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Y. Isagi · Y. Suyama
Editors

Single-Pollen Genotyping

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Single-Pollen Genotyping

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Cover
Front cover: A beetle on a composite flower of *Erigeron annuus*

Back cover:
Left: Microscopic view of a single pollen grain crushed with a sterile plastic pipette tip on the internal wall of a PCR reaction tube
Middle: A thrip on a *Spiranthes sinensis* flower
Right: Microscopic view of a crushed single pollen grain

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Preface

Pollination is one of the most critical steps influencing the reproductive success of angiosperms and gymnosperms. Determination of the amount of pollen and the pattern and process of pollen dispersal during plant reproduction will contribute to our understanding of the ecology and evolution of gymnosperms and angiosperms. This information is also essential to achieve sustainable and optimal crop yield and is important in agriculture and forestry. The haploid nature of pollen DNA can be utilized in evolutionary genetic studies and genomic analyses. However, direct observation of the movements of pollen grains during pollination is not feasible owing to their small size; therefore, we have been unable to ascertain the pattern of pollen movements. Thus, pollen movement has been estimated by indirect methods, and only limited information is available despite the importance of this information for studies in various disciplines.

Recent developments in molecular biology techniques have enabled the amplification of single-copy DNA from a locus into an amount that can be used to determine the genotype by polymerase chain reaction (PCR). When we published the first basic protocol for single-pollen genotyping in 1996, the focus of the application of this technique was not in the field of ecological research but in paleobotany. However, we were convinced that the technique would open new opportunities in various research areas, especially in pollination ecology. Several years after publication of the protocol for this method, some pioneer ecologists became interested in this technique. They collaborated with us and started using it successfully in their research, and subsequently many other researchers developed an interest in the technique. In 2007, we organized the symposium "Single-Pollen Genotyping" at the 54th Annual Meeting of the Ecological Society of Japan, and compilation of the presentations at that symposium made it possible for the attendees to have a published record of the studies. This volume is mainly based on the material presented at the symposium, but most articles have been considerably revised after the symposium. We are pleased that many of the pioneer researchers have contributed to this publication. The range of topics presented here reflects the wide variety of applications of the single-pollen genotyping technique, and these applications will undoubtedly increase in the near future. We hope that this volume will facilitate sharing of the information about single-pollen genotyping with as many researchers as possible, and that the technique will be used extensively in various disciplines in the future.

We are very grateful to the authors of each chapter for their interesting and valuable contributions. We would also like to thank Lynn Adler, Keith D. Bennett, Elizabeth Elle, Shun Hirota, Akihiko Hoya, Ayako Izuno, Takashi Masaki, Akiko Sakai, Makoto Takahashi, Yoshihiko Tsumura, and Yasuyuki Watano for their helpful comments and their assistance in improving the manuscripts.

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Chapter 1
Significance of Single-Pollen Genotyping in Ecological Research

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1.1 Introduction

Pollination – the transfer of pollen from the anther to the stigma – results in genetic exchange, recombination, and successful reproduction in plants; therefore, it is one of the most important processes that facilitate sexual reproduction in angiosperms and gymnosperms. Because pollen grains are extremely small, direct observation of pollination is impossible, and this limitation imposes constraints on the conduct of pollination studies. However, significant advances in molecular biology techniques in the past two decades have facilitated the analysis of even small amounts of DNA, such as that from a single pollen grain. In this volume, we study the significance and application of single-pollen genotyping in ecological research.

1.2 Pollination Methods Have Diversified During the Past 130 Million Years

Pollen grains develop from microspores, which are formed through meiosis in the stamen (male reproductive organ) and are involved in sexual reproduction in gymnosperms and angiosperms. In animals, mosses, ferns, and several gymnosperms, the sperms transfer genes to the next generation, whereas in angiosperms and most gymnosperms, the sperm nuclei pass on the genes during the process of fertilization. This difference may be attributed to plant adaptation to dry environments, as the male gametes of angiosperms have lost the ability to move using flagella. Therefore, plants were required to develop methods to transfer pollen grains from the anther to the stigma. Pollen grains were transferred by wind in most gymnosperms, which dominated and flourished in terrestrial ecosystems more than 100 million years ago. However, various methods of transfer of microspores or pollen grains in plants have evolved over the years. Various kinds of organisms are known to enhance pollination or fertilization efficiency in organisms other than angiosperms, such as gymnosperms (e.g., Norstog and Nicholls 1997) and mosses (Cronberg et al. 2006). Angiosperms appeared around 130 million years ago (Soltis et al. 2005), and they rapidly spread to various environments and evolved tremendous adaptations to fulfill pollination. Therefore, we can observe a variety of pollination modes in nature, including pollination by wind (anemophily), water (hydrophily), birds (ornithophily), mammals (zoophily), and insects (entomophily). Among these, entomophily may be one of the most diverse methods of pollination, and the ecological and evolutionary success of angiosperms is largely attributable to their coevolution with advanced orders of insects, such as Diptera, Lepidoptera, and Hymenoptera, over the past 100 million years (Schoonhoven et al. 2005). In the first stage of entomophily, insects feed on tissues, secretions, and pollen grains of plants. When the insects that are carrying pollen grains move from one flower to another, they incidentally transfer the pollen grains to the other flower, thereby facilitating cross-pollination and promoting plant fitness. The more attractive the flowers and rewards of plants became, the more frequently they were visited by pollinators and thus enjoyed greater fitness.

Presently, we can observe a wide range of specialization in the relationships between the pollinator and flowering plants. Many plants attract a variety of pollinators, whereas some plants attract only specific pollinators. Each type of relationship has its advantages and disadvantages. The former plants have the advantage of frequent visits from pollinators, but the disadvantage is that their pollen grains will not necessarily be transferred to the stigma of the same plant species. The latter plants attract a narrow range of pollinators, thereby ensuring pollen transfer to the stigma of the same plant species. However, the stronger dependence on these pollinators might cause some vulnerabilities to environmental variation.

1.3 Importance of Studying Pollen Movement

Several studies have been conducted to elucidate the relationships among flower characteristics (morphology, flowering time, duration, etc.), pollinator attraction, and plant fitness. In addition to the findings of these studies, information on the genetic traits of each pollen grain will facilitate better understanding of ecology and evolution of angiosperms. However, to date, few studies have been performed on this aspect. Gene flow is a spatial movement of alleles, and a large proportion of gene flow in plants is achieved by pollen transfer. Therefore, analysis of the patterns and amount of pollen transfer is important for studying the many fundamental aspects needed to understand the regeneration process of a plant population, the amount of gene exchange between individuals and communities, and the degree of genetic isolation in a population. Human impact (habitat loss or degradation, overexploitation, usage of agrochemicals, global warming, etc.) will result in reduction or changes in the number of pollinators. The consequent changes in pollen movement can be used as an indicator to evaluate the effects of human activities, such as forest degradation or fragmentation, on plant communities.

Pollination is also important in applied aspects because it is a requisite for development of fruit- and seed-sets, thereby ensuring an optimal and a sustainable crop yield. Some crop plants are self-incompatible; therefore, their yield is highly dependent on pollen grains from other plant individuals. Moreover, even self-compatible plants require adequate agents to bring a sufficient amount of self-pollen. Knowledge of the pattern and amount of pollen movement is also essential for evaluating one of the risks associated with transgenic crops – escape of the transgenes into related wild species through pollen movement.

1.4 Traditional and Novel Methods to Infer Pollen Movement

Direct determination of pollen movement is complicated, and pollen transfer from the anther to the stigma has been evaluated indirectly by measuring various parameters at the several stages of pollination, such as visitation frequency of pollinators,

amounts of pollen removed from the anthers and/or deposited on the stigma, and number of fruit- or seed-sets. Gene flow through pollen movement has been estimated indirectly by comparing the genotypes of putative parents and the offspring (Devlin and Ellstland 1990; Smouse et al. 2001; Isagi et al. 2004, 2007), observing the frequency of the visitations of pollinators (Galen and Newport 1987; Herrera 1989), counting the number of pollen grains removed/deposited by pollinators (e.g., Herrera 1989; Conner et al. 1995; Muchhala and Potts 2007), and by using a fluorescent dye as a pollen analogue (Murawski and Gilbert 1986; Adler and Irwin 2006). Most of these methods provide no information on the genetic traits or diversity of the transferred pollen grains. Genetic composition will differ among pollen grains that are (1) transferred by pollinators, (2) deposited on the stigma, and (3) inherited to the cohorts of seedlings under the influence of self-incompatibility, pollen-tube competition, pollen viability, and inbreeding depression. Therefore, the pattern and amount of effective gene flow may differ from those of pollen flow.

The single-pollen genotyping technique resolves some of the aforementioned difficulties in pollination studies. This method was developed on the basis of a study conducted by Suyama et al. (1996), who sequenced the DNA of a single fossil pollen grain from Pleistocene peat. Matsuki et al. (2007) modified the method of Suyama et al. (1996) and succeeded in conducting multilocus genotyping of a single pollen grain in a polymerase chain reaction (PCR) tube (the method is described in Chap. 2). This method enabled various analyses, such as analysis of parentage, discrimination of self-pollen, and evaluation of the genetic diversity of pollen grains collected from a pollinator. In this book, we have provided the following examples of ecological analyses conducted with this novel method: pollen transfer in a plant population (Chaps. 3–6), evaluation of the functions of different pollinators (Chap. 3), analysis of the genetic traits of pollen grains obtained from pollinators (Chaps. 3, 5, and 6) and styles (Chap. 4), comparison of genetic traits between pollen grains and seeds (Chaps. 4 and 6), and effect of biological invasion during competition for pollination (Chap. 7). Single-pollen genotyping provides useful information about not only pollen grains of the biota of this age but also about those belonging to ancient times. Information from fossil samples can be used to study the processes of migration, microevolution, and the demographic histories of plants. The method for DNA analysis from fossil pollen is described in detail in Chap. 8.

By taking advantage of the haploid nature of pollen grains and the large numbers of pollen grains produced, single-pollen genotyping would enable us to do recombination analysis and construct a linkage map more easily than with conventional pedigree analysis of inbred lines from experimental crossing. The haploid genome of the pollen grains also facilitates DNA sequencing of nuclear loci. The possible applications of single-pollen genotyping in evolutionary genetic and genomic research are explained in Chap. 9.

Readers will find the examples described in each chapter interesting, and single-pollen genotyping will be useful for studies in pollen biology, reproduction biology, plant breeding, forestry, agriculture, horticulture, forensic science, conservation ecology, and other disciplines.

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Chapter 2
Procedure for Single-Pollen Genotyping

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2.1 Introduction

The single-pollen genotyping technique has opened new opportunities in various research areas such as pollination ecology and evolutionary and genomic research. This technique will facilitate breakthroughs in a wide range of disciplines. For example, in plant population analyses, this technique will allow the estimation of pollen flow by directly tracking the actual movement of individual pollen grains. Further, in genomic studies this technique will enable direct analysis of the haploid DNA sequence of single pollen grains. The protocol described in this chapter is simple yet efficient, and it can be applied to a large variety of pollen grains from different origins. This report provides a comprehensive description of the laboratory protocol for this technique, thereby facilitating sharing of this information with as many researchers as possible.

The basic protocol for this technique was originally developed more than a decade ago by Suyama et al. (1996) and then modified by Matsuki et al. (2007). Details of the methods used for the extraction of ancient DNA (aDNA) from fossil pollen are presented in the study performed by Parducci et al. (2005). Recently, several approaches have been used for DNA amplification from pollen (Petersen et al. 1996; Ziegenhagen et al. 1996; Aziz et al. 1999; Matsunaga et al. 1999; Widmer et al. 2000; Cozzolino et al. 2005; Paffetti et al. 2007; Zhou et al. 2007; Aziz and Sauve 2008; Chen et al. 2008; Ito et al. 2008; Matsuki et al. 2008; Hasegawa et al. 2009). In this chapter, I describe additional modifications to the original method. Further, it is important to emphasize that although the basic technique is easy to perform and amenable to modification, reproducible and reliable results are obtained only if the researchers follow appropriate laboratory procedures, particularly during the polymerase chain reaction (PCR) analyses, and handle the pollen grains with great care to avoid contamination. This technique can be applied to other small biological samples such as mycological (conidial mass: Iwamoto et al. 2002, 2005; spores: Sato et al. 2005) and microbiological materials (oocysts: Honma et al. 2007).

2.2 Materials

2.2.1 Clean Workspace

The sample manipulations preceding PCR should be performed in a separate room isolated from the one in which DNA extraction and amplification are performed. At least two rooms (one pre-PCR and one post-PCR room), and ideally three rooms (a third room for sample handling), are required to effectively avoid contamination during the entire process. Separate sets of clean equipment and laboratory coats should be used in each room. The benches (vibration-free type) and workspace for sample handling must be clean. The bench surface should be regularly and properly cleaned.

2.2.2 Stereomicroscope

A stereomicroscope with a 10×–40× zoom lens and an appropriate light source is highly recommended. A wide working space is also required for proper manipulation of the samples.

2.2.3 Laboratory Tools and Equipment

Laboratory tools and equipment include a micropipette and disposable tips, disposable petri dishes, glass microscope slides, PCR tubes, and a thermal cycler. The other basic laboratory tools for molecular biology analyses are not listed here. All glassware, plasticware, and solutions should be regularly sterilized or autoclaved. Clean lab coats and disposable sterilized gloves should be always worn while performing experiments.

2.3 Methods

2.3.1 Sample Handling

All the pollen samples (e.g., pollen from insect bodies, flowers, birds, soil from sediments, or microscope slides) are normally stored in a freezer before laboratory manipulation. Depending on the nature of the sample, the pollen grains are collected from the sample by using a needle, a pipette tip, or a stretched plastic pipette tip using a washing solution. Details of the protocol for collection of pollen samples from insect bodies (Chaps. 3 and 6), styles or stigmas of female flowers (Chaps. 4 and 7), beaks of birds (Chap. 5), microscope slides smeared with Vaseline grease (Box 2.1), and lake sediments (Chap. 8) are presented in this book.

After collection, pollen grains are individually separated and morphological examination is performed. Subsequently, after identification of the pollen grains, those with an intact structure are selected under the stereomicroscope and separated using standard micropipettes and tips. Each selected pollen grain is then transferred to a sterile distilled water drop placed on a clean hydrophobic microscope slide or a disposable petri dish and washed repeatedly with several drops of sterile distilled water (Fig. 2.1). During this step, the individual pollen grains can be separated by moving them from one water drop to the next. Manipulation of the pollen grains in less than 0.2 μl water is facilitated by optimal pipetting using standard micropipettes and tips with 0.5–10 μl capacity under a standard stereomicroscope. It is important to hold the micropipette and the tip extremely steady during the process and to press/release the piston of the pipette slowly and as smoothly as possible.

After collection, individual pollen grains are transferred to a DNA-free PCR tube with less than 0.2 μl water (Fig. 2.2).

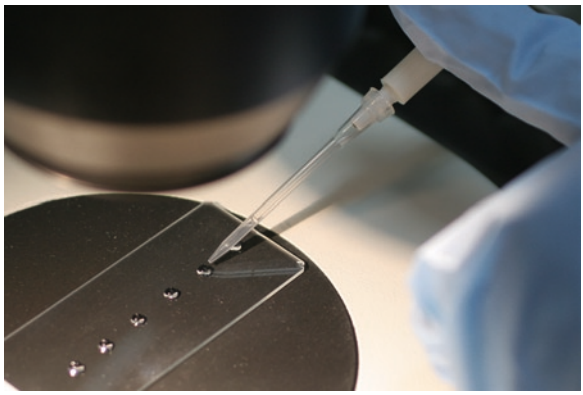

Fig. 2.1 Pollen grains are repeatedly washed in sterile water drops on a hydrophobic microscope slide or a disposable petri dish and then transferred to a polymerase chain reaction (PCR) tube

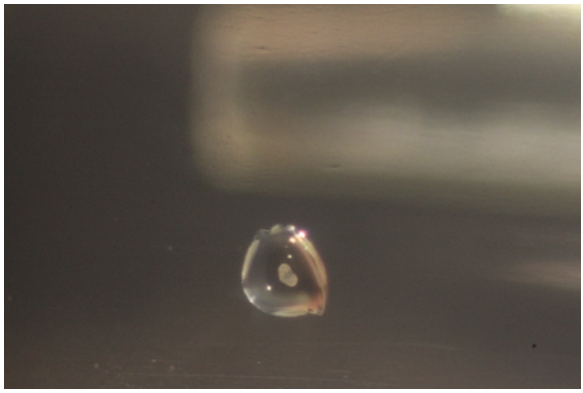

Fig. 2.2 A single pollen grain of *Picea abies* present in a drop of sterile water (<0.2 μl) is falling into a DNA-free PCR tube. The picture was taken through the wall of the PCR tube with a sterile plastic pipette tip (20–200 μl) in the background at approximate 30-fold magnification

2.3.2 DNA Extraction

To minimize the loss of DNA and to avoid possible contamination during DNA extraction, this process is simplified as much as possible. To avoid the loss of pollen material, the entire extraction step is performed in the same PCR tube used for PCR amplification.

Subsequently, 1–2 μl extraction buffer containing 0.01% sodium dodecyl sulfate (SDS), 0.1 g/l proteinase K, 0.01 M Tris–HCl (pH 7.8), and 0.01 M ethylenediaminetetraacetic acid (EDTA) is added to the PCR tube containing the pollen grain. The PCR tube is then closed and incubated at 37ºC or 56ºC for 60 min and at 95ºC for 10 min. During extraction, the pollen grains are differently affected depending on the plant species and the characteristic features of the grain; therefore, optimization of the extraction processes for individual systems should be encouraged. In some cases, pollen grains can be used directly as PCR templates, thereby avoiding the need for an extraction step. However, if the pollen wall is resistant to the DNA extraction agents, the grains may have to be crushed before

extraction. In this case, the grain is pressed against the wall of the tube by using a disposable pipette tip (20–200 µl) (Fig. 2.3), and the disrupted particles in the water drop can be visualized under the microscope. To avoid the loss of particles, the pipette tip should be carefully washed in the PCR tube before disposal (Fig. 2.4). The crushing step is not easy and requires some practice. The solution can then be directly used for PCR or as a template for whole-genome amplification (Matsuki et al. 2007; see Chap. 9).

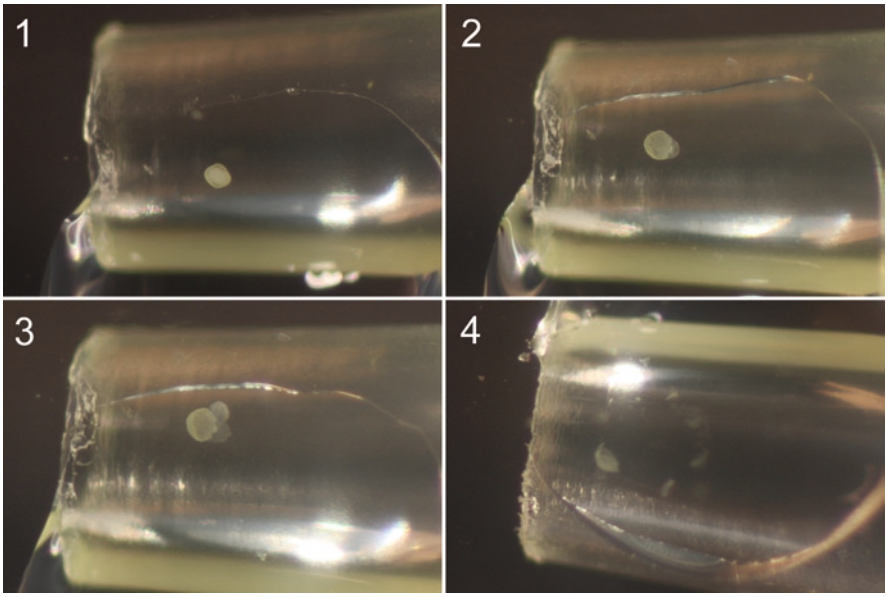

Fig. 2.3 A single pollen grain of *Hemerocallis* is pressed against the wall of the PCR tube and is crushed using a sterile plastic pipette tip (20–200 µl) (steps *1* through *4*). The picture was taken through the wall of the PCR tube with the pipette tip in the background

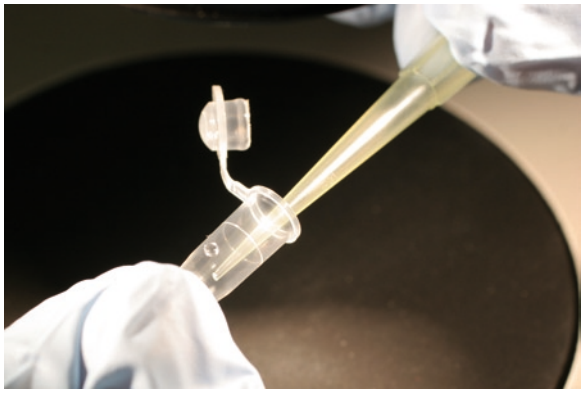

Fig. 2.4 The pipette tip is washed with the extraction solution in the PCR tube to avoid loss of pollen material

2.3.3 PCR

All PCR conditions, including primer design, annealing temperature/time, number of cycles, and selection of polymerase should be optimized for the amplification of extremely small amounts of template DNA.

The primers must be target specific and show 100% matching with the target sequence. Normally, a low level of mismatch does not cause serious problems if PCR is initiated using a large amount of template DNA. However, in the case of pollen, the amount of template DNA is extremely low, and common or universal primers are not recommended because even a low level of mismatch can affect the experiment (see Box 2.1) (Ito et al. 2008). The primer pair should also meet other general requirements for optimal PCR, such as the absence of dimer and hairpin structure formation and the ability to form a stable duplex with the template sequence. Therefore, designing optimal PCR primers and using primer design software is highly recommended. Finally, high success rates of amplification are generally achieved when the target region for the amplification is short (less than ~200 bp) (see Chap. 9).

The annealing temperature should also be optimized for each system depending on the choice of primers, polymerase, and the type of thermal cycler used. As a general rule, the number of cycles is increased up to 40 cycles (5–10 cycles more than the standard PCR protocol) because of the low amount of template DNA.

Box 2.1 Airborne-Pollen Pool Compositions Revealed by Single-Pollen PCR Technique

The single-pollen PCR technique was used to reveal airborne-pollen pool composition in a hybrid zone between *Pinus pumila* and *Pinus parviflora* var. *pentaphylla* on Mount Apoi (810 m above sea level, 42º06′N, 143º02′E), Hokkaido, Japan (Ito et al. 2008). *P. pumila* is a creeping shrub that dominates the vegetation zone above the forest limit of the high mountains in Japan, whereas *P. parviflora* var. *pentaphylla* is a tall tree in the subalpine to montane zones. Hybrid zones, which are spread over several mountains, are observed in the ecotonal zone of the two species, and these zones provide an opportunity to investigate the nature of interspecific gene flow.

Airborne pollen grains were collected by using Durham pollen samplers (Fig. 2.5) at four stations at altitudes of 200 m, 365 m (*P. parviflora* var. *pentaphylla* zone), 520 m (the transitional zone of the two species), and 590 m (*P. pumila* zone). The pollen grains were collected on two microscope slides smeared with Vaseline grease. The slides were replaced by new ones every day, except on rainy days, during the flowering period in June 2003. The slides on which pollen grains had been collected were immediately protected by coverslips to prevent contamination and then stored in a refrigerator at 4ºC. One of the two microscope slides at each site was used for counting pollen density, and the pollen on the other slide was used for single-pollen

(continued)

Box 2.1 (continued)

Fig. 2.5 A Durham pollen sampler is used to capture airborne-pollen grains in the field. Two microscope slides smeared with Vaseline grease are set in the sampler

PCR analysis. Because chloroplast (cp) DNA is paternally inherited in *Pinus* species, the pollen pool compositions of each species can be identified by determining the frequencies of the cpDNA haplotypes of the pollen grains. The cpDNA types of pollen grains were determined by PCR-single-strand conformation polymorphism (SSCP) analysis of the region from *trn*L (UAA) 3′-exon to *trn*F (GAA). The universal PCR primer set (Taberlet et al. 1991) was suitable for DNA from adult plants, but it was not suitable for single-pollen PCR; therefore, a PCR primer set designed for Pinaceae species (Suyama et al. 2000) was employed. A single *Pinus* pollen grain was selected from the microscope slide by using a needle under a stereomicroscope. PCR amplification from a single pollen grain was performed using the method of Suyama et al. (1996). On the basis of the pollen density counts and the flowering phenology, we selected 5 days (9, 11, 13, 16, and 19 June) for performing single-pollen genotyping. On each day, more than 30 pollen grains from each pollen collection site were haplotyped.

The frequencies of *pumila*- and *parviflora*-type cpDNAs in the airborne-pollen pool were well reflected both by species composition at each pollen collection site and by the difference between the flowering times of the two pine species. Pollen grains with *parviflora*-type cpDNA, a mixture of the two types, and *pumila*-type cpDNA were predominant at lower altitudes, the transitional zone, and higher altitude, respectively. The results showed that the two pine species may form F_1 hybrids. Further, the data obtained from comparison between the airborne-pollen pool and paternal parents of sired seeds (data not shown here) indicate the existence of a post-pollination hybridization barrier.

However, excessive cycles should also be avoided because they can produce nonspecific amplification fragments and chimera products. The optimal reaction volume during PCR may vary depending on the amount of extract solution available. Generally, a reaction volume of about 10–20 μl containing 1–2 μl extract solution gives good results. Further, the final concentration of SDS in the reaction volume during PCR should not exceed 0.005% or the reaction will be inhibited.

A large number of DNA polymerases and PCR kits designed to produce high yields of DNA are now available in the market. For multilocus genotyping, a multiplex PCR kit may be the kit of choice.

2.4 Notes

Contamination is the most serious problem for any researcher working with single-pollen genotyping techniques.

Physical isolation between pre-PCR and post-PCR analyses is the standard method to avoid contamination. Physically separated rooms/space/equipment should be also used for the different steps of the process, that is, sample handling, PCR preparation, and post-PCR analysis.

Temporal isolation is also effective against contamination. For example, the researchers should not return to the sample handling room and the pre-PCR room during the same day after treating the PCR product. Further, laboratory work should be avoided during the period corresponding to pollen release in the natural environment, particularly when researchers are working with wind-pollinated species.

Finally, experiments should be always designed to include negative controls (i.e., samples that do not contain DNA template), and these controls should yield negative results.

2.5 Conclusions

The single-pollen genotyping technique presented in this chapter is relatively easy and only requires a few basic molecular biology techniques. Therefore, this technique can be easily performed by any researcher new to this method. However, the researcher should be aware that the major problems or difficulties associated with this technique are caused by the extremely small amounts of template DNA available for PCR. Therefore, adequate care should be taken during all procedures, or contaminations can become persistent.

Although the single-pollen genotyping technique presents some difficulties, it already has produced an impressive list of accomplishments. Recent studies performed by many of the researchers who pioneered the application of this technique are presented in this volume (Chaps. 3–8). The range of subjects presented in these studies reflects the wide variety of applications of this technique, and these applications will undoubtedly expand in the near future.

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Chapter 3
Pollination Efficiencies of Insects Visiting *Magnolia obovata*, as Determined by Single-Pollen Genotyping

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3.1 Introduction

To exchange and transmit genes to the next generation, flowering plants need to transfer their pollen to the stigmas of other conspecifics and, reciprocally, receive pollen grains on their own stigmas. In this pollination process, quantitative and qualitative factors of transported pollen grains (e.g., amount of transported pollen and the genetic composition of these pollen grains) are key elements, because these factors will determine the reproductive success and fitness of plant individuals and the genetic structure of the population. The cost of self-pollination and the advantage of outcross pollination could differ depending on the characteristics of each plant species, such as degree of inbreeding/outbreeding depression and self-incompatibility, and the effective population size (e.g., Lloyd 1992; Goodwillie et al. 2005; Moeller and Geber 2005). The traits of transported pollen grains will influence the pollination systems and the reproductive strategies of each plant species (Barrett 2003). Pollen grains are transported by biotic and abiotic vectors, such as insects, birds, water, and wind. In particular, angiosperms and pollinating insects have coevolved over the past 100 million years (Schoonhoven et al. 2005), and diversified systems of insect pollination have been successful. To understand the evolution of interaction between plants and pollinators, to design conservation and management strategies, and also to adapt plants for economic uses, it is necessary to clarify the various and complex plant mating systems and plant–pollinator interactions.

Here, we show the latest approach, which directly determines the genotypes of pollen grains transported by insects and evaluates the pollination efficiencies of each flower-visiting insect (Matsuki et al. 2007, 2008).

3.2 Traditional Approaches to the Study of Pollination Ecology

Clarifying the interactions between plants and pollinators in the pollination process is a central topic in plant ecology and evolutionary biology. Many researchers have tried to track pollen movement, and several methods for the visualization of pollen movements have been attempted. Because it is difficult to observe pollen grains and pollen movement with the naked eye, in early experiments, biologists used radioisotopes (Colwell 1951) or neutron activation bombardment (Gaudreau and Hardin 1974) to mark pollen and to track pollen movement. Fluorescent dyes have also been used as a pollen label to estimate the movement of pollen and pollinators (Murawski and Gilbert 1986; Adler and Irwin 2006). In addition to tracking pollen, one of the long-standing challenges for pollination ecologists has been the distinction of pollen donor plants. Color polymorphisms in pollen grains (Jorgensen et al. 2006) and differences of ploidy (Williams et al. 1999) can be assessed, but the discrimination is relatively poor and is restricted to certain species.

Recently, gene transformation techniques that can distinguish specific pollen genotypes in vivo have also been used (Havens and Delph 1996; Hudson et al. 2001). These measurement techniques, including marking pollen, using pollen analogues, or using transforming genes, have clarified parts of the pollination process. However, even using these techniques, it is hard to provide information on the genetic composition or diversity of the pollen grains that are transported to flowers in natural populations. To determine the genetic traits of pollen grains, we have to distinguish among many pollen donors spread across a wide area.

The past 20 years have seen remarkable developments in the application of molecular biology techniques to ecological research. It has become easy to genetically distinguish hundreds of individuals using genetic markers with high polymorphism. Recently, molecular techniques have been applied to address many ecological phenomena. For example, parentage analysis between progenies and candidate parents enables inferences about the distance and direction of seed or pollen dispersal, allowing for the direct examination of gene flow patterns (Campbell 1998; Isagi et al. 2007). Statistical methods have also been improved, and we can now detect pollen movement on a large scale (Dick et al. 2003; Latouchee-Hallé et al. 2004; Kenta et al. 2004).

In conventional approaches of parentage analysis of plants, seeds collected from mother trees, established seedlings, or saplings have been used as the progeny samples. However, the progeny of plants often undergo pollen-tube competition and inbreeding depression. Thus, the genetic traits of the progeny do not necessarily directly reflect the genetic traits of pollen grains that are transported to flowers. In the pollination study, clarification of the genetic composition of pollen grains transported by flower visitors is important for the evaluation of the pollination efficiencies of each pollinator species. The differences of pollination efficiencies among pollinators closely relate to the evolution of the pollination system and coevolution between plants and pollinators. To introduce molecular technique to pollination studies and assess the pollination process from the genetic aspect, Matsuki et al. (2007) developed a method for direct genetic analysis of pollen grains that allows pollen from different pollen donors to be distinguished. This method will facilitate a deeper understanding of pollen movement and the roles of pollinators in various pollination systems.

3.3 Target System and Genetic Analysis

3.3.1 Study Species

Angiosperms have achieved great success, and one of the factors that caused their prosperity is the adaptation of their reproductive organs to pollination by animals. In insect-pollinated plants, flowers are often visited by a variety of insect species (Herrera 1987; Adler and Irwin 2006). These insects vary widely in morphological and behavioral features; thus, they may have different effects on the reproductive

success, fitness, and population structures of pollinated plants. Although various groups of insects, such as bees (Hymenoptera), butterflies (Lepidoptera), and flies (Diptera), contribute to plant reproduction as pollinators, beetle pollination is considered to be one of the most long-standing systems among insect pollination. Beetles primarily pollinate plant species that retain primitive characteristics (Thien et al. 2000). The Magnoliaceae is a well-known example; beetle pollination syndrome in the genus *Magnolia* was recognized in the late 1800s. However, in the flowers of *Magnolia obovata*, a common *Magnolia* species in Japan, it is often observed that not only beetles but also bees, flies, and some thrips visit the flowers. How do these flower-visiting insects contribute to reproduction in *M. obovata*? We focused on the pollination system of *M. obovata* and analyzed the pollen transfer patterns in this species using a pollen genotyping method.

M. obovata Thunb. (Magnoliaceae) is a large (up to 20–30 m in height), common deciduous tree species that is native to temperate forests in Japan. The standing density of adult trees is relatively low, with only a few trees per hectare (Isagi et al. 2000). The flowers are hermaphroditic and protogynous. On the first day of flowering, the female-phase flower opens during the daytime. The female phase lasts for an hour to a half-day of daytime, after which the petals and the stigmas close in the afternoon or evening and no longer receive pollen (Kikuzawa and Mizui 1990). The next day, the petals open again, the stamens release, and pollen is available. Although the flowering period of each flower is 3–4 days, flowering persists on individual trees for up to 40 days (Kikuzawa and Mizui 1990; Ishida et al. 2003). Geitonogamy (the transfer of pollen between flowers on the same plant) occurs frequently because of the simultaneous presence of female- and male-phase flowers on an individual tree (Ishida et al. 2003). Thus, the rate of self-pollination is high, reaching approximately 80% (Ishida 2006). On the other hand, the early life stages of *M. obovata* exhibit substantial inbreeding depression (Ishida 2006), and so it is essential that a tree receive pollen transported from other reproductive trees (outcrossing) for effective reproduction.

Similar to other beetle-pollinated plants, flowers of *M. obovata* emit a strong odor to attract pollinators (Dieringer et al. 1999; Gottsberger 1999; Sakai and Inoue 1999). Not only beetles (Scarabaeidae and Lagriidae), but also bees (Apidae, Andrenidae, and Halictidae), flies (Syrphidae), and some thrips were observed visiting or staying in the flowers of *M. obovata*. Isagi et al. (2000) conducted parentage analysis between saplings and reproductive trees of *M. obovata* and calculated the average pollen movement distance to be 157.1 ± 21.9 m (maximum, 500 m). What kind of insect contributes to such interplant pollen movements and effective pollination? We identified the donors of pollen grains that were transported by flower-visiting insects and assessed the pollen transfer characteristics of each insect group.

3.3.2 Sample Collection and Counting of Adhered Pollen

A field survey was carried out at Ogawa Forest Reserve, Ibaraki Prefecture, central Japan (36º56′N, 140º35′E; 610–660 m a.s.l). The study site consisted of

a preserved area (98 ha) and its surrounding area (29 ha, including fragmented broad-leaved forest, young secondary forest, and coniferous plantations). The annual mean air temperature and precipitation over 10 years (1986–1995), as measured at a meteorological station in Ogawa (36º54′N, 140º35′E), were 10.7°C and 1,910 mm, respectively (Mizoguchi et al. 2002). The preserved area is covered by deciduous broad-leaved forest, and the dominant woody species in the canopy are *Quercus serrata*, *Fagus japonica*, and *Fagus crenata*. The reproductive *M. obovata* trees (DBH ≥ 20 cm) were located and their genotypes were determined (Isagi et al. 2000; Matsuki, unpublished data). Six reproductive *M. obovata* trees with trunk shapes suitable for safe climbing to the canopy were selected (Fig. 3.1a).

From late May to mid-June 2005, observations and collections of flower-visiting insects were conducted for a total of 51.7 h. Visitation frequencies (individuals/hour) of flower visitors were as follows: flower beetles (*Protaetia cataphracta*: Fig. 3.1b, and *Eucetonia* spp.): 1.55; small beetles (Lagriidae and Elateridae): 2.30; bumblebees (Apidae, *Bombus* spp.): 2.05; other bees (Andrenidae and Halictidae): 0.56; and flies (Syrphidae): 0.29 (Matsuki et al. 2008). Among the

Fig. 3.1 A *Magnolia obovata* tree that was surveyed. (**a**) The ladder was attached to a tree to be surveyed, and observation and collection of flower-visiting insects were conducted at about 15 m in height above ground. (**b**) Flower beetles can fly rapidly by spreading their wings without raising the elytra. (**c**) Pollen grains of *M. obovata* that adhered to leg of the beetle and a pipette tip which was stretched and cut off to obtain a sharp end

insects, flower beetles (Scarabaeidae, subfamily Cetoniinae: *P. cataphracta*), small beetles (Lagriidae: *Arthromacra sumptuosa*), and bumblebees (Apidae: *Bombus ardens* and *Bombus diversus*) were used for later analysis.

The number of adhering pollen grains was measured for all insects collected. To remove adherent pollen from the insect body, each insect was washed in 2 ml 1% sodium dodecyl sulfate (SDS) by vortexing for 1 min. The pollen present in 10 μl of resulting solution was counted under a stereomicroscope. The total number of pollen grains adhering to each insect was estimated from the average of three replicate counts. For bumblebees, the pollen loads packed onto the bumblebee's hindleg pollen transport structure were not included in the counting, because such packed pollen was no longer available for pollination as a result of the addition of nectar and oil by the bumblebee (Thorp 1979, 2000). The average numbers of pollen grains adhering to flower beetles ($n=38$), small beetles ($n=118$), and bumblebees ($n=41$) were 27,761 ± 20,770 (range, 1,267–79,400), 916 ± 2,099 (range, 0–16,167), and 16,354 ± 12,227 (range, 0–54,867), respectively (Matsuki et al. 2008). The number of pollen grains adhering to each insect differed among insect groups ($F=100.5$, $df=2$, $P<0.001$). Flower beetles had more pollen than bumblebees and small beetles. As for the insects that were used for the genetic analysis of adhered pollen, the pollen grains for genotyping were removed before the counting of pollen.

3.3.3 Genotyping of Transported Pollen Grains

Under a stereomicroscope, the pollen grains were separately picked from the insect surface in a random manner, using a plastic pipette tip (for manipulating 0.5–10 μl of liquid) that had been stretched and cut off to obtain a sharp end (Matsuki et al. 2007) (Fig. 3.1c). From 11 flower beetles (*P. cataphracta*), 9 small beetles (*A. sumptuosa*), and 11 bumblebees (6 *B. ardens* and 5 *B. diversus*), 1,417 pollen grains were removed for analysis (average, 46 pollen grains/insect). Pollen grains were picked and separately put into 0.2-ml polymerase chain reaction (PCR) tubes. Multiple microsatellite loci of each pollen grain were amplified in a PCR reaction using eight pairs of microsatellite primers (*M6D1*, *M6D3*, *M6D8*, *M10D6*, *M10D8*, *M15D5*, *M17D3*, and *M17D5*; Isagi et al. 1999). PCR amplifications were performed using a thermal cycler (GeneAmp PCR System 2700; Applied Biosystems) under the following conditions: initial activation at 95°C for 15 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 90 s, and extension at 72°C for 1 min, followed by a final incubation at 60°C for 30 min. The volume of the reaction mixture was 6 μl, containing 1 μl extracted pollen DNA, 0.2 μM of each microsatellite primer labeled with fluorescent phosphoramidites (6-FAM, VIC, NED, and PET), and 3 μl QIAGEN Multiplex PCR Master Mix (QIAGEN). The genotypes were determined using an ABI PRISM 3100 genetic analyzer, the GENESCAN software version 3.7, and the GENOTYPER software version 2.0 (Applied Biosystems). The percentage of successful genotype determinations among the microsatellite loci ranged from 25.5% (*M17D3*) to 86.5% (*M15D5*). The failures of genotype determination

were unevenly distributed in the particular loci, suggesting that these failures resulted from the characteristics of each locus or the PCR primers. Pollen samples for which genotypes were determined for more than four microsatellite loci (1,174 samples: 83%) were used for further analysis.

3.4 Origin of Pollen Grains: Where was the Source of the Pollen?

3.4.1 Proportion of Self-Pollen in the Transported Pollen

Paternity analyses between 1,174 pollen grains and 326 candidate fathers were conducted. The Mendelian probabilities, which were calculated for the genotype of each pair of pollen grains and candidate trees, could assign a single pollen donor for 716 pollen grains (61%). For 386 pollen grains, multiple trees remained as candidates of donor, and the LOD scores (the logarithm of the likelihood ratio) of these donors did not differ significantly, so single donors could not be determined for these pollen. These pollen samples were excluded from further analysis because we cannot analyze the patterns of the pollen movements. For 72 pollen grains, no donors were assigned; these pollen grains were considered to come from donors outside the research site.

To accurately estimate the pollen movement in our study area, it is required to evaluate the amount of "cryptic gene flow" – the probability that an adult tree within the study area is assigned as the donor of unrelated pollen samples. The total paternity exclusion probability over eight loci was 0.9999959, calculated using the computer program CERVUS 3.0.3 (Marshall et al. 1998). Therefore, the probability of excluding correctly all unrelated adults (326 trees) within our study area was $0.9999959^{326} = 0.9987$. The probability of cryptic gene flow was estimated as 0.001 $(1 - 0.9987)$. This value was comparably low compared with other studies (for example; 0.37: eight microsatellite loci, 38 putative trees: Bittencourt and Sebbenn 2008; 0.0064: six microsatellite loci, 161 putative trees: Carneiro et al. 2009). In our estimates, 716 pollen grains came from pollen donors in the study site, so that the amount of cryptic gene flow was estimated as 716 $(1 - 0.9987) = 0.93$. Therefore, the total number of pollen grains brought from outside the study area that was corrected for cryptic gene flow was estimated to be $72 + 0.93 = 72.93$.

For nine flower beetles, six small beetles, three *B. ardens*, and five *B. diversus*, we could assign single donors for more than 50% of analyzed adherent pollen. Using these insects, the proportions of self-pollen among adherent pollen were calculated. The proportion of self-pollen per insect differed among insect groups [$F = 27.31$, $df = 2$, $P < 0.001$; one-way analysis of variance (ANOVA)]. The result of a Sheffé's multiple comparison test indicated that flower beetles had significantly lower proportions of self-pollen (mean ± SD, 0.30 ± 0.26; range, 0.00–0.86) than small beetles (0.97 ± 0.02; range, 0.93–1.00; $P < 0.001$) or bumblebees (0.88 ± 0.16; range, 0.54–1.00; $P < 0.001$). The proportion of self-pollen on flower beetles varied

widely among individual insects, and there were some beetles with an extremely low proportion of self-pollen. For most of the small beetles, more than 90% of the adhered pollen grains were self-pollen. Self-pollen also constituted a majority of the pollen that adhered to the bumblebees. There was no significant difference in the proportion of self-pollen transported by *B. ardens* and *B. diversus* (Mann–Whitney *U* test, $P = 0.39$). The genetic composition of pollen on the insect bodies suggested that bumblebees and small beetles move primarily within a tree and mainly carry self-pollen; in contrast, flower beetles certainly move between plants and transport outcross pollen to flowers of *M. obovata*.

3.4.2 Composition of Pollen Donor of Adherent Pollen Grains

The composition of pollen donors of adherent pollen grains per insect is shown in Fig. 3.2. The number of pollen donors per insect was generally low. The average number of detected outcross pollen donors the pollen adhered to a flower beetle was 3.3 donors (range, 2–5). However, it should be noted that eight of nine flower beetles had pollen that was likely transported from outside the study area (maximum, 48%; insect ID: YR257-11). Although five of six small beetles had pollen that came from outside the study area (on average, 2.9%), the majority of adherent pollen of small beetles was self-pollen (Fig. 3.2). Two *B. ardens* (PD306-39 and YR257-20) and one *B. diversus* (PD300-22) transported the outcross pollen within the study area. Examples of pollen transfer patterns for each insect are shown in Fig. 3.3. For flower beetles, typical patterns of pollen movements were illustrated, while for bumblebees and small beetles, the insects that had outcross pollen were selected, because most of these insects had only self-pollen. It was often found that insects moved inter-plant, skipping some reproductive trees. This phenomenon might be influenced by the differences in the phenology and flowering intensity of each tree. Distances between assigned pollen donors and the trees from which the insects were collected are shown in Fig. 3.4. In the case of flower beetles, although approximately 52% of outcross pollen movements were limited to within 200 m, long-distance movements, up to 1,124 m, were also recognized. The maximum distance of pollen movement by small beetles was approximately 130 m. Pollen movements by bumblebees were limited to within 300 m. However, for small beetles and bumblebees the pollen that was brought from outside the study area was also estimated.

3.5 Pollen Transfer Characteristics of Flower-Visiting Insects

3.5.1 Flower Beetles and Small Beetles

The flowers of the Magnoliaceae are thought to be adapted to beetle pollination (Thien 1974; Bernhardt 2000). The large protogynous and odoriferous flowers, the timing of flowering, and the large quantities of pollen produced are typical

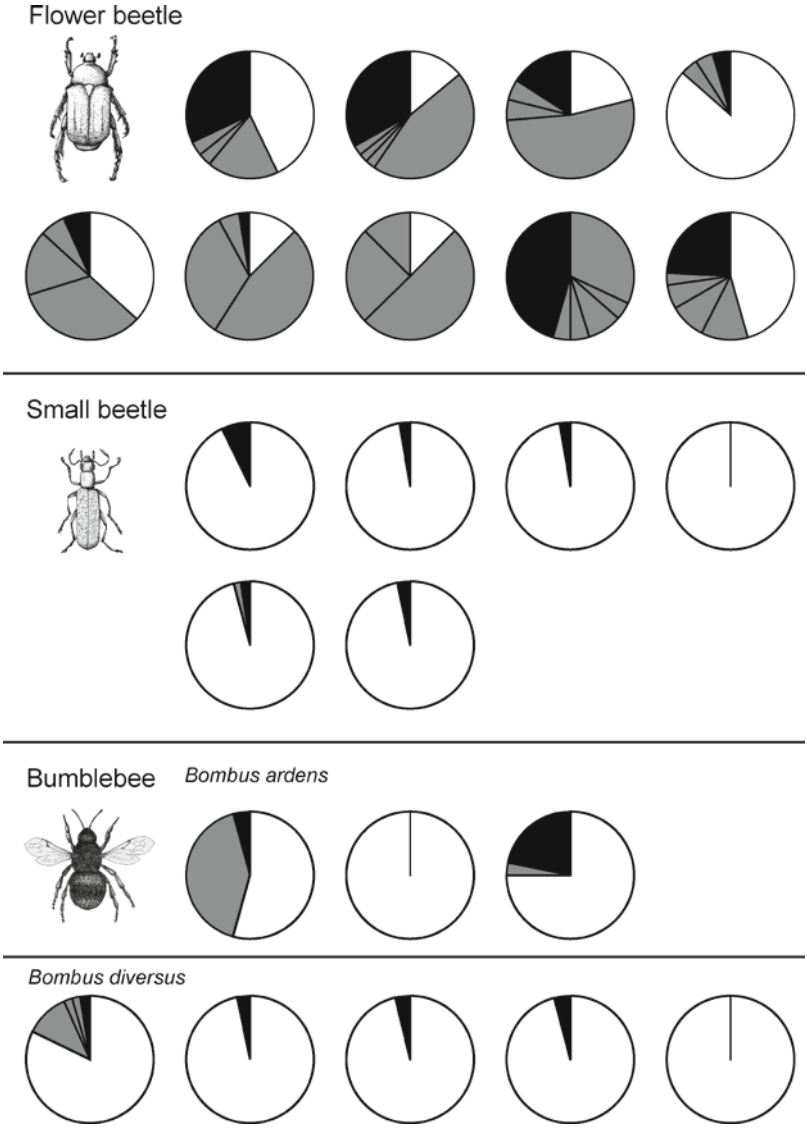

Fig. 3.2 Composition of pollen donor of adherent pollen grains. Each *circle graph* represents an individual insect. Insects for which the adhered pollen grains were assigned as single donors more than 50% were described. *White graph segment* represents self-pollen; *gray demarcated graph segment* indicates the proportion of each assigned pollen donor; *black segment* indicates pollen donors were outside the research site

features of beetle-pollinated species. In our analysis, some flower beetles had proportionally less self-pollen compared with small beetles and bumblebees. Moreover, paternity analyses of pollen grains and candidate paternal trees showed that flower beetles actively traveled between trees and brought pollen from distant

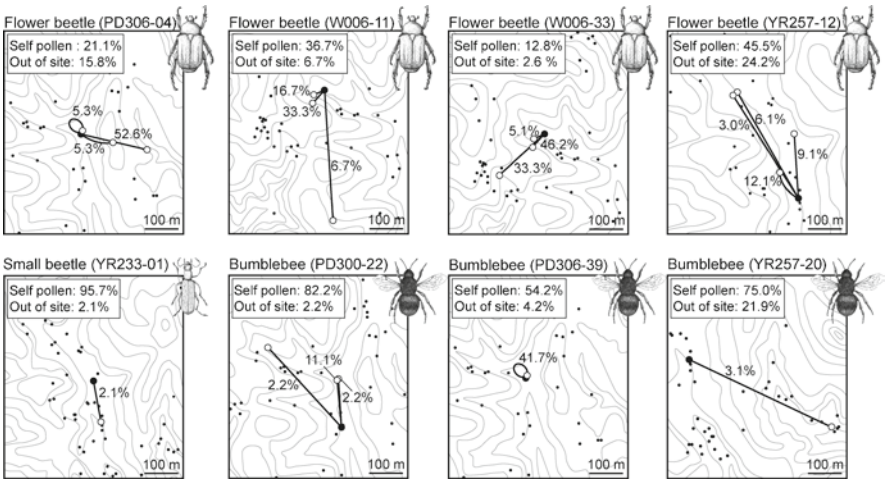

Fig. 3.3 Examples of pollen transfer pattern by each insect. *Large filled circles* and *large open circles* represent the trees from which the insect was collected and the pollen donors, respectively. *Small filled circles* indicate other reproductive trees. *Percentage values* represent the proportion of each pollen donor among the pollen load of each insect. Contour interval, 10 m

pollen donors (maximum, approximately 1,100 m), while bumblebees and small beetles moved less far and mostly moved pollen within trees. Englund (1993) observed frequent interplant flights of flower beetles (*Cetonia* spp.) in European temperate zones. Studies concerning the flight patterns of beetles are scarce; however, it seems that most beetles of the subfamily Cetoniinae can fly more readily than other beetles because they are able to spread their wings without raising the elytra (Haas and Beutel 2001; Fig. 3.1b). The genetically diverse pollen donors of the pollen transported by flower beetles are consistent with previous assessments of the contribution of the Cetoniinae to pollination (Englund 1993).

Although the beetle pollination syndrome had been thought to be primitive (Diels 1916; Crepet and Friis 1987) and relatively inefficient (Faegri and van der Pijl 1971), it is widely acknowledged that beetle pollination is a highly specialized pollination system, as are other insect pollination syndromes (Bernhardt 2000). For example, Scarabaeidae play an important role in the pollination of the Araceae and Dipterocarpaceae in tropical regions (Young 1988; Gibernau et al. 1999; Sakai et al. 1999). Similarly, the pollination systems of the Magnoliaceae are considered highly specialized systems that operate in cooperation with beetles (Thien 1974). Because in the early life stages of *M. obovata* there is substantial inbreeding depression (Ishida 2006), flower beetles are likely to contribute significantly to the outcrossing of *M. obovata* by transporting a large number of genetically diverse outcross pollen grains.

On the other hand, almost all the pollen grains adhering to small beetles were self-pollen. Anthophilous beetles visit flowers not only to obtain edible resources,

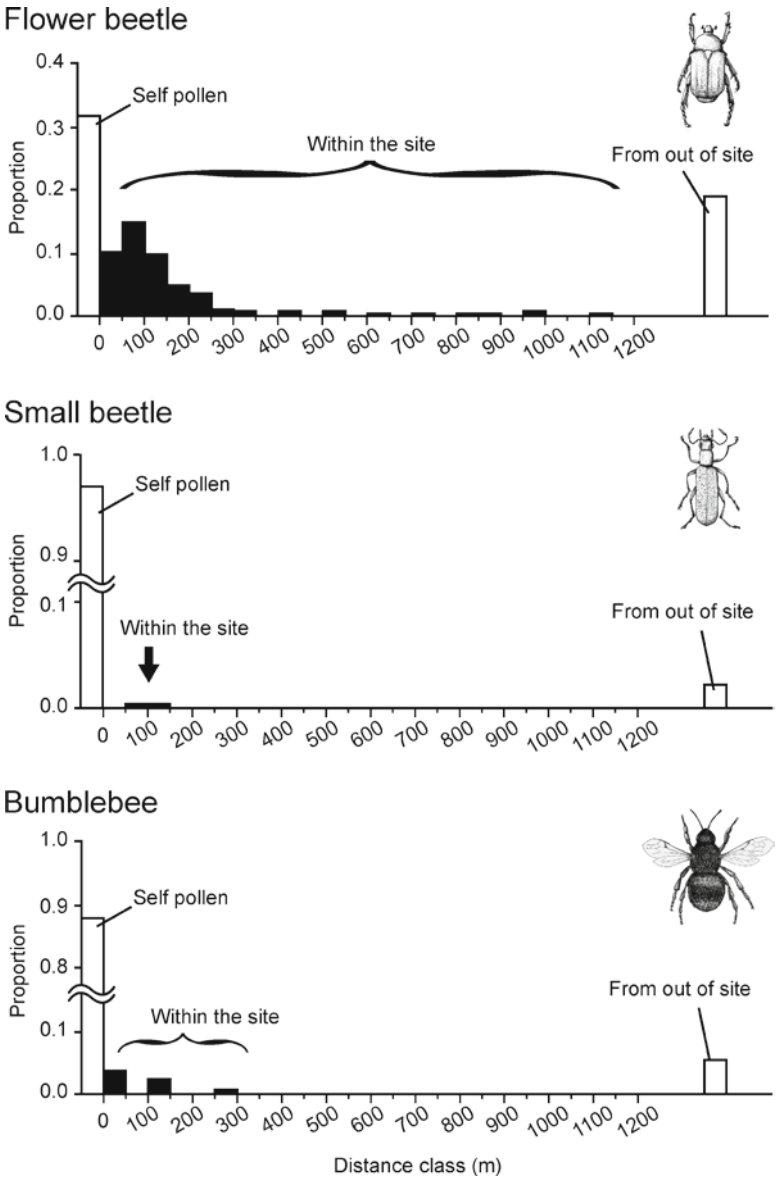

Fig. 3.4 Histograms of distances between assigned pollen donors and the trees from which the insects were collected

but also to carry out mating activities, to hide from predators, and for thermogenesis (Young 1988; Gottsberger 1999; Bernhardt 2000). Small beetles were observed staying in *M. obovata* flowers throughout the female and male phases. Because small beetles tend to stay in the flower for a long time and move mainly within the

trees, these insects might promote the self-pollination of *M. obovata*. However, the existence of pollen that was not assigned suggests occasional interplant movement of small beetles.

3.5.2 Bumblebees

The number of pollen donors for bumblebees' pollen loads was few and the distance of pollen movement was short compared with those of flower beetles. Although some bumblebees, such as *Bombus terrestris*, tend to actively move more than 500 m (Walther-Hellwig and Frankl 2000; Darvill et al. 2004), the foraging behavior of bumblebees generally consists of frequent short-distance movements in a narrow patch and infrequent long-distance movements (Dramstad 1996; Saville et al. 1997). The foraging patterns of bees are strongly affected by resource distribution patterns (Widén and Widén 1990; Utelli and Roy 2000). The standing density of adult *M. obovata* trees was relatively low, with only a few trees per hectare (Isagi et al. 2000), and individual trees produce many flowers simultaneously. These features of *M. obovata* might cause the bumblebees to use one *M. obovata* tree intensively and to move between trees infrequently. In addition to bumblebee movement behavior, their grooming behaviors may also affect the genetic composition of the pollen load. Bumblebees often groom their bodies during foraging, packing the collected pollen with nectar and oil onto transport structures on their hindlegs and thereby effectively making the pollen unavailable for pollination (Rademaker et al. 1997; Thorp 2000). Although these pollen grains were not included in our analysis, reduction of pollen carryover as a result of grooming (Thomson 1986) could explain the low diversity of the pollen donors. Because of grooming, most of the available pollen on the bumblebee's body would have recently adhered and, coupled with intensive foraging within the same tree, this process may result in the dominance of self-pollen and low diversity of pollen donors.

Bumblebees have been reported to be effective pollinators that contribute to the pollination and reproduction of many herbaceous pollinated plants (Mayfield et al. 2001; Schulke and Waser 2001). However, bumblebee visits may negatively affect reproduction in *M. obovata*, causing self-pollination and thereby increasing the risk of inbreeding depression. Bumblebees may be undesirable visitors for plants that, as does *Magnolia*, stand sparsely, have relatively large crowns, and produce many female and male flowers simultaneously.

3.5.3 Conclusions and Future Directions

The female phase of *M. obovata* is short (from 1 h to at most a half-day) and does not provide any reward for foraging insects (Kikuzawa and Mizui 1990). The opportunity for successful pollination is restricted because of a shortage of pollinators

(Kikuzawa and Mizui 1990) and pollen (Ishida et al. 2003). Isagi et al. (2004) conducted a genetic analysis of germinated seedlings of *M. obovata* and determined that the composition of pollen donors and the rates of self-pollination differed among fruits. The current study showed that insects of various taxonomic groups had transported pollen, the genetic composition of which varied widely. We suggest that a flower of *M. obovata* is pollinated by a few insects that happen to visit the flower, and the genetic composition of the seed-set will be directly determined by the pollen that was transported by these insects. The foraging behaviors of insects tend to be influenced by the distribution of flower resources, such as nectar and pollen (Elliott and Irwin 2009). Further analyses that take phenology and flower intensity of individual trees and population into account will facilitate our understanding of gene flow via pollen.

The paternity analysis between seedling arrays and candidate trees showed that the pattern of pollen movement was composed predominantly of self-pollination with a few long-distance movements (maximum, 500 m) (Isagi et al. 2004). The patterns of pollen movement detected by the current paternity analysis between pollen grains and candidate trees also showed a similar tendency. From the traditional point of view concerning beetles, it was suggested that self-pollination of *M. obovata* was promoted by beetles (Isagi et al. 2004). However, the current results suggest that a certain beetle group, namely flower beetles, also contributes to long-distance pollen movement. The direct genetic analysis of pollen that adhered to visitors enabled us to identify the pollinators that effectively contributed to plant reproduction.

Information on the behaviors of pollinators is important for understanding the reproduction process of plants, assessing the role of flower visitors as pollinators, and planning conservation strategies for plants and pollinators. However, because pollinators sometimes show unexpected movements (Dick et al. 2003), it is difficult to thoroughly determine the pollinator's behavior by observation alone. The direct genotyping of pollen grains makes up for the shortcomings of traditional approaches.

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Chapter 4
Difference in Pollen Donor Composition During the Early Phases of Reproduction as Revealed by DNA Analysis of Pollen Grains and Seeds in the Monoecious Tree *Castanea crenata*

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4.1 Introduction

Plants are immobile and consequently depend on abiotic and biotic vectors to transport their pollen for sexual reproduction. Under field conditions, however, the pollen grains deposited on female flowers are not always adequate for seed production (Wilcock and Neiland 2002), such as when self-pollination or pollination from closely related neighboring plants occurs, which can reduce the performance of the next generation through inbreeding depression (Heywood 1993; Nason and Ellstrand 1995; Herlihy and Eckert 2004). In many angiosperms, pollen selection mechanisms that reduce inbreeding depression and promote outcrossing have evolved. These mechanisms include self-incompatibility, in which only outcross-pollen grains are used for seed production (Seavey and Bawa 1986; de Nettancourt 1997), and inbreeding avoidance, in which pollen grains from more distant and nonrelated parents are more likely to sire seeds (Waser and Price 1993; Souto et al. 2002; Glaettli et al. 2006). In these systems, outcross-pollen grains typically have a much greater reproductive advantage compared to self-pollen or related-pollen grains at prezygotic stages such as pollen germination (de Nettancourt 1997) or pollen-tube growth (cryptic self-incompatibility: Bateman 1956; inbreeding avoidance: Waser and Price 1993; Souto et al. 2002; Glaettli et al. 2006). Ovarian inhibition of self-pollen tubes or self-embryo abortion (late-acting self-incompatibility: Seavey and Bawa 1986) and early-acting inbreeding depression also enhance the outcrossing rate. As a result, these traits may cause a difference in pollen donor composition between the pollination stage (i.e., reach stigmas) and seed stage (i.e., sire seeds).

To demonstrate experimentally the effectiveness and evolution of pollen selection mechanisms, it is important to evaluate the extent of self- and related-pollen grain depositions in plant populations exhibiting fine-scale genetic structure. Under natural conditions, individual female flowers may receive a greater proportion of pollen grains from neighboring plants compared to distant plants. If closely related individual plants (i.e., with fine-scale genetic structure) are distributed in a spatially aggregated manner, most of their seeds are likely to derive from the relatively unfavorable outcross-pollen grains from neighboring plants. Furthermore, pollen limitation is frequently observed in natural populations (Burd 1994; Wilcock and Neiland 2002; Ashman et al. 2004), especially in self-incompatible tree species (Larson and Barrett 2000), in which, as a result, outcross-pollen grains from related neighboring plants may compensate for the limitation of more favorable pollen grains from more distant plants.

Therefore, in this study, we evaluated the process of pollen selection in the early phases of reproduction, and we analyzed fine-scale genetic structure using microsatellite genotyping of pollen grains, seeds, and potential paternal trees in the self-incompatible monoecious tree species *Castanea crenata*. DNA amplification from a single pollen grain or pollinarium (i.e., a pollen package) has been reported in several recent studies (Petersen et al. 1996; Suyama et al. 1996; Ziegenhagen et al. 1996;

Aziz et al. 1999; Matsunaga et al. 1999; Widmer et al. 2000; Cozzolino et al. 2005; Parducci et al. 2005; Matsuki et al. 2007, 2008; Paffetti et al. 2007; Zhou et al. 2007; Aziz and Sauve 2008; Chen et al. 2008; Ito et al. 2008; Hasegawa et al. 2009; Matsumoto et al. 2010). Multiplex polymerase chain reaction (PCR) techniques that amplify some microsatellite regions in a single reaction allow for easy paternity analysis from a single pollen grain (Matsuki et al. 2007, 2008; Hasegawa et al. 2009).

Here we specifically addressed the following questions: (1) What is the composition of pollen donors in each of the individual flowers and individual plants in a natural population of *C. crenata*? (2) Does pollen donor composition differ between the pollination and seed stages? (3) If so, which pollen donors (i.e., self-, related, or nonrelated individuals) are more likely to sire seeds?

4.2 Study Species

Castena crenata Sieb. et Zucc. (Japanese chestnut) is a hardwood tree common in the temperate forests of Japan (Jaynes 1975). Because *Castanea* spp. are important tree species for fruit production on farms, many microsatellite markers have been developed in this genus for breeding (Buck et al. 2003; Marinoni et al. 2003; Yamamoto et al. 2003), and they can be used for the ecological studies of *C. crenata*.

Castanea spp. are late-acting, self-incompatible plants (Seavey and Bawa 1986) with two types of inflorescences: unisexual staminate catkins at proximal positions of the shoot and bisexual catkins at terminal positions (Klinac et al. 1995) (Fig. 4.1a). Multiple female flowers with three ovaries are present at the bases of the bisexual catkins, and, on pollination, each flower develops into a single cupule (Fig. 4.1b,c). Flowering occurs between late June and early August at our study site. On individual trees, the flowering periods of both male and female flowers generally overlap. The flowers typically require pollinators for seed production (de Oliveira et al. 2001) and are visited by various insects (e.g., honeybees, bumblebees, syrphid flies, butterflies, moths, and beetles; Y. Hasegawa et al., unpublished data). The seeds (three per cupule) mature in mid-October of the same year that flowering occurred.

4.3 Study Site

The study site was located in a deciduous broad-leaved forest dominated by *Quercus crispula*, *Fagus crenata*, *Aesculus turbinata*, and *C. crenata* in Ippitsu Forest Reserve, Miyagi, Japan (38°49′N, 140°45′E; 718 m above sea level). Diameter at breast height (DBH) of the largest individual in these four species was greater

Fig. 4.1 (**a**) Flowers of *Castanea crenata* with current-year shoot. Female flower (**b**) and seeds (**c**) within a cupule of *C. crenata*

than 100 cm (Terabaru et al. 2004). Furthermore, age of a large individual tree of *A. turbinate* was more than 300 years, as revealed by analysis of yearly growth rings (R. Takahashi and K. Seiwa, unpublished data). This study site is an old-growth forest that has not suffered human disturbance.

We established a 6-ha (240×250 m) plot in which all stems of *C. crenata* greater than 5 cm in DBH were mapped (range, 8.1–99.4 cm; $n=281$; Fig. 4.2). As all these trees reach the canopy layer, we considered them to be potential pollen donors in this study. This sample plot was located in the middle of a large population of *C. crenata*, with numerous reproductive trees of *C. crenata* outside the plot edge.

Individual trees of *C. crenata* were preferentially distributed on ridges and west-facing slopes, resulting in three main patches of trees within the study plot (Fig. 4.2). The diameter of individual patches was approximately 90 m. Significantly positive kinship coefficients were observed within a range of 80 m, and significantly negative kinship coefficients were found more than 104 m apart (randomization test,

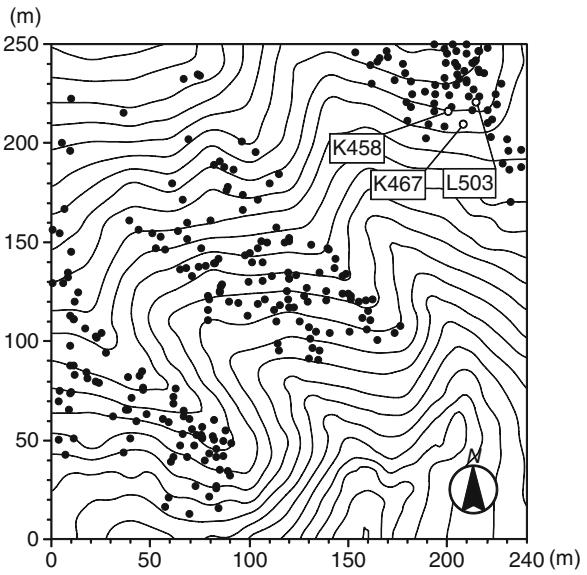

Fig. 4.2 Location of the three maternal trees (*open circles* with ID numbers) and the other 278 trees (*filled circles*) of *Castanea crenata* in the 6-ha plot. *Contour lines*, 5-m elevation change. (From Hasegawa et al. 2009)

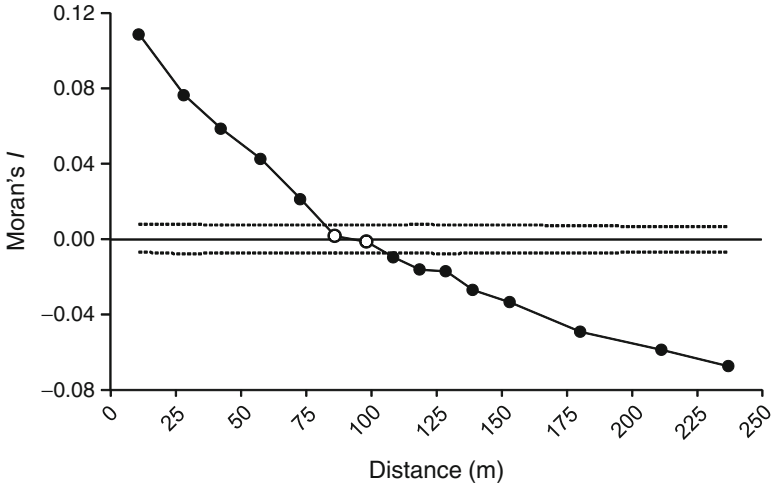

Fig. 4.3 Correlogram showing the fine-scale genetic structure in the study plot based on Moran's *I* calculated using SPAGeDi 1.2b (Hardy and Vekemans 2002). *Broken lines* are the upper and lower 95% confidence intervals for the null hypothesis of no genetic structure, which was obtained after 10,000 permutations of the multilocus genotypes. *Filled circles* indicate significant deviation from zero ($P<0.05$). (From Hasegawa et al. 2009)

$P<0.05$; Fig. 4.3). Therefore, individual trees within each patch were closely related to each other.

The fine-scale genetic structure within the study plot seems to reflect the restricted pollen and seed dispersal distances. The seed of *C. crenata* is dispersed

by rodents (Seiwa et al. 2002). In this study plot, most seeds of *C. crenata* were dispersed within the individual patch revealed by maternity analysis of seedlings using the pericarp DNA (Y. Hasegawa et al., unpublished data).

4.4 Methods

4.4.1 Sampling Design

Leaf tissues for DNA microsatellite genotyping were collected from each of the 281 trees representing the entire (100%) reproductive population in the plot. In late July 2004, female flowers were collected from three trees (K458, K467, L503) located in the northeast corner of the plot to acquire the longest possible measurement of the pollen dispersal distance (Fig. 4.2). For paternity analysis at the pollination stage, pollen samples ($n = 1{,}219$, $n = 912$, $n = 1{,}440$) were isolated from 33, 17 and 20 female flowers from the three trees. For paternity analysis at the seed stage, individual seeds ($n = 102$, 101, 101) were isolated from 40, 35, and 43 cupules from the three trees in mid-October 2004. Tissue samples were stored at −20°C before DNA analysis.

Pollen grains were collected from styles of female flowers. In *C. crenata*, pollen grains germinate only on the tip of the needle-shaped style, indicating that this is the stigma (Nakamura 1992). If a pollen tube has started to grow on a stigma, DNA cannot be extracted from the pollen grain. Thus, we collected pollen grains at the pollination stage from the surface of styles.

The styles were placed in 0.01% sodium dodecyl sulfate (SDS) solution on slides with a water-repellent finish. Thereafter, pollen grains that were morphologically identified as *C. crenata* and bore no structural damage were collected with a micropipette under a stereo dissecting microscope.

4.4.2 DNA Extraction and Amplification

One pollen grain and 0.5 μl 0.01% SDS solution were placed in a PCR tube that contained 1.0 μl reaction buffer (10 mM Tris–HCl, pH 8.3 at 20°C; 1.5 mM $MgCl_2$; 50 mM KCl; 0.01% Proteinase K), incubated for 60 min at 54°C, and heated for 10 min at 95°C. The extract was used directly as a PCR template. Total DNA from leaf and seed tissues was isolated using a DNeasy 96 Plant Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol.

The forward primers were labeled with fluorescent dye (G5 dye set: 6-FAM, VIC, NED, or PET; Applied Biosystems) to simultaneously analyze 11 microsatellite loci (CsCAT2, CsCAT5, CsCAT14: Marinoni et al. 2003; EMCs2, EMCs17: Buck et al. 2003; KT001b, KT004a, KT005a, KT020a, KT024a: Yamamoto et al. 2003; KT030a: T. Yamamoto, personal communication) of similar allelic size and to avoid overlaps among loci with the same dye. Multiplex PCR amplification was carried out using a Multiplex PCR Kit (Qiagen).

4.4.3 Paternity Analysis

For paternity analysis, we used 11 loci for pollen grains and 10 loci for seeds. Apparent null alleles were detected as genotype mismatches between the mother tree and seed for the KT004a locus. We eliminated the KT004a locus from the overall paternity analysis of seeds because a heterozygote seed bearing a null allele shows the same pattern as a homozygous seed for the other allele. We still used this locus in the analysis of pollen grains because null alleles can be recognized by the absence of an allele.

As several loci were not amplified by pollen genotyping for several pollen grains, we added a locus (KT004a) in the analysis of pollen grains to increase the exclusion probability. For the following analysis, we used pollen samples that had more than eight genotyped loci (mean exclusion probability = 0.989). All sampled seeds were used for analysis because they had all ten genotyped loci (exclusion probability = 0.995).

Pollen grains and seeds were assigned as self if they did not contain nonmaternal alleles. The paternity of each outcross-pollen grain and outcross-seed was assigned by a simple exclusion approach based on the multilocus genotypes of the 281 trees. If a pollen grain or seed lacked any potential pollen donor genotypes among the 281 candidate trees, we assumed that the pollen donor came from outside the study plot. If a pollen grain or seed had two or more possible pollen donor candidates, we inferred paternity based on the maximum likelihood paternity assignment using CERVUS 2.0 (Marshall et al. 1998).

4.4.4 Statistical Analysis

We compared the self-pollen rates at the pollination stage and the selfing rates at the seed stage by applying a generalized linear mixed model (GLMM) with binomial errors and a logistic link, using the glmmML package in R (R Development Core Team 2006). Here, individual trees were treated as a random effect, stage (pollination or seed) as a fixed effect, and the pollen donor (self- or outcross-) as the response variable.

4.5 Pollen Donor Composition at the Pollination Stage

For 946 (26.5%) of the 3,571 pollen samples isolated from female flowers, DNA fragments were successfully amplified and genotyped at more than eight loci. In 169 (17.9%) of the 946 samples, we obtained two alleles at one locus, and in 4 (0.4%) of the 946 samples, we found three alleles at one locus. As pollen grains are haploid, these results indicate that two or three pollen grains had been placed in one PCR tube and then amplified at the same time. Therefore, we did not use the 173 multi-allele samples in the following analyses, and we obtained microsatellite

genotypes for a total of 773 pollen grains ($n = 270$, 153, and 350 from K458, K467, and L503, respectively).

At the pollination stage, the self-pollen rate (i.e., proportion of self-pollen to the total number of pollen grains) was high for all flowers (40–100%; Fig. 4.4) and in each of the three maternal trees (98.9%, 77.1%, and 94.6% from K458, K467, and L503, respectively). The rates were almost identical to the self-fertilization rate of an insect-pollinated tree, *Magnolia obovata* (82–86%; Ishida 2006), but were greater than those of several herbaceous plants (0–70%; reviewed by de Jong et al. 1993). Floral displays of individual trees could promote a high frequency of pollinator movements within an individual tree (Frankie et al. 1976). Also, we observed that most pollinators (e.g., bumblebees, syrphid flies, and beetles) moved frequently within individual trees but only rarely between trees (Y. Hasegawa et al., unpublished data). Furthermore, we documented significant overlap of the flowering periods for both male and female flowers within individual trees (Y. Hasegawa et al., unpublished data). These traits may in part account for the high rate of geitonogamous self-pollination.

Castanea spp. are late acting and self-incompatible (Seavey and Bawa 1986), and female flowers of *C. crenata* have 16–22 ovules per ovary, which develops into a seed (i.e., overproduction of ovules; Nakamura 2003). In this self-incompatible system, bundles of self-pollen tubes reach the ovules and disable them (Kawagoe and Suzuki 2005). Although the self-pollen rate was very high for all female flowers in *C. crenata*, the proportion of matured cupules to total number of female flowers is relatively high under natural conditions (36%; Y. Hasegawa et al., unpublished data).

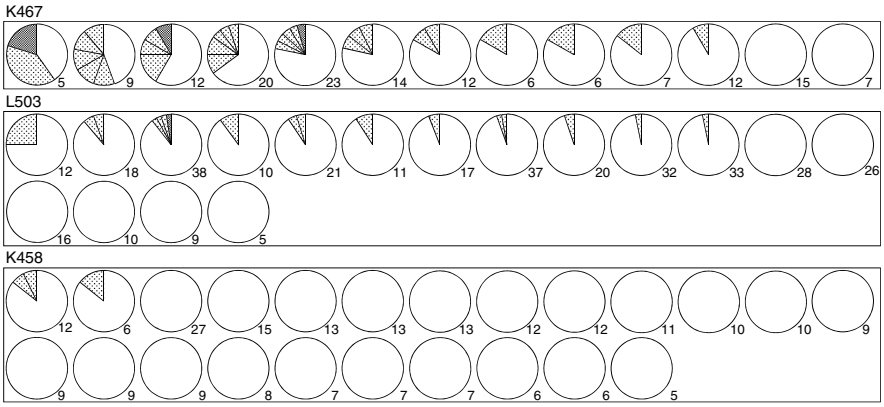

Fig. 4.4 Rates of outcross-pollen (*light gray areas*, pollen dispersal from pollen donor within the study plot; *dark gray areas*, pollen dispersal from outside the study plot) and self-pollen (*white areas*) at the pollination stage for each female flower from three adult trees (K467, L503, and K458, respectively) of *Castanea crenata*. Each *circle* indicates a pie chart of an individual female flower. Pieces of the same pattern in a pie chart indicate the pollen dispersal from different pollen donors in the same category. The *numbers* indicate the number of parentaged pollen grains. Female flowers with more than five parentaged pollen grains are shown

Therefore, the overproduction of ovules in *C. crenata* may be an adaptation for compensation of seed production under the high self-pollination rate. However, several factors for evolution in the overproduction of ovules have been hypothesized, such as selective ovule abortion to enhance seed quality (Marshall and Folsom 1991) or uniform resource absorption of seeds (Sakai 2007); thus, further studies are needed.

4.6 Pollen Selection

In *C. crenata*, the selfing rate at the seed stage was very low (mean ± SE, 0.3 ± 0.3%, $n = 3$), although the self-pollen rate at the pollination stage was very high (90.2% ± 6.7%, $n = 3$; GLMM, $P < 0.01$; Fig. 4.5). Such a drastic decrease in the selfing rate indicates self-incompatibility, which is very important for the rejection of selfing in *C. crenata*.

The genetic relatedness of the outcross-pollen donors and the maternal trees was not significantly different between the pollination stage (0.035 ± 0.038) and the seed stage (0.076 ± 0.032) (one-tailed bootstrap test, $P = 0.53$; Fig. 4.6). The frequency distributions of the distance between the outcross-pollen donors and the maternal trees (i.e., pollen dispersal distances) were not significantly different between the pollination and seed stages (Kolmogorov–Smirnov test: $D = 0.11$, $P = 0.62$; Fig. 4.7). At the seed stage, the crossing rate between individual trees in the same patch was much higher (71%) than that for trees in two different patches and outside the study plot (29%), most probably because the pollen dispersal distance was much shorter within patches (range of dispersal distance, 2.9–56.5 m) than between patches (range, 92.4–242.6 m; see Figs. 4.2 and 4.7).

If outcross-pollen grains from related individuals are underutilized for seed production in *C. crenata*, it is expected that the pollen dispersal distance will be increased in the seed stage, relative to the pollination stage, simply because individual trees are more closely related to their immediate neighbors (i.e., fine-scale

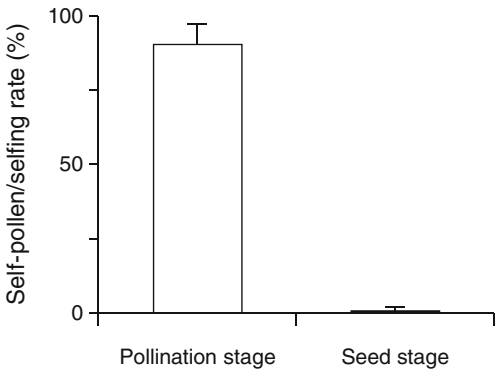

Fig. 4.5 Difference between the self-pollen rate (mean ± SE for three *Castanea crenata* trees) at the pollination stage and the selfing rate (mean ± SE) at the seed stage. (From Hasegawa et al. 2009)

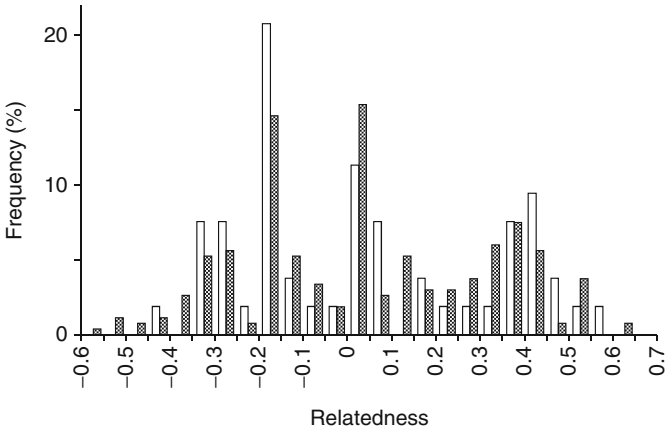

Fig. 4.6 Frequency distributions for relatedness between paternal and maternal parents of *Castanea crenata* at the pollination stage (*white bars*) and the seed stage (*gray bars*) calculated using MER 3.0 (Wang 2002). Analyses were conducted using only outcross-pollen grains ($n = 53$) and seeds ($n = 267$) from a pollen donor within the study plot. (From Hasegawa et al. 2009)

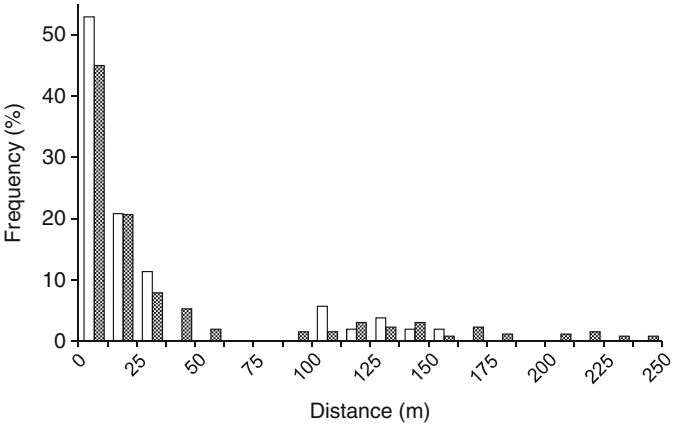

Fig. 4.7 Frequency distributions of distances between paternal and maternal parents of *Castanea crenata* (i.e., pollen dispersal distances) at the pollination stage (*white bars*) and seed stage (*gray bars*). Analyses were conducted using only outcross-pollen grains ($n = 53$) and seeds ($n = 267$) that had a pollen donor within the study plot. (From Hasegawa et al. 2009)

genetic structure), as demonstrated for the patches at our study site. However, there was little difference in the pollen dispersal distance between the pollination and seed stages. We also observed no difference in the relatedness of paternal and maternal parents in the outcross events between the pollination and seed stages. These observations strongly indicate that related outcross-pollen grains are used for seed production during these periods and therefore suggest that biparental inbreeding avoidance was not operating in *C. crenata*.

In addition, the crossing rate between individual trees at the seed stage was much higher within patches (71%) than between patches (29%). Such distance-dependent crossing may promote biparental inbreeding depression after seed production (Heschel and Paige 1995; Koelewijn et al. 1999; Ubukata et al. 1999; Richards 2000; Ishida 2006; Isagi et al. 2007). If the biparental inbreeding depression reduces the number of offspring that are produced by related outcross pollination, the genetic structure of the recruitments within a population will be weakened as they grow. Thus, the related outcross pollination for seed production may have a weak effect on creating the genetic structure in this population.

Inbreeding depression is thought to be strong in long-lived perennial tree species (Ishida 2006), probably as a result of the high rate of genomic mutation per generation (Morgan 2001). Furthermore, *Q. crispula*, belonging to the same family (Fagaceae), exhibits biparental inbreeding depression at both the seed germination and seedling stages (Ubukata et al. 1999). However, we did not investigate inbreeding depression in *C. crenata*. Thus, further studies are needed to clarify the effect of biparental inbreeding on seed germination and seedling establishment in *C. crenata*.

In several hand-pollination experiments, the pollen germination and pollen-tube growth rates were usually lower for outcross-pollen grains from related individuals than for those from nonrelated ones (Waser and Price 1993; Souto et al. 2002; Glaettli et al. 2006). For the observation of such a reproductive bias, even under natural conditions, the proportion of outcross-pollen grains from related individuals would be expected to decrease from the pollination to the seed stage. As noted here, however, little difference was observed in the pollen donor compositions between the pollination and seed stages in *C. crenata* under natural conditions.

Tree species tend to exhibit pollen limitation (Larson and Barrett 2000). If pollen limitation occurred in *C. crenata*, a mother tree would have few chances to select nonrelated individuals. Thus, related outcross-pollen grains may compensate for the limitation of nonrelated outcross-pollen grains in *C. crenata*. However, it remains unclear whether related pollen grains contribute to seed production even under conditions for which pollen is not limited. To further clarify the pollen selection mechanisms of *C. crenata*, additional studies, including hand-pollination experiments, are needed.

4.7 Conclusion

We investigated the pollen donor composition of early phases of reproduction in a natural population of *C. crenata*. Using microsatellite analysis, we showed a drastic decrease in self-pollen rates from the pollination stage to the seed stage. In crossing events, however, relatedness between the pollen donor and maternal tree was not different between pollination and seed stages. These traits suggest that tree species may have evolved pollen selection mechanisms for increasing both quality and quantity of seeds under limited pollen dispersal events caused by limited movement of pollinators.

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Chapter 5
Expanded Home Range of Pollinator Birds Facilitates Greater Pollen Flow of *Camellia japonica* in a Forest Heavily Damaged by Volcanic Activity

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5.1 Introduction

Ecological observations during and after volcanic activity provide valuable opportunities to study how organisms respond to environmental devastation. Previous studies of volcanic islands have mainly addressed the colonization processes of pioneer plants to examine the primary succession of plant communities (e.g., Kitayama et al. 1995; Tsuyuzaki and del Moral 1995; Thornton et al. 1996; Shanahan et al. 2001; Kamijo et al. 2002; Kamijo and Hashiba 2003). However, no study has focused on the recovery processes of late climax forest communities by secondary succession according to various symbiotic species interactions. Because secondary succession dominates the recovery process, a better understanding of the detailed interactions between organisms during recovery is important.

In an effort to study these community recovery processes, we examined the effects of volcanic activity on a plant–animal system comprising a common broad-leaved evergreen tree species, *Camellia japonica*, from climax forests, and a pollinating bird, the Japanese white-eye, *Zosterops japonica*. We examined the hypothesis that *Camellia* trees are sufficiently robust to serve as a core species in natural forest recovery, even in severely damaged areas. We previously reported the effects of volcanic activity on the maternal reproductive success of *C. japonica* (Abe and Hasegawa 2008). We found that the net fruit production of *C. japonica* in a heavily damaged area was nearly the same as that in a less damaged area; this was true because the heavily damaged area had better pollination and seed-set rates compared to the less damaged area, which compensated for the suppressed blossoming and fruit growth in the heavily damaged area where poisonous volcanic gas exposure was intense. Despite the similar maternal reproductive success in terms of the final fruit-set rates in heavily damaged and less damaged sites, the genetic diversity of subsequent generations (seeds) may be different among sites. Knowledge about the genetic diversity of pollen transported by pollinators will deepen our understanding of population maintenance mechanisms and reproductive strategies in *C. japonica*. These types of studies are currently quite limited for bird-pollinated species (Ward et al. 2005).

In this study, we show how pollinator behavior affects the pollen dispersal of *C. japonica* at sites having variable flower densities. We do this using a single-pollen genotyping method and radio tracking of pollinator birds. Our previous study (Abe and Hasegawa 2008) suggested that because pollinator birds transport *C. japonica* pollen grains collected from various areas with low flower density, genetic diversity should increase in *C. japonica* progeny as a consequence of long-distance pollen dispersal vectors. Recently, direct genotyping of single pollen grains was developed (Matsuki et al. 2007, 2008). In the present study, we reveal characteristics of pollen movement of *C. japonica* pollinated by *Z. japonica* by comparing the genetic diversity of pollen grains on the birds between sites having different flower densities. In addition, we also compare the results from radio tracking of pollinator birds with pollen haplotyping. We examined the stability of plant–pollinator systems with regard to volcanic disturbance by combining the single-pollen genotyping method (Matsuki et al. 2007, 2008) with radio tracking,

which demonstrated that genetic diversity in pollen grains found on the birds was more diverse in the heavily damaged area that had low flower density. The consequences of these results for plant–pollinator system stability following volcanic disturbances are discussed.

5.2 Materials and Methods

5.2.1 Study Areas

The Izu Islands are a group of volcanic islands located on the western rim of the Pacific Ocean. They are characterized by a humid warm temperate climatic zone with an annual rainfall of more than 2,000 mm and an average air temperature ~17°C. The volcanically active island Miyake-jima (Fig. 5.1), located about 180 km south of Tokyo, was selected as the study island because the volcano explosively erupted during the summer of 2000. The main vegetation is evergreen broad-leaved dominated by species such as *Castanopsis sieboldii*, *Machilus thunbergii*, and *Camellia japonica* before the 2000 volcanic eruption (Kamijo et al. 2002; Kamijo and Hashiba 2003).

In the winter of 2006, we established three study sites on Miyake-jima [Tubota (TU), Igaya (IG), and Nanto-road 2 (N2); Figs. 5.1 and 5.2] to examine the genetic aspects of reproductive success under various flowering conditions related to volcanic activity.

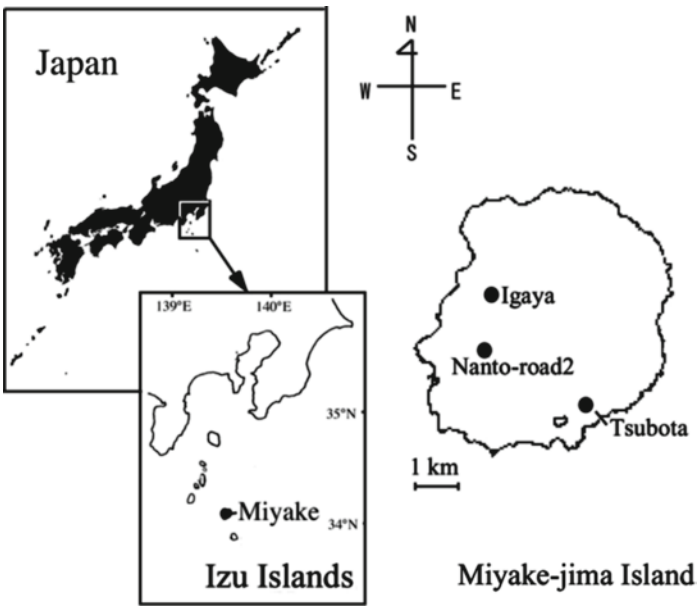

Fig. 5.1 Three study sites on Miyake-jima Island, Japan

Fig. 5.2 *Camellia japonica* blossoming at the area (Nanto-road 2, N2) heavily damaged by volcanic gases

5.2.2 Study Species

Camellia japonica was selected as the study species. In eastern Asia, *C. japonica* is a common broad-leaved evergreen tree species in climax forests (Wendel and Parks 1985; Katsuta 1999; Nagamasu 2006). This species is monoecious in its reproductive system. It produces conspicuous red flowers with a large quantity of nectar that attracts birds (*Z. japonica* and *Hypsipetes amaurotis*). It flowers during November to March with a peak during January to February on Yakushima Island (Yumoto 1988) and the Izu islands (Abe et al., unpublished). *C. japonica* is also self-incompatible (Shibata and Ieyumi 1991), and flowers isolated from pollinating birds do not bear fruit (Kunitake et al. 2004). Kunitake et al. (2004) experimentally demonstrated that *Z. japonica* (Fig. 5.3) is the most effective pollinator of *C. japonica* on Niijima Island adjacent to Miyake-jima. Although *H. amaurotis*, *Cettia diphone*, and *Parus major* are also potential pollinators at Miyake-jima, *Z. japonica* was always dominant and represented 78–96% of the flower-visiting birds (Abe and Hasegawa 2008). *Z. japonica* migrates to warmer places in winter, and some birds fly from mainland Honshu to the Izu islands. Resident birds on the Izu islands are classified as subspecies *Z. japonica* var. *stejnegeri*. Kikkawa and Kakizawa (1981) determined that about 9% of *Z. japonica* caught in winter on Miyake-jima were mainland subspecies. Abe et al. (2006) reported secondary seed dispersal by the Japanese wood mouse (*Apodemus speciosus*) on Niijima.

Fig. 5.3 *Zosterops japonica* visiting *Camellia* flower. (Photograph by Akio Ogura)

5.2.3 Flowering Conditions of C. japonica and Home Ranges of Z. japonica

During winter 2006, the number of flowering trees and flowers per flowering tree were counted, and the flower density (per hectare, ha) was estimated at each study site to analyze the relationship among flowering conditions, pollen genetic diversity, and the home range size of the pollinator bird *Z. japonica*. Home range sizes were estimated using radiotelemetry and analyzed for birds tracked for more than 1 week.

In the radiotelemetry tracking, birds were captured with 12- to 18-m mist nets (mesh size, 36 mm) that were placed at a central location between the radio-tracking sites (~100 ha). The capture of birds using mist nets was permitted by the Ministry of the Environment, Government of Japan (permit no. 051118001). Transmitters (model LB-2; Holohil Systems, Canada; http://www.holohil.com) weighing less than 0.5 g were glued to the backs of adult birds heavier than 11 g in body mass, based on the methods of Raim (1978). Any two birds captured at the same time were determined to be a pair. Therefore, we only attached a transmitter to one of them. The life of a transmitter was 12 days (lifespan range, 8–15 days), and its dimensions were $13 \times 6.5 \times 3.5$ mm (L × W × H). We radio-tracked birds with transmitters between 5 January and 5 February 2006 at high- and low-flower-density sites at TU, IG, and N2 on Miyake-jima (see Fig. 5.1). The birds were tracked more than three different times per day, between sunrise and sunset (normally at 0700, 1200, and 1600) for active birds and once during the night for inactive birds. The home range of the birds that were continually tracked for more than 1 week was depicted using minimum convex polygons. Radio-tracking equipment comprised a receiver (FT-817; Vertex Standard) that had three handheld Yagi aerial-antenna elements.

5.2.4 Pollen Grain Sampling and Haplotyping

For all birds captured using the methods just described, we first checked for the presence or absence of pollen grains on the beak and then wiped any visible pollen grains off the beak using a cotton-tipped swab (Fig. 5.4). Pollen grains were dropped on a glass slide from the swab, and a single pollen grain was picked up using a needle under a stereomicroscope.

DNA extraction and microsatellite haplotyping of a single pollen grain were conducted according to the method described by Matsuki et al. (2007). Haplotypes were determined using eight pairs of microsatellite polymerase chain reaction (PCR) primers: *TMSE-27B03T*, *TMSE-9E07S*, *TMSE-25E07T*, *TMSE-11D02T*, *TMSE-4B07S*, *TMSE-10C05T* (Taniguchi et al., personal communication), *MSCjaH38* (Ueno et al. 1999), and *MSCjaR2* (Abe et al. 2006). Microsatellite amplification was then performed using a Multiplex PCR Mix according to the manufacturer's protocol (Takara Bio). Reactions were incubated in a TaKaRa PCR Thermal Cycler Dice Gradient TP600 (Takara Bio). After an initial denaturation phase of 15 min at 95°C, 30 thermal cycles were performed under the following conditions: denaturation for 30 s at 94°C, annealing for 90 s at 58°C, and extension for 90 s at 72°C, with final extension for 10 min at 72°C. The PCR amplification products were detected using a genetic analyzer (ABI Prism 3100; Applied Biosystems). Individual genotypes were determined using Genotyper software (Applied Biosystems).

5.2.5 Mature Tree Sampling and Genotyping

Because the distribution of *C. japonica* at the study sites tended to be clumped, we selected a dense area of *C. japonica* trees as the study plot within each site. From late July to early August 2005, we collected leaves from all mature trees that had

Fig. 5.4 Wiping pollen from the beak of *Cettia diphone* using a cotton swab

floral buds in each 50×60 m area (0.3 ha) at the three study sites. The number of mature trees within each population was 48, 24, and 17 for TU, IG, and the N2 population, respectively. Genomic DNA was extracted from dried or frozen leaves using a modified CTAB method (Murray and Thompson 1980). Genotypes were determined using the same method as in the pollen grain analysis already described.

5.2.6 *Analysis of Genetic Diversity*

We calculated three genetic parameters: number of alleles (*Na*), gene diversity (*H*), and allelic richness (A_r). *Na* and *H* were estimated using the FSTAT 2.9.3 software (Goudet 2000). The number of gene copies [*g*] was standardized to 20 for the calculation of A_r, according to El Mousadik and Petit (1996). To compare genetic diversity among the study sites, the partitioning of allelic richness among pollen grains ($A_{st}[g]$) was calculated as $A_{st}[g]=1-(A_s[g]-1)/(A_t[g]-1)$ (El Mousadik and Petit 1996; Petit et al. 1998; Comps et al. 2001), where $A_s[g]$ and $A_t[g]$ signify the mean allelic richness within each pollen pool adhering to a bird and the total allelic richness in the pollen pools adhering to birds within each study site, respectively. Values of A_{st} depend on the distribution of alleles among populations. If rare alleles are clustered in some populations (pollen pools, here), high values of A_{st} are expected. On the other hand, even distribution of alleles among pollen pools will give low A_{st} values. From a conservation point of view, more emphasis should be placed on rare alleles, and therefore A_{st} is a more suitable measure than F_{st} (El Mousadik and Petit 1996). Comparisons of genetic diversity among sites were carried out using $A_{st}[g]$ values. Differences in the mean values of the analyzed genetic parameters between bird species and among study sites were analyzed using Mann–Whitney *U* tests and Kruskal–Wallis tests after a test of equal variances.

5.3 Results

5.3.1 *Flowering Conditions and Home Range of Zosterops japonica*

The flower and flowering tree densities were highest at TU and lowest at N2 (Table 5.1). For the three sites described above, a total of 18 *Zosterops* birds were captured (Table 5.2). One bird at TU was determined, by the length of its beak, to

Table 5.1 Flowering conditions of *C. japonica* in the Miyake-jima Island study sites

Study site	Census area (ha)	Flower density (/ha)	Flowering tree density (/ha)	Number of flowers per flowering tree	(SD)
TU (Tubota)	0.3	10,106.7	193.3	207.6	(156.1)
IG (Igaya)	0.5	206.0	36.0	16.2	(18.7)
N2 (Nanto-road 2)	1.0	53.0	12.0	16.6	(8.1)

Table 5.2 Number of birds captured and those from which pollen grains on their beak were analyzed

	Zosterops japonica		*Hypsipetes amaurotis*		*Cettia diphone*		*Parus major*	
Study site	Captured	Analyzed	Captured	Analyzed	Captured	Analyzed	Captured	Analyzed
TU (Tubota)	13 (3)	16	0	0	6	0	1	0
IG (Igaya)	3 (1)	4	1	1	7	3	9	1
N2 (Nanto-road 2)	2 (0)	2	0	0	2	1	3	3
Total	18 (4)	22	1	1	15	4	13	4

Number of recaptured birds is in parentheses

be a *Z. japonica* that had migrated from the Honshu mainland. The others were identified as a nonmigratory resident subspecies from the Izu Islands, *Z. japonica* var. *stejnegeri*.

In total, ten birds were tracked for 7–20 days ($n=7$, 16.6 days at TU; $n=3$, 7.5 days at IG). The home range sizes were estimated using the minimum convex polygon method (Table 5.3). The mean home range size at the high-flower-density site (TU) was smaller than at the low-flower-density site (IG), although no significant differences were found (Welch's T test, $P=0.13$; Table 5.3 and Fig. 5.5).

The mean number of flowers estimated to be within all home ranges was greater at TU than at IG (Table 5.3). In contrast, the mean number of flowering trees estimated to be within all home ranges at TU tended to be smaller than at IG (Table 5.3). However, these differences were not significant (Mann–Whitney U test: $Z=1.03$, $P=0.31$).

Table 5.3 Mean home range sizes of *Zosterops japonica* and estimated mean number of flowers and flowering trees at two study sites based on the home range sizes and flowering density in Table 5.1

		Mean home range size		Estimated number within the home range			
Study site	Number of birds	Hectares (ha)	(SD)	Flowers	(SD)	Flowering trees	(SD)
TU (Tubota)	7	0.26	0.24	2,599	2,466	49.7	47.2
IG (Igaya)	3	1.97	1.20	405	247	74.7	45.7

No birds were analyzed at site N2 (Nanto-road 2)

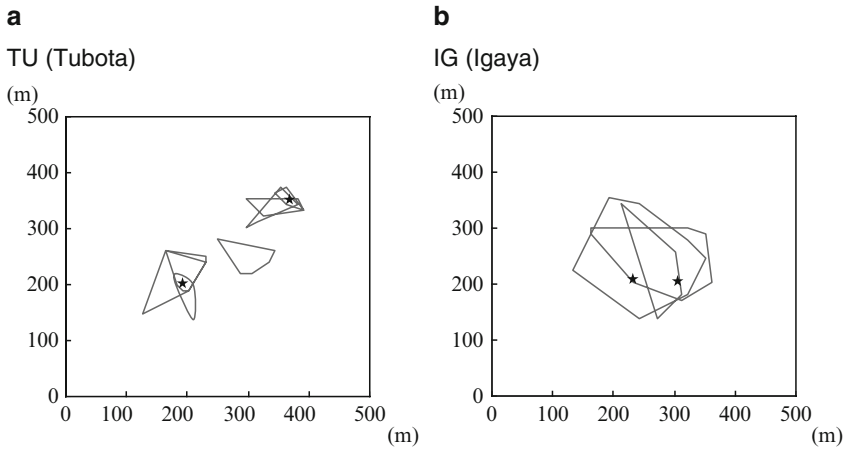

Fig. 5.5 Home ranges of *Zosterops japonica* individually tracked using radiotelemetry in winter 2006 on Miyake-jima. Outermost points of locations tracked for more than 1 week were connected by *lines* to form minimum convex polygons of home ranges. (**a**) High-flower-density area of Tubota (*TU*). (**b**) Low-flower-density area of Igaya (*IG*). *Stars* represent points where birds were captured

5.3.2 Microsatellite Haplotyping from Single Pollen Grains

All *Zosterops* captured using nets ($n=22$) had pollen grains adhered to their entire beak surfaces. Four *Cetta diphone* ($n=15$; 27%) and four *Parus major* ($n=13$; 31%) individuals had small amounts of pollen on their beaks. Only one *Hypsipetes amaurotis* individual was captured with a small quantity of pollen on its beak (see Table 5.2).

The haplotypes of pollen grains found on the 18 captured *Z. japonica* and 4 recaptured birds were determined (Table 5.2). Additionally, we determined the haplotypes of pollen grains on 1 *H. amaurotis*, 4 *C. diphone*, and 4 *P. major* captured in the low-flower-density areas of IG and N2 (Table 5.2).

In total, 878 pollen grain samples were haplotyped for more than five microsatellite loci and used in the genetic analyses ($n=599$, mean 27.2, and range 16–48 for *Z. japonica*; $n=279$, mean 31.0, and range 22–92 for other birds). All microsatellite loci were amplified in 83% of the pollen grains.

5.3.3 Genetic Diversity

The genotypes of all 89 mature trees were determined at the three study plots (each 0.3 ha: $n=48$ at TU; $n=24$ at IG; $n=17$ at N2). The genetic parameters of the pollen grains found on *Z. japonica* are summarized in Table 5.4. We summed the data at IG and N2 in the low-flower-density area because of the small number of birds analyzed for pollen. A comparison of the diversity parameters (*Na*, *H*, and *Ar*) for adult trees in the high-flower-density site (TU) to those in the low-flower-density sites (IG and N2) revealed that diversity only slightly differed among different densities and that this difference was not significant (Table 5.4a). On the other hand, the three genetic parameters for the pollen were significantly higher in the low-flower-density sites (IG and N2) than in the high-flower-density site (TU) (Table 5.4b). In addition, A_{st} were significantly lower in the low-flower-density sites (Table 5.4b and Fig. 5.6). Higher genetic diversity parameters in the low-flower-density sites were probably caused by the larger numbers of pollen donors sampled by *Z. japonica* (see Table 5.3), whereas the lower values of A_{st} in the low-flower-density sites were considered to be the result of more homogeneous distribution of alleles among pollen pools on beaks of *Z. japonica* in the low-flower-density sites.

The genetic parameters for the pollen found on *H. amaurotis*, *C. diphone*, and *P. major* are also summarized in Table 5.4. A comparison of the genetic diversity of the pollen found on *Z. japonica* and the other birds in the low-flower-density sites (IG and N2) showed a significant difference in three parameters (Table 5.4c). However, the mean A_{st} of *Z. japonica* did not differ from that of the other birds (Table 5.4c).

Table 5.4 A comparison of genetic diversity between study sites (**a** and **b**) and among bird species (**c**), based on eight microsatellite markers

a Mature trees

	Study sites		
Gene diversity	TU	IG and N2 (IG | N2)	Significance[a]
Na	7.3	7.2 (8.0 | 6.3)	NS
H_e	0.68	0.70 (0.72 | 0.67)	NS
A_r	5.71	5.26 (5.42 | 5.10)	NS

b Pollen pools on *Z. japonica* beaks

	Study sites		
Gene diversity	TU	IG and N2 (IG | N2)	Significance[a]
Na	3.6	4.7 (4.2 | 5.2)	**
H	0.48	0.54 (0.53 | 0.56)	*
A_s	3.28	3.95 (3.75 | 4.15)	***
A_t	4.48	4.37 (4.40 | 4.33)	NS
A_{st}	0.35	0.15 (0.19 | 0.06)	***

c Pollen pools on beaks of *Z. japonica* and other bird species

	Bird species		
Gene diversity	*Z. japonica*	Other birds	Significance[a]
Na	4.5	3.5	*
H	0.54	0.43	**
A_s	3.95	2.84	**
A_t	4.37	3.71	–
A_{st}	0.15	0.23	NS

For comparison between study sites, the high-flower-density site (TU) and low-flower-density sites (IG and N2) were considered. Values for IG and N2 are also listed separately

Each genetic diversity parameter indicates *Na*, no. of alleles; H_e, expected heterozygosity; *H*, gene diversity; A_r, allelic richness; A_s, mean allelic richness within each pollen pool adhering to a bird; A_t, total allelic richness in the pollen pools adhering to birds within each study site; A_{st}, partitioning of allelic richness among pollen grains

Gene copies of allelic richness were standardized at 20

[a]Significance: NS, nonsignificant; $*P<0.05$, $**P<0.01$, $***P<0.005$ (Mann–Whitney *U* test)

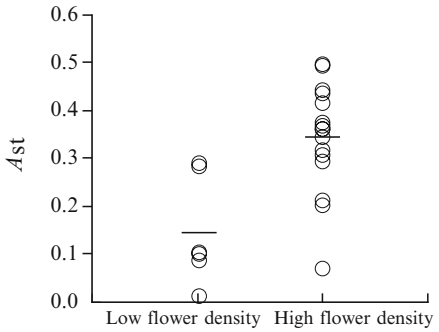

Fig. 5.6 Relationship between flower density and the partitioning of allelic richness among pollen grains on each *Zosterops* individual (A_{st}). Site TU had a high flower density, whereas sites IG and N2 had low flower densities. *Circles* and *bars*, each sampled bird and the mean value for each site, respectively

5.4 Discussion

5.4.1 *Resilience of the Camellia japonica–Zosterops japonica System Against Environmental Perturbation*

Cascading negative impacts of reduced flower density on pollination, fruit-set, and seed-set rates have been observed in many plant species (Ågren 1996; Mustajärvi et al. 2001; Leimu and Syrjänen 2002). Low fruit-set rates have generally been attributed to pollen or resource limitation (Stephenson 1981; Bawa and Webb 1984; Zimmerman and Pyke 1988; Sperens 1996). Even with efficient seed production that produces a sufficient quantity of pollen, the genetic variation of progeny might decrease because of a decrease in pollen donors. Although long-distance pollen dispersal by insects occurs when there is low tree density as a consequence of sparse distribution or fragmented landscapes (Stacy et al. 1996; Konuma et al. 2000; Dick et al. 2003), the low population numbers or low flowering density increases selfing and inbreeding depression (Aldrich and Hamrick 1998; Obayashi et al. 2002; Latouche-Hallé et al. 2004; Naito et al. 2008). For *Shorea leprosula*, it was demonstrated that flowering tree density was negatively correlated with the rare allele frequency in the seedling population as well as with pollen dispersal distance, without reference to the biological systems that caused these results (Fukue et al. 2007). In contrast to the conclusions of numerous previous studies, reduced flower density in *C. japonica* engendered markedly high genetic diversity in pollen grains associated with *Zosterops* (see Table 5.4) as well as an expansion in the pollen dispersal area (see Fig. 5.5). Moreover, the F_{st} values, or the partitioning of heterozygosity among pollen grains, were lower in the low-flower-density areas (0.267 at IG; 0.169 at N2) compared to the high-flower-density area (0.301 at TU). We propose that specialized interactions between *C. japonica* and *Z. japonica* along with the foraging behavior of *Z. japonica* account for the resilience within the *C. japonica–Z. japonica* system against environmental perturbation, such as volcanic eruption and anthropogenic habitat destruction, which is discussed next.

Because *Z. japonica* strongly depends on *Camellia* flower nectar as a food resource in winter, it is considered a seasonal specialist (Kunitake et al. 2004). During the winter season, invertebrates, fruits, and other flowering plants are scarce, whereas *C. japonica* flowers that secrete large amounts of nectar are abundant. This phenological concordance between the pollinator's resource shortage and *C. japonica* flowering might have stimulated seasonal specialization by omnivorous birds that produced effective pollination services. This specialization may never stop *Z. japonica* from visiting *Camellia* flowers, even if volcanic disturbance severely limits the flowering activities of *C. japonica*; that is, birds without access to high-flower-density areas were forced to seek *Camellia* flowers in low-flower-density areas. Consequently, the shortage of flower resources increases the home range size of the pollinator (Table 5.3 and Fig. 5.5) and the number of visited flowering trees (Table 5.3) under low flower densities. The corresponding increase in pollen donors not only enhanced

genetic diversity of the pollen grains on *Zosterops* individuals (Table 5.4), but also increased the proportion of flowers visited by pollinators, thereby ensuring higher pollination rates in low-flower-density areas (Abe and Hasegawa 2008).

At the high-flower-density site (TU), 70% of tracked *Zosterops* moved within the study area, while at the low-flower-density sites (IG and N2) 100% of tracked birds disappeared from the study plot for 9 days or fewer (data not shown). The home ranges in the low-flower-density sites may be more flexible than those in high-flower-density sites. The existence of pollinator birds with a large home range in low-flower-density habitats (Fig. 5.5) must be one of the factors preventing genetic differentiation among pollen grains, because the number of trees visited by *Z. japonica* in low-flower-density areas was larger than in the high-flower-density area, which would increase the number of pollen donors for each mother tree. High genetic diversity of pollen grains in low-density sites is therefore likely a result of the resilient relationship between *C. japonica* and *Z. japonica*. The enlarged area is generated in response to environmental perturbation, leading to an increase in potential pollen donors for recipient *Camellia* trees in low-flower-density areas.

5.4.2 Conclusions and Future Research

This study examined how reduced flower density affected the pollen movement of *Camellia japonica* through a combination of single-pollen haplotyping and radio tracking of the specialized pollinator bird, *Zosterops japonica*. Greater foraging movements of the pollinator in areas with low flower density promote higher genetic diversity of pollen grains transported by *Zosterops*. These results corresponded to an enhanced efficiency in maternal reproductive success (pollination rate and seed-set rate) of *C. japonica* in low-flower-density areas (Abe and Hasegawa 2008). A greater dependence on nectar, which results in an increase in the transporting rates of *C. japonica* pollen grains, makes *Z. japonica* the most effective pollinator of *C. japonica* among the potential avian pollinators. The greater genetic diversity within the transported pollen (see Table 5.4) and higher visitation rates by pollinator birds (Kunitake et al. 2004; Abe and Hasegawa 2008) together provide solid evidence that the *C. japonica–Z. japonica* system, with its innate mechanisms, is robust against environmental perturbation such as volcanic eruption and anthropogenic habitat destruction.

Finally, there are two other issues that should be addressed in future studies. One concern is to determine whether the high genetic diversity found within the pollen adhering to pollinator birds is successfully delivered to the trees by examining the genetic diversity of pollen donors within a fruit or seedling, which would determine the genetic diversity of the next generation in a more direct way. The second issue is to examine how spatial differences in flowering phenology (i.e., flowering time and the number of flowers) among individual trees affect both maternal and paternal reproductive success via pollen flow. Variations in flowering phenology

might induce spatial and temporal variation in flower density and affect the degree of spatial restriction of pollen flow. In fact, previous studies have reported that flower density can strongly affect pollen flow (Levin and Kerster 1969). For example, at the TU site with high flower density, the number of flowers per flowering tree varied greatly (see Table 5.1). There may be large variations in genetic diversity among pollen pools that individual trees have received. If *Z. japonica* were only attracted to *Camellia* trees that had large amounts of flowers, the number of pollen donors received per tree would decrease, which would lead to genetic differentiation. Therefore, the differences in genetic diversity of pollen pools between the low- and high-flower-density sites might be caused by variations in flowering phenology within each population. To better understand the *C. japonica–Z. japonica* system, we must determine the consequences of variable pollen flow on genetic differentiation within the populations considering the interactions among flowering time, the number of flowers, and visitation rates of pollinators.

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Chapter 6
Can Tiny Thrips Provide Sufficient Pollination Service During a General Flowering Period in Tropical Rainforest?

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6.1 Introduction

Lowland tropical rainforests in western Malaysia are characterized by a high diversity of tree species (Whitmore 1984) and the dominance of the Dipterocarpaceae in the canopy and emergent layers (Ashton 1982, 1988). These forests regenerate through the unique and spectacular phenomenon termed masting, or "general flowering" (Fig. 6.1a). During the general flowering period (GFP), which occurs at irregular intervals of 2–10 years and over a period of a few months, nearly all species of the Dipterocarpaceae and many species of other families come into flower synchronously, whereas many of these species hardly bloom during the intervening periods (Ashton et al. 1988; Appanah 1993; Sakai et al. 1999b; Numata et al. 2003). Synchronized mast fruiting after the intense flowering of the GFP satiates seed predators, and irregular flowering causes their population size to decrease owing to shortage of food resources during the non-GFP. Thus, mast flowering is considered to be an adaptive strategy that increases the survival rates of seeds and seedlings in tropical rainforests with diverse and massive numbers of predators (Janzen 1971, 1974; Kelly 1994; Curran and Leighton 2000; Curran and Webb 2000). On the other hand, such irregular and intense flowering can place immense demands on pollinators for seed production, because pollination of most tropical plant species is achieved by animal pollen vectors (Momose et al. 1998b).

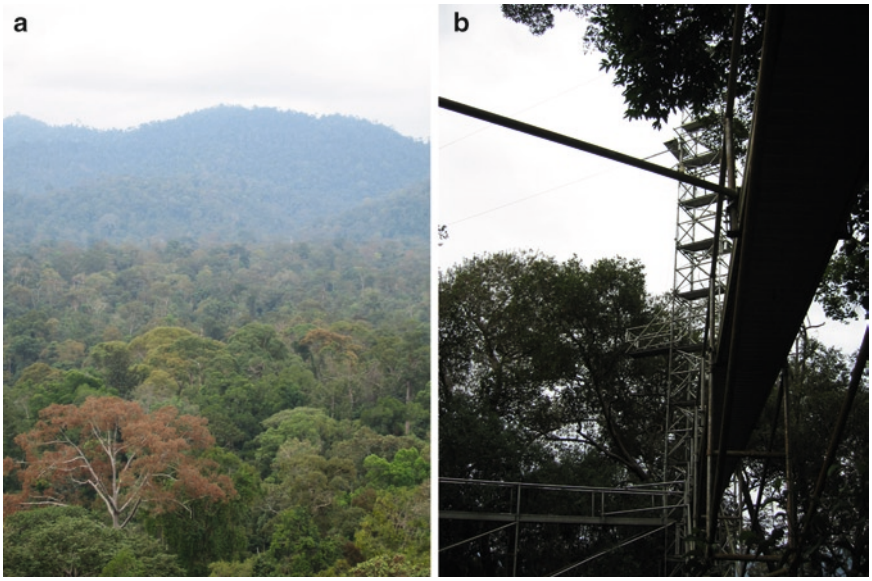

Fig. 6.1 A view of the mast fruiting event after general flowering in 2005 (**a**) and the canopy observation systems (**b**) in a 40-ha ecological plot in the Pasoh Forest Reserve. During the general flowering period in 2005, more than 80% of the mature *Shorea acuminata* trees in the 40-ha plot came into full flower. The three canopy towers (two of which are 32 m tall; one is 52 m) are interconnected by the canopy walkway at a height of 30 m

The shortage of floral resources in the non-GFP also leads to a decrease of pollinators, which mainly feed on floral tissues as a reward for the transition of pollen grains. Pollinator shortage might also occur during the GFP unless there are pollinators that can quickly respond to this phenomenon. Thus, one of the most interesting and important questions in tropical rainforest research is what pollination systems are adapted to these general flowering species (Ashton et al. 1988; Sakai 2002).

Appanah and Chan (1981) studied pollination systems during the GFP in tropical rainforest by investigating the floral visitors in six emergent tree species of *Shorea* section *Mutica* in the Pasoh Forest Reserve in Peninsular Malaysia. They ascertained that thrips (Thysanoptera), which oviposit on unopened flower buds and feed on floral tissues, accounted for 97.7% of all floral visitors (Fig. 6.2). A density of 2.9 adult thrips per flower was observed in *Shorea lepidota*, and a single adult was found to carry as many as 27 pollen grains on its body, with the average per adult being 2.43 grains (Appanah and Chan 1981). Because of the short generation time (~8 days) and high reproductive rate of thrips (average fecundity of 27 eggs per female), Appanah and Chan (1981) argued that thrips were ideal pollinators during the GFP. Thrips can sharply increase their population size to provide sufficient pollination service by feeding on scattered flower resources during the GFP. Such thrips pollinations were also observed in another section of mast-fruiting *Shorea* (*Richetioides*) and the genus *Hopea*, which are by far the largest genera of the Dipterocarpaceae (Appanah 1979).

The contribution of flower visitors to pollination, however, cannot be measured solely by their visitation frequency (Schemske and Horvitz 1984, and references therein), the amount of body pollen (Inouye et al. 1994), and the patterns and levels of pollen deposition on stigmata (Muchhala and Potts 2007). The fertilization success and offspring fitness of plants are strongly affected by the genetic composition and diversity of transported pollen through the features

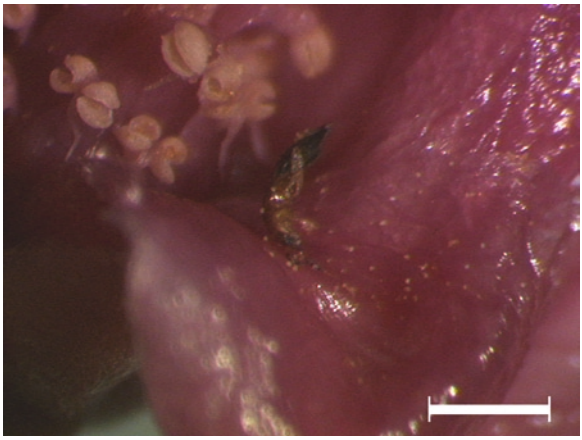

Fig. 6.2 Flower thrips hidden within a corolla of *Shorea acuminata*. Pollen grains of *S. acuminata* were observed on and around its body. In this research, insects were collected together with a small branch that had more than 100 flowers; they were killed by placing them at −30°C in a refrigerator with flowers and branch. *Bar* = 1 mm

of self-incompatibility and inbreeding depression. Most plant species in tropical rainforests grow at low population density, and they have self-incompatibility (Gan et al. 1977; Hamrick and Murawski 1990; Tsumura et al. 2003) and strong spatial genetic structure over short distances (Ng et al. 2004, 2006; Takeuchi et al. 2004), wherein efficient seed production requires long-distance cross-pollination (Bawa 1990). Therefore, it remains unclear whether minute thrips with an inferior flying ability that depends on local air currents can provide sufficient pollination service in tropical rainforest, where most plant species grow at low population density.

Methods using microsatellite DNA markers have been used to determine the pattern and distance of pollen dispersal by comparing the genotypes of offspring and putative parents (e.g., paternity analysis: Isagi et al. 2007; two-generation analysis: Smouse et al. 2001), but studies were unable to trace the actual movement of each pollen grain or to detect the pollination efficiencies of each pollinator. However, a recently developed method for determining multiple microsatellite genotypes from a single pollen grain (Matsuki et al. 2007, 2008) should provide insight into the specific questions in plant–animal interactions and pollination systems during the GFP in tropical rainforests.

In this study, we investigated the pollination system of an emergent and canopy tree, *Shorea acuminata* Dyer (Dipterocarpaceae, section *Mutica*), during the GFP in 2005 using a canopy observation system. In addition to measuring the flower visitation frequency of thrips and their seasonal changes throughout the GFP, we used microsatellite DNA analyses of single pollen grains adhering to pollinators and genotyped seeds to evaluate the pollination efficiency of thrips. Based on our findings, we discuss what pollination systems or plant–pollinator interactions are adapted to the phenomenon of general flowering, which is characterized by irregular and intense flowering.

6.2 Materials and Methods

6.2.1 Study Site, Target Plant Species, and Mast Flowering in 2005

The study site was a primary lowland dipterocarp forest in the Pasoh Forest Reserve, Negri Sembilan state, Peninsular Malaysia (latitude 2°59′N, longitude 102°19′E; 75–150 m above sea level). In a 40-ha ecological plot in the reserve, we observed flowering and fruiting phenology of dipterocarp trees with the aid of three canopy towers (Fig. 6.1b: two at 32 m; one at 52 m) interconnected by a canopy walkway at a height of 30 m. All dipterocarp trees more than 30 cm in diameter at breast height (dbh) in this plot were mapped and identified to species level.

Shorea acuminata is a common canopy tree that is widely distributed in mixed dipterocarp forests of Malaya, Sumatra, and Lingga (Ashton 1982). This species

is known for supraannual reproduction during the GFP. *S. acuminata* trees bear hermaphrodite flowers, which showed self-incompatibility in a controlled pollination experiment (Chan 1981). Individual crimson-red flowers open at dusk, and most corollas are pushed off and drop to the forest floor the next day when new flowers start to open (Appanah 1979). The blooming duration of *S. acuminata* is nearly 3 weeks (Ashton et al. 1988), and an individual tree of the close relative *Shorea leprosula* was estimated to produce more than 600,000 flowers in 1 day during peak bloom (Appanah and Chan 1981). The flowers release a strong sweet scent but do not secrete nectar. The pollen production of *Shorea* section *Mutica* species is generally low, varying from approximately 3,000 to 5,500 grains per flower, and the size of pollen grains ranges from 2.5 to 3.0 μm (Appanah and Chan 1981). The pollen grains are sticky and are not significantly dislodged by wind (Appanah and Chan 1981).

In the 40-ha plot, the flowering phenology of 27 dipterocarp species with greater than 30-cm dbh, including 61 mature *S. acuminata* trees, was observed during the GFP in 2005. Ten flowering censuses were performed at intervals of approximately 2 weeks until the end of mature seed dispersal (S. Nishimura, unpublished data). During the GFP in 2005, more than 80% of *S. acuminata* trees (49 individuals) came into full flower. The first flowering event of *S. acuminata* was observed on two trees during the fourth flowering census (11–19 April). Flowering reached a peak (with 42 flowering trees) during the fifth census (20–29 April), and all *S. acuminata* trees had stopped flowering during the seventh census (20 May–6 June).

6.2.2 Collection of Insect Visitors to Flower and Tree

The pollination of one *S. acuminata* individual (G221, 104-cm dbh), which was accessible by the 52-m canopy tower, was studied from 19 April to 29 June 2005 from before flowering to the fruiting season. Insects were collected from flowers and branches in the following manner. We quickly covered a small branch that had more than 100 flowers with a sealable plastic bag and cut the branch off inside the bag. As for the nonflowering seasons (two collection periods: before flowering, and fruiting period), we collected a small branch that had more than 200 flower buds or more than 25 seeds, respectively. The bag was placed on ice to stop insect movement and brought back to the laboratory. Insects were killed by placing the bag at −30°C in a refrigerator and then removed from the flowers and branches. Flower and tree visitors were collected at five phenological periods of tree G221: before flowering, 19 April; start of flowering, 24 April; peak of flowering, 29 April; late flowering, 4 May; fruiting period, 29 June. We collected five branches per 6-h interval (at 0600, 1200, 1800, and 2400) in each collection period, to a total of 100 branches. The number of each type of insect collected from each branch was calculated. Differences in the number of thrips among phenological periods and times were examined by one-way analysis of variance (ANOVA) followed by Scheffé's multiple comparison test.

6.2.3 Genotype Determination of Pollen Grains Adhering to Thrips

The microsatellite genotypes of pollen grains adhering to thrips were analyzed to determine their pollination efficiency. For DNA extraction from a single pollen grain, we modified the methods described by Suyama et al. (1996) and Matsuki et al. (2007). Into a 0.2-ml polymerase chain reaction (PCR) tube was placed 0.5 μl extraction buffer containing 0.025% sodium dodecyl sulfate (SDS), 0.1 μg/μl proteinase K (Takara), and 1× Ampli*Taq* Gold PCR buffer (containing 1.5 mM $MgCl_2$; Applied Biosystems). Under a stereomicroscope, a single pollen grain was picked from the insect surface on a plastic pipette tip (for manipulating 0.5–10 μl of liquid) that had been stretched and cut to obtain a sharp end. The single pollen grain was washed in sterile distilled water and transferred with 0.5 μl water to a prepared PCR tube with extraction buffer. The reaction buffer was incubated at 55°C for 2 min, 37°C for 90 min, and 95°C for 5 min.

For the determination of multiple microsatellite genotypes from a single pollen grain, the multiplex PCR method (Chamberlain et al. 1988) was used (see Matsuki et al. 2007). Genotypes of each pollen grain were scored by using eight pairs of microsatellite primers previously developed for *Shorea* species (Ujino et al. 1998 for *Shorea curtisii*: *Shc*03, *Shc*07, and *Shc*09; Lee et al. 2004 for *S. leprosula*: *Sle*280, *Sle*392, *Sle*465, and *Sle*566; Lee et al. 2006 for *Shorea lumutensis*: *Slu*044*a*). These eight pairs of microsatellite primers were screened in *S. acuminata* by Naito (2008). Multiplex PCR amplifications were performed in a thermal cycler (GeneAmp PCR System 9600, Applied Biosystems) under the following conditions: initial activation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 90 s, and extension at 72°C for 1 min; followed by a final incubation at 60°C for 30 min. The 6-μl reaction mixture contained 1 μl extracted pollen DNA, 0.2 μmol each microsatellite primer labeled with fluorescent phosphoramidites (6-FAM, VIC, NED, PET), and 3 μl Qiagen Multiplex PCR Master Mix. The genotypes were determined on an ABI PRISM 3100 Genetic Analyzer with GeneScan version 3.7 and Genotyper version 2.0 software (Applied Biosystems).

6.2.4 Proportions of Self-Pollen and Genetic Diversity in Pollen Grains Adhering to Thrips

Those pollen samples for which genotypes were determined for more than five microsatellite loci were used for further analysis. The genotypes of pollen grains were compared with those of the *S. acuminata* tree (G221) from which the insects were sampled. Pollen samples that had the subset of alleles at all analyzed loci as G221 were considered self-pollen (pollen transported from a different region on the

tree). Pollen samples that had alleles different from those of G221 were considered outcross pollen.

The proportion of self-pollen in the total pollen load was calculated for each thrips. The genetic diversity of the pollen adhering to thrips was expressed in terms of the probability that two alleles chosen randomly are different (Nei 1973). Differences in the proportion of self-pollen grains and genetic diversity for adherent pollen grain on each thrips among three different phenological periods (start, peak, and late flowering periods) were examined using one-way ANOVA followed by Scheffé's multiple comparison test.

6.2.5 *Selfing Rates of Seeds*

We determined the proportions of cross-pollinated seeds and their genetic diversity within 48 immature seeds collected from the crown of tree G221. These seeds originated from the mast flowering in 2005. Total DNA was extracted using a modified cetyltrimethyl ammonium bromide (CTAB) method (Tsumura et al. 1995), and the seed genotypes were scored using the eight pairs of microsatellite primers noted above. The genotypes of seeds were compared with that of the collection tree. Seed samples that had alleles different from those of G221 were considered to be of outcross origin.

6.2.6 *The Contribution of Thrips to Seed Production*

To examine the contribution of thrips to seed production, differences in both the number of observed alleles and allelic richness at each locus between immature seeds and pollen grains adhering to thrips were examined. Because half of the total number of alleles observed among immature seeds originates from the *S. acuminata* tree (G221) from which seeds and insects were sampled, the value of allelic richness within immature seeds, which is dependent on allele frequency within sampled group, is undervalued when we make a comparison with that of pollen grains with a haploid gene. To eliminate the effects from the comparison, we calculated the modified allelic richness within pollen grains by adding the observed alleles of the collection tree as many as the number of pollen grains at each locus. If the collection tree had a heterozygous locus, we added these alleles as many as half of the number of pollen grains, respectively. This method allows the estimation of the thrips contribution to seed production by comparison of allelic richness. In addition to the modified allelic richness, we also calculated the normal allelic richness based on the observed alleles within pollen grains.

6.3 Results

6.3.1 Flower-Visiting Insects and Their Seasonal Variation

In our study of flower- and tree-visiting insects on *S. acuminata*, we collected a total of 718 insects. Most insects (621) were collected during the flowering season (three collection periods: start, peak, and late flowering periods), and of these most (500) were collected during the flowering time (from 1800 to 0600). During the nonflowering seasons (two collection periods: before flowering, and fruiting period), only 97 insects were collected (Table 6.1).

The principal flower-visiting insects were thrips (Thysanoptera), accounting for 76.2% of 500 insects collected during the flowering time, followed by plant and lygaeid bugs (Hemiptera: 12.2%) and small beetles (Coleoptera: 8.2%) (Table 6.1). There were two types of thrips with different behavioral features. Flower thrips (Thripidae, *Thrips* spp.) were collected from corollas and accounted for 63.4% of the 500 collected insects. Although we did not identify them to species, in another study 75% of all the thrips collected from the close relative *Shorea*

Table 6.1 Insect visitors to a *Shorea acuminata* tree during the flowering season (three collection periods: start, peak, and late flowering periods) and nonflowering season (two collection periods: before flowering, and fruiting period)

Order	Flowering season		Flowering time during flowering season		Nonflowering season	
Family	Average *n*	(%)	Average *n*	(%)	Average *n*	(%)
Hemiptera						
Miridae	4.0	1.9	3.3	2.0	1.0	2.1
Lygaeidae	20.7	10.0	16.0	9.6	7.0	14.4
Cicadellidae	1.7	0.8	1.0	0.6	9.0	18.6
Thysanoptera						
Thripidae	126.0	60.9	105.7	63.4	0.5	1.0
Phloeothripidae	28.0	13.5	21.3	12.8	26.5	54.6
Coleoptera						
Nitidulidae	3.3	1.6	2.3	1.4	0.5	1.0
Chrysomelidae	5.3	2.6	3.7	2.2	2.0	4.1
Curculionidae	10.0	4.8	7.7	4.6	1.0	2.1
Hymenoptera	8.0	3.9	5.7	3.4	1.0	2.1
Average number of insects per collection period	207.0	100.0	166.7	100.0	48.5	100.0
Total number of insects collected	621		500		97	

Average number sampled per collection period and the relative prevalence in each collection period (%) are shown. These values were also calculated during the flowering time (three collection times from 1800 to 0600), except for samples collected at 1200

parvifolia section *Mutica* was a single species, *Thrips hawaiiensis* (Sakai et al. 1999a). Stipule thrips (Phloeothripidae, *Haplothrips* spp.) always occurred in the capsule-shaped stipules and accounted for 12.8% of the 500 collected insects. Among the flower-visiting insects collected during the flower and branch collection, flower thrips showed the highest visiting frequency to *S. acuminata* during the flowering time.

The visiting patterns of flower thrips, the principal floral visitor, and the seasonal changes in the number of flower thrips are shown in Fig. 6.3a. The number of flower thrips per collected branch differed among collection periods ($F = 34.1$, $df = 4$, $P < 0.001$; one-way ANOVA). Scheffé's multiple comparison test indicated that the highest number of flower thrips visited *S. acuminata* during the peak of the flowering period (mean ± SD, 11.40 ± 6.84), followed by the late flowering period (3.85 ± 3.01) and start of the flowering period (3.65 ± 2.70). Only one individual flower thrips was observed before the flowering period, and no thrips visited *S. acuminata* during the fruiting period. By contrast, stipule thrips and bugs showed a different visiting pattern. Although these insects increased in number during the peak of flowering, they also occurred on *S. acuminata* trees frequently during the nonflowering seasons (before flowering and fruiting seasons; see Table 6.1).

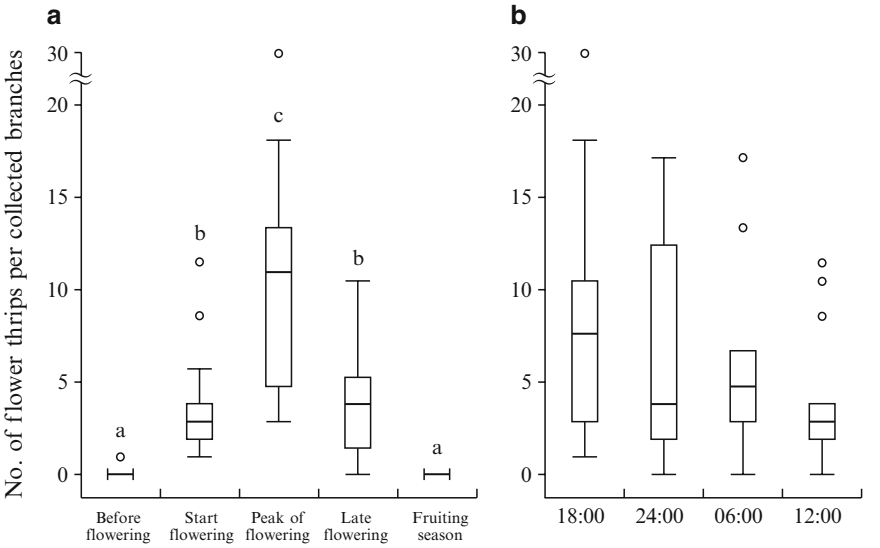

Fig. 6.3 Seasonal (**a**) and intraday (**b**) changes in the number of flower thrips per collected branch. Maximum and minimum in each sample (except for the outliers represented by *circles*) are shown by the upper and lower ends of the *vertical bars*; 75% and 25% points are given by the upper and lower ends of the *boxes*; and the *middle line within each box* represents the median value. Values denoted by the *same letters* are not significantly different from each other at the $P \leq 0.05$ level (Scheffé's multiple comparison test)

The intraday changes in the number of flower thrips collected after flower opening (1800) are shown in Fig. 6.3b. Although there were no significant differences among the four collection times ($F = 1.6$, $df = 3$, $P = 0.210$; one-way ANOVA), flower thrips visitation to *S. acuminata* was concentrated during the flowering time from 1800 (flowers start opening) to 0600 (flowers start dropping); their number quickly decreased at noon, when there were no fresh flowers. Visitors during the evening flowering time (8.60 ± 7.74 at 1800) were twice as numerous as at 1200 (4.07 ± 3.63). Thus, the visitation of flower thrips to *S. acuminata* was concentrated during the flowering seasons and was synchronous with flower opening.

6.3.2 Microsatellite Genotyping from Single Pollen Grains

In total, 398 pollen grains were removed from 44 adult flower thrips, and the genotypes of eight microsatellite loci were determined. Among the 3,184 combinations of eight loci and 398 pollen grains, 2,514 genotypes (79.0%) were successfully determined. The percentage of successful genotype determinations among the microsatellite loci ranged from 40.7% (*Sle*465 locus) to 88.9% (*Shc*09; see Table 6.2). In total, 355 pollen grains (89.2%) were successfully genotyped for more than five microsatellite loci and used for further genetic analysis.

6.3.3 Proportions of Self-Pollen and Genetic Diversity in Pollen Grains Adhering to Flower Thrips

Among the 355 pollen grains successfully genotyped, the proportion of self-pollen was 0.92 (Table 6.3). The proportions of self-pollen grains adhering to each flower thrips ranged from 0.56 to 1.00 (mean ± SD, 0.91 ± 0.12; Fig. 6.4a) and 27 of 44 flower thrips (61.4%) only had self-pollen. The mean Nei's genetic diversity values of pollen load of each flower thrips ranged from 0.08 to 0.38,

Table 6.2 Number of pollen grains for which each microsatellite locus was successfully amplified

Microsatellite loci	*Shc* 03	*Shc* 07	*Shc* 09	*Sle* 280	*Sle* 392	*Sle* 465	*Sle* 566	*Slu* 044a
Number of pollen grains	354	344	353	371	337	162	267	326
Percentage of success	88.9	86.4	88.7	93.2	84.7	40.7	67.1	81.9

A total of 398 pollen grains were analyzed

Table 6.3 Proportion of self-pollen grains (or selfing rate of immature seeds) and number of alleles observed at each locus

Proportion of self-pollen (selfing rate)		The number of alleles									
		Shc 03	*Shc* 07	*Shc* 09	*Sle* 280	*Sle* 392	*Sle* 465	*Sle* 566	*Slu* 044a	Mean	Total
Flower thrips	0.915	3	3	4	3	3	10	2	4	4.00	32
Immature seeds	0.417	3	7	6	4	7	8	8	8	6.38	51
Collection tree		2	1	2	2	1	1	1	2	1.50	12

Flower thrips, $n=44$ (355 pollen grains); immature seeds, $n=48$; collection tree, $n=1$

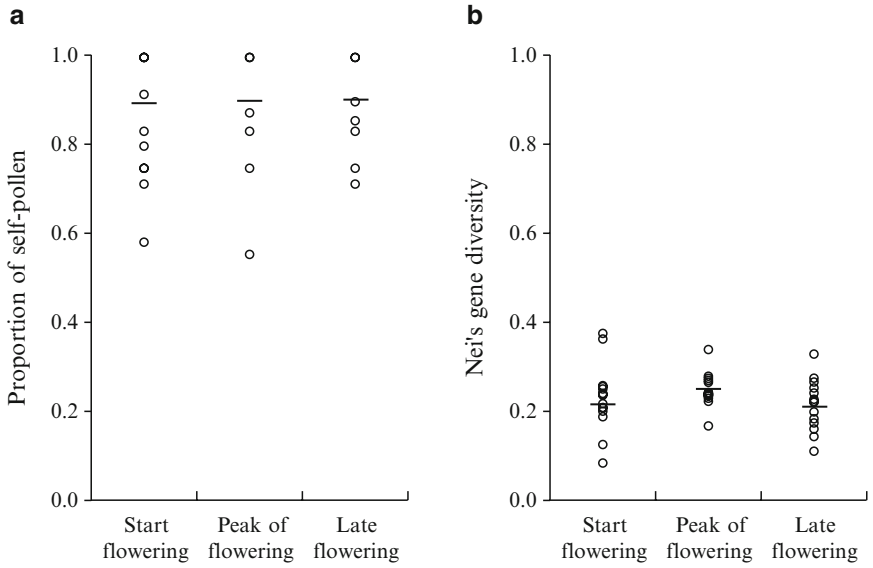

Fig. 6.4 Seasonal changes in the proportion of self-pollen grains (**a**) and genetic diversity of adherent pollen (**b**) on flower thrips. *Circles* and *bars* represent each individual sample and the average value for each phenological period, respectively. Start of flowering (SF), $n=15$; peak of flowering (PF), $n=15$; late flowering (LF), $n=14$

with an average of 0.23 (Fig. 6.4b). There were no significant differences in either the proportion of self-pollen grains or genetic diversity in adherent pollen on each thrips among the three phenological periods (selfing rate: $F=0.43$, $df=2$, $P>0.05$; genetic diversity: $F=1.17$, $df=2$, $P>0.05$; one-way ANOVA; Fig. 6.4a,b, respectively). The proportion of outcross pollen grains and genetic diversity of adherent pollen on each flower thrips were extremely low throughout the flowering period.

Table 6.4 Values of allelic richness at each locus

	Shc 03	*Shc* 07	*Shc* 09	*Sle* 280	*Sle* 392	*Sle* 465	*Sle* 566	*Slu* 044a	Mean
Flower thrips (normal)	2.51	1.93	2.57	2.17	1.93	6.98	1.22	2.51	2.73
Flower thrips (modified)	2.24	1.55	2.26	2.09	1.55	4.16	1.12	2.28	2.15
Immature seeds	2.94	5.95	5.36	3.58	5.91	8.00	7.69	7.21	5.83

Flower thrips, $n=44$ (355 pollen grains); immature seeds, $n=48$

Notes: To compare allelic richness between pollen grain and immature seeds which were collected from a seed parent, we calculated the modified allelic richness within pollen grains by adding the observed alleles of collection tree as many as the number of pollen grains at each locus. For details, please refer to Sect. 6.2 (Materials and Methods)

6.3.4 *Selfing Rates of Seeds and the Contribution of Thrips to Seed Production*

Among 48 immature seeds gathered from the crown of tree G221, from which the insects were sampled, the proportion of self-pollinated immature seeds (selfing rate) was 0.417 (see Table 6.3). Fifty-one alleles were observed across the microsatellite loci analyzed. The number of alleles ranged from 3 (*Shc*03) to 8 (*Sle*566 and *Slu*044*a*), with an average of 6.38 alleles per locus. The values of allelic richness in immature seeds ranged from 2.94 (*Shc*03) to 8.00 (*lep*465), with an average of 5.83 (Table 6.4). On the other hand, the number of alleles observed among 355 pollen grains ranged from 2 (*Sle*566) to 10 (*Sle*465), with an average of 4.00 alleles per locus (Table 6.3). The values of modified allelic richness ranged from 1.12 (*lep*566) to 4.16 (*lep*465), with an average of 2.15 (Table 6.4). The number of observed alleles and allelic richness among 48 immature seeds was significantly higher than among 355 pollen grains adhering to flower thrips (for the number of observed alleles, $P<0.05$; allelic richness, $P<0.001$: paired t test).

6.4 Discussion

6.4.1 *Pollination Efficiency of Flower Thrips*

Researchers in the field of tropical ecology are working to elucidate what pollination systems are adapted to mast flowering species, which flower massively at multiyear intervals (Ashton et al. 1988; Sakai 2002). A study of *Shorea* section *Mutica* (Appanah and Chan 1981) found that highly fecund flower thrips are the primary pollinators and can rapidly respond to an abrupt increase of flowers during the GFP. However, because thrips are minute and rather weak flyers, it had remained

unclear to what extent they could contribute to cross-pollination of mast flowering species growing at low population density. In the present study, microsatellite DNA analyses of single pollen grains adhering to pollinators combined with direct observation of flower visitors clearly revealed the pollination efficiency of flower thrips during the GFP.

As compared with previous research conducted at the same site, which clarified that thrips accounted for 97.7% of all floral visitors (Appanah and Chan 1981), several additional flower visitors aside from flower thrips were observed by the aid of the canopy observation system (see Table 6.1). However, our findings also indicate that flower thrips are the principal flower visitors to *S. acuminata*, accounting for 63.4% of collected insects during the flowering time, and their visiting pattern was highly synchronous with the flowering phenology of *S. acuminata*. Flower thrips visited *S. acuminata* only during the flowering period, and their abundance quickly increased during the peak of flowering (see Fig. 6.3a). Furthermore, flower thrips visitation was concentrated during the evening flowering time (flowers start opening) when thrips were twice as numerous as at noon, and their number quickly decreased at noon when flowers started dropping and there were no fresh flowers (Fig. 6.3b). Thus, flower thrips is a typical flower visitor and the synchronization of their visitation with flower opening can be attributed to their strong dependence on the floral resources of *S. acuminata* as food and oviposition sites. Because such behaviors increase the opportunity for contact of thrips with flowers and transition of pollen grains, it seems likely that flower thrips will make a greater contribution to *S. acuminata* pollination than other insects.

However, the genetic analysis of pollen grains suggested geitonogamous self-pollination by flower thrips. Almost all pollen grains (325/355, 91.5%) adhering to flower thrips were self-pollen transported from a different part of the tree from which insects were sampled (see Table 6.3). Furthermore, the proportion of self-pollen grains adhering to each flower thrips was extremely high throughout the flowering period (mean ± SD, 0.91 ± 0.12; see Fig. 6.4a). Such large proportions of self-pollen in the adherent pollen load were also observed among pollen grains adhering to bumblebees (Matsuki et al. 2008). Matsuki et al. (2008) evaluated the pollination efficiencies of bumblebees and flower beetles that visited the flowers of *Magnolia obovata* (Magnoliaceae), which are thought to have been adapted to beetle pollination (Thien 1974; Bernhardt 2000), and they clarified that flower beetles had a much lower proportion of self-pollen (mean ± SD, 0.40 ± 0.31) compared with bumblebees (0.88 ± 0.13). They concluded that such geitonogamous self-pollination by bumblebees could be attributed to both their grooming behavior, which brings about the reduction of pollen carryover, and their effective foraging behavior, that tends to travel shorter distances in places where the standing density and flowering intensity are high (Larson and Barrett 1999; Utelli and Roy 2000). The pollination efficiency of flower thrips observed in this study was comparable to that of bumblebees. Thus, it was considered that flower thrips may not serve as efficient pollinators of *S. acuminate* despite their high visitation frequency on *S. acuminate* synchronous with flowering.

Throughout the flowering period, we observed remarkable geitonogamous pollen movement by flower thrips and corresponding low levels of genetic diversity of

pollen load on thrips (Fig. 6.4a,b). These findings suggest that flower thrips make a small contribution to cross-pollination, instead promoting selfing and low-frequency mating restricted to particular conspecifics. In fact, research on the gene flow of *S. acuminata* in Pasoh Forest Reserve revealed that the mean overall selfing rate of seeds was 38.7% (Naito et al. 2008a), which is comparable to the result of this study (41.7% at immature seed stage; Table 6.3), and outcrossing events were predominantly (76.5%) short-distance events with a mating distance of 100 m or less. Furthermore, Naito et al. (2008a) also clarified that the selfing rate sharply decreased with an increase in the number of neighboring flowering conspecifics, and such density-dependent selfing was also observed in other *Shorea* section *Mutica* species (*S. leprosula*: Fukue et al. 2007) and thrips-pollinated tree species in the understory of lowland dipterocarp forest (*Popowia pisocarpa*: Momose et al. 1998a). These findings can be attributed to both the short-distance pollen movement by flower thrips, revealed by the adherent pollen DNA analysis, and the limitation of outcrossing events resulting from the insufficient number of efficient pollinators on the isolated trees.

Such geitonogamous pollen movement by flower thrips would negatively affect the reproduction of *S. acuminata*, causing inbreeding depression. Naito et al. (2008a) also clarified that seed mass of outcrossed progeny was heavier than that of selfed progeny, and heavier seeds showed higher success rates in germination and seedling establishment. Thus, flower thrips, whose visitation resulted in frequent geitonogamous self-pollination, made a small contribution to seedling establishment and regeneration of *S. acuminata*. Their high visitation frequency on *S. acuminata* synchronous with flowering, however, brought about vast production of self-pollinated seeds when cross-pollination was limited by the insufficient number of efficient pollinators. In fact, vast production of self-pollinated seeds resulting from density-dependent selfing was observed in *Shorea* section *Mutica* species despite their feature of self-incompatibility (Fukue et al. 2007; Naito et al. 2008a). Furthermore, the high ratio of predispersal seed predation by insects was also observed in *S. acuminate* trees in Pasoh Forest Reserve (mean, 53.7%; range, 27.9–74.9%; Naito et al. 2008b), and such predispersal seed consumption by insects has been reported in various dipterocarp species (Nakagawa et al. 2005). In the tropical rainforest with diverse and massive numbers of predators, the vast production of self-pollinated seeds would decrease the predation pressure on cross-pollinated seeds compared to when only a small amount of seeds was produced, originated from cross-pollination. Thus, it is likely that flower thrips contribute to forest regeneration indirectly by producing a large amount of self-pollinated seeds, which brought about the satiation of numerous seed predators.

6.4.2 Pollination System of S. acuminata

The genetic analysis of pollen grains suggested that flower thrips make a small contribution to cross-pollination, instead promoting selfing and low-frequency

mating restricted to particular conspecifics. Furthermore, the genetic analysis of seeds clarified that the number of observed alleles and allelic richness among pollen grains adhering to flower thrips was significantly lower than that among the seeds (see Tables 6.3 and 6.4). Such remarkable geitonogamous pollen movement by flower thrips and the greater genetic diversity of seeds than that of their adherent pollen load implies the existence of other pollinators that provide sufficient pollination service for *S. acuminata*. In fact, long-distance pollen dispersal of *S. acuminata* with a mating distance of 500 m or greater was observed in Pasoh Forest Reserve, although outcrossing events were predominantly (76.5%) short-distance events within 100 m (Naito et al. 2008a). Such long-distance pollen dispersal serves an important role in seed production and subsequent seedling establishment of *S. acuminata*, which suffers inbreeding depression (Chan 1981; Naito et al. 2008a).

Sakai et al. (1999a) studied the pollination system of *S. parvifolia* (section *Mutica*) by field observations and the pollination experiments in Sarawak, Malaysia. They found that herbivorous beetles (Coleoptera: Chrysomelidae and Curculionidae), which have superior flying ability to thrips, accounted for about 60% of flower visitors collected by a net-sweeping method. They also reported that introduced beetles from *S. parvifolia* trees into bagged inflorescences of other *S. parvifolia* trees were more successful than thrips at depositing pollen on stigmata and fertilizing the flowers. Because these beetles were collected outside the GFP by beating on *Shorea* leaves, Sakai et al. (1999a) noted that herbivorous beetles were ideal pollinators that may respond to an abrupt increase of floral resources during the GFP by changing their food from dipterocarp leaves to flowers. However, they also suggested that different pollination systems exist in dipterocarp forests between two regions divided by the South China Sea, because the density of thrips differed greatly between Peninsular Malaysia and Sarawak.

In this study, the principal visitors to *S. acuminata* flowers were flower thrips (Thripidae: *Thrips* spp.), accounting for 63.4% of flower visitors collected during the flowering period (see Table 6.1), followed by stipule thrips (Phloeothripidae: *Haplothrips* spp., 12.8%), which always occurred in the capsule-shaped stipules, and lygaeid and plant bugs (Hemiptera, 12.2%). The density of thrips was much higher than that in Sarawak (Sakai et al. 1999a), and the density of small beetles (Coleoptera, 8.2%) was much lower. The visitation of flower thrips to *S. acuminata* was concentrated during the flowering season and was highly synchronous with flower opening (see Fig. 6.3a,b), whereas stipule thrips and lygaeid bugs (Lygaeidae) showed a different visitation pattern. Similar to flower thrips, the number of these insects increased during the peak of flowering, but they also occurred in *S. acuminata* trees during nonflowering seasons (before flowering and fruiting seasons; Table 6.1). This observation suggests that stipule thrips and lygaeid bugs do not always depend on the floral resources of *S. acuminata*. The capsule-shaped stipules of *S. acuminata* provide a nest for aphids (Ashton et al. 1984), and stipule thrips also reproduced in these stipules (T. Kondo, personal observation). Furthermore, lygaeid bugs typified by big-eyed bugs (*Geocoris* and *Piocoris*) are known to prey on thrips (Hirose et al. 1999). Because lygaeid bugs prey on thrips on the opened flowers, such behaviors

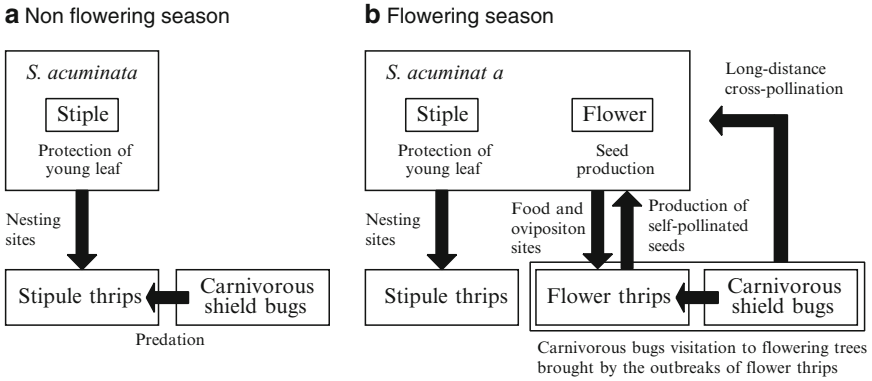

Fig. 6.5 Conceptual diagram of plant–insect interactions during nonflowering season (**a**) and flowering season (**b**)

as a predator increase the opportunity for contact of lygaeid bugs with flowers and transportation of pollen grains among flowering *S. acuminata* trees. In fact, pollen grains adhering to lygaeid bugs were observed (T. Kondo, unpublished research). Ishida et al. (2009) also presented an example of evolution from predatory flower visitors feeding on the pollinators to the main pollinators. They reported the pollination systems of *Macaranga tanarius* (Euphorbiaceae) in which flower bugs are the main pollinators. Similar to the genus *Shorea*, *Macaranga* is a large genus including approximately 260 species with a wide distribution in tropical and subtropical regions. Because abundant thrips have been observed on inflorescences of close relatives of *M. tanarius* and the observed flower bugs belong to a predacious group, whose major prey includes thrips, they noted that hemipteran visitors, which had been originally predators of thrips pollinators, became the main pollinator. Thus, such plant–insect relationships may provide sufficient pollination service for *S. acuminata* in Peninsular Malaysia. Outbreaks in the number of flower thrips may attract the visitation of lygaeid bugs to flowering *S. acuminata* trees as edible resources during the GFP, and this phenomenon would promote long-distance pollen dispersal (Fig. 6.5b). In addition, lygaeid bugs can respond quickly to this abrupt flowering phenomenon by preying upon stipule thrips that inhabit the stipule during the nonflowering seasons (Fig. 6.5a). Thus, it appears that both flower and stipule thrips indirectly contribute to the long-distance cross-pollination of *S. acuminata* by maintaining the population of pollinators with superior flying ability during the non-GFP and attracting their visitations to flowering trees during the GFP by serving as food resources.

Further DNA analysis of adherent pollen on other flower-visiting insects will help to reveal what pollination systems or plant–pollinator interactions are adapted to the unique phenomenon of mast flowering in tropical rainforests, which is characterized by irregular and intense flowering.

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Chapter 7
Alien Dandelions Displace a Native Related Species Through Interspecific Pollen Transfer

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7.1 Introduction

Recently, the number of invading species has been increasing and their habitat ranges have been expanding (Pyšek and Prach 1995; Vitousek et al. 1996; Pyšek et al. 2005). Thus, the invasion of alien species is gaining attention as a major threat to biodiversity and ecosystem functioning (Pimentel et al. 2001; Gurevitch and Padilla 2004). Moreover, to prevent the loss of native biodiversity and ecosystem integrity, an urgent need exists to understand precisely how invasive exotics outcompete native species. However, knowledge pertaining to the mechanisms that allow an invasive species to displace indigenous natives from their habitats is very limited, and the question is mostly unresolved.

To date, interspecific competition is regarded as one of the most important processes determining the likelihood of plant invasion (Crawley 1990), and such competition for resources has been the primary focus of studies directed at clarifying the mechanisms responsible for the impact of invasive plants. Invasive alien species are frequently considered superlative competitors for nutrients (Wardle et al. 1994; Minchinton et al. 2006), water (Grace and Wetzel 1981, 1982; Delph 1986; Huenneke and Thomson 1995; Weihe and Neely 1997), and space (Ágren and Fagerström 1980; Newsome and Noble 1986).

In contrast to competition for resources, little is known about whether invasives compete for pollinators against native species. This uncertainty persists despite the fact that for most flowering plants, sufficient pollen availability via animal pollination is an essential process for the long-term persistence of populations, as it directly affects seed production and genetic variability (Stanton et al. 1986; Burd 1994; Corbet 1998; Ashman et al. 2004; Bell et al. 2005). Competition for pollinators between native plant species has been extensively reported as one of the most important factors determining the fitness of their populations (Free 1968; Waser 1978b; Gross and Werner 1983; Rathcke 1983; Armbruster and Herzig 1984; Galen and Gregory 1989). Few studies (except Chittka and Schürkens 2001; Brown et al. 2002), however, have reported an adverse effect on native fitness resulting from competition for pollinators between invasive and native species.

The endemic *Taraxacum japonicum* occurs in the lowlands of western Japan and has been threatened by the exotic *Taraxacum officinale*, a European invasive plant, especially in suburban areas (Ogawa 1979; Ogawa and Mototani 1985). Hybrid vigor (Morita et al. 1990b), seedling germination rates (Hoya et al. 2004), and superiority for resistance to artificial disturbance caused by agamospermy (Serizawa 1995) are thought to be responsible for the displacement of the indigenous *T. japonicum* by the nonindigenous *T. officinale*. No studies, however, except for those by Takakura et al. (2009) and Matsumoto et al. (2010), have investigated the mechanisms actually operating in the field. These congeneric species have similar floral morphologies with the indigenous *T. japonicum* flowering phenologies completely covered up by the exotic *T. officinale* (Takakura et al. 2009). These species share common general pollinators (Hymenoptera, Diptera, Coleoptera, Lepidoptera; Matsumoto et al., unpublished data). Thus, the occurrence of competition for pollination service between *Taraxacum* species may be

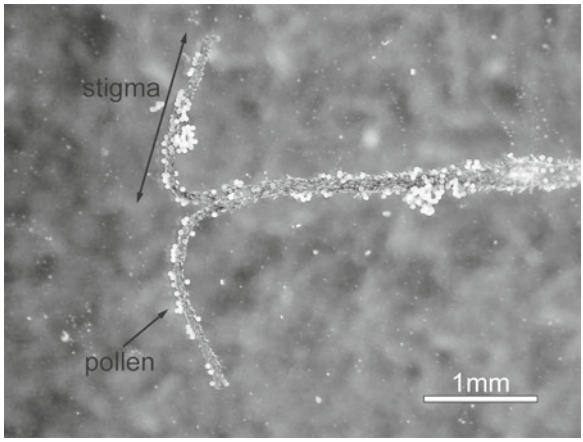

Fig. 7.1 Photograph of the pistils of *Taraxacum japonicum* with pollen grains on the stigma

expected. In fact, Takakura et al. (2009) suggested this type of competition in the *Taraxacum* system, finding that co-occurrence of exotic *T. officinale* flowers reduced the fitness of native *T. japonicum*, and that removal of the *T. officinale* flowers allowed recovery of *T. japonicum* fitness, although the precise mechanisms causing the observed reduction in fitness remained unclear. Two important aspects of pollination service exist: quantity and quality (Waser 1978a; Rathcke 1983). The quantity of pollination service refers to the number of visits or amount of pollen received, and the quality of pollination service refers to the effects of pollinator sharing on interspecific pollen transfer. Preliminary observations of the stigmas of native *T. japonicum* have revealed that the impact of the quantity of pollination service was almost negligible because every stigma had sufficiently large amounts of pollen (Fig. 7.1). In this study, I therefore focused on the quality aspect of pollination service.

The objective of the study was to examine whether reduction in fitness of indigenous *T. japonicum* is caused by pollen of the exotic *T. officinale* in the field. To that end, I raised the following three questions: Is the ratio of native *T. japonicum* seed development negatively associated with the number of invasive *T. officinale* in the vicinity of the native individual? Is the frequency of invasive *T. officinale* pollen on the stigmas of native *T. japonicum* positively related to the number of the invasive *T. officinale* in the vicinity of the native individual? Does a negative relationship exist between the ratio of native *T. japonicum* seed development and the amount of invasive *T. officinale* pollen on the stigmas of native *T. japonicum*?

7.2 Materials and Methods

7.2.1 Study Sites

I carried out field observations and sampling at two sites located in Osaka City, Japan. Exotic *T. officinale* and native *T. japonicum* co-occurred, and both species were the dominant flowers among flower flora in late April and early May. The first

site was a green tract of land within the Nature Experiment and Observation Garden at Tsurumi Park (34°42′32″N, 135°34′55″E). The garden has been managed for nature study and related amenities since 1998 (Takakura et al. 2009). The second site was a plum garden within Osaka Castle Park (34°41′9″N, 135°31′43″E) approximately 5 km from Tsurumi Garden (see Takakura et al. 2009 for detailed descriptions).

7.2.1.1 Materials

Taraxacum japonicum, native to the lowlands of western Japan, is a self-incompatible sexual diploid (Richard 1973; Morita et al. 1990a) that flowers in the spring. It is a perennial herb and forms a radial rosette of leaves producing solitary heads composed of many yellow ligulate florets. The habitat range of *T. japonicum* has decreased, especially in urban areas, since the 1960s (Hotta 1977; Ogawa and Mototani 1985).

T. officinale was first recorded in Japan in 1904 (Makino 1904) and is now distributed throughout Japan. It flowers almost throughout the year and is an agamospermous triploid (Richard 1973; Mogie and Ford 1988), although it produces viable pollen (Morita et al. 1990a). It has similar morphology to *T. japonicum*, but the external involucral bracts of *T. officinale* are turned downward whereas those of *T. japonicum* closely contact the flower head.

Shibaike et al. (2002) and Shibaike and Morita (2002) revealed three types of hybrids between *T. japonicum* and *T. officinale* – triploid, tetraploid, and male parthenogenesis – and the hybrids have been spreading in urban areas (Watanabe et al. 1997a,b; Shibaike and Morita 2002). The morphologies of these hybrids are closely similar to pure *T. officinale*. Only the triploid hybrid type produces as much pollen as *T. officinale*, while the tetraploid produces little pollen and male parthenogenesis none (Shibaike 2005). My aim was to examine the adverse effect of invasive *T. officinale* pollen on indigenous *T. japonicum* during reproductive stages. I therefore recognized pure *T. officinale* and the triploid hybrid as putative *T. officinale* for reasons of their production of possibly harmful pollen. During the study period, putative *T. officinale* had overwhelming numbers of flowers compared to the tetraploids and male parthenogenesis at our research sites (Takakura et al., unpublished data).

7.2.1.2 Sampling Methods

To examine whether the pollen of putative *T. officinale* posed adverse effects on *T. japonicum*, 12 *T. japonicum* were randomly selected and three pistils were sampled from each individual with tweezers. Sampled pistils were separately stuck onto thin paper using double-faced adhesive tape and wrapped with the paper to prevent contamination. At both sites, pistil sampling was performed on 20 April 2007, at the peak of *T. japonicum* flowering. Sampled individuals were marked with

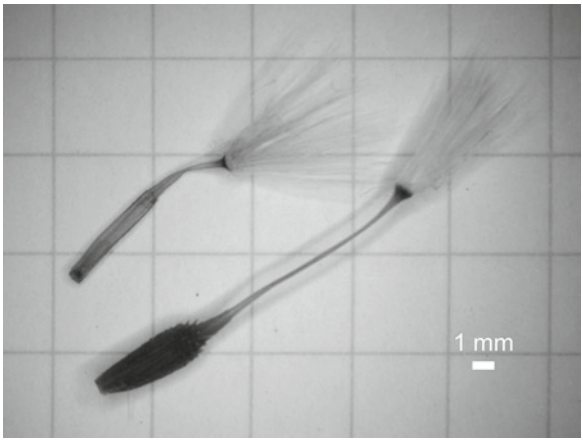

Fig. 7.2 Photograph of developed *Taraxacum japonicum* seeds (*lower right*) and undeveloped seeds (*upper left*)

red tape for future seed development rate research. To test whether a negative effect of *T. officinale* on *T. japonicum* was frequency dependent, I counted the number of putative *T. officinale* and *T. japonicum* capitulums flowering within a 2-m radius from the sampled individuals. The ratio of putative *T. officinale* flowers was defined as the local alien proportion where the local alien proportion = the number of putative *T. officinale* flowering capitulums/(the number of putative *T. officinale* flowering capitulums + the number of *T. japonicum* flowering capitulums). Two meters was the average distance for effective pollen transfer in dandelions (Takakura et al., in press). For future genetic discrimination between putative *T. officinale* and *T. japonicum*, an approximately 1-cm piece from the tips of the leaves was cut from 30 *T. japonicum* and 30 *T. officinale* at each site and silica gel dried in a plastic box. Approximately 2 weeks after pistil sampling, on May 2, 2007, in Osaka Castle, and on May 4, 2007, in Tsurumi Garden, when most of the fertilized ovules had developed into mature seeds, a capitulum was collected from a different scape of the same individual that had been used for the pistil sampling and brought to the laboratory. I dissected the capitulums, counted the number of developed and undeveloped seeds, and estimated the rate of seed development as the number of developed seeds/(the number of developed seeds + the number of undeveloped seeds). Developed seeds were easily distinguished from undeveloped seeds (Fig. 7.2).

7.2.1.3 Molecular Methods

Native pollen had to be distinguished from exotic pollen to determine whether putative *T. officinale* interferes with the reproduction of native *T. japonicum* through interspecific pollen transfer. However, the morphology of most of the pollen from both *T. japonicum* and the putative *T. officinale* was highly similar,

and thus distinguishing between them was almost impossible, although some *T. officinale* pollen had a characteristic polygonal shape. Therefore, genetic discrimination of the pollen from the two plants was performed according to the methods described by Matsuki et al. (2007). Because the method is explained in detail in another chapter of this book, here it is described briefly. One microliter of Proteinase-K buffer containing 0.01% sodium dodecyl sulfate (SDS), 0.1 μg/μl Proteinase K (Takara, Tokyo, Japan), and 1× PCR buffer (containing 1.5 mM $MgCl_2$ Ampli*Taq* Gold; Applied Biosystems, Foster City, CA, USA) was placed on the inner wall of a 0.2-ml polymerase chain reaction (PCR) tube. A grain of randomly selected pollen from the stigma of *T. japonicum* was carefully transferred to the buffer drop under a binocular microscope at 10× magnification using a stretched-out plastic pipette tip. DNA was extracted from 20 pollen grains per pistil. Pistils with fewer than 20 pollen grains attached to them were excluded from the analysis, although almost all the sample pistils had more than 20 pollen grains. The sample was incubated at 37°C for 60 min and then at 95°C for 10 min. Three pistils from the same individual were combined for the analysis. For DNA extraction from leaves, I followed a standard cetyltrimethyl ammonium bromide (CTAB) protocol.

Eight microsatellite loci and a chloroplast locus were used for genetic discrimination of *T. japonicum* from putative *T. officinale* (Table 7.1). Shibaike et al. (2002) found pure *T. officinale* and *T. japonicum* were distinguishable using a 77-bp-ins/del in a chloroplastidic intergenic region between the *trn*L (UAA) 3′-exon and *trn*F (GAA). I designed a primer (MSTAtrn-F) with the ins/del contained between the universal primer F of Taberlet et al. (1991) and the primer (MSTAtrn-F) using the DDBJ database (accession numbers: AB070902 and AB070922). Discriminating the hybrid from *T. japonicum*, however, was impossible because the hybrid had the same chloroplast type as *T. japonicum* (Shibaike et al. 2002). Therefore, using the eight microsatellite loci developed by Falque et al. (1998) and Vašut et al. (2004), I genotyped leaf samples and searched for species-characteristic alleles at each site (see Table 7.1). Species discrimination was performed in cases in which the genotyped pollen had at least one species-characteristic allele. No individual has two kinds of species-characteristic alleles at different loci. Pollen with two alleles per locus was considered to be putative *T. officinale*. Undiscriminated pollens were excluded from the analysis.

All nine loci were simultaneously PCR amplified with a Qiagen (Basel, Switzerland) multiplex PCR kit using 1 μl template DNA, 0.2 μM of each primer, and 3 μl 2× Qiagen Multiplex PCR Master Mix on a GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA, USA). PCR conditions for amplification were as follows: an initial denaturation step at 95°C for 15 min, 30 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C, and a final extension at 72°C for 12 min for leaf samples; an initial denaturation step at 95°C for 15 min, 40 cycles of 30 s at 94°C, 90 s at 48°C, and 1 min at 72°C, and a final extension at 60°C for 30 min for pollen. PCR products were visualized on an ABI 3100 sequencer (Perkin-Elmer), and fragment sizes were scored using the Genotyper software package (Applied Biosystems).

Table 7.1 Characterization of eight *Taraxacum* microsatellite loci and a chloroplast locus

			Number of alleles (Size range, bp)			
			putative *T. officinale*		*T. japonicum*	
Primer Name	Sequences (5′ → 3′)	Source	Osaka Castle	Tsurumi Garden	Osaka Castle	Tsurumi Garden
MSTA54-F	GTAACGGTCATGAAAGTGGC	Falque et al. (1998)	4 (217–238)	4 (217–238)	9 (217–241)	8 (217–252)
MSTA54-R	TCTTCTAATGAGGCAGTATGGC					
MSTA53-F	CAATTATTATGGTCTCGTCCTT	Falque et al. (1998)	5 (197–226)	5 (197–226)	9 (189–211)	8 (191–213)
MSTA53-R	CCAGTTGAAGCAAAAACAGT					
MSTAtrn-F	CATATTATAGGCCCGGGATG	Shibaike et al. (2002)	2 (234–311)	2 (234–311)	1 (311)	1 (311)
Universal Primer TrnF	ATTTGAACTGGTGACACGAG	Taberlet et al. (1991)				
MSTA61-F	CTACGCTCTTCCCCATTGAT	Falque et al. (1998)	6 (117–143)	5 (117–140)	8 (117–143)	8 (132–147)
MSTA61-R	TGGTGGTTGATGCTCTGTTT					
MSTA78-F	TGATTGATTCTGCCCTAAACC	Falque et al. (1998)	3 (143–159)	4 (143–163)	3 (143–155)	3 (121–146)
MSTA78-R	TGCCAAGACATCCGAAAAG					
MSTA60-F	TTTGCCAAATAAATGCTACA	Falque et al. (1998)	5 (299–327)	2 (299–327)	7 (295–311)	3 (303–309)
MSTA60-R	CGTTGGAATGGATAAGAAAG					
MSTA93-F	GTTTTGTTTGGGTTTGGTATTGTC	Vašut et al. (2004)	3 (280–286)	4 (280–291)	8 (280–319)	5 (303–333)
MSTA93-R	ATGCCCCCTCTATGTCCTAT					
MSTA67-F	TTCGGATATGACCCTTCACT	Falque et al. (1998)	6 (202–231)	6 (211–233)	13 (185–225)	12 (185–221)
MSTA67-R	GACATCTTGCACCTAAAAACAAT					
MSTA58-F	CCACTTTGAACGACATTGTTA	Falque et al. (1998)	6 (120–143)	5 (120–143)	11 (110–146)	10 (106–130)

7.2.1.4 Statistical Analysis

I analyzed the relationship between the proportion of alien pollen on native stigmas and the local alien proportion using a generalized linear model (GLM; Milton and Arnold 1995), assuming that the responsive variable conformed to a binomial distribution and designating a logit link function. In the analysis, the responsive variable was the alien pollen on native stigmas, while the explanatory variables were the local alien proportion and the total number of *Taraxacum* head flowers indexed as microhabitat. I also analyzed the relationship between the seed-development rate and the local alien proportion, and between the seed-development rate and the alien pollen proportion on native stigmas using a generalized linear mixed model (GLMM; Wolfinger and O'Connell 1993), with a binomial error structure and a logit link function. Responsives were the normally developed seeds in the two analyses, whereas fixed effects included the local alien proportion and the total number of *Taraxacum* head flowers in the former and the alien pollen proportion on native stigmas and the total number of *Taraxacum* head flowers in the latter. Individual factors (e.g., light, soil condition, or pollinator activity) were incorporated into the model as random effects in both analyses. All analyses were conducted using R version 2.7.1 (R Development Core Team 2008).

7.3 Results

The trends observed between the two sites were nearly the same. A strongly negative relationship was detected between the rate of seed development and the local alien proportion at both sites (GLMM, $p<0.001$ for both sites; Fig. 7.3). The effect of the total number of *Taraxacum* within 2 m on the rate of seed development was different between sites. At Osaka Castle, a significantly positive relationship was found, although the coefficient was extremely small, whereas at Tsurumi Garden, the total number of *Taraxacum* was not significantly related to the rate of seed development (Table 7.2).

GLM showed that the frequency of occurrence of alien pollen on native stigmas had a strongly positive relationship with the local alien proportion at both sites ($p<0.001$ for both sites; Fig. 7.4), whereas the total number of *Taraxacum* had no significant effect on the frequency of occurrence of alien pollen on native stigmas (Table 7.3).

The rate of occurrence of alien pollen on native stigmas significantly depressed the seed-development rate at both sites (GLMM: $p=0.03$ for Osaka Castle, $p=0.04$ for Tsurumi Garden; Fig. 7.5 and Table 7.4).

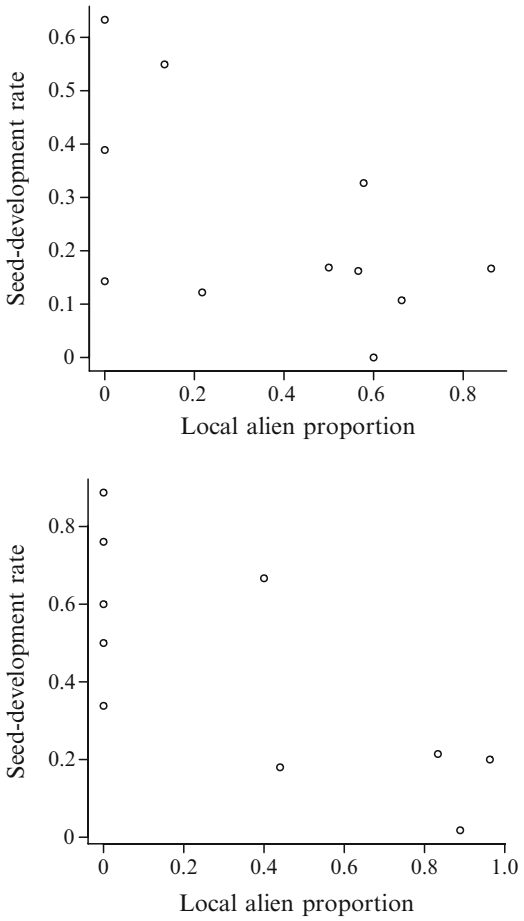

Fig. 7.3 Relationship between rate of seed development in *T. japonicum* and local alien (*T. officinale*) proportion within 2 m at two sites (*upper graph*, Osaka Castle; *lower graph*, Tsurumi Garden)

Table 7.2 Results of generalized linear mixed model (GLMM) for the seed development rate of native *Taraxacum japonicum*

		Factor			
		Local alien proportion within 2 m		Total *Taraxacum* within 2 m	
Site	*df*	Estimated coefficient ± S.E.	*p*	Estimated coefficient ± S.E.	*p*
Osaka Castle	7	−4.120 ± 1.218	<0.001	0.034 ± 0.014	0.01
Tsurumi Garden	6	−2.939 ± 0.864	<0.001	−0.002 ± 0.023	0.93

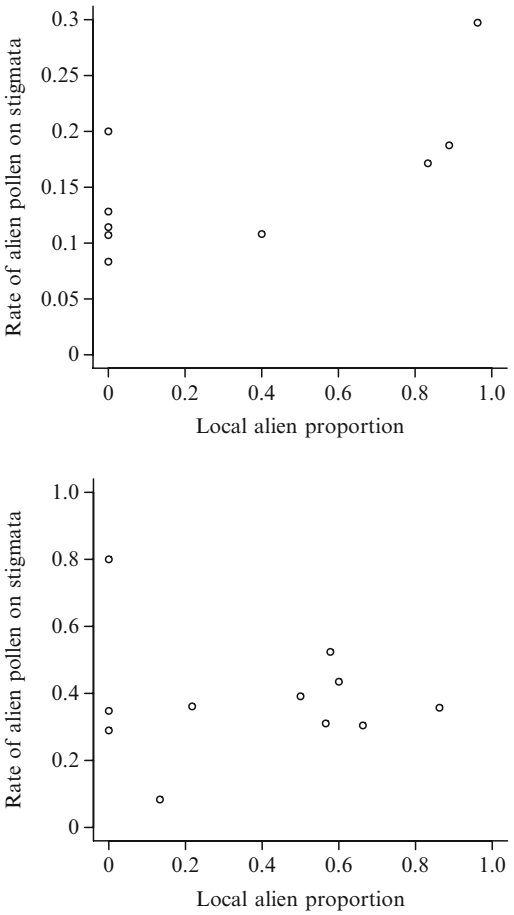

Fig. 7.4 Relationship between frequency of occurrence of alien (*T. officinale*) pollen on native *T. japonicum* stigmas and local alien (*T. officinale*) proportion within 2 m at two sites (*upper graph*, Osaka Castle; *lower graph*, Tsurumi Garden)

Table 7.3 Results of generalized linear model (GLM) for the alien *T. officinale* pollen rate on native *T. japonicum* stigma

		Factor			
		Local alien proportion within 2 m		Total *Taraxacum* within 2 m	
Site	*df*	Estimated coefficient ± SE	*p*	Estimated coefficient ± SE	*p*
Osaka Castle	8	4.602 ± 0.829	<0.001	−0.001 ± 0.005	0.91
Tsurumi Garden	7	6.410 ± 1.846	<0.001	−0.002 ± 0.013	0.85

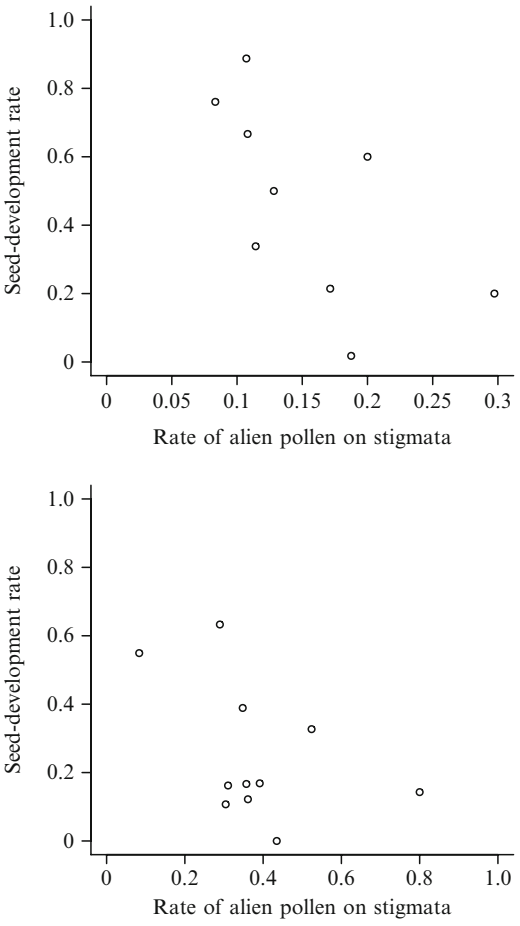

Fig. 7.5 Relationship between rate of seed development in *T. japonicum* and occurrence of alien (*T. officinale)* pollen on native *T. japonicum* stigmas at two sites (*upper graph*, Osaka Castle; *lower graph*, Tsurumi Garden)

Table 7.4 Results of GLMM for the seed development rate of native *T. japonicum*

		Factor			
		Local alien proportion within 2 m		Total *Taraxacum* within 2 m	
Site	*df*	Estimated coefficient ± S.E.	*p*	Estimated coefficient ± S.E.	*p*
Osaka Castle	7	−4.572 ± 2.142	0.03	−0.036 ± 0.027	0.18
Tsurumi Garden	6	−10.575 ± 5.281	0.04	−0.021 ± 0.053	0.69

7.4 Discussion

This study showed that the presence of invasive species pollen mediated by shared pollinators heavily impaired the reproductive success of the native species and that the strength of the adverse effect caused by the invasives was density dependent. The observed reduction of native seed-set was caused not by the competition for resource against invasives but by alien pollen for these reasons. First, because the effects of total *Taraxacum* numbers were insignificant or the strength of effect was very small even in the only one case when significance was detected, and second because previous studies revealed that hand-pollinating of alien pollen remarkably reduced the native seed-set (Morita et al. 1990a) and that removal of neighboring alien flowers led to a higher seed-development rate than control (Takakura et al. 2009). Invasive plant species are often considered to be potential competitors of native species, usually because of their greater capacity for colonization and utilization of resources, but few studies have focused on how alien species may affect an essential part of the life cycle of native flowering plant species, namely, their pollination and subsequent reproductive success (Bjerknes et al. 2007). As pollinator sharing is common within plant communities (Waser et al. 1996; Richardson et al. 2000), the negative effects of invasive pollen grains landing on the stigmas of native species is expected to become a more commonly observed mechanism through which exotic invading species displace native species.

A reduced quality of pollinator service has been implicated as a cause of reduced seed-set in many other studies, primarily of native species (Thomson et al. 1982; Campbell and Motten 1985; Kohn and Waser 1985; Waser and Fugate 1986; Galen and Gregory 1989; Murphy and Aarssen 1995a,b,c,d; Murcia and Feinsinger 1996). The presence of heterospecific pollen leads to stigma clogging (Waser 1978b; Kohn and Waser 1985; Waser and Fugate 1986), stylar clogging (Shore and Barrett 1984; Galen and Gregory 1989), and stigma closing (Waser and Fugate 1986), and inhibits pollen germination or tube growth (Sukhada and Chandra 1980a,b, Murphy and Aarssen 1989, 1995a,b), respiration (Jimenez et al. 1983; Ortega et al. 1988), seedling germination or growth (Anaya et al. 1992a,b), leaf chlorophyll (Sukhada and Chandra 1980a), and seed-set (Sukhada and Chandra 1980a; Murphy and Aarssen 1995c,d). How the exotic *T. officinale* pollen grains prevent seed development of the indigenous *T. japonicum* in this *Taraxacum* system is unclear and requires further research.

To date, interspecific pollen transfer between relatives has been considered in the context of interspecific gene flow and hybridization (Ellstrand and Schierenbeck 2000). Many ecologists have focused on the genetics of introduced species, recognizing the importance of the role that new genotypes may play in species spread (Vilá and D'Antonio 1998; Ellstrand and Schierenbeck 2000; Vilá et al. 2000; Daehler and Carino 2001; Pyšek et al. 2003). Genetic assimilation has also been proposed to contribute to the displacement of native *T. japonicum* by exotic *T. officinale* (Morita et al. 1990b; Hoya et al. 2004). However, the advantage (seed germination ability) of the hybrid against native species was very small in comparison

with the reduction of reproductive success observed in the study and was detected under unnatural conditions (much lower or higher temperatures than under field conditions) in a laboratory (Hoya et al. 2004). Further, many colonies of native species remain stable even in urban areas (Serizawa 1995), which contradicts the hypothesis that an invasive is superior to a native species in an artificially disturbed area. Thus, the primary factor causing the displacement in *Taraxacum* systems would be the reduction of native seed by exotic pollen transfer observed in the field.

Hybrids with few pollen (male parthenogenesis) or no pollen (tetraploid) are also distributed over a wide geographic range formerly inhabited by native *Taraxacum* (Shibaike 2005), which is not explainable by interspecific pollen transfer. These two types might be introduced after pure *T. officinale* and/or triploid hybrids, here putative *T. officinale*, repel the native *T. japonicum* through interspecific pollen transfer. Or, mechanisms other than interspecific pollen transfer might contribute to the success of the hybrids. Further research observing the displacement process is needed to resolve this problem.

Reproductive interference (RI), defined as interactions between species associated with their mating systems that adversely affects the fitness of at least one species (Hochkirch et al. 2007), has begun to attract attention as a factor in the determination of habitat range between competitive species (Hochkirch et al. 2007; Thum 2007; Takakura et al. 2009; Matsumoto et al. 2010; Nishida et al., in manuscript). The important point of the RI is that strength of RI depends on the frequency of interaction (Levin and Anderson 1970; Kuno 1992). Kuno (1992) showed in a theoretical model that RI facilitated the exclusion of interfered species even when resource competition alone could not, because the strength of the interference will accelerate more rapidly because of the frequency dependence, once one species gains an advantage over another. This observation successfully explains that most displacements of natives by invasive have occurred rapidly. My study combined with that of Takakura et al. (2009) was the first to elucidate a detailed mechanism and mode for RI between prospering invasive species and diminishing native plants and strongly suggested that RI was responsible for the replacement. Many invasive alien species have been shown to affect plant and animal communities (Braithwaite et al. 1989; Alvarez and Cushman 2002). However, most invading species pose little or no threat to native species or ecosystems. Only a few of these species are problematic. The results reported here and in previous related studies suggest that RI should be taken into account when determining which kinds of invasive species have an influence on the native flora and fauna. Environmental perturbations can teach us much about natural systems. Biological colonization can be viewed as a natural experiment for the examination of habitat range or abundance of species and should be used more frequently to inform more general ecological theory and understanding.

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Chapter 8
Single-Pollen Genotyping of Holocene Lake Sediments

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8.1 Ancient DNA Analysis

Genetic analyses of fossilized materials are now possible by means of new techniques such as polymerase chain reaction (PCR) and the more recent next generation sequencing techniques. These techniques have attracted much attention in all areas of biology during the past two decades. By directly analyzing DNA from fossil samples of different ages collected from different sites, it is now possible to study the history of ancient taxa, clarify the migration routes followed after glaciation, and understand the microevolutionary processes that occurred in the recent past. In the past two decades, ancient DNA (aDNA) has been successfully extracted from fossilized organisms, and results from specimens of late Quaternary remains (up to 100 thousands of years, ka) have provided insights into many evolutionary processes, particularly in animal species (Krings et al. 1997; Cooper et al. 2001; Lambert et al. 2002; Shapiro et al. 2004; Gilbert et al. 2008). In addition, the combination of coalescent methods (Hein et al. 2005) and aDNA sequences is a very powerful tool to reconstruct detailed demographic histories, test models of population genetics, and understand the interplay between microevolution and environment (Hadly et al. 2004; Ramakrishnan and Hadly 2009). In plants, unfortunately, methodological difficulties and the rarity of suitable well-preserved samples have often prevented the widespread application of aDNA studies, especially at the population level, because well-preserved hard tissues such as wood are difficult to obtain in sufficient numbers over sufficiently large geographic areas.

8.2 Why Fossil Pollen?

Fossilized pollen grains of vascular plants are the most abundant and among the best preserved remains of many groups of plants (Faegri and Iversen 1989). During the past 50 years, fossil pollen have been the key tools used by paleoecologists for elucidating the environmental conditions during the previous thousands of years. The sediments of lakes at highly elevated regions, such as in the mountains, contain pollen deposits from local plants; columnar sediment cores from the bottom of such lakes can be extracted (Fig. 8.1) and analyzed to reconstruct the structure of the land cover for thousands of years earlier. The cores of these sediments are often horizontally stratified because new sediments are regularly deposited into the lake each year. After establishing the age of a layer through radiocarbon dating, the abundance and type of pollen preserved within the sediment are analyzed. Routine analysis of the pollen usually reveals information about the genus or family, thereby providing a generalized view of the vegetation history of the region. Analysis of fossil pollen reveals that the distributions of species dispersal at the end of the last glaciation period were related to the modern patterns of genetic variation in many plant species, providing important linkages between temporal and modern patterns of variation in plants (e.g., in *Picea abies*; Tollefsrud et al. 2008).

Fig. 8.1 A sediment core sample (**c**) was collected (**a**) at Lake Holtjärnen (**b**)

Pollen retrieved from Holocene lake sediments from the cold regions of northern latitudes offers many advantages for aDNA studies. If the lake is confined to a specific area, favorable depositional conditions and fast burial rate reduce physical damage and exposure of the grains to biotic degradation by protecting the environment of the fossil and preventing percolation through the sediment sequence. Further, Holocene lake sediments contain high concentrations of pollen grains (>100,000 grains/cm^3), and as a result of the low temperatures these grains are more likely to have preserved DNA molecules because they are less affected by diagenesis. Another advantage is the relatively young age of pollens, which allows tracing of the lineages of Holocene specimens directly to the living taxa by using appropriate molecular markers. Thus, extant sequences can easily be compared with the ancient ones, and direct links can be established with modern populations. Finally, the fine degree of chronological precision that can be obtained from the Holocene specimens using radiometric dating methods enables the establishment of a detailed timescale using small amounts of material.

Pollen contains haploid DNA and is the means of its dispersal to the haploid DNA of the ovules. In flowering plants, the pollen disseminated from the flowers contains either two or three haploid cells. Both pollen types possess a large vegetative cell that encloses either a single or two generative cells. The vegetative cells comprise the bulk of pollen grain cytoplasm, including numerous plastids and mitochondria, and are responsible for the development of the pollen tube and delivery of the generative cells to the embryo sac together with the nuclear haploid DNA.

Therefore, multiple copies of organellar DNA (i.e., DNA of plastids and mitochondria) are present in the pollen of flowering plants. In addition, depending on the mode of inheritance of the organelles (paternal or maternal), there is a selective increase or decrease in the amount of organellar DNA in each pollen grain during pollen maturation (Nagata et al. 1999). Hence, pollen from species with paternal inheritance of plastid DNA, which include the majority of conifers, should be particularly rich in plastid DNA at maturation; that is, plastid DNA sequences are present in high copy number.

Because fossil DNA is the repository of the genetic history of an organism, this DNA is the direct consequence of evolution. In 1996, Suyama et al. succeeded in amplifying a short region of chloroplast DNA from the pollen grains of a 150,000-year-old *Abies* spp. plant collected from a Quaternary peat deposit at Kurota Lowland, Fukui, Japan. Some years later, by using the same technique we succeeded in isolating and analyzing short chloroplast DNA regions (<200 base pairs, bp) from Holocene pollen of Scots pine (*Pinus sylvestris* L.) retrieved from a postglacial lake sediment from central Sweden (Parducci et al. 2005). The method has also been recently applied to an angiosperm species (*Fagus sylvatica*; Paffetti et al. 2007) to analyze chloroplast variation in pollen grains as old as 45,000 years.

8.3 Pollen Treatment and DNA Extraction

The samples should be obtained from lake sediments with optimal and continuous preservation conditions (e.g., deep lakes from cold environments, which have a fast sedimentation rate). The pollen type should be as abundant as possible and easily distinguished from other pollen types present in the sediment. For example, the pollen grain of *P. sylvestris* has a 50-μm-long body with two laterally placed large bladders, and this pollen can be easily distinguished from that of *P. abies* with large body size, commonly longer than 75 μm, and a smooth transition between bladders and body.

Fieldwork, including drilling the core, and laboratory analyses must be performed during autumn and winter, to avoid the pollination period (i.e., spring), and specific methods should be used to avoid contamination (for details, see Parducci et al. 2005). We removed about 5 g wet weight of sediment samples from the core using a sterile scalpel in a DNA-free pollen laboratory that was located in a building physically separated from the one in which DNA analysis was performed. The upper 2-cm part from the surface of the sediment was previously discarded to avoid contamination during sampling. The samples were stored in sterile plastic bags in a freezer (−20°C). Subsequently, the sediment core was analyzed for pollen and macro remains, and then radiocarbon dating was performed. Before DNA analysis, about 0.5 g of each sample was dissolved in a few drops of sterile distilled water and the solution was sieved through filter cloths using distilled water. In the end, we collected approximately 2 ml of final solution containing the majority of the target pollen grains in a sterile petri dish and stored this solution at 5°C for no more than 2 days.

Next, the petri dishes were transferred to a second building in a pre-PCR lab equipped with a positive-pressure flow hood irradiated with ultraviolet radiation; 10–15 drops of soil solution was placed on glass slides and observed under a microscope with 10–40× magnification. Using standard micropipettes of 0.5–10 µl capacity, single pollen grains were selected under the microscope and transferred on clean glass slides. Each grain was washed 30–50 times with sterile distilled water drops, transferred to a clean glass slide, washed again 10–20 times, and finally moved to a sterilized PCR tube containing less than 0.5 µl sterilized water. For each sample, contamination by exogenous DNA was monitored by using a PCR blank that contained 1 µl solution from the last drop of water used for washing the grains.

For DNA extraction, we use a modified version of the extraction method described by Suyama et al. (1996) and Matsuki et al. (2007). To the PCR tube containing the pollen grain, we added 2 µl extraction buffer containing 0.01% sodium dodecyl sulfate (SDS), 0.1 g/l proteinase K, 0.01 M Tris–HCl (pH 7.8), and 0.01 M ethylenediaminetetraacetic acid (EDTA). The grain was crushed under the microscope using a sterile plastic pipette tip. The PCR tube was then closed and incubated at 37 or 56°C for 60 min and at 95°C for 10 min.

8.4 Choice of Markers and Amplification

PCR is a fundamental technique used in aDNA analysis. There are more chances of recovering aDNA from mitochondrial or chloroplast genomes of fossil pollen than from nuclear DNA because both mitochondria and chloroplasts are present in multiple copies in the vegetative cells of the grains and the plastid membranes afford extra protection to their DNA. In addition, the organellar DNA is conserved in its priming sites. The availability of a large database facilitates the designing of primers and selection of DNA regions with the appropriate level of polymorphism for testing of different hypotheses. However, because significant degradation of aDNA complicates the amplification of fragments longer than a few hundred base pairs, the amplification of target fragments longer than 200 bp is usually avoided.

Nevertheless, pollen grains are not a good source of aDNA because only one DNA region (nuclear, plastid, or mitochondrial) can be amplified from one pollen grain at one time; in contrast, several DNA regions can be analyzed at different times from DNA extracts obtained from multicellular samples (such as bone, tissue, or wood). This problem of pollen grains can be partially circumvented by analyzing several DNA regions in a single PCR reaction (multiplex PCR method) by using multiple primers (Chamberlain et al. 1988). In this way, the analysis of multiple regions can provide more detailed information on the nature of genetic change that occurred within individuals from different ages. At the same time, the amplification of regions from different genomes facilitates data validation in aDNA analysis. If more information can be simultaneously obtained from multiple assays, there is much less probability that the results are influenced by contamination. We performed multiplex PCR amplifications using a Multiplex PCR kit (Qiagen) with an

initial activation step at 95°C for 15 min, 35–40 cycles of denaturation at 94°C for 30 s, annealing at 55–60°C (depending on the primer pair mix) for 90 s, and extension at 72°C for 1 min, and a final step at 72°C for 10 min. The volume of the reaction mixture was 10 μl, including 2 μl extracted pollen DNA solution, 1× Multiplex PCR Master Mix (Qiagen), 0.2 μM each primer, and water (for adjusting the final volume). Using internal primers specific to the target sequence of interest, we performed a secondary amplification on the PCR products separated as single bands on 2% agarose gel. The 20-μl reaction mixture used for the latter procedure contained 1× HF PCR Buffer (Phusion; Finnzymes), 0.2 mM each dNTP, 0.5 μM each primer, and 0.02 unit/μl of Phusion DNA polymerase (Phusion; Finnzymes). The amplification conditions were as follows: initial activation at 98°C for 30 s, 30 cycles of denaturation at 98°C for 10 s, annealing at 50–65°C for 10 s, and finally incubation at 72°C for 5 min. Secondary PCR products were purified, and 4–8 μl of this product was used for direct sequencing using internal primers. Each sequence was read from both strands. In addition, 3 μl of the purified secondary product was used for cloning, and a minimum of six clones per fragment were amplified, aligned, and their sequences compared with those obtained from direct dideoxy sequencing (forward and reverse sequences). Amplifications using fresh plant material were performed after those of aDNA.

The prerequisite for successful multiplex analysis is a good primer design that does not allow unspecific product generation, formation of extensive primer dimers, and preferential amplification that may result from unbalanced primer characteristics. In our case, we tried to design primers to obtain products that are as short as possible (<200 bp). In this type of analysis, DNA degradation is presumably the most common reason for PCR failure because most pollen grains do not contain significant amounts of intact targets more than 200 bp in length. The second important reason for amplification failure is the presence of inhibitors, and this can be easily identified by the lack of formation of primer dimer or the presence of any other unspecific amplification products on agarose gel. The inhibiting substances may come from soil, which contains components derived from decomposing plant material that inhibit the *Taq* polymerase activity.

8.5 Contamination

The studies on aDNA are complicated by several technical difficulties and require careful execution and practical expertise. Contamination is the most serious hazard for any researcher working with PCR techniques. The power and sensitivity of PCR, which generates large amounts of amplified product from as samples as small as less than a single cell, are the factors that increase the chances of contamination. Researchers working on modern DNA do not face this problem because their target DNA samples outnumber the other possible contaminating DNA molecules present in the PCR environment; this allows them to keep the number of PCR cycles as low as 25–30. However, the fewer numbers of cycles is not enough for the analysis of ancient sequences wherein the cycle numbers are usually up to 35–40.

Contamination may occur at different points during the analyses. First, it may occur during the handling of samples before PCR or while transferring the reagents during PCR. Second, contamination may occur within the PCR reaction tubes (contaminated reagents or disposable laboratory equipment purchased directly from the manufacturer). The latter is more difficult to avoid because it is beyond the laboratory's control, while contamination during handling can be detected by using an adequate set of control samples (blanks). Carryover contamination occurs when amplification products are introduced in the pre-PCR analysis step and is usually easy to detect. Such contamination can be disastrous and can be avoided by effectively separating the pre- and post-PCR areas, including all assigned equipment; irradiating workbenches and equipments with UV light; or simply by cleaning all surfaces with alkaline or acidic solutions (more environmentally friendly). On the other hand, cross-contamination or sample-to-sample contamination is usually more difficult to detect; however, this type of contamination is less frequent when handling pollen grains because no isolated DNA is involved in the pre-PCR steps. It requires, however, that all pre-PCR steps should be performed meticulously, such as carefully separating and handling grains, using effective cleaning techniques, changing disposable gloves on a regular basis, and using filter tips. In general, contamination that occurs because of personal handling of sediments, pollen grains, and reagents is detected by using sets of control samples consisting of negative controls; the approach to detect such contamination will vary with the mode of introduction of the contamination.

8.6 Validation of Results

To prove that only indigenous DNA and not the contaminating substance is amplified, it is necessary to repeat the entire experiment by using different DNA extracts (from the same sample) in a different laboratory (criterion of reproducibility). However, when pollen is the source of aDNA, only one type of DNA can be amplified from a pollen grain at one time. In such a case, reproducible results can be obtained by ensuring that a very large number of grains can be procured, thereby allowing statistical evaluation of the population genotypes, rather than giving primary importance to the successful extraction of genetic material from a few specimens (or even one specimen). In addition, when a very large number of samples can be obtained, the validation power of the reproducible results mainly depends on the particular information content of the obtained sequences. For example, the discriminating power of repeatedly obtained results is high when targeting variable regions, and comparable haplotypes can be obtained from the same population in two different laboratories. Hence, different results obtained from different individuals (grains) become a tool of self-validation if they can be obtained consistently (Montiel et al. 2001).

8.7 Conclusion

The analysis of pollen DNA from samples of different ages collected from different sites makes it possible to directly assess the history and dynamics of ancient plant taxa and to understand the microevolutionary processes that occurred during the recent past. In particular, the combination of ancient sequences and coalescent methods is a powerful tool to reconstruct detailed demographic histories, test models of population genetics, and understand the interplay between microevolution and environment. For example, by analyzing DNA extracted from ancient pollen, one should be able to investigate how ancient populations responded to specific climate changes in the past and to answer specific questions, such as these: When exactly did tree species lose or gain genetic diversity during the postglacial population migration from the southern refugia? or, Is there any correlation between changes in genetic diversity and climate events that occurred during postglacial migration?

Because pollen is probably the most widely distributed Holocene fossil and is available in large numbers, further developments in the extraction and analysis of DNA from fossil material will provide answers to the questions of plant movement in space and time by reducing the taxonomic resolution from species or generic level to population or species level. Although the study of plant aDNA is still in its youth as compared to that of animals, the availability of suitable material for the former has been demonstrated. The timescale for aDNA analysis covers the last glacial–interglacial transition, and for a considerable portion of this period, accurate and precise chronological data can be obtained by using radiocarbon dating methods. Additionally, several studies on the phylogeography of modern plants, especially trees (Lascoux et al. 2004), provide adequate baseline data for comparison with aDNA results. Therefore, it is possible to create a dated record of changes in phylogeography in time as well as space.

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Chapter 9
Potential Application of Pollen Genotyping for Evolutionary Genetic and Genomic Studies: Linkage/Recombination Analysis and Haplotype Sequencing

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9.1 Previous Studies of Recombination and Linkage Analysis Using Haploid Cells

In this section, we describe some examples in which haploid tissues or cells were used for genotyping. One technique that takes advantage of the haploid genome is recombination/linkage analysis. Recombination, or the exchange of DNA segments between pairs of homologous chromosomes during meiosis, enables various types of gene combinations to occur and may result in genotypic and phenotypic variation. Understanding the patterns of recombination also provides insights into the historical evolutionary process such as selection events that occurred in the past.

In human and livestock genetics, recombination analysis using meiotic gametophytes, that is, sperm, was developed more than 20 years ago (reviewed in Carrington and Cullen 2004; since Li et al. 1988). A sperm cell is haploid and contains the complete genetic information required for meiotic recombination. A large number of sperm can be obtained from each donor. This method has been successfully used to generate linkage maps, particularly for locus ordering and high-resolution recombination analysis in humans and livestock, allowing the observation of crossovers and recombination in the meiotic process (Clark et al. 2007; Coop et al. 2008; Cui et al. 1989; Jeffreys and May 2004; Jeffreys et al. 1990, 1994, 2005; Lien et al. 1999). Human linkage disequilibrium and sperm-typing studies have revealed that recombination rates vary tremendously across the human genome and that most recombination events occur in narrow "hotspots" of about 1–2 kb (Jeffreys and May 2004; Jeffreys et al. 2005; Webb et al. 2008). One sperm cell contains a single copy of the genome, which is less than that found in a single pollen grain (Box 9.1). Although it may appear difficult to analyze a large number of markers with one single sperm, most studies have overcome this problem by using efficient whole-genome amplification (Kittler et al. 2002; Snabes et al. 1994; Zhang et al. 1992).

In ferns and mosses, a haploid gametophyte is usually composed of a large number of cells. Thus, it is relatively easy to isolate DNA from haploid cells, which has facilitated genetic analysis and genomic sequencing (Rensing et al. 2008). In some gymnosperm coniferous species, linkage maps have already been constructed using the megagametophyte, which has a haploid genome ($1n$) derived from the female parent in gymnosperms (cf. $3n$ in angiosperms). The haploid genome directly reveals the products of meiosis (equivalent with a cross to a homozygous test or strain), allowing efficient mapping of genes even for long-lived trees (Gurles et al. 1978; Bahrman and Damerval 1989; Tulsieram et al. 1992; Nelson et al. 1993, 1994). Moreover, problems associated with using dominant markers for mapping, which have been often used in linkage analysis such as the amplified fragment length polymorphism (AFLP) technique, can be overcome using megagametophytes. The megagametophytes allow dominant markers to be scored clearly, and linkage can be determined using haplotype data from arrays of half-sib offspring (Raeder and Broda 1986; Hulbert et al. 1988). This method has already been successfully used for

constructing linkage maps in some coniferous species (e.g., *Picea*: Tulsieram et al. 1992; Binelli and Bucci 1994; *Pinus*: Travis et al. 1998). In angiosperms, haploid individuals can be regenerated from microspores or pollen grains by a technique called anther culture (Bhojwani and Bhatnagar 1999). Although the regenerated plants are generally genetic chimeras derived from multiple haploid cells, regeneration from a single haploid cell is sometimes possible. However, the application of this technique to genetic analysis is limited because of strong segregation distortion and technical difficulties in many plant species (Pink et al. 2008).

9.2 Potential Application of Pollen Genotyping

Pollen grains of angiosperms are haploid; thus, pollen genotyping would allow the analysis described above. Because a pollen grain is composed of two to three cells (see Box 9.1), the isolation of DNA is easier than for sperm, which is a single nucleus. Here we describe two potential applications of pollen genotyping for genetic and genomic analysis.

9.2.1 Recombination Analysis and Construction of Linkage Map

Genetic linkage maps are useful for genetics-based studies, such as genome structure analysis, map-based cloning of agriculturally valuable and evolutionarily interesting genes, and mapping of quantitative trait loci (QTLs) (Lynch and Walsh 1998). Molecular markers linked to important traits can be applied to agricultural breeding programs and

Box 9.1 Structure of a Pollen Grain

The pollen grain, or male gametophyte, contains two or three cells that are generated by stereotypical cell divisions. The larger cell is called a vegetative cell and the smaller cell is called a generative cell. The vegetative cell does not divide any further and does not directly participate in fertilization; however, it eventually forms the pollen tube. The generative cell is engulfed by the vegetative cell and is contained in its cytoplasm. The generative cell undergoes mitosis, which is sometimes termed second or pollen mitosis, to form two sperm cells that directly fertilize the female gametophyte. The timing of this second or pollen mitosis varies among different plant species. It commonly occurs during pollen-tube growth; however, it sometimes occurs within the anther. Thus, a single pollen grain contains two or three genetically identical nuclei: in this respect, it differs from a simple animal-type gamete, which contains only one nucleus.

medical purposes as well as basic research studies. Genetic linkage maps traditionally have been developed based on pedigree analysis of inbred lines derived from experimental crossing (Fig. 9.1a) or the pseudo-testcross method using a natural population (Grattapaglia and Sederoff 1994). This method detects gene recombination and linkages by analyzing the genotype segregation in the offspring of individuals, consequently providing information about meiosis (Fig. 9.2). Because recombination rates correlate with the distance between loci, a large number of offspring are required to obtain accurate estimations (e.g., 73–150 offspring for *Cryptomeria*: Tani et al. 2003; 30 offspring for each of four *Betula* families: Pekkinen et al. 2005; 243 offspring for *Populus*: Woolbright et al. 2007). However, similar to sperm in animals and megagametophytes in coniferous species, pollen genotyping can be potentially applied to these analyses.

There are several advantages in pollen genotyping compared with traditional offspring genotyping. First, plenty of pollen can be obtained from one flower, which potentially produces a sufficient number of samples to provide accurate estimates of recombination frequencies (Fig. 9.2b). Obviously it is not necessary to cross flowers

Fig. 9.1 (**a**) Inbred lines of the model organisms, *Arabidopsis thaliana*. The plants flower about 4 weeks after sowing. (**b**) Flowering of many tree species, including dipterocarps, occurs every 2–7 years in Southeast Asia tropical rainforest. It is difficult to produce inbred lines in those species even though using the canopy access system by the crane (*left*) and by the ladders (*right*), which are permanently set in Lambir Hills National Park, Sarawak, Malaysia

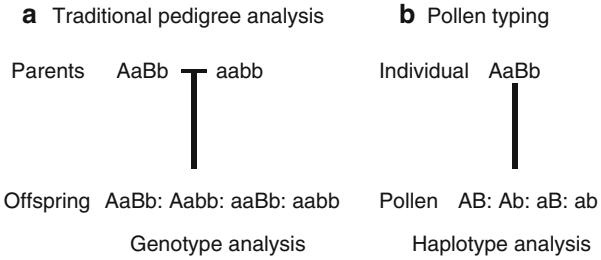

Fig. 9.2 Linkage analysis by the simplest form of traditional pedigree analysis (**a**): one must determine the pedigrees of heterozygous and homozygous parents. Linkage disequilibrium can provide an estimate of the segregation distortion ratio of the genotypes of offspring. For pollen genotyping (**b**), one simply estimates the segregation distortion from the haplotype of the pollen. In both methods, the parents should contain informative heterozygous loci that can be mapped

and obtain offspring, even for long-lived organisms such as trees (Figs. 9.1b and 9.3a), in which it would take a very long time to produce controlled progenies, F_1, or subsequent generations (Fig. 9.3b) (Pekkinen et al. 2005; Tani et al. 2003). Second, pollen genotyping prevents segregation distortion caused by lethality and sterility in F_1 and F_2 plants after fertilization. It is common for particular genomic regions from parent plants to be found at lower levels than expected in the subsequent generation because of genomic incompatibility, such as Dobzhansky–Muller incompatibility (Lynch and Walsh 1998). The accuracy of the estimation of genetic linkage and QTLs around genomic regions with a segregation distortion is low. Segregation distortion is particularly pronounced in interspecific crosses. In contrast, the pollen haplotype method can provide less-biased gamete samples that at least avoid segregation distortion after fertilization. A third advantage of pollen genotyping is that the haplotype can be directly accessed; that is, the haplotype can be obtained without treatment (see Box 9.2). Direct haplotype determination enables simpler assessment of the combination of linked loci, even when using dominant markers such as AFLP.

When many loci are being analyzed, genotyping data from all loci should ideally be derived from a single pollen grain. The small amount of DNA in a single pollen grain is, however, a limiting factor for this approach. There are two possible solutions for this problem: one is to increase the amount of DNA by whole-genome amplification, which is quite effective; however, subsequent polymerase chain reaction (PCR) may be difficult because of biased genome amplification. Another solution is to combine adequate amounts of partial genotyping data that determine a few loci per pollen grain (Table 9.1). It should be possible to analyze relationships using statistical methods together with pollen analysis, although little work has exploited this method. Robust analysis requires an increased sample size; however, in the case of pollen genotyping, the number of samples is usually not a limiting factor because one flower generally provides plenty of pollen grains. Pollen genotyping does have limitations; for example, the pollen grains cannot be used directly for QTL mapping because of the absence of individual phenotypes. Despite these limitations, pollen genotyping can be a useful first step because it provides a genetic map of target

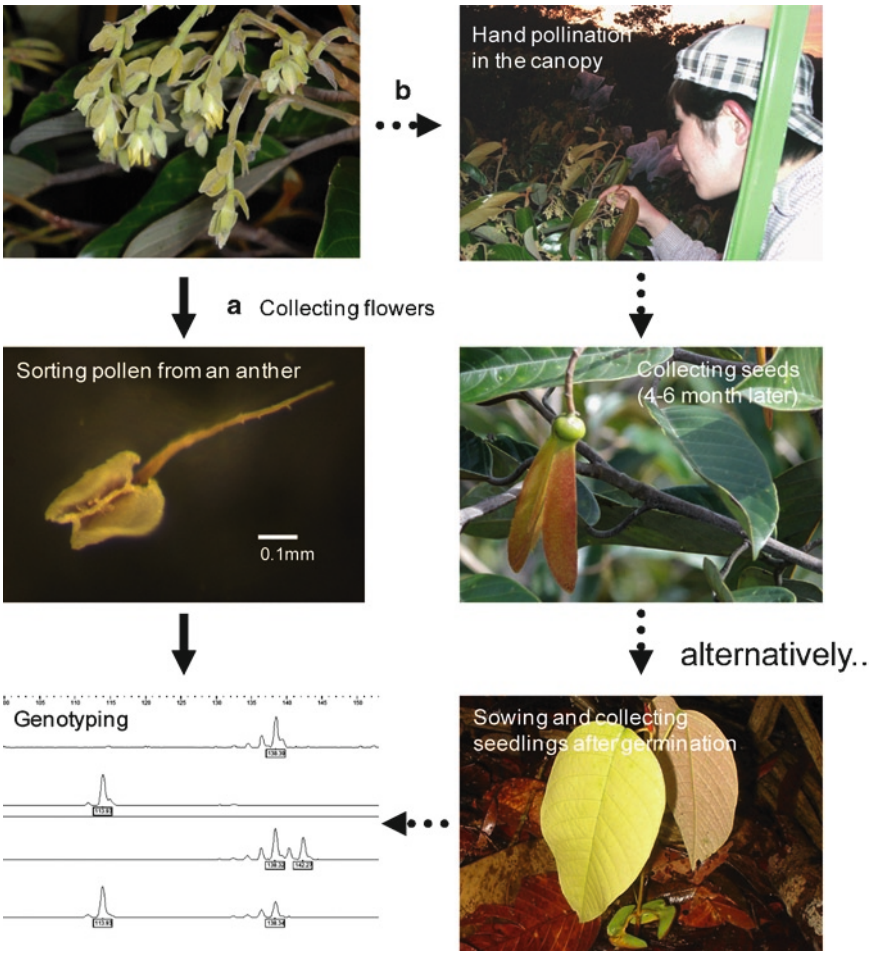

Fig. 9.3 Examples of sample preparation to generate a linkage map by (**a**) the pollen genotyping method and (**b**) traditional pedigree analysis. The former is easier and saves time and space

Box 9.2 Haplotype Determination Strategies

When a specific site in a DNA sequence has more than two different nucleotides (G, A, T, or C) within a diploid individual or a population, this polymorphic site and the variations are called single nucleotide polymorphisms (SNPs: Hartl and Clark 2006). In the direct sequencing of heterozygote individuals, double peaks are observed at the positions of SNPs, and after the position of indel polymorphisms, double peaks continue (Shimizu-Inatsugi et al. 2009). Because these phenomena have been found frequently in most diploid organisms in the wild, many strategies are known for the experimental and statistical determination of haplotypes.

(continued)

Box 9.2 (continued)

1 Experimental Methods

Experimental haplotype determination is the direct solution to assess the genetic phase. However, most methods are time- and cost consuming and have technical limitations, as discussed next.

1.1 Cloning

Cloning is the most common strategy for obtaining haplotypes. However, this method has several critical problems. First, cloning requires significant time and financial cost. Most importantly, artificial recombination between alleles and even between duplicated genes is frequently found. The frequency of occurrence of recombinant clones is between 0% and 89% (Bradley and Hillis 1997; Cronn et al. 2002; Lihova et al. 2006). It is thought that recombination occurs because incompletely extended PCR products act as primers for heterologous sequences and because of heteroduplex excision repair in *Escherichia coli* (Cronn et al. 2002; Thompson et al. 2002). Thus, efficient numbers of clones must be sequenced to form a true sequence consensus from the artificial recombinants of one sequence (Ishiyama et al. 2008).

1.2 Allele-Specific PCR

In this PCR method, only one specific allele should be amplified when using primers based on the position of an SNP (Pettersson et al. 2003). Because Taq DNA polymerase lacks 3′- to 5′-exonuclease activity, a primer including a mismatch at the 3′-terminus with respect to the template amplifies the mismatching template at a greatly reduced efficiency, allowing the discrimination between matched and mismatched templates. However, this method is not effective in all cases, and the primers must be optimized.

1.3 Haplotype-Specific Primer in Sequencing Reaction

In a cycle sequencing reaction, only one haplotype among many PCR products will be amplified using a haplotype-specific primer. First, the mixture of haplotype sequences is directly sequenced and SNP positions are identified. The haplotype-specific sequencing primers are designed to bind to the SNP position (Stadler et al. 2008). As the site mismatch occurs between the first and the fifth base from the 3′-end, primers that contain multiple mismatches increase specificity. This approach allows the verification of SNPs and indel variations and the establishment of haplotypes based on overlapped sequence information.

(continued)

Box 9.2 (continued)

1.4 Haplotype Separation Using Restriction Enzymes

If the target heterozygous amplicon contains a restriction site at the SNP position, two haplotypes can be separated by digesting with the restriction enzyme (Smirnova et al. 2007). The PCR product containing the two haplotypes can then be treated with the restriction enzyme and used for a second round of PCR with inner primers. Otherwise, the haplotypes can be separated by gel electrophoresis, after which the products can be sequenced again.

1.5 Next-Generation Sequencers

Pyrosequencing (Bentley 2006) also provides haplotype sequences. The sequencing strategy of pyrosequencing is fundamentally different from that of Sanger DNA sequencing. Each haplotype is obtained even when the whole genome is used as a template. The latest 454 sequencer (GS FLX Version 2.0) can sequence up to 400–500 bp for one read and can provide 400–600 million bases per run. If several reads can be concatenated properly by bioinformatics techniques, longer haplotype sequences will be obtained. However, the data would contain the errors in the sequence and chimeras resulting from recombination between sequences, even though error rates was relatively low (Hamady and Knight 2009). Moreover, because the cost per run is relatively high, efficient experimental design is fundamental.

2 Statistical Methods

Over the past two decades, inferring haplotypes from haplotype-unknown genotype data through statistical algorithms has enabled haplotype reconstruction. This is also a cost-effective solution, and numerous practical algorithms are available as software packages (Niu 2004).

2.1 Parsimony-Based Methods

This method is a rule-based haplotype reconstruction algorithm, from genotype data, based on the principle of maximum parsimony (Clark 1990). Under this method, distinct sequence haplotypes such as homozygote and single-site-heterozygote sequences are referred as “known haplotypes,” which are probably common. The rationale for this approach is that ambiguous genotypes likely contain the known haplotypes. When known common haplotypes were compatible with the unphased genotype, haplotypes could be resolved. This algorithm determines the minimum set of a haplotype. A modified program, HAPINFERX, is computationally very efficient and is one of the most popular algorithms for haplotype reconstruction (Antunes et al. 2002; Bartish et al. 2006; Clark et al. 1998).

(continued)

Box 9.2 (continued)

2.2 Likelihood-Based Methods

Expectation–maximization (EM) methods estimate population haplotype probabilities under the maximum-likelihood principle based on the assumptions of Hardy–Weinberg equilibria such that the result maximizes the probabilities of the observed genotype data (Excoffier and Slatkin 1995; Long et al. 1995). Subsequently, many EM programs have been developed to improve the computational efficiency by using various more efficient techniques (e.g., EM method software: ARLEQUIN 3.11-EM, Excoffier et al. 2005; PL-EM, Qin et al. 2002; GCHap, Thomas 2003).

In contrast, numerous Bayesian methods are available as alternative likelihood-based approaches that incorporate different models and initial assumptions, thus extending their applicability (Lin et al. 2002; Stephens et al. 2001; Zhang et al. 2006). The state-of-the-art program of the Bayesian method is based on the assumption of conditional distribution of the inferred haplotypes. The next inferred haplotype tends to be identical or similar to a haplotype that has already been observed or inferred (cf. Bayesian method software: HAPLOREC 2.3, Eronen et al. 2006; HAPLOTYPER 1.0, Niu et al. 2002; PHASE 2.1.1, Stephens and Donnelly 2003).

Table 9.1 Example of a data set for linkage analysis that allows for missing data

	Pollen1	Pollen2	Pollen3	Pollen4	Pollen5	Pollen6	Pollen7	Pollen8	Pollen9	Pollen10
LocusA	○	×	×	○	×	○	×	○	○	○
LocusB	○	○	×	○	○	×	○	×	○	×
LocusC	○	○	○	×	○	○	×	×	×	○
LocusD	×	○	○	○	×	○	○	○	×	×
LocusE	×	×	○	×	○	×	○	○	○	○

○, data; ×, no data

Supposing that genotype data can only be obtained from three loci of a targeted five loci from a single pollen grain, the genotyped loci should overlap with other pollen samples. A large sample size for each locus is necessary for robust statistical analysis

species that allows us to assess various kinds of markers such as linked, unbiased, or linkage-group-specific markers.

9.2.2 Haplotype Sequence Analysis Using a Single Pollen Grain

The basic datum in genetic and genomic analysis is the haplotype, which is the array of alleles present in a chromosome (Hartl and Clark 2006). Haplotypes may determine a given phenotype in which gene expression is regulated over long distances, considering the combination of single nucleotide polymorphisms (SNPs)

as functional evidence (Altshuler et al. 2005). Moreover, the variation and frequency of haplotypes within a population can be affected by evolutionary events, and the signature of natural selection can be detected through haplotype analysis.

Despite its critical importance, haplotype data are difficult to obtain for diploid outcrossing organisms because most natural populations contain heterozygous individuals (Lihova et al. 2006; Shimizu-Inatsugi et al. 2009). Direct sequencing can result in the overlap of two haplotype sequences and thus may not be adequate for separating the haplotype sequences. In cloning procedures, artificial recombination between alleles is frequently found, and again, it is not easy to obtain haplotype sequences. Thus, determining the haplotype over a long range is one of the challenges of haplotype-based studies, such as in evolutionary genetics.

A number of experimental and statistical methods for determination or estimation of the haplotype have been developed, such as cloning and allele-specific PCR (AS-PCR) (reviewed in Box 9.2). Some experimental methods provide effective strategies for obtaining distinct haplotypes. However, because their success strongly depends on the sequence around the SNP position, it is sometimes not possible to design functional primers. Statistical estimation methods (see Box 9.2) are usually reliable; however, the estimation relies on assumptions. Pollen genotyping can solve these problems because pollen is haploid and without the limitation of combining haplotypes. It could be the most cost- and time-effective technique for determining haplotypes.

9.3 Conclusions: Application of Pollen Genotyping to Evolutionary Genetic and Genomic Studies

The use of pollen genotyping analysis has just begun in ecological studies. As we have shown, pollen genotyping could be applicable to basic genetic studies as well as studies on sperm and the megagametophyte. The main advantages of using single pollen are (1) obtaining most of the haploid genome with less-biased segregation proportion and (2) saving time, space, and cost with a potentially unlimited number of meiotic samples. We emphasize that genotyping and linkage mapping might be efficiently attainable through pollen genotyping. This innovative method provides us with further knowledge of the genomic background of model organisms. Moreover, it can provide fundamental knowledge of genomic information in nonmodel organisms, including long-lived organisms, because most genetic studies are limited to model organisms and their relatives owing to the availability of genomic information.

However, there are some limitations to these techniques. First, the sequence-reading length has been reported to be relatively short, even though there have been only a few studies so far; for example, 220 bp (Suyama et al. 1996) and 316, 446 bp in the chloroplast region (Matsuki et al. 2007). In the nuclear region, after whole-genome amplification (GenomiPhi Kit; GE Healthcare), only about 150 bp were read (Takeuchi, unpublished data). Second, although whole-genome amplification is often useful, it is not trivial to obtain genotypes of a large number of loci from single pollen,

and specifically devised methodology would be needed (see 9.2.1). Despite these limitations, pollen genotyping has great potential for the future, combined with the development of other molecular biological techniques and bioinformatics.

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