Bull. Natl. Mus. Nat. Sci., Ser. B, **37**(1), pp. 23–30, February 22, 2011

Genetic Structure and Diversity of the Eelgrass *Zostera marina* L. (Zosteraceae) in Sagami Bay, Japan

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(Received 16 November 2010; accepted 22 December 2010)

Abstract Over the past few decades, seagrass beds are declining worldwide. The eelgrass species *Zostera marina* L. is the main component of seagrass beds and has been declining in some areas of Japan, including Sagami Bay. This study examined the genetic structure and diversity in populations of this eelgrass in the Sagami Bay to establish guidelines for its restoration. Genotypes of 260 individuals from nine eelgrass beds were determined using five microsatellite markers. Genetic differentiation was observed among all eelgrass beds. A neighbor-joining tree based on F_{ST} values among eelgrass beds revealed that they were grouped in two areas: the Miura Peninsula and the Izu Peninsula. Significant positive correlation between genetic and geographical distances was detected among all beds as well as among beds of the Miura Peninsula. We suggest that restoring beds by transplanting seeds and/or plants of eelgrass to other sites should be avoided; however, if eelgrass beds have disappeared or severely declined, neighboring beds, except those of the Tenjin Island and Nabeta, should be used as donors. The beds of the Tenjin Island and Nabeta are not suitable donors since they lack genetic diversity.

Kew words : eelgrass, gene flow, genetic diversity, microsatellite marker, restoration, Sagami Bay, seagrass, *Zostera marina.*

Introduction

The eelgrass *Zostera marina* L. is an angiosperm that inhabits soft-bottom marine habitats ranging from the intertidal zone to depths of approximately 10 m in temperate latitudes (den Hartog, 1970). It is widely distributed in the northern Pacific and northern Atlantic Oceans, and occurs from Kyusyu to Hokkaido in Japan. Because of their high primary productivity and provision of habitats for various associated fauna, eelgrass beds are regarded as one of the most important components of coastal ecosystems (Kikuchi and Pérès, 1980; Jernakoff *et al.*, 1996; Duarte and Chiscano, 1999; Hemminga and Duarte, 2000; Williams and Heck, 2001). However, they are becoming more scarce worldwide due to direct and indirect effects of human activity (Fortes, 1988; Shorts and Wyllie-Echeverria, 1996; Duarte, 2002; Moore and Jarvis, 2008; Rueda *et al.*, 2009; Waycott *et al.*, 2009; Martin *et al.*, 2010). Seagrass beds have been restored at many sites worldwide (Zimmerman *et al*., 1995; van Katwijk, 2000; Orth *et al.*, 2006); however, some problems associated with artificial transplantation have become apparent. One concern is that transplantation of eelgrass seeds and/or plants from remote areas may violate the genetic composition of regional populations. In addition, eelgrass beds with high genetic diversity are ideally suited as donors for restoration by reintroduction. It has been demonstrated that levels of genetic diversity in species determines the success of reintroduction (Martins and Jain,

1979; Calero *et al.,* 1998). This is also true for eelgrass, which demonstrates better survivorship and reproductive success with higher genetic diversity (Williams, 2001; Hughes and Stachowicz, 2004; Ehlers *et al.,* 2008). Therefore an assessment of the genetic diversity and gene flow of wild eelgrass populations is required before projects for restoration are initiated.

The Sagami Bay is located between the Miura and Izu Peninsulas. All eelgrass beds are confirmed to be present only in the eastern part of the Bay (Tanaka *et al.*, 2006). Kudo (1999) reported that eelgrass beds in the Odawa Bay have reduced within the last two decades due to the decline in water transparency, with the largest remaining in the Sagami Bay. A possibility that eelgrass beds will continue to decrease exists.

In this study, we performed population genetic analysis of eelgrass beds in the Sagami Bay using microsatellite marker, codominant molecular markers that are suitable for detecting genetic structure and diversity within a population and among populations (Reusch, 2001; Olsen *et al*., 2004). Based on this data, we discussed the genetic structure of eelgrass in the Sagami Bay and suggested guidelines for its restoration.

Materials and Methods

Between 2003 and 2004, seven eelgrass beds on the west side of the Miura Peninsula and two on the east side of the Izu Peninsula were sampled (Fig. 1 and Table 1). From each bed, 27 to 35 shoots of eelgrass were randomly collected. To avoid collection of the same genet, only one shoot was collected within a diameter of 3 m. In the Tenjin Island and Nabeta beds, shoots were collected at >1 m intervals because the beds were small and their distribution was fragmented. Collected shoots were rinsed with fresh water to remove epiphytic algae, and pieces of leaves were cut and frozen at −80°C within 24 h of collection.

Genomic DNA was extracted from approximately 0.02–0.04 g dry weight of eelgrass leaf tissue using the CTAB (hexadecyltrimethyl ammonium bromide) method of Doyle and Doyle (1987). Crude extracts of total DNA were purified with the Plasmid Mini Kit (QIAGEN, Hilden, Germany) and preserved at −20°C.

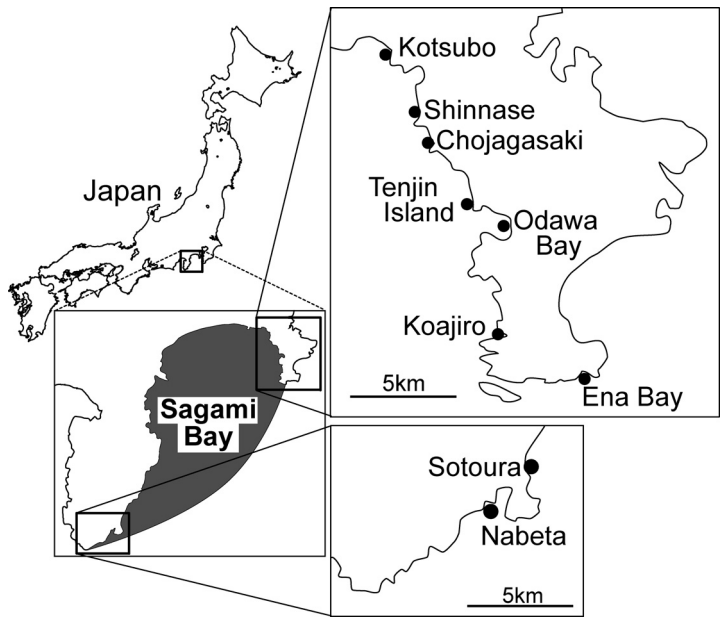

Fig. 1. Collection sites of eelgrass (*Zostera marina*) in this study. Closed circles indicate the positions of eelgrass beds. Related information of each site is described in Table 1.

Table 1. Sample collection data and habitat characteristics for eelgrass used in this study. The positions of collection sites are indicated in Fig. 1

Collection sites	Number of individuals	Locality	Area (m^2)	Latitude	Longitude	Voucher*
Miura Peninsula						
Kotsubo	30	Kotsubo, Zushi, Kanagawa Pref.	500	N35°17′46″	E139°33′22″	TNS-9524126-9524131
Shinnase	29	Shinnase harbor, Hayama, Kanagawa Pref.	1600	N35°16′18″	E139°34′12″	TNS-9524132-9524137
Chojagasaki	29	Chojagasaki, Hayama, Kanagawa Pref.	2300	N35°15′13″	E139°34′39″	TNS-9524138-9524142
Tenjin Island	35	Tenjin-island, Yokosuka, Kanagawa Pref.	200	N35°13′17″	E139°36′18″	TNS-9524180-9524183
Odawa Bay	27	Odawa Bay, Yokosuka, Kanagawa Pref.	40000	N35°12′34″	E139°37′15″	TNS-9524143-9524148
Koajiro	29	Koajiro Bay, Miura, Kanagawa Pref.	3400	N35°39′44″	E139°37′36″	TNS-9524149-9524155
Ena Bay	28	Ena Bay, Miura, Kanagawa Pref.	8700	N35°08′43″	E139°39′55″	TNS-9524156-9524162
Izu Peninsula						
Sotoura	30	Sotoura Bay, Shimoda, Shizuoka Pref.	800	N34°40′24″	E138°58′27″	TNS-9524163, 9524169
Nabeta	29	Nabeta Bay, Shimoda, Shizuoka Pref.	100	N34°39′53″	E138°56′43″	TNS-9524170-9524173

* All voucher specimens are deposited in National Museum of Nature and Science, Japan (TNS).

A preliminary test using polymerase chain reaction (PCR) amplification was conducted for 12 microsatellite loci (Zosmar CT-3, Zosmar GA-1, Zosmar GA-2, Zosmar GA-3, Zosmar GA-4, Zosmar GA5-5, and Zosmar GA-6; Reusch *et al.*, 1999: Zosmar CT-12, Zosmar CT-19, Zosmar CT-35, Zosmar CT-20, and Zosmar CT-17H; Reusch, 2000). Based on the degree of polymorphism and stability of amplification, five loci (Zosmar CT-3, Zosmar GA-1, Zosmar GA-3, Zosmar GA-4, and Zosmar CT-19) were selected for the following analyses.

PCR was carried out in a volume of 6.0 μl containing 3 ng of template DNA, 1.2 pmole of each primer, 0.2 mM of each dNTP, 1x PCR buffer, and 0.3 units of ExTaq polymerase (Takara Bio Inc., Shiga, Japan). PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR program employed consisted of a 5 min denaturing step at 95°C, followed by 30 cycles at the following times and temperatures: 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C.

Size separation of PCR products was carried out using capillary electrophoresis on an ABI PRISM 3100Avant genetic analyzer (Applied Biosystems). Size sorting of banding patterns and genotyping was performed in a semi-automated way using the software program GeneMapper version 3.5 (Applied Biosystems).

Genetic diversity of each eelgrass bed was measured using clonal diversity, which was expressed as a function of the number of ramets and genets sampled (Olsen *et al.*, 2004), the number of alleles, allelic richness (Pettitt *et al.,* 1998), and average heterozygosity (Nei, 1987). Deviation from the Hardy–Weinberg equilibrium (HWE) was tested using the Markov chain algorithm developed by Guo and Thompson (1992). These calculations were performed using GENEPOP on the Web 3.4 (Raymond and Rousset, 1995) and by hand.

Genetic differentiation and distances were quantified by calculating the proportion of genetic variance (F_{ST}) (Weir and Cockerham, 1984) using Arlequin 2.001 (Schneider *et al*., 2001) and

GENEPOP on the Web 3.4. A neighbor-joining (Saitou and Nei, 1987) tree based on F_{ST} values was constructed using PAUP* 4.0 (Swofford, 2001). To calculate variance components between groups, among beds, and among individuals, an analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) was performed. Isolation by distance (IBD) was detected using the Mantel test (Mantel, 1967) between two matrices: F_{ST} and geographical distance in kilometers between pairs of populations. Geographical distances between populations were manually measured from a chart as either direct distance (when there was no land) or coastal distance (when land was located in a direct line connecting the two points of collection). The Mantel test was performed using the ISOLDE program of GENEPOP on the Web (Rousset, 1997).

Results

Clonal diversity varied from 0.37 (Tenjin Island) to 1.00 (Odawa Bay). The mean number of alleles per locus and bed ranged from 2.0 (Nabeta) to 6.4 (Odawa Bay; Table 2). Allelic richness ranged from 1.29 to 1.69. Private alleles were observed in beds from Kotsubo, Chojagasaki, Odawa Bay, Ena Bay, and Sotoura. Average observed heterozygosity for all beds was between 0.379 and 0.690 and average expected heterozygosity was between 0.467 and 0.690 and was lowest at 0.364 (Nabeta) (Table 2). All beds, except the Tenjin Island beds ($P=0.0089$) were in Hardy-Weinberg equilibrium (HWE). Although F_{ST} value varied from 0.033 to 0.468, significant genetic differentiation was detected among all beds ($P<0.001$; Table 3). Analysis of the genetic relationship among the nine beds using the

Table 2. Genetic diversity and clonality in eelgrass of the Sagami Bay

Site	n	G	C	A	Ar	Pa	H_O	H_E	HWE
Kotsubo	30	23	0.77	5.6	1.64	1	0.653	0.639	ns
Shinnase	29	25	0.86	4.8	1.61	0	0.634	0.611	ns
Chojagasaki	29	25	0.86	6.0	1.69	2	0.648	0.690	ns
Tenjin Island	35	13	0.37	3.6	1.58	0	0.669	0.585	P=0.0089
Odawa Bay	27	27	1.00	6.4	1.64	1	0.607	0.635	ns
Koajiro	29	25	0.86	3.0	1.52	0	0.552	0.518	ns
Ena Bay	28	25	0.89	5.2	1.47	2	0.464	0.467	ns
Sotoura	30	28	0.93	6.2	1.59	1	0.627	0.594	ns
Nabeta	29	16	0.55	2.0	1.29	0	0.379	0.364	ns
average	29.6	23.0	0.8	4.8	1.6	0.8	0.581	0.567	

n, number of ramets; G, number of genotypes; C, clonal diversity; A, number of alleles; Ar, allelic richness; Pa, number of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, deviation from Hardy-Weinberg equilibrium (ns=not significant).

Table 3. Pairwise F_{ST}-values (Weir and Cockerham, 1984) among nine eelgrass (*Zostera marina*) beds in the Sagami Bay

	Kotsubo	Shinnase	Chojagasaki	Tenjin Island	Odawa Bay	Koajiro	Ena Bay	Sotoura
Shinnase	0.033							
Chojagasaki	0.057	0.107						
Tenjin Island	0.119	0.092	0.114					
Odawa Bay	0.078	0.100	0.043	0.083				
Koajiro	0.085	0.125	0.082	0.188	0.132			
Ena Bay	0.209	0.253	0.201	0.328	0.244	0.186		
Sotoura	0.101	0.131	0.112	0.145	0.066	0.193	0.266	
Nabeta	0.319	0.359	0.294	0.379	0.223	0.430	0.468	0.167

Genetic differentiation was significantly detected among all beds ($P<0.05$).

neighbor-joining tree revealed that the eelgrass beds were grouped into two clusters, seven in the waters of the Miura Peninsula and two in the waters of the Izu Peninsula (Fig. 2). AMOVA revealed significant genetic variation at two spatial levels (among eelgrass beds within each geographical group and among individuals within each bed), excluding the Miura Peninsula and the Izu Peninsula among groups ($P=0.026$; Table 4). Significant positive correlation between genetic and geographical distances was detected among all beds as well as among beds of the Miura Peninsula (Mantel test, $P<0.01$; Fig. 3).

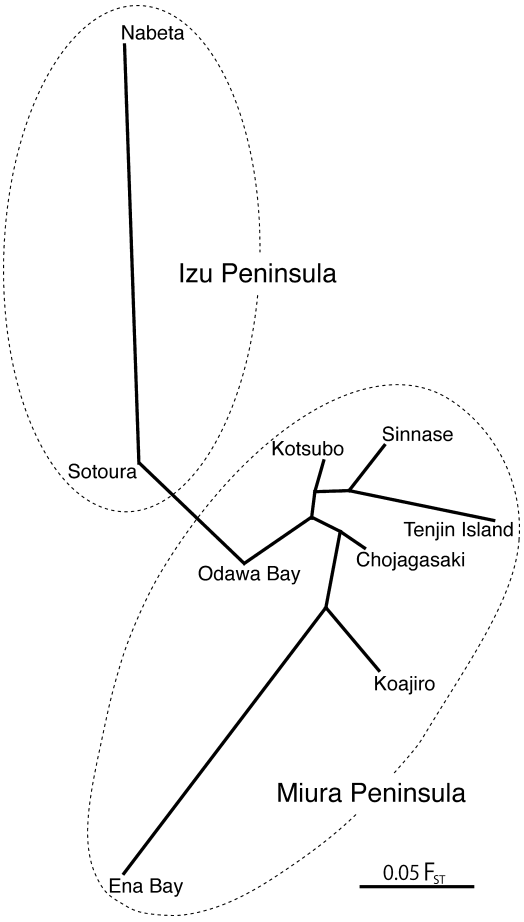

Fig. 2. Neighbor-joining (NJ) tree based on F_{ST} values (Weir and Cockerham, 1984) among eelgrass beds in the Sagami Bay.

Discussion

Genetic diversity of eelgrass beds

In previous studies of eelgrass, heterozygosity ranged from 0.15 to 0.69 in North America and Europe (Olsen *et al*., 2004), 0.491 to 0.563 (average, 0.539) in the San Quintin Bay, Mexico (Muniz-Salazar *et al*., 2006), 0.312 to 0.541 in the Southern California Bight, Mexico (Coyer *et al*., 2008), and 0.464 to 0.708 (average, 0.620) in the Tokyo Bay (Tanaka *et al.,* 2011). In comparison with these data, the genetic diversity of eelgrass in the Sagami Bay (heterozygosity: 0.364–0.690: Table 2) did not differ from that at other sites.

Procaccini *et al*. (2007) suggested the importance of considering both genetic (allelic) and genotypic (clonal) diversity for a seagrass ecosystem. The Odawa Bay had above average allelic diversity (allelic richness and heterozygosity) and high clonal diversity despite the noticeable decline in the eelgrass beds in the 1980s and 1990s (Kudo, 1999). Among the Miura Peninsula, above average allelic diversity and low clonal diversity were detected in the Tenjin Island beds. These results indicate that the Tenjin Island beds may have been affected by the method by which shoots were collected at intervals closer than those in other beds; however, the Tenjin Island beds, consisting of several small patches, deviated from HWE. Several causes for deviation are known (e.g., inbreeding, assortative mating, subdivision of a population, and selection) (Nei, 1987). Deviation in the Tenjin Island beds probably occurred because of subdivision of the population. A contrast was observed between the two beds of the Izu Peninsula. The Nabeta beds showed low genetic and clonal diversities compared to the other beds, while above average statistics were observed in the Sotoura beds, which are located in the neighborhood of Nabeta. There are few eelgrass beds within approximately 200 km west of Nabeta (Tanaka *et al*., 2009) and the strong sea current, Kuroshio, flows south of the Izu Peninsula from west to east. Although low clonal diversity may be affected by the sampling

Table 4. Analysis of molecular variance (AMOVA) for eelgrass (*Zostera marina*) beds in the Sagami Bay

Source of variation	d.f.	Variance component	%Total variance	*P*-value
Between groups	1	0.197	10.76	0.026
Eelgrass beds/groups	7	0.238	12.97	<0.001
Individuals/eelgrass beds	266	1.440	78.52	<0.001

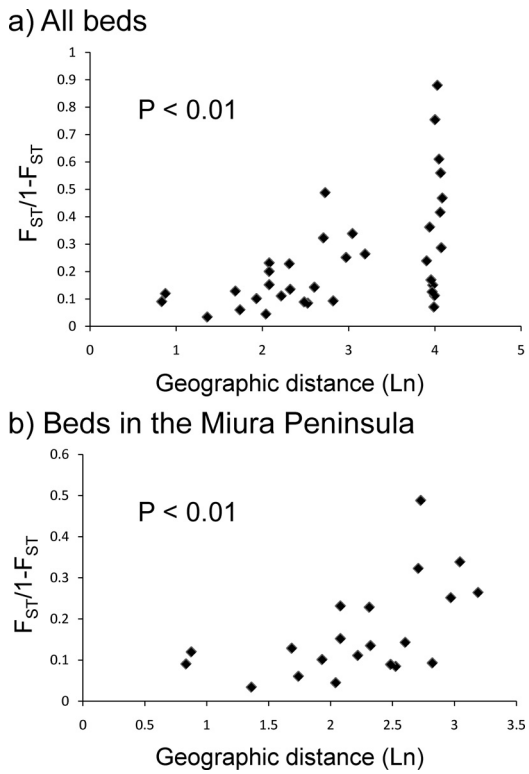

Fig. 3. Isolation-by-distance (IBD) used Mantel tests for eelgrass populations in the Sagami Bay based on FST as genetic distance. a) All nine eelgrass beds: b) Seven beds within the Miura Peninsula (Kotsubo, Shinnase, Chojagasaki, Tenjin Island, Odawa Bay, Koajiro, Ena Bay). Pairwise genetic differentiation between populations were linearized and plotted against the geographic distance.

method, low allelic diversity may be caused by the small population size (area = 100 m^2; Table 1) and the geographical location. Consequently, the eelgrass beds, except for those of Tenjin Island and Nabeta, fulfill the necessary conditions as donor sites for restoration in terms of allelic and clonal diversities.

Genetic differentiation and gene flow among eelgrass beds

In this study, genetic differentiation was observed among all eelgrass beds (Table 3). The eelgrass beds are grouped into two clusters: those in the waters of the Miura Peninsula and those in the water of the Izu Peninsula, although the genetic difference is not strong because AMOVA analysis does not support a significant genetic variation between the two geographical groups. Isolation by distance (IBD) analysis showed significant correlation between genetic and geographic distances among all beds as well as among beds of the Miura Peninsula. This result is in contrast to that obtained in the Tokyo Bay, located east and adjacent to the Sagami Bay. In the inner part of the Tokyo Bay, correlation between genetic and geographic distances was not detected among eelgrass beds (Tanaka *et al.*, 2011). No IBD was observed when strong gene flow occurred among populations within a slightly closed region such as a gulf or a bay (Muniz-Salazar *et al*., 2005, 2006). This could be due to the nearly saturated gene flow that easily occurs in a closed environment. The Sagami Bay is not as closed as the Tokyo Bay. We believe that these geographical traits of the Sagami Bay caused the genetic differences among the beds and the genetic structure following IBD.

Implications for the restoration of eelgrass beds in the Sagami Bay

In the Sagami Bay, all eelgrass beds are genetically different from each other; therefore, we believe that transplanting seeds and/or plants of eelgrass to other sites should be avoided. However, if eelgrass beds have disappeared or severely declined, neighboring beds, except for those of Tenjin Island and Nabeta, should be used as donors.

The beds of Tenjin Island and Nabeta are not suitable donors since they lack genetic diversity.

Acknowledgements

The authors thank Shimoda Floating Aquarium and fisheries cooperative associations of the Miura Peninsula for help with collecting plant materials, and Chikako Ishii, Shoh Nagata, Haruka Matsuyoshi for analysis of the data and drawing figures.

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