



Genetic variation and population structure of *Asarum misandrum* (Aristolochiaceae) in Korea

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각시족도리풀(*Asarum misandrum*)의 유전적 다양성 및 집단 구조

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ABSTRACT: Genetic variation in *Asarum misandrum*, a woodland herb in Korea, was investigated based on allozyme analysis with starch gel electrophoresis. All of previously reported populations in Korea were sampled and seven loci from six enzymes were analyzed. Overall genetic variation of *A. misandrum* population showed considerably high levels of genetic variation within the species ($A = 2.05$, $P = 71.4$, $H_E = 0.294$). A positive F_{IS} value of *A. misandrum* indicated overall deficiency of heterozygotes, and a low F_{ST} value (0.112) meant very little differentiation among populations. Factors contributing to the high levels of genetic diversity found within populations of *A. misandrum* include population maintenance via wide distribution range from Korea to Japan and primarily outcrossing breeding system. Although it showed moderate genetic diversity level, most habitats of the species were scattered and discontinuous. Besides, low numbers of individuals were found in the most habitats and individuals are collected frequently from the wild due to the unique shape of the flowers as well as the rarity of the species. Thus, there is a need to set up a reasonable conservation strategies including the maintenance mechanism of genetic diversity of *A. misandrum*.

Keywords: *Asarum misandrum*, Genetic variation, Conservation, Korean peninsula, Aristolochiaceae

적 요: 한국과 일본에만 한정 분포하는 각시족도리풀(*Asarum misandrum*) 4개 집단의 자생지 상태와 합리적인 보전전략의 수립을 위하여 7개의 allozyme marker를 이용하여 유전적 다양성과 구조를 분석하였다. 각시족도리풀 집단의 대립 유전자의 수(A)는 2.05개, 다형적 유전좌위의 비율(P)은 71.4%, 이형접합체의 평균 기대치(H_E)는 0.294를 나타내어 분포 역이 넓고 주로 내륙에 분포하는 특산식물과 유사하거나 다소 높은 수준의 유전적 다양도를 유지하고 있는데, 그 이유는 자가수분이 가능하지만 타가수정을 주로 하기 때문인 것으로 판단된다. 유전적 구조분석 결과 집단간 F_{IS} 는 양의 값을 나타내었고 집단간 유전적 분화도는 매우 낮은 결과(0.112)를 보였다. 각시족도리풀의 자생지가 비록 적정 수준의 유전적 다양도를 보이고 있지만, 한국과 일본의 남쪽지방에만 한정 분포하는 특징, 불연속적이고 분산되어있는 자생지의 상태, 대부분의 자생지에서 적은 개체가 나타나며 특이하고 희귀한 꽃 때문에 남획될 가능성이 높다는 점 등은 이 식물의 보전을 위한 관심을 필요로 한다. 각시족도리풀은 최근에 기록되어 현재 희귀식물로 지정된 종은 아니지만 보전적 가치가 높다고 판단되며 이 종의 보전을 위해 종 생물학적 정보를 수집하고 유전적 변이의 유지기작을 포함한 합리적인 보전전략의 수립이 요구된다.

주요어: 각시족도리풀, 유전적 변이, 보전, 쥐방울덩굴과

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Genetic diversity, the level of biodiversity, plays a very important role in species' survival and adaptability of a species. Genetic diversity is required for populations to evolve in response to environmental changes (Reed and Frankham, 2003) and thus, the vulnerability of a population to certain types of historical events can also increase with a reduction in genetic diversity. In plants, breeding system can significantly affect genetic diversity and its partitioning within and among populations (Hamrick and Godt, 1996).

The genus *Asarum* consists of 70-90 species of low-growing perennial herbs in the family Aristolochiaceae, and is widely distributed in temperate and warm temperate zone of Europe, North America and Asia in the Northern Hemisphere (Huber, 1993; Kelly, 1998). Of them, 3 species and 7 forma of the genus *Asarum* occur in Korea (So and Kim, 2008).

According to the phylogenetic study, *Asarum* is well supported as monophyletic and is distinguished from the other genera of Aristolochiaceae by 2 foliage leaves, 2-cataphylls, and a fleshy or spongy capsule (Kelly, 1997; Kelly, 1998). Based on a study of morphology on *Asarum*, several new taxa were recently discovered in Korea and subsequently described (e.g., Yamaki et al., 1996; Yook and Kim, 1996; Oh et al., 1997; So et al., 2010). Also, all members of the taxa are poisonous and have been used traditionally as a herbal medicine for medicinal purpose. For this reason, researches on *Asarum* were mainly focused on the taxonomic study and medicinal researches. Therefore, there is little information about genetic variation in population, genetic structure and conservation.

Plant-animal interactions often play a strong role in determining plant abundance and persistence of a plant population in the ecosystem (Knight, 2007). Shin (1991) reported significance of the ecological niche of *Asarum* species. *Asarum* is known to be a food source specific to the larvae of *Luehdorfia puziloi* (Lepidoptera: Papilionidae). Also, *L. puziloi* is a major pollinator of the spring ephemeral herb *Erythronium japonicum* (Liliaceae) (Ishizaki et al., 2010; Shin, 1991). It is thought that *Asarum* species plays a very important role in the ecosystem as a keystone species.

Of three Korean *Asarum* species, *A. misandrum* is a perennial herb which has been described as a new species by Oh et al. (1997) and this species is distinguished from the other species within the genus by strongly recurved calyx lobes which adhere closely to calyx tube, no trichomes on adaxial leaf surface and petiole, and smaller size of plant.

Through our field research, *Asarum misandrum* is found mainly in the moist and shaded sites of the woodland in Korea and this species occurs with lower trees, *Quercus acutissima*, *Lindera obtusiloba* and *Lindera erythrocarpa*, and herbaceous

plants, *Hedera rhombea*, *Trachelospermum asiaticum*, *Liriope platyphylla* and etc. Current status of *A. misandrum* populations is limited to the south-western coastal area of Korea and low numbers of individuals were found. Also, most habitats are discontinuous and scattered (S. So, pers. obs.). However, no research has examined yet the genetic variation of *A. misandrum* populations in order to construct an effective conservation plan.

The purpose of the present study was to investigate the genetic diversity and differentiation within and among natural populations of *A. misandrum* in Korea based on the investigation of their allozymes. The main objectives of the study were to (a) access levels of genetic diversity of populations, (b) evaluate the degree of differentiation among populations, and (c) provide baseline information for establishing management practices to preserve this species and habitats.

Materials and Methods

Asarum misandrum sample groups of 18 individuals on average were collected from four natural populations in Korea (Fig. 1; Table 1). Those four sites were the representative habitats of *A. misandrum* in Korea and they were living mainly in the shaded sites on the mountain sides. Collections of individuals from each sites were made at least 5 m apart to

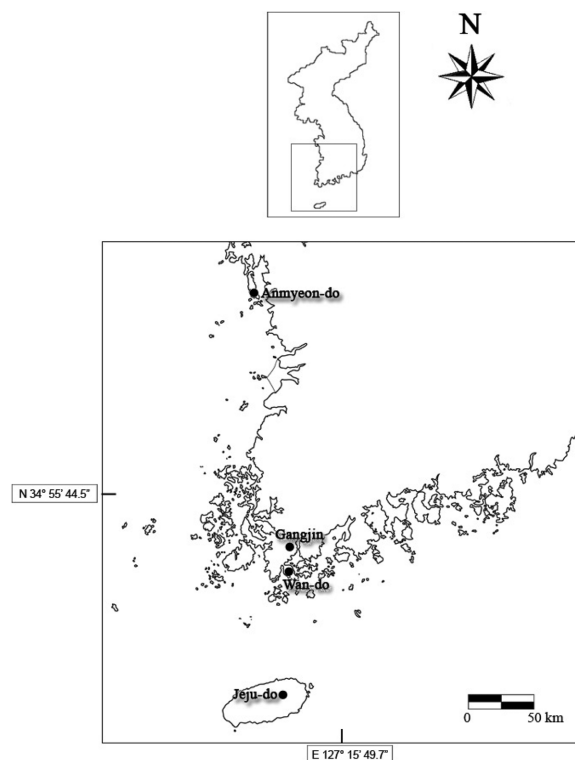


Fig. 1. Distribution of *Asarum misandrum* in Korea.

Table 1. Collection sites for four populations of *Asarum misandrum* for the allozyme analyses.

Population name	Location	Number of samples
1 Anmyeon-do	Anmyeon-do, Taean-gun, Chungnam, Korea	18
2 Gangjin	Gangjin-gun, Jeonnam, Korea	18
3 Jeju-do	Jeju-do, Korea	18
4 Wan-do	Wan-do, Wando-gun, Jeonnam, Korea	18

reduce the likelihood of collecting clones. Young leaves are collected and stored at 4 until the experiment.

For the survey of allozymes within the species using the starch gel electrophoresis, samples were pulverized and ground in porcelain dish using extracting buffer containing 0.1 M tris-HCl, pH 7.5, 1 mM EDTA (tetrasodium salt), 10 mM MgCl₂, 10 mM KCl, 14 mM 2-mercaptoethanol, and 5-10 mg/ml solid polyvinylpyrrolidone (Gottlieb, 1981). Four chromatography paper wicks approximately 15 mm long and 3-4 mm wide were dipped into each sample solution, and were stored under -60°C refrigerator before the actual electrophoresis.

Each wick was then processed through electrophoresis with 13% starch gel using two buffer systems. An electrode buffer of 0.065 M L-histidine and 0.007 M citric acid, adjusted to pH 6.5 with NaOH was diluted by 1 : 3 ratio for System I. Another electrode buffer of 0.18 tris, 0.1 M Boric acid, and 0.004 M EDTA, pH 8.6, also diluted according to 1 : 3 ratio was used for System II. The whole process of electrophoresis was carried out inside a refrigerator under 4°C. Gel systems were run at 40 mA for about 5 hours. Enzyme-activity staining and agarose overlays generally followed the protocols of Soltis et al. (1983). Loci and alleles were numbered sequentially and lettered alphabetically beginning with the most anodal form. Total of eleven enzymes were assayed by using System I and II; glyceraldehydes-3-phosphate dehydrogenase (GA3PD), 6-phosphogluconate dehydrogenase (6PGD), malate dehydrogenase (MDH), aldolase (ALD) and shikimate dehydrogenase (SKDH) were assayed with System I, while malic enzyme (ME), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), aspartate aminotransferase (AAT) and alcohol dehydrogenase (ADH), menedione reductase (MNR) were resolved with System II.

The mean number of alleles per locus (A), percentage of polymorphic loci (P), average observed heterozygosity (H_o), and mean expected heterozygosity (H_e) of the 20 samples from each group were studied by using BIOSYS-1 program (Swofford and Selander, 1981). Also, to study the population differentiation,

Wright's F-statistics were calculated. It includes F_{IS} , an index of inbreeding, F_{IT} , the overall inbreeding coefficient, and F_{ST} , a measure of the genetic differentiation among populations (Wright, 1965). UPGMA tree was produced by input of Nei's genetic identity values into the BIOSYS-1 program.

Results

Of the six enzymes evaluated, one (ADH) could be consistently resolved and scored for two interpretable loci (ADH-1 and ADH-3) while only one isozyme locus of the remaining enzymes could be scored. Out of seven loci, all seven were polymorphic among four populations of *Asarum misandrum* inhabiting in Korean peninsula; allele a of ME-2 was unique to population 2 (Gangjin); allele b of ADH-1 was unique to population 1 (Anmyeon-do); allele a of SKDH2 was unique to population 3 (Jeju-do) (Table 2).

All populations had 71.4 % of polymorphic loci. The mean number of alleles per locus ranged from 2.0 to 2.1 with the

Table 2. Summary allele-frequency of *Asarum misandrum* in Korea.

Locus	Anmyeon-do	Gangjin	Jeju-do	Wan-do
PGM-1				
a	0.412	0.353	0.833	0.500
b	0.529	0.471	0.111	0.375
c	0.059	0.176	0.056	0.125
ME-2				
a	0.000	0.176	0.000	0.000
b	1.000	0.765	1.000	0.813
c	0.000	0.059	0.000	0.188
ADH-1				
a	0.765	1.000	1.000	1.000
b	0.235	0.000	0.000	0.000
ADH-3				
a	0.528	0.438	0.722	0.000
b	0.472	0.563	0.278	1.000
PGI-1				
a	0.583	0.559	0.536	0.607
b	0.139	0.206	0.179	0.071
c	0.278	0.235	0.286	0.321
6PGD-2				
a	0.111	0.059	0.111	0.176
b	0.889	0.941	0.889	0.824
SKDH2				
a	0.000	0.000	0.059	0.000
b	1.000	1.000	0.882	0.875
c	0.000	0.000	0.059	0.125

lowest being population 1 and 4 (Anmyeon-do and Wan-do) and the highest being population 2 and 3 (Gangjin and Jeju-do). The mean heterozygosity expected by Hardy-Weinberg equilibrium ranged from 0.251 to 0.318 and averaged 0.294. The mean heterozygosity observed ranged from 0.112 to 0.212 and averaged 0.162. All populations showed lower observed heterozygosity than expected by Hardy-Weinberg equilibrium (Table 3). This clearly indicated severe heterozygote deficit.

Observed genotype proportions were compared to those expected by the Hardy-Weinberg equilibrium by calculating the fixation index (F) for each polymorphic locus in each population. The statistical difference of the F value from 0 was then calculated by using the chi-square test (Table 5). Of the 20 valid tests, 6 loci showed accordance to Hardy-Weinberg proportions, while most of the remaining loci were significantly different from the Hardy-Weinberg equilibrium and the value of F exceeded 0, indicating heterozygote deficiency.

F statistics was used to measure the degree of genetic differentiation among four populations of *A. misandrum*. F_{IS} value indicates the degree of divergence from Hardy-Weinberg expectations within populations. F_{IT} value indicates the degree

Table 3. Mean sample size per locus (N), mean values of alleles per locus (A), percentage of polymorphic loci (P), mean observed heterozygosity (H_O), and mean expected heterozygosity (H_E) in four populations of *Asarum misandrum* in Korea.

Population	N	A	P	H_O	H_E
1 Anmyeon-do	16.7	2.0	71.4	0.143	0.318
2 Gangjin	16.1	2.1	71.4	0.180	0.323
3 Jeju-do	17.1	2.1	71.4	0.212	0.251
4 Wan-do	15.0	2.0	71.4	0.112	0.285
Mean	16.2	2.1	71.4	0.162	0.294

Table 4. Summary of F-statistics at 7 loci from four populations of *Asarum misandrum*. F_{IS} , an index of inbreeding, F_{IT} the overall inbreeding coefficient, and F_{ST} , a measure of the genetic differentiation among populations.

Locus	F_{IS}	F_{IT}	F_{ST}
PGM-1	1.000	1.000	0.109
ME-2	1.000	1.000	0.119
ADH-1	1.000	0.438	0.187
ADH-3	0.212	-0.496	0.287
PGI-1	-0.507	1.000	0.007
6PGD-2	1.000	1.000	0.017
SKDH2	1.000	1.000	0.061
Mean	0.672	0.706	0.112

of divergence from Hardy-Weinberg expectation among total population. F_{ST} indicates allele frequency divergence among populations (Allendorf and Luikart, 2007). Although few loci (PGI-1 and ADH-3) of *A. misandrum* had negative F_{IS} and F_{IT} values, the mean F_{IS} and F_{IT} value of all seven loci of *A. misandrum* studied were 0.672 and 0.706, respectively, which indicated overall deficiency of heterozygote compared to the Hardy-Weinberg expectation. The mean F_{ST} value of seven loci of *A. misandrum* was 0.112, which means very little differentiation among populations (Table 4).

The Nei's unbiased genetic identity (Nei, 1978) showed that four populations studied were close to each other in pairwise comparison. The genetic identity values ranged from 0.872 for the comparison between population 3 (Jeju-do) and 4 (Wan-

Table 5. Value of the fixation index F for polymorphic loci in populations of *Asarum misandrum*. Consistency with the Hardy-Weinberg equilibrium, i.e., statistical difference of fixation indices from 0, was evaluated using chi-square analysis and is indicated via asterisks: * $P < 0.005$

Locus	Anmyeon-do	Gangjin	Jeju-do	Wan-do
PGM-1	1.000*	1.000*	1.000*	1.000*
ME-2	-	1.000*	-	1.000*
ADH-1	1.000*	-	-	-
ADH-3	0.666*	0.238	-0.385	-
PGI-1	-0.479	-0.496	-0.549	-0.502
6PGD-2	1.000*	1.000*	1.000*	1.000*
SKDH2	-	-	1.000*	1.000*

Table 6. Mean values for Nei's (1978) unbiased genetic identity coefficient for pair-wise comparisons of populations of *Asarum misandrum* in Korea.

	1	2	3	4
1 Anmyeon-do	-			
2 Gangjin	0.974	-		
3 Jeju-do	0.945	0.936	-	
4 Wan-do	0.917	0.944	0.872	-

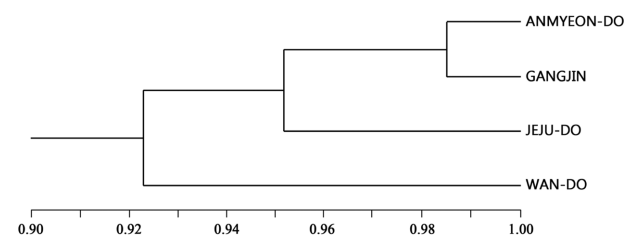


Fig. 2. UPGMA phenogram derived from Nei's genetic identity of four populations of *Asarum misandrum* in Korea.

do) to 0.974 for the comparison between population 1 (Anmyeon-do) and 2 (Gangjin) (Table 6). Further investigation showed that population 4 (Wan-do), although still genetically close to other population, was the farthest from other populations in terms of Nei's genetic identity values. A UPGMA phenogram based on genetic identity values were constructed to examine genetic relationships among four populations of *A. misandrum* (Fig. 2).

Discussion

Genetic variation and population structure

Asarum misandrum maintains considerably higher levels of genetic variation ($A = 2.05$, $P = 71.4$, $H_E = 0.294$), similar to that of continental endemic species. In case of Korean continental endemic plants, for example, it showed higher level of genetic variation than that of *Hosta minor* ($A = 1.68$, $P = 52.59$, $H_E = 0.230$) (Chung, 1994) and lower level than that of *Eranthis byunsanensis* ($A = 2.4$, $P = 90.0$, $H_E = 0.311$) (So et al., 2013) and *Hemerocallis hakuimensis* ($A = 3.17$, $P = 75.0$, $H_E = 0.253$) (Kang and Chung, 1997).

Generally, selfing species tend to have lower genetic diversity and heterozygosity within populations, as well as higher genetic differentiation among populations, than mixed or outcrossing ones (Hamrick and Godt, 1989; Nybom and Bartish, 2000). Although breeding system of *A. misandrum* has not been studied previously, there has been much speculation about the pollination biology of *Asarum* species due to unique shape of flowers. According to previous pollination studies of *Asarum* species, it was proposed that these species are allogamous and pollinated by carrion insects including flies (Peattie, 1940; Lu, 1982). Also, it was observed that in flowering period of *A. caudatum*, the anthers dehisce near the stigmas and self-pollination occurs (Lu, 1982). Thus, *A. misandrum* is more likely to be a self-compatible but pollinator-dependent outcrosser and these are factors contributing to the high levels of genetic diversity found within populations.

All four populations of *A. misandrum* had a low F_{ST} value, which indicates a close similarity between populations. It ranged from 0.007 (PGI-1) to 0.287 (ADH-3) and the mean value was 0.112. In comparison with the average value of perennial herbs ($G_{ST} = 0.213$) (Hamrick and Godt, 1989), it was lower than that of *A. misandrum* populations and showed that not much divergence among populations and it suggested that this species has not had much time yet to differentiate among populations since arriving at Korean peninsula. According to phylogeny and evolution study of *Asarum*, interactions including migration,

hybridization, and introgression presumed Korean *Asarum* species had migrated to Japan via a southern route from Korea (Yamaji et al., 2007). Additionally, *Asarum* species in Korea and Japan mainly had slightly or no polymorphism of the ITS sequences and these results supported the evidence for the origin of *A. misandrum*.

Conservation implication

Information on the genetic and demographic structure of populations of the species is essential for establishing comprehensive conservation plans (Wallace, 2002). The measurement and maintenance of genetic variability is one of the most important part of conservation biology and knowledge on its levels and pattern provides essential background information for effective conservation strategies (Avice and Hamrick, 1996; Zhou et al., 2010).

Historical factors might provide one possible explanation for the high genetic variation and low levels of genetic differentiation detected in *A. misandrum*. Earlier it was known to occur only in the southern parts of Korean peninsula (Oh et al., 1997) but they have been lately found in some isolated localities in Korea such as Anmyeon-do and also in Kyushu, Japan (Yamaji et al., 2007; So and Kim, 2008).

Smaller populations, which can be typical of endangered species, are especially vulnerable to loss of variation through stochastic events (Wallace, 2002). Although it showed high genetic diversity, most habitats in Korea were small, discontinuous and were scattered. Besides, low numbers of individuals were found in most habitats and individuals are collected frequently from the wild due to the unique shape of the flowers as well as the rarity of the species.

Plant-animal interactions often play a strong role in determining plant abundance and persistence of a plant population (Knight, 2007). A decrease of *Asarum* species as a keystone species in the ecosystem, is more likely to affect *L. puziloi*, a major pollinator of the spring ephemeral herb *Erythronium japonicum*, which may lead to its extinction thereby affecting *E. japonicum* continuously in the future.

Consequently, *A. misandrum* which has narrow and restricted distribution range must be conserved for maintenance of genetic variation. Especially, the habitat of population 1 (Anmyeon-do), which is one of the most famous tourists destinations in Korea and this place is not fall under any legal protection. Moreover, this habitat is placed near Anmyeon Natural Recreation Forest, which is a famous place for visitors to take a stroll in Anmyeon-do. Thus, a reasonable conservation of the populations and all biological information, which are continuous monitoring of natural habitats, seed dispersal study

and ecological study, must be collected and this species could be designated as a rare species by IUCN categories and criteria. Also, due to low genetic differentiation among populations, the establishment of active conservation strategies to increase the number of individuals and their genetic variation including *ex-situ* and *in-situ* conservation is needed.

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