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Conservation Genetics of Endangered *Brasenia schreberi* Based on RAPD and AFLP Markers

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***Brasenia schreberi* J.F. Gmelin is a declared endangered species found in the lakes and ponds of South Korea. For planning its conservation strategy, we examined the genetic diversity within and among six populations, using randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). Polymorphisms were more frequently detected per loci with AFLP (69.3%) than RAPD (36.8%). High genetic diversity was recognized within populations: polymorphic loci (*PPL*) values ranged from 36.3% in the CJM population to 74.5% in the GGT population, with a mean value of 47.8% based on AFLP markers. Great genetic differentiation (θ^B) was detected among the six populations (0.670 on RAPD and 0.196 on AFLP), and we calculated a low rate of gene flow (N_em), i.e., 0.116 on RAPD and 0.977 on AFLP. Furthermore, a Mantel test revealed that no correlation existed between genetic distances and geographical distances among the six local populations, based on RAPD or AFLP markers. These results are attributed to a number of factors, including an insufficient length of time for genetic diversity to be reduced following a natural decline in population size and isolation, adaptation of the genetic system to small population conditions, and a restricted gene flow rate. Based on both its genetic diversity and population structure, we suggest that a strategy for conserving and restoring *B. schreberi* must focus on maintaining historical processes, such as high levels of outbreeding, while monitoring increased gene flow among populations. This is because a reduction in genetic diversity as a result of genetic drift is undesirable.**

Keywords: AFLP, *Brasenia schreberi*, Conservation, Endangered species, Genetic diversity, RAPD

Studies on the conservation genetics of rare and endangered plant species are necessary when establishing management plans to preserve their biodiversity (Schneller and Holderegger, 1996; Zawko et al., 2001; Liu et al., 2007). Diversity and population structure are determined by several factors, such as mating system, gene flow, evolutionary history, and distribution pattern (Lesica et al., 1988; Lee et al., 2004; Dong et al., 2007; Koga et al., 2007; Liu et al., 2007; Lopez-Pujol et al., 2007). A species without an appropriate amount of genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites. Therefore, investigations within a species may not only illustrate the evolutionary process and mechanism but also provide information useful for biological conservation (Fischer et al., 2000; Tang et al., 2006; Chen et al., 2007).

Brasenia schreberi J.F. Gmelin, the single species within that genus, has wide but sporadic distribution in freshwater ponds and lakes in temperate and tropical regions of eastern Asia, Australia, Africa, West Indies, and the Americas (Raymond and Dansereau, 1953; Aston, 1977; Choi, 2007). Plants are characterized by floating, alternate, entire, and peltate leaves and by small, purple flowers. The thick and transparent mucilage covers all underwater organs, including the upper portions of stems, and the lower surfaces of leaves, petioles, and developing floral buds (Chrysler, 1938; Osborn and Schneider, 1988). This mucilage has phytotoxic properties and may function in the allopathic plant management of aquatic weeds (Elakovich and Wooten, 1987). *B. schreberi* combines sexual reproduction with vegetative propagation via rhizomes and winter buds (Chrysler, 1938; Osborn and Schneider, 1988). Based on its floral and pollen features, it also is anemophilous and protogynous, with individual flowers blooming for 2 d (Osborn and Schneider, 1988; Taylor and Osborn, 2006). Protogyny is widely thought to have evolved as a means for preventing self-pollination (Bertin, 1993; Griffin et al., 2000).

In Korea, *B. schreberi* is currently threatened with extinction due to a decline in the area covered by wetlands and deterioration in water quality that has resulted either directly or indirectly from human activities (Kim, 1996). It is listed as a critically endangered species (Lee et al., 2005). To support its conservation and management programs, knowledge about its genetic variability and structure in extant populations is necessary. However, such information has not been addressed previously.

Molecular markers (e.g., RAPD, AFLP, and ISSR) are now widely used to detect the genetic diversity and population structure of endangered species (Kang et al., 2005; Uehara et al., 2006; Koga et al., 2007; Zhang et al., 2007; Kim et al., 2008). Randomly amplified polymorphic DNA (RAPD) analysis is one of the most convenient methods for characterizing genetic polymorphism because it does not require probe DNA and details about genomic and population polymorphisms (Perez et al., 1998; Lee et al., 2006; Liu et al., 2007). Amplified fragment length polymorphism (AFLP) is another DNA fingerprinting technique that approaches the ideal as a marker system for resolving genetic diversity among individuals, populations, and species (Palacios and Gonzalez-Candelas, 1999; Archak et al., 2003; Sehgal and Raina, 2005). Hence, we performed RAPD and AFLP to evaluate 48 ramets of Korean *B. schreberi* from six locations to determine their population-level genetic diversity and differentiation. We also analyzed the genetic distances between populations to determine if they corresponded to geographical distances between locations.

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MATERIALS AND METHODS

Plant Materials and DNA Extraction

For RAPD and AFLP analyses, we collected 48 ramets of *Brasenia schreberi* from six locations in South Korea (Fig. 1). Distances between sites ranged from 4 to 550 km. Fresh leaves were randomly collected from each ramet (at least 5 m apart) to increase the possibility of detecting potential among individual variations. Areas were directly measured for each local population.

Total genomic DNA was extracted from fresh leaves by a rapid DNA minipreparation method (Chen and Ronald, 1999). It was re-suspended in Tris-EDTA buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)], and the DNA concentration of each sample was determined with a spectrophotometer (Geneflow Ltd., Staffordshire, UK).

RAPD Analysis

RAPD-polymerase chain reactions (PCR) were conducted in a volume of 25 μL, with final concentrations of 200 ng of template DNA; 100 μM each of the four dNTPs, 1.9 mM $MgCl_2$, 1× PCR reaction buffer (Solgent Co.), 1 unit of *Taq* DNA polymerase (Solgent Co.), and 10 pmole of decamer primer. PCR was performed in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA) with the protocol including 4 min of initial denaturation at 94°C; followed by 40 cycles of denaturation for 30 s at 94°C, 30 s of annealing at 37°C, and 2 min of extension at 72°C; then a final extension step of 10 min at 72°C. A total of 60 primers (Primers 1 to 20 of Kits A, O, and AF; Operon Technologies Inc., Alameda, CA, USA) were screened using 2 representatives from each of the 6 populations. To avoid biasing our estimates of polymorphism, the selection of primers for band-scoring depended only on the clarity and repeatability of the RAPD fragments, not on the level of polymorphism. In all, 18 primers that gave clear and reproducible fragment patterns were selected for our final analysis (Table 1).

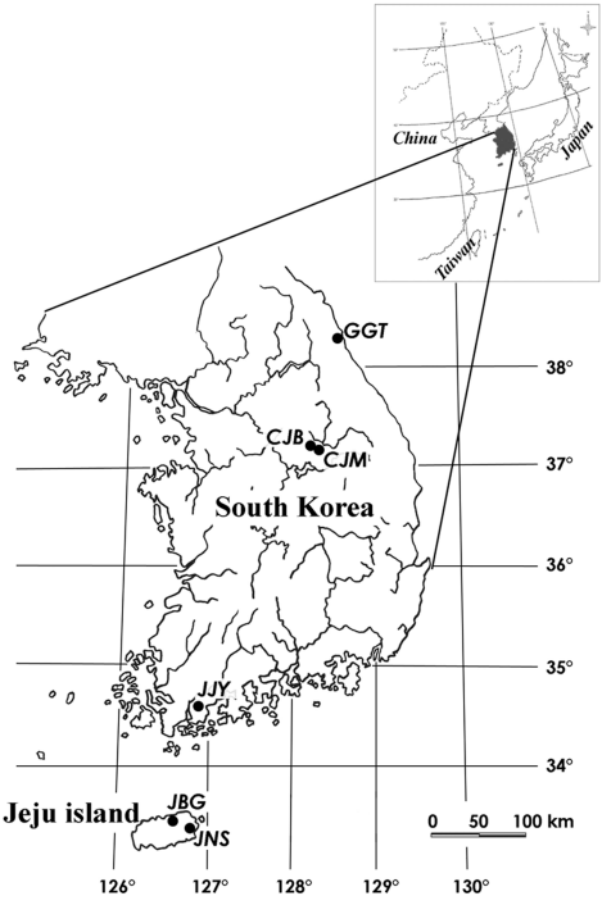

Figure 1. Locations of six populations of *Brasenia schreberi* in South Korea. GGT: Cheonjin lake, Toseong-myeon, Goseong-gun, Gangwon-do; CJB: Midang-ri, Bongyang-eup, Jecheon city, Chungcheongbuk-do; CJM: Mosan-dong, Jecheon city, Chungcheongbuk-do; JJY: Yuchi-myeon, Jangheung-gun, Jeollanam-do; JNS: Galme pond, Seongsan-eup, Namjeju-gun, Jeju-do; JBG: Dongbok-ri, Gujoa-eup, Bukjeju-gun, Jeju-do.

AFLP Analysis

AFLP analysis was performed essentially as described by Vos et al. (1995), but florescence technology was used for imaging the labeled DNA bands. Total genomic DNA (2 μg) was digested with 5 units of an *Eco* RI and *Mse* I endonuclease mixture in a total volume of 50 μL for 6 h at 37°C; digestion was checked by electorphoresis on a 1.2% agarose gel. The 10 μL of ligation mixture contained 5 pmole of *Eco* RI adaptor, 50 pmole of *Mse* I adaptor, 1× T4 DNA ligase buffer, and 1 unit of T4 DNA ligase (Promega Co.). This mixture was added to the digests in a total volume of 40 μL, and the ligation mixture was incubated overnight at 4°C in a cold chamber. The reaction primer pair *Eco* RI + A/*Mse* I + C was used, and amplification was carried out for 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 56°C), and extension (1 min at 72°C). A sample run on a 1.5% agarose gel showed a visible smear in the range of 100 to 1000 bp. The pre-amplification products were diluted 10-fold with distilled water as template for further selective amplification, which was performed with an *Eco* RI-primer tagged with a 6-FAM dye and *Mse* I-primer (see Table 1 for combinations). PCR conditions included one cycle at 94°C for 3 min; then 13 cycles of 94°C for 30 s, 65°C for 30 s (with a reducing ramp of 0.7°C per cycle), and 72°C for 1 min; followed by 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. Reaction products were fractionated with an ABI 3100 DNA sequencer (Applied Biosystems). The AFLP-GeneScan files were further scored with Genotyper 3.7 (Applied Biosystems).

Statistical Analysis

RAPD and AFLP bands were scored as '1' (present) or '0' (absent) in a binary matrix for each primer. Afterward, monomorphic bands across all individuals were discarded from further analysis (Keiper and McConchie, 2000). To estimate genetic diversity and population differentiation based on RAPD and AFLP markers, we used the following computer programs: TFGPA 1.3 (Miller, 1997) for percentages of polymorphic loci (*PPL* at the 0.99 level), and Nei's (1978) unbiased expected heterozygosity (H_E); and POPGENE 1.31 (Yeh et al., 1997) for Shannon and Weaver's (1949) index (I). We also took a Bayesian approach (see Holsinger et al.,

Table 1. Polymorphic bands generated by effective RAPD and AFLP primers in six populations of *Brasenia schreberi*.

NO.	Primer name	Total bands	Polymorphic bands	Polymorphism(%)
RAPD				
1	OPA9	5	2	40.0
2	OPA14	8	1	12.5
3	OPA16	6	3	50.0
4	OPA17	8	4	50.0
5	OPA19	9	4	44.4
6	OPAF1	7	5	71.4
7	OPAF2	6	1	16.7
8	OPAF4	4	1	25.0
9	OPAF8	7	1	14.3
10	OPAF10	7	2	28.6
11	OPAF14	12	6	50.0
12	OPAF16	9	2	22.2
13	OPAF18	9	2	22.2
14	OPAF19	9	5	55.6
15	OPO1	7	4	57.1
16	OPO7	9	2	22.2
17	OPO12	8	3	37.5
18	OPO15	7	3	42.9
	Mean	7.6	2.8	36.8
	Total	137	51	
AFLP				
1	ECO-CAAC / MSE-ACTG	107	67	62.6
2	ECO-CAAC / MSE-ACTC	109	79	72.5
3	ECO-CACT / MSE-ACTC	99	59	59.6
4	ECO-CATG / MSE-ACTG	145	114	78.6
5	ECO-CACC / MSE-ACTG	122	89	73.0
	Mean	116.4	81.6	69.3
	Total	582	408	

2002) to determine genetic diversity (*hs*, analogous to H_E) and population differentiation (θ^B), using Hickory 1.0.4 (Holsinger and Lewis, 2005). To ensure consistency of results (nBurnin = 50,000, nSamples = 250,000, thin = 50 in each run), we used five runs for each of three models (full model, f = 0 model, and f-free model). Model selection was based on the deviance information criterion (DIC) (Spiegelhalter et al., 2002). Although models with smaller DICs are preferred, a difference of >6 DIC units among models is required in selecting one model over another (Holsinger and Lewis, 2005).

We assessed the hierarchical genetic structure of a population on a Euclidean distance matrix, using WinAMOVA 1.55 (Excoffier et al., 1992; Excoffier, 1993). Input files for this program were generated by the program AMOVA-PREP (Miller, 1998). Significant levels of variance were obtained with tests that included 1000 permutations per analysis. We divided the six populations into two regions according to locations: 1) Mainland (GGT, CJB, CJM, and JJY), and 2) Jeju Island (JBG and JNS) (Fig. 1). Pair-wise genetic distances (Φst) among local populations and their levels of significance were also obtained by applying WinAMOVA 1.55.

To illustrate the genetic relationships between individuals within a single population and between populations, we analyzed the matrix of RAPD, AFLP, and combined RAPD and AFLP bands with neighbor-joining (NJ) based on pair-wise distances, according to PAUP 4.02 (Swofford, 1998).

Linear regression was used to assess the relationship between the amount of genetic variation (*PPL*, H_E, *hs*, and *I*) and population area (m^2), using SAS 8.0 (SAS Institute Inc., 1999). A Mantel test (Mantel, 1967) was conducted to identify any correlations between geographical distance and genetic distance (pair-wise Φst) in our six populations. Significance of that test was determined by TFGPA 1.3, with 5000 permutations (Miller, 1997). The amount of gene flow (N_em) among the six populations was estimated based on the mean value of pair-wise genetic distances. This involved the formula $N_em = (1/4)[(1/\Phi st)-1]$, where N_e is effective population size and m is migration rate (Wright, 1951).

RESULTS

RAPD and AFLP Polymorphisms

To assess the genetic variation and population structure of

Table 2. Population-genetics diversity within six local populations of Korean *Brasenia schreberi*, based on RAPD and AFLP markers.

Populations[a]	P_A	N	RAPD				AFLP			
			PPL	H_E	*hs*	*I*	*PPL*	H_E	*hs*	*I*
GGT	8,004	13	21.6	0.062	0.073	0.094	74.5	0.212	0.226	0.323
CJB	254	6	11.8	0.046	0.065	0.064	45.6	0.177	0.211	0.245
CJM	131	6	11.8	0.051	0.077	0.069	36.3	0.142	0.198	0.196
JJY	340	10	13.7	0.040	0.051	0.061	51.2	0.183	0.203	0.264
JNS	310	10	17.7	0.069	0.076	0.097	55.6	0.191	0.209	0.277
JBG	321	3	15.7	0.084	0.097	0.100	36.5	0.195	0.225	0.233
Mean		8.0	15.4	0.059	0.073	0.081	47.8	0.190	0.212	0.258

[a]Abbreviations for populations are indicated in Fig. 1; P_A, population area (m^2); *N*, sample size; *PPL*, percentage of polymorphic loci; H_E, Nei's (1978) unbiased expected heterozygosity; *hs*, genetic diversity using Bayesian approach; *I*, Shannon and Weaver's (1949) index.

Table 3. Population differentiations (θ^B) calculated by three models, using Bayesian approach.

Model	RAPD			AFLP		
	f[a]	θ^B	DIC[b]	f[a]	θ^B	DIC[b]
Full	0.870	0.670	273.2	0.972	0.196	5735.2
f=0	0.000	0.648	274.4	0.000	0.146	5804.3
Free	0.570	0.670	273.7	0.511	0.185	5825.7

[a]inbreeding index within populations; [b]Deviance information criterion

Table 4. Summary of Analysis of Molecular Variance (AMOVA), based on RAPD and AFLP markers for six local populations of *Brasenia schreberi* in South Korea.

Levels of variation	df	RAPD			AFLP		
		Variance component		P[a]	Variance component		P[a]
		Absolute	%		Absolute	%	
Two-hierarchical level							
Among populations	5	4.64	75.0	<0.001	9.67	20.8	<0.001
Within populations	42	1.55	25.0	<0.001	41.05	79.2	<0.001
Three-hierarchical level							
Between regions[b]	1	0.77	11.8	0.1578	2.28	4.3	0.074
Among populations	4	4.26	64.7	<0.001	9.67	18.2	<0.001
Within populations	42	1.55	23.5	<0.001	41.05	77.5	<0.001

[a]Levels of significance are based on 1000 interaction steps; [b]Two regions: mainland vs. Jeju island.

Table 5. Genetic distance matrix based on combined RAPD and AFLP markers, using pair-wise estimated values of Φst (RAPD / AFLP below diagonal) and geographical distances (above diagonal) between six local populations of *Brasenia schreberi* in South Korea.

Populations	GGT	CJB	CJM	JJY	JNS	JBG
GGT		126	125	418	563	550
CJB	0.782 / 0.257***	–	4	294	437	424
CJM	0.812 / 0.257***	0.788 / 0.347***	–	296	438	425
JJY	0.794 / 0.205***	0.456 / 0.069***	0.805 / 0.233***	–	151	136
JNS	0.788 / 0.212***	0.649 / 0.203***	0.775 / 0.241***	0.619 / 0.189***	–	18
JBG	0.780 / 0.209***	0.632 / 0.207***	0.765 / 0.244***	0.651 / 0.208***	0.140 / 0.031***	–

[a]Abbreviations for populations are indicated in Fig. 1. Statistic significance is based on 1000 permutations: ***, P < 0.001.

Brasenia schreberi in South Korea, we collected 48 ramets from six local populations. From our RAPD analysis, 18 decamer primers produced a total of 137 RAPD reproducible bands. Of these, 51 were polymorphic across all ramets, with the percentage of polymorphic bands for those primers ranging from 12.5% (OPA14) to 71.4% (OPAF1), and averaging 36.8%. The number of polymorphic fragments for each primer also varied, from 1 (OPA14, OPAF2, 4, and 8) to 6 (OPAF14), with an average of 2.8 fragments (Table 1). In our AFLP analysis, 5 primer combinations resulted in 582 amplification products, i.e., 116.4 bands per combination. Polymorphic products in those combinations ranged from 59 to 114, with an average of 81.6 per combination (Table 1).

Genetic Diversity within Populations

The genetic data for within-populations, based on RAPD and AFLP markers, is summarized in Table 2. With RAPD markers, the percentage of polymorphic loci (*PPL*) for the six populations ranged from 11.8% (CJB and CJM) to 21.6% (GGT), with a mean value of 15.4%. However, population JBG exhibited the highest heterozygosity in the other parameters (H_E = 0.084; *hs* = 0.097; *I* = 0.100). Mean values for H_E, *hs*, and *I* (Shannon and Weaver's [1949] index) across all populations were 0.059, 0.073, and 0.081, respectively. In the case of AFLP markers, the *PPL* percentages ranged from 36.3% (CJM) to 74.5% (GGT), with a mean value of 47.8%. Population GGT also exhibited the highest heterozygosity in the other parameters (H_E = 0.212; *hs* = 0.216; *I* = 0.323). Mean values for H_E, *hs*, and *I* across populations were 0.190, 0.212, and 0.258, respectively. Genetic diversity within populations was higher when analyzed by AFLP than by RAPD markers (Table 2). Population areas ranged in size from 131 m^2 (CJM) to 8004 m^2 (GGT) (Table 2). Although no significant correlations were detected between three parameters (H_E, *hs*, and *I*) and area, a highly significant correlation was detected between levels of genetic variation (*PPL* value) and population area (R^2 = 0.651, *P* = 0.05 on RAPD; R^2 = 0.719, *P* = 0.03 on AFLP).

Genetic Differentiation among Populations

We applied three different models of the Bayesian procedure to determine population differentiation (θ^B), based on RAPD and AFLP markers (Table 3). The smallest value for DIC (deviance information criterion) was obtained with the full model (DIC = 273.2 on RAPD and 5735.2 on AFLP) -- results that suggested only a small degree of inbreeding in populations (*f* = 0.8701 on RAPD and 0.9723 on AFLP). Among our six populations, values for θ^B were 0.670 on RAPD and 0.196 on AFLP.

The Analysis of Molecular Variance (AMOVA) is summarized in Table 4. In the first model, analyzed with regional grouping into the Korean mainland and Jeju Island, the total variation among groups was not significant (*P* = 0.1578 on RAPD and *P* = 0.074 on AFLP), implying that this model was inadequate. In contrast, the model that employed only two hierarchical levels showed that variance components among and within populations were highly significant (*P* < 0.001 on both RAPD and AFLP). Based on RAPD, 75.0% of the total genetic diversity was distributed among populations, with the remainder (25.0%) residing within populations. However, 79.2% of the total genetic variation was attributed to differentiation within populations, the rest to that among populations, based on our AFLP analysis.

Gene Flow and Correlation between Geographical and Genetic Distances

Pair-wise genetic distances (Φ*st*) based on RAPD and AFLP markers and the geographic distances between populations are shown in Table 5. All Φ*st* values for each comparison were highly significant (*P* < 0.001). The pair-wise genetic distance among populations, based on RAPD bands, ranged from 0.140 (between Populations JBG and JNG) to 0.812 (populations GGT and CJM), with a mean value of 0.682. For AFLP, the mean pair-wise genetic distance among populations was 0.204, with values of 0.031 (JBG and JNS) to 0.347 (CJM and CJB). No correlations were detected between genetic and geographical distances (Mantel's test: *r* = 0.136, *P* = 0.05 on RAPD; *r* = 0.005, *P* = 0.43 on AFLP), a finding that did not support the isolation by distances between populations (Fig. 2). The amount of gene flow (N_em) estimated among the six populations was 0.116 on RAPD and 0.977 on AFLP.

Genetic Relationships among Populations

A Mantel test revealed a significant correlation between the RAPD- and AFLP-based genetic distances among populations (*r* = 0.845, *P* = 0.006). Therefore, those two data sets were combined for cluster analysis. A relatively high level of pair-wise distance values generated by those combined sets was observed among our 48 ramets, with most distance

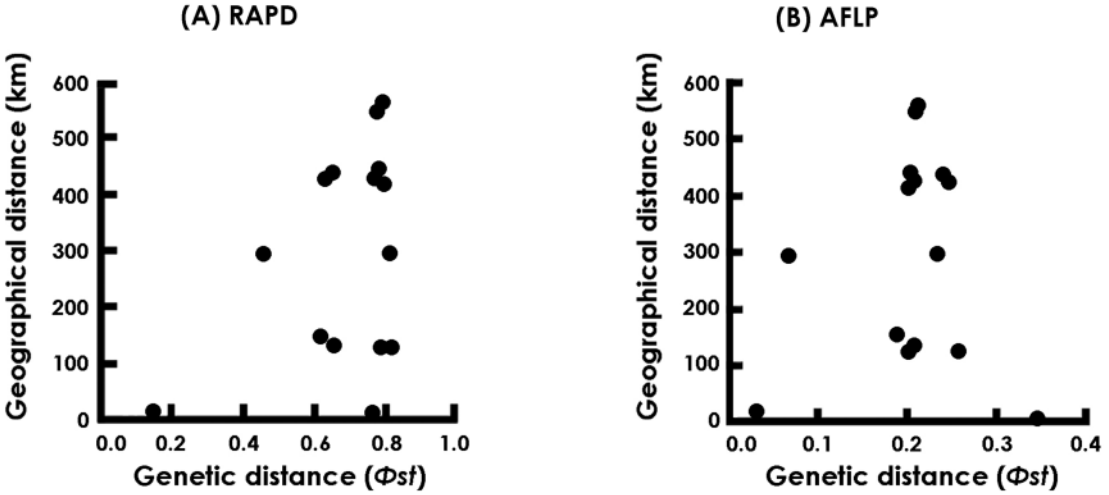

Figure 2. Relationship between pair-wise geographical and pair-wise genetic distances (Φ*st*) among six local populations of *Brasenia schreberi* based on (A) RAPD and (B) AFLP markers (see Table 5). No correlation was found between geographical distance (km) and genetic distance among populations when using Mantel test (*r* = 0.136, *P* = 0.05 on RAPD; *r* = 0.005, *P* = 0.43 on AFLP)

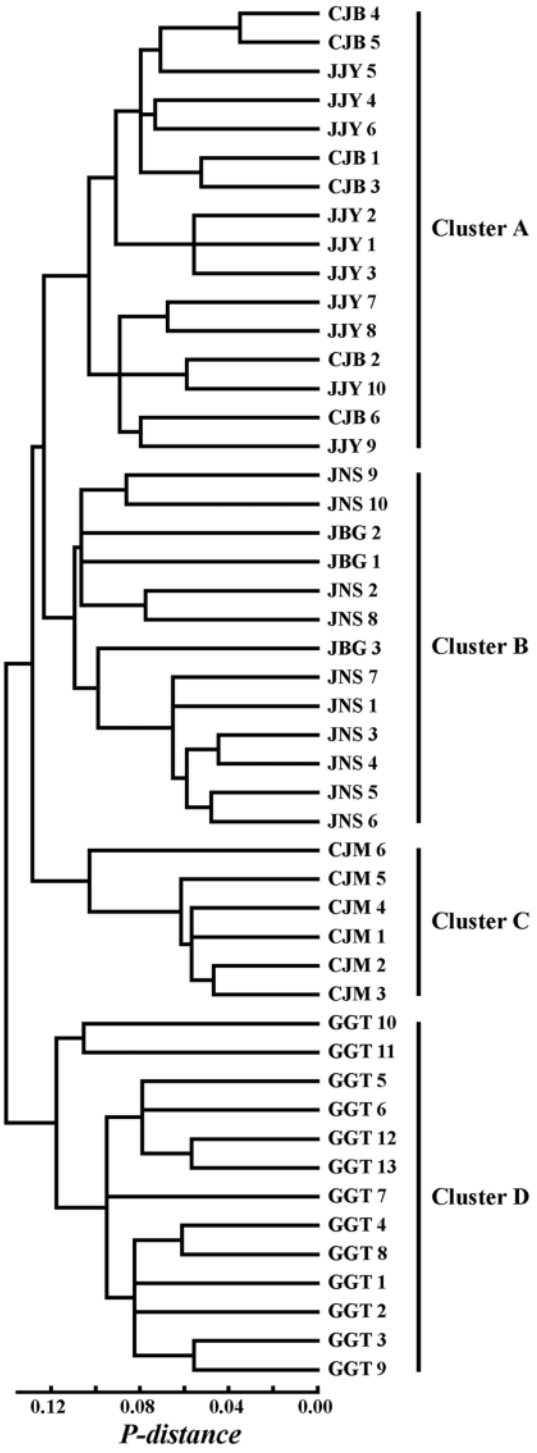

Figure 3. Neighbor-joining dendrogram for 48 ramets from six populations of *Brasenia schreberi* based on combined 459 RAPD and AFLP markers, using PAUP 4.02 (Swofford, 1998). The *x*-axis represents pair-wise genetic distance (*P-distance*). Dendrogram shows six populations being clustered into 4 clusters: A, comprises CJB and JJY populations; B, is constituted with individuals of two populations (JNS and JBG) from Jeju Island; C and D, are formed by CJM and GGT populations, respectively. Population abbreviations are given in Fig. 1.

measures falling between 0.070 and 0.440 (mean value = 0.241; data not shown). We constructed an NJ dendrogram on 48 ramets from the sis populations, based on 459 RAPD and AFLP markers with pair-wise genetic distances (Fig. 3). Four clusters were defined at a *p*-distance of ca. 0.12. Here, Cluster A comprised individuals from populations JJY and CJB. Cluster B contained populations JBG and JNS from Jeju Island. Finally, Clusters C and D were formed by the CJM and the GGT populations, respectively (Fig. 3).

DISCUSSION

Variation in RAPD and AFLP Markers from *Brasenia schreberi*

Polymorphic markers are important when planning recovery strategies and the *in situ* and *ex situ* conservation of endangered *Brasenia schreberi* (Palacios and Gonzalez-Candelas, 1999; Chen et al., 2008). The polymorphism that is obtained with RAPD and AFLP markers has several underlying causes at the molecular level, and thus may provide differing degrees in the analysis of genetic diversity among populations. Here, 81.6 polymorphisms were generated per primer in the case of AFLP; 2.8 with RAPD (Table 1). This indicates that polymorphisms were more frequently detected with AFLP than with RAPD. The former also detected polymorphism more frequently (69.3%) per locus than did the latter (36.8%). These results demonstrate the greater discriminating power of AFLP analysis, whose superiority has also been reported with other species (Palacios and Gonzalez-Candelas, 1999; Archak et al., 2003).

Genetic Diversity within Populations

We used RAPD and AFLP bands to determine genetic diversity within populations, and obtained the following data: *PPL* = 15.4%, H_E = 0.059, *hs* = 0.073, and *I* = 0.081 for RAPD versus *PPL* = 47.8%, H_E = 0.190, *hs* = 0.212, and *I* = 0.258 for AFLP. Based on our RAPD markers, the level of genetic diversity within *Brasenia* populations was similar to that found with other endangered aquatic plants. For example, Kim et al. (2008) have shown that diversity within the population of *Isoetes coreana* in South Korea has a mean *PPL* value of 15.5%, ranging from 3.4% to 33.9%. Using ISSR markers, Koga et al. (2007) have reported genetic variation for thirteen populations of *Ranunculus nipponicus* (*PPL* = 9.6% [0.0% – 34.0%]) while Chen et al. (2008) have found the percentage of polymorphism in Chinese populations of threatened aquatic plant *Ottelia alismoides* to be 13.0%, ranging from 2.3% to 18.5%. However, our *B. schreberi* showed low genetic diversity within populations when compared with aquatic plants that are not endangered, c.f., *Nelumbo nucifera* from China (*PPL* = 50.4% for wild and 54.0 for cultivated; Xue et al., 2006) and *Potamogeton maakianus* in the Yangtze River, China (*PPL* = 24.2% and *I* = 0.15; Li et al., 2004), based on RAPD markers.

Using AFLP markers, we determined that *B. schreberi* populations had similar or slightly higher levels of genetic diversity compared with that of other critically endangered plants. Kang et al. (2005) have reported the intra-population diversity for seven populations of *Isoetes sinensis*, an endangered aquatic species in China, with a mean *PPL* value of 35.2%, but ranging from 20.0% to 53.8%. Tero et al. (2003)

have calculated that the genetic diversity for a predominantly outcrossing plant, *Silene tatarica*, is 25.9% to 54.9% (*PPL*) and 0.075 to 0.176 (H_E, Nei's estimate). However, for *Myricaria laxiflora*, which has a mixed reproduction system of insect-outcrossing pollination, selfing, and vegetative clonal propagation in the Yangtze River, China, its *PPL* values reveal relatively lower genetic diversity, from 12.6% to 27.24% (Liu et al., 2006). Therefore, our data the present results of that show a high level of genetic variation for *Brasenia* can most likely be attributed to its breeding system. *B. schreberi* is a predominantly outcrossing species (Osborn and Schneider, 1988; Taylor and Osborn, 2006), a group that commonly has greater genetic diversity than do selfing plants (Lesica et al., 1988; Zawko et al., 2001).

Among our six populations, that from GGT maintained higher genetic diversity than the others, based on AFLP markers. In contrast, population CJM had the lowest when all genetic variation was estimated. The level of that variation within populations should be related to their area of coverage. That is, a larger population area would tend to harbor more variation. However, except for *PPL* values, we detected no significant correlation between area and the three parameters of H_E, *hs*, and *I*. This suggests that migration of propagules within populations was related to the spatial scale of its isolated wetland habitat (Kang et al., 2005).

Genetic Differentiation among Populations

In this study, θ^B values indicated great genetic differentiation among the six populations (0.670 on RAPD and 0.196 on AFLP). An *Fst* (analogous to θ^B) value >0.15 also means that genetic differentiation is high among populations (Hartl and Clark, 1997). This has been noted for several other endangered aquatic species. For example, using 59 RAPD markers, Kim et al. (2008) have reported a high estimate (θ^B = 0.742) among seven populations of *I. coreana* in South Korea. Similarly, a high value for *Fst* (0.326) has been calculated among populations of the rare *Helmholtzia glaberrima* when AFLP markers are used (Prentis and Mather, 2008). In contrast, Xue et al. (2006) have found via RAPD markers that genetic differentiation is relatively low (*Gst* = 0.131) for *Nelumbo nucifera*, a non-endangered aquatic species. High differentiation is attributed to low inter-population gene flow and genetic drift (Loveless and Hamrick, 1984). Generally, N_em values greater than 1 imply that gene flow between populations is at a sufficient level to counterbalance genetic drift, whereas values less than 1 mean that genetic drift will result in substantial differentiation (Slatkin, 1987). Our study demonstrated a lower level of inter-population gene flow (N_em = 0.116 on RAPD and 0.977 on AFLP) in the remaining populations of *B. schreberi* when compared with other submerged or floating-leaved aquatic species, including *Zostera japonica* (N_em = 0.26 on RAPD), *Thalassia testudinum* (N_em = 1.7 on AFLP), and *Nelumbo nucifera* (N_em = 3.32 on RAPD) (Waycott and Barnes, 2001; Lee et al., 2004; Xue et al., 2006). Our present data indicating that *Brasenia* displays a high degree of genetic variation and population differentiation can most likely be attributed to several factors, e.g., insufficient length of time for genetic diversity to be reduced following a natural decline in population size and isolation; adaptation of the genetic system to conditions of a small population; and restricted gene flow (Loveless and Hamrick, 1984; Rossetto et al., 1995).

Conservation Implications

The level of genetic variation and population structure found in our *B. schreberi* populations provides an important guideline toward the conservation of this species. Its high genetic diversity indicates that it may be capable of adapting to changes in its environment. However, extant *Brasenia* populations have faced a declined in the number of individuals due to human activity as well as intense competition from other hydrophyte species (Kim, 1996). Therefore, it is urgent that we pay more attention to the diminishing size of its populations. Because we found a significant correlation between levels of genetic variation (*PPL*) and population area (see Table 2), larger extant population would be more effective for conserving its genetic resources. Protecting all remaining individuals of an endangered *B. schreberi* is frequently not possible, and decisions about which populations being preserved must be made. Considering the population genetic diversity and area of coverage, *in situ* conservation is suitable for populations GGT on the mainland and JBG on Jeju Island (Table 2). Moreover, it is important to restore as many individuals as possible propagated *in vitro* from seeds. On-going efforts must continue for producing *Brasenia* plants via tissue culture; artificial germination of seeds in the laboratory has already met with considerable success (Oh et al., 2008). Our study demonstrates that the distributional pattern of this aquatic plant is random regardless of the distances between populations (Fig. 2). However, based on RAPD and AFLP analyses, those six populations clustered into four groups (Fig. 3), with each keeping its own genetic identity. Therefore, any plan for conservation management should be carefully designed to avoid the potential risk for outbreeding depression that can result from the admixture of individuals from different regions.

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