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# The Genetic Basis of Flower-related Phenotypic Differences between Closely Related Species of Asian *Mitella* (Saxifragaceae)

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**Abstract** We crossed *Mitella pauciflora* (pollen donor) with *M. furusei* (pollen recipient) and constructed an F2 population consisting of 222 plant individuals. Two phenotypic characters, namely, number of stigma lobes per flower and number of flowers per inflorescence, were selected among several traits that differentiate the two species, and scored for these F2 plants in 2012 and 2013. We confirmed that these traits are stable within individuals or across different flowering seasons (2012 and 2013), indicating that these are under the strong control of the plant genotype. We also found that there was an association between these two traits both in 2012 and 2013, suggesting the genetic linkage of the genes controlling these traits. Our present preliminary analyses indicate that this F2 population would be potentially useful for understanding the genetic basis of speciation-associated phenotypic divergence, especially if combined with high-resolution genetic mapping.

**Key words**: *Asimitellaria*, F2 population, genetic mapping, linkage, species differences.

## Introduction

Studying the genetic basis of phenotypic differences between closely related species is a critical step to understand how these species had given rise to independent evolutionary lineages that are reproductively isolated from each other (Noor and Feder, 2006). *Asimitellaria* is especially a useful model system to address this issue, as it is a monophyletic group of perennials that have diversified into 13 species in Japan archipelago and Taiwan each with distinct life-history traits. Among these species, *Mitella pauciflora* and *M. furusei* constitute an ideal species pair for studying ecological speciation process, as they naturally co-occur in many populations and markedly different in various characters, including pollination systems and associated floral traits, yet they are one of the closest relatives in *Asimitellaria* (Okuyama *et al.*, 2005, 2008, 2012; Okuyama and Kato, 2009). These observations indicate that the speciation of the two species involves divergent selection and niche differentiation that are the major mechanisms shaping biodiversity.

To genetically dissect the differences of life-history traits between these two species, we generated F2 progeny from a cross between these species (Fig. 1). In this paper, to illustrate the patterns of character variations generated by this cross, we selected two phenotypic traits, namely, number of stigma lobes per flower and number of flowers per inflorescence, among several traits that differentiate the two species and assessed whether this system is suitable for analyzing the genetic basis underlying these variations.

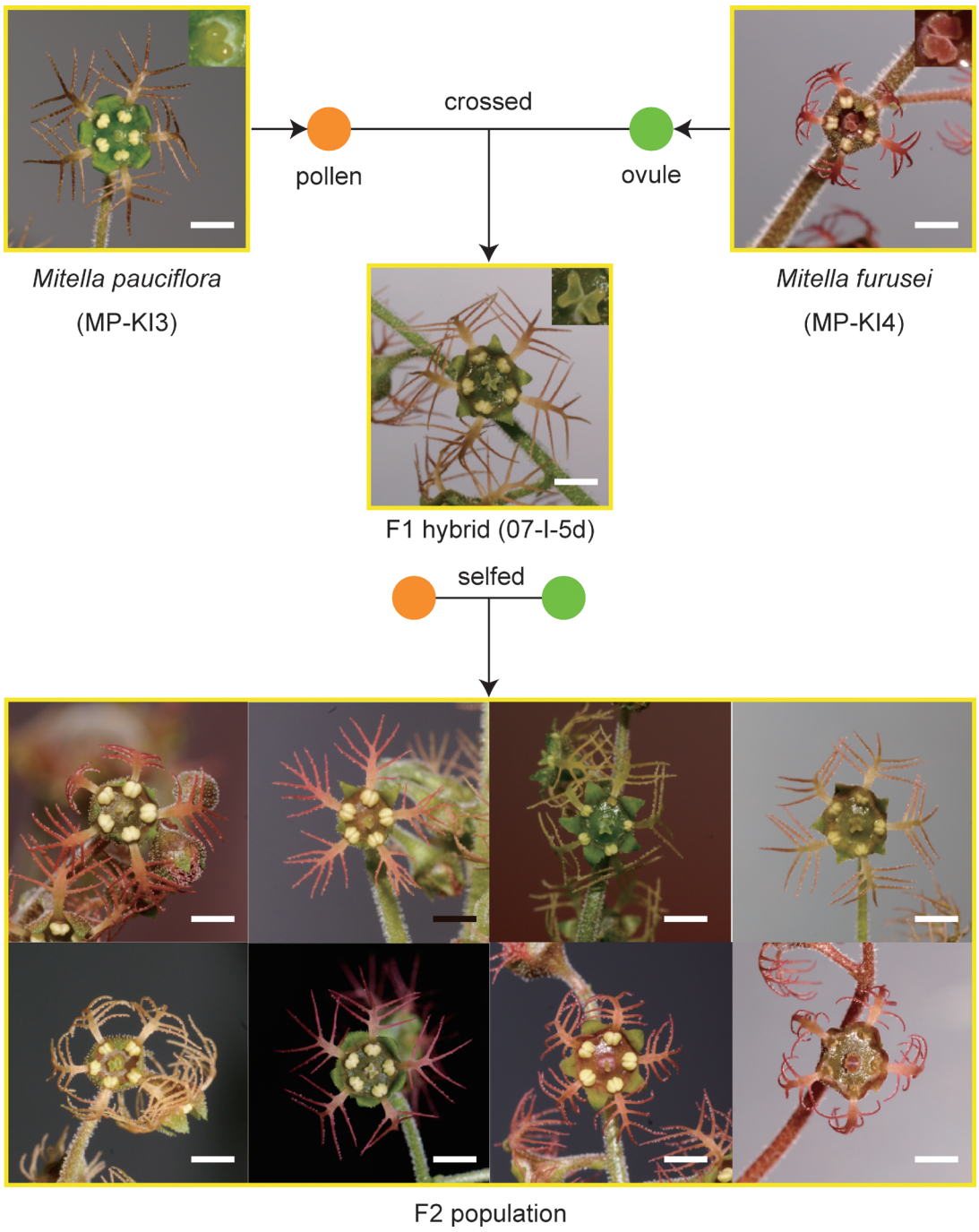


Fig. 1. A schematic presentation of the experimental cross used in this study. The presented flower pictures are the subset of those used for phenotyping on stigma lobes. The magnified pictures of the stigmatic lobes of the parental plants and F1 plants are also shown on the top right of each picture. Bar = 2 mm.

## Materials and Methods

In March, 2007, *M. furusei* strain MF-KI4 (collected from Ibi-kyo, Ibigawa, Gifu, Japan) was crossed with *M. pauciflora* strain MP-KS3 (collected from Shibakura-dani, Ibigawa, Gifu, Japan), where MP-KS3 was used as the pollen donor and MF-KI4 was used as a pollen recipient (Fig. 1). The resultant seeds were grown into F1 plants, and an individual "07-I-5d" was selected as a source of F2 plants examined for phenotypic variation. In April 2010, more than 300 seeds were collected from selfed 07-I-5d, sowed and grown using the method described elsewhere (Okuyama and Kato, 2009). Each seedling was transplanted in a plastic pot (10.5 cm in diameter) with soil optimized for *Mitella* cultivation and grown for flowering in spring. As the result, 222 F2 plant individuals were obtained for potential phenotyping. Every plant individual was replanted into the new soil on 2012 autumn.

A total of 208 flowering individuals were scored for number of stigma lobes throughout 2012 and 2013. A digital camera (Konica Minolta ALPHA 7D, Sony, Tokyo, Japan) fitted with a macro lens (Tamron AF 90mm f/2.8 Di SP A/M 1 : 1 Macro Lens, Tamron, Tokyo, Japan) was used to take photographs of two arbitrary selected flowers per F2 individual. Each flower was photographed frontally from an object distance of 30.48 cm and the resultant pictures were used for the scoring.

As for the number of flowers per inflorescence, 169 and 191 individuals were scored in 2012 and 2013 respectively, where the number of flowers (including flower buds and fruits) was counted and averaged for every inflorescence present at the time.

All statistical analyses were conducted using R (R Development Core Team, 2011).

## Results

*Phenotypic diversity of F2 plants*

As expected, F2 plants showed a variety of phenotypes for floral morphology (Fig. 1). For example, the number of stigma lobes of F2 progenies ranged from 2 to 10 (average; 4.86, variance: 3.11), while that of *M. pauciflora*, *M. furusei*, and their F1 hybrid is each fixed to 2, 4, and 8–10, respectively. Overall, the majority of F2 individuals had four stigma lobes per flower, which is an intermediate phenotype between those of the parental species, and much less individuals had 8 stigma lobes (as *M. furusei*) or 2 stigma lobes (as *M. pauciflora*) (Fig. 2). The number of stigma lobes was stable among flowers in an individual and between flowering seasons (2012 and 2013; data not shown), indicating strict genetic control of the trait.

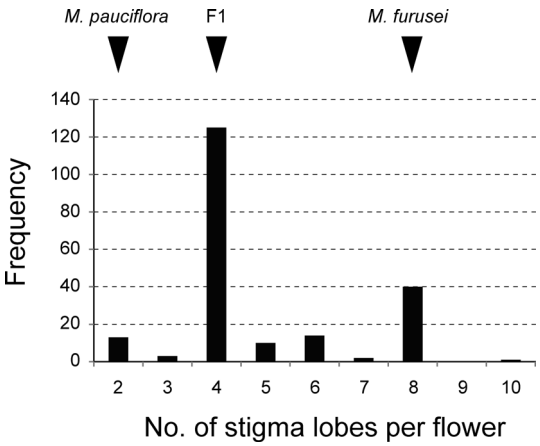


Fig. 2. The frequency distribution of the number of stigma lobes in F2 population. Arrows on the top indicate the phenotypes of parental plants and the F1 plant.

The average number of flowers per inflorescence of F2 progenies also varied largely, and the phenotype range exceeded that between the parental species (Fig. 3). Overall, the frequency distribution of the trait among F2 progenies was deviated from normality both in 2012 (Shapiro-Wilk normality test, $p = 4.0 \times 10^{-5}$) and 2013 (Shapiro-Wilk normality test, $p = 6.0 \times 10^{-4}$). We also confirmed that the trait was stable between flowering seasons. A simple linear regression of the phenotypic traits in 2013 with those in 2012 was significant at $p = 2.2 \times 10^{-16}$ with function $y = 0.89139x$ (intercept was set to zero, Fig. 4). This indicates that the trait is also

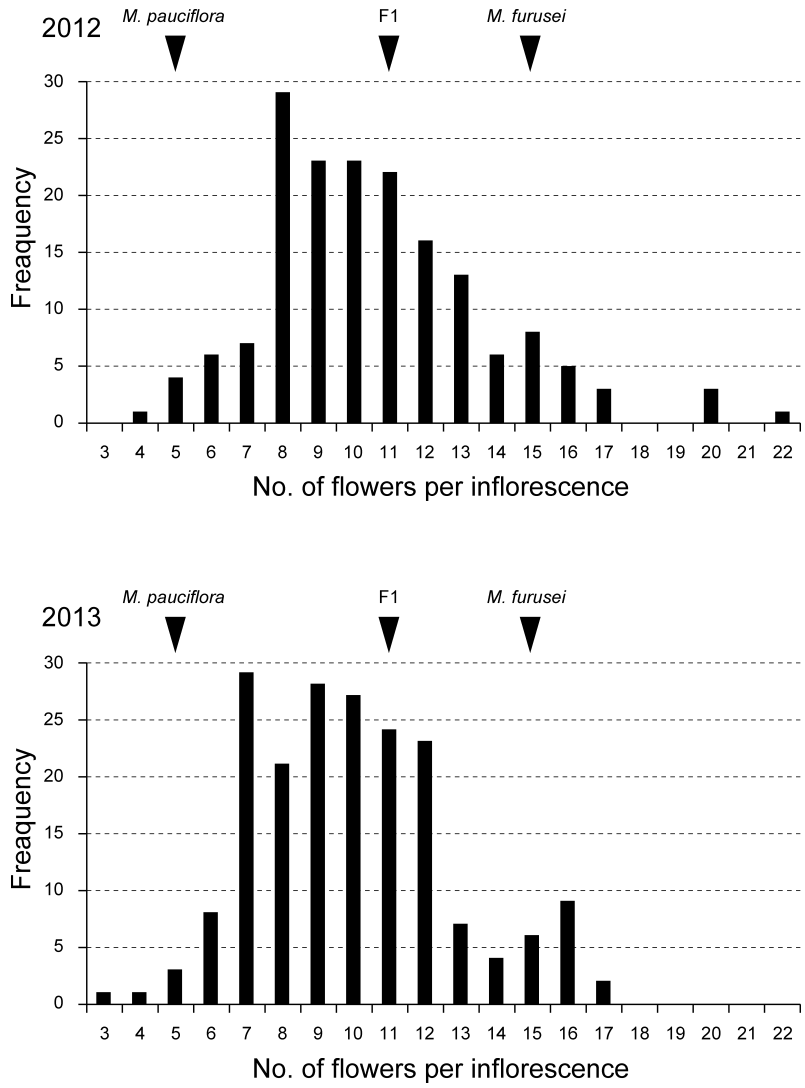


Fig. 3. The frequency distribution of the average number of flowers per inflorescence in F2 population in 2012 and 2013. Arrows on the top indicate the phenotypes of parental plants and the F1 plant.

under the strong control of plant genotype.

*Genetic association between the number of stigma lobes and the number of flowers per inflorescence*

We examined whether there is any association between the number of stigma lobes and the number of flowers per inflorescence in the F2 population. A significant association between the number of stigma lobes and the number of flowers per inflorescence was observed both in 2012 and 2013 (linear regression with nonzero intercept, $p = 0.018$ in 2012 [Fig. 5A] and $p = 1.4 \times 10^{-5}$ in 2013 [Fig. 5B]).

## Discussion

In this study, we have illustrated part of the phenotypic characteristics of the F2 population resulted from the cross between *M. pauciflora* and *M. furusei*, which constitutes one of the closest species pair in *Asimitellaria* with strikingly

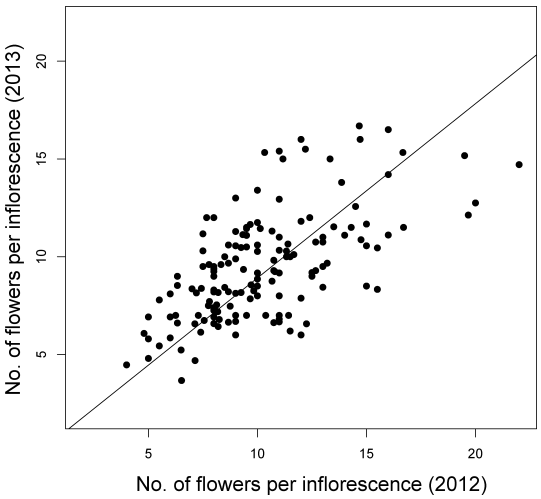


Fig. 4. A strong correlation of the average number of flowers per inflorescence of F2 plant individuals between 2012 and 2013. Each plot represents an F2 individual. The line indicates the linear regression with intercept set to zero ($y = 0.89139x$).

different life-history traits. We first confirmed that the two phenotypic traits, namely the number of stigma lobes and the number of flowers per inflorescence are largely determined genetically, based on the observation that these traits are stable within an F2 plant individual and across different flowering seasons. Moreover, we found that the frequency distribution of these traits was significantly deviate from normality (Figs. 2, 3), indicating that these traits are under the control of a small number of genes with large effect, rather than a large number of genes with small effect. Applying the traditional estimate of the number of genes affecting the quantitative traits (equation 8.50, Hartl and Clark, 2007), we obtained the number of genes affecting the number of stigma lobes as 1.5 (using the number of stigma lobes as 2 and 8 for *M. pauciflora* and *M. furusei*, respectively), and the number of genes affecting the number of flowers per inflorescence in 2012 and 2013 as 1.4 and 1.7, respectively (using the number of flowers per inflorescence as 4.3 and 14.6 for *M. pauciflora* and *M. furusei*, respectively; scored from the parental plants used for the cross).

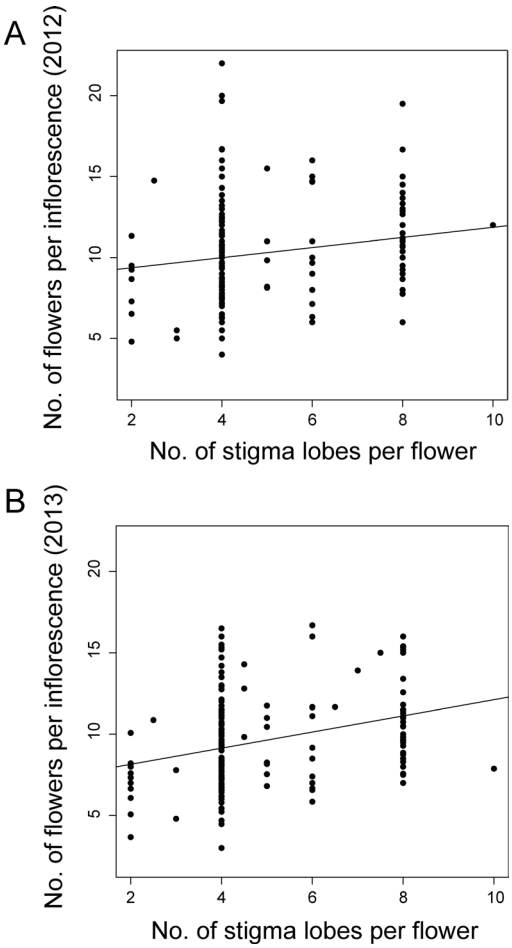


Fig. 5. The correlation between the number of stigma lobes and the average number of flowers per inflorescence of F2 plant individuals. Each plot represents an F2 individual. The correlation is observed for both the number of flowers per inflorescence in 2012 (linear regression: $y = 0.3113x + 8.7469$) and in 2013 (linear regression: $y = 0.4958x + 7.1577$)

Unexpectedly, we also found the association between the two traits examined in this study (Fig. 5). Currently we cannot determine whether this association is originated from the genetic linkage, or the same gene controlling the two traits, although it is difficult to assume that the same developmental mechanism determines the two very different traits, i.e., the number of stigma lobes and the number of flowers per inflorescence. Therefore, the observation that, two

arbitrary chosen traits might have the genetic linkage to each other, might indicate that the number of linkage groups determining the species difference is small.

Whatever the case, our present, preliminary research has proven the potential use of our F2 population for genetic analyses for speciation-associated phenotypic divergence. Now, a high-resolution genetic mapping using sequenced RAD markers (Baird *et al.*, 2008) is underway for these F2 population, and it will shed light on how the striking differences of life-history traits between *M. pauciflora* and *M. furusei* had formed and were coded in their genomes through the speciation process.

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