea*) and a tabanid fly in the Nepalese Himalayas. *New Phytologist*, **211**, 1402–1411.

Penny J.H.J. (1983) Nectar guide color contrast: a possible relationship with pollination strategy. *New Phytologist*, **95**, 707–721.

Pérez-Barrales R., Arroyo J., Armbruster W.S. (2007) Differences in pollinator faunas may generate geographic differences in floral morphology and integration in *Narcissus papyraceus* (Amaryllidaceae). *Oikos*, **116**, 1904–1918.

Schemske D.W., Bradshaw H.D.Jr. (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 11910–11915.

Schiestl F.P. (2015) Ecology and evolution of floral volatile-mediated information

transfer in plants. *New Phytologist*, **206**, 571–577.

Schiestl F.P., Johnson S.D. (2013) Pollinator-mediated evolution of floral signals. *Trends in Ecology & Evolution*, **28**, 307–315.

Shi S.D., Qiu Y.X., Li E.X., Fu C.X. (2006) Phylogenetic relationships and possible hybrid origin of *Lycoris* species (Amaryllidaceae) revealed by ITS sequences. Biochemical Genetics, **44**, 198–208.

Simón-Porcar V.I., Santos-Gally R., Arroyo J. (2013) Long-tongued insects promote disassortative pollen transfer in style-dimorphic *Narcissus papyraceus* (Amaryllidaceae). *Journal of Ecology*, **102**, 116–125.

Stebbins G.L. (1970) Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. *Annual Review of Ecology and Systematics 1*, 307–326.

Van der Niet T., Peakall R., Johnson S.D. (2014) Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Annals of Botany*, **113**, 199–211.

Van Der Niet T., Johnson S.D. (2012) Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology & Evolution*, **27**, 353–361.

Waser N.M., Chittka L., Price M.V., Williams N.M., Ollerton J. (1996) Generalization in pollination systems, and why it matters. *Ecology*, **77**, 1043–1060.

Webb C.J., Lloyd D.G. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zealand Journal of Botany*, **24**, 163–178.

Willmer P. (2011) *Pollination and floral ecology*. Princeton: Princeton University Press.

Wilson P., Thomson J.D. (1996) How do flowers diverge? In: Lloyd D.G., Barrett S.C.H. (Eds), *Floral biology: studies on floral evolution in animal-pollinated plants.* Chapman & Hall; New York, US: 88–111.

Yamaji F., Ohsawa A.T. (2015) Breaking-bud pollination: a new pollination process in partially opened flowers by small bees. *Journal of plant research*, **128**, 803–811.

Yamaji F., Ohsawa A.T. (2016) Field experiments of pollination ecology: The case of *Lycoris sanguinea* var. *sanguinea*. *Journal of Visualized Experiments*, **117**, e54728.

Zhang B., Li Q.J. (2014) Phenotypic selection on the staminal lever mechanism in *Salvia digitaloides* (Labiaceae). *Evolutionary Ecology*, **28**, 373–386.

Table 2-1. Population codes, locations, research dates, number of observed flowers, observation periods, and observation-based variety identification.

Population code	Locality	Observation date	Observation flower (n)	Observation period (h)	Variety identification
1	Tsushima-city, Nagasaki Pref.	27, 28 Aug. 2015	31	16	var. *koreana*
2	Nishisonogi-gun, Nagasaki Pref.	15, 16 Sep. 2015	24	10	var. *koreana*
3	Fujitu-gun, Saga Pref.	19-21 Jul. 2013	11	27	var. *kiushiana*
4	Itoshima-city, Fukuoka Pref.	16-18 Jul. 2013	13	26	var. *kiushiana*
5	Touon-city, Ehime Pref.	21-24 Jul. 2015	8	30	var. *kiushiana*
6	Saijou-city, Ehime Pref.	23, 24 Jul. 2015	6	16	var. *sanguinea*
7	Shoubara-city, Hiroshima Pref.	25-28, 31 Jul. 2015	15	23	var. *kiushiana*
8	Shoubara-city, Hiroshima Pref.	26-28, 31 Jul. 2015, 3 Aug. 2015	19	22	var. *sanguinea*
9	Ibaraki-city, Osaka Pref.	7-10 Aug. 2015	14	26	var. *sanguinea*
10	Shin-shiro-city, Aichi Pref.	3, 4 Aug. 2015	20	16	var. *sanguinea*
11	Okaya-city, Nagano Pref.	10, 11 Aug. 2015	8	18	var. *kiushiana*
12	Osato-gun, Saitama Pref.	8, 9 Aug. 2015	14	16	var. *sanguinea*
13	Chiba-city, Chiba Pref.	5, 6 Aug. 2015	16	16	var. *sanguinea*

Table 2-2. Simultaneous tests for general linear hypotheses of interactions between floral morphologies and pollinator groups.

	Estimate	Standard error	Z-value	P-value
STL				
small bee	**-0.12801**	**0.03807**	**-3.363**	**0.00236**
large bee	**-0.127**	**0.03375**	**-3.763**	**0.0005**
large butterfly	0.01299	0.02244	0.579	0.91022
PEL				
small bee	**-0.12364**	**0.03804**	**-3.251**	**0.003405**
large bee	**-0.12221**	**0.03376**	**-3.619**	**0.000877**
large butterfly	0.01147	0.02237	0.513	0.935175
PPL				
small bee	**-0.12389**	**0.03815**	**-3.248**	**0.003447**
large bee	**-0.12858**	**0.03374**	**-3.81**	**0.000417**
large butterfly	0.01245	0.02243	0.555	0.919596
PIL				
small bee	**-0.126**	**0.03803**	**-3.313**	**0.00273**
large bee	**-0.12459**	**0.03375**	**-3.691**	**0.00066**
large butterfly	0.01302	0.02243	0.58	0.90967

P-values were adjusted by Holm corrections. Bold means significant differences from zero at the $P < 0.05$ level.

Table 2-3. Simultaneous tests for general linear hypotheses of interactions between floral morphology and biotic/abiotic factors.

	Estimate	Standard error	Z-value	P-value
Mor1				
small bee	**0.82718**	**0.2247**	**3.681**	**0.00116**
large bee	0.2011	0.30422	0.661	1
large butterfly	0.1635	0.11825	1.383	0.66705
bio1	-0.26893	1.09081	-0.247	1
bio2	-0.08471	0.06645	-1.275	0.66705
Mor2				
small bee	-0.3479	2.1805	-0.16	1
large bee	-1.1528	2.9522	-0.39	1
large butterfly	0.1585	1.1475	0.138	1
bio1	-5.4602	10.5854	-0.516	1
bio2	-0.1846	0.6449	-0.286	1

P-values were adjusted using Holm corrections. Bold indicates a significant difference from zero at the $P < 0.05$ level.

Table 2-4. Simultaneous tests for general linear hypotheses of interactions between floral morphology and biotic factors at the breaking-bud stage.

	Estimate	Standard error	Z-value	P-value
Stigma-anther				
small bee	**-0.11038**	**0.03792**	**-2.911**	**0.0108**
small bee to breaking bud	**0.11608**	**0.05151**	**2.254**	**0.04845**
large bee	**-0.12216**	**0.03864**	**-3.162**	**0.00627**
large butterfly	0.01153	0.02248	0.513	0.60799
Stigma-petal				
small bee	**-0.11351**	**0.03774**	**-3.008**	**0.00789**
small bee to breaking bud	0.11484	0.05145	2.232	0.05122
large bee	**-0.12192**	**0.03862**	**-3.157**	**0.00638**
large butterfly	0.0119	0.02246	0.53	0.5963

P-values were adjusted using Holm corrections. Bold indicates a significant difference from zero at the $P < 0.05$ level.

Table S2-1. Numbers of pollen-deposited floral visitors and positions of pollen deposition.

Pop. code	Smal bee					Large bee					Large butterfly				
	H	T	A	L	W	H	T	A	L	W	H	T	A	L	W
1	4/10	7(**2**)/10	8(**5**)/10	6(**4**)/10	4/10	2/5	0/5	1/5	0/5	3/5	4/5	2/5	2/5	5/5	5/5
2	3/5	2/5	3(**2**)/5	2(**2**)/5	1/5	3/4	0/4	4(**3**)/4	2(**1**)/4	1/4	2/3	1/3	1/3	3/3	3/3
3	4/5	1(**1**)/5	3(**2**)/5	2(**1**)/5	1/5	1/1	1/1	1(**1**)/1	1/1	0/1	1/1	1/1	0/1	1/1	1/1
4	0/1	1(**1**)/1	1(**1**)/1	1(**1**)/1	0/1	-	-	-	-	-	1/1	0/1	0/1	1/1	1/1
5	2/3	2/3	2(**1**)/3	1/3	1/3	1/1	0/1	1/1	1/1	0/1	-	-	-	-	-
6	1/7	1/7	1(**1**)/7	2(**1**)/7	0/7	2/2	1/2	1(**1**)/1	1/2	1/2	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	2/5	2/5	2/5	5/5	5/5
8	0/3	1/3	1(**1**)/3	1(**1**)/3	1/3	-	-	-	-	-	-	-	-	-	-
9	1/7	4/7	5(**2**)/7	5(**2**)/7	1/7	2/2	0/2	0/2	0/2	0/2	-	-	-	-	-
10	5/11	6(**1**)/11	9(**4**)/11	6(**5**)/11	4/11	-	-	-	-	-	-	-	-	-	-

11	0/1	0/1	0/1	0/1	0/1	-	-	-	-	-	-	-	-	-	-
12	3/12	5/12	9(**2**)/12	8(**2**)/12	1/12	1/1	0/1	0/1	0/1	0/1	-	-	-	-	-
13	2/10	3(**1**)/10	7(**3**)/10	9(**4**)/10	2/10	4/8	0/8	4/8	3/8	0/8	-	-	-	-	-

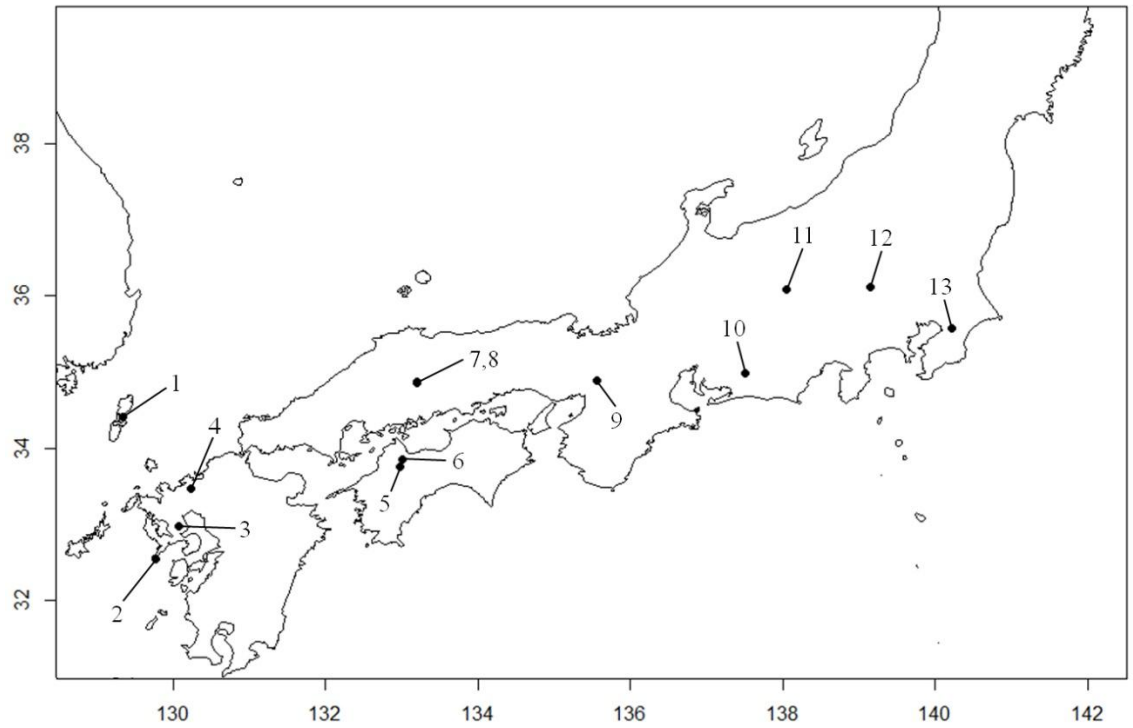
Numbers to the right of the slash indicate total captured individuals, and numbers to the left indicate pollen-deposited individuals. Numbers in parentheses indicate individuals with > 100 pollen grains. H = head, T = thorax, A = abdomen, L = legs, and W = wings. Dashes indicate no data.

Table S2-2. Results of self-pollination experiments in 11 populations.

Population code	Variety identification	n	FS	SS
1	var. *koreana*	-	-	-
2	var. *koreana*	-	-	-
3	var. *kiushiana*	8	12.5	30.0
4	var. *kiushiana*	10	40.0	25.0
5	var. *kiushiana*	7	71.4	36.0
6	var. *sanguinea*	25	48.0	25.8
7	var. *kiushiana*	15	46.7	32.9
8	var. *sanguinea*	25	64.0	23.1
9	var. *sanguinea*	15	46.7	28.6
10	var. *sanguinea*	54	74.1	34.5
11	var. *kiushiana*	30	90.0	45.6
12	var. *sanguinea*	19	57.9	24.5
13	var. *sanguinea*	28	50.0	28.6

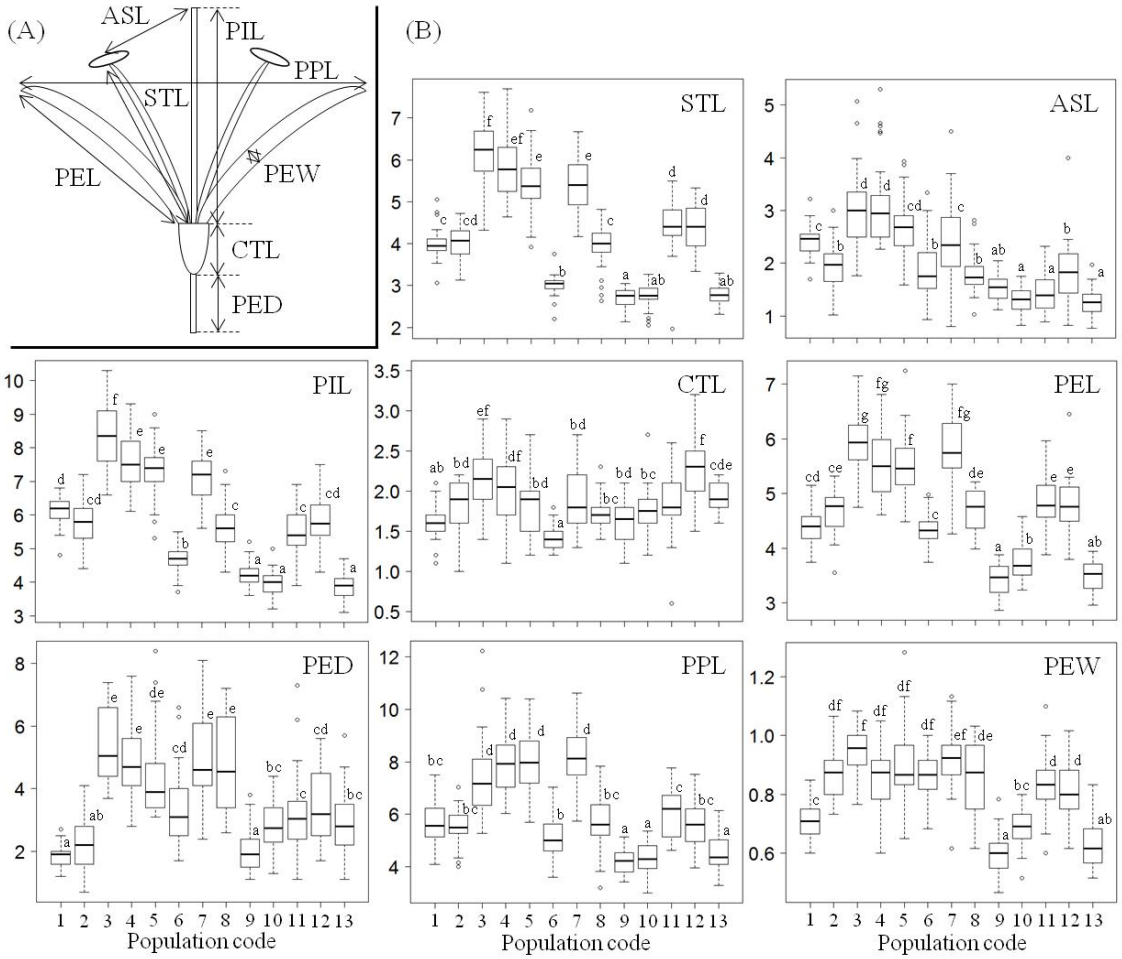
n indicates the flower number for the experiment. FS and SS indicate fruit-set and seed-set ratios (percentages), respectively.

Figure 2-1. Study site locations.



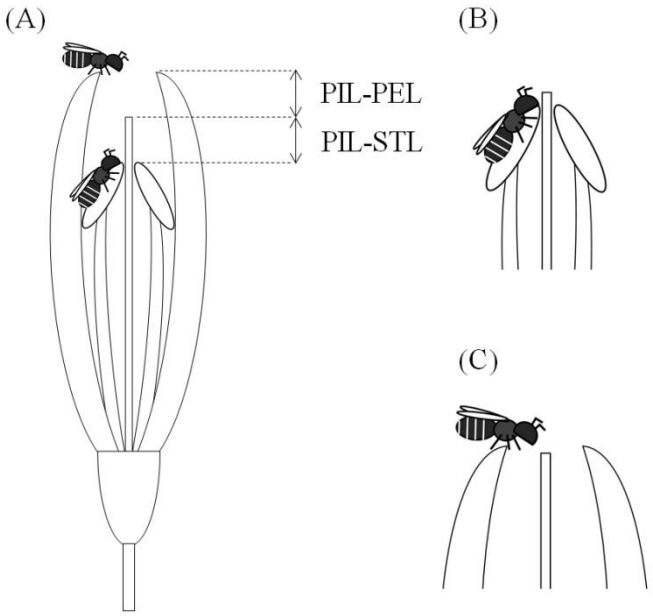
Each number represents a research population code. Population codes and locations are listed in Table 2-1.

Figure 2-2. The results of morphological measurements for eight floral parts.



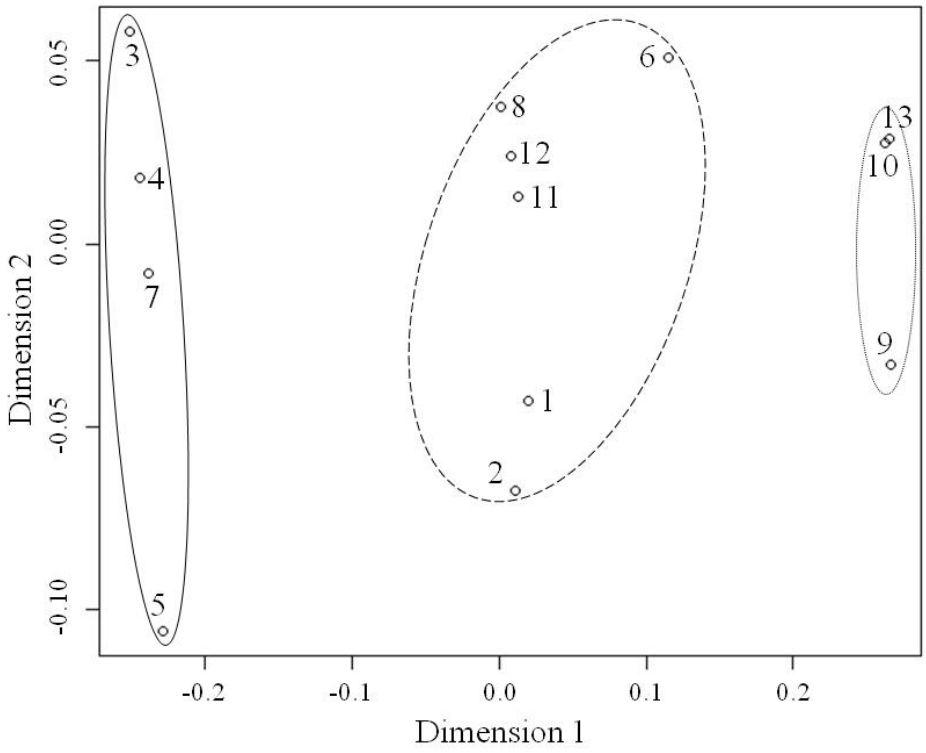
(A) Schematics of the measured floral parts. ASL = anther-stigma length, CTL = corolla tube length, PED = pedicel length, PEL = petal length, PEW = petal width, PIL = pistil length, PPL = petal-petal length, STL = stamen length. (B) Boxplots of the eight measured traits.

Figure 2-3. Schematics for the test of breaking-bud pollination effects.



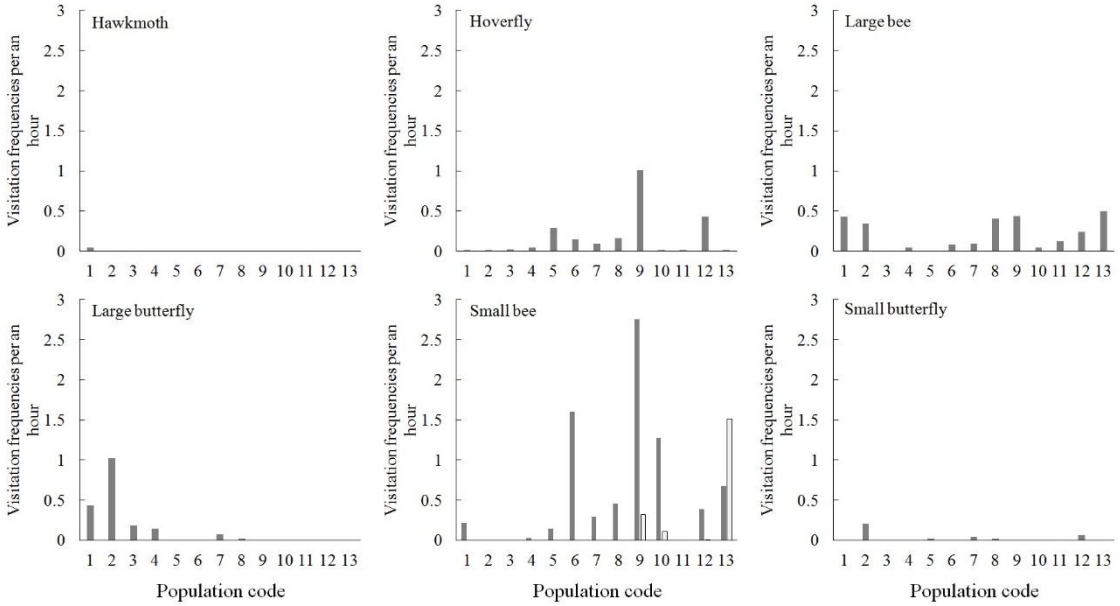
(A) Possible process of pollination at the breaking-bud stage. (B) Stigma-anther hypothesis. (C) Stigma-petal hypothesis.

Figure 2-4. Nonmetric multidimensional scaling (NMDS) based on the results of morphological cluster analysis.



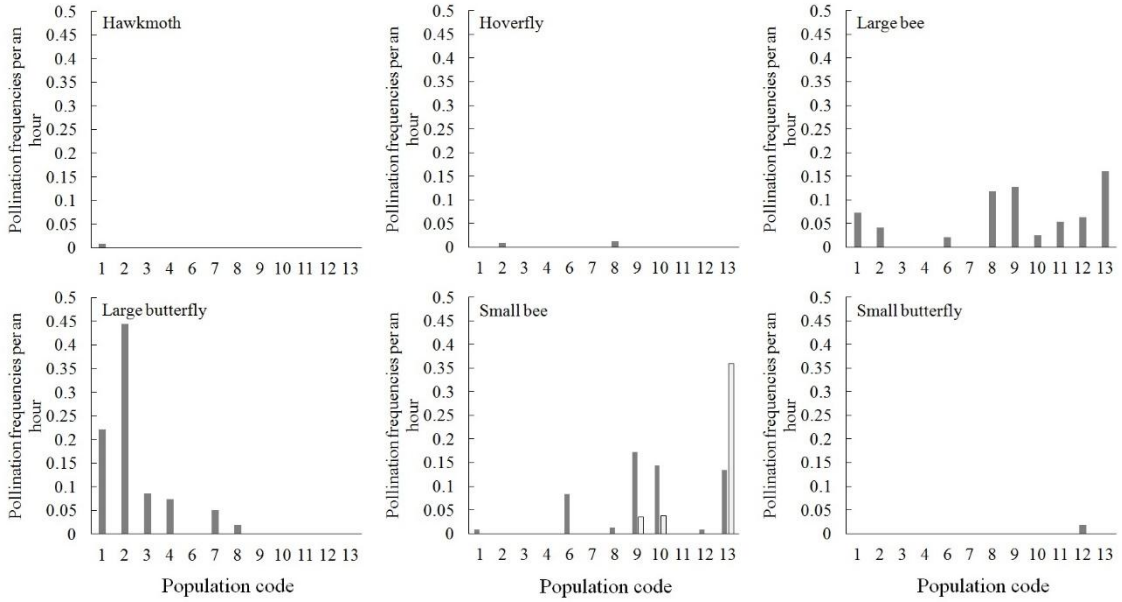
Numbers indicate population codes. The three circles described by different lines indicate significantly different clusters.

Figure 2-5. Visitation frequencies and frequency ratios of each population by pollinator functional group.



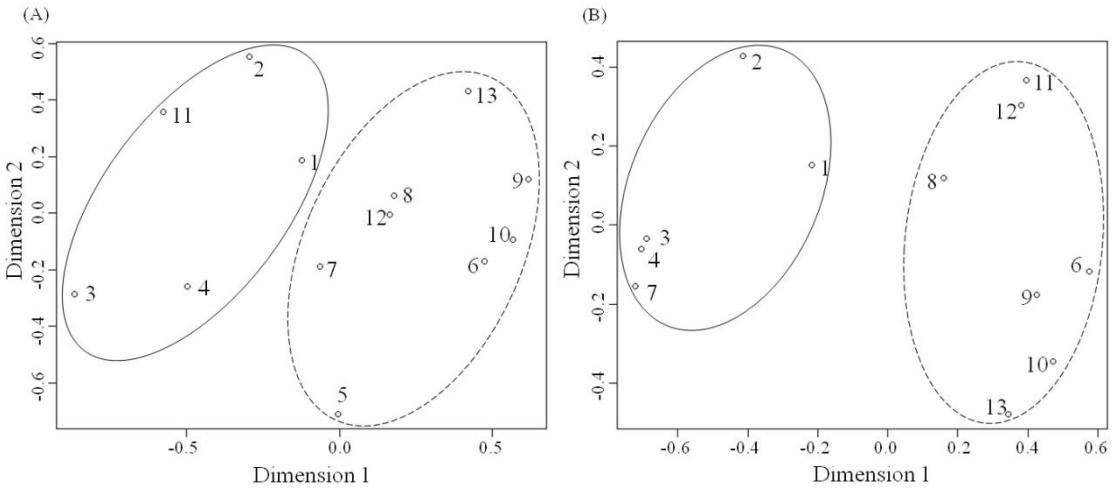
Each bar shows average frequencies of pollinators in each population. White bars in "Small bee" indicate frequencies to breaking buds.

Figure 2-6. Pollination frequencies and frequency ratios of each population by pollinator functional group.



Each bar shows average frequencies of pollinators in each population. White bars in "Small bee" indicate frequencies to breaking buds.

Figure 2-7. Nonmetric multidimensional scaling (NMDS) based on the results of cluster analysis using visitation and pollination frequencies of each floral visitor.



Numbers indicate population codes. (A) Visitation frequencies. (B) Pollination frequencies.

Chapter 3: Transplant experiments in *Lycoris sanguinea* var. *sanguinea*: floral adaptation to breaking-bud pollination

Introduction

Animal-pollinated flowers prepare floral resources to attract pollinators. One of these resources, pollen, provides nutrition for pollen-collecting animals (Roulston & Cane 2000; Hargreaves *et al.* 2010). Generally, less than 1% of removed pollen grains are used for pollination (Harder & Johnson 2008), and this pollen limitation could limit seed production (Ashman *et al.* 2004). These conditions could promote plant diversification by floral adaptation (e.g. Vamosi *et al.* 2006; Harder & Aizen 2010). Flowering plants experience a variety of floral visitors, and some of them consume floral resources, such as nectar or pollen without transporting conspecific pollen to a stigma (Hargreaves *et al.* 2009; Irwin *et al.* 2010). Pollen thieves may directly decrease seed production of the plant population by consuming pollen grains without pollination, and they may greatly influence fitness (Hargreaves *et al.* 2009). Previous studies suggest that pollen theft is a more common phenomenon than reported (Hargreaves *et al.* 2010); however, the harmful effects on plant reproduction, specific influences on flowers (e.g. Solís-Montero *et al.* 2015), and plant responses to pollen theft have been seldom reported.

Pollen-thieved plants could adapt floral traits to tolerate or resist thieves, or convert them into pollinators (reviewed in Hargreaves *et al.* 2009). Pollen theft can cause a pollen limitation, and may promote floral adaptation against the thieves. In hermaphroditic flowers, herkogamous or dichogamous flowers tend to have pollen

stolen because of mismatches between their visiting phases and pollinator body sizes (e.g. Armbruster *et al.* 1989; Ish-Am & Eisikowitch 1993). To attract floral visitors to female-phase flowers, is important for flowers with dichogamy to reduce the effects of pollen thieves, and some plants mimic floral rewards or provide feeding pollen as attractive materials (Jesson & Barrett 2003; Lunau 2000). However, to our knowledge, there are no reports of a pollen thief converted into an effective pollinator until now. The evolutionary forces mediated by pollen thieves are likely significant to plants, and future empirical studies would resolve these questions.

In this study, I investigated the possibility of floral adaptation mediated by pollen thieves in *Lycoris sanguinea* Maxim. var. *sanguinea* Koyama (Amaryllidaceae). This plant is visited by various insect species, but the main visitor is the small bee *Lasioglossum japonicum*. The bees visit breaking buds, which are partially-opened flowers. Although these small bees tend to be pollen thieves because they are too small to contact the stigma, they collect pollen grains from undehisced anthers of breaking buds by their mandibles and can pollinate these buds (termed “breaking-bud pollination” in Yamaji & Ohsawa 2015). Furthermore, I demonstrated that individuals can be artificially pollinated one to two days before flowering (Yamaji & Ohsawa 2015). This novel pollination process, “breaking-bud pollination,” was observed in a part of the distributed area of *L. sanguinea* var. *sanguinea* (Chapter 2). This plant does not need to adapt to pollinators at the breaking-bud stage. The ancestral pollinators were likely butterfly species based on floral traits that are partly consistent with butterfly-pollinated syndromes (Faegri & van der Pijl 1979). Butterflies visit fully-opened flowers to land stably on opened corolla. Other larger insects pollinate opened flowers due to their body sizes, but only *L. japonicum* can enter to breaking buds. *L. japonicum* collect most of

the pollen grains of *L. sanguinea* var. *sanguinea* at the breaking-bud stage, possibly causing a pollen limitation. Such conditions would decrease the pollination efficiency by floral visitors in later flowering stages, and could reduce the fitness of *L. sanguinea* var. *sanguinea* populations. Under these conditions, the improvement of pollination efficiencies by small bees could increase floral fitness. Therefore, I hypothesized that small bees were originally pollen thieves and floral adaptation increased pollination efficiency, changing the bees' function from thief to pollinator.

To test this hypothesis, I conducted transplant experiments and following pollinator observations and bagging experiments of field populations with breaking-bud pollination. Reciprocal transplant experiments are the ideal approach for detecting the local effects of biological interactions, including plant-pollinator relationships (Kawecki & Ebert 2004; Blanquart *et al.* 2013; Sun, Gross & Schiestl 2014; Newman *et al.* 2015). However, there are practical limitations to studies of short-flowering plants in multiple populations. The flowering season of *L. sanguinea* var. *sanguinea* was approximately late-July to mid-August, and the period of breaking-bud stage approximately half a day or less. Common garden experiments (de Villemereuil *et al.* 2016) with *L. japonicum* are difficult, and because ecological information on *L. japonicum*, such as nest sites, is scarce, it is difficult to collect them in populations with breaking-bud pollination. For these reasons, I conducted transplant experiments in one population with breaking-bud pollination. I compared fruit-set and seed-set ratios and visitation and pollination frequencies between populations with different pollinator faunas.

One of the possible reasons for floral adaptation caused by small bee visits to breaking buds is to improve the pollination efficiencies by small bees (e.g. Castellanos *et al.* 2003). At the fully-open stage, small bees have few chances to contact the stigma

because of the spacing between the anther and stigma. In contrast, at the breaking-bud stage, small bees may easily touch the stigma because the space between anthers and stigma are smaller than in the fully-open stage. These differences could promote the selection of individuals in populations that are adapted to small bee visits to breaking buds. To test this hypothesis, I artificially restricted insect visitation to flowers at several flowering stages. One treatment enabled only small bees to visit the flowers for the entire flowering season. I prepared iron-wire cages with gaps that were larger than small bees, but smaller than other insect visitors, which were placed over individuals with unopened flowers and remained until the end of the season. Another treatment was to allow visitation of small bees at the fully-open stage. In addition, I attached insect-eliminating covers on cages that were removed after the flowers completely opened. To compare the floral fitness between treatments, I estimated the differences in pollination efficiencies through fruit- and seed-set ratios.

Materials and methods

Plant material

L. sanguinea var. *sanguinea* is a perennial herb that lives on deciduous forest floors and is mainly distributed in the Honshu region of Japan (Kurita 1988; Kawano 2009). The flowering season occurs from approximately late-July to late-August, and there are typically two to six opened flowers on their scapes. The flowers are bright orange-red without floral scent and the stamens are shorter than the perianths, but the pistil is slightly exserted from the corolla (Hsu *et al.* 1994; Kawano 2009). Various insect species, such as larger bees and butterflies, visit these flowers; however, the main visitor is the small bee *L. japonicum* (Yamaji & Ohsawa 2015). *L. sanguinea* var. *sanguinea* is self-compatible and requires pollinators for seed production (Yamaji & Ohsawa 2015). Pollen-mediated gene flow ranges were limited (Chung *et al.* 1999), and I can infer that there is no seed dispersal mechanism because of their small black seeds (Willson & Traveset 2000).

Transplant experiments

To investigate the floral adaptation of *L. sanguinea* var. *sanguinea* at the breaking-bud stage, I transplanted individuals from populations without breaking-bud pollination to a field where breaking-bud pollination had been observed. The main experimental field was Izumi Nature Park (Noro-cho, Chiba Pref., 35°34’38”N, 140°13’50”E) where breaking-bud pollination is observed and there is a large space for transplant experiments. I selected four populations based on a previous study (Chapter 2). Data on floral visitor frequencies, potential pollinators, and presence or absence of breaking-bud

pollination of these populations were previously collected. I observed two populations of *L. sanguinea* var. *sanguinea* with breaking-bud pollination (Aichi and Chiba) and two without (Ehime and Hiroshima). In mid- to late-July of 2016, I collected 30 individuals from each population. I selected individuals that were beginning to elongate their scapes, and the tips were just on the ground. Six meters separated each individual to prevent collecting clonal individuals (Chung *et al.* 1999). I carefully dug in the ground approximately 20-cm around individuals not to damage their bulbs and roots. The samples were transplanted into 15-cm pots with commercially available culture soil, and transported to Izumi Nature Park. To reduce the effects of gene flow from other populations to those native to Izumi Nature Park, I located the experimental plot at least 150 m from other populations based on the estimated foraging ranges of small bees (Gathmann & Tscharntke 2002; Greenleaf *et al.* 2007). I arranged collected samples in a regular grid of 30 × 4 plots (Figure 3-1). Arranged samples were separated from each other by 0.5 m. Our preliminary experiments showed that transplanted individuals in Chiba populations have no significant differences in fruit-set or seed-set ratios with those planted in the ground in natural conditions (generalized linear models with binomial errors: fruit set, coefficient = -0.024, z = -0.066, P = 0.95; seed set, coefficient = -0.025, z = -0.135, P = 0.89).

Bagging experiment

To evaluate the fitness of each population to breaking-bud pollination, I conducted various bagging experiments based on our previous work (Yamaji & Ohsawa 2015). Treatments were the following: (A) Control: no treatment; (B) Breaking bud: observed small bee visits to breaking buds, then removed these anthers, and bagged using

insect-excluded bags; (C) Pollen supplementation: selected flowers and pollinated them with pollen grains of other individuals; (D) Bud pollination: selected unopened buds which will open one or two days after, then removed anthers of them, and pollinated them with pollen of other individuals; and (E) Pollinator exclusion: unopened buds were bagged until the end of flowering season. To reduce the individual effects of fruit and seed sets, I treated a flower per each treatment in an individual, as possible. At the end of the flowering seasons, I collected treated samples and recorded the fruit set and seed number of each flower.

Pollinator observation

To compare the floral visitors and pollinators between populations and pollination types, I recorded floral visitors using digital video cameras. Our main objective was to determine whether breaking buds of individuals from other populations were visited by small bees or not. In the flowering season, digital video cameras (GZ-E220 and GZ-E265, JVC Kenwood, Japan) with tripods were placed approximately 50 cm from the target flowers. Target flowers included both completely open flowers and breaking buds in each observation day, as possible. I recorded insect visitors to selected flowers from 06:00 to 10:00. After that, I viewed the footage to record visiting insect numbers and species. I also checked whether each floral visitor touched a stigma or not, and I identified formers as pollinators. I calculated visitation and pollination frequencies of each insect per hour. Target flowers were decided at the start of observations in each day, and I ensured that flowering phenologies were not different between populations (Figure S3-1).

Cage-cover experiment

To reveal the differences of pollination efficiencies by small bees, I manipulated flowers not to be visited at breaking-bud stage but to be at fully-open stage. I first prepared smaller mesh cages that only small bees could pass through. I made preliminary cages with several sizes of mesh holes; 10 mm × 10 mm, 7 mm × 6 mm, 5 mm × 5 mm, and 4 mm × 4 mm. I caught small bees and large bees in June, and released them into the prepared cages. I determined that the 7 mm × 6 mm mesh was suitable because small bees could pass through smoothly, but large bees could not. In Izumi Nature Park, I selected individuals with no opening flowers, transplanted them into pots with commercially available soil, and transported them to the experimental site. Pots were placed 0.5 m apart, and I covered them before opening with pollinator-excluded covers. In next day, I removed the covers and exposed cages for small bees. Unopened or partially-opened buds and fully-open flowers coexisted in each cage, and I checked each flower for buds or opening flowers. After anthesis, I removed the cages and collected fruits and seeds. Fruit-set and seed-set ratios were calculated and compared using the following statistical methods.

Data analyses

To compare populations in the bagging experiments, I used a generalized linear model (GLM) with binomial errors in R software. I compared fruit set and seed number per fruit between pollination types and populations in each treatment. In the case of zero values in any group, I prepared hypothetical samples. This virtual sample had a seed per fruit, and I added a sample per population. I also tested differences of each value between pollination types using Wald tests. I further used statistical analyses to reveal

which population pairs had significant differences in fruit set and seed number per fruit. I adopted multiple comparison tests, adjusted using the Holm correction, in the glht function of the multcomp package (Hothorn *et al.* 2008).

To compare the floral visitor frequencies between pollination types, we used generalized linear mixed models (GLMMs) with Poisson errors in the glmer function of the package lme4. I compared visitation and pollination frequencies of five visitor types: small bees, small bees to breaking buds, larger bees, hoverflies, and large butterflies. I used visitation and pollination counts of each flower as response variables and pollination types as explanatory variables. I also set observed days and populations as random effects. Statistical differences between pollination types were evaluated by Wald tests. Additionally, I added an offset variable to scale frequencies by the hour of observation in each period. I also compared both frequencies between populations using GLMMs with observed days as random effects and observed hours as the offset variable. Multiple comparisons with Holm adjustment were used in the glht function.

Finally, I compared the results of cage experiments using GLMMs with binomial errors in the glmer function of the package lme4. I compared fruit-set ratio and seed numbers per fruit using treatment type (breaking buds or opening flowers) as a fixed effect and individuals as random effects. I also considered the emerged times of flowers and small bees. In our experiment, breaking buds had one additional day for pollination compared to opening flowers. The flowering periods of *L. sanguinea* var. *sanguinea* individuals were approximately five days (personal observation). Therefore, I added an offset variable for GLMMs with various values A (A = 3~7) to breaking buds and A-1 to opening flowers. I checked significant differences using Wald tests.

Results

Bagging experiments

The results of the bagging experiments are shown in Figures 3-2 and 3-3. The breaking bud treatment showed that population type significantly affected fruit-set ratio (GLM with binomial error: $Z = -3.716$, $P < 0.001$), indicating differences in floral fitness to small bees at the breaking-bud stage. The bud pollination treatments showed the reason for the lower fruit set of the two populations; they had no pollination abilities at the bud stage (Figure 3-2: fruit set, GLM; $Z = -3.616$, $P < 0.001$). This data suggested that the individuals in populations with breaking-bud pollination had more matured stigmas compared with other populations. Both treatments resulted in similar seed sets among populations (Figure 3-3). Other treatments revealed smaller gaps of fitness between populations; control and outcross treatments showed no significant differences between populations (Figure 3-2: Control, GLM: $Z = -0.702$, $P = 0.483$; Outcross, GLM: $Z = -0.647$, $P = 0.518$), and the pollinator exclusion treatment showed no reproductive success of *L. sanguinea* var. *sanguinea* without pollinators, consistent with previous reports.

Pollinator observation

I recorded 44 h over 10 days and 1484 floral visitors in 4 functional groups were recorded; 1243 small bees (including 516 ones for breaking buds), 48 large bees, 190 hoverflies, and 3 large butterflies. Visitation frequencies to breaking buds by small bees were not different between pollination types (GLMM with Poisson error; $Z = -0.786$, $P = 0.432$), suggesting no floral signal to attract small bees to breaking buds. In contrast,

in the Hiroshima population, visitation frequencies of small bees to fully-opened flowers and breaking buds were significantly lower than other three (Figure 3-4: GLMM with Poisson error; $P < 0.01$). Pollination frequencies of small bees to breaking buds were significantly different between pollination types and between populations (Figure 3-5: GLMM; pollination types: $Z = -2.013$, $P < 0.05$; populations: $P < 0.05$). Visitation and pollination frequencies of other pollinator groups were not significantly different between pollination types or between populations.

Cage-cover experiments

A total of 60 individuals and 229 flowers were used for the cage experiments (102 flowers for breaking buds and 127 for open flowers). The fruit-set ratio in the breaking-bud treatment was higher than in the flowering treatment (Figure 3-6, Table 3-1: GLMM, offset = log (A, A-1|A = 3~7); $P < 0.01$). In contrast, the seed-set number per fruit was lower in breaking buds (GLMM; $P < 0.01$).

Discussion

This study investigated the hypothesis that breaking buds of *L. sanguinea* var. *sanguinea* can adapt to pollen theft by small bees. Although the small bees visited breaking buds of all four plant populations (Figure 3-4), populations with breaking-bud pollination had higher fruit-set ratios in the "breaking bud" treatment (Figure 3-2). This suggested that the individuals in these populations adapted to small bees at the breaking-bud stage. This study also demonstrated that the trait of premature development of the stigma could be selected for in these populations ("bud pollination" in Figure 3-2). The ability of the stigma to accept pollen at the breaking-bud stage is important to establish breaking-bud pollination and the selection for earlier-maturing stigma could promote higher fruit sets in *L. sanguinea* var. *sanguinea*. Only small bees can visit breaking buds; thus, it is unlikely that the selection of stigma prematurity is due to other factors. Furthermore, the pollination efficiency of the small bees changed in response to flowering stage. Results of the cage experiments showed a lower fruit-set ratio in the fully-open stage mediated by small bees (Figure 3-6A). These differences could promote floral adaptation in breaking buds because consumed pollen is not used for pollination (Thorp 2000), and pollen consumption in breaking buds could decrease the efficiencies of other insects indirectly (Hargreaves *et al.* 2009). Therefore, these results positively support the hypothesis that small bees changed from pollen thieves to pollinators by floral adaptation at the breaking-bud stage.

The evolutionary impacts of pollen theft on flowering plants have scarcely been studied. This study records a possible process for converting pollen thieves into pollinators. In previous studies, the main reason of why floral visitors act as pollen

thieves was the separation of male/female reproductive parts in space, such as herkogamy (Hargreaves *et al.* 2009). In our case, however, the anthers and stigma are closer in breaking buds than in completely open flowers, and small bees visiting breaking buds overcomes the challenge of mismatches between reproductive parts and insect body sizes. Although another barrier to small bees being effective pollinators was stigma immaturity in breaking buds, natural selection could resolve it. This might have shown the difficulties of converting thieves into pollinators because both time and space separations could be prohibitive. A previous study suggested that floral traits, not the behaviours of floral visitors influenced whether they acted as pollen thieves (Hargreaves *et al.* 2012). However, in *L. sanguinea* var. *sanguinea*, the visitation of small bees to breaking buds was undoubtedly a key factor for the bees to be effective pollinators, and our results suggest the importance of changes in visitor behaviours. Therefore, behavioural changes that could reduce the separation of reproductive organs would be required for pollen thieves to become pollinators. Breaking buds contain a large amount of pollen that is collected only by small bees, and the visitation to breaking buds would be advantageous for small bees. These conditions could be produced by increases in pollen collectors including small bees or decreases other plant abundances as resources for pollen grains. In both cases, an excessive demand for pollen might promote the visitation of breaking buds and the adaptation to small bees.

It has been suggested that a reduction in herkogamy or dichogamy could increase the efficiency of pollen thieves (Hargreaves *et al.* 2009). However, it also could increase self-pollination, which reduces genetic diversity and lowers fitness via inbreeding depression. Therefore, these selective forces may conflict with each other. A previous study demonstrated the self-compatibility of *L. sanguinea* var. *sanguinea*

(Yamaji & Ohsawa 2015), and *L. sanguinea* var. *kiushiana* was observed to produce fruits and seeds by self-pollination (Table S2-2). Although seed germinated ratios or viabilities have not been researched, this plant might have received little influence by selfing, and the selection against to pollen thieves was likely to act to breaking buds. Self-compatibility might be another condition that changes visitor functions.

To the best of our knowledge, this is the first record of pollen thieves becoming pollinators due to floral adaptation. Populations without breaking-bud pollination showed slightly decreasing ratios of reproductive success under natural conditions ("Control" in Figures 3-2 and 3-3), suggesting that other floral visitors, such as larger bees, contributed greatly to floral fitness. It is possible that large bees carried pollen grains with higher quality or quantity on their bodies than the small bees. This study did not examine pollen quality or quantity; therefore, further research on the differences in efficiencies is required. Additionally, visitation frequencies of small bees differed between populations (Figure 3-4). I previously observed differences in visitation frequencies and visitor types between these populations (Chapter 2). Some floral traits that attract visitors, such as floral scent, may vary in *L. sanguinea* var. *sanguinea*, and these floral traits could have influenced the results in this study.

References

Armbruster W.S., keller S., Matsuki M., Clausen T.P. (1989) Pollination of *Dalechampia magnoliifolia* (Euphorbiaceae) by male euglossine bees. *American Journal of Botany.* 76, 1279–1285.

Ashman T.L., Knight T.M., Steets J.A., Amarasekare P., Burd M., Campbell D.R., Dudash M.R., Johnston M.O., Mazer S.J., Mitchell R.J., Morgan M.T., Wilson W.G. (2004) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology.* 85(9), 2408–2421.

Blanquart F., Kaltz O., Nuismer S., Gandon S. (2013) A practical guide to measuring local adaptation. *Ecology Letters.* 16, 1195–1205.

Castellanos M.C., Wilson P., Thomson J.D. (2003) Pollen transfer by hummingbirds and bumblebees, and the divergence of pollination modes in *Penstemon*. *Evolution.* 57, 2742–2752.

Chung M.Y., Chun C.P., Chung M.G. (1999) Clonal and spatial genetic structure in a population of the endangered herb *Lycoris sanguinea* var. *koreana* (Amaryllidaceae). *Genes & Genetics Systems.* 74, 61–66.

de Villemereuil P., Gaggiotti O.E., Mouterde M., Till-Bottraud I. (2016) Common garden experiments in the genomic era: new perspectives and opportunities. *Heredity.* 116, 249–254.

Faegri K., van der Pijl L. (1979) *The principles of pollination ecology.* Oxford: Pergamon.

Gathmann A., Tscharntke T. (2002) Foraging ranges of solitary bees. *Journal of Animal Ecology.* 71, 757–764.

Greenleaf S.S., Williams N.M., Winfree R., Kremen C. (2007) Bee foraging ranges and their relationship to body size. *Oecologia.* 153, 589–596.

Harder L.D., Aizen M.A. (2010) Floral adaptation and diversification under pollen limitation. *Philosophical Transactions of the Royal Society B, Biological Sciences*. 365, 529–543.

Harder L.D., Johnson S.D. (2008) Function and evolution of aggregated pollen in angiosperms. *International Journal of Plant Sciences.* 169, 59–78.

Hargreaves A.L., Harder L.D., Johnson S.D. (2009) Consumptive emasculation: the ecological and evolutionary consequences of pollen theft. *Biological Reviews.* 84, 259–276.

Hargreaves A.L., Harder L.D., Johnson S.D. (2010) Native pollen thieves reduce the reproductive success of a hermaphroditic plant, Aloe maculate. *Ecology.* 91, 1693–1703.

Hargreaves A.L., Harder L.D., Johnson S.D. (2012) Floral traits mediate the vulnerability of aloes to pollen theft and inefficient pollination by bees. *Annals of Botany.* 109(4), 761–772.

Hothorn T., Bretz F., Westfall P. (2008) Simultaneous Inference in General Parametric Models. *Biometrical Journal*. 50, 346–363.

Hsu P.S., Kurita S., Yu Z.Z., Lin J.Z. (1994) Synopsis of the genus *Lycoris* (Amaryllidaceae). *Sida*. 16, 301–331.

Irwin R.E., Bronstein J.L., Manson J.S., Richardson L. (2010) Nectar robbing: ecological and evolutionary perspectives. *Annual Review of Ecology, Evolution, and Systematics.* 41, 271–292

Ish-am G., Eisikowitch D. (1993) The behavior of honey bees (*Apis mellifera*) visiting avocado (*Persea americana*) flowers and their contribution to its pollination. *Journal of*

Apicultural Research. 32, 175–186.

Jesson L.K., Barrett S.C.H. (2003) The comparative biology of mirror-image flowers. *International Journal of Plant Sciences.* 164, 237–249.

Kawano S. (2009) Life-history monographs of Japanese plants. 13: *Lycoris sanguinea* Maxim. (Amaryllidaceae). *Plant Species Biology.* 24, 139–144.

Kawecki T., Ebert D. (2004) Conceptual issues in local adaptation. *Ecology Letters.* 7, 1225–1241.

Kurita S. (1988) Variation and Evolution in the Karyotype of *Lycoris*, Amaryllidaceae VI. Intrapopulational and/or intraspecific variation in the karyotype of *L. sanguinea* Max. var. *kiushiana* and *L. sanguinea* Max. var. *koreana* (Nakai) Koyama. *Cytologia.* 53, 307–321

Lunau K. (2000) The ecology and evolution of visual pollen signals. *Plant Systematics and Evolution.* 222, 89–111.

Newman E., Manning J., Anderson B. (2015) Local adaptation: mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities. *Evolution.* 69, 2262–2275.

Roulston T.H., Cane J.H. (2000) Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution.* 222, 187–209.

Solís-Montero L., Vergara C.H., Vallejo-Marín M. (2015) High incidence of pollen theft in natural populations of a buzz-pollinated plant. *Arthropod-Plant Interactions.* 9, 599–611

Sun M., Gross K., Schiestl F.P. (2014) Floral adaptation to local pollinator guilds in a terrestrial orchid. *Annals of Botany.* 113, 289–300.

Thorp R.W. (2000) The collection of pollen by bees. *Plant Systematics and Evolution.*

222, 211–223.

Vamosi J.C., Knight T.M., Steets J.A., Mazer S.J., Burd M., Ashman T.L. (2006) Pollination decays in biodiversity hotspots. *Proceedings of the National Academy of Sciences of the United States of America.* 103(4), 956–961.

Willson M.F., Traveset A. (2000) *The ecology of seed dispersal.* In: Seeds: the ecology of regeneration in plant communities (ed. Fenner, M.). CABI Publishing, New York. Pp 85–109.

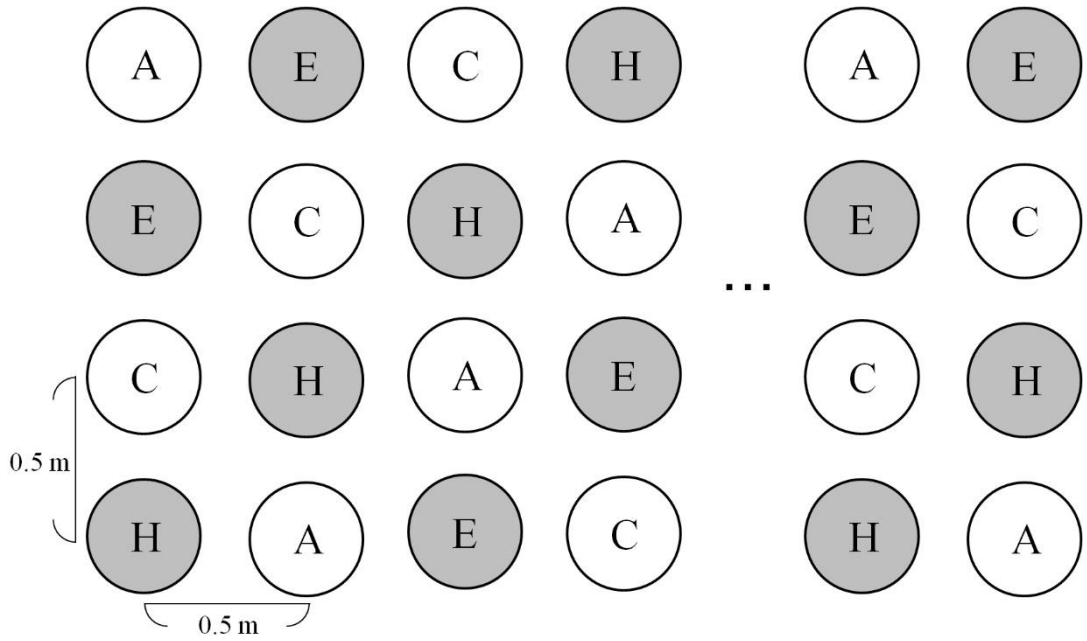
Yamaji F., Ohsawa A.T. (2015) Breaking-bud pollination: a new pollination process in partially opened flowers by small bees. *Journal of plant research.* 128, 803–811.

Table 3-1. The results of statistical analyses in cage-cover experiments.

Offset value (log(x))	Estimate	Std. Error	Z value	P value
Fruit set ratio				
x=3	-0.7861	0.2939	-2.674	**0.0075**
x=4	-0.9039	0.2939	-3.075	**0.0021**
x=5	-0.9684	0.2939	-3.295	**0.0010**
x=6	-1.0092	0.2939	-3.434	**0.0006**
x=7	-1.0374	0.2939	-3.53	**0.0004**
Seed number per fruit				
x=3	0.8144	0.1972	4.131	**0.0000**
x=4	0.6966	0.1972	3.533	**0.0004**
x=5	0.6321	0.1972	3.206	**0.0014**
x=6	0.5913	0.1972	2.999	**0.0027**
x=7	0.5631	0.1972	2.856	**0.0043**

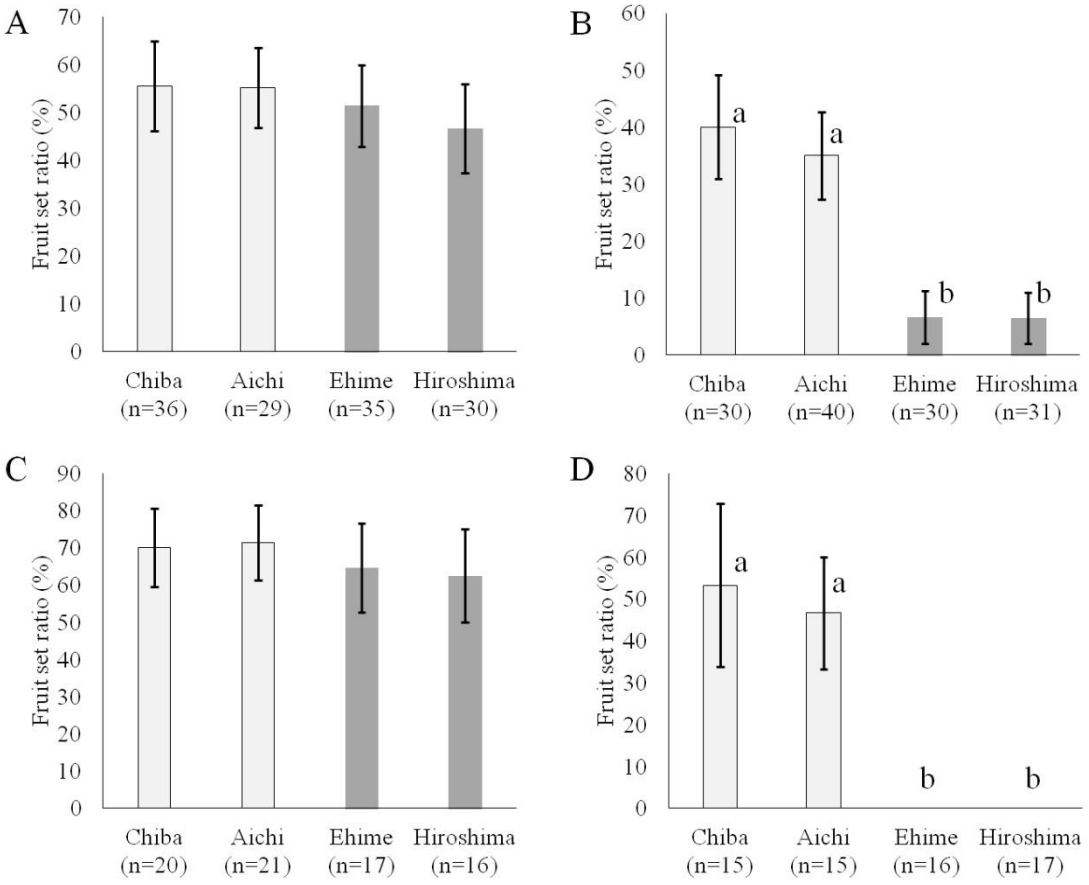
Bold means significant differences at the $P < 0.05$ level.

Figure 3-1. Individual arrangements for transplant experiments.



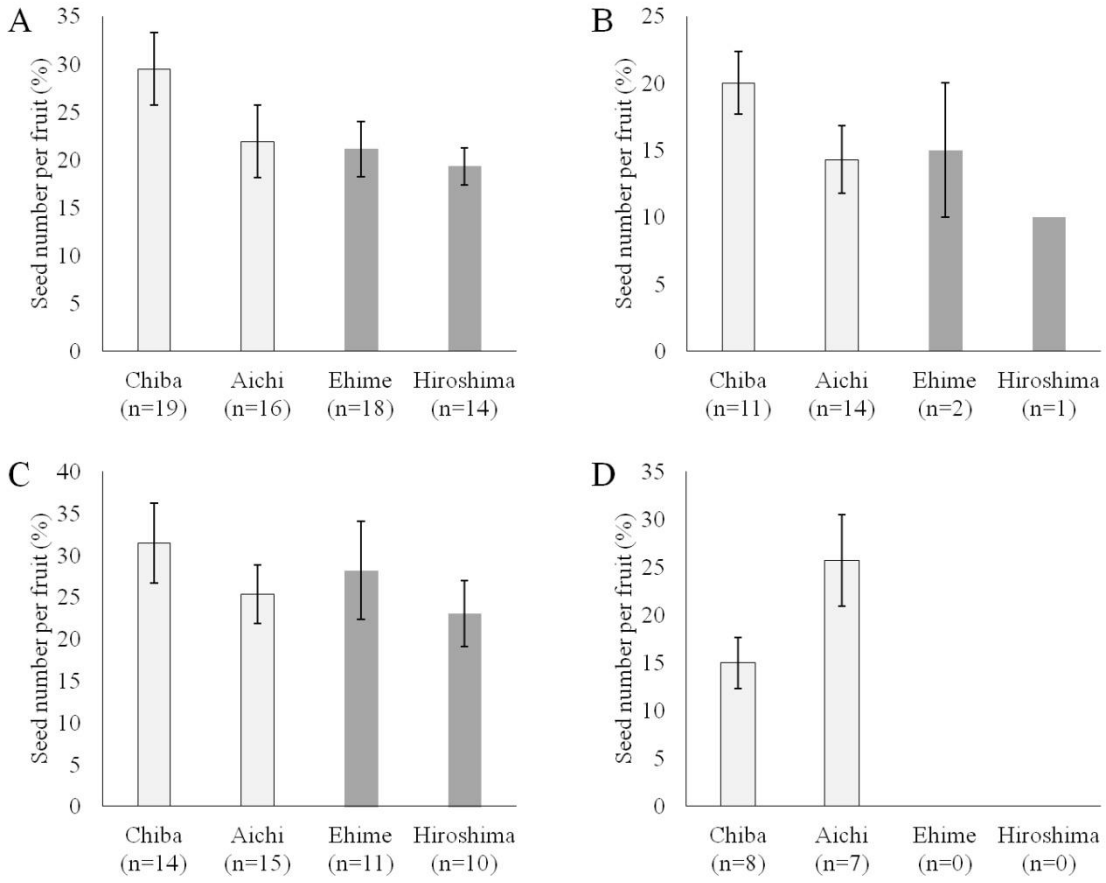
Each circle means individuals, and alphabets in circles show populations. C, Chiba, A, Aichi, E, Ehime, and H, Hiroshima.

Figure 3-2. Fruit set ratios for bagging experiments.



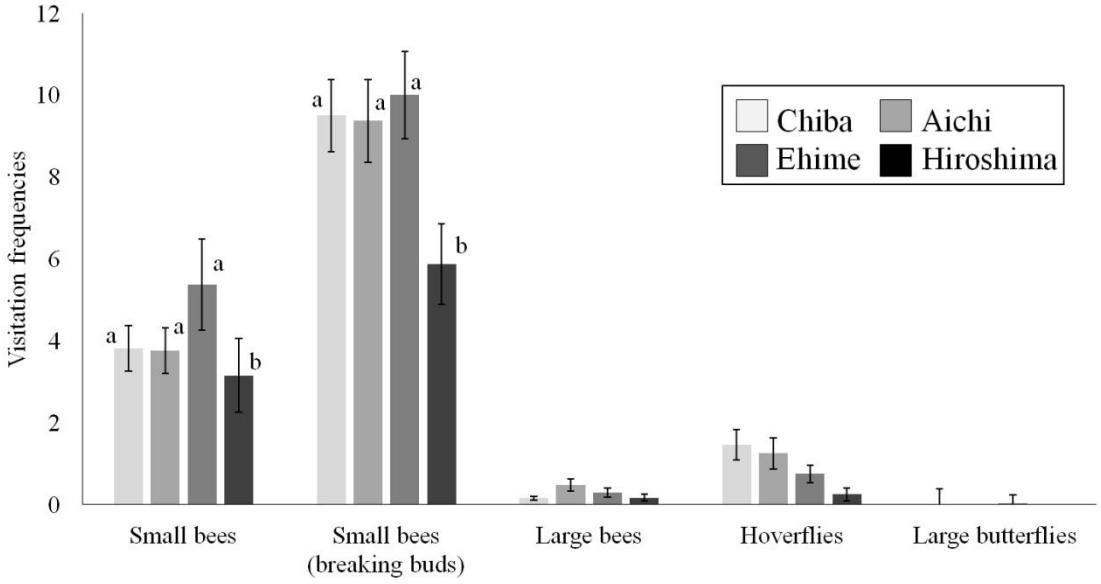
Each bar shows average ratios of each population. Error bars show 1 SE. Same alphabets on average bars show no significant differences between these populations. (A) Control, (B) Breaking bud, (C) Pollen supplementation, (D) Bud pollination.

Figure 3-3. Seed numbers per fruit for bagging experiments.



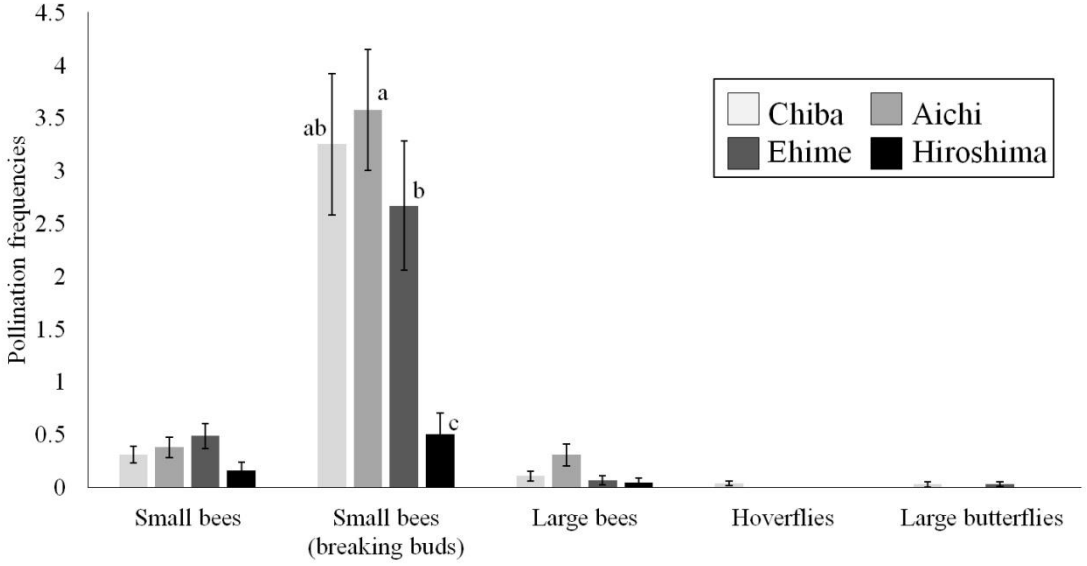
Each bar shows average ratios of each population. Error bars show 1 SE. (A) Control, (B) Breaking bud, (C) Pollen supplementation, (D) Bud pollination.

Figure 3-4. Visitation frequencies of each pollinator group.



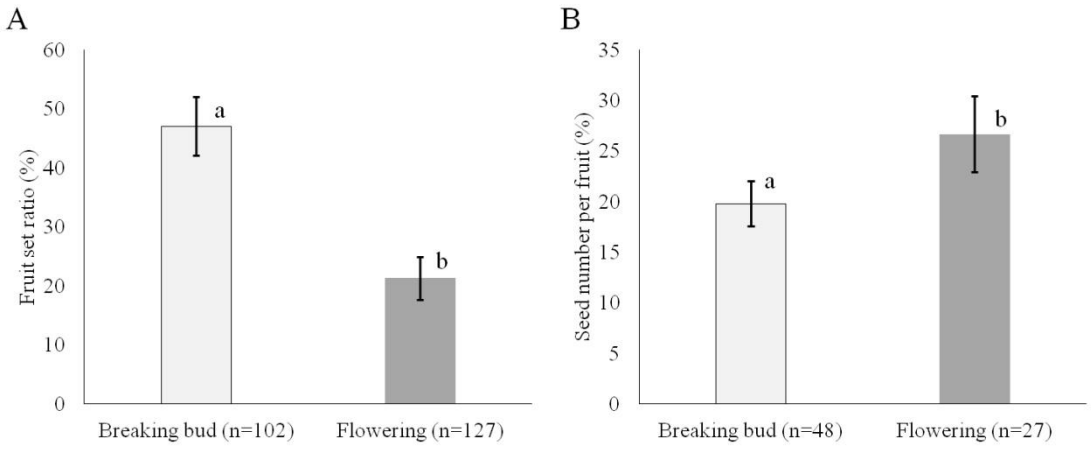
Each bar means average frequencies calculated by visitation numbers per flower per hour. Error bars show 1 SE. Same alphabets on the bars mean no significant differences between these populations.

Figure 3-5. Pollination frequencies of each pollinator group.



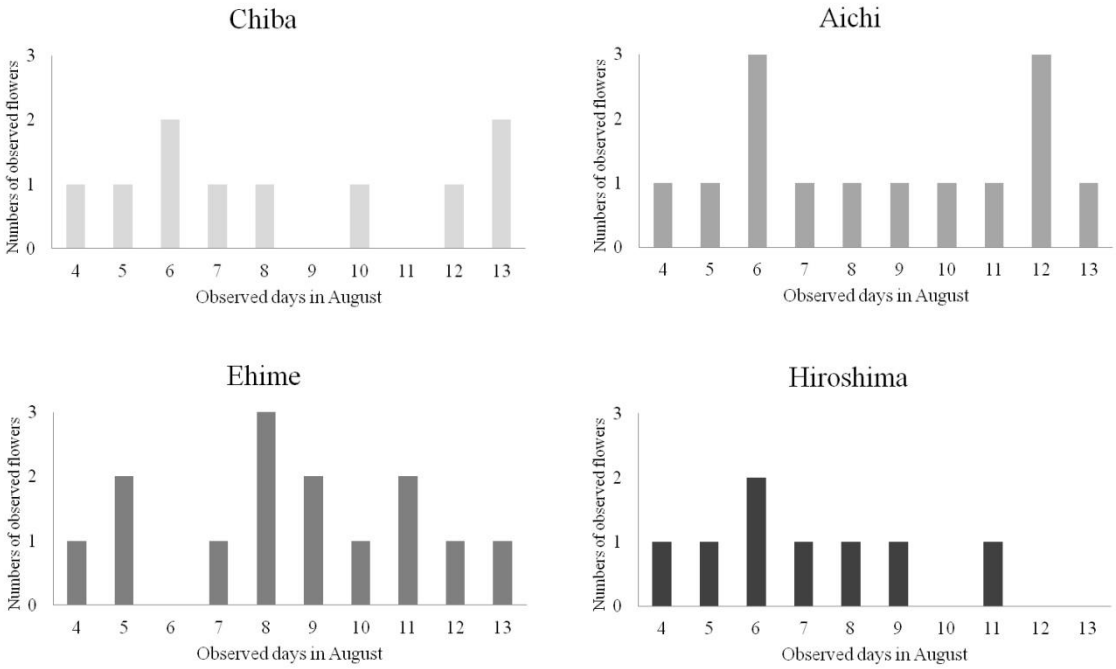
Each bar means average frequencies calculated by pollination numbers per flower per hour. Error bars show 1 SE. Same alphabets on the bars mean no significant differences between these populations.

Figure 3-6. The results of cage experiments.



Each bar means average values and error bars mean 1 SE. (A) Fruit set ratios, (B) Seed number per fruit.

Figure S3-1. Flower numbers for observations of floral visitors in each day.



Each bar showed the numbers of observed flowers.

Chapter 4: Inferences of population structure and divergences in *Lycoris sanguinea*: relationships between population genetics and pollination system

Introduction

Plant-pollinator interactions have been well studied for evolutionary biology and ecology. The differences of pollinator species or functional groups could promote prezygotic reproductive isolation and cause speciation (Kay & Sargent 2009; Willmer 2011). However, the specific mechanisms of pollinator-mediated speciation have remained unclear (van der Niet & Johnson 2014). For understanding the mechanisms of plant-pollinator interactions, genetic or genomic studies in closely related taxa could have been effective methods (e.g. Milano *et al.* 2016).

Lycoris sanguinea var. *sanguinea* has the rare pollination process named as breaking-bud pollination (Yamaji & Ohsawa 2015). This process happens at partially opened stage by small bees *Lasioglossum japonicm*. Some populations of *L. sanguinea* var. *sanguinea* adapt to this pollination system, possibly by moving up the period of stigma receptivity as discussed in the previous chapter. However, we don't know whether these regional floral adaptation to breaking-bud pollination is accompanied with neutral genetic differentiation among populations.

Next generation sequencing technologies enables us to obtain huge amounts of DNA sequence fragments (Baird *et al.* 2008; Peterson *et al.* 2012). Especially, restriction site associated DNA sequencing (RADseq) can be adopted to organisms without genomic information by reading flanking regions of restriction sites for phylogenetic, phylogeographic and population genetic studies (i.e. Andrews *et al.*

2016).

Here we focused on the patterns of genetic structures and population divergences between populations of *L. sanguinea* varieties. We selected nine populations which included three breaking-bud pollinated populations. We used RAD sequencing method for genome-wide single nucleotide polymorphism (SNP) detection. We analyzed genetic data for basically genetic information of each population, population genetic structures and population divergent patterns.

Materials and methods

Plant samples

We selected nine populations of *Lycoris sanguinea* varieties based on the cluster analysis of morphological data, which was conducted in chapter 2 (Figure 4-1). We collected leaf or pericarp samples from 10 individuals from each population. Each sample was dried by silica gel and total DNA of each sample was extracted by CTAB method (Doyle & Doyle 1987). DNA amounts were calculated by Quantus Fluorometer (Promega, USA), and we adjusted concentration of the DNA samples to 20 ng/μl.

Rad sequencing

We adopted double-digested RAD methods (Peterson *et al.* 2012) for the collection of genetic data. RAD sequencing was conducted in Clockmics (Osaka, Japan) as the collaborative research research with Dr. Atsushi J. Nagano, Ryukoku University. Genomic DNA was digested using two restriction enzymes EcoRI and BglII, and the library constructed were sequenced on Hiseq (Illumina), at one lane in single end 50bp reads mode.

RADseq data processing

Processes of RAD-seq data were performed by PyRAD v2.0 (Eaton 2014). PyRAD is de novo assembly software which utilized sequence clustering program VSEARCH, enabling consideration of indel variation. Our raw FastQ data have already been separated by sample barcodes, and we first conducted quality filtering with removal of barcodes, restriction sites and sequence adaptors. We replaced base calls with a quality

scores below 33 to “Ns”, and reads with more than four “Ns” were discarded. Second, we clustered filtered data within and across samples. Within-sample and consensus sequence clusters were built with 85% clustering threshold. Minimum depth of coverage for a cluster was set to four. Finally, we constituted alignment files with filtering for paralog. We set minimum sample numbers in a final locus to four and maximum individuals with shared heterozygous sites to 48.

After the de novo assembly by PyRAD, we used TASSEL software (Bradbury *et al.* 2007) to remove SNPs with Ns in more than 20% of individuals, SNPs with a minor allele frequency less than 5%, and individuals with Ns at more than 20% of SNPs. We also generated SNPs dataset with different filtering parameters, but results of PCoA on the other datasets showed lower resolution. To remove the F_{ST} outlier loci, we used BayeScan v2.1 (Foll & Gaggiotti 2008) with default parameters and a false discovery rate of 0.05. BayeScan estimated F_{ST} coefficient of each loci in global and population levels, and detected candidates which have been subject to selection using a Bayesian method.

Genetic diversity and population genetic analyses

After the remove of outlier loci, we used SPAGeDi software (Hardy & Vekemans 2002) to estimate genetic diversity (He) and individual inbreeding coefficient (Fis) with 1000 permutations of each population. Additionally, pairwise population F_{ST} values were calculated by using GenAlex v6.5 (Peakall & Smouse 2012) with an option of “interpolate missing” and 999 permutations. The graph of Principal Coordinate Analyses (PCoAs) was constructed based on not-standardized F_{ST} distance values by using GenAlex v6.5. Testing of isolation by distance pattern of genetic divergence was

conducted by using Mantel test (Mantel 1967) with 999 permutations.

Bayesian clustering was performed using STRUCTURE 2.3 (Pritchard *et al.* 2000). The admixture model with correlated allele frequencies was used. The model was run with the likely number of clusters (K) set to values from 2 to 9, using a burn-in of 30,000 Markov ChainMonte Carlo (MCMC) iterations followed by 100,000 MCMC iterations. The optimal K was determined based on the method of Evanno *et al.* (2005) implemented in STRUCTURE HARVESTER (Earl and vonHoldt, 2012), by using the data of 10 independent runs. Bar charts representing the proportion of cluster membership in each individual were obtained using CLUMPAK (Kopelman *et al.* 2015).

A number of clusters (*K*) varying from 2 to 9, was evaluated under Admixture and LOCPRIOR models (Hubisz *et al.* 2009) by running 100,000 burn-in Markov Chain Monte Carlo (MCMC) repetitions and 1,000,000 subsequent repetitions based on the LOCPRIOR model. The probabilities of each *K* were averaged over 10 runs. We employed the CLUMPAK server (ref, http://clumpak.tau.ac.il/index.html) to evaluate multimodality among runs at each *K*. The optimum *K* value was determined based on ΔK, evaluating the probability of the data (Ln P(D)) for each *K* value using STRUCTURE HARVESTER. Bar charts representing the proportion of cluster membership in each individual were obtained using CLUMPAK .

Population genetic divergences based on phylogenetic tree

We used SplitsTree v 4.14.4 (Huson 1998) to visualize the phylogenetic networks between populations. We adopted neighbor-net algorithms (Bryant & Moulton 2004)

with default parameters (uncorrected P, ambiguous states ignored, variance with Ordinary Least Squares).

Result

RAD sequencing

Generated total read numbers were 181.9 million reads from 90 individuals. Average read counts per sample were 2.02 million (0.30 – 6.00 million) and average ratio of Q30 scored reads were 95.38% (94.15 – 96.05%). After the filtering of assembled data, 574 loci of 72 samples remained. 25 loci among them were detected as non-neutral loci by BayeScan, and they were removed for the following analyses.

Basic population genetic parameters

The expected heterozygosity (He) and individual inbreeding coefficient (Fis) values in nine populations examined were given in Table 4-1. Pairwise F_{ST} values between populations were shown in Table 4-2.

Population structure

Principal coordinate analysis (PCoA) showed clear genetic differentiation between populations (Figure 4-2). The breaking-bud pollinated populations (Pop9, 10 and 13) were separated from the other populations on the first axis (coord. 1), which explained 21.9% of the variation. On the second axis (coord. 2, 12.73% explained), Pop1 was separated and the other populations were located adjacently from east to west.

In the STRUTURE analysis, ΔK suggested K = 7 as optimal. In k=2, the breaking-bud pollinated populations (Pop9, 10 and 13) were grouped into cluster 1 and the remaining populations were assigned to cluster 2 (Figure 4-3). In k=3, Pop1 was separated from the non breaking-bud pollinated populations, but showed significant admixture with two Kyushu populations (Pop3 and 4). The result in this cluster number

coincided with that of PCoA. In k=4-7, clinal patterns of genetic divergence from east to west were revealed.

The Mantel test showed significant correlations between geographic distances and pairwise F_{ST} values between populations, indicating clear pattern of isolation of distance (Figure 4-4).

Phylogenetic networks

Neighbor-net graphics visualized a pattern of genetic differentiation among the nine populations examined. Populations with breaking-bud pollination (Pop9, 10 and 13) appeared as a distinct group (Figure 4-5). As with PCoA and STRUCTURE, individuals of Pop1 were clearly separated from others. Populations in Kyusyu regions (Pop3 and 4) were located closely. In Shikoku and Chugoku regions, Pop6 and 8 lied next to each other, and an individual of Pop6 was located within an aggregate of Pop5.

Discussion

To reveal the relationships between population genetics and pollination system, we estimated genetic structures among populations by using RADseq data. All of genetic cluster analyses and phylogenetic network showed clear genetic divergence between three eastern populations and the other six ones. Such genetic divergence between eastern and western regions in Japan has been frequently observed in plants and animals (Table 2 in Aoki *et al.* 2011). For example, in glacial and interglacial periods, plant distributions could be restricted in eastern and western refugia. After these periods, populations expanded distribution areas and present distribution patterns were formed (e.g. Aoki *et al.* 2011). Another hypothesis is that genetic divergence could be generated by the events of generic drift through the distributional shift from the past center of distribution (e.g. Tsuda *et al.* 2015; Uchida *et al.* 1997). In this theory, peripheral populations are expected to have lower genetic diversities than the central populations. In the present study, however, genetic diversities (He) did not vary so much among populations, suggesting that severe genetic drift events would be unlikely.

In mantel test and STRUCTURE, we could detect the genetic patterns of isolation by distance. *Lycoris sanguinea* have very limited dispersal abilities of seeds and clonal bulbs (Chung *et al.* 1999). It is known that body sizes of bees and their foraging distances are highly correlated (Greenleaf *et al.* 2007). *Lasioglossum japonicum*, a main pollinator in some populations, has approximately 5-mm body sizes, suggesting short dispersal abilities of pollens (Gathmann & Tscharntke 2002; Greenleaf *et al.* 2007). Other larger bees such as *Amegilla florea* have 15 to 20-mm sizes and they may also carry pollen grains in limited ranges. Therefore, although the long distance

dispersal of pollen grains could be rarely happened (Ahmed *et al.* 2009; Nathan 2006), small and large bees would contribute to gene flow between populations. In contrast, butterfly species could have longer movement distances than bees (e.g. Hovestadt *et al.* 2011). Pollen dispersal abilities between bees and butterflies have not been compared yet, but it could be possible that large butterflies have higher abilities of pollen dispersal distances than bees. They might promote geographical clusters of population genetic structures in *L. sanguinea* observed in STRUCTURE results.

Although an apparent association between breaking-bud pollination and genetic divergence at neutral loci is observed, it is difficult to determine whether the pollinator differences influenced genetic structures of study populations. All of the three populations with breaking-bud pollination were located in eastern areas, and thus it would be also possible that pollinator divergence occurred after the eastern versus western genetic divergence caused by putative geographical isolation events in glacial period(s), as discussed above. In order to resolve this, additional genetic studies including Nagano and Saitama populations (Pop11 and 12 in chapter 2), in which breaking-bud pollination was not observed, would be desired.

In the nine populations examined in this chapter, Pop6 had frequent pollination chances by small and large bees (Figure 2-5). This population showed smaller sizes of floral traits, and can be classified to *L. sanguinea* var. *sanguinea* based on the criteria of Hsu *et al.* (1994). In STRUCTURE analyses, Pop6 was assigned to the same cluster with the nearest population, Pop5 of *L. s.* var. *kiushiana* (Table 2-1), from k=2 to k=5. Neighbor-net phylogenies showed that Pop5 was distantly related to other *L. s.* var. *sanguinea* populations (Pop9, 10 and 13). In the same way, Pop5 and 8 were closely clustered in a group in all analyses, but other two larger-flower populations classified to

L. s. var. *kiushiana* (Pop3 and 4) were separated in phylogenetic networks. These results suggested that patterns of floral morphologies could have been caused independently to the history of population divergences at neutral genetic markers. Our previous works suggested the interactions between floral morphologies and pollination frequencies. Therefore, we suggested that floral morphologies could be affected by regional pollinator differences among *L. sanguinea* populations, and not by population genetic divergences.

References

Ahmed S., Compton S.G., Butlin R.K., Gilmartin P.M. (2009) Wind-borne insects mediate directional pollen transfer between desert fig trees 160 kilometers apart. *Proceedings of the National Academy of Sciences of the United States of America*. 106, 20342-20347.

Andrews K.R., Good J.M., Miller M.R., Luikart G. Hohenlohe P.A. (2016) Harnessing the power of RADseq for ecological and evolutionary genomics. *Nature Review Genetics*. 17, 81-92.

Aoki K., Kato M., Murakami N. (2011) Phylogeography of Phytophagous Weevils and Plant Species in Broadleaved Evergreen Forests: A Congruent Genetic Gap between Western and Eastern Parts of Japan. *Insects*. 2, 128-150.

Baird N.A., Etter P.D., Atwood T.S., Currey M.C., Shiver A.L., Lewis Z.A., Selker E.U., Cresko W.A., Johnson E.A. (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS One*. 3, e3376.

Bradbury P.J., Zhang Z., Kroon D.E., Casstevens T.M., Ramdoss Y., Buckler E.S. (2007) TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics*. 23, 2633-2635.

Bryant D., Moulton V. (2004) Neighbor-net: an agglomerative method for the construction of phylogenetic networks. *Molecular Biology and Evolution*. 21, 255-265.

Chung, M.Y., Chun, C.P., Chung, M.G. (1999) Clonal and spatial genetic structure in a population of the endangered herb *Lycoris sanguinea* var. *koreana* (Amaryllidaceae). *Genes & Genetics Systems*, 74, 61–66.

Doyle J., Doyle J.L. (1987) Genomic plant DNA preparation from fresh tissue - CTAB

method. *Phytochemical Bulletin* 19, 11-15.

Earl D.A., vonHoldt B.M. (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. 4, 359-361.

Eaton D.A.R. (2014) PyRAD: assembly of de novo RADseq loci for phylogenetic analyses. *Bioinformatics*. 30, 1844-1849.

Evanno G., Regnaut S., Goudet J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*. 14, 2611-2620.

Foll M., Gaggiotti O. (2008) A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: A bayesian perspective. *Genetics*. 180, 977-993.

Gathmann A., Tscharntke T. (2002) Foraging ranges of solitary bees. *Journal of Animal Ecology*. 71, 757–764.

Greenleaf S.S., Williams N.M., Winfree R., Kremen C. (2007) Bee foraging ranges and their relationship to body size. *Oecologia*. 153, 589–596.

Hardy O.J., Vekemans X. (2002) SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes*. 2, 618-620.

Hovestadt T., Binzenhöfer B., Nowicki P., Settele J. (2011) Do all inter-patch movements represent dispersal? A mixed kernel study of butterfly mobility in fragmented landscapes. Journal of Animal Ecology. 80, 1070-1077.

Hsu P.S., Kurita S., Yu Z.Z., Lin J.Z. (1994) Synopsis of the genus *Lycoris* (Amaryllidaceae). *Sida*. 16, 301–331.

Hubisz M.J., Falush D., Stephens M., Pritchard J.K. (2009) Inferring weak population

structure with the assistance of sample group information. *Molecular Ecology Resources*. 9, 1322-1332.

Huson D.H. (1998) SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics*. 14, 68-73.

Kay K.M., Sargent R.D. (2009) The role of animal pollination in plant speciation: Integrating ecology, geography, and genetics. *Annual Review of Ecology Evolution and Systematics*. 40, 637-656.

Kopelman N.M., Mayzel J., Jakobsson M., Rosenberg N.A., Mayrose I. (2015) CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*. 15, 1179-1191.

Mantel N. (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*. 27, 209-220.

Milano E.R., Kenney A.M., Juenger T.E. (2016) Adaptive differentiation in floral traits in the presence of high gene flow in scarlet gilia (Ipomopsis aggregate). *Molecular Ecology*.

Nathan R. (2006) Long-distance dispersal of plants. Science. 313, 786-788.

Peakall R., Smouse P.E. (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software foer teaching and research-an update. *Bioinformatics*, 28, 2537-2539.

Peterson B.K., Weber J.N., Kay E.H., Fisher H.S., Hoekstra H.E. (2012) Double digest RADseq:An inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS One*. 7, e37135.

Pritchard J.K., Stephens M., Donnelly P. (2000) Inference of population structure using multilocus genotype data. *Genetics*. 155, 945-959.

Tsuda Y., Nakao K., Ide Y., Tsumura Y. (2015) The population demography of *Betula*

maximowicziana, a cool-temperate tree species in Japan, in relation to the last glacial period: its admixture-like genetic structure is the result of simple population splitting not admixing. *Molecular Ecology*. 24, 1403-1418.

Uchida K., Tomaru N., Tomaru C., Yamamoto C., Ohba K. (1997) Allozyme variation in natural populations of hinoki, *Chamaecyparis obtusa* (Sieb. et Zucc.) Endl., and its comparison with the plus-trees selected from artificial stands. *Breeding Science*.47, 7–14.

Van der Niet T., Peakall R., Johnson S.D. (2014) Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Annals of Botany.* 113, 199-211.

Willmer (2011) *Pollination and floral ecology*. Prinston University Press.

Yamaji F, Ohsawa A.T. (2015) Breaking-bud pollination: a novel pollination process in partially opened flowers by small bees. *Journal of Plant Research*. 128, 803–811

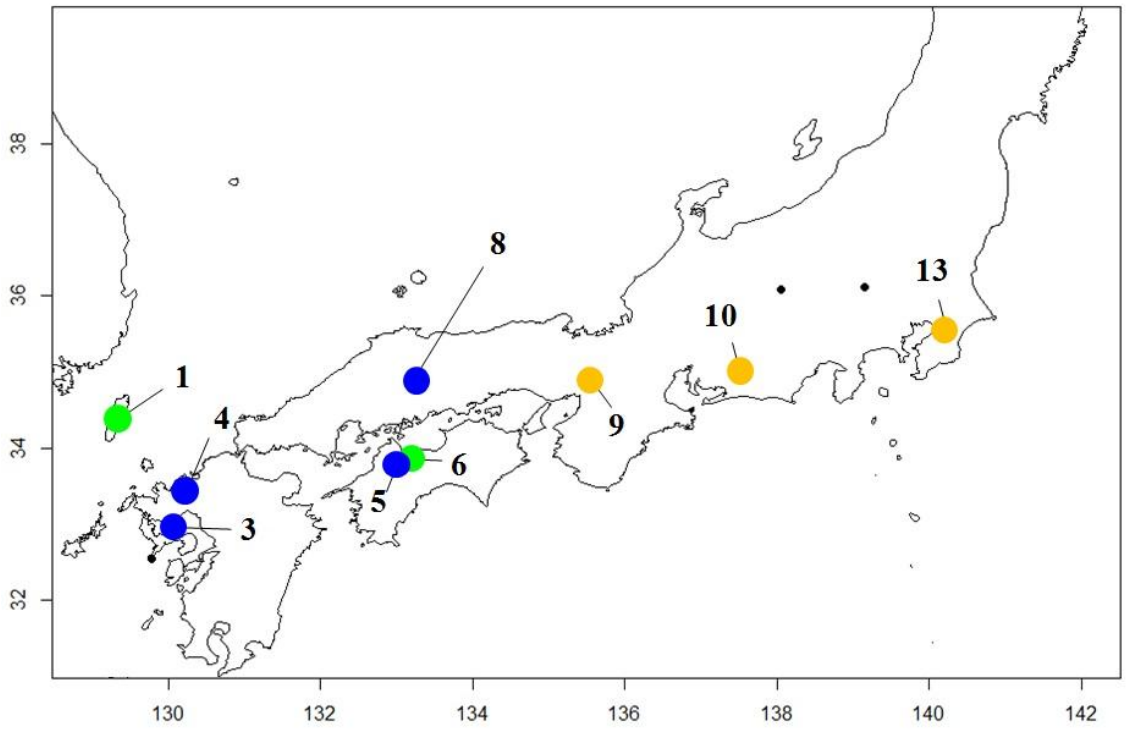
Table 4-1. Genetic diversity of each population.

Populations	Sample size	He	F_{IS}
Pop1	7	0.1966	-0.039
Pop3	9	0.2529	0.238
Pop4	9	0.2388	0.26
Pop5	10	0.1998	0.204
Pop6	7	0.1986	0.198
Pop8	6	0.2223	0.322
Pop9	9	0.1837	0.054
Pop10	9	0.1982	0.129
Pop13	6	0.144	0.216

Table 4-2. Pairwise population F_{ST} values.

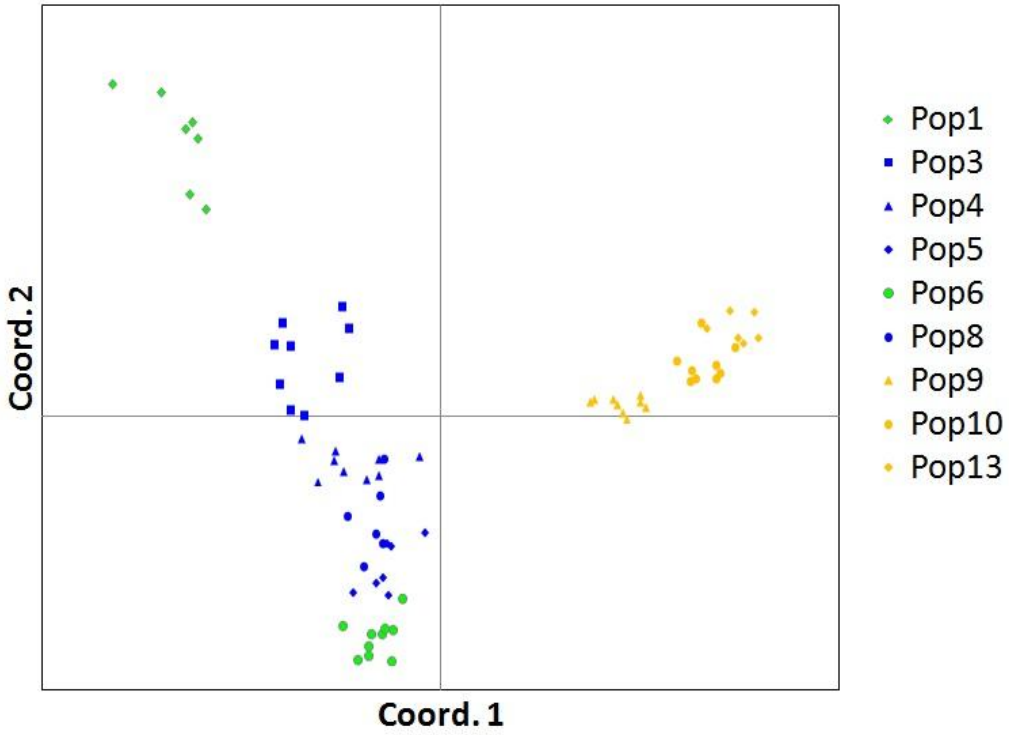
Pop1	Pop3	Pop4	Pop5	Pop6	Pop8	Pop9	Pop10	
0.220								Pop3
0.237	0.062							Pop4
0.340	0.179	0.115						Pop5
0.310	0.161	0.092	0.105					Pop6
0.263	0.134	0.086	0.102	0.079				Pop8
0.355	0.222	0.193	0.240	0.209	0.184			Pop9
0.410	0.250	0.226	0.296	0.276	0.255	0.168		Pop10
0.449	0.303	0.275	0.344	0.336	0.309	0.223	0.179	Pop13

Figure 4-1. Localities of this study sites.



Each number means research population codes. Different color circles mean clusters based on floral morphologies. Blue, large flower groups, Yellow, small flower groups, Green, intermediate groups.

Figure 4-2. The graph of Principal Coordinate Analyses (PCoAs).



Each population is divided by color and shapes. Colors correspond to morphological clusters drawn in Figure 4-1.

Figure 4-3. STRUCTURE results. Each cluster shows different versions of k values.

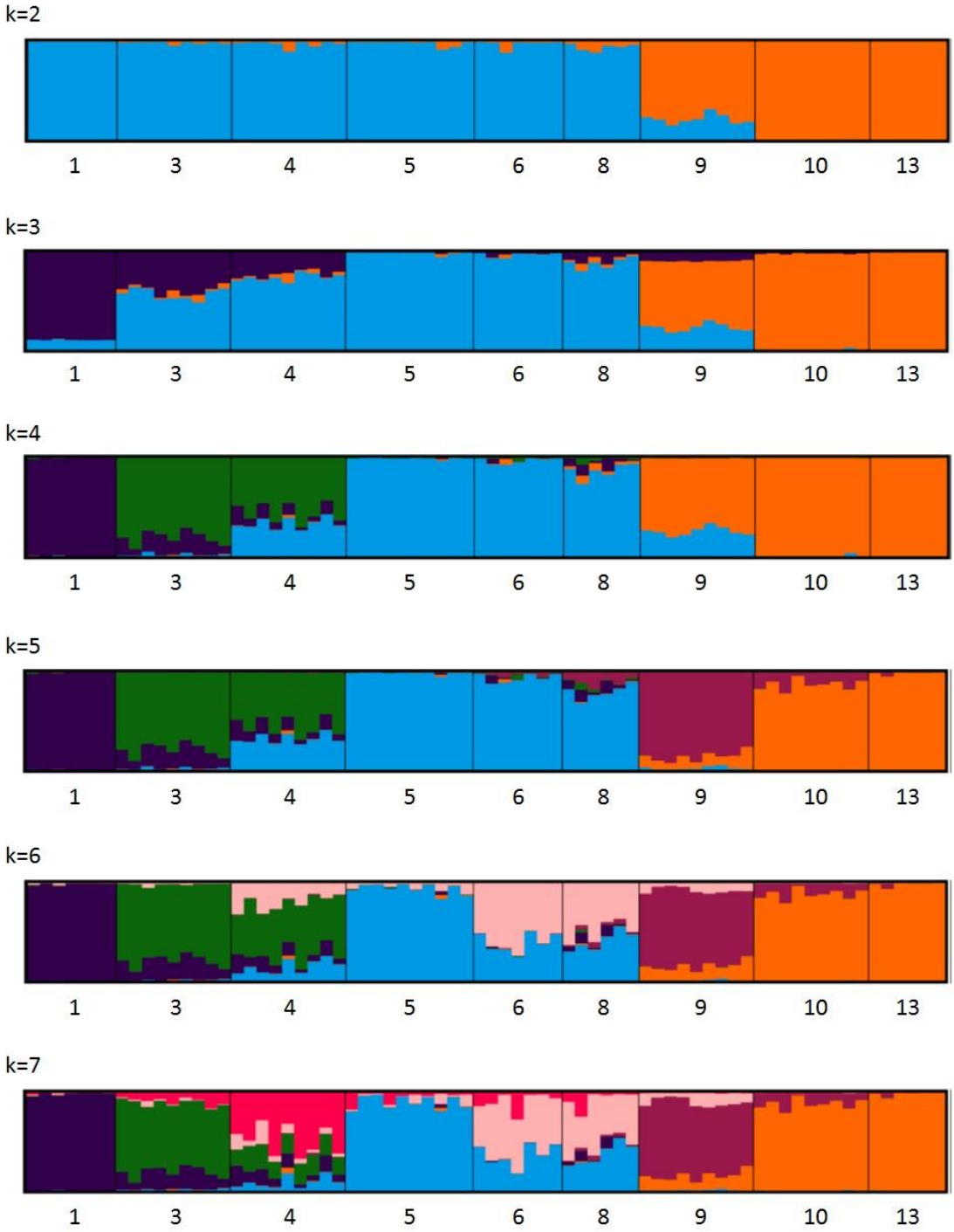


Figure 4-3. Continued.

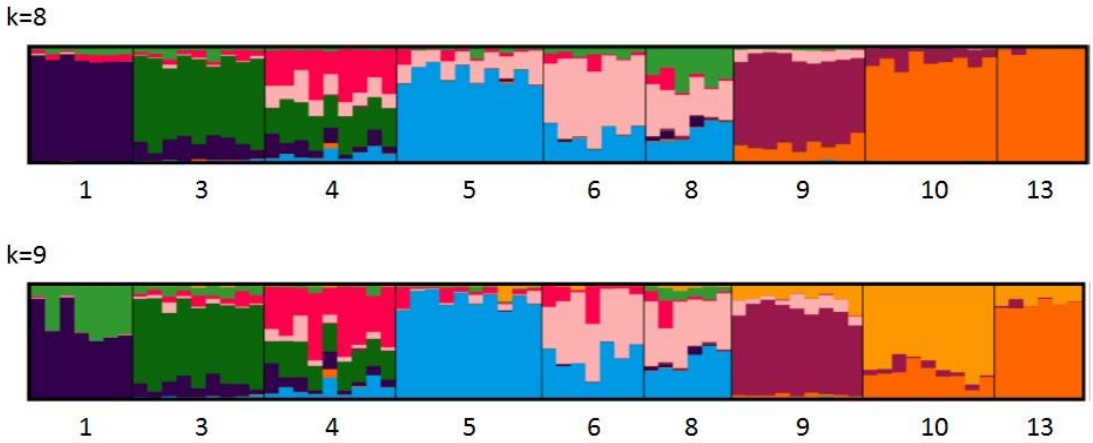


Figure 4-4. Isolation-by-distance relationship between all population pairs.

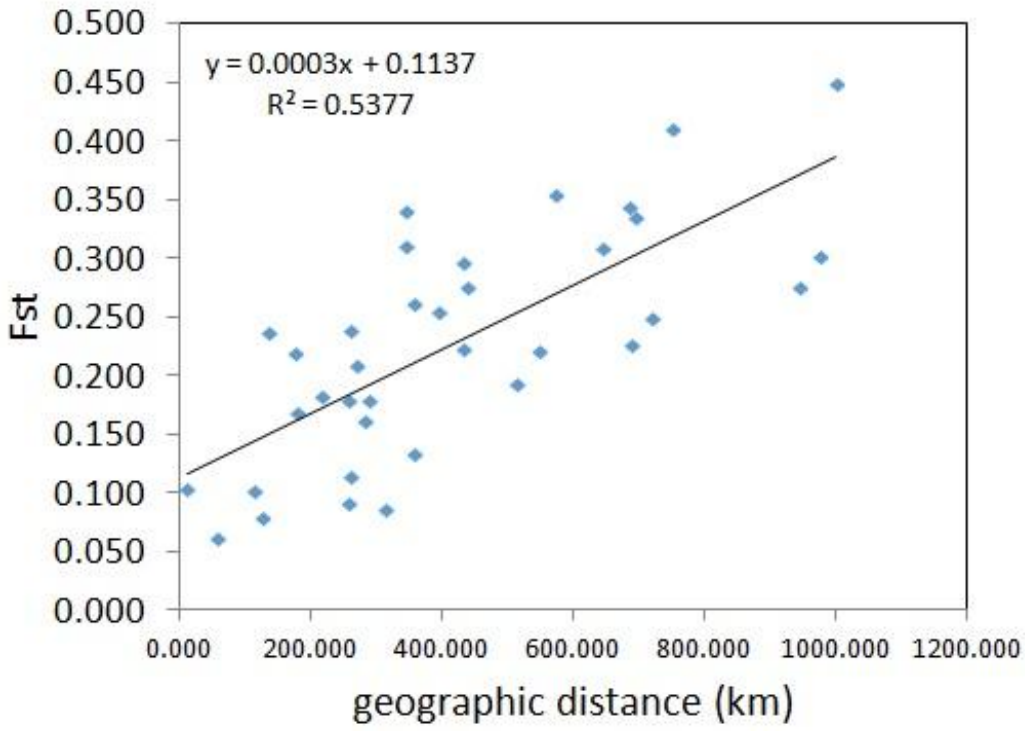
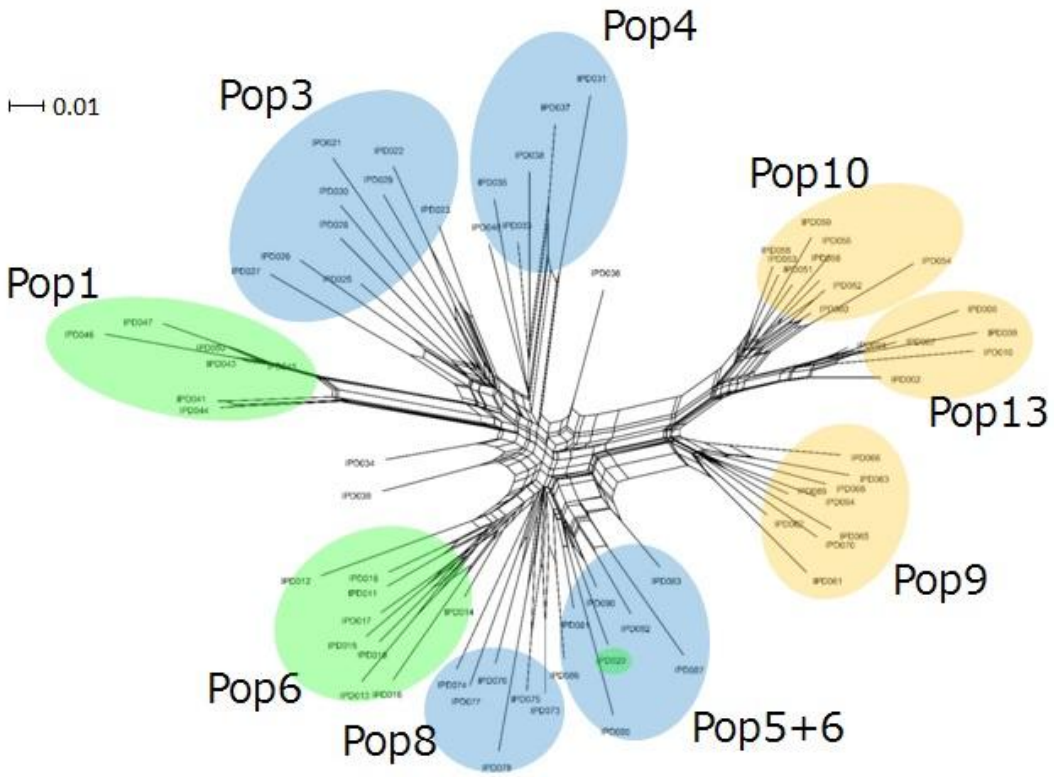


Figure 4-5. Phylogenetic networks built by SplitsTree.



General discussion

Breaking-bud pollination is the first case of a pollination process that is mediated by insect species at partially opened stage. Previous studies about pollinator-associated floral traits were focused on following characteristics; floral color, scent, morphologies and flowering times (e.g. Bradshaw & Schemske 2003; Byers *et al.* 2014; Hall & Willis 2006; Schemske & Bradshaw 1999). These traits promote reproductive isolation by attracting different pollinators or delimiting pollination chances by maladaptive ones. Even when the same pollinators are shared, interspecific pollen transfer can be reduced by the positions of pollen placement and stigma contact on the bumblebee's body surface in *Pedicularis* (Huang & Shi 2013). Compared to these cases, breaking-bud pollination is made possible by hastening the receptive period of stigma, one or two day(s) shift of stigma maturation. In populations with breaking-bud pollination, stigma has already matured in bud stage, and this shift would be favored by natural selection by converting "just a pollen thief" to "effective pollinator". At the breaking-bud stage, small bees collected most of the pollen grains of flowers (Figure 1-3). These pollens collected by small bees could not be used for reproduction because the bees consumed them as the energy and nutrition of their larvae or themselves (Hargreaves *et al.* 2009; Roulston & Cane 2000). This would cause pollen limitation and decrease fitness of *L. sanguinea* var. *sanguinea* individuals. Additionally, differences of pollination effectiveness between breaking-bud and fully-opening stages could also affect fitness. Compared to fully-opened stage, anthers and stigma at breaking-bud stage are spatially closer. Therefore, contact with stigma by small insects collecting pollen grains from anthers could be more likely to occur at breaking-bud stage (Thomson & Plowright

1980). We showed in Chapter 3 that small bees had lower effectiveness at fully-opening stage (Figure 3-6), and hypothesized that these conditions could cause the maturation of stigmas at breaking bud stage. This type of floral adaptation has never been reported and we suggest that this pollination process has novel perspectives in ecology and evolutionary biology.

For the adaptation to small bees at the breaking-bud stage in *L. sanguinea* var. *sanguinea*, the behaviors of small bees visiting breaking buds are the key factor. Previous study suggested that floral traits determined whether the floral visitors acted as pollen thieves because they can smoothly change the behavioral patterns for visiting flowering plants (Hargreaves *et al.* 2012). The present study showed the acceleration of stigma maturation was an important trait for enabling breaking-bud pollination (Chapter 3). Furthermore, entering behavior of small bees into breaking buds is undoubtedly essential and a prerequisite for the evolution of breaking-bud pollination. However, it remains to be answered why breaking-bud pollination is observed only in some of eastern populations in Japan. One hypothesis is that regional abundance of the small bees could bring about the entering behavior into breaking buds through the competition between the small bee individuals for pollen resources of *L. sanguinea*. Another one is that the entering behavior of small bees into breaking buds could be heritable. I tried to examine the phylogenetic relationships of small bees between study populations using mitochondrial DNA regions COI (Figure S1). The result showed the populations 5 and 8 were genetically differentiated from other populations. It should be noted that visitation of small bees to open flowers was observed but not at breaking-bud stage in the two population. Therefore, the genetic backgrounds could reflect the visitation patterns to *L. sanguinea*.

The present study also gave taxonomic implications for *L. sanguinea* varieties. Three varieties of *L. sanguinea* have been classified by the floral morphologies and flowering period. I classified my study populations based on these traits (Table 2-1), and nine populations, which included four populations of *L. s.* var. *sanguinea*, four ones of *L. s.* var. *kiushiana*, and one of *L. s.* var. *koreana* (Figure 4-1), were used for the estimation of genetic structures. However, the taxonomic groups and morphological clusters or the phylogenetic network were not consistent (Figure 2- , Figure 4-5). In contrast, cluster 3, which had the smallest flowers in study populations, was grouped together in neighbor-net tree of populations. Other two clusters were not consistent to the network, and the differences of local pollinator faunas in these populations might be reflected to floral morphology variations as suggested in chapter 4. At least, the populations included in cluster 3 might be identified as another variety in *L. sanguinea*, although the reassessment of floral traits for taxonomical groupings would be needed.

On the other hand, Population 1 in Chapter 4 had clear genetic differentiation to other eight populations. Tsushima Island has been thought to limited distribution area of *L. s.* var. *koreana* (Ministry of the Environment Japan 2015), and the genetic analyses could suggest that individuals of Population 1 were *L. s.* var. *koreana*. However, this population was separately located across the sea and the results of genetic analyses might only reflect the effects of geographic barriers. I also need to compare the individuals of Tsushima Island to those of type localities of *L. s.* var. *koreana* for the identification of species. Further analyses of genetic structures and morphological traits using more samples would be requested.

References

Bradshaw H.D., Schemske D.W. (2003) Allele substitutions at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature*. 426, 176-178.

Byers K.J.R.P., Bradshaw H.D., Riffell J.A. (2014) Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). *The Journal of Experimental Biology.* 217, 614-623.

Hargreaves A.L., Harder L.D., Johnson S.D. (2009) Consumptive emasculation: the ecological and evolutionary consequences of pollen theft. *Biological Reviews*. 84, 259-276.

Hargreaves A.L., Harder L.D., Johnson S.D. (2012) Floral traits mediate the vulnerability of aloes to pollen theft and inefficient pollination by bees. *Annals of Botany.* 109(4), 761–772.

Huang S.Q., Shi X.Q. (2013) Floral isolation in *Pedicularis*; how do congeners with shared pollinators minimize reproductive interference? *New Phytologist*. 199, 858-865.

Kurita, S., Hsu, P.S. (1996) Hybrid complexes in *Lycoris,* Amaryllidaceae. *American Journal of Botany*. 89, 207

Lee, N.S., Kim, M., Lee, B.S., Park, K.R. (2001) Isozyme evidence for the allotroploid origin of *Lycoris flavescens* (Amaryllidaceae). Plant Systematics and Evolution. 227, 227–234.

Ministry of the Environment Japan. (2015) *Red Data Book 2014–Threatened wildlife of Japan, Vol. 8: Vascular Plants.* Gyousei, Tokyo, Japan.

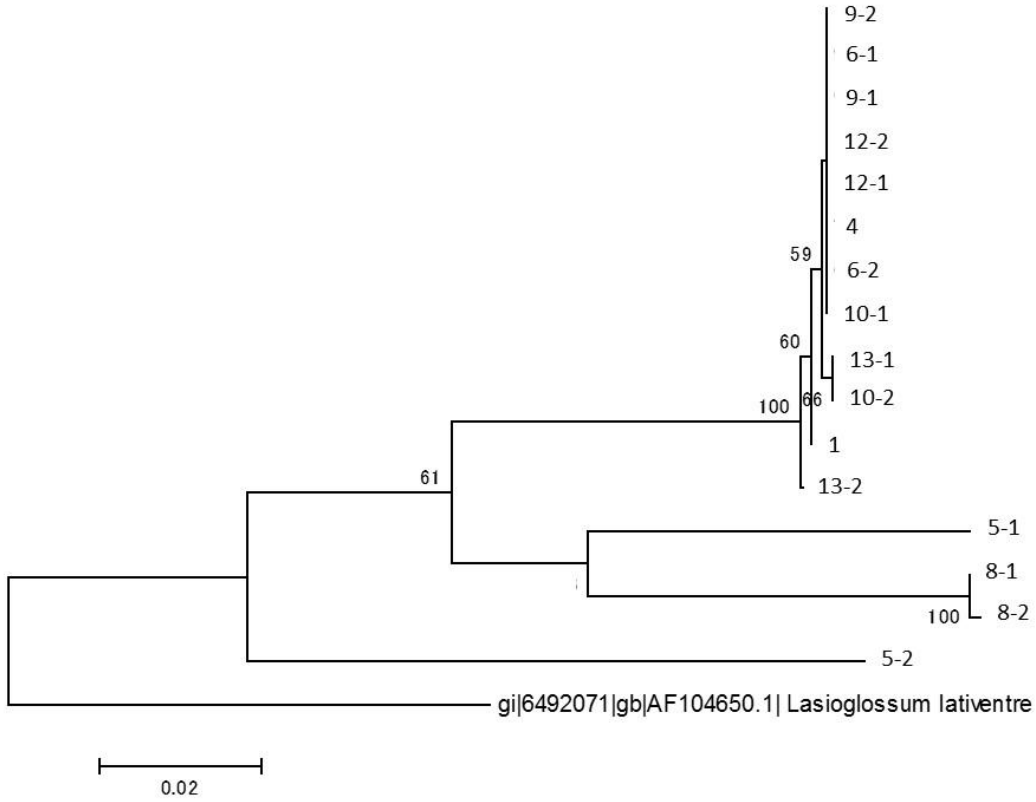
Roulston T.H., Cane J.H. (2000) Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution*. 222, 187-209.

Schemske D.W., Bradshaw H.D. (1999) Pollinator preferences and the evolution of

floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America.* 96, 11910-11915.

Thomson J.D., Plowright R.C. (1980) Pollen carryover, nectar rewards, and pollinator behavior with special reference to *Diervilla lonicera. Oecologia*. 46, 68-74.

Figure S1. Phylogeny of *Lasioglossum japonicum* estimated with a maximum likelihood (ML) analysis of an intergenic mitochondrial region.



The analysis includes 16 samples in 9 populations. Support values are indicated > 50% supported.

Supporting methods

DNA extraction

Lasioglossum japonicum was sampled in nine populations. The bees were collected by pooter (aspirator) when they visited to flowers of *L. sanguinea*. These samples were killed quickly by ethyl acetate and then preserved in 70% ethanol. DNA extraction was conducted based on the method in Montero-Pau *et al.* (2008). I cut a hind leg of each sample and put them in 1.5 mL individual tubes. Then I aliquot 75uL of alkaline lysis reagent (NaOH 25mM, disodium EDTA 0.2mM, pH 8.0) into each tube. After that, I incubate for 1 hour at 95℃ and then cool on ice. Finally, I aliquot 75 uL of neutralization reagent (Tris-HCl 40mM, pH 5.0) and vortex the tubes.

PCR amplification and sequencing

I amplified mitochondrial COI region using the primers named as Jerry-Pat (approximately 900 bp length, Danforth *et al.* 1999). PCR was performed using 2.0 uL DNA extract with 0.03 uL of ExTaq (TaKaRa), 0.5 uL of dNTPs, 0.625 uL of 10×PCR buffer, 1.25 uM of each primer for 6.25 uL volume. PCR conditions were as follows: 5 min for initial denaturation at 94℃, followed by 3 amplification cycles of 1 min denaturation at 94℃, 1 min annealing at 53℃, 1.5 min extension at 72℃, followed by 3 cycles of 1 min denaturation at 94℃, 1 min annealing at 50℃, 1.5 min extension at 72℃, then followed by 25 cycles of 1 min denaturation at 94℃, 1 min annealing at 47℃, 1.5 min extension at 72℃, and a final 8 min extension at 72℃. Amplified DNA was purified using Exonuclease and Alkaline Phosphatase (illstra^{TM}) according to the manufacture's instruction. Cycle sequencing reactions were performed using BigDye

Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Each sample was purified by ethanol precipitation and sequenced in an ABI3500 Sequencer (Applied Biosystems).

Alignment and phylogenetic analysis

Sequence alignments were performed in CLUSTAL W (Larkin *et al.* 2007) and then modified manually. Maximum likelihood analysis was performed using MEGA version 6.06 (Tamura *et al.* 2013). Mitochondrial sequence of *L. lativentre* was extracted from GenBank (accession number AF104650.1) and used as an outgroup for phylogenetic analysis.

Reference

Danforth B.N. (1999) Phylogeny of the bee genus *Lasioglossum* (Hymenoptera: Halictidae) based on mitochondrial COI sequence data. *Systematic Entomology*. 24, 377-393.

Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J., & Higgins D.G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948.

Montero-Pau J., Gómez A. & Muñoz J. (2008) Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs. *Limnology and Oceanography: Methods*. 6, 218-222.

Tamura K., Stecher G., Peterson D., Filipski A., & Kumar S. (2013) MEGA6:

molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution.* 30, 2725–2729.

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