

Evolutionary history and mechanisms for generating floral morphological diversity of *Asarum* (Aristolochiaceae) in East Asia

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Summary

The Sino Japanese Floristic Region (SJFR) harbours the world's most diverse temperate flora. Historically, this diversity has been interpreted as the outcomes of geographic isolations caused by the climatic oscillations during the Pleistocene period (< 2.6 million years ago). Many phylogeographic studies have revealed that the present interspecific and intraspecific genetic structures of temperate plants in this region reflected the past range shifts caused by climatic oscillations during the Pleistocene period. It has been hypothesised that allopatric speciation would be major mode of speciation in this region. Although the importance of geographic isolation as a major isolation mechanism has been addressed in plants, recent studies in other regions have implied that biotic factors also promote species diversification. However, many studies conducted in the SJFR have only discussed the role of allopatric fragmentation due to geographic and climatic events, and few studies have considered other factors as drivers of the diversification of the temperate plants. In addition, most phylogeographic studies in the region have focused on individual species or only small groups, including fewer than 10 taxa. Thus, our knowledge of the diversification process of temperate plants in the SJFR remains fragmentary, due to a lack of integrative multidimensional studies of morphology, phylogeny, biogeography, and ecology with adequate sampling of diversified groups.

Section *Heterotropa* in genus *Asarum* (Aristolochiaceae) is endemic to the SJFR and comprises 85 species. All species are perennial herbs growing in the shady evergreen-forest understory. *Heterotropa* species are considered to have very limited dispersal ability due to the myrmecochorous seeds, and this section includes a number of local endemics. These lead to hypothesis that an allopatric speciation would be major mode in this section. In addition, this section is also characterised by high divergence in floral traits, while their vegetative traits show almost no differences, implying that biotic factors would be concerned with the diversification of this section. In *Heterotropa*, most species inhabit almost the same environments and the floral difference and/or geographic isolation would act as reproductive barriers rather than habitat differences. Thus, I considered that this section would be an ideal system to reveal relative contribution of biotic and abiotic drivers to the diversification of temperate plants in this region. My primal objectives were to reveal the diversification history and to obtain implication to

the mechanisms for generating the floral morphological diversity of this section.

I first conducted phylogenetic analysis using ITS and *matK* sequences to confirm the monophyly of *Heterotropa* and to infer phylogenetic relationships among the sections within the genus. The constructed trees showed *Heterotropa* was a monophyletic group and sister to the clade including species distributed in North America (Chapter 1). Then, using ddRAD-seq and chloroplast genome data, I built a time-calibrated phylogenetic tree including 79 *Heterotropa* species. The results indicated that *Heterotropa* diverged into two clades (continental clade and insular clade) during the Miocene. The major subclades almost corresponded to geographic entities (Sichuan basin, other parts of mainland China, Taiwan to the islands of Amami Groups, southern and northern parts of mainland Japanese), and colonisation to other regions after formation of the regional lineages was observed only in several species. Most speciation events within the regional lineages would have occurred during the Pleistocene period. The range fragmentation and contractions during the Pleistocene period would have triggered diversification of *Heterotropa* especially in eastern insular systems. In addition, several sister pairs showed floral trait divergence without geographic overlap. Thus, the diversification of *Heterotropa* would have been triggered by the geographic and climatic events during the Miocene, and subsequent repeated floral trait evolution with and without geographic isolations in the regional lineages during the Pleistocene (Chapter 2).

Subsequently, I focused on series *Sakawanum* in *Heterotropa*. This series is a monophyletic group and consists of four taxa; *A. costatum*, *A. sakawanum* var. *sakawanum*, *A. sakawanum* var. *stellatum*, and *A. minamitanianum*. These taxa are allopatrically distributed in Kyushu and Shikoku islands in Japan and this series exhibits inter-taxonomic clinal variation in the calyx lobe length. I considered that revealing the formation mechanisms of the clinal variation would help us understand the mechanisms generating the floral diversity of *Heterotropa*. Firstly, I investigated evolutionary history of the four taxa by employing approximate Bayesian computation method based on both nuclear microsatellite loci and chloroplast sequences, and I also inferred the past distributions of the series by using ecological niche modelling. The estimated evolutionary history involved secondary contacts between previously isolated taxa triggered by the geographic range shifts to refugia during the Pleistocene period. These secondary contacts would have contributed to formation of morphologically intermediate taxa within the series (Chapter 3). To test whether the neutral process or natural selection shaped the calyx lobe length of each taxon, I conducted Q_{CT} - F_{ST} comparison. In all taxa pairs, the degrees of neutral genetic differentiation were lower than these of morphological differentiation, indicating that natural selection may have driven and

maintained the calyx lobe length variation in the series (Chapter 4). I hypothesised flower-pollinator interactions would be concerned with the calyx lobe variation in the series. Finally, in order to obtain the evolutionary insights into the calyx lobe extension, I investigated the reproductive ecology of two *Sakawanum* species; *A. costatum* and *A. minamitanianum*, which have the shortest and longest calyx lobes in the series. I conducted fine-scale spatial genetic analysis, multi-year pollinator observations, paternity analysis of seeds, estimation of genetic diversity, and crossing experiments. The results showed that the visitation frequency of flies was higher in *A. minamitanianum*, and that *A. minamitanianum* conducted predominantly outcrossing, while *A. costatum* showed a wide range of selfing rate. It has been reported that elongated filiform appendages could increase the display size, or act as landing platforms of flies. Thus, I considered that the extended calyx lobe of *A. minamitanianum* might be maintained by natural selection and have evolved possibly to facilitate the attraction of flies, while its attraction mechanisms remain unclear. In addition, both species showed self-compatibilities, long flowering time, low fruiting rates due to pollen limitation, strong fine-scale spatial genetic structures caused by limited seed dispersal distances, and inbreeding depression at the late-stage (Chapter 5). To the best of my knowledge, my study is the first comprehensive study reporting reproductive ecology of perennial herbs growing under warm-temperate evergreen forests in the SJFR. These reproductive characters are also observed in long-lived tree species. During the Pleistocene period, the climatic oscillations strongly impacted the vegetation and the insect fauna of the SJFR. Although the low dispersal ability promoted the population isolation of *Heterotropa*, the self-compatibility and the long flowering time would have contributed to sustaining populations even in environments with limited pollinators. The late-stage inbreeding depression and the long life-span would mitigate loss of the genetic diversity. Thus, these reproductive characters could also contribute to the diversification of this section in the SJFR.

Although both biotic and abiotic factors have long been recognized as fundamental drivers of diversity, many studies conducted in the SJFR have only discussed the role of abiotic drivers and their relative contributions to the diversification had been rarely investigated especially by using specious plant groups. Overall, my study revealed that geographic range shifts caused by the past climatic events would have caused isolations and secondary contacts of populations, and biotic drivers, especially the interactions of floral morphology and pollinators would have subsequently promoted the diversification of *Heterotropa*. My finding would contribute to the understanding of the diversification process and the mechanisms of temperate plants in the SJFR.