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Genetic Diversity and Its Implications in the Conservation of Endangered *Zostera japonica* in Korea

Sunhwa Lee[1], Sunmi Ma[1], Yongseok Lim[1], Hong-Keun Choi[2], and Hyunchur Shin[1]*
[1]Department of Biology, Soonchunhyang University, Chungnam 336-745, Korea
[2]Division of Natural Sciences, Ajou University, Gyeonggi 443-749, Korea

As part of the on-going effort to conserve endangered *Zostera japonica* Ascher. & Graebn. in Korea, we have used RAPD band patterns to analyze its genetic structure and diversity. Out of 50 primers tested, 45 formed amplified bands with its genome, including 814 polymorphic and 28 monomorphic bands. The highest number (120) was found in the population of Geoje-do; the smallest (58), in Anmyeon-do. An examination of its genetic structure with AMOVA revealed that about 50% of all variations could equally be assigned to within and between populations. The statistical value G_{ST} (index of genetic differences) was 0.49, and the average number of individuals exchanged between populations per generation (N_em) was calculated as 0.26. Although the habitats of *Z. japonica* in Korea are disappearing at an alarming rate, significant levels of genetic variation still exist, especially in the Geoje-do population. Therefore, any recovery strategy for this endangered species should be planned on the basis of this genetic diversity among populations.

Keywords: AMOVA, endangered species, RAPD, seagrass, *Zostera japonica*

Z. japonica Ascherson and Graebner is a marine angiosperm that inhabits sandy or muddy sea-beds in the photic zones (1 to 3 m deep) of broadly sheltered bays (Miki, 1933). Its distribution stretches from the subtropical seacoast of eastern Asia to Vietnam and Sakhalin, Russia (Shin and Choi, 1998). The subgenus *Zosterella* consists of about 10 species (Hartog, 1970), of which *Z. japonica* is primarily distributed in Korea and Japan (Shin and Choi, 1998). This species has also been introduced to the west coast of North America (Harrison, 1976). One of its diagnostic features is an open-sheathed and relatively narrow leaf blade with three veins.

Marine angiosperms, including *Z. japonica*, play a pivotal role in the estuarine ecosystem, not only because they provide food and shelter for diverse organisms, but also because they stabilize coastlines and the structure of the food web in seacoast communities (Larkum et al., 1989). In particular, the canopy of *Z. japonica* significantly affects the composition and abundance of associated fauna as well as the amount of detritus accumulated on sediment surfaces (Lee et al., 2001). Its pattern of growth also influences the chemical characteristics of sediment in the coastal ecosystems (unpublished data).

*Corresponding author; fax +82-41-530-1256
e-mail shinhy@sch.ac.kr

Unfortunately, the habitats of marine angiosperms have declined worldwide due to urban, tourist, industrial, and economic pressures (Christine and Pergent, 1996). Because the habitats of *Z. japonica* are generally so shallow, those areas have easily been reclaimed and heavily impacted by human activity. For example, a population of this species once coexisted with *Z. marina* at Hansilpo, Tong-yeong city, Gyeongnam, Korea (Kong, 1984), but both species have completely disappeared due to land reclamation. This has also occurred with a population at Anmyeon-do Island, at Chungnam, Korea, while plants have barely escaped the same fate and now grow only in the waterways between salt fields.

These environmental situations have made *Z. japonica* an endangered species in Hong Kong (Lee, 1997), Korea, and Japan; some Japanese provinces have included this species in the "Data Deficient" category in their Red Data Book (Anonymous, 2001a, b), and another listing can be found in the "Lower Risk" in Korea (Anonymous, 2001c). A rising sense of urgency means that, unless immediate and forceful countermeasures are taken, *Z. japonica* will soon disappear from its natural habits along the Korean coastlines. Therefore, understanding the genetic diversity of this species constitutes an integral part of conservation programs required for its long-term survival (Kirsten et al., 1998; Bouza et al., 2002).

To this end, we have used RAPD markers in this study and have estimated genetic diversity in terms of the amount and structure of Z. *japonica* populations on the Korean peninsula. The RAPD markers utilized here are the same as those for research on the population genetics of two other seagrass genera, *Posidonia* and *Thalassia* (Waycott, 1995; Kirsten et al., 1998; Jover et al., 2003).

MATERIALS AND METHODS

Plant Materials

Plants of *Z. japonica* were sampled between September 2002 and October 2003 from three different populations in Korea -- Hwajinpo, Anmyeon-do Island, and Geoje-do Island (Fig. 1, Table 1). At each location, the population was divided into five widely separated 30- x 30-cm quadrats. After all the plants were collected from each quadrat, the number of shoots and shoot lengths were recorded, and their DNA was extracted for RAPD analyses. To investigate changes in genetic diversity caused by disturbances at Hwajinpo, individual specimens were gathered in 2002 before and after repairs were made on a local bridge.

DNA Isolation and RAPD Analysis

For each RAPD analysis, one plant per quadrat was chosen and transported to the laboratory on ice in a plastic bag. There, the leaf tissues were washed with distilled water and the epiphytes were removed. Samples were then wrapped in aluminum foil and kept frozen at -65°C. Total DNA was extracted according to the CTAB method (Doyle and Doyle, 1987), then measured with a Biotech photometer and agarose gel electrophoresis, and stored at 4°C. DNA amplifications were carried out using a GeneAmp PCR system 9700, following the method of Williams et al. (1990). Sixty primers (OPA 1 through 20, OPB 1 through 20, and OPAF 1 through 20) were

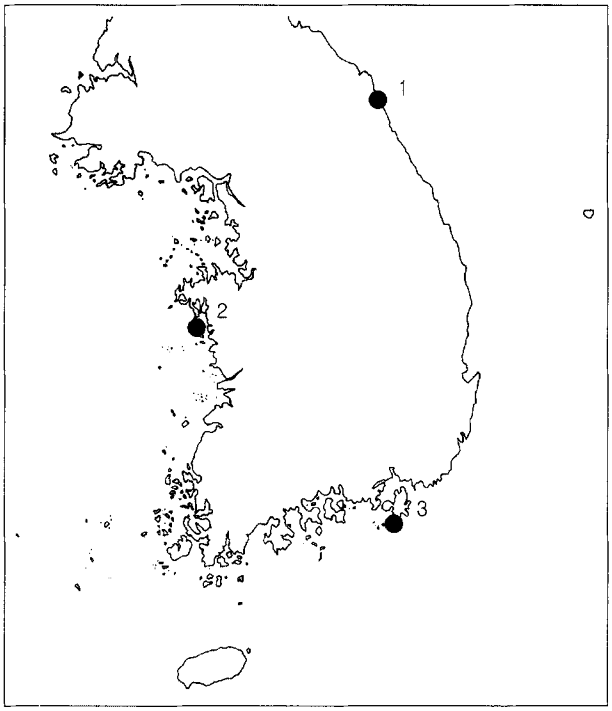

Figure 1. Populations of *Z. japonica* in Korea. 1: Hwajinpo, 2: Anmyeon-do Island, 3: Geoje-do Island.

purchased from Operon Technologies, and the amplification products were separated on a 1.4% agarose gel. The resulting RAPD bands were visualized with ethidium bromide and photographed over ultraviolet light.

Data Analysis

Bands were scored manually on the photographs and confirmed with stained gels. Polymorphic amplification products were scored with a 1 when present and a 0 when absent; monomorphic and ambiguous bands were excluded. The genetic diversity and structure were also assessed by Arlequin ver. 2.000 (Stefan et al., 2000), an adapted analysis of molecular variance (AMOVA) analysis (Stewart and Excoffier, 1996).

Table 1. Collection sites for *Z. japonica* to analyze genetic diversity.

Population	Locality	Collection date	Remark
Anmyeon Isl.	Chungnam, Taean-gun, Anmyeon-eup, Jungjang-ri	Jun 7, 2003	N 36° 29' 17.7" E 126° 22' 44.7"
Geoje Isl.	Gyengnam Geoje city, Nambu-myeon, Dadai-ri	Sep 29, 2003	N 34° 43' 57.8" E 128° 37' 55.4"
Hwajinpo	Kwangwon, Kosung-gun, Hyunnae-myeon, Chodo-1-ri	Jun 9, 2003 Sep 7, 2002	N 38° 28' 40.4" E 128° 26' 20.6"

RESULTS

Population Size Variations

The average number of shoots per quadrat ranged from 42.8 at Hwajinpo to 259.0 at Geoje-do Island in 2003 (Table 2). Populations had declined between 2002 and 2003 at Hwajinpo and at Geoje-do Island from 1997 to 1998 (data not shown). Shoots tended to be longer when the plants were constantly submerged in seawater, as was the case at Anmyeon Island and Hwajinpo, while shoots were shorter when plants were intermittently exposed to air and sunlight. The latter situation was typical at Geoje Island, where the population was found in the upper tidal regions.

RAPD Profile and Genetic Diversity

Of the 60 primers tested, 45 generated 212 amplified bands, for an average of 4.7 per primer. Among them, 184 bands (~87%) were polymorphic (Table 3). The number of polymorphic bands varied from 1 (OPAF-1 and 20) to 20 (OPA-4 and 15, and OPB-16), with an average of 4.1 polymorphic bands per primer. The Geoje-do population produced 120 polymorphic bands, whereas the smallest number, 58, was identified in samples from the Anmyeon-do population (Table 3). In the case of the Hwajinpo plants, 91 polymorphic bands were found in the 2002 population, but only 70 in 2003 (Table 4). Using AMOVA (Arlequin ver. 2.000), we estimated a mean genetic diversity of 0.193 for the overall *Z. japonica* population in Korea; the highest was in the Geoje-do Isl. population at 0.278, the lowest, 0.135, from Anmyeon-do Island.

Table 2. Lengths and number of shoots per quadrat.

Population		Number of shoot per quadrat*	Shoot length
Anmyeon		143.8±84.7**	31.0±5.3
Geoje		259.0±53.1	10.9±2.1
Hwajinpo	2003	42.8±26.7	26.0±4.1
	2002	65.4±26.7	19.5±3.6

*quadrat size, 30 x 30 cm; **mean±SD (n=5).

Genetic Structure of *Z. japonica*

When all three populations were grouped together, approximately 51% of the total genetic variation was found within populations, and 49% between populations (Table 5). The G_{ST} index, which describes that genetic difference among populations, was 0.49, and the average number of individuals exchanged between populations per generation (N_em) was 0.26. The average genetic distance among populations was 0.46, but that value was longest between Anmyeon-do Island and Hwajinpo, at 0.63, and closest between

Table 3. Percentage of polymorphic RAPD bands and Nei's gene diversity within *Z. japonica*.

Populations	Number of individuals	Number of polymorphic bands		Genetic diversity*
		Number	%	
Anmyeon	5	58	31.5	0.135±0.084
Hwajinpo	5	70	38.0	0.166±0.102
Geoje	5	120	65.2	0.278±0.170
Total	15	184	100.0	0.193**

*Genetic diversity was estimated using Arlequin ver. 2.000, **mean value.

Table 4. Comparison of genetic diversity and number of individuals in Hwajinpo population between 2002 and 2003.

Year	Number of polymorphic bands	Total number of bands	% of polymorphic bands	Genetic diversity	Average number of shoots
2002	91	168	54.2	0.187±0.114	65.4±26.7
2003	70	184	38.0	0.166±0.102	42.8±26.7

Table 5. Genetic (below diagonal) and geographic (above diagonal) distances among three populations of *Z. japonica* in Korea.

	Anmyeon	Geoje	Hwajinpo
Anmyeon	*	$300^1(500^2)$	300(900)
Geoje	0.54	*	400(450)
Hwajinpo	0.63	0.21	*

Unit: Km; 1: shortest distance across the land; 2: distance along coastline.

Hwajinpo and Geoje-do Island, at 0.21 (Table 5). In terms of the physical coastline, the farthest geographic distance was between the Anmyeon-do Island and Hwajinpo populations, a fact that seemed also to be reflected in genetic distance (Fig. 1).

Annual Changes in Genetic Diversity for the Hwajinpo Population

In 2002, the average number of shoots per quadrat was 65.4 in the Hwajinpo population. However, after that habitat was disturbed by bridge repairs, the count decreased within one year to 42.9. This trend was also noted in the number of polymorphic bands, the ratio of polymorphic to total bands, and the AMOVA value for genetic diversity (Table 4).

DISCUSSION

Reductions in *Zostera* Population Numbers

In Korea, the distribution of *Z. japonica* was once thought to be limited to the area near Sea-Diamond, Nambu-myeon, and Geoje city, in the Province of Kyungnam. However, further studies have revealed several other habitats in the southern coastal area, including Tong-yeong city, Kyungnam (Kong, 1984) and in the eastern coastal region, namely Hainam Gun, Chunnam (Shin and Choi, 1998) and Hwajinpo, Kwangwon (Kim and Lee, 2000). While conducting this research, we also found another population at Anmyeon, Chungnam, in the western coastal region, which demonstrates that *Z. japonica* can be located throughout the coastline of the Korean peninsula. Unfortunately, the population that once grew at Tong-yeong has now completely disappeared due to reclamation, so that in our surveys we were able to find this species only at our three study sites.

Threatening Factors in Three Populations

Z. japonica plants grow in the intertidal region of the Korean seacoast, and along many parts of the coastline of Southeast Asia. These habitats are under severe stress, such as from increased sedimentation, which is one common cause for the extinction of seagrass populations (Lee, 1997). Other impacts to these environments include an increase in human activities along the coast, e.g., the discharge of urban and industrial waste, construction projects, beach regeneration, illegal fishing techniques (Moreno et al., 2001), and isolation of the remaining seagrass populations (Angel, 2002).

Korean populations of *Z. japonica* are also affected by these factors. As a result, plants of that species at Anmyeon-do Island now exist only in waterways that supply seawater to salt farms. If frequent digging of the waterway continues, there is the distinct possibility that *Z. japonica* could soon be extinct in that region. In fact, when we visited the site in January 2003, we could find no plants and, upon our return in June of that year, we found a population growing only in very crowded conditions. In the Hwajinpo area, a group of *Z. japonica* plants was living in an entry lagoon near the bridge. However, following repairs to that structure, the population was seriously influenced by increased turbidity, a smaller habitat area, and isolation from the rest of the ecosystem that existed before the repairs were begun.

The habitat of the Geoje-do population spreads over an area of about one hectare, possibly the largest known in Korea. However, its future is threatened by two factors -- drainage of untreated sewage and the construction of a breakwater that interferes with free flow of the currents. That breakwater localizes a variety of pollutants near the *Zostera* plants and damages water quality. Unfortunately, this situation could reduce the size of the population, if not kill it off entirely.

Genetic Diversity within *Zostera* Populations

Genetic variations in aquatic angiosperms are generally considered low (Laushman, 1993) for a variety of reasons, such as the uniform nature of their habitats, a high frequency of asexual reproduction, and long-distance dispersal of seeds or shoots (Santamaria, 2002). However, several RAPD studies have revealed much higher levels of genetic diversity (Table 6). For example, based on the proportion of polymorphic to total bands for three Korean populations of *Z. japonica*, their diversities were 27% at Anmyeon-do Island, 33% at Hwajinpo, and 57% at Geoje-do Island. Nevertheless, these values are lower than those of other seagrasses and some hydrophytes in mangroves (Table 6). In the case of *Aldrovanda vesiculosa*, an endangered hydrophyte, genetic diversity is 37.1% (Martin et al., 2003). Such results further demonstrate that *Zostera* populations in Korea face an uncertain future.

Until now, clonal organisms with facultative sexual reproduction, such as the seagrasses, were believed capable of maintaining higher levels of genetic variability than that associated with either obligate sexual

Table 6. Number of polymorphic RAPD bands and their percentages in various hydrophytes.

Taxa	Total no. of bands	No. of polymorphic bands	% of polymorphic bands	Reference	Remark
Halodule wrightii	80	58	72.5	Angel, 2002	Seagrass
Thalassia testudinum	?	?	80	Kirsten et al., 1998	Seagrass
Posidonia australis	?	?	54	Waycott, 1995	Seagrass
Aldrovanda vesiculosa	213	79	37.1	Martin et al., 2003	Endangered species
Potamogeton pectinatus	164	99	60.4	Mader et al., 1998	Submerged
"	87	65	74.7	Hangelbroek et al., 2002	"
Bruguiera gymnorhiza	29	21	72.4	Abeysinghe et al., 2000	Mangrove
Bruguiera sexangula	29	14	48.3	"	"
Hydrilla verticillata	121	91	75.2	Hofstra et al., 2000	Invasive plant

or clonal species (Kersten et al., 1998). Indeed, in the past, greater diversity has been reported in *Z. marina*, *Posidonia australis*, and *Thalassia testudinum* (Kirsten et al., 1998). However, at the same time, a low amount of clonal genetic diversity has been noted in the less structured and youngest prairies of *P. oceanica*, perhaps because of the pioneering effect of colonizing new habitats, the existence of young populations (Jover et al., 2003), or the nature of their reproduction system (Kirsten et al., 1998).

Although records for the natural existence of *Zostera* in Korea were unclear to us, its populations are not very young, except for the plants at Anmyeon-do Island, which appear to have arrived after the construction of the salt fields and are now affected by frequent digging in the waterways. That man-made influence may be keeping the diversity of that particular population especially low. Because *Z. japonica* is monoecious and equivalent to *Z. marina* (a species that shows high diversity), the decrease in values for the former may be due to recent isolation mechanisms that block gene flow between populations, all of which are initiated by human activities, e.g., reclamation and water pollution.

Population Size and Genetic Diversity

In general, small populations tend to maintain low genetic diversity, i.e., size is positively correlated with the level of diversity (Jung et al., 2003), primarily due to random genetic drift over generations (Hartl and Clark, 1997). However, *Z. japonica* in Korea did not reflect this trend, with the population of Anmyeon-do Island being larger than that of Hwajinpo (Table 2), but its genetic diversity, as estimated by RAPD, being lower (Table 4). A similar lack of positive correlation has been reported with other endangered species (Maki et al., 1996; Maki, 2003), possibly resulting from fluctuations in size due to frequent extinctions and re-colonization (Maki, 2003). For example, plants at Anmyeon-do Island often disappeared and were re-colonized during the process of digging the waterway. In contrast, the Hwajinpo population showed the expected positive correlation between size and diversity; there, the average number of shoots in 2002 was 65.4, but only 42.8 in 2003, while the number of polymorphic bands decreased from 91 to 70 in those same years. This suggests that genetic diversity among Anmyeon-do Island plants was mainly affected by fluctuations in suitable habitat, whereas those at Hwajinpo were influenced by the population size itself.

Genetic Diversity among Populations

Our analysis of molecular variance (AMOVA) showed that the genetic variation within populations was 51%, which is somewhat higher than reports of 40.2% for *Zostera caespitosa* (Tanaka et al., 2002) and 41% for *Halodule wrightii* (Angel, 2002), but lower than the 82% recorded for *T. testudinum* (Kirsten et al., 1998). Such high diversity is a consequence of outbreeding (Bouza et al., 2002), whereas lower values denote a highly structured species (Angel, 2002). In addition, our research showed that the average number of individuals exchanged between populations per generation (N_em) was relatively low, which suggests that populations of *Z. japonica* are not fully differentiated from each other and that gene flow between them is steadily restricted due to the loss of habitat from human activities.

Generally, an F_{ST} value >0.25 indicates very great genetic differentiation among populations (Hartl and Clark, 1997). RAPD-based G_{ST} values, which are direct estimates of F_{ST} (Excoffier et al., 1992), are now available for 35 plant species; an average value of 0.16 represents outbreeding and 0.60, inbreeding (Bussell, 1999). Based on that index, the population of *Z. japonica* (G_{ST}=0.49) would be considered an inbreeding species, with gene flow between populations seemingly restricted and differentiation promoted within populations. Alberte et al. (1994) have also reported that gene flow can be limited between populations of seagrasses separated by as little as 30 km. Likewise, strong gene flow exists between populations of *Z. marina* because of seed dispersal (Ruckelshaus, 1996, 1998), an action that may be very restricted in *Z. japonica*. Nevertheless, the precise reason for low gene flow in the latter species is unclear, except for the fact that plants growing in polluted estuaries do not produce any flowering shoots (Miki, 1933).

Conservation Implications for *Z. japonica* in Korea

Conservation biology requires that plans be made for protecting a particular species before its habitat disappears and its genetic variation is disrupted. Such an endeavor also requires an understanding of genetic differences and population structures to sustain current levels of genetic diversity (Angel, 2002). Quantifying diversity is a useful tool for optimizing sampling strategies aimed at conserving and managing genetic resources (Bouza et al., 2002). Although RAPD analysis data must be interpreted with caution (Stewart and Excoffier, 1996; Lynch and Milligan, 1994; Jover et al., 2003; Maki, 2003), especially with regard to its reproducibility and dominance, our results have demonstrated that *Z. japonica* populations in Korea face serious threats.

Generally, populations with high genetic diversity could be used for restoring genetically poor populations (Jung et al., 2003). Despite the enormous losses of *Zostera* in Korea, a significant level of genetic variation still exists, especially in the Geoje-do Island populations. Therefore, restoration plans should be put in place, without hesitation, using that population. In addition, many lagoons on the eastern coast, in which seagrasses grow in seriously polluted water, should have conservation programs for maintaining the genetic diversity of the *Z. japonica* population. Improving the water quality is also urgently needed. Finally, to design the most appropriate and effective conservation and environmental management strategies for that species, further research is required on its population genetics and reproductive biology.

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