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Genetic variation in endangered *Scrophularia takesimensis* (Scrophulariaceae) from Ulleung Island

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ABSTRACT. Genetic variation of *Scrophularia takesimensis*, an endangered species endemic to Ulleung Island, was studied using starch gel electrophoresis. All known populations were sampled for allozyme electrophoresis of eight enzymes coded by nine loci. Overall genetic variation was very low. Genetic polymorphism was rarely found among populations, and its estimated values were far smaller than those of other insular species. A positive F_{IS} value of *S. takesimensis* indicated overall deficiency of heterozygotes, and a low F_{ST} value meant very little differentiation among populations. High genetic identity between populations showed that they are recently originated, and did not undergo much differentiation. The extremely low genetic variation of *S. takesimensis* calls for effective conservation measures to be set up as soon as possible.

Keywords: Conservation; Genetic variation; *Scrophularia takesimensis*.

INTRODUCTION

Generally, genetic variation enables a species to increase its possibility of survival in case of future environmental change (Schaal et al., 1991). Thus, the measurement and maintenance of genetic variation are essential parts of conservation biology. The dominant characteristic of a rare or endangered plant species is comparatively low genetic variation since its populations experience strong genetic drift and directional selection (Karron, 1987; Williamson and Werth, 1999; Park, 2004; Wei et al., 2008). Hence, most of its members have no other choice but to inbreed, which eliminates individuals with unique alleles and heterozygotes within the population. This, in turn, causes the species to become genetically less variable and more vulnerable to environmental change (Allendorf and Luikart, 2007).

Scrophularia takesimensis (Scrophulariaceae), endemic and endangered species, consists of only a small number of individuals distributed exclusively in four different places along the coast of Ulleung Island, a small volcanic island located in the East Sea approximately 150 km from the mainland of Korea (Sun and Stuessy, 1998). *Scrophularia takesimensis* is not known to habitate anywhere other than this island, and Korea's Department of the Environment classified it as a second-class endangered plant species.

Scrophularia takesimensis grows to a height of almost 3 feet with square stems and opposite leaves with saw-like edges, which are generally 4 to 7.5 cm long. The flower blooms between June and July. There are five overlapping receptacles, approximately 1.5 mm long and 3 mm wide, and violet corolla about 1 cm long with three divided ends. Tiny fruits, about 8 to 9 mm long, are generally spherical with pointed ends. The most closely related species, *S. grayanoides,* is also a limited distribution in the northeastern part of Japanese mainland Honshu (Kamada et al., 2007).

The main subject of this study, *S. takesimensis*, is also an endangered species, and a significant portion of its population is continuously being threatened by heedless development in the surrounding environment. Because *S. takesimensis* inhabits the coast of Ulleung Island, road construction, a current objective of its regional government, can irreversibly destroy *S. takesimensis'* habitat, resulting in serious population decreases. However, not much research has examined the genetic variation of *S. takesimensis* groups in order to construct an effective conservation plan.

This study compares the genetic variation of samples from four representative S. *takesimensis* groups located on Ulleung Island using starch gel electrophoresis in order to elucidate its genetic variation and make a plan to conserve genetic diversity of this species.

MATERIALS AND METHODS

Collection

Scrophularia takesimensis sample groups of 20 individuals on average were obtained from four different

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Table 1. Collection sites and number of individuals sampled of *S. takesimensis* populations for starch gel electrophoresis.

Number	Location	Number of individuals sampled
1 (Chusan)	Chusan, Ulleung-do, Korea	16
2 (Sadong)	Sadong, Ulleung-do, Korea	26
3 (Dodong)	Dodong, Ulleung-do, Korea	21
4 (Pyungri)	Pyungri, Ulleung-do, Korea	16

populations on Ulleung Island (Table 1). Those four sites were the only remaining *S. takesimensis* habitat in Korea. They were living mainly on the sea shore, but also in various environments, including cliffs, mountain sides, and high and low grounds. Population samples consisted mainly of young leaves and were stored in an icebox until the experiment.

Allozyme Electrophoresis

For running the starch gel electrophoresis, samples were ground in a porcelain dish using extracting buffer containing 0.1 M tris-HCl, pH 7.5, 1 mM EDTA (tetrasodium salt), 10 mM MgCl, 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 stored at -60°C in a refrigerator.

Each wick then underwent electrophoresis with a 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 to a 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 to a 1:3 ratio was used for System II. The whole process of electrophoresis was carried out inside a refrigerator at -4°C. Gel systems were run at 40 mA for about 5 h. Enzyme-activity staining and agarose overlays generally followed the protocols of Soltis et al. (1983). Loci and alleles were numbered sequencially and lettered alphabetically beginning with the most anodal form. Four enzymes were assayed using System I; glyceraldehydes-3-phosphate dehydrogenase (GA3PD), 6-phosphogluconate dehydrogenase (6PGD), malate dehydrogenase (MDH), and shikimate dehydrogenase (SKDH) were resolved. Four enzymes were assayed using System II; malic enzyme (ME), phosphoglucose isomerase (PGI), aspartate aminotransferase (AAT), and alcohol dehydrogenase (ADH) were resolved.

Data Analysis

The mean number of alleles per locus (A), percentage of polymorphic loci (P) (0.95 criterion), average observed heterozygosity (H_O), and mean expected heterozygosity (H_E) of the 20 samples from each group were studied by using the BIOSYS-1 program (Swofford and Selander, 1981). Also, to study the population differentiation, Wright's F-statistics were calculated. This includes F_{IS}, an index of inbreeding, F_{IT}, the overall inbreeding coefficient, and F_{ST}, a measure of the genetic differentiation among subpopulations (Wright, 1965). Fixation indices (F) were calculated as a measure of inbreeding, and a Chi-square test was conducted to test for significant deviations from Hardy-Weinberg expectation. A UPGMA tree was produced by input of Nei's genetic identity values into the BIOSYS-1 program.

RESULTS

Of the nine enzymes evaluated, one (PGI) could be consistently resolved and scored for two interpretable loci (PGI-1 and PGI-2) while only one isozyme locus of the remaining enzymes could be scored. Five out of nine loci were polymorphic among four populations of *S. takesimensis* inhabiting Ulleung Island, Korea; allele a of PGI-2 was unique to population 1 (Chusan); allele b of ME-1 was unique to population 2 (Sadong); allele b of AAT-2 and allele a of ADH-1 were unique to population 3 (Dodong) (Table 2).

Table 2. Summary allele-frequency data for nine polymorphic loci among four of populations of *S. takesimensis* in Ulleung Island, Korea.

Locus	Chusan	Sadong	Dodong	Pyungri
AAT-2				
a	1.000	1.000	0.975	1.000
b	0.000	0.000	0.025	0.000
ADH-1				
a	0.000	0.000	0.143	0.000
b	1.000	1.000	0.857	1.000
GA3PD				
a	1.000	1.000	1.000	1.000
ME-1				
a	1.000	0.962	1.000	1.000
b	0.000	0.038	0.000	0.000
MDH-1				
a	1.000	1.000	1.000	1.000
6PGD1				
a	1.000	1.000	1.000	1.000
PGI-1				
a	0.969	0.600	1.000	0.857
b	0.031	0.400	0.000	0.143
PGI-2				
a	0.063	0.000	0.000	0.000
b	0.938	1.000	1.000	1.000
SKDH1				
a	1.000	1.000	1.000	1.000

All populations had 11.1% of polymorphic loci. The mean number of alleles per locus ranged from 1.1 to 1.2, with the lowest being population 4 (Pyungri) and the remaining populations being the highest. For population 1 (Chusan) and 2 (Sadong), the mean observed heterozygosity was higher than expected by Hardy-Weinberg predictions while population 3 (Dodong) and 4 (Pyungri) had lower observed heterozygosity than expected. The highest observed heterozygosity was found in population 4 (Pyungri), which was 0.032. The lowest observed heterozygosity was found in population 2 (Sadong), which showed no heterozygote (Table 3).

By computing the fixation index F for each polymorphic locus in each population, genotype proportions were compared to those expected in the Hardy-Weinberg equilibrium. Statistical difference of the fixation index from 0 was calculated by the chi-square test (Table 4). Over the four populations, seven tests could be validly conducted. Of the valid tests, four showed accordance to Hardy-Weinberg proportions while three were significantly different from Hardy-Weinberg equilibrium. In each statistically significant case, the value of F exceeded 0, indicating heterozygote deficiency.

Table 3. Mean sample size per locus (N), mean number 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 *S. takesimensis* in Ulleung Island, Korea.

Population	N	A	P	H_O	H_E
1. Chusan	16.0	1.2	11.1	0.021	0.020
2. Sadong	25.9	1.2	11.1	0.000	0.063
3. Dodong	20.9	1.2	11.1	0.006	0.033
4. Pyungri	15.7	1.1	11.1	0.032	0.028

*A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95.

Table 4. Values of the fixation index F for polymorphic loci in populations of *S. takesimensis*. Consistency with Hardy-Weinberg equilibrium, i.e., statistical difference of fixation indices from 0, was evaluated using chi-square analysis and is indicated via asterisks: *P<0.001.

Locus	Chusan	Sadong	Dodong	Pyungri
AAT-2	------	------	-0.026	------
ADH-1	------	------	1.000*	------
ME-1	------	1.000*	------	-----
PGI-1	-0.032	1.000*	------	-0.167
PGI-2	-0.067	------	------	------

Table 5. Summary of F-statistics at 9 loci from four populations of *S. takesimensis*. F_{IS}, an index of inbreeding, F_{IT}, the overall inbreeding coefficient, and F_{ST}, a measure of the genetic differentiation among subpopulations.

Locus	F_{IS}	F_{IT}	F_{ST}
AAT-2	-0.026	-0.006	0.019
ADH-1	1.000	1.000	0.111
ME-1	1.000	1.000	0.029
PGI-1	0.557	0.646	0.201
PGI-2	-0.067	-0.016	0.048
Mean	0.588	0.653	0.158

Table 6. Mean values for Nei's (1978) unbiased genetic identity coefficient for pair-wise comparisons of four populations of *S. takesimensis* in Ulleung Island, Korea.

Population	Chusan	Sadong	Dodong	Pyungri
1 CHUSAN	------			
2 SADONG	0.985	------		
3 DODONG	0.998	0.980	------	
4 PYUNGRI	0.999	0.993	0.996	------

F statistics were used to measure the degree of genetic difference among four populations of *S. takesimensis*. The F_{IS} value indicates the degree of divergence from Hardy-Weinberg expectations within subpopulations. F_{IT} indicates the degree of divergence from Hardy-Weinberg expectation among total population. F_{ST} indicates allele frequency divergence among subpopulations (Allendorf and Luikart, 2007). Although few loci (AAT-2 and PGI-2) of *S. takesimensis* had negative F_{IS} and F_{IT} values, the mean F_{IS} and F_{IT} value of all nine loci of *S. takesimensis* studied were 0.588, which indicated an overall deficiency of heterozygote compared to the Hardy-Weinberg expectation. The mean F_{ST} value of nine loci of *S. takesimensis* was 0.158, higher than those for previous outcrossed species, but lower than the value for species with an explosive seed dispersal mechanism like *S. takesimensis* (Hamrick, 1989).

The Nei's unbiased genetic identity (Nei, 1978) showed that four populations studied were apparently close to each other in pairwise comparison. The genetic identity values ranged from 0.980 for the comparison between population Dodong and Sadong to 0.999 for the comparison between population Chusan and 4 Pyungri (Table 6). Further investigation showed that population Sadong, although still genetically close to other populations, was the farthest from them in terms of Nei's genetic identity values. A UPGMA phenogram based on genetic identity values was constructed to examine genetic relationships among four populations of *S. takesimensis* (Figure 1).

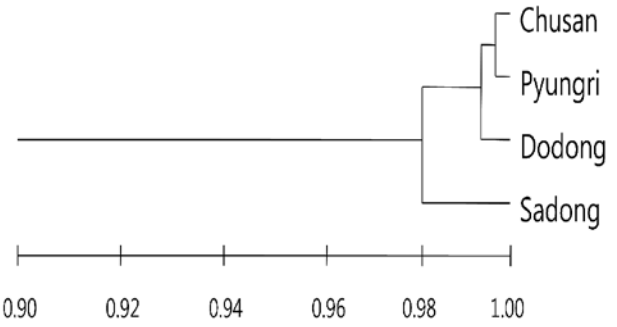

Figure 1. UPGMA phenogram derived from Nei's genetic identity of four populations of *S. takesimensis* in Ulleung Island, Korea.

DISCUSSION

Genetic variation is the most influential value in determining survival of a population or a species in both the long and short term. A decrease in heterozygosity results in reduced individual and population viability, and a drop in allelic diversity significantly undermines a species' ability to acclimate to changing selection (Huenneke, 1991). Hence, being fully aware of genetic diversity and its distribution is the first and foremost step in setting conservation plan for endangered species such as *S. takesimensis*.

According to much genetic diversity research, endemic island species generally have lower genetic variation than related continental species (Park and Jung, 2000). In the case of the Bonin Islands (Ito et al., 1998), for example, endemic species belonging to genus *Crepidiastrum*, *Pittosporum*, and *Symplocos* showed very low heterozygosity compared to continental species. Additionally, in the case of Hawaiian endemic species (Dejoode and Wendel, 1992), mean alleles per locus of 64 species were 1.32, and the percentage of heterozygotes of endemic island angiosperms was found to be 0.25. Because of a small founder population and a severe bottleneck effect and inbreeding, endemic island species display significantly low genetic variation (Ito et al., 1998).

Scrophularia takesimensis, an endangered endemic island species, shows very low genetic variation. In comparison with other island species such as Hawaiian and Bonin species, *S. takesimensis* has relatively low genetic variation. In the case of Hawaiian endemic species (*B. insignis* and *B. rockii*) (Gemmill et al., 1998), the mean number of alleles per locus was 1.3, and their percentage of polymorphic loci was 22.8%. Additionally, the expected heterozygosity was 0.040. However, *S. takesimensis* showed even lower genetic variation, with the mean number of alleles per locus being 1.1 and their percentage of polymorphic loci being 11.1% while the expected heterozygosity was only 0.028. The most probable reason for this aberrational result is the recent introduction of *S. takesimensis* to Ulleung Island. First of all, four populations of *S. takesimensis* had a low F_{ST} value, which indicates a close similarity between subpopulations. However, *S. takesimensis* also showed a large deficiency of heterozygotes, which directly indicates ongoing, intensive inbreeding, which must result in high differentiation among subpopulations (Table 5). These seemingly contradictory results of a low F_{ST} value and heterozygote deficiency can be explained by hypothesizing that although *S. takesimensis* is currently undergoing intensive inbreeding, it has not had much time yet to differentiate among subpopulations since arriving at Ulleung Island.

In addition, the fact that Ulleung Island is closer to the mainland than other islands such as Hawaii or Bonin adds support to the aforementioned hypothesis. The closer an island is to the mainland, the more likely it is that it will receive seeds or living individuals from there, which can possibly introduce a new allele to the insular gene pool, increasing the genetic variation as time passes by. These kinds of explanations were well supported in case of *Campanula takesimana* which is also a species endemic to Ulleung Island (Park and Jung, 2000). The relatively high genetic variation of the species suggested that it may be originate from multiful genetically polymorphic progenitor populations.

However, the *S. takesimensis* shows low genetic variation even though Ulleung Island is close to mainland Korea. This obviously indicates that there has not been enough time for mainland individuals to cross the East Sea and migrate there, which supports the short insular history of *S. takesimensis*. This could be also explained by postulating that *S. takesimensis* arrived at Ulleung Island by a single introduction from remote regions via long-distance dispersal rather than from the Korean mainland. One possible scenario is that *S. takesimensis* originated from Japanese endemic *S. grayanoides*, which has a limited distribution in the northeastern part of Honshu (Kamada et al., 2007). Recent molecular phylogenetic analysis using ITS data showed the sister-group relationship between *S. takesimensis* and *S. grayanoides* (M. Kim, unpublished data).

Another possible reason for the extraordinarily low genetic variation of *S. takesimensis* is fragmentation of population. Despite the already small number of individuals of *S. takesimensis,* they are divided into even smaller subpopulations throughout Ulleung Island. Since small populations caused by habitat fragmentation tend to inbreed and are affected by genetic drift, smaller subpopulations undergo even more intense inbreeding and genetic drift, resulting in a significant reduction in the number of heterozygotes, which ultimately lowers the genetic variation of *S. takesimensis* (Neel and Ellstrand, 2001).

To effectively conserve *S. takesimensis* and prevent future extinction, the population size should be increased to a certain amount in an effort to reduce their vulnerability to possible environmental changes. Furthermore, a larger

number of individuals means more room for genetic diversity since both the chance of mutation and the amount of gene flow among subpopulations increase. The genetically wisest choice of increasing total population is selecting the most genetically diverse subpopulation as the first target for artificial proliferation. In this way, genetic variation as well as population size could be increased since the most genetically diverse population holds the largest potential for genetic diversity. In the case of *S. takesimensis* on Ulleung Island, it is true that the population 3 (Dodong) would be the most genetically diverse, and deserve to be our first and foremost target for conservation, but the conservation of total populations would be the most effective plan, because the total population of *S. takesimensis* consisted of only four, and little genetic difference appeared among them.

Increasing the size and strategic distribution of those increased portions of the *S. takesimensis* population will not suffice. More should be taken into account. Currently, one of the main causes of low variation of *S. takesimensis* is its fragmentation into several subpopulations. This seriously accelerates inbreeding and worsens the low diversity situation. Therefore, newly established seedlings from *Ex situ* collections of *S. takesimensis* should be planted strategically in order to amend those fragmentations. For instance, a new subpopulation could be made between the original subpopulations so that the gap between them diminishes. The ultimate goal of this process would be constructing a continuous population of *S. takesimensis* within Ulleung Island.

Although not one of the genetically effective solutions, preventing commercial development in *S. takesimensis* habitats is also very important. Currently, road construction projects around the island are seriously endangering the seashore habitats of *S. takesimensis*. Also, exhaust from human activities is polluting other habitats such as mountain sides. These dangers should be eliminated beforehand in order to establish a firm foundation for the long-term conservation of *S. takesimensis*. Additionally, the establishment of new populations in suitable habitats should be also considered. In this case, intensive harvest of seeds from genetically variable natural population is required for an effective establishment (Brown and Briggs, 1991).

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韓國 Ulleung 島之瀕臨絕種 *Scrophularia takesimensis* (Scrophulariaceae) 的遺傳變異

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使用澱粉膠質電泳技術，我們研究了 Ulleung 島土生的瀕臨絕種之 *Scrophularia takesimensis* 的遺傳變異。所有族群均取樣以分析位於 9 個基因座之 8 種酵素的電泳式樣。整體之基因變異很低。各族群間很難找到基因之多型性，而且它之估計值遠小於其他島上品種。*S. takesimensis* 之正 F_{IS} 值指出差異接合子的整體缺乏性質，同時低 F_{ST} 值表示族群間極少分化。族群間之高度遺傳相同性顯示它們最近才形成，同時尚未進行顯著之分化。*S. takesimensis* 如此極端低的遺傳變異告訴我們要儘快地建立有效的保育措施。

關鍵詞：保育；遺傳變異；*Scrophularia takesimensis*。