Direct Genetic Analysis of Single Pollen Grains in Pollination Studies

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Introduction

Habitat fragmentation is a major threat to the biodiversity of forest ecosystems. During plant reproduction, habitat fragmentation disturbs gene flow and affects both the genetic structure and genetic diversity of progeny. Because insects are sensitive to habitat disturbances (Aizen and Feinsinger 2002), the pollination of insect-pollinated plants is often negatively affected by fragmentation (Young *et al*. 1996, Ghazoul *et al*. 1998, Benítez-Malvido and Martínez-Ramos 2003). In contrast, Dick *et al*. (2003) reported that long-distance pollen movement was enhanced in a fragmented site. These results reflect the complex and unpredictable responses of animal pollinators to fragmentation.

The genetic diversity and genetic structure of plant progeny are directly affected by both the quality and quantity of pollen grains that are transported to the stigma. Despite a wealth of data concerning the quantity of pollen transported to flowers (e.g., Herrera 1987, Mayfield *et al*. 2001), no studies have directly analyzed the genetic quality of transported pollen. The determination of genotypes from pollen grains carried by insects can reveal the genetic structure and diversity of transported pollen as well as the movement patterns of flower-visiting insects. This method may also provide a novel approach for evaluating human impacts on forest ecosystems.

We developed a new method of multiple microsatellite genotyping to analyze the pollination of insect-pollinated plants. We confirmed the reliability of the method by analyzing the genotypes of pollen grains transported by flower-visiting insects.

Materials and Methods

Plant species

Magnolia obovata Thunb. (Magnoliaceae) is a large (up to 20–30 m in height), common deciduous tree species native to temperate forests in Japan. The standing density of adult trees is relatively low, with only a small number of trees per hectare (Isagi *et al*. 2004). Flowers of this species are hermaphroditic and protogynous. The primary pollinators of *M. obovata* are beetles (Kikuzawa and Mizui 1990, Isagi *et al*. 2004), which crawl through the flowers for long periods of time (Thien 1974). Bees, hover flies, and thrips also visit the flowers of *M. obovata* (Tanaka and Yahara 1998).

Study site

A field survey was conducted at the Ogawa Forest Reserve, Ibaraki Prefecture, central Japan (36˚56’ N, 140˚35’ E). The average annual mean air temperature and precipitation over 10 years (1986–1995) at a meteorological station in Ogawa (36˚54’ N, 140˚35’ E) were 10.7°C and 1910 mm, respectively (Mizoguchi *et al*. 2002). The area is covered by a deciduous broad-leaved forest, and the dominant woody species in the canopy are *Quercus serrata*, *Fagus japonica*, and *F. crenata*. Research in the reserve has included intensive

studies of the structure and dynamics of the plant community (Nakashizuka *et al*. 1992) and the population dynamics of *Carpinus* (Shibata and Nakashizuka 1995), *Acer* (Tanaka 1995), and *Cornus* (Masaki *et al*. 1994) species. Several types of land use (e.g., conifer plantations, paddy fields, pastures) surround the forest reserve.

Sampling

During the flowering period of *M. obovata* in 2004 and 2005, fresh stamens and flower-visiting insects were collected from six adults of *M. obovata*. Each insect was collected immediately after visitation. Samples were stored at –30˚C prior to DNA analysis.

DNA extraction from a single pollen grain

For DNA extraction from a single pollen grain, we modified the extraction method described by Suyama *et al*. (1996). Extraction buffer (1 μL) containing 0.01% SDS, 0.1 μg/μL proteinase K (TaKaRa, Tokyo. Japan), 1× PCR buffer (containing 1.5 mM $MgCl_2$) of Ampli*Taq* Gold (Applied Biosystems, Foster City, CA) was placed into a 0.2 mL PCR tube. Under a stereomicroscope, a single pollen grain was removed from the stamen surface using a plastic pipette tip (for manipulating 0.5–10 μL of liquid) that had been stretched and cut to obtain a sharp end. One pollen grain was then placed into the buffer and crushed using a sterile plastic pipette tip (for manipulating 20–200 μL of liquid). The reaction buffer was incubated at 37˚C for 60 min and then at 95˚C for 10 min.

Determination of multiple microsatellite genotypes

Because a single pollen grain contains only a small amount of haploid nuclear material, it is impossible to divide template DNA into multiple reaction tubes to amplify multiple microsatellite loci. Therefore, to obtain multiple microsatellite genotypes from a single pollen grain, we had to either conduct whole genome amplification before microsatellite genotyping or genotype several loci simultaneously in a single reaction tube. We tested both methods for obtaining genotypes of multiple microsatellite loci from single pollen grains.

LL-DOP PCR for whole genome amplification

LL (long products from low DNA quantities)-DOP (degenerate oligonucleotide-primed) PCR (Kittler *et al*. 2002) is one method for whole genome amplification from a small amount of DNA. This method can generate long products from a small amount of template DNA with high fidelity (Kittler *et al*. 2002). The entire genomes of 16 pollen grains were amplified separately using the Expand High Fidelity PCR System (Roche, Basel, Switzerland) following the manufacturer's protocol. Using amplified DNA from a single pollen grain as a template, 11 microsatellite loci developed by Isagi *et al*. (1999) were amplified separately in 11 reaction tubes.

Multiplex PCR

The multiplex PCR method (Chamberlain *et al*. 1988) can amplify multiple loci simultaneously in a single reaction tube using a small quantity of DNA. Genotypes of nine microsatellite loci of 20 pollen grains were scored using a Multiplex PCR kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

Genotypes were determined using an ABI PRISM 3100 Genetic Analyzer, GENESCAN™ analysis software version 3.7, and GENOTYPER™ analysis software version 2.0 (all from Applied Biosystems). The genotypes of pollen grains were compared to those of the pollen parent from which the stamen was sampled.

Genotyping of pollen grains adhering to flower-visiting insects

Among the observed flower-visiting insects, bumblebees (Apidae, *Bombus*), flower beetles (Scarabaeidae, Cetoniinae), and small Coleoptera (Ligriidae, *Arthromacra*) were used to confirm the effectiveness of our analysis method. We removed 47–48 pollen grains (143 grains total) from the surfaces of a bumblebee (*Bombus diversus*), a flower beetle (*Protaetia cataphracta*), and a small beetle (*Arthromacra sumptuosa*). The genotypes of nine microsatellite loci of each pollen grain were determined using the multiplex PCR method. The pollen samples for which genotypes were determined for more than five microsatellite loci were used for analysis. The percentage of self-pollen (i.e., pollen transported within a tree) was calculated for each insect. The genetic diversity of transported pollen grains was expressed in terms of gene diversity (Nei 1973).

Results

LL-DOP PCR

Among 176 combinations of 11 loci and 16 pollen grains, 71 genotypes (40.0%) were successfully determined. The proportion of successful genotyping differed among the microsatellite loci (Fig. 1), ranging from 0.06 (locus: *M15D5*) to 0.94 (locus: *M6D1* and *M6D8*).

Multiplex PCR

Among 180 combinations of nine loci and 20 pollen grains, 163 genotypes (approx. 90.6%) were successfully determined, and all of the amplified alleles were consistent with those of the pollen parent from which the stamen was collected (Fig. 2). Although the proportion of successful genotyping differed among the microsatellite loci (Fig. 1), the variances were smaller than those of the LL-DOP PCR method.

Genotyping of pollen grains adhering to flower-visiting insects

In the analysis of pollen grains that adhered to flower-visiting insects, we were able to determine genotypes for more than five microsatellite loci of 134 pollen grain samples. The percentage of self-pollen in the pollen load was 93.6% on bumblebees, 12.5% on flower beetles, and 95.7% on small beetles. The gene diversity of pollen grains from bumblebees, flower beetles, and small beetles was 0.36, 0.79, and 0.47, respectively.

Discussion

Comparison of analysis methods

We compared two methods of PCR amplification for determining the genotypes of multiple microsatellite loci from a single pollen grain. The proportions of successful genotyping in the LL-DOP PCR method were lower than those in the multiplex PCR method. In addition, the variance in the success rate of each locus was high in the LL-DOP PCR method. Whole genome amplification methods do not always amplify the entire genome, and a portion of genome may often be lost (Wells *et al.* 1999). Therefore, it may be difficult to amplify multiple

regions that include microsatellite loci from one copy of the haploid nuclear genome of a single pollen grain. Thus, we determined that the LL-DOP PCR method was unsuitable for the genetic analysis of pollen grains.

In contrast, the multiplex PCR method successfully genotyped a single pollen grain with high fidelity. Failed genotyping was unevenly distributed among individual loci, suggesting that these failures resulted from traits of each locus or the PCR primers. The multiplex PCR method is suitable for analyzing pollen grains because it amplified pollen DNA with high success rates and fidelity. In addition, this method can be completed in less time and at a lower cost than the LL-DOP PCR method.

Genotyping of pollen grains adhering to flower-visiting insects

Among the 143 pollen grains analyzed, the genotypes of 134 pollen grains (93.7%) were determined for more than five microsatellite loci. This result indicates that the method is practical and effective for detailed studies on pollination. We found large differences in the percentage of self-pollen and gene diversity among insect species, suggesting that the visitation behavior of insects varies with species. Differences in visitation behavior can result in varying effects on the reproduction and fitness of pollinated *M. obovata*. The present method of genetically analyzing a single pollen grain may facilitate more detailed pollination studies, including research on pollinator efficiency and human impacts on pollination systems.

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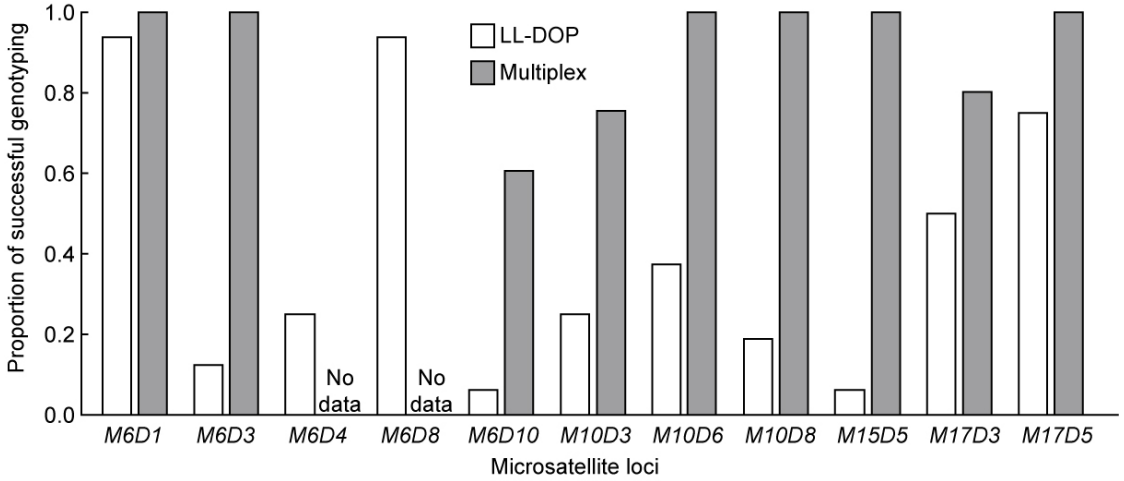

Fig. 1 The proportion of successful genotyping of microsatellite loci using the LL-DOP PCR and multiplex PCR methods. We examined 16 and 20 pollen grains using the LL-DOP PCR and multiplex PCR methods, respectively. Genotype determination was considered successful when the allele corresponding to the pollen parent was obtained. 1

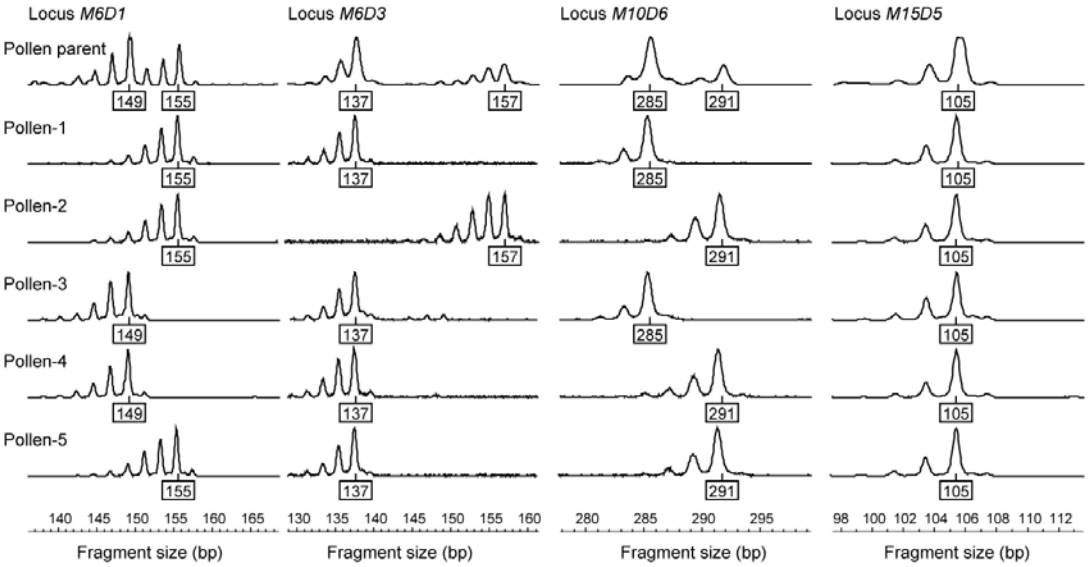

Fig. 2 Electropherograms of alleles at four microsatellite loci (*M6D1*, *M6D3*, *M10D6*, *M15D5*) of pollen parents and pollen grains amplified using the multiplex PCR method (modified from Matsuki *et al*. 2007).