

# **Conservation ecology of endangered species in semi-natural ecosystems based on genetic analysis of extant and specimen samples**

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## **Summary**

### **Chapter 1**

From approximately 10,000 years ago to the early 20th century, the total global area of semi-natural grasslands expanded due to increases in anthropogenic activities that tended to maintain grasslands such as burning, grazing and mowing. In recent decades, in contrast, semi-natural grassland areas have been rapidly decreasing because of drastic land-use changes; this has caused a rapid loss of grassland biodiversity. Accordingly, many semi-natural grassland species are now at risk for decline or extinction. Although the use of specimens collected in the past is one possible means of restoring past genetic characters, few studies have addressed this possibility because genetic analysis of historic specimens is difficult due to the deterioration of DNA over time. In addition, germinated seeds in herbarium specimens could be useful as reintroduction resources, though the restorative effects of their use on genetic diversity have not yet been studied. Using specimens, I elucidated the demographic history, recent decline mechanisms, and effective conservation methods for endangered semi-natural grassland species. This chapter offers an overview of the study and introduces its aims.

### **Chapter 2**

Using molecular markers, I elucidated the historic and recent demographic history and the factors contributing to the recent decline of the endangered grassland butterfly *Melitaea ambigua* (Lepidoptera; Nymphalidae) in Japan. First, I estimated the past demography (12,000 years~) based on 1378 bp of mitochondrial COI gene. Second, I examined changes in genetic diversity and structure change during the last 30 years from specimen DNA using nine microsatellite markers. The effective population size of this species drastically increased about 2,000~7,000 years ago, when human impact had begun to expand in Japan. From the 1980s to the 2010s, in contrast, the genetic diversity

of *M. ambigua* in many populations has significantly declined, indicating a recent decline in population size of the species. In addition, the genetic differentiation between populations within the species has also increased in recent decades. These findings suggest that the demography of *M. ambigua* has followed the historical rise and fall of semi-natural grassland areas during the Holocene period.

### Chapter 3

The monitoring of genetic characters and construction of effective conservation units are important for conservation for endangered semi-natural grassland species. Using molecular markers, I elucidated the recent transitions in genetic diversity and developed an effective conservation strategy for the endangered grassland butterfly *Melitaea protomedia* (Lepidoptera; Nymphalidae) in Japan. First, I examined changes in genetic diversity and structure change from the 1980s to the 2010s from specimen and current DNA samples using eight microsatellite markers. Second, I estimated the genetic structure based on 1374 bp of mitochondrial COI gene from current samples. The genetic diversity of *M. protomedia* has significantly declined from the 1980s to the 2010s in all populations. Although genetic differentiation is very strong among populations in c. 2010, there were only weak genetic structures in c. 1990. In addition, the number of haplotypes based on mitochondrial DNA is now very low due to recent declines. These findings suggest that effective conservation units for critically endangered species should be determined based on historic genetic diversity and structure prior to population declines, because genetic differentiation increases along with declines in genetic diversity and bottlenecks. Genetic analysis of historic specimen DNA is useful to restore historic genetic information.

### Chapter 4

Recent global land-use changes have led to reductions in many herbaceous plant species in semi-natural grassland landscapes. Changes in management frequency and intensity are known to cause declines in plant populations. I determined a suitable management (mowing) schedule for *Vincetoxicum pycnostelma* Kitag. (Apocynaceae; Asclepiadoideae), an endangered summer- and autumn-blooming semi-natural grassland herb. I examined 15 *V. pycnostelma* populations to assess the effects of mowing timing

on the genetic diversity of each population using nine microsatellite markers as well as its effects on pollination and reproductive success. Pollination success was not affected by flowering timing. Mowing during the middle to late flowering and/or fruiting periods of *V. pycnostelma* (July–September) had a significant negative effect on the number of inflorescences and total fruits produced, whereas mowing before the flowering and growing periods (April–May and November–March) had positive effects on the number of inflorescences and fruits, respectively. Furthermore, mowing during the mid to late flowering and fruiting periods also caused a significant decrease in genetic diversity. My results demonstrated that mowing during the mid to late flowering and fruiting periods caused significant declines in the genetic diversity and/or reproductive success of *V. pycnostelma*. By contrast, mowing before the flowering period significantly enhanced reproductive success. To conserve semi-natural grassland herb diversity, mowing should be avoided during seasons when the flowering and fruiting periods of many endangered species overlap.

## Chapter 5

The reintroduction of *ex-situ* conserved individuals is an important approach to the conservation of threatened plants and the reduction of extinction risk. I examined the effects of reintroduction of *ex-situ* conserved individuals on the genetic diversity of wild populations of *Vincetoxicum pycnostelma* Kitag. by modelling the genetic consequences of reintroducing plants using germinated seeds of herbarium specimens. This semi-natural grassland herb is threatened in Japan. First, the germinability of seeds from herbarium specimens collected from nine sites in the Kinki and Tokai districts of Japan (one specimen per site, total 206 seeds) were tested. Next, I analysed the genetic diversity and structure of the germinated seedlings and current wild individuals using nine polymorphic microsatellite markers. Germination was observed for 38 seeds (18.4%) from four specimens collected 3–18 years prior to the study. Although the genetic diversity of the specimens' seeds was lower than that of the wild population because of the small sample size, the seedlings from specimens taken from three sites had unique alleles that did not exist in the wild populations. Consequently, viable herbarium specimen seeds with unique alleles could be useful resources for restoring genetic diversity in threatened wild plant populations.

## **Chapter 6**

My series of studies revealed the demographic histories of several semi-natural grassland species, the effects of the quantitative and qualitative decline of semi-natural grassland habitats on their genetic diversity and structure, and several important points to consider in developing effective conservation methods (construction of conservation units and restoration effects by germinated specimen seeds). The results of this study suggested three general management issues for semi-natural grassland species. 1) The maintenance of semi-natural grassland habitats of sufficient quantity and quality through human activities such as burning and mowing is essential for species conservation. 2) Construction of conservation units should be based on each species' extant and historic genetic diversity and structure. Historic genetic information can be recovered through genetic analysis of specimen DNA collected in the past. 3) Because germinated seeds in herbarium specimens have unique alleles, their reintroduction could contribute to the restoration of genetic diversity in wild populations. Although I only had one target species in each study, comparative conservation genetic studies using multiple species might contribute to more detailed knowledge of the decline and/or extinction mechanisms affecting endangered species. In addition, conservation genomics using next-generation sequencers could also contribute to our understanding of the effects on functional genes of the recent changes in endangered semi-natural grassland species due to human activity.