Timing of mowing influences genetic diversity and reproductive success in endangered semi-natural grassland plants

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ABSTRACT

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. However, little is known about the impact of changes in the timing of management practices on the genetic diversity as well as the reproductive success of rare semi-natural grassland species. We determined the suitable management (mowing) timing for *Vincetoxicum pycnostelma* Kitag. (Apocynaceae; Asclepiadoideae), an endangered summer- and autumn-blooming semi-natural grassland herb. We examined 15 *V. pycnostelma* populations to assess the effects of mowing timing on the genetic diversity of each population using nine microsatellite markers and on pollination and reproductive success. Pollination success was not affected by flowering timing. Mowing during the mid- to late flowering and fruiting periods of *V. pycnostelma* (July–September) had a significant negative effect on the number of inflorescences and total fruits produced, whereas mowing before 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. Our results demonstrated that mowing events 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 flowering periods 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.

Keywords: Anthropogenic disturbance regime; Conservation; Microsatellite; Rare grassland

plants; Suitable management; *Vincetoxicum pycnostelma*

1. Introduction

In recent decades, the area and biodiversity of semi-natural grasslands within agricultural landscapes have been globally and rapidly decreasing because of drastic changes in traditional and extensive land-use systems, garnering much attention from basic and applied ecologists (IUCN, 2012; Kleijn et al., 2011; Krebs et al., 1999; McNeely et al., 1995; Tilman et al., 2001; Tscharntke et al., 2005). Together with recent changes in anthropogenic disturbance (e.g., mowing, burning, and grazing) regimes, abandonments of threatened semi-natural habitats, which have been caused by degradation and conversion of rural life styles and cultures, have decreased plant diversity, particularly the number of rare endangered species in European and Asian semi-natural grasslands (Albrecht and Haider, 2013; Babai and Molnár, 2014; Kleijn et al., 2011; Uchida and Ushimaru, 2014; Uematsu and Ushimaru, 2013).

Although several studies have elucidated the negative impacts of both increases and decreases in disturbance frequency and/or intensity on semi-natural grassland plant diversity (Ekroos et al., 2010; Kleijn et al., 2011; Pöyry et al., 2006; Uchida and Ushimaru, 2014; Uematsu et al., 2010), little is known about the effects of changes in the timing of management practices on plant diversity and the reproductive success of individual plant species (Brys et al., 2004; Endels et al., 2007). Because plant species usually exhibit seasonal reproductive activity (i.e., flowering and fruiting), anthropogenic disturbances during flowering and fruiting periods tend to diminish reproductive success (Brys et al., 2004; Endels et al., 2007; Jantunen et al., 2007). If semi-natural grassland plants have adapted their reproductive periods to traditional management timing, changes in these timings may also negatively impact plant fitness via a reduction in seed production.

The above hypothesis has been tested by several researchers (Brys et al., 2004; Endels et

al., 2007; Jantunen et al., 2007); however, the process by which changes in management timings cause declines in populations has not been sufficiently examined for plants living within agricultural landscapes. Anthropogenic disturbances during the reproductive period of animal-pollinated flowers may diminish seed production in several ways. The first is a basic reduction in the number of reproductive units. Mowing during flowering and fruiting periods inevitably reduces the numbers of flowers and fruits (Brys et al., 2004; Endels et al., 2007; Jantunen et al., 2007), leading to reduction in seed sets. Second, a reduction flower number results in decreased pollination success. Because both individual- and population-level numbers play important roles in pollinator attraction (Ebeling et al., 2008; Potts et al., 2006), a reduction in flower number can result in lower seed production in both respects. Furthermore, pollinator abundance and richness could be decreased by changes in disturbance regimes, independent of flower reduction (Hudewenz et al., 2012; Kearns et al., 1998; Söderström et al., 2001). Thus, to determine the effects of changes in disturbance regime on reproductive success in animal-pollinated plants, these scenarios should be examined simultaneously.

Natural and anthropogenic disturbance regimes can also affect genetic diversity in plant populations (Marchi et al., 2013; Rüdinger et al., 2008). Genetic diversity within a given population is considered important for the conservation of endangered plant species, as genetic diversity is usually positively correlated with fitness: low genetic diversity within a population greatly diminishes fitness through inbreeding depression and qualitative pollen limitation, particularly in self-incompatible species (Johansson et al., 2007; Leimu et al., 2006; Young and Pickup, 2010). Reduced seed recruitment due to mowing and grazing during the annual reproductive period may lower genetic diversity in small and isolated populations. Thus, changes in management practices can cause local extinction of species through a loss

of genetic diversity. However, the effects of the timing of management practices on genetic diversity in semi-natural grassland plants have rarely been investigated and remain unclear.

In the present study, we examined the reproductive success and genetic diversity of 15 *Vincetoxicum pycnostelma* Kitag. (Apocynaceae; Asclepiadoideae) populations subjected to different management practices maintained for at least last 10 years. Using this endangered perennial herb species as the study subject, we aimed to elucidate the impact of the timing of an anthropogenic disturbance (mowing) on the sexual reproduction and genetic diversity of a semi-natural grassland plant. Although this species was very common in the region a few decades ago, it has experienced rapid population declines due to changes in land-use in semi-natural grasslands throughout Japan (Environment Agency of Japan, 2000; Uematsu et al., 2010). *Vincetoxicum pycnostelma* is a representative example of many native herbaceous plants that reproduce from summer to autumn but that have rapidly declined in semi-natural grasslands (Koyanagi and Furukawa, 2013). Assessing the effects of mowing timing is also essential for planning the conservation of endangered semi-natural grassland herbs as well as entire plant communities. We predict that intensive mowing during their flowering and fruiting periods will significantly reduce not only reproductive success but also the genetic diversity of this endangered grassland species. Based on our results, we discuss the importance of traditional management practices and suitable management timings for the conservation of semi-natural grasslands and the diversity of endangered plants, including *V. pycnostelma*.

2. Materials and methods

2.1. Study species

Vincetoxicum pycnostelma is a perennial herb species endemic to the semi-natural grasslands

of Japan, Korea, China, and Russia (Kitamura et al., 1957). The flowers of *V. pycnostelma* produce pollinia, which have a sticky appendage called a corpusculum. The flowers are self-incompatible and open at night, and small- and intermediate-sized moths (Lepidoptera) and crane flies are recorded as pollinators (Nakahama et al., 2013a, b; Yamashiro et al., 2008). Relatively large follicle fruits (7 mm in diameter, 5–7 cm in length, including several tens of seeds) mature during September to October. The life-form of the species is geophyte, based on Raunkiaer (1934): rhizomes have many shoot meristems so that above-ground shoots can quickly regrow even after mowing. During recent decades, this species has experienced rapid population declines due to natural succession after land abandonment and development of grasslands. Therefore, it was categorised as Near Threatened (NT) on the Japanese Red List (Ministry of the Environment, Government of Japan, 2012). Moreover, this species is also at risk of regional extinction, as it is listed in 45 of 47 prefectural Red Lists in Japan (Koyanagi and Furukawa, 2013).

2.2. Study site

Vincetoxicum pycnostelma populations were investigated at 15 study sites in the Kinki and Tokai districts in Japan (Fig. A1, Table A1). Semi-natural grasslands have been maintained by anthropogenic activities such as mowing and burning at the study sites. The 15 populations were isolated from other populations by the developed lands, forests and mountains. The geographical distances among the study sites ranged from 5 to 222.9 km. The grassland area of each site was measured using Google Earth (http://earth.google.com/). The total grassland area varied among the sites from 67.7 to 1486.7 m^2 (Table 1). To measure population size, we walked around and carefully searched for *V. pycnostelma* plants and counted the number of ramets within each study site, as the species exhibits a phalanx-type clonal growth, and

ramets were almost identical to genets. The timing and frequency of mowing at each study site were elucidated by interviewing land managers (Table 1). The frequency and timing of mowing management had remained unchanged for at least the most recent decade at each study site, except for sites i and o (Table 1). Mowing (including burning) events in each study year were categorised into five groups based on their timing: during November and March (pre-growing period), during April and May (onset of the growing period), June (early flowering period), July (peak flowering), and during August and September (late flowering and fruiting period) (Table 1). Selective mowing practices (the land manager mowed approximately half the area of each semi-natural grassland) during June and September were also observed, which are commonly observed in traditionally managed grasslands (K. Uchida and A. Ushimaru, personal observation).

2.3. Sampling of pollination and reproductive success

To examine male and female pollination success, we examined pollinia removal and deposition on the stigmas of selected flowers. The pollinia of *V. pycnostelma* attach to the proboscis or leg of pollinators and are removed from flowers. Pollinia on the pollinators are then inserted into the stigmatic chamber of other conspecific flowers (Nakahama et al., 2013a; Wyatt and Broyles, 1994). In each population, we arbitrarily selected 10 ramets that had flowers 1–2 months after the most recent mowing, and all flowers in a single inflorescence were collected from each ramet in 2013. If fewer than 10 flowering ramets were available, flower samples were collected from all flowering ramets. In four populations (sites a, d, e, and k), samples could not be collected because flowering ramets were not found; thus, flower samples were obtained for the remaining 11 populations. In the two selective-mowing populations (site f and h), flowers growing in unmown area were also

193 collected before mowing. We examined all collected flowers under a stereomicroscope to
194 count both the number of flowers in which at least one pollinium had been removed and the
195 number of pollinated flowers for each inflorescence.
196 The numbers of inflorescences and total fruits were used as indicators of flowering and
197 fruiting success for each ramet. We examined these metrics in all populations except that of
198 site n, where wild deer foraged the aboveground parts of many *V. pycnostelma* individuals in
199 2013. In each population, we arbitrarily selected 20–25 ramets (5–15 ramets at the sites with
200 small population sizes) that had regrown after mowing from the whole habitat area and
201 counted the total number of inflorescences and fruits in September or October, when *V.*
202 *pycnostelma* produces mature fruits (Table A2). Because *V. pycnostelma* retains inflorescence
203 stalks even after anthesis, we were able to count the number of inflorescences even if flowers
204 no longer remained. Inflorescence and fruit numbers were counted in 2012 (nine populations)
205 and 2013 (14 populations). In addition, we recorded stem diameters at ground level in 2013
206 as indicators of vegetative growth.
207
208 *2.4. Sampling for microsatellite analysis*
209 In 2012 and 2013, we collected leaf samples from 432 *V. pycnostelma* individuals in 14
210 different populations (Table 2). Site j was not examined in the microsatellite analysis due to it
211 very small population. Sample size (12–58 individuals) varied among populations depending
212 on the population size (Table 2).
213 Genomic DNA was extracted using a modified CTAB method (Milligan, 1992). Seven of
214 the nine loci developed by Nakahama et al. (2012) were used in this study: i.e. *Vpy002*,
215 *Vpy006*, *Vpy012*, *Vpy013*, *Vpy016*, *Vpy018* and *Vpy022*. These loci were suitable for the
216 present study because they did not exhibit significant deviation from Hardy–Weinberg

equilibrium (HWE) (except *Vpy022* in one population) and significant linkage equilibrium for any pairs of loci (except *Vpy022*/*Vpy016* in one population) (Nakahama et al., 2012). Two of the nine loci could not be used because they were not amplified from some samples. We designed two additional microsatellite primer pairs (Table A3) using the same protocol as Nakahama et al. (2012). None of the nine loci pairs exhibited deviation from HWE or significant linkage equilibrium. The genotyping protocol is described in Appendix A1.

2.5. Statistical analysis of genetic diversity

The genetic diversity of each population was evaluated in terms of Nei's unbiased expected heterozygosity (H_E; Nei, 1987), observed heterozygosity (H_O), the average number of alleles per locus (A), allelic richness (A_R; El Mousadik and Petit, 1996) and the inbreeding coefficient (F_{IS}). H_E and A_R were corrected for differences in sample size for each population. However, because of a large among-population variation in sample size, we used data from 22 randomly selected individuals for the populations with more than 22 samples in microsatellite analyses (Appendix A2; Tables 2, A4). All parameters were calculated using FSTAT ver. 2.9.3 software (Goudet, 2001). Nei's genetic distance (D_A; Nei et al., 1983) was calculated using MSA analyzer ver.4.05 (Dieringer and Schlötterer, 2003). Deviation from HWE was also examined using FSTAT.

A Mantel test (Mantel 1967) was used to assess correlations between D_A and the logarithmically transformed geographic distance, performing 9999 permutations in GenAlEx ver. 6.41 (Peakall and Smouse, 2006). We evaluated genetic relationships among populations using Bayesian clustering in STRUCTURE ver. 2.3.4. (Prichard et al., 2009), which assigns individuals into K clusters. Population structure was simulated with values of K = 1–14 under an admixture model, i.e., the correlated allele frequency model (Hubisz et al., 2009). All runs

involved 1,000,000 Markov chain Monte Carlo generations after a burn-in period of 1,000,000 iterations. Ten runs were performed for each value of *K*. The number of clusters was determined by comparing mean values and variability of log likelihoods in each run. To select the optimal value of *K*, STRUCTURE Harvester was used. The *F* value, amount of genetic drift between each cluster and a common ancestral population, and expected heterozygosity were calculated.

2.6. Statistical analysis of pollination success, reproductive success and genetic diversity

Generalised linear mixed models (GLMMs) with binomial errors and a logistic-link function were used to examine the impact of flowering timing on pollination success. In the full model, grassland area (m^2), population size, sampling date (the number of days after 1 June), and its square were selected as explanatory variables, and population identity was chosen as a random term. The response variables were the percentage of pollinia-removed flowers and pollinated flowers of all flowers for each observed inflorescence in 2013.

In parallel, to examine the impact of mowing timing on flowering and fruiting success, we used GLMMs (Poisson errors and a log-link function). Population and survey year identities were used as random terms. In the full model, the explanatory variables were area, population size and presence/absence (1/0) data of current mowing (including burning) events: November–March, April–May, June, July and August–September. The selective mowing of a given period was treated as 0.5. We also examined the effects of environmental factors (mean temperature and total precipitation during April and October in 2012 or 2013) as covariates. The response variable was the number of inflorescences in 2013 or fruit number in 2012 and 2013. In addition, we examined linear mixed models (LMMs) to examine the effects of population size and mowing timing on ramet size (stem diameter) as

well.

Finally, we examined the effects of mowing timing on genetic diversity with LMMs, in which population and locus identities were included as the random terms. In the full model, explanatory variables were grassland area, population size, mowing timing during the last 10 years and environmental factors (mean temperature and total precipitation from April to October during 1981–2010). In this analysis, the mowing categories at sites i and o were those conducted during years prior to 2010 or 2011 and were different from their current mowing categories because their mowing timings were changed in 2010 or 2011, respectively (Table 1). The response variable was mean A_R or arcsin-transformed H_E.

For all the analyses, we conducted model selection based on Akaike's Information Criterion (AIC) to clarify factors strongly influencing response variables. The model with the lowest AIC was regarded as the best model approximating the data for each analysis. All statistical procedures were performed using R software (ver. 2.15.1; R Development Core Team, 2012) and the package lme4.

3. Results

3.1. Pollination, reproductive success and vegetative growth

The numbers of pollinia-removed and pollinated flowers did not vary with sampling date and population size, whereas they increased with the grassland area in the best model (Fig. 1; Table 3).

Mowing events during July–September had significant negative effects on the numbers of inflorescences and fruits (Fig. 2a, Tables 3, A5). In the best model for inflorescence number, mowing events during April–May and total precipitation had significant positive effects, whereas mowing during July–September had a significantly negative effect on the number of

289 inflorescences in 2013 (Table 3). Based on the best model for fruit set, mowing events during
290 November–March had a significant positive effect, whereas mowing during July–September,
291 the area and mean temperature had significant negative effects (Table 3).
292 Similarly, stem diameter at ground level was also significantly negatively affected by
293 mowing events during June–September, whereas the variable was positively affected by
294 mowing events during November–May (Tables A6, A7). In 2013, stem diameter at ground
295 level was also significantly correlated with the number of inflorescences ($P < 0.001$),
296 indicating that smaller ramets formed fewer inflorescences.
297
298 *3.2. Genetic diversity*
299 A_R and H_E for the populations with mowing events during July–September tended to be lower
300 than those of other populations, which were mown during the pre-growing to early flowering
301 period (November–June) or mown selectively (Fig. 2b). In the best models for both A_R and
302 H_E, mowing events during July–September and population size had significant negative
303 effects (Tables 3, A8).
304 Genetic distance (D_A) and geographic distance were not significantly correlated among
305 the 14 populations ($P = 0.057$, $R^2 = 0.093$; Fig. A2). STRUCTURE analysis indicated that *V.*
306 *pycnostelma* populations did not exhibit clear spatial genetic clusters between populations
307 (Fig. A3). The variance in log likelihood among runs was high, at $K > 7$ (Fig. A3a). The ΔK
308 value was clearly highest, at $K = 6$ (Fig. A3b). Thus, results obtained with $K = 6$ are shown
309 (Fig. A3c). Sites a, d, i and o were assigned to clusters 1, 2, 3 and 4, respectively, for which
310 the $F = 0.080$, 0.117, 0.246 and 0.073 and $H_E = 0.749$, 0.721, 0.633 and 0.747, respectively.
311 The remaining populations were not assigned to specific clusters.
312

4. Discussion

We found that mowing during the peak-to-late flowering and fruiting periods (i.e., during July through September) diminished genetic diversity as well as reproductive success in *V. pycnostelma*, which was consistent with our predictions. Furthermore, four populations mown during July–September had unique genetic structures. In contrast, mowing events during the pre-growing (pre-March) or pre-flowering (April–May) periods enhanced reproductive success in *V. pycnostelma*. These results suggest that the timing of mowing greatly influences not only reproductive success but also genetic diversity in endangered semi-natural grassland plant populations. In contrast, the timing of mowing did not affect pollination success in *V. pycnostelma*, suggesting that the cause of low reproductive success and consequent reduction in genetic diversity could be a simple reduction in flowers and fruits due to mowing.

4.1. Effects of mowing timing on reproductive success

Mowing events during July–September had a severe negative impact on inflorescence, fruit production and stem diameter at ground level (Tables 3, A6 and A7). These results indicate that mowing during peak flowering and fruiting periods of the species reduced shoot size and produced fewer inflorescences. Although mowing timings have been unchanged during the last decade in most sites, a quick adaptation of growing and reproductive phenology due to the recent mowing timing is not considered to occur. This is because *V. pycnostelma* is perennial so that the same individuals can reproduce for several years. Furthermore, the species requires a few years to start reproduction after seed germination, likely leading to the moderate or long regeneration time in this species. Previous reports have suggested that mowing during flowering and fruiting periods negatively affects the flower and seed

337 production of other flowering species (Brys et al., 2004; Endels et al., 2007). Taken together,
338 our current and the previous findings suggest that low reproductive success following
339 mowing during the reproductive period is caused primarily by a simple reduction in the
340 numbers of flowers and fruits and not by a reduction in pollination success.
341 In contrast, mowing during April–May and November–March enhanced inflorescence and
342 fruit production, respectively. The removal of the above-ground parts of other plant species
343 before the peak growing season might promote growth and underground resource storage of *V.*
344 *pycnostelma* in spring. The positive effects of mowing before flowering on reproductive
345 success in summer- and autumn-blooming species should be examined in other species and/or
346 in different semi-natural grassland ecosystems.
347 Pollination success was not affected significantly by flowering timing but instead by the
348 grassland area of each site in *V. pycnostelma*. The flowers of this species are pollinated by
349 very common small- and intermediate-sized moths, the adults of which usually have an active
350 period of more than 3 months (Inoue et al., 1982; Nakahama et al., 2013a, b; Yamashiro et al.,
351 2008). Therefore, a delay in the flowering period due to mowing should not lead to a
352 phenological mismatch between flowering and pollinator activity. Furthermore, *V.*
353 *pycnostelma* would likely selectively abort fruits, as fruit sets in naturally and hand-pollinated
354 flowers were usually very low (Nakahama et al., 2013b). Consequently, even if pollination
355 success can vary depending on the number of flowers, a variation in fruit set would be
356 diminished by selective abortion.
357
358 *4.2. Effects of mowing timing on genetic diversity*
359 Our results demonstrated that mowing events during peak flowering and fruiting periods had
360 significant negative effects on genetic diversity (Table 3). The reduction of flower number

361 and, consequently, of fruit and seed production in every year caused by mowing during this
362 period could decrease genetic diversity in three ways. First, disturbance events at peak
363 flowering would diminish the number of potential mates, likely causing a loss in the genetic
364 diversity of seeds within a given population. Second, the reduction in subsequent fruit
365 production would result in lower genetic diversity in seeds. Because the pollen of
366 Asclepiadoideae plants exists as a pollinium (Endress, 1994), the diversity of paternally
367 inherited genes per individual fruit should be low, corresponding to a single father.
368 Consequently, a reduced number of fruit per population would lead to low genetic diversity in
369 seeds. The study populations are isolated from one another, and therefore gene flow between
370 populations might be limited. Thus, low genetic diversity in seedlings in the next year would
371 gradually diminish the genetic diversity of the population, as gene flow is limited by a
372 reliance on intrapopulation reproduction. Third, reduced fruit production would also result in
373 low genetic diversity in seed banks. Many grassland herbs produce seed banks, which act to
374 restore their populations after anthropogenic disturbances (Willems and Biks, 1998).
375 *Vincetoxicum pycnostelma* would also form a long-term soil seed bank because its seeds form
376 large embryos and exhibit physiological dormancy (Martin, 1946; Zhou et al., 2003). Seed
377 banks contribute to the maintenance of genetic diversity at post-disturbance stages of
378 population growth (Honnay et al., 2008; Zaghloul et al., 2013). Therefore, less genetic
379 diversity in seed banks would reduce the genetic diversity of frequently disturbed plant
380 populations.
381 The genetic diversity of populations that were mown before the flowering peak (July)
382 when plants produced more fruits, tended to be greater compared with the other populations
383 (Fig. 2b). Increased seed production would provide higher levels of genetic diversity in
384 seedlings and/or seed banks. Therefore, to maintain the genetic diversity of *V. pycnostelma*, it

385 is clearly important to avoid mowing during the peak flowering and fruiting periods. In
386 particular, the reproductive success of self-incompatible species is often positively correlated
387 with genetic diversity (Leimu et al., 2006; Young and Pickup, 2010). Thus, in
388 self-incompatible *V. pycnostelma*, a reduction in genetic diversity could subsequently result in
389 a further reduction in reproductive success, likely leading to a negative feedback loop
390 between reproductive success and genetic diversity. In this study, we could not determine
391 why population size had a negative effect on genetic diversity. Mean temperature and total
392 precipitation from April to October had no significant effects, although environmental factors
393 such as temperature, precipitation and soil moisture potentially influence genetic diversity
394 (Avolio and Smith, 2013; Huang et al., 2015).
395 In the present study, neither clear spatial genetic structure nor significant isolation by
396 distance existed among the populations of *V. pycnostelma* in the Kinki and Tokai districts in
397 Japan, although some populations had unique genetic structures (Fig. A2, A3c). The extent of
398 genetic differentiation of common grassland species such as *Miscanthus sinensis* and
399 *Artemisia indica*, which dominate in semi-natural grasslands and whose habitats are the same
400 as *V. pycnostelma*, is low due to the rapid expansion of their distribution range and frequent
401 historical gene flow between populations (Shimono et al., 2013a, b). Because *V. pycnostelma*
402 was also common in semi-natural grasslands in the past, it may have also rapidly expanded its
403 distribution, as it shares the same grassland habitat and co-exists with the aforementioned
404 grassland species. This rapid expansion may explain the observed low genetic differentiation
405 and unclear spatial genetic structure in *V. pycnostelma*. At sites a, d, i and o, where mowing
406 during the peak flowering or fruiting periods have been conducted, loss of allele diversity
407 might be the cause of a unique genetic structure (Fig. 2b, A3c).
408

4.3. Decline mechanisms of semi-natural grassland plants

We demonstrated that mowing events before the flowering period are favourable for the reproductive success of endangered *V. pycnostelma*, whereas those during the peak flowering and fruiting periods would cause significant reductions of not only reproductive success but also genetic diversity. We also found reduced flowering and/or fruiting of other summer- and autumn-blooming endangered species at our study sites where mowing events occurred during their flowering periods (Tables A9, A10). In Japan, traditional mowing with a sickle had been considerably time-consuming and labour-intensive. Therefore, intensive mowing from mid-summer to early autumn had been uncommon to avoid physical overexertion (Baba et al., 1991). Furthermore, selective mowing, which kept unmown grassland areas, had been commonly observed because living plant biomass for livestock fodder and organic fertiliser was constantly necessary throughout the seasons (Arita and Kimura, 1993; Baba et al., 1991; Itoh and Baba, 1999). Consequently, traditional mowing practices had been generally extensive in summer, maintaining many summer- and autumn-blooming rare plants in Japanese semi-natural grasslands (Itoh and Baba, 1999). During recent decades, however, mowing from mid-summer to early autumn has become common practice because of the popularisation of motorised shoulder-type grass-cutting machines, which are compact and mobile. Using the machines, farmers can mow easily and intensively, avoiding physical overxertion even in mid-summer (Arita and Kimura, 1993; Itoh and Baba, 1993). Thus, these changes in mowing timing and frequency may be one factor causing the decline in semi-natural grassland herbs, including *V. pycnostelma*.

5. Conclusion

To the best of our knowledge, this study is the first to demonstrate that the timing of mowing

affects the genetic diversity of endangered semi-natural grassland herb populations, although the frequency of management practices (mowing and/or burning) has been well documented as being crucial for the conservation of endangered semi-natural grassland herbs (Kleijin et al., 2011; Uchida and Ushimaru, 2014; Zechmeister et al., 2003).

Of the 186 species defined as Japanese grassland herb species by Koyanagi and Furukawa (2013), flowering phenology has been described for 168 (Kitamura et al., 1957; Kitamura et al., 1964; Kitamura and Murata, 1961), 69 of which are currently listed as endangered (described in the Red Lists of over four prefectures). Approximately 70% of the total species and 75% of the endangered species have a flowering peak from summer to autumn like *V. pycnostelma*. Thus, the timing of mowing that enhanced the reproductive success and genetic diversity of *V. pycnostelma* would be effective to conserve other endangered grassland herbs. In contrast, mowing during mid-summer to early autumn (July–September) should be avoided. Based on our findings, we suggest that future studies will investigate different species in different semi-natural grasslands throughout the world to confirm the importance of timing management for the conservation of grassland plant species.

ACKNOWLEDGEMENTS

We thank Y. Hashimoto, E. Imazumi, M. Kagohashi, T. Matsumoto, T. Murase, K. Nagase, K. Ohtoshi, K. Sakai, Y. Sawada, T. Yamaguchi, S. Yoshida, and A. Yoshino, who acted as guides within the *V. pycnostelma* habitats. We also thank A. Takayanagi, M. Yamasaki, and M. Yokogawa for valuable discussions about and helpful support with this manuscript. This work was supported by a 2012 Grants-in-Aid from the Kansai Organization for Nature Conservation.

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Appendix

Additional supplemental material can be found in the online version of this article.

Appendix A1. The protocol of microsatellite analysis.

Appendix A2. The protocol of random resampling for genetic analysis.

Table A1. Mean temperature and total precipitation during the growing season of *V. pycnostelma* (April to October) at each site.

Table A2. Reproductive success and vegetative growth of each *V. pycnostelma* population.

Table A3. Characteristics of two novel microsatellite loci.

Table A4. Genetic diversity (Allelic richness and expected heterozygosity) calculated from all samples and randomly selected samples.

Table A5. Selected models for the reproductive success of *V. pycnostelma*.

Table A6. Estimated coefficients of explanatory variables in the best GLMMs for the vegetative growth parameters of *V. pycnostelma*.

Table A7. Selected models for the vegetative success of *V. pycnostelma*.

649

650 **Table A8.** Selected models for the genetic diversity of *V. pycnostelma*.

651

652 **Table A9.** Presence/absence of other coexisting rare endangered species for each population

653 of *V. pycnostelma*.

654

655 **Table A10.** Estimated coefficients of explanatory variables in the best GLMMs for coexisting

656 endangered species.

657

658 **Figure A1.** Locations of *V. pycnostelma* study populations.

659

660 **Figure A2.** Relationships between genetic distance and geographic distance.

661

662 **FigureA3.** Results of STRUCTURE analysis

Table 1. Characteristics of the fifteen *V. pycnostelma* populations examined in this study. Population ID, area (m^2), population size, the mean vegetation height, and mowing practice (the presence or absence of the mowing event during the each period and total number of mowing events per one year) are indicated for each population. Mowing timings were divided into five groups depending on mowing practice (see text for details). Selective mowing is indicated as 0.5. [a]Prior to 2010, population i was mowed in July (in italic) unlike recent mowing practices that take place in June. [b]Prior to 2011, population o was selectively mowed in July (in italic).

ID	Prefecture	Area (m^2)	Population size	Mean vegetation height (cm)	Mowing events					Total number of mowing events per one year
					Pre growing period (Nov.–Mar.)	Growing period (Apr.– May)	Early flowering period (Jun.)	Flowering peak (Jul.)	Late flowering and fruiting period (Aug.–Sept.)	
a	Gifu	67.7	92	9.58	0	0	0	1	0	1
b	Aichi	333.3	260	54.17	1	0	1	0	0	2
c	Shiga	1486.7	300	60.83	1	0	1	0	0	2
d	Osaka	320.0	220	12.92	0	0	1	0	1	2
e	Osaka	154.18	100	31.67	0	0	0	0	1	1
f	Hyogo	277.73	180	66.25	1	1	0.5	0	0.5	4
g	Hyogo	200.48	200	40.83	0	0	1	0	0	1
h	Hyogo	180.06	42	38.75	0	0	0	0	0.5	2
i	Hyogo	332.48	58	43.75	0	0	1	*1*[a]	0	1
j	Hyogo	75.0	5	34.58	0	1	0	1	0	2
k	Hyogo	430.6	24	38.75	1	1	1	0	1	4
l	Hyogo	340.9	30	86.25	1	0	0	0	0	l
m	Hyogo	237.6	100	43.33	1	1	1	0	1 + 0.5	5
n	Hyogo	823.0	60	52.5	1	0	0	0	0	1
o	Hyogo	753.9	50	34.17	1	0	0	1(0.5 [b])	0	3

Table 2. Genetic diversity measurements of fourteen *V. pycnostelma* populations. No. of samples; numbers outside and in parentheses indicate the numbers of samples used for genetic analysis and all samples, respectively, *A*; number of alleles per locus, A_R; allelic richness,H_O; observed heterozygosity, H_E; expected heterozygosity, F_{IS}; inbreeding coefficient

Population ID	No. of samples	*A*	A_R	H_O	H_E	F_{IS}
a	22 (27)	6.78	5.86	0.758	0.702	-0.107
b	22 (50)	7.56	6.44	0.737	0.742	-0.012
c	22 (58)	8.44	7.10	0.717	0.763	0.036
d	22 (28)	6.89	5.78	0.753	0.694	-0.115
e	22 (26)	8.00	6.66	0.732	0.745	-0.012
f	22 (22)	8.44	6.95	0.742	0.754	-0.006
g	22 (54)	8.44	7.08	0.773	0.759	-0.041
h	22 (35)	8.67	7.09	0.808	0.781	-0.056
i	22 (30)	4.67	4.33	0.652	0.621	-0.052
j	–	–	–	–	–	–
k	16 (16)	7.56	6.75	0.701	0.748	0.030
l	12 (12)	7.78	7.78	0.843	0.783	-0.116
m	22 (22)	7.22	6.22	0.798	0.746	-0.097
n	22 (29)	9.44	7.79	0.793	0.795	-0.019
o	22 (23)	8.44	7.02	0.758	0.760	-0.020

Table 3. Estimated coefficients of explanatory variables in the best generalized linear mixed model for the pollination success, reproductive success and genetic diversity of *V. pycnostelma*. Bold numbers indicate that the 95% confidence interval (CI) for the partial regression coefficient did not include zero. AIC: Akaike's information criterion.

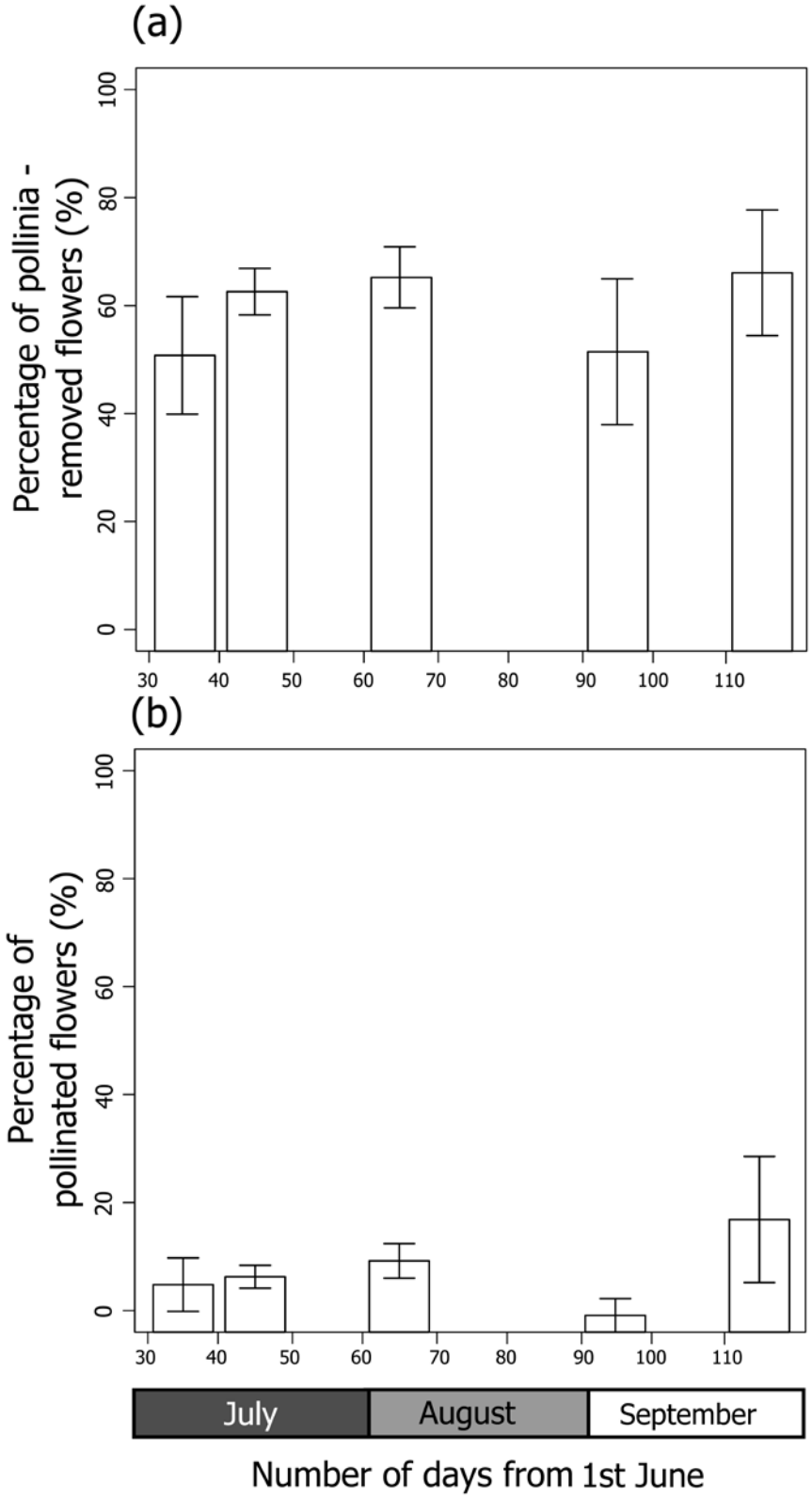
Response variable		Explanatory variable	Coefficient	95% CI		AIC of the model	
				Lower	Upper	Null	Best
Pollination success	No. of pollinia-removed flowers	Area (m^2)	**0.001**	0.001	0.002	449.6	442.8
		Intercept	-0.027	-0.298	0.245		
	No. of pollinated flowers	Area (m^2)	0.001	-0.000	0.001	254.9	254.3
		Intercept	**-2.457**	-2.885	-2.028		
Reproductive success	No. of inflorescences in 2013	Mowing during Apr. and May.	**1.276**	0.647	1.905	1965.9	1953.5
		Mowing in Jul.	**-1.841**	-2.519	-1.162		
		Mowing during Aug. and Sep.	**-1.718**	-2.337	-1.098		
		Total precipitation	**0.003**	0.001	0.006		
		Intercept	-1.830	-4.38	0.719		
	No. of fruits in 2012 and 2013	Mowing during Nov. to Mar.	**0.889**	0.566	1.212	879.7	858.1
		Mowing in Jul.	**-1.912**	-2.533	-1.291		
		Mowing during Aug. and Sep.	**-0.851**	-1.176	-0.526		
		Area (m^2)	**-0.0005**	-0.001	-0.000		
		Mean temperature	**-0.426**	-0.600	-0.252		
		Intercept	**8.527**	4.897	12.157		
Genetic diversity	H_E (arcsin–transformed)	Mowing in Jul.	**-0.172**	-0.239	-0.105	-128.1	-140.1
		Mowing during Aug. and Sep.	**-0.060**	-0.105	-0.014		
		Population size	**-0.0003**	-0.0001	-0.0000		
		Intercept	**0.946**	0.842	1.049		
	Allelic richness	Mowing in Jul.	**-2.438**	-3.155	-1.721	474.3	460.2
		Mowing during Aug. and Sep.	**-1.010**	-1.498	-0.521		
		Population size	**-0.004**	-0.007	-0.001		
		Intercept	**7.956**	6.707	9.205		

FIGURE LEGENDS

Fig. 1. Relationship between pollination success and flowering timing. Bar plots indicate the percentages of pollinia-removed flowers (a) and pollinated flowers (b) in inflorescences in each 10-days period. Error bars indicate the standard errors.

Fig. 2. Comparison of reproductive success and genetic diversity among study populations: mean numbers of inflorescences and fruits in 2013 (a) and mean expected heterozygosity and allelic richness (b). Black circles represent populations mowed during July and September. Grey circles represent populations mowed selectively during July and September. Open circles represent populations mowed in other months. The bars indicate standard errors.

711 Fig. 1

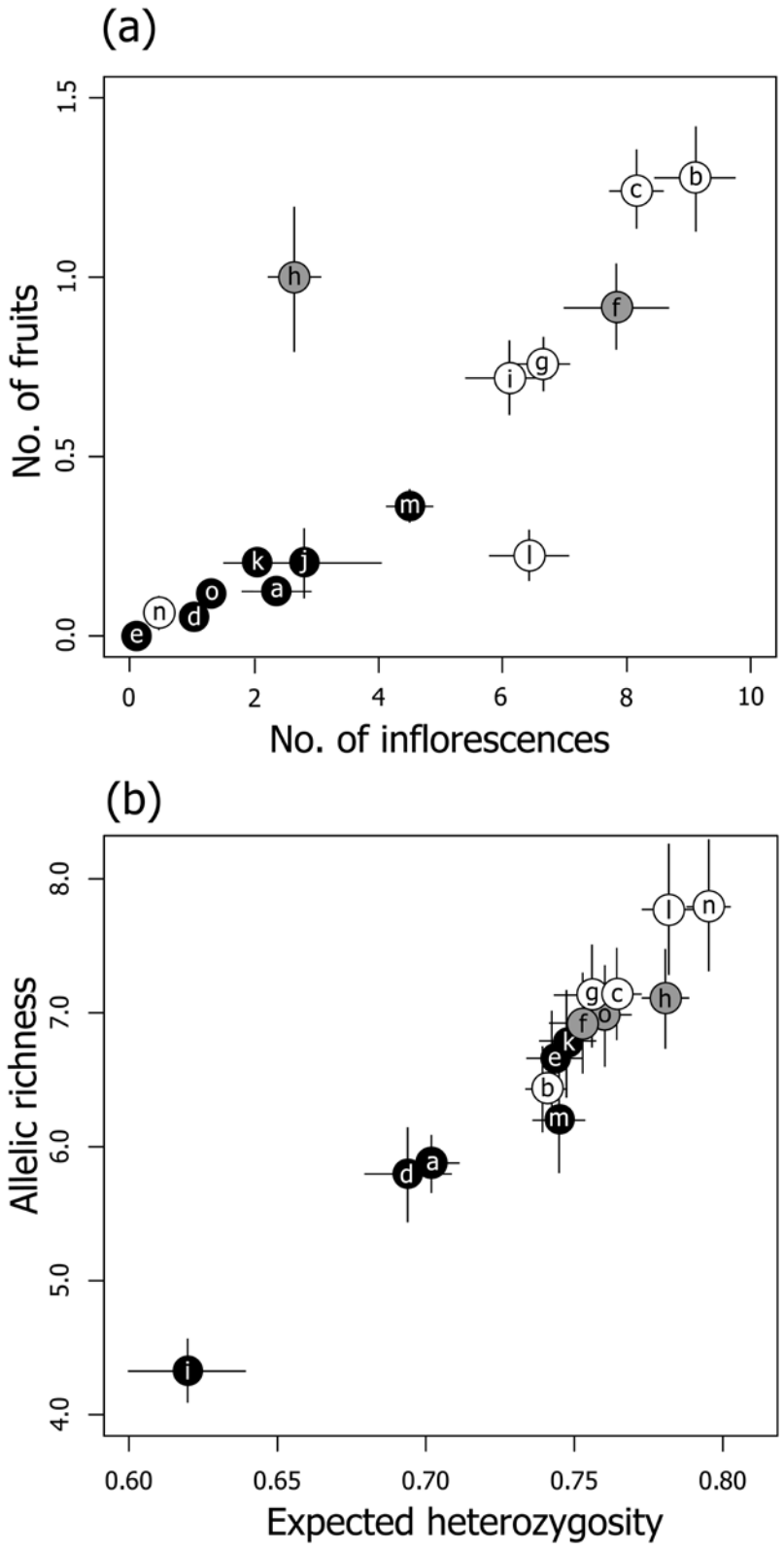


712

713

714

715 Fig. 2


(a)
No. of fruits
No. of inflorescences
0.0
0.5
1.0
1.5
0
2
4
6
8
10
(b)
Allelic richness
Expected heterozygosity
4.0
5.0
6.0
7.0
8.0
0.60
0.65
0.70
0.75
0.80

716

Supplementary data

Appendix A. Supplementary Material and Methods

Appendix A1

With the exception of *Vpy025* and *Vpy031*, PCR amplifications were performed following the standard protocol of the Qiagen Multiplex PCR kit (Qiagen): the final 5 μL volume contained 16 ng of extracted DNA, 2.5 μL of 2× Multiplex PCR Master Mix (Qiagen), and 0.2 μM of each multiplexed primer. For *Vpy025* and *Vpy031*, the forward primer was synthesised using an M13 tag sequence (*Vpy025*: 5′-CACGACGTTGTAAAACGAC-3′, *Vpy031*: 5′-TGTGGAATTGTGAGCGG-3′; Boutin-Ganache et al., 2001). The PCR of the *Vpy025* and *Vpy031* reaction mixture had a final volume of 5 μL, which contained 16 ng of extracted DNA, 2.5 μL of 2× Multiplex PCR Master Mix, 0.01 μM of forward primer, 0.2 μM of reverse primer, and 0.1 μM of M13 (fluorescently labelled) primer. The PCR amplifications of all loci were performed using a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) with the following protocol: initial denaturation at 95°C for 15 min, followed by 25 cycles of 30 s at 94°C, 1.5 min at 57°C, 1 min at 72°C, and a final extension for 30 min at 60°C. PCR product sizes were measured using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) and GeneMapper (Applied Biosystems).

Reference

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Appendix A2

We randomly resampled 22 individuals for 10 populations with more than 22 samples (except sites f, k, l and m) in order to avoid the effect of the variation in sample size among populations on genetic analyses. The random resampling was repeated for 1000 times from the all samples using R software (version 2.15.1; R Core Development Team, 2012) and 1000th resampling data set was used for the microsatellite analyses. In order to assess the validity of selected samples, we compared the allelic richness and Nei's unbiased expected heterozygosity calculated from the all samples (n =23–58) and the random selected samples (n=22) in 10 populations using the package hierfstat. In addition, the mean value and standard deviations were calculated using the 1000 resampling data sets. There were very little differences in the genetic variables among the data sets (Table A4).

Table A1. Mean temperature and total precipitation during the growing season of *V. pycnostelma* (April to October) in 1981-2010, 2012, and 2013 in each site. The data were collected from nearest AMeDAS (Automated Meteorological Data Acquisition System; http://www.data.jma.go.jp/obd/stats/etrn/index.php) point for each study site.

ID	Temperature (°C)			precipitation (mm)			Point
	1981–2010	2012	2013	1981–2010	2012	2013	
a	20.9	21.5	22.0	1,350.5	1,102.0	1,230.5	Mino–Kamo
b	20.9	21.5	22.0	1,350.5	1,102.0	1,230.5	Mino–Kamo
c	19.8	19.6	19.8	1,085.2	1,223.0	1,242.0	Maibara
d	21.8	22.7	22.9	869.9	896.5	842.0	Sakai
e	21.2	21.5	21.9	917.7	859.0	1,008.5	Kumatori
f	20.0	20.4	20.4	942.2	816.0	1,059.5	Sanda
g	20.0	20.4	20.4	942.2	816.0	1,059.5	Sanda
h	20.0	20.4	20.4	942.2	816.0	1,059.5	Sanda
i	20.0	20.4	20.4	942.2	816.0	1,059.5	Sanda
j	20.6	21.2	21.2	884.9	823.0	1,080.0	Miki
k	22.4	22.9	23.0	918.8	855.0	1,014.5	Kobe
l	22.4	22.9	23.0	918.8	855.0	1,014.5	Kobe
m	21.4	21.6	21.8	818.7	951.0	1,209.0	Gunge
n	19.1	19.6	19.7	1,481.4	1,563.5	1,699.5	Ikuno
o	20.3	21.2	21.1	1,078.7	958.0	1,252.5	Toyo–Oka

Table A2. Reproductive success (number of inflorescences and fruits per ramets) and vegetative growth (maximum stem height and stem diameter at ground level) of each *V. pycnostelma* population. The numbers of fruits and the maximum stem height were measured from September to October in 2012 and 2013. The number of inflorescences and stem diameter at ground level of the tallest stem were measured from September to October in 2013.

			Reproductive success			Vegetative growth		
	No. of examined ramets		Mean no. of inflorescences per ramets	Mean no. of fruits per ramets		Mean maximum stem height (cm)		Mean stem diameter at ground level (mm)
Population ID	2012	2013	2013	2012	2013	2012	2013	2013
a	25	25	2.32	0.00	0.12	25.2	40.7	0.95
b	25	25	9.04	0.92	1.28	57.8	62.8	1.39
c	–	25	8.16	–	1.24	–	59.2	1.54
d	–	25	0.92	–	0.04	–	29.6	0.72
e	–	25	0.08	–	0.00	–	24.0	0.77
f	22	25	7.84	1.14	0.92	54.8	63.6	1.52
g	25	25	6.64	0.76	0.76	54.8	62.7	1.43
h	15	13	2.62	0.53	1.00	60.5	50.1	1.12
i	–	25	6.04	–	0.72	–	55.9	1.22
j	5	5	2.80	0.00	0.20	31.8	34.0	1.24
k	11	24	2.08	0.30	0.20	40.5	40.6	1.12
l	–	9	6.44	–	0.22	–	82.4	2.26
m	25	25	4.52	0.36	0.36	27.4	49.0	1.18
n	20	20	0.50	0.05	0.10	35.8	31.0	1.22
o	20	25	1.28	0.20	0.12	33.0	28.3	1.07

Table A3. Characteristics of two novel microsatellite loci for *V. pycnostelma* and their variability. Deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between loci were tested using FSTAT ver. 2.9.3 software (Goudet, 2001) with genotype data of site c. Significance levels were tested using Bonferroni correction for multiple testing. No significant deviations from HWE were detected for either locus. There was no evidence of significant linkage disequilibrium between the two loci. T_a, annealing temperature; *A*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

Locus	Repeat motif	Primer sequence (5'–3')	T_a (°C)	Size range (bp)	*A*	H_O	H_E	Accession No.
Vpy 025	$(GAT)_8$	CCACTCTGCGTTTGGACTTG CGTTCCCTAACATATCGCGG	57	232–265	7	0.741	0.740	AB948217
Vpy 031	$(AT)_{11}$	ATGTTGGCATGTTCTAAGGC AGCAAGTAGGCAAACGGTG	57	168–188	7	0.759	0.717	AB948218

Table A4. Genetic diversity indices calculated from 1000 resampling data sets, 1000th resampling and full data sets. Resampling was not conducted for populations f, k, l and m, because their sample size were equal or fewer than 22. A_R; Allelic richness; H_E; expected heterozygosity

Population ID	1000 resampling data sets (n = 22)		1000th resampling data set (n = 22)		Full data set (n = 12–58)	
	A_R (Mean±SD)	H_E (Mean±SD)	A_R (Mean)	H_E (Mean)	A_R (Mean)	H_E (Mean)
a	5.84±0.12	0.703±0.0005	5.86	0.702	5.85	0.703
b	6.38±0.21	0.736±0.0121	6.44	0.742	6.38	0.735
c	7.06±0.22	0.769±0.0126	7.10	0.763	7.06	0.769
d	6.02±0.16	0.714±0.0082	5.78	0.694	6.03	0.715
e	6.74±0.13	0.745±0.0054	6.66	0.745	6.75	0.746
f	no resampling	no resampling	no resampling	no resampling	6.95	0.754
g	7.13±0.20	0.779±0.0107	7.08	0.759	7.14	0.779
h	6.97±0.20	0.781±0.0072	7.09	0.781	6.97	0.781
i	4.44±0.09	0.638±0.0089	4.33	0.621	4.44	0.638
k	no resampling	no resampling	no resampling	no resampling	6.75	0.748
l	no resampling	no resampling	no resampling	no resampling	7.78	0.783
m	no resampling	no resampling	no resampling	no resampling	6.22	0.746
n	7.52±0.15	0.781±0.0081	7.79	0.795	7.52	0.781
o	7.02±0.08	0.760±0.0042	7.02	0.76	7.02	0.761

Table A5. Results of model selections for the reproductive success of *V. pycnostelma* based on AIC. Estimated coefficient of each explanatory variable, AICs and ΔAICs (the difference between each AIC value and the smallest value) are indicated for the top ten models with lower AICs. Bold numbers indicate that the 95% confidence interval (CI) for the partial regression coefficient did not include zero.

		Mowing timing										
Model	Intercept	During Apr. and May	In Jun.	In Jul.	During Aug. and Sep.	During Nov. to Mar.	Area (m^2)	Population size	Mean temperature	Mean precipitation	AIC	ΔAIC
The number of inflorescences												
Best model	-1.830	**1.276**		**-1.841**	**-1.718**					**0.003**	1953.5	-
2nd	-1.656	**1.211**	0.416	**-1.492**	**-1.709**					**0.003**	1953.8	0.5
3rd	-2.100	**1.154**	0.469	**-1.572**	**-1.731**		-0.0004			**0.003**	1954.5	1.0
4th	-2.169	**1.240**		**-1.930**	**-1.734**		-0.0003			**0.004**	1954.8	1.3
5th	-3.561	**1.304**		**-1.905**	**-1.790**				0.072	**0.004**	1955.2	1.7
6th	-1.721	**1.301**		**-1.756**	**-1.706**			0.0006		**0.003**	1955.3	1.8
7th	-1.942	**1.298**		**-1.868**	**-1.720**	-0.048				**0.003**	1955.4	1.9
8th	**8.921**		**1.148**		**-1.195**	**1.330**	**-0.0014**		**-0.377**		1955.4	1.9
9th	-3.283	**1.238**	0.410	**-1.557**	**-1.777**				0.067	**0.003**	1955.6	2.1
10th	-1.710	**1.188**	0.459	-1.512	-1.717			-0.0004		**0.003**	1955.8	2.3
The number of fruits												
Best model	**8.527**			**-1.912**	**-0.851**	**0.889**	**-0.0005**		**-0.426**		858.8	-
2nd	**8.677**			**-1.927**	**-0.797**	**0.864**	**-0.0006**		**-0.464**	0.0007	859.4	0.8
3rd	**8.984**		0.234	**-1.693**	**-0.805**	**0.910**	**-0.0006**		**-0.457**		859.7	0.9
4th	**10.090**	-0.272		**-1.872**	**-0.669**	**1.06**	**-0.0007**		**-0.502**		860.2	1.4
5th	**9.044**		0.191	**-1.745**	**-0.767**	**0.884**	**-0.0007**		**-0.484**	0.0006	860.7	1.9
6th	**8.615**			**-1.946**	**-0.875**	**0.907**	**-0.0005**	-0.0002	**-0.429**		860.8	2.0
7th	**10.82**	-0.311	0.249	**-1.631**	**-0.593**	**1.104**	**-0.0008**		**-0.546**		860.9	2.1
8th	**9.659**	-0.171		**-1.897**	**-0.691**	**0.976**	**-0.0007**		**-0.508**	0.0006	861.2	2.4
9th	**9.449**		0.306	**-1.755**	**-0.885**	**0.990**	**-0.0006**	-0.0009	**-0.476**		861.2	2.4
10th	**8.836**			**-1.991**	**-0.840**	**0.900**	**-0.0006**	-0.0004	**-0.470**	0.0007	861.3	2.5

Table A6. Estimated coefficients of explanatory variables in the best generalized linear mixed model for vegetative growth of *V. pycnostelma*. In 2012, data from nine populations were analyzed (sites c, d, e, i, l, and n were excluded); in 2013, data from 14 populations were used (site n was excluded). Bold numbers indicate that the 95% confidence interval (CI) for the partial regression coefficient did not include zero. AIC, Akaike's information criterion.

			95% Confidence interval		AIC	
Response variable	Explanatory variable	Coefficient	Lower limit	Upper limit	Null model	Best model
Stem diameter at ground level in 2013	Mowing during Nov. to Mar.	**0.320**	0.142	0.498	361.3	347.9
	Mowing during Apr. and May	0.238	-0.002	0.479		
	Mowing in Jun.	**-0.237**	-0.446	-0.028		
	Mowing in Jul.	**-0.553**	-0.824	-0.282		
	Mowing during Aug. and Sep.	**-0.568**	-0.769	-0.368		
	Intercept	**1.490**	1.280	1.699		

Table A7. Results of model selections for the vegetative growth of *V. pycnostelma* based on AIC. Estimated coefficients for selected explanatory variables, AICs and ΔAICs (the difference between each AIC value and the smallest value) are indicated for the lowest ten models of AICs. Bold numbers indicate that the 95% confidence interval (CI) for the partial regression coefficient did not include zero.

		Mowing timing									
Model	Intercept	During Apr. and May	In Jun.	In Jul.	During Aug. and Sep.	During Nov. to Mar.	Area (m^2)	Population size	Mean temperature	Mean precipitation	AIC	ΔAIC
Stem diameters at ground level in 2013												
Best model	**1.490**	0.238	**-0.237**	**-0.553**	**-0.568**	**0.320**					347.9	-
2nd	**1.481**			**-0.464**	**-0.513**	**0.402**		**-0.0011**			348.2	0.3
3rd	**1.532**	0.193	-0.183	**-0.565**	**-0.575**	**0.340**		-0.0005			349	1.1
4th	**1.524**		-0.131	**-0.529**	**-0.502**	**0.413**		-0.0009			349.1	1.2
5th	**1.475**	0.131		**-0.472**	**-0.566**	**0.350**		-0.0010			349.2	1.3
6th	**0.722**	**0.265**	**-0.241**	**-0.586**	**-0.622**	**0.308**			0.037		349.2	1.3
7th	**1.494**			**-0.458**	**-0.535**	**0.453**	-0.0002	-0.0009			349.2	1.3
8th	**1.439**		-0.214	**-0.489**	**-0.456**	**0.406**					349.3	1.4
9th	**1.499**	0.203	-0.215	**-0.542**	**-0.572**	**0.365**	-0.0001				349.5	1.6
10th	**1.475**		-0.176	**-0.487**	**-0.499**	**0.473**	-0.0002				349.6	1.7

Table A8. Results of model selections for the genetic diversity of *V. pycnostelma* based on AIC. Estimated coefficients for selected explanatory variables, AICs and ΔAICs (the difference between each AIC value and the smallest value) are indicated for the lowest ten models of AICs. Bold numbers indicate that the 95% confidence interval (CI) for the partial regression coefficient did not include zero.

		Mowing timing										
Model	Intercept	During Apr. and May	In Jun.	In Jul.	During Aug. and Sep.	During Nov. to Mar.	Area (m^2)	Population size	Mean temperature	Mean precipitation	AIC	ΔAIC
H_E (arcsin-transformed)												
Best model	**0.945**			**-0.172**	**-0.060**			**-0.0003**			-140.1	-
2nd	**0.886**			**-0.173**	-0.047			**-0.0003**		0.00005	-138.8	1.3
3rd	**0.924**		-0.049	**-0.170**	-0.037						-138.7	1.4
4th	**1.139**			**-0.173**	**-0.051**			**-0.0003**	-0.009		-138.7	1.4
5th	**0.933**			**-0.162**	**-0.056**	0.015		**-0.0003**			-138.5	1.6
6th	**0.889**		**-0.060**	**-0.139**		0.035					-138.4	1.7
7th	**0.937**			**-0.168**	**-0.054**		0.00002	**-0.0003**			-138.4	1.7
8th	**0.944**	0.016		**-0.171**	**-0.067**			**-0.0003**			-138.4	1.7
9th	**0.944**		**-0.016**	**-0.175**	-0.054			-0.0002			-138.3	1.8
10th	**0.927**	**0.041**	**-0.057**	**-0.174**	-0.056					0.00008	-138.2	1.9
Allelic richness												
Best model	**7.956**			**-2.438**	**-1.010**			**-0.004**			460.2	-
2nd	**7.130**			**-2.447**	**-0.839**			**-0.004**		0.0007	461.1	0.9
3rd	**7.736**			**-2.264**	**-0.948**	0.246		**-0.004**			461.2	1.0
4th	**7.359**		**-0.751**	**-2.182**	**-0.595**	0.429					461.2	1.0
5th	**7.802**			**-2.357**	**-0.917**		0.0003	**-0.004**			461.4	1.2
6th	**7.705**	0.567	**-0.810**	**-2.471**	**-0.959**						461.7	1.5
7th	**7.934**		-0.258	**-2.486**	**-0.919**			-0.003			461.7	1.5
8th	**7.940**	0.207		**-2.426**	**-1.110**			**-0.004**			461.8	1.6
9th	**7.661**		**-0.696**	**-2.423**	**-0.696**						462.0	1.8
10th	**8.581**			**-2.441**	**-0.982**			**-0.004**	-0.030		462.1	1.9

154 **Table A9.** Presence of other flowering and fruiting endangered species that coexisted with study *Vincetoxicum pycnostelma* populations in autumn
155 2013. Endangered species were defined as species listed as more than near threatened (NT) in at least 5 out of all 47 prefectural Red Lists in Japan
156 (Association of Wildlife Research and Envision Conservation Office, 2007 (http://www.jpnrdb.com/index.html, last accessed 01/10/2013)). 1,
157 presence; 0, absence, *, selectively conserved by land managers.

Family name	Species name	Flowering month	No. of Prefectural Red lists	Populations														
				a	b	c	d	e	f	g	h	i	j	k	l	m	n	o
Apiaceae	*Sium ninsi*	8~10	24	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Asteraceae	*Atractylodes ovata*	9~10	21	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Doellingeria rugulosa	8~9	18	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Serratula coronata subsp. *insularis*	8~10	10	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Campanulaceae	*Platycodon grandiflorus*	8~9	44	0	0	0	1*	0	0	1	0	0	0	0	0	0	0	0
Caryophyllaceae	*Dianthus superbus* var. *longicalycinus*	6~9	5	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Cyperaceae	*Scleria parvula*	7~10	21	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Fabaceae	*Dunbaria villosa*	8~9	9	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Lespedeza virgata	8~9	18	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gentianaceae	*Gentiana scabra var. buergeri*	9~11	9	0	0	0	1*	0	1	0	0	0	0	0	0	0	1	0
	Swertia japonica	10~11	13	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
Iridaceae	*Iris ensata* var. *spontanea*	6~7	26	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Liliaceae	*Allium thunbergii*	9~10	9	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
	Hemerocallis citrina var. *vespertina*	7~8	18	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Lilium leichtlinii f. *pseudotigrinum*	7~9	7	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Hosta longissima	9~10	9	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Saxifragaceae	*Parnassia palustris* var. *palustris*	8~10	20	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Scrophulariaceae	*Siphonostegia laeta*	8	20	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Valerianaceae	*Patrinia scabiosifolia*	8~10	18	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0
	Total species number			**4**	**2**	**1**	**1**	**0**	**5**	**7**	**1**	**2**	**0**	**0**	**1**	**0**	**2**	**0**

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Table A10. Estimated coefficients of explanatory variables in the best generalized linear model with Poisson errors and log–link function for endangered species found near *V. pycnostelma* in the study sites. Bold numbers indicate that the 95% confidence interval (CI) for the partial regression coefficient did not include zero. AIC, Akaike's information criterion.

Explanatory variable	Coefficient	95% Confidence interval		AIC	
		Lower limit	Upper limit	Null model	Best model
Mowing in Jun.	0.676	-0.156	1.508	62.5	54.5
Mowing during Aug. and Sep.	-1.170	-2.396	0.056		
Area (m^2)	-0.001	-0.003	-0.000		
Mean temperature	-0.460	-0.956	0.036		
Intercept	10.756	0.239	21.274		

Figure A1. Location map of studied *Vincetoxicum pycnostelma* populations in the Kinki and Tokai districts (a). Location of the Kinki and Tokai districts in Japan (b).

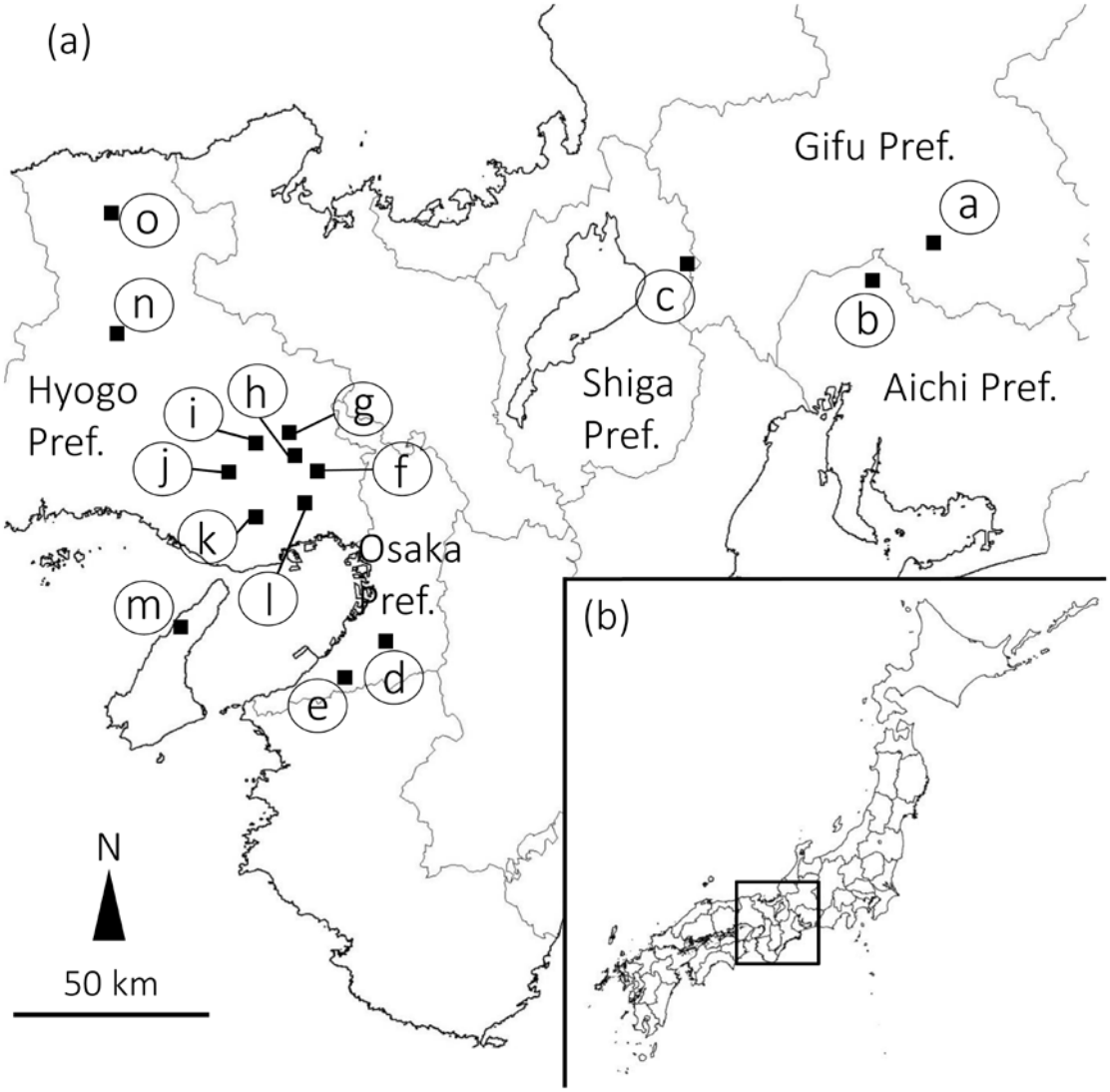


Figure A2. The relationships between genetic (D_A) and geographic distances (logarithmic transformed kilometers) between two sites. There were no significant correlation ($P = 0.057$, $r^2 = 0.093$).

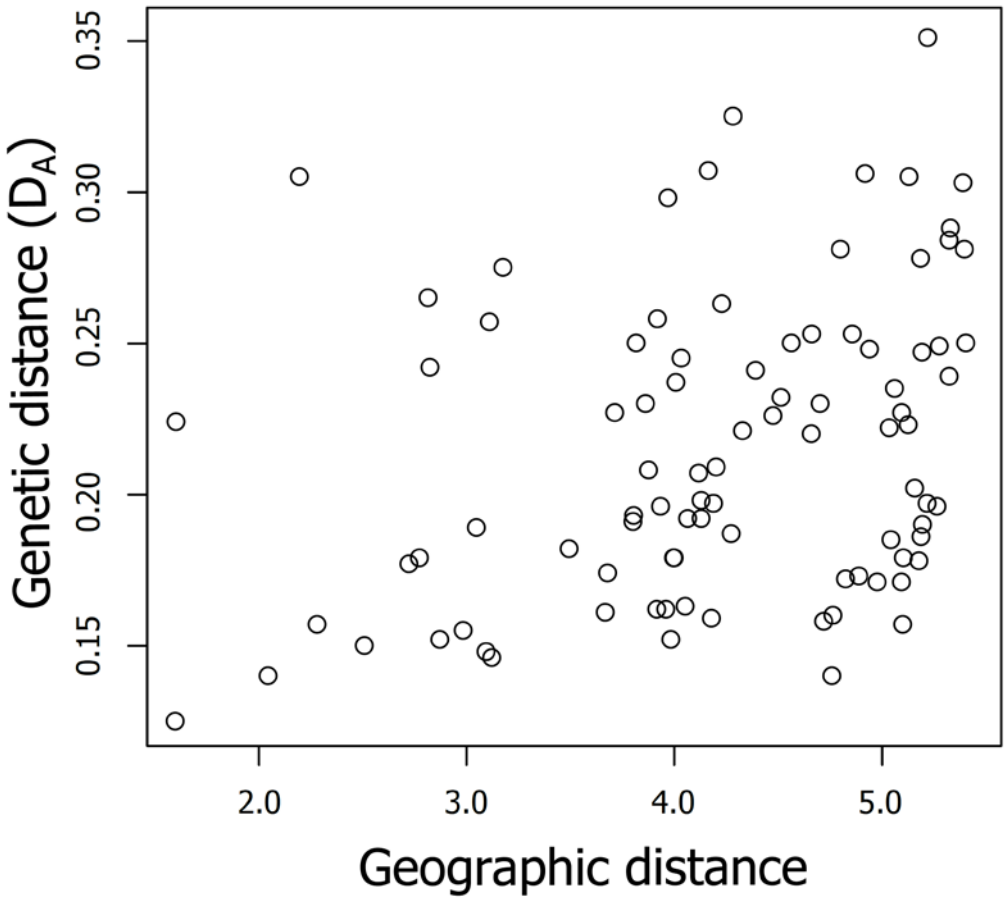
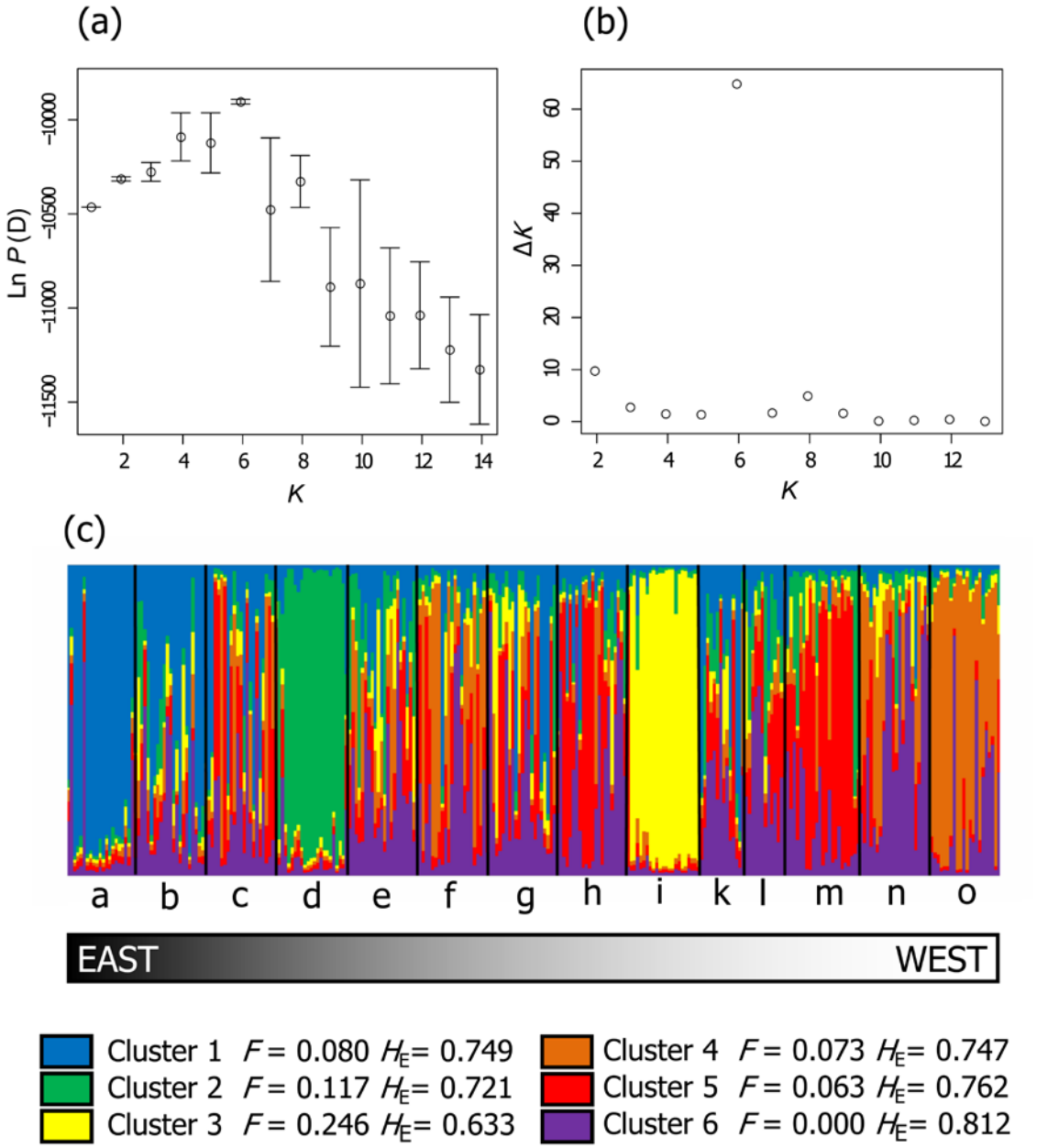


Figure A3. Genetic structure using STRUCTURE analysis (Pritchard et al., 2000).



(a) Value of the estimated ln probability of data (ln P (D)) for K = 1– 14 (means ± SD). (b) ΔK based on the rate of change in the log probability of data between successive K values (Evanno et al., 2005). (c) The proportion of the membership coefficient of 292 in 14 populations for each of the inferred clusters for K = 6. Each column represents an individual.

225 Reference

226 Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of

227 individuals using the software Structure: a simulation study. Mol. Ecol. 14,

228 2611–2620.

229