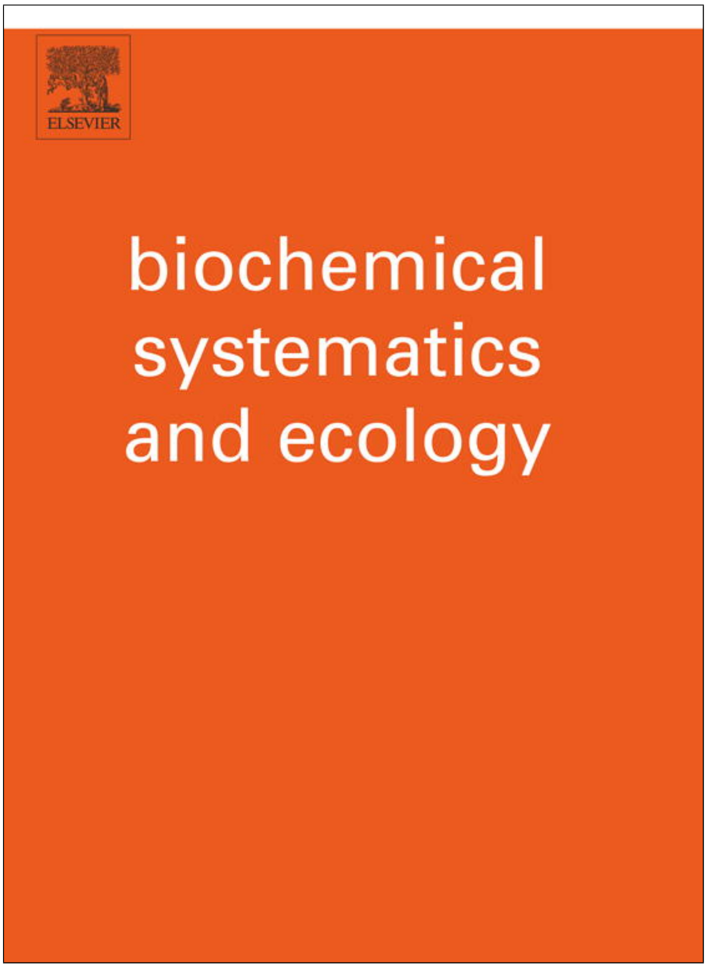
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Fine scale spatial genetic structure of the endangered *Heptacodium miconioides* endemic to China

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A B S T R A C T

A study on the spatial genetic structure of endangered plants can provide basic information to support species management and conservation. *Heptacodium miconioides* Rehder is an endangered plant endemic to China. The fine-scale spatial genetic structure of *H. miconioides* individuals and within population age classes was studied to elucidate population history and selection pressures and to predict the species evolutionary potential. Significant fine-scale spatial genetic structure of *H. miconioides* populations at a distance less than 8.06 m may occur because of limited pollen dispersal, asexual reproduction and micro-environmental selection. The significant fine-scale spatial genetic structure of *H. miconioides* populations from four different age classes might be due to self-thinning and human disturbance.

1. Introduction

The spatial genetic structure (SGS) within a population is a recent hot topics in population genetics. Fine-scale SGS (i.e., the non-random spatial distribution of genotypes or genetic diversity) has important consequences for population biology (Vekemans and Hardy, 2004; Troupin et al., 2006). SGS within a population can be affected by different selection pressures and historical events, such as seed and pollen dispersal, environmental heterogeneity, mating system, intra- and interspecific competition, genetic drift or population history (Troupin et al., 2006). Alternatively, SGS within a population can affect the mating system, effective population size, inbreeding depression and fitness of progeny. Accurate estimation of SGS within a population could effectively obtain information on population history, life history traits, genetic drift, gene flow and other genetic processes (Epperson, 1993; Vekemans and Hardy, 2004; Troupin et al., 2006).

SGS of forest trees species have been reported, and the results have contributed to the management of these species (Ng et al., 2004); however, there are only a few studies on the SGS of endangered species of plants such as *Sinojackia rehderiana* Hu (Styracaceae, Yao et al., 2011), *Coccoloba cereifera* Schwacke (Polygonaceae, Moreira et al., 2010), *Echinosophora koreensis* Nakai (Fabaceae, Chung et al., 2006), *Lycoris sanguinea* Maxim. var. *koreana* (Nakai) T. Koyama (Amaryllidaceae, Chung et al., 1999) and *Cerastium fisherianum* var. *molle* Ohwi (Caryophyllaceae, Maki and Yahara, 1997). Successful conservation strategies partly depend on detailed knowledge of the genetic structure within and among populations (Holsing and Gottlieb, 1991).

Recently, researchers have realised that SGS can provide information across life stages that can provide further indirect information about the demographic structure of populations, elucidating potential ecological and evolutionary processes that

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shape the genetic composition of populations (Futch et al., 2010). Analysis of the SGS within age classes can couple the population succession history and selection issues with pollen and seed dispersal pattern, which can provide clues to the mechanism of genotypes spatial distribution. Therefore, studies on the SGS at different life stages or age classes were enhanced (Fuches et al., 2010).

Heptacodium miconioides Rehder (Rehder, 1916) is a deciduous, long-lived species endemic to China, and it belongs to a monotypic genus: *Heptacodium* (Caprifoliaceae). Because of long-term deforestation and the slow regeneration of populations, the species has declined in recent decades, and the distribution of this species is restricted to only a few localities in eastern China, such as Zhejiang Province, Anhui Province and Hubei Province. Some populations (e.g., Xingshan City in Hubei Province, where the first model specimen was collected) have been reduced to almost zero (Yu et al., 2003). *H. miconioides* is at risk of extinction and is listed as a national second class protected plant species in China (Fu and Jin, 1992). The application of *H. miconioides* in forestry is valuable for its phylogenetic status in the family Caprifoliaceae as well as its beautiful and ornamental flower. In recent decades, we have systematically studied the population structure and dynamics, community ecology, physiological ecology, reproductive ecology, biochemistry and genetics of *H. miconioides* (Jin et al., 2009). We found that the species is becoming more endangered due to such factors as declining populations, inverted pyramid-shaped age structure, impaired natural regeneration, high genetic differentiation among populations, low genetic diversity within populations and low gene flow (Jin et al., 2009). It is urgent to establish conservation strategies for *H. miconioides*.

The conservation of an endangered plant requires an understanding of not only population structure and dynamics but also genetic structure within a population over space and time (Setsuko et al., 2004). Information on the SGS of an endangered plant at different age classes is useful to explore evolutionary history and endangered mechanisms (Kalisz et al., 2001; Ng et al., 2004), providing basic information for the conservation of endangered species (Maki and Yahara, 1997). We analysed fine-scale SGS of *H. miconioides* individuals and different age classes to elucidate population history, selection pressures and predict the evolutionary potential of the species. The results could provide information for the establishment of conservation strategies for this endangered species.

2. Materials and methods

2.1. Study species

H. miconioides, the only species belonging to the *Heptacodium* genus (family Caprifoliaceae), is endangered and endemic to China. It is a deciduous tall shrub to small tree (usually around seven meters tall at its maximum). The tree is elegantly shaped, and the flower is beautiful. The terminal paniculiform inflorescence is composed of several whorls per head, as with cymose inflorescence, which has the form of a tower, with each whorl containing seven tiny flowers, hence the common name, “seven-son flower.” The flowers are fragrant and can be either white or soft pink. The sepals continue to grow as the flower fades, which results in a unique formation. *H. miconioides* is naturally distributed in Zhejiang Province, Anhui Province (Jingxie City and Yicheng City) and Hubei Province (Xingshan City). It usually grows on cliffs, valleys and under forests on the hillsides. The populations of *H. miconioides* have dramatically shrunk in recent decades due to habitat destruction and long-term deforestation.

2.2. Study site

The study was conducted at Shiziyankeng in the northern region of Huading Mountain (29°15′N, 121°06′E) in Tiantai County, Zhejiang Province, China. It is in a tropical monsoon climate zone with a mean annual precipitation of 1700 mm, mean annual temperature of 13 °C, mean relative humidity of 85% or more and frost-free period of approximately 230 d. *H. miconioides* is distributed naturally on the two sides of Shiziyankeng valley at an altitude between 500 m and 1000 m. The habitat is poor, and the soil is shallow with many exposed rocks. The vegetation is an ever-green deciduous forest composed of a tree layer, a shrub layer and a grass layer. *H. miconioides* is distributed at the second sub layer (sometimes at the first sub layer) of the tree layer with a canopy density of 70∼90%. *H. miconioides* is a significant dominant species in the community with an importance value of 39.19%. The main accompanying tree species are *Fraxinus insularis* Hemsl. (Forbs and Hemsley, 1889), *Lindera rubronervia* Gamble (Gamble, 1914) and *Alniphyllum fortunei* (Hemsl.) Makino (Makino, 1906).

2.3. Population sample

In July 2006, all 204 individuals of *H. miconioides* were accurately positioned using the point-pattern method (Yang et al., 2006) in an area of 20 × 60 m (Fig. 1). The diameter at breast height (DBH) of each individual was measured simultaneously. DBH is an important tree characteristic and a statistical analysis shows that the age of trees can be determined with DBH (Lukaszklewlca and Kosmala, 2008). All individuals were divided into four age classes according to the range of DBH. The numbers of individuals at different age classes are listed in Table 1. The distribution maps of individuals for the four age class are shown in Fig. 1. Fresh, tender leaves were collected randomly from every tree. The sampled leaves were kept in a bag with dry ice and were delivered to the laboratory immediately. The leaves were stored at −70 °C until DNA extraction.

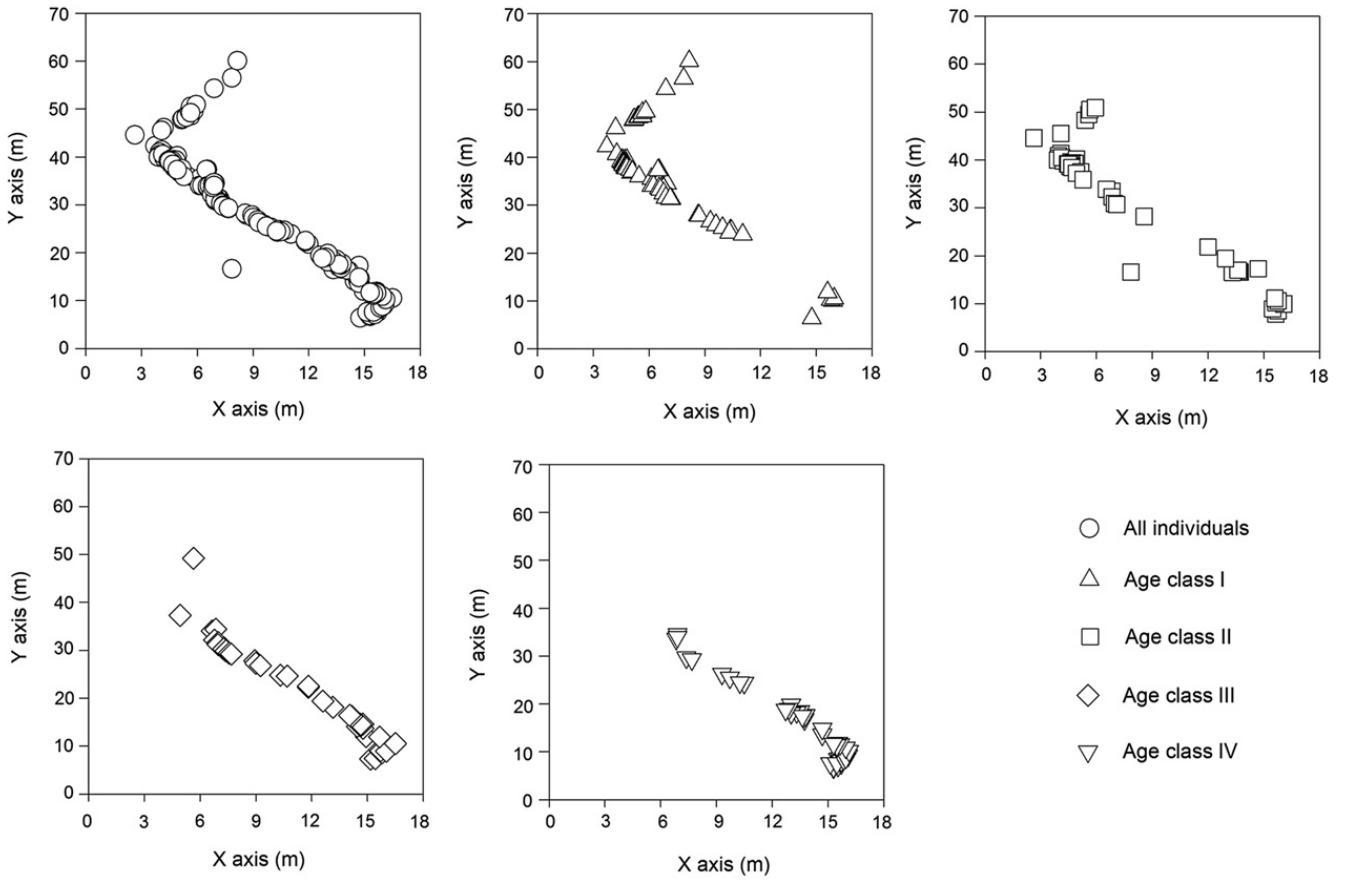

Fig. 1. Spatial distribution map for all individuals of *Heptacodium miconioides*.

2.4. Total DNA extraction and ISSR amplification

Frozen leaves were ground in liquid nitrogen, and genomic DNA was extracted from 0.1 g powder following the optimised SDS (sodium dodecyl sulphate) method (Li et al., 2002). DNA concentration was determined by comparing the sample with a reference DNA in 0.8% agarose gel using Quantity One software with Gel Doc XR image analysis system (Bio-Rad, Inc., Hercules, California, USA). DNA was diluted to 10 ng μl^{-1} and stored at −20 °C for ISSR amplification.

Amplification reaction with optimal reaction conditions (Jin and Li, 2007) was performed in a PTC 220 Thermal Cycler (Bio-Rad, Inc., Hercules, California, USA). The touchdown cycle program included an initial 5 min denaturation at 94 °C, followed by 10 cycles of 1 min at 94 °C, 1 min at 56 °C (touchdown for 1 °C every cycle) and 1.5 min at 72 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 50 °C and 1.5 min at 72 °C, and 5 min final extension at 72 °C. The PCR product was electrophoresed in 1.6% agarose gel at 100 V for 2 h and stained with ethidium bromide. The electrophoresis buffer was 0.5 × TBE. Images were taken with Gel Doc XR image analysis system.

The negative control was run by replacing template DNA with ddH_2O. For every primer, triplicate amplification was conducted. A subset of 12 primers (Table 2) was chosen from 100 primers (UBC primer set No. 9, Biotechnology Laboratory, University of British Columbia) for further analysis because they provided consistent and strong amplification products, uniform and reproducible fragments and lack of amplification in the negative control.

2.5. Data analysis

The data concerning band presence/absence in the ISSR analysis were used to examine the spatial distribution of genotypes. The spatial genetic software was used to perform a test on geographical structure (Degen, 2000; Degen et al., 2001). The spatial genetic structure of 10 sample classes was evaluated by Taminoto's genetic distance (D_G) (Degen, 2000). To assess statistical significance, 95% confidence intervals were generated from Monte Carlo Permutations (1000 replications) (Degen et al., 2001). Spatial autocorrelation was visually examined using autocorrelograms that plotted D_G values (observed and expected) and the 95% confidence intervals as a function of geographical distance.

Table 1
Four age classes of *Heptacodium miconioides* according to the diameter at breast height.

Age classes	Diameter at breast height (cm)	Number of individuals
I	<2.5	68
II	$\geq 2.5, <7.5$	49
III	$\geq 7.5, <12.5$	37
IV	≥ 12.5	50

Table 2
Sequences of 12 random primers used in ISSR analysis of *Heptacodium miconioides*.

Primer	5′–3′ sequences	Primer	5′–3′ sequences
UBC 807	$(AG)_8T$	UBC 824	$(TC)_8G$
UBC 808	$(AG)_8C$	UBC 825	$(AC)_8T$
UBC 810	$(GA)_8T$	UBC 840	$(GA)_8YT$
UBC 811	$(GA)_8C$	UBC 844	$(CT)_8RC$
UBC 822	$(TC)_8A$	UBC 856	$(AC)_8YA$
UBC 823	$(TC)_8C$	UBC 857	$(AC)_8YG$

3. Results

3.1. Fine-scale spatial genetic structure within a population

Significant fine-scale genetic structure within a *H. miconioides* population was found at a distance less than 8.06 m (Fig. 2). The genetic distance increased with geographical distance across the *X*-axis until 11.57 m, and genetic distance decreased slowly with geographical distance.

3.2. Fine-scale spatial genetic structure at different age classes

Significant fine-scale SGS were also found in *H. miconioides* at four age classes (Fig. 3). The maximum geographical distance among individuals was different for the four age classes with the shortest distance at age class I (only 21.8 m), the largest distance at age class IV (40.3 m), and the intermediate distances at age class II and age class III (32.1 m and 33 m, respectively). For age classes I, II, and IV, significant fine-scale SGS were found at a distance of less than 4.36 m, 6.42 m, and 8.76 m, respectively. For age class III, significant fine-scale SGS was found at a distance less than 4.14 m, and genetic distance increased with geographical distance across the *X*-axis at approximately 5.64 m. The genetic distance decreased quickly and significant fine-scale SGS was also found at a distance of 9.90 m.

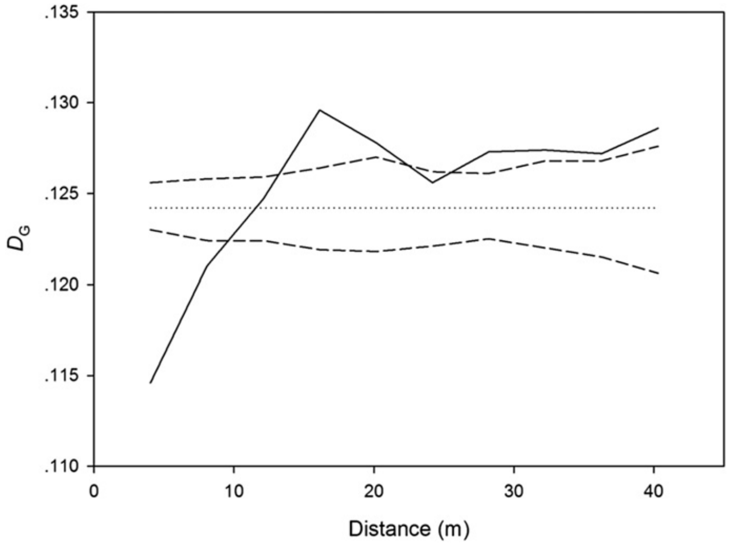

Fig. 2. Spatial genetic structure within a *Heptacodium miconioides* population. The solid line represents genetic distance for each distance class. The dotted line represents the mean reference genetic distance for each distance class after permutations. The dashed lines represent the upper and lower 95% confidence limits for each distance class after permutations.

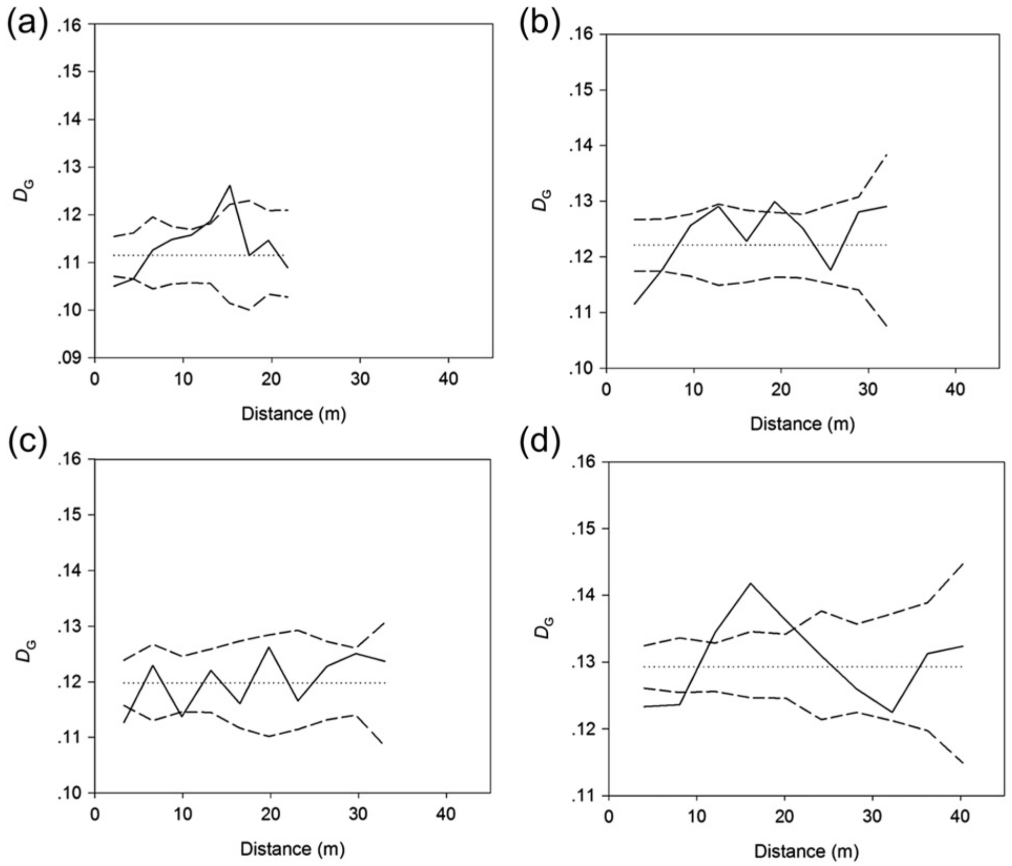

Fig. 3. Spatial genetic structure of *Heptacodium miconioides* at age class I (a), age class II (b), age class III (c) and age class IV (d). The solid line represents the genetic distance for each distance class. The dotted line represents the mean reference genetic distance for each distance class after permutations. The dashed lines represent the upper and lower 95% confidence limits for each distance class after permutations.

4. Discussion

In this study, significant fine-scale genetic structure was found within a *H. miconioides* population at a distance less than 8.06 m. *H. miconioides* is a long-lived woody species with a mixed mating system (Bian et al., 2002). A comparative analysis of SGS in 47 plant species showed that insect-pollinated species had stronger SGS than species pollinated and dispersed by wind (Vekemans and Hardy, 2004). A few trees with a mixed mating system showed similar results. For example, Trapnell et al. (2008) found that a large, long-lived, evergreen and dioecious shrub, *Ceratiola ericoides* Michaux (Empetraceae), had significant SGS at distances less than 5 m. Ueno et al. (2002) found the SGS of an over-green woody, *Camellia japonica* L., in Japan was significant at a distance less than 20 m. Krauss et al. (2009) found woody, *Banksia hookeriana* Meisn, showed significant SGS at a distance less than 8 m.

It has been demonstrated that limited pollen and seed dispersal might be the main factors shaping significant SGS (Vekemans and Hardy, 2004). The mechanism was reported in *Ceratiola ericoides* (Trapnell et al., 2008), *C. japonica* (Ueno et al., 2002) and *B. hookeriana* (Krauss et al., 2009). *H. miconioides* is entomophilous. Field observation showed there were scarcities of pollination agents in the community dominated by *H. miconioides*, which might decrease pollen flow and might play an important role in the evolution of self-pollination (Bian et al., 2002). Based on the results of the bag experiment, there was no significant difference in the seed setting rate between autogamy and xenogamy, indicating that *H. miconioides* might be selfing (Bian et al., 2002). The gene flow within a *H. miconioides* population was very low based on the results of the population genetics revealed by the ISSR technique, which might attribute to the clustering of individuals with similar genotypes and the shaping of the significant SGS (Chen, 2001). Therefore, limited pollen dispersal might be a main contributing factor to the shaping of the significant SGS of *H. miconioides* at small distances.

Genetic structure within a population is affected by the seedling recruitment dynamics (Ohsako, 2010). The seed setting rate of *H. miconioides* is high, but most of the seeds are immature (Bian et al., 2002). The dominancy of *H. miconioides* seeds is long, and the germination rate of seeds is extremely low (Wang et al., 1995). Field observation shows that the seedlings are lacking in the community (Jin, 1997, 1998), indicating that *H. miconioides* rarely reproduces sexually. The seedlings sprouted from the base or root of *H. miconioides* are the main origin for population regeneration. Sometimes, broken branches in wet sand near the valley can survive and mature. The asexual propagation could also be responsible for the shaping of the significant SGS at small distances (Geburek, 1993) and might be another important cause for the shaping of the significant SGS of *H. miconioides* at small distances.

In addition, local selection pressure could govern the formation and maintenance of SGS within a population (Kalisz et al., 2001). The post-dispersal selection of the micro-environment would increase the SGS (Kalisz et al., 2001). The *H. miconioides* population is located in a valley with an inhospitable habitat. The *H. miconioides* population is located in a valley with an inhospitable habitat: the soil is barren and full of large rocks. The micro-environmental selection pressure could reduce the mortality of seedlings after seed dispersal leading to the clustering of seedlings with similar competitive and adaptive abilities.

In this study, significant fine-scale SGS were also found in *H. miconioides* at four age classes. Significant fine-scale SGS were found in age classes I, II and IV at a distance less than 4.36 m, 6.42 m and 8.76 m, respectively. These were determined by limited pollen dispersal, asexual reproduction and micro-environmental selection. Yao et al. (2011) found significant fine-scale SGS of the endangered plant *S. rehderiana* in different life-history stages at a distance of 19 m, indicating short-distance gene dispersal and limited gene flow played an important role in maintaining the population genetic structure. As individuals mature, self-thinning occurs, and any clumped pattern will disappear, which would eliminate spatial genetic structure (Hamrick et al., 1993; Aldrich et al., 1998). Genetic distance increased with age of *H. miconioides*, indicating that self-thinning might play an important role in the formation of SGS at different developing stages. Self-thinning is often the case with the SGS of forest tree species and is found in many trees, such as *Magnolia tomentosa* (Magnoliaceae, Setsuko et al., 2004), *C. japonica* (Theaceae, Chung et al., 2003) and *Pinus halepensis* (Pinaceae, Troupin et al., 2006). However, significant fine-scale SGS was found in age III at a distance less than 3.3 m and emerged again at a distance of 9.9 m. The reason remains unclear. One possible mechanism might be due to human disturbance. Most of the individuals at ages III and IV are located along a valley, where a small tray has been established for residuals to go back to for their life necessities. The number of individuals at age III was the smallest, indicating that seedlings were destroyed by human disturbance during the development of individuals at age III. Furthermore, the tray was abandoned for the culture of the forest during the development of individuals at ages I and II. The mortality of seedlings affected by human disturbance might reduce SGS (Chung et al., 2003).

In summary, significant SGS of *H. miconioides* at small distances might be due to limited pollen dispersal, asexual reproduction and micro-environmental selection. The SGS at different age classes could be influenced by self-thinning and human disturbance. Further studies should focus on the effect of SGS on the adaptation and evolution of the endangered species *H. miconioides* to predict the evolutionary potential of this species and establish suitable conservation strategies for this species.

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