

Combining field and molecular approaches to understand the regeneration processes of tropical tree species

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Abstract Reproductive and regeneration processes comprising flowering, pollination, seed development, seed dispersal, and seedling establishment are crucial in determining the abundance and spatial distribution pattern of species in a community. Investigators have tried to assess the ecological and genetic consequences of these processes in tropical tree species, particularly focusing on dipterocarps and using combined field and molecular approaches such as ecological genetics. Here we review the findings obtained using ecological genetics in the Lambir Hills National Park, Sarawak, Malaysia. Through field surveys during several general flowering (GF) events, the demography of flower to seedling, the pollination process, and patterns of seed/seedling survival have been investigated. Gene flow and mating systems of dipterocarp trees have been investigated using DNA markers such as microsatellites. These studies have revealed the reproductive process of dipterocarp trees and attempted to identify the ultimate cause of GF. In addition, recent advances in knowledge and techniques of molecular biology enable us to characterize the detailed genetic background of GF. We review applied studies pertaining to the physiological and developmental pathways underlying flowering, which have been elucidated in model plants. A large amount of genomic information was obtained from a flowering dipterocarp tree by high-throughput and cost-effective next-generation sequencing, and flowering genes were identified. The information obtained supported the hypothesis that the trigger of flowering is drought. Finally, we discuss the perspectives for future research using an ecological genetics approach in tropical forests.

Keywords Ecological genetics, Ecological transcriptome, General flowering, Promotion-of-pollination hypothesis, Predator saturation

An ecological genetics approach to identify the ecological and genetic consequences of regeneration in tropical tree species

Reproductive and regeneration processes comprising flowering, pollination, seed development, seed dispersal, and seedling establishment are crucial for determining the abundance and spatial distribution pattern of species in a community. These processes determine genetic diversity and genetic structure through gene flow, leading ultimately to population differentiation and speciation. Molecular analysis to investigate genetic variation is a direct approach to elucidate these processes, particularly the gene flow. Ecological studies incorporating molecular methods, sometimes called “ecological genetics,” have become popular in the last two decades owing to the development of molecular technologies. For example, analyses of neutral genetic markers such as microsatellites have revealed mating systems (Nagamitsu et al. 2000; Naito et al. 2005), genetic structures (Harata et al. 2012; Takeuchi et al. 2004), and gene flow patterns (Kenta et al. 2004; Takeuchi et al. 2013) in tropical forests. More recent advances in sequencing technologies, including next-generation sequencing, have provided more detailed genetic information (Ekblom and Galindo 2011); genome-wide single-nucleotide polymorphism studies show the fine-scale population genetic structure and inbreeding level (Andrews et al. 2016; Davey et al. 2011; Morin et al. 2004), and transcriptome analysis can reveal how organisms respond immediately to the environment (Kobayashi et al. 2013, also see below). Such technical advances have also led to further progress in tropical ecology, which still presents mysteries and unanswered questions. One of the most mysterious phenomena of tropical forests in Southeast Asia is synchronous flowering at the community level, so-called “general flowering (GF),” which has been observed only in Southeast Asian lowland tropical forests (Appanah 1985; Sakai 2002). The Lambir Hills National Park (LHNP), Sarawak, Malaysia, is one of the main stations worldwide for studying GF because it has been a site of long-term monitoring of flowering since 1992 (Sakai 2002; Sakai et al. 2006). This research station is the best site in the world for testing hypotheses pertaining to the ultimate and proximate causes of GF (Appendix 1), and major insights into GFs have already been obtained from this site (Roubik et al. 2005). Furthermore, coupled with ecological genetics, analyses of the genetic and ecological consequences of GF have also been performed recently.

Here we review ecological genetics studies conducted at the LHNP, which focused particularly on the reproductive and generation processes and GF in dipterocarp species. Our focus is on how an ecological genetic approach helps in identifying the consequences of the regeneration process in plant populations. We first review studies on the pollination stage of dipterocarp species, including their mating system and pollen dispersal. We also describe the results of testing the proximate and ultimate causes of GF, conducted using a field method and a recent technology, ecological transcriptomics. Finally, we discuss the perspectives of future research aimed at elucidating the biodiversity and evolution in tropical forests.

Mating systems of dipterocarp species

Pollination of tropical trees including dipterocarps is largely mediated by small insects (Momose et al. 1996; Momose et al. 1998; Sakai et al. 1999b). The presence of a large insect pollination guild indicates strong interaction between tropical trees and insect pollinators in evolution and may influence the mating system of trees. The predominance of outcrossing and inbreeding depression in dipterocarp species has been established based on field and genetic experiments (Konuma et al. 2000; Murawski et al. 1994; Naito et al. 2005; Obayashi et al. 2002), suggesting

that dipterocarps have at least partial self-incompatibility to avoid inbreeding depression (Chan 1981; Nagamitsu et al. 2000). Sakai et al. (1999a) performed a hand-pollination experiment using a tree of *Shorea parvifolia* and showed that no fruits of self-pollen grew, indicating strong self-incompatibility. Kenta et al. (2002) first demonstrated self-incompatibility in the stigmas of *Dipterocarpus tempehes*; they showed that pollen tube guidance failed in self-pollinated pistils because only a few pollen tubes entered the hollow, whereas the other pollen tubes continued to elongate in the stigmatic exudate. Thus, outcrossing dominates in dipterocarp trees showing self-pollen avoidance.

Pollen dispersal of dipterocarp species

The success of outcrossing pollination directly determines fitness (number of seeds), given that dipterocarps have self-incompatibility or inbreeding depression mechanisms. Among the family Dipterocarpaceae, the main pollinators are giant honey bees (*Apis dorsata*), stingless bees and moths for the genera *Dipterocarpus* and *Dryobalanops*, and small beetles and thrips for most *Shorea* species (Momose et al. 1998). Giant honey bees migrate only to forests with flowering trees and do not always live there (Itioka et al. 2001). Pollination success by giant honey bees is crucial for dipterocarp trees pollinated by bees because self-pollination is disadvantageous in most trees. Furthermore, the giant honey bee is believed to be an effective pollinator because of its flight ability. This leads to the promotion-of-pollination hypothesis of GF (Sakai 2002, Appendix 1), which postulates that GF evolved to promote pollinators' visits to their flowers and achieve effective pollen dispersal. In tropical rain forests, most tree species are present at low density, and a display of a few species flowering is too weak to attract pollinators such as nomadic giant honey bees. In fact, few giant honey bees were observed during an event with low flowering intensity in 1998, and the pollinators of *D. tempehes* were moths (Kenta et al. 2004). Kenta et al. (2004) compared the pollen dispersal distances between two events with different flowering intensity and pollinators: in 1996 (strong, giant honey bee) and 1998 (weak, several species of moths). They performed parentage analysis using microsatellite genotype matching between candidate father trees and offspring. They calculated each allele frequency within the population and estimated the probability of partiality exclusion over multiple loci. They found that both giant honey bees and moths mediated pollen dispersal over a large area, although mean dispersal distances were longer in 1996, when pollen was carried by giant honey bees, than in 1998, when the pollinators were moth species. This finding suggests that nomadic giant honey bees were more effective pollinators than other small resident insects in the forest, although both pollinators were effective. Another study also found that small beetles, which are pollinators of *Shorea* trees, dispersed pollen as far as giant honey bees did (Takeuchi et al. unpublished). Although these results were not suggested to reject the promotion-of-pollination hypothesis, they suggest the importance of a pollinator community rather than a specific species as a key to the evolution of GF in the region.

Ecological genetics to elucidate the ultimate cause of GF

Besides the promotion-of-pollination hypothesis, the predation saturation hypothesis was examined. The intensity of GF in 2004 was low, whereas the subsequent GF in 2005 was more intense (Takeuchi et al. 2010). In these 2 years, seed traps were placed beneath the crowns of trees of *S. laxa* to determine the numbers of flowers and seeds. The seeds were categorized into four classes: immature, mature and sound, predated by insects, and predated by vertebrates, and the

mortality among events was compared. The proportions of sound seeds did not differ between the two events, so that the predator saturation hypothesis was not supported. Takeuchi et al. (2010) also demonstrated a density effect on offspring mortality; the directions of density dependency were opposite, being positive in 2004 and negative in 2005. This process determines the spatial distribution pattern, and the abundance of the species in the community.

In *S. laxa*, the effect of forest fragmentation on the regeneration processes was investigated (Takeuchi et al. 2013). Flower–seed–seedling demography in a primary forest (LHNP) and one fragmented forest near the LHNP were compared. The authors assessed survivorship during the flower-to-seedling stage and found no clear differences among the forests. They also compared seed survival and predation; sound seeds were more abundant in the fragmented forest, because predation by vertebrates was lower there, possibly because of a lower density of seed-predating vertebrates. The genetic diversity of seed arrays was similar to that of the primary forest, and the selfing rate was lower in the fragmented than in the primary forest. This finding indicates that there is no strong negative effect of forest fragmentation on flower-to-seedling mortality in *S. laxa*. The authors found long pollen dispersal distances in the primary forest. In contrast, in the fragmented forest, pollen exchange was limited within the forest. This limitation could lead to homogenizing the genetic component within the forest, with a possible long-term negative effect. Thus, genetic tools make ecological processes visible and reveal the genetic component of tree populations. Applying the method to disturbed forests would be useful for evaluating the effect of forest disturbance on ecological processes and also provide information for sustainable forest management.

Ecological transcriptome approach to investigation of the proximate cause of GF

Several environmental factors have been suggested as triggers of GF. However, the mechanisms connecting the environmental factors and GF are still not clear. Kobayashi et al. (2013) studied gene expression changes in combination with meteorological data to characterize physiological and developmental changes during flowering. This approach is called ecological transcriptome analysis. The target species was *S. beccariana*, which flowered in 2009. Before the flowering of this species, two consecutive drought periods were observed. The authors collected two samples during the drought periods and an additional two samples before and after the drought. These samples were used for gene expression analysis, yielding the expression patterns of ~30,000 genes whose sequences showed similarities to genes in the model plant *Arabidopsis thaliana*. Consistently with the meteorological data, drought response genes changed their expression during the drought periods. Sucrose response genes also changed their expression under drought. Interestingly, increases in sucrose level have been observed under flowering-inducing conditions in several plant species (Lejeune et al. 1993). Two interesting flowering genes were found, both of which changed their expression during the drought period. One was *SbSVP*, whose sequence was similar to that of a floral repressor, the *SVP* gene in *A. thaliana*. The other was *SbFT*, a homolog of the *FT* gene in *A. thaliana*, which is thought to act as a floral activator. The authors then constructed transgenic *A. thaliana* that overexpressed these flowering genes, to confirm their functions. As they expected, the transgenic *A. thaliana* overexpressing *SbSVP* showed delayed flowering, suggesting that the gene acted as a floral repressor. In contrast, the transgenic *A. thaliana* overexpressing *SbFT* showed early flowering, suggesting that the gene acted as a floral activator. In summary, under drought conditions, sucrose response genes changed their expression

levels. At the same time, two important flowering genes, *SbFT* and *SbSVP*, also changed their expression levels. These are the major physiological changes observed during GF. These results are consistent with the hypothesis that drought is a trigger of GF (Sakai et al. 2006).

Concluding remarks

As we have suggested, an ecological genetics approach would be useful for elucidating ecological processes such as pollen dispersal and mating system, proximate and ultimate factors of GF, and forest fragmentation effects on ecological processes. As genetic components of populations are expected to be influenced directly and indirectly by disturbance (Banks et al. 2013), the approach may also provide useful information for prediction of future dynamics of genetic diversity and population sustainability in human-modified landscapes, which are common in tropical regions. In particular, ecological transcriptome and whole-genome analysis may help in understanding genetic mechanisms of traits and evolution of tree species. Future research will include the evaluation of genetic consequences for understanding demographic and evolutionary responses to human disturbances and climate change. For example, ecological transcriptome and genomics approaches can be applied to assess plant response to environmental changes at the transcriptional level and evaluate the risks of tree populations under climate change (Neale and Kremer 2011).

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Appendix 1. Ultimate and proximate causes of general flowering (GF)

Evolutionary consequences of GF

It has been unclear why the phenomenon of GF has evolved in this region. There are two major hypotheses about the ultimate cause of GF: the “promotion-of-pollination” and the “predator saturation” hypotheses (Sakai 2002). In the first, GF evolves to enhance display effects. GF-type plants are usually pollinated by insects, birds, and mammals. Because effective pollen transfer depends on these pollinators, plants promote pollinator visits to their flowers. However, because most tree species are at low density, display of a few species’ flowers is too weak to attract pollinators. Especially large amounts of flower resources are needed to attract the most important pollinator, the giant honey bee, which migrates to the lowland tropical forest only when GF occurs (Itioka et al. 2001). As numerous tree species depend on the bee, GF evolved to attract the giant honey bee from outside the forest. The other hypothesis postulates that synchronous mass fruiting after GF is advantageous for avoidance of predation (Janzen 1971). Mass fruiting after GF releases many seeds into the forest, and they are available for seed predator insects and animals. However, if seed predators consume most seeds, few offspring of plants can survive. Thus, plants do not

always bear seeds, thereby keeping predators hungry and their population sizes small. Under low predation pressure, seed predators cannot consume all seeds and even during mass fruiting cannot inflict high mortality by predation. Under this hypothesis, the predation rate is expected to be lower in larger GF events. The two hypotheses are not mutually exclusive.

The trigger of GF

The trigger of flowering, that is, the proximate cause of GF, has also been discussed. Generally, seasonal weather conditions are important cues for flowering; most plants respond to changes in day length, daily temperature, and amount of rainfall to flower. However, these environmental cues would be useless for trees in Borneo, which has no clear day length change, seasonal warm–cool change, or rainy–dry change. Thus, it is not apparent what environmental factors act as a trigger of GF. Two important hypothetical factors are drought and temperature drop, which occur irregularly in Southeast Asian regions. Sakai et al. (2006) found that drought is likely to be a trigger of flowering, as all flowering peaks were preceded by dry periods during their observation of GF in the LHNP. However, there remains a lack of empirical evidence that drought stimulates flower initiation. How plants respond to environmental and endogenous factors to initiate GF remains to be investigated.