

Genetic Diversity of Regenerated Eelgrass Population at Nojima Coast in Tokyo Bay

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Abstract The genetic diversity of the regenerated eelgrass (*Zostera marina* L., Zosteraceae) population at Nojima Coast, Kanagawa Prefecture, Japan, was detected using five microsatellite markers. Comparison of the diversity with other sites revealed that the Nojima population is included within the genetic group of inner bay populations in Tokyo Bay and has a high genetic diversity and little low clonal diversity. The result showed successful regeneration at the initial stage and the necessity of long-term genetic monitoring.

Key words : eelgrass, genetic diversity, microsatellite marker, restoration, seagrass, Tokyo Bay.

Introduction

The eelgrass *Zostera marina* L. is a seagrass species that 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, seagrass 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). Because they are becoming increasingly scarce worldwide as a result of 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).

Tokyo Bay is one of the most industrialized coastal areas of Japan. However, numerous eelgrass communities existed in the bay 100 years ago (Izumi, 1908). Environmental deteriorations due to the reclamation of coastal zones and eutrophication of waters and sediments in the second half of the 20th century caused the eelgrass beds to decrease (Tanaka *et al.*, 2006).

Some problems associated with artificial transplantation have become apparent. One concern is that the transplantation of eelgrass seeds and/or plants from remote areas may violate the genetic composition of regional populations. In addition, eelgrass beds with a high genetic diversity are ideally suited as donors for restoration by reintroduction. In fact, better survivorship and reproductive success have been shown in eelgrass populations with a higher genetic diversity (Williams, 2001; Hughes and Stachowicz, 2004;

Ehlers *et al.*, 2008). The genetic diversity of eelgrass populations in Tokyo Bay was assessed to be average compared with that of the species in other areas (Tanaka *et al.*, 2011).

At Nojima Coast, located in Yokohama, Kanagawa Prefecture, the restoration of eelgrass beds using seeding and transplanting was conducted by the Kanagawa prefectural government from 2003 to 2005. The Nojima Coast is one of the few natural tidelands existing in Tokyo Bay. Although eelgrasses were recorded there around 100 years ago (Izumi, 1908) and also in 1994, 1997, 2000 and 2003 (Tanaka and Murakami 1996; Tanaka *et al.*, 1999; Hasegawa *et al.*, 2001; Takahashi *et al.*, 2005), eelgrass of Nojima Coast was almost gone by large-scale red tide of 2003 (Morita, 2013). The seeds for seeding and for

cultivating the seedlings for transplanting to Nojima Coast were collected from the eelgrass bed in Hashirimizu (HA), which is assessed to be grouped into the genetic cluster, including all eelgrass populations of the inner bay of Tokyo Bay and to have a high genetic diversity (Tanaka *et al.*, 2011). After the introduction of eelgrass to the north-western area of Nojima Coast from 2003 to 2005, it gradually eelgrass propagated and expanded throughout the coast by 2006 (Fig. 1). The area of the eelgrass bed reached 18,322 m² (Morita, 2013). The increase is an important essence of the success of the restoration work. However, if the increased population has a scarce genetic diversity, the success is superficial.

In this study, we aimed to assess the regenerated eelgrass population at Nojima Coast in

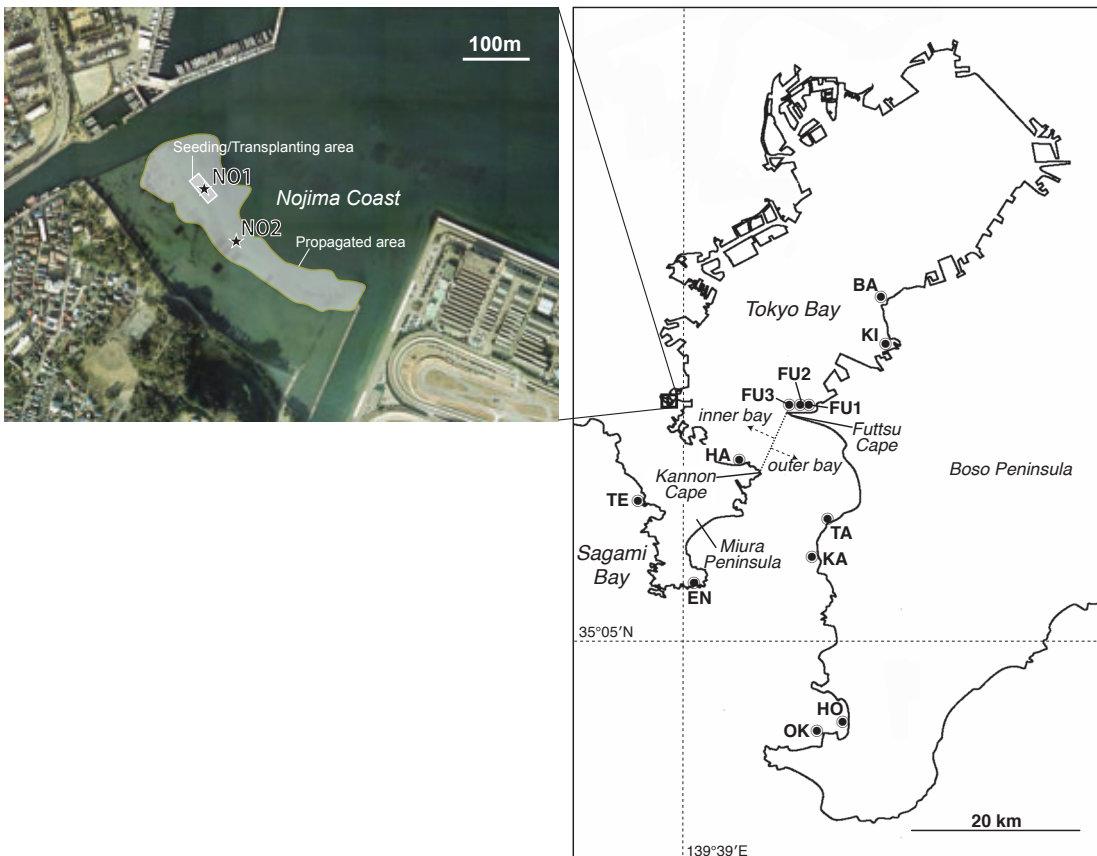


Fig. 1. Location of collected eelgrass beds around Tokyo Bay. Filled stars and filled circles show collecting sites in this study and in the previous study, respectively. The photograph of collected sites of Nojima Coast was taken in December 2000.

terms of the genetic diversity. We detected the genetic variation in the Nojima population using microsatellite markers, calculated the genetic and clonal diversity, and compared it with that of the other populations around Tokyo Bay.

Materials and Methods

Samples from two sites at Nojima Coast were collected in June 2006 (Table 1 and Fig. 1). Samples of seedling/transplanting area of Nojima Coast (NO1) were collected from the center of seeding/transplanting area in 2003 and NO2 were collected from individuals that propagated out of the seeding/transplanting area. The samples and genetic data for the other sites around Tokyo Bay for comparison have been cited from Tanaka *et al.* (2011).

From each site, dozens of shoots of eelgrass were randomly collected. To avoid collection of the same genet, only one shoot was collected within a diameter of 3 m. 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 .

Five loci (Zosmar CT-3, Zosmar GA-1, Zosmar GA-3, Zosmar GA-4: Reusch *et al.*, 1999a and Zosmar CT-19 : Reusch, 2000) selected by the preliminary test for degree of polymorphism and stability of amplification (Tanaka *et al.*, 2011) were used for this study.

PCR was carried out in a volume of $6.0\mu\text{L}$ 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

Table 1. Collection data for eelgrass used in this study

Sites	Number of samples	Locality	Latitude	Longitude
NO1	27	Seeding/Transplanting area, Nojima Coast, Yokohama, Kanagawa Pref.	N35°19'51"	E139°38'09"
NO2	26	Propagated area, Nojima Coast, Yokohama, Kanagawa Pref.	N35°19'47"	E139°38'15"
BA	23	Banzu-higata, Kisarazu, Chiba Pref.	N35°25'35"	E139°54'28"
KI	17	Kisarazu harbour, Kisarazu, Chiba Pref.	N35°19'44"	E139°53'38"
FU1	23	Futtsu-higata, Futtsu, Chiba Pref.	N35°19'06"	E139°48'45"
FU2	26	Futtsu-higata, Futtsu, Chiba Pref.	N35°18'59"	E139°47'56"
FU3	13	Futtsu-higata, Futtsu, Chiba Pref.	N35°19'06"	E139°47'05"
HA	27	Hashirimizu, Yokosuka, Kanagawa Pref.	N35°15'39"	E139°43'39"
TA	26	Tsuhama seaside, Futtsu, Chiba Pref.	N35°12'18"	E139°49'15"
KA	27	Ushiyama, Futtsu, Chiba Pref.	N35°10'37"	E139°49'21"
HO	20	Hojoh Coast, Tateyama, Chiba Pref.	N35°00'13"	E139°51'43"
OK	16	Okinoshima eastside, Tateyama, Chiba Pref.	N34°59'00"	E139°49'17"
EN	28	Ena Bay, Miura, Kanagawa Pref.	N35°08'43"	E139°40'12"
TE	35	Tenjin-islands, Yokosuka, Kanagawa Pref.	N35°13'17"	E139°36'18"

The positions of collection sites are indicated in Fig. 1.

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 FSTAT (Goudet, 2001). Genetic differentiation was quantified by calculating the propor-

tion of genetic variance (F_{ST}) (Weir and Cockerham, 1984) using GENEPOP on the Web 3.4.

Results

Clonal diversity of NO1 and NO2 were 0.85 and 0.69 respectively and were a little low in comparison with other eelgrass population around Tokyo Bay (average: 0.88) (Table 2). As indices of genetic (allelic) diversity, the mean

Table 2. Genetic diversity and clonality of eelgrass populations in this study

Site	<i>n</i>	G	C	A	Ar	H_O	H_E	F_{IS}
Nojima Coast (this study)								
NO1	27	23	0.85	5.6	4.8	0.593	0.630	0.172
NO2	26	18	0.69	5.4	4.8	0.519	0.624	0.079*
Around Tokyo Bay (previous study)								
BA	23	23	1.00	5.6	5.0	0.600	0.648	0.075
KI	17	16	0.94	5.8	5.4	0.576	0.657	0.126
FU1	23	23	1.00	6.8	5.9	0.696	0.700	0.007
FU2	26	26	1.00	7.2	5.8	0.608	0.656	0.075
FU3	13	10	0.77	5.8	5.8	0.677	0.703	0.039
HA	27	27	1.00	6.0	5.7	0.688	0.682	-0.009
TA	26	18	0.69	4.0	3.6	0.526	0.560	0.066
KA	27	26	0.96	4.8	4.4	0.708	0.595	-0.194*
HO	20	19	0.95	4.6	4.1	0.511	0.578	0.117
OK	16	16	1.00	5.2	4.7	0.640	0.613	-0.046
EN	28	25	0.89	5.2	4.3	0.464	0.467	0.007
TE	35	13	0.37	3.6	3.3	0.669	0.585	-0.146
Average without Nojima Coast	23	20	0.88	5.4	4.8	0.614	0.620	0.028
Total average	23.9	20.2	0.9	5.4	4.8	0.605	0.621	0.040

n, number of ramets; G, number of genotypes; C, clonal diversity; A, number of alleles; Ar, allelic richness; H_O , observed heterozygosity; H_E , expected heterozygosity; F_{IS} , inbreeding coefficient.

* Indicates a significant F_{IS} value (e.g., departure from Hardy-Weinberg equilibrium, $P < 0.01$)

Table 3. Pairwise F_{ST} values (Weir and Cockerham, 1984) among 14 eelgrass sites in this study

	NO1	NO2	BA	KI	FU1	FU2	FU3	HA	TA	KA	HO	OK	EN
NO2	0.019*												
BA	0.013*	0.016*											
KI	0.018*	0.019*	0.019*										
FU1	0.015*	0.009*	0.000*	0.007*									
FU2	0.023	0.016*	0.005*	0.006*	0.000*								
FU3	0.010*	0.017*	0.031	0.006*	0.005*	0.025							
HA	0.000*	0.004*	0.000*	0.000*	0.000*	0.013*	0.007*						
TA	0.146	0.163	0.096	0.128	0.104	0.087	0.150	0.123					
KA	0.102	0.141	0.120	0.127	0.111	0.124	0.080	0.117	0.233				
HO	0.216	0.238	0.233	0.209	0.197	0.242	0.168	0.188	0.284	0.217			
OK	0.173	0.186	0.18457	0.182	0.145	0.198	0.140	0.141	0.267	0.161	0.063		
EN	0.139	0.205	0.19188	0.159	0.172	0.198	0.126	0.175	0.330	0.119	0.266	0.236	
TE	0.204	0.226	0.22486	0.225	0.177	0.224	0.191	0.187	0.271	0.263	0.180	0.108	0.328

* Differentiation between pairwise populations was not detected ($P \geq 0.05$)

Abbreviation; see Table 1.

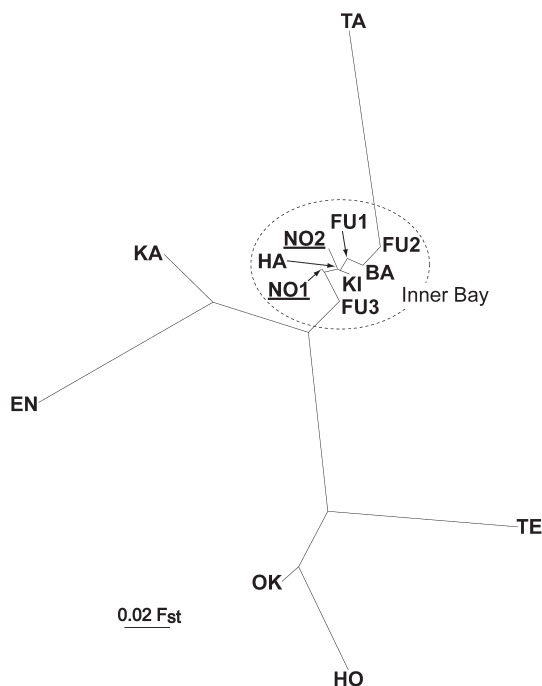


Fig. 2. NJ tree based on F_{ST} values (Weir and Cockerham, 1984) among eelgrass beds around Tokyo Bay.

number of alleles per locus and site (NO1: 5.6, NO2: 5.4) and allelic richness (NO1 and NO2: 4.8) were almost average compared to other population (Table 2). The average expected heterozygosity (Nei's genetic diversity) was 0.630 (NO1) and 0.624 (NO2). Deviation from the Hardy-Weinberg equilibrium was detected in NO2 as heterozygote deficiency ($P = 0.0000$). The F_{ST} value varied from 0 to 0.329 among all populations. Significant genetic differentiation was not detected in most of pairs among eelgrass beds of the inner bay (Table 3). A neighbor-joining (NJ) tree among the 12 beds was constructed on the basis of F_{ST} values. The NJ tree showed that NO2 and NO1 were included within the closely related group consisting of the six eelgrass beds of the inner bay: FU1, FU2, FU3, KI, BA, and HA populations (Fig. 2).

Discussion

For transplantation of eelgrass to Nojima

Coast, the seeds and plants from the HA population of the inner bay of Tokyo Bay were used. The NJ tree constructed using F_{ST} values among eelgrass populations revealed that the two sites of regenerated eelgrass bed are closely related to the inner bay populations, including HA (Fig. 2). The closely relationship of NO1 (seeding/transplanting area) with inner bay populations probably reflect the genetic component of the donor site (HA). On the other hand, three causes why NO2 (propagated eelgrass beds out of the seeding/transplanting area) is included within the genetic group of inner bay populations are assumed. First possible cause is that the propagated eelgrasses are derived from seeds dispersed from seeding/transplanting area, second cause is they from buried seeds in the sediments of Nojima Coast, and last cause is that they from the dispersed seeds from the other eelgrass populations mainly in the inner bay of Tokyo Bay. We can not evaluate these hypotheses, because there are almost no significant differentiation among the inner bay populations. However, even any cause, propagated eelgrass communities have the genetic composition of the inner bay populations can be evaluated to be close to natural population in Nojima Coast.

Nei's genetic diversities (expected heterozygosity: H_E) of the two sites in Nojima Coast are considered to be average for eelgrass populations around Tokyo Bay and high compared with those of the other regions, which ranged from 0.491 to 0.563 (average = 0.539) in San Quintin Bay, Mexico (Muniz-Salazar *et al.*, 2006) and from 0.312 to 0.541 in the Southern California Bight, Mexico (Coyer *et al.*, 2008). The number of alleles and allelic richness at Nojima Coast are average, which shows that the regenerated eelgrass population has a genetic diversity equivalent to that of other natural populations.

Procaccini *et al.* (2007) suggested the importance of considering both genetic (allelic) and clonal (genotypic) diversity for a seagrass ecosystem. The clonal diversity of NO1 was almost average in this study; however, that of NO2 was lower than average (0.69) (Table 2). In general,

the low clonal diversity indicates the presence of a few large clones in the bed (Olsen *et al.*, 2004). In addition, heterozygote deficiency was detected in NO2; this is also probably affected by high clonality of individuals with homozygotes.

In general, eelgrass propagates by both seedling and elongation of rhizomes. In rare cases, however, one eelgrass population in the Baltic Sea consisted of a single clone, whereas some beds in Germany consisted of many clones (Reusch *et al.*, 1999b). Because better survivorship and reproductive success are shown in eelgrass beds with a higher genetic diversity (Williams, 2001; Hughes and Stachowicz, 2004; Ehlers *et al.*, 2008), if the regenerated eelgrass population propagates only by vegetative growth, the population may have a problem in terms of its stability.

In this case, however, the genetic (allelic) diversity was high. This means that a high proportion of clonal individuals is found; however, the variation in the genetic component is large within the population. We observed the intermittent expansion of eelgrass beds in the area, which probably represents propagation by seedling. In addition, because the other eelgrass populations in Tokyo Bay have a high genetic diversity and the gene flow by seed dispersal occurs in the inner bay (Tanaka *et al.*, 2011), seed dispersal from other populations to Nojima are highly expected. Consequently, the restoration of the Nojima population can be assessed to be successful in terms of the genetic diversity at this time. However, because there are concerns about excessive vegetative propagation, the long-term monitoring of the genetic and ecological transition is necessary.

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