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Gene flow among populations of *Zostera caespitosa* Miki (Zosteraceae) in Sanriku Coast, Japan

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The genetic variance and genetic similarity within and among populations of *Zostera caespitosa* Miki (Zosteraceae) in Sanriku Coast, Japan, were detected using RAPD variation, and the gene flow among populations and among bays was estimated. Analysis of molecular variance (AMOVA) showed that 40% of the genetic variation was within populations and 60% was among populations using all populations, and that 45% of the variation was among populations when using only two bays that are located close to each other. These values show that the gene flow among populations of *Z. caespitosa* is restricted, and that the populations are in a state in which differentiation is promoted compared with other seed plants, including *Z. marina*. A UPGMA dendrogram of populations based on Euclidian distances calculated from RAPD variation showed that the populations of Otsuchi Bay and Yamada Bay did not form separate clusters. In addition, AMOVA analysis showed that the genetic variation between Otsuchi Bay and Yamada Bay was very small (3.5%). These results reveal that the modes of gene flow within Otsuchi and Yamada Bays differ.

Key words: gene flow, genetic variation, RAPD, seagrass, *Zostera caespitosa*

INTRODUCTION

In seagrasses (marine angiosperms), pollen and seeds are dispersed on the water surface or in the water (Den Hartog 1970). Therefore, gene flow among populations is probably influenced by environmental parameters, such as the movement of water (sea currents and so on), that differ from those affecting terrestrial plants. In an allozyme analysis of *Posidonia australis* on the South Coast of Australia, Waycott et al. (1997) reported a geographical gradient, with more closely located populations clustering with each other. However, an allozyme analysis of *Thalassia testudinum* by Schlueter and Guttman (1998) found no genetic relationships among distant populations, while closely located populations were related to each other. In Zosteraceae species, several studies have found no relationship between geographic and genetic distance (Alberte et al. 1994, Ruckelshaus 1998). In addition, Alberte et al. (1994) showed that gene flow between populations separated by as little as 30 km is limited. Since the gene flow of seagrasses could not be characterized in previous studies, the nature of gene flow likely differs according to each species and its environment; therefore, further data on other species and regions are needed.

Many seagrasses develop creeping rhizomes in all directions and form dense beds (Den Hartog 1970); so distinguishing individual plants in a population is difficult and may impede sampling for population analysis. In one study, 30% of the individuals investigated in populations of *Zostera marina* originated from clones (Reusch et al. 1999). *Zostera caespitosa* Miki (Zosteraceae) is a perennial seagrass that is distributed in northern Japan, Korea, and northeast China (Miki 1934, Den Hartog 1970). The rhizomes of *Z. caespitosa* grow diagonally and do not creep; as the name suggests, their growth form is caespitose. In seagrasses, this is a very rare character, and one that makes it easy to distinguish individuals. Therefore, *Z. caespitosa* is suitable for population genetic analysis.

The sites chosen for this study were in Otsuchi and Yamada Bays, in Iwate Prefecture, northeastern Japan; the distribution of *Z. caespitosa* in these bays has been investigated in detail (Omori et al. 1996). In addition, Otsuchi and Yamada Bays have similar dimensions and are connected only by the Pacific Ocean, making it possible to discuss gene flow between the bays, as well as within the bays' populations.

Random amplified polymorphic DNA (RAPD) as well as allozyme analysis has been widely used to carry out genetic analyses of populations of wild species (Dawson et al. 1995, Gillies et al. 1997, Fischer et al. 2000). Many allozyme loci are difficult to detect in wild plant species and enzyme activity deteriorates with time after sampling (Wendel and Weeden 1989). In contrast, RAPD detects genetic polymorphism (Edwards 1998) and at low temperatures the extracted DNA remains stable for a long time.

This study assessed the genetic variation within and among populations of *Z. caespitosa* using RAPD, and calculated genetic variance and similarity using Euclidian distance. We also discuss the gene flow within and among populations and among bays. We analyzed the RAPD data using the phenotypic approach, Shannon's index, and an analysis of molecular variance (AMOVA), rather than using the genotypic approach of Lynch and Milligan (1994), which calculates the gene frequency of RAPD loci assuming Hardy-Weinberg equilibrium.

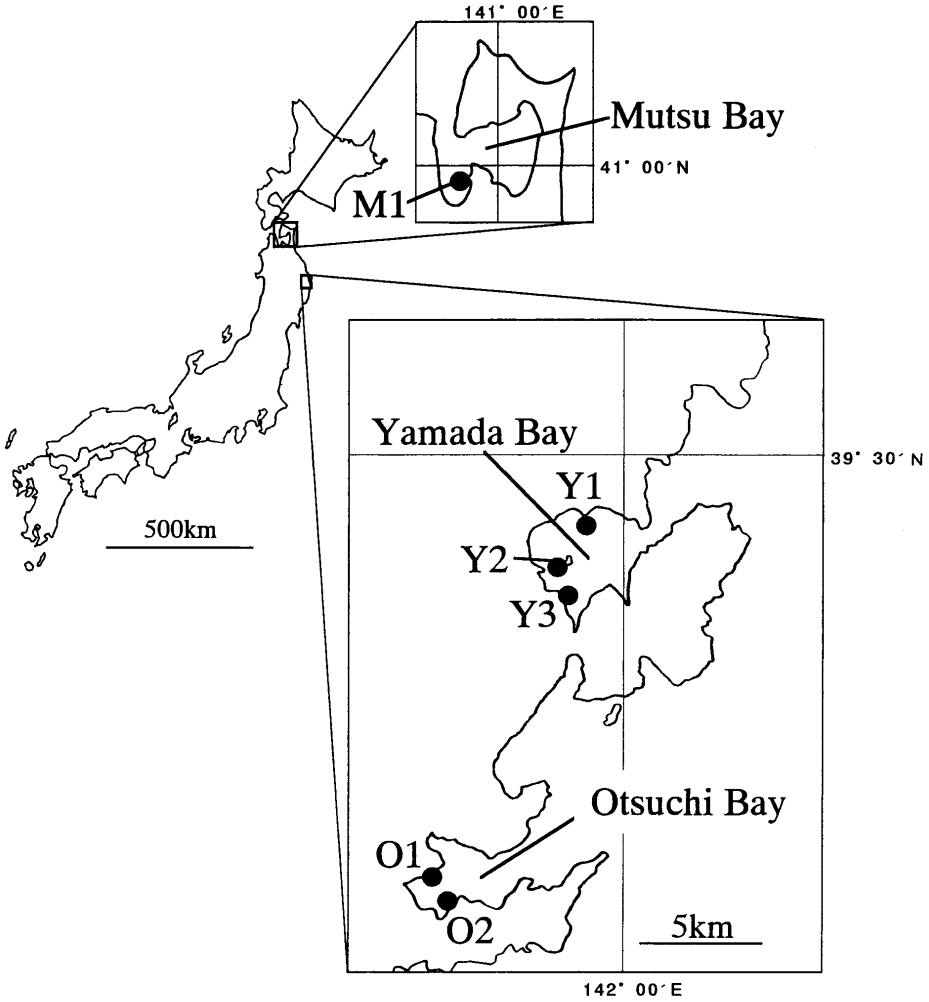

Fig. 1. Locations of the six populations of *Zostera caespitosa* in Japan. M1: Asamushi, Mutsu Bay, Aomori Pref. O1: Murohama, Otsuchi Bay, Iwate Pref. O2: Hakosaki, Otsuchi Bay, Iwate Pref. Y1: Kumagasaki, Yamada Bay, Iwate Pref. Y2: Ohshima, Yamada Bay, Iwate Pref. Y3: Namiitasaki, Yamada Bay, Iwate Pref.

MATERIALS AND METHODS

PLANT MATERIALS

Representative samples from about 20 plants were collected from each of the six populations of *Zostera caespitosa* Miki investigated (Fig. 1), resulting in a total of 260 individual samples. Several leaves were collected from each individual, and other organisms were removed. The leaves were washed in seawater, and then stored at −80°C. Of the six populations, two were located in Otsuchi Bay, three in Yamada Bay, and one was in Mutsu Bay (Fig. 1). Mutsu Bay is in Aomori Prefecture, northern Japan and is used to compare with Otsuchi and Yamada Bays.

DNA EXTRACTION AND RAPD AMPLIFICATION

Total DNA was extracted from fresh or deep-frozen leaves according to Tanaka et al. (1997) and purified with a Plasmid Mini kit (QIAGEN, Germany). RAPD amplifications were performed using arbitrary primers according to the method of Williams et al. (1990). The following cycling profile was used for all reactions: 2 min at 94°C, 1 min at 36°C, 2 min at 72°C for 1 cycle; 1 min at 94°C, 1 min at 36°C, 2 min at 72°C for 44 cycles. Nine primers from Operon Technologies Inc. were used (Table 1). The products were analyzed by electrophoresis on 1.3% agarose gels and visualized by staining with ethidium bromide. Each DNA sample was replicated running amplifications to confirm the banding pattern.

STATISTICAL ANALYSIS

The presence or absence of each fragment was scored in a binary data matrix, and the frequency of each band in each population was determined. A matrix of pairwise distances between all individuals within each species was calculated using Euclidian distance (Excoffier et al. 1992) and the matrix was applied to AMOVA analysis (Excoffier et al. 1992, Huff et al. 1993). These calculations were performed using Arlequin ver. 2.000 (Schneider et al. 2000). A matrix of the average number of pairwise differences between populations was used in an UPGMA cluster analysis. The genetic diversity was measured using Shannon's diversity index (Lewontin 1973) $H=-\Sigma p_i \log_2 p_i$, where p_i is the frequency of a given RAPD fragment. Shannon's diversity index is suited to the analysis of RAPD data because of its insensitivity to the bias that can be introduced into the data by the inability to detect heterozygous individuals (Dawson et al. 1995).

RESULTS

The nine primers used in this study generated 20 informative fragments that gave consistent results (Table 1). Of

these, six bands did not vary within each population, and five, one, and one bands were specific to populations M1, O1, and Y2, respectively. Five and eight bands did not vary within Otsuchi and Yamada Bays, respectively. The 127 individuals belonged to 96 different RAPD phenotypes.

The results of the AMOVA analysis are shown in Table 2. In all populations, 60% of the total variation was maintained among populations, and the remaining variation was maintained within populations. Excluding population M1, which is located at a distance from the other populations, 45% of the total variation was maintained among populations, and the remaining variation was maintained within populations. Two-level variance partitioning of the total variation between these five populations showed that the variance between Otsuchi and Yamada Bays was small (3.53%).

A UPGMA dendrogram based on Euclidian distances between populations was constructed (Fig. 2). The five populations (O1, O2, Y1, Y2, Y3) in Otsuchi and Yamada Bays formed a cluster, but the two populations from Otsuchi Bay (O1, O2) did not form a single cluster, nor did the three populations from Yamada Bay (Y1, Y2, Y3).

Shannon's diversity index (H) was calculated to quantify the level of genetic diversity for each population. The values ranged from 0.1517 (Y3) to 0.2835 (O1) (Table 3).

Table 1. List of primers, sequences and the approximate lengths of polymorphic markers observed

Primer	Sequence (5′-3′)	Marker bands (bp)
OPAA1	AGACGGCTCC	1000, 1350
OPAA6	GTGGGTGCCA	850, 1100
OPAA11	ACCCGACCTG	950, 1480
OPAA17	GAGCCCGACT	400
OPN20	GGTGCTCCGT	600, 1000
OPV7	GAAGCCAGCC	900, 1750
OPV8	GGACGGCGTT	1200, 1250, 1300
OPV19	GGGTGTGCAG	550, 700, 730
OPV20	CAGCATGGTC	920, 950, 1200

DISCUSSION

In this study, 40% of the genetic variation was found within populations and 60% was among populations when all populations were examined together. Excluding population M1, which is remote, 45% of the variation was among populations (Table 2). In their review of >400 plant species, Hamrick and Godt (1990) used G_{st} values to indicate the proportion of isozyme diversity residing among

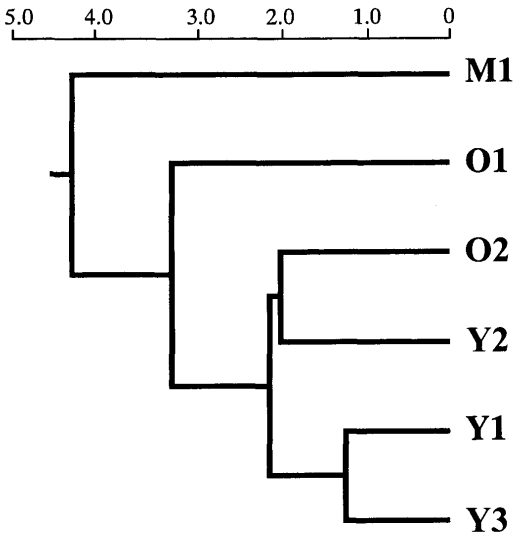

Fig. 2. UPGMAdendrogram of six populations of *Zostera caespitosa* based on average number of Euclidian distance between populations. Locations of populations are shown in Fig. 1.

Table 2. Analysis of molecular variance (AMOVA) for populations of *Zostera caespitosa*.

Source of variation	d.f.	Variance component	% Total variance	P-value
All populations				
Global				
Among populations	5	2.104	59.79	<0.001
Within populations	121	1.415	40.21	
Hierarchical				
Among bays	2	1.275	33.28	0.053
Populations/bays	3	1.142	29.81	<0.001
Individuals/populations	121	1.415	36.91	<0.001
Otsushi bay and Yamada bay				
Global				
Among populations	4	1.196	44.73	<0.001
Within populations	96	1.478	55.27	
Hierarchical				
Between bays	1	0.096	3.53	0.103
Populations/bays	3	1.139	41.99	<0.001
Individuals/populations	96	1.478	54.47	<0.001
Otsuchi bay				
Between populations	1	2.363	60.11	<0.001
Within populations	37	1.568	39.89	
Yamada bay				
Among populations	2	0.557	28.16	<0.001
Within populations	59	1.421	71.84	

Table 3. Shannon's diversity indices based on RAPD for six populations of *Zostera caespitosa*

Population	n	Shannon's diversity index
M1	25	0.1650
O1	21	0.2835
O2	19	0.1965
Y1	25	0.1522
Y2	21	0.2174
Y3	16	0.1517
Overall	127	0.4292

populations. They reported an average G_{st} of 20% for animal-pollinated outcrossers compared with 51% for selfers. To date, RAPD-based G_{st} values are available for 35 plant species, and average 15.5% for outbreeding species and 59.6% for inbreeding species (Bussell 1999). Compared with these values, the populations of *Zostera caespitosa* examined are generally closer to inbreeding species than to outcrossing species. This shows that gene flow among the populations is restricted and differentiation is promoted among the populations. According to overviews of the genetic variation within and among plant populations, high interpopulation variation is found in selfing breeding systems and in systems with attached seed dispersal as opposed to systems using outcrossing and wind or explosive dispersal (Loveless and Hamrick 1984, Hamrick and Godt 1990). There is little information on the characters that affect gene flow in *Z. caespitosa*. Although *Z. caespitosa* is protogynous (authors' observation) like *Z. marina* (De Cock 1980), it may carry out self-fertilization, because several flowering shoots are generated from an individual simultaneously. However, it is not known whether *Z. caespitosa* is self-incompatible, or how often *Z. caespitosa* is actually fertilized by self-pollination. Regarding seed dispersal, it has been reported that its fruit is not buoyant and is not suitable for dispersal by currents, but the generative shoots often detach at the time of fruiting, and then float on the surface and are transported by currents and wind action (Den Hartog, 1970). In a review on ecology of *Zostera* spp. in Japan, number of seeds per spathe of *Z. caespitosa* is shown to be fewer than that of other subsp. *Zostera* species (Nakaoka and Aioi 2001). This may affect on the degree of gene flow. However, the precise reason for low gene flow in *Z. caespitosa* is not clear at the present.

Ruckelshaus (1996) reported that seed dispersal in *Z. marina* is greater than in terrestrial angiosperms because of its high gene flow. In an allozyme analysis of *Z. marina*, Ruckelshaus (1998) also calculated the effective number of migrants per generation between bays as $N_em=2.9$. This value means that there is strong gene flow between bays. These reports on *Z. marina* are inconsistent with our results for *Z. caespitosa*, which found that the gene flow in *Z. caespitosa* is generally restricted compared to other seed plants, including *Z. marina*.

The UPGMA dendrogram among populations based on RAPD variation showed that the populations of Otsuchi Bay and Yamada Bay did not form single clusters (Fig. 2). The genetic variation between Otsuchi Bay and Yamada Bay was very small (3.5%) in the AMOVA analysis (Table 2). These results shows that only population O1 was dissimilar, while the other four populations in Otsuchi Bay and Yamada Bay were similar. In addition, the genetic variation between populations in Otsuchi Bay was 60%, and that within Yamada Bay was 28%. From this it is clear that gene flow between populations in Otsuchi Bay is very restricted, while more gene flow takes place in Yamada Bay. The two populations in Otsuchi Bay are 1.7 km apart, and Y1 & Y2 and Y2 & Y3 are 2.2 and 1.2 km apart, respectively. These distances are not large, so some reason other than geographical distance causes the difference in gene flow between Otsuchi Bay and Yamada Bay, although the reason cannot be showed in this study. Schlueter and Guttman (1998) presumed that environmental factors (ocean depth, current, and light) caused a few sites to be genetically different from all other sites in *Thalassia testudinum*. Waycott et al. (1997) discussed how patterns of genetic variability in different populations of *Posidonia australis* are affected by the founder effect and inbreeding at one site, and by paleogeographic factors at another.

Based on genetic variation within and among populations, the gene flow between and the origin of the Otsuchi and Yamada Bay populations of *Z. caespitosa* can be interpreted in four ways. 1) No gene flow has taken place within Otsuchi Bay, while there has been gene flow from Yamada Bay to population O2, but not to population O1. 2) No gene flow has taken place within Otsuchi Bay, and the two populations in Otsuchi Bay have different origins: population O2 is derived from Yamada Bay lineages and O1 is derived from other lineages. 3) Population O2 resulted from a recent migration from Yamada Bay, and there has been no subsequent gene flow between O1 and O2. 4) Population O1 resulted from a recent migration from some region other than Yamada Bay, and there has been no subsequent gene flow between O1 and O2. In populations originating from recent migrations, as in hypotheses 3 and 4, genetic diversity should be low due to the founder effect (Bonnell and Selander 1974). Therefore, a lower genetic diversity would be expected in population O1 or O2. However, Shannon's Index of genetic diversity was 0.2835 for O1 and 0.1965 for O2, which are not low compared to the values for the other populations studied (Table 3). Therefore, hypotheses 3 and 4 are unlikely, but we cannot discuss the other hypotheses any further at this time. To clarify this requires a phylogenetic study including populations in other regions. Nevertheless, this study has shown that the modes of gene flow in Otsuchi and Yamada Bays differ.

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三陸海岸におけるスゲアマモ集団間の遺伝子交流

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三陸海岸におけるスゲアマモ*Zostera caespitosa* Miki (Zosteraceae) の集団内および集団間の遺伝的変異および類似度をRAPDから検出し，集団間および湾間の遺伝子交流について評価した．AMOVAの結果，全集団の解析では遺伝的変異の40%が集団内に，60%が集団間に，また，近隣の2湾による解析では45%が集団間に維持されていることが明らかになった．これは，アマモ (*Zostera marina*) を含めた他の種子植物と比較して，スゲアマモ集団の遺伝子交流は制限されており遺伝的分化が促進される状態にあることを示している．ユークリッド距離に基づく集団間のUPGMA類似度図では，大槌湾と山田湾の集団はそれぞれ単一のクラスターを形成しなかった．さらに，この2湾間に維持される遺伝的変異は非常に小さかった (3.5%, AMOVA)．これらから，大槌湾と山田湾では，湾内の遺伝子交流の動きがそれぞれ異なることが明らかになった．

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