

# Flower color polymorphism in *Hepatica nobilis* var. *japonica* with reference to genetic backgrounds and reproductive success

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## Summary

Because both purifying selection and genetic drift reduce genetic and phenotypic variations in local populations, how the polymorphism can be maintained in natural local populations is one of the important issues of evolutionary biology. Here, I use the word “polymorphism” to refer to variation within a local population in a frequency high enough not be the result of recurrent mutations.

In plant, flower color polymorphism has been used as a suitable material for elucidation of maintenance mechanism. From historical perspective, mechanisms to maintain flower color polymorphism include both non-adaptive process and selective forces. However, at least theoretically, it was suggested that spatial heterogeneity could be related to the frequency of polymorphism, but is insufficient to maintain polymorphism within a population.

Although some of the studies showed neutral processes contribute to patterns of spatial variation for flower color polymorphism, most supported the idea that balancing selection (i.e., negative frequency-dependent selection and opposing selection), rather than neutral processes, plays a major role in the maintenance of flower color polymorphism. This is because genetic drift will eventually lead to allele loss or fixation in finite populations, even if the fitness of all polymorphic traits were equivalent.

As a mechanism for maintaining flower color polymorphisms, negative frequency dependent

selection, the rare morphs increase their fitness, is considered as the most powerful selection. However, such a mechanism has been successfully demonstrated only in a few cases in both food-deceptive and rewarding plant, and others failed to detect evidences for the selection processes. Thus, mechanisms other than negative frequency-dependent selection are required to explain the co-existence of flower colors. In recent years, it has been speculated that other selective pressure rather than negative frequency dependent selection may be involved in situations where three or more morphs are maintained; it is called opposing selection. Opposing selection is brought mainly by pollinators and herbivores. Previous study suggested that polymorphisms will be maintained when pollinators and herbivores have similar color preferences. Actually, the study of *Claytonia virginica* (Portulacaceae) suggested that since the darker color is favored by pollinators and herbivores, four types of flower color polymorphisms are consequently maintained.

Certainly, some studies showed that opposing selection works effectively in maintaining the flower color polymorphism. However, these experiments were only performed in experimental population in greenhouse or fields. Thus, the role and effectivity of opposing selection in the maintenance of flower color polymorphism in natural population is poorly understood. In this study, to highlight the mechanism of the maintenance of flower color polymorphism in plant populations, I examined the i) neutral process, ii) negative frequency dependent selection, and iii) opposing selection by focusing on natural populations of *Hepatica nobilis* var. *japonica*.

In chapter 1, to determine the relationships between flower color polymorphism, within and

among populations, and the genetic structure of *H. nobilis* var. *japonica*, I estimated the genetic variation using simple sequence repeat (SSR) markers. Here, I revealed two things. First, we examined whether cryptic lineages corresponding to distinct flower colors contribute to the flower color polymorphisms in *H. nobilis* var. *japonica*. SSR analyses revealed that 18% of the genetic variance was explained by differences among populations, whereas no genetic variation was explained by flower color hue or intensity (0% for both components). These results indicate that the flower color polymorphism is likely not explained by cryptic lineages that have different flower colors. In contrast, populations in the SJ and PO regions were genetically distinguishable. As with the other plant species in these regions, refugial isolation and subsequent migration history may have caused the genetic structure as well as the spatially heterogeneous patterns of flower color polymorphisms in *H. nobilis* var. *japonica*.

In chapter 2, I aimed to categorize the flower color polymorphism of this species based on anthocyanin component using high performance liquid chromatograph (HPLC), and identify the candidate genes involved in the anthocyanin biosynthetic pathway. In the results, I categorized these variegated flower colors into three distinct types based on HPLC analysis: white (no anthocyanin), red (containing only cyanidin) and blue (containing cyanidin and delphinidin). Flower color transcripts using RNA-Seq revealed candidate genes for flower color polymorphism. The transcripts were compared among the three flower colors and differentially expressed genes were determined. Transcripts encoding the enzymes involved in the flavonoid biosynthesis pathway were detected in

DEGs. The difference between red- and blue-colored flowers was attributable to differentiation of expression in *F3'5'H*, whereas the differential transcription factor R2R3-Myb was involved in the determination of white and pigmented (blue and red) flowers. Since *F3'5'H* is necessary for the production of delphinidin and R2R3-Myb controls genes working in the anthocyanin biosynthetic pathway, the data obtained from DEGS are consistent with the results of HPLC. Thus, these data suggested the flower color types of *H. nobilis* var. *japonica* may genetically different.

In chapter 3, to clarify the relationship between the flower color polymorphism maintained in a population and flower visitors as pollinators or herbivores, I observed visiting insect fauna, focusing on visual color discrimination ability and feeding damage to blue, red, and white *H. nobilis* var. *japonica* flowers. Although clear differences in the color recognition abilities of Hymenoptera and Diptera by color model estimate were confirmed for white and pigmented flowers, no difference was found in the number of visits by these orders. By contrast, although there is some annual variation in visitation rates, Coleoptera visited more white flowers than pigmented flowers. In addition, blue flowers were the least often damaged by herbivores. Although the plants with flowers with damaged tepals, stamens, or gynoecia produced significantly fewer seeds, the total numbers of seeds did not differ among flower colors, presumably because herbivores also act as pollinators, and may promote autogamy. These results indicate that herbivores had antagonistic functions in terms of feeding damage and pollination, and their influence differed by flower color type. Taken together, these data suggest that the mechanism by which flower color variation is maintained may include both opposing and

fluctuating selection.

In chapter 4, I aimed to test whether negative frequency dependent selection or opposing selection acting in this species. Here I found there was significant variation in the composition of flower color among the populations, and blue was the minority in most cases. However, flower color frequency failed to predict the individual variations in to the reproductive success. Finally, the damaged tepals area was positively correlated with reproductive success while the undamaged tepals area was not. The ratio of damaged flower was significantly different between blue and white, while blue-red and red-white were not. No relationships between flower color frequency and reproductive success indicated that the negative frequency dependent selection was not acting, despite being said that it is one of the powerful selection maintaining the flower color polymorphism. The effects of damaged tepals area to reproductive success suggested the opposing selection of Coleoptera. These results imply that flower color polymorphism in this species may be maintained not by negative frequency dependent selection but opposing selection.

Overall, the result of this study suggests that not negative frequency dependent selection but opposing selection and neutral population dynamics may involve in the maintenance of flower color polymorphisms in this species. Indeed, theoretical studies has shown that three or more flower color polymorphisms cannot be maintained with negative frequency dependent selection by pollinators (Kagawa and Takimoto 2016). Several empirical studies also suggest the acting of opposing selection by pollinator and herbivore (or pathogens) in three or more flower color types (Frey 2004; Strauss and

Whittall 2006; Caruso et al. 2010). Flower color polymorphism including multiple flower color types, rather than two color types, may be maintained more often by not negative frequency dependent selection but opposing selection.