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Genetic Diversity and Gene Flow of East Asian Seagrass, *Zostera caespitosa* Miki (Zosteraceae), in Northeast Japan

Norio Tanaka[1,*], Masahiro Nakaoka[2], Yuji Omori[3] and Keiko Aioi[4]

[1] Department of Botany, National Museum of Nature and Science, Amakubo 4–1–1, Tsukuba, Ibaraki 305–0005, Japan
[2] Hokkaido University, Akkeshi Marine Station, Field Science Center for Northern Biosphere, Hokkaido University, Aikappu, Akkeshi, Hokkaido 088–1113, Japan
[3] Yokosuka City Museum, Fukadadai 95, Yokosuka, Kanagawa 238–0016, Japan
[4] Wetlands International Japan, Nihonbashi-Ningyocho 3–7–3, Chuo-ku, Tokyo 103–0013, Japan
* E-mail: ntanaka@kahaku.go.jp

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Abstract The genetic diversity and gene flow of *Zostera caespitosa* Miki (Zosteraceae) in northeast Japan were evaluated. Twelve microsatellite markers designed for *Z. marina* were surveyed for their applicability to *Z. caespitosa*. For four of these loci, 167 individuals from nine sites were genotyped. Although a neighbor-joining tree based on F_{ST} values among *Z. caespitosa* sites grouped them into four geographical areas: Otsuchi Bay, Yamada Bay, Mutsu Bay, and Notoro Lake, analysis of molecular variance (AMOVA) did not reveal significant genetic differentiation among the four areas. No significant correlation between genetic and geographical distances was detected within either Otsuchi Bay or Yamada Bay. It was concluded that strong gene flow occurs in *Z. caespitosa*, at least in nearly closed environments such as bays.

Kew words : gene flow, genetic diversity, microsatellite marker, seagrass, *Zostera caespitosa.*

Introduction

The genus *Zostera* (Zosteraceae), consisting of 12 species, is representative of seagrasses adapted to brackish to salt water (den Hartog, 1970; Tanaka *et al.*, 2003). The genus is distributed from the intertidal to subtidal zone, mainly in subarctic to subtropical zones of both hemispheres. Most species show local distributions, except for eelgrass *Z. marina*, which is widespread in the temperate to subarctic coastal areas of the northern hemisphere.

Because *Z. marina* beds are considered 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), the genetic structure and diversity of *Z. marina* have been well studied in various regions by several approaches. The development of microsatellite markers has improved the understanding of the genetic structure (Reusch, 2001; Olsen *et al.*, 2004). Intensive research in both Europe and North America has revealed that the genetic clonal structure of a single bed and genetic similarity among local populations vary greatly from site to site (Reusch *et al.*, 1999a). The study found that the size of *Z. marina* beds and their genetic clonal structure were not always related. Genetic similarity among *Z. marina* populations decreases monotonically with geographic distance in some regions, such as the western Baltic and the Gulf of California, whereas isolation by distance (IBD) was not obvious for populations in southern Europe and the Pacific coast of Baja California (Olsen *et al.*, 2004; Muñiz-Salazar *et al.*, 2005).

While studies of *Z. marina* are abundant, those

of other *Zostera* species are scarce. The genetic structure of *Z. noltii*, a European species, was characterized with microsatellite markers (Coyer *et al.*, 2004), while the genetic structures of *Z. muelleri* in New Zealand (Jones *et al.*, 2008) and *Z. caespitosa* in Japan (Tanaka *et al.*, 2002) were characterized with random amplified polymorphic DNA (RAPD) markers.

Four *Zostera* species are endemic to the ocean surrounding the Japanese archipelago. Of these species, *Z. caespitosa* Miki is distributed in northern Japan, Korea, and northeast China (Miki, 1933; Tanaka *et al.*, 2009). The rhizomes sub-erect and do not creep; their growth habit is caespitose, which is rare character in seagrasses. Because it is probable that individuals cannot propagate vegetatively such as other *Zostera* species, comparison of the genetic structure of *Z. caespitosa* with that of other *Zostera* species will have important implications for the population genetics of seagrasses.

In this study, we surveyed the applicability of microsatellite markers described for *Z. marina* (Reusch *et al.*, 1999b; Reusch, 2000) to *Z. caespitosa*. Using these markers, we performed population genetic analysis of *Z. caespitosa* in northeast Japan.

Materials and Methods

Three *Zostera caespitosa* sites in the Otsuchi Bay and three in the Yamada Bay, two in the Mutsu Bay and one in the Notoro Lake were sampled between 1999 and 2002 (Fig. 1 and

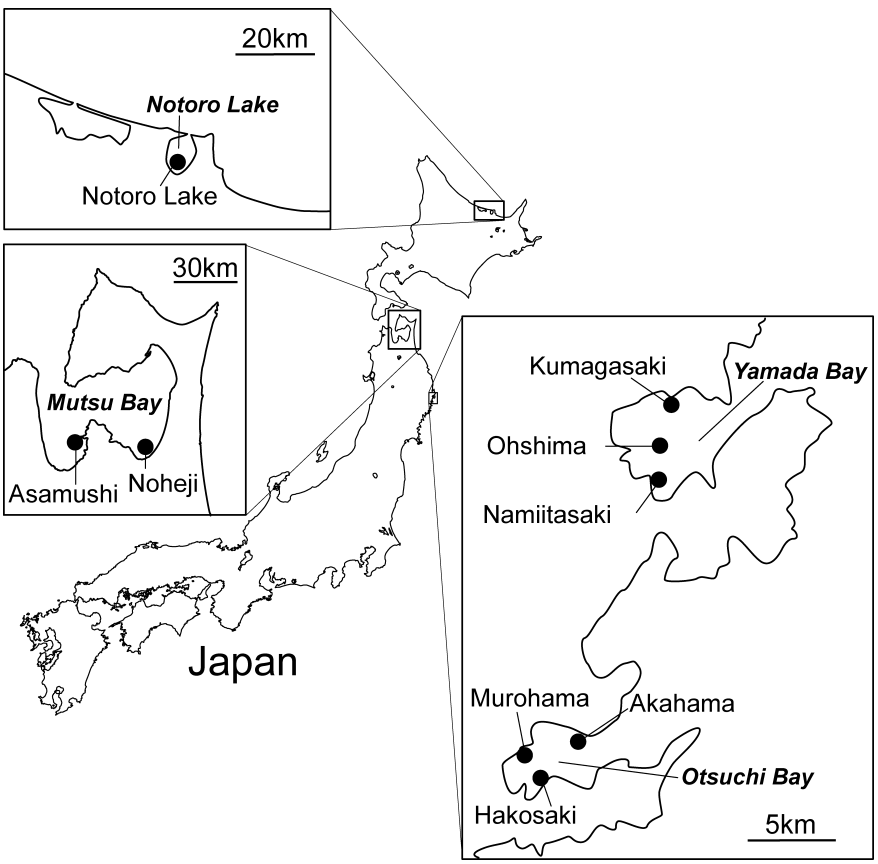

Fig. 1 Collection sites of *Zostera caespitosa* in this study. Closed circles indicate the positions of *Z. caespitosa* sites. Related information of each site is described in Table 1.

Table 1. *Zostera caespitosa* sites used in this study. The positions of collection sites are indicated in Fig. 1

Collection sites	Locality	Latitude	Longitude
Otsuchi Bay			
Akahama	Akahama, Otsuchi, Iwate Pref.	N39°21′02″	E141°55′56″
Hakosaki	Hakosaki, Kamaishi, Iwate Pref.	N39°19′54″	E141°55′16″
Murohama	Murohama, Kamaishi, Iwate Pref.	N39°20′25″	E141°54′49″
Yamada Bay			
Kumagasaki	Kumagasaki, Yamada, Iwate Pref.	N39°26′57″	E141°58′57″
Namiitasaki	Namiitasaki, Yamada, Iwate Pref.	N39°26′57″	E141°58′08″
Ohshima	Ohshima, Yamada, Iwate Pref.	N39°27′46″	E141°58′21″
Mutsu Bay			
Asamushi	Asamushi, Aomori, Aomori Pref.	N40°53′58″	E140°51′20″
Noheji	Noheji, Aomori Pref.	N40°52′25″	E141°07′46″
Notoro Lake			
Notoro Lake	Notoro Lake, Abashiri, Hokkaido Pref.	N44°00′59″	E144°07′31″

Table 1). From each site, 8 to 24 plants of *Z. caespitosa* were randomly selected and their leaves were collected. 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 leaf tissue using the CTAB (hexadecyltrimethyl ammonium bromide) method of Doyle and Doyle (1987).

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.*, 1999b: Zosmar CT-12, Zosmar CT-19, Zosmar CT-35, Zosmar CT-20, and Zosmar CT-17H; Reusch, 2000).

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 3100 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 site 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), average heterozygosity (Nei, 1987), and level of inbreeding (F_{IS}; Weir and Cockerham, 1984). 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, 1995) only for allelic richness.

Genetic differentiation and distances were quantified by calculating the proportion of genetic variance (F_{ST}) (Weir and Cockerham, 1984) using Arlequin 3.11 (Excoffier *et al.*, 2005). 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 among bays and lake (Otsuchi Bay, Yamada Bay, Mutsu Bay and Notoro Lake), among sites, 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

Of the 12 microsatellite markers tested for PCR amplification, four markers (Zosmar CT-3, Zosmar GA-1, Zosmar GA-2, and Zosmar GA-6) showed stable amplification and polymorphism. These four loci were selected for subsequent analyses.

Clonal diversity varied from 0.48 (Notoro Lake) to 1.00 (Noheji). The mean number of alleles per locus and site ranged from 8 (Akahama) to 17 (Asamushi; Table 2). Allelic richness ranged from 1.77 (Akahama) to 2.46 (Ohshima). Private alleles were observed in sites from Ohshima, Asamushi, and Noheji. Average observed heterozygosity for all sites was between 0.375 (Kumagasaki) and 0.600 (Noheji), and average expected heterozygosity (H_E) was between 0.343 (Kumagasaki) and 0.544 (Noheji; Table 2). All sites were in Hardy–Weinberg equilibrium (HWE). Although F_{ST} value varied from 0.002 to 0.258, significant genetic differentiation was detected among all sites, except between Akahama and Murohama, Namiitasaki and Ohshima, and Asamushi and Noheji ($P > 0.05$; Table 3). Analysis of the genetic relationship among the nine sites using a neighbor-joining (NJ) tree revealed that the sites fell into four groups: three in Otsuchi Bay, three in Yamada Bay, two in Mutsu Bay, and the Notoro Lake site (Fig. 2). AMOVA revealed significant genetic variation at two spatial levels: among sites within

Table 2. Genetic diversity and clonality of *Zostera caespitosa* in this study

Site	n	G	C	A	Ar	Pa	H_O	H_E	F_{IS}
Akahama	8	7	0.88	8	1.77	0	0.500	0.453	−0.113
Hakosaki	19	13	0.68	11	1.98	0	0.500	0.411	−0.225
Murohama	21	12	0.57	10	1.74	0	0.429	0.379	−0.136
Kumagasaki	24	20	0.83	15	2.03	0	0.375	0.343	−0.096
Namiitasaki	17	16	0.94	16	2.30	0	0.456	0.436	−0.048
Ohshima	21	18	0.86	16	2.46	1	0.571	0.501	−0.144
Asamushi	24	20	0.83	17	2.25	2	0.417	0.392	−0.064
Noheji	10	10	1.00	14	2.33	1	0.600	0.544	−0.11
Notoro Lake	23	11	0.48	10	1.82	0	0.435	0.420	−0.037
Average	18.6	14.1	0.79	13.0	2.08	0.4	0.476	0.431	−0.108

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; F_{IS}, inbreeding coefficient.

Table 3. Pairwise F_{st}-values (Weir and Cockerham, 1984) among nine *Zostera caespitosa* sites in this study

	Akahama	Hakosaki	Murohama	Kumagasaki	Namiitasaki	Ohshima	Asamushi	Noheji
Hakosaki	0.128							
Murohama	0.029*	0.109						
Kumagasaki	0.066	0.223	0.183					
Namiitasaki	0.112	0.173	0.222	0.021				
Ohshima	0.064	0.120	0.138	0.038	0.020*			
Asamushi	0.059	0.149	0.084	0.051	0.090	0.034		
Noheji	0.077	0.139	0.108	0.065	0.079	0.053	0.002*	
NotoroLake	0.153	0.258	0.216	0.059	0.128	0.092	0.042	0.078

* Genetic differentiation was not detected ($P > 0.05$).

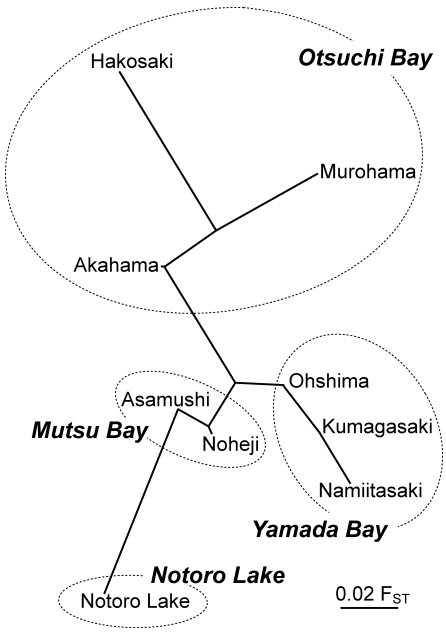

Fig. 2 Neighbor-joining (NJ) tree based on F_{ST} values (Weir and Cockerham, 1984) among *Zostera caespitosa* sites in this study.

Table 4. Analysis of molecular variance (AMOVA) for *Zostera caespitosa* sites

Source of variation	d. f.	Variance component	%Total variance	*P*-value
Among bays and lake	3	0.065	7.54	$P = 0.002$
Sites/bays and lake	5	0.039	4.47	<0.001
Individuals/ sites	325	0.759	87.99	<0.001

each bay and lake and among individuals within each site. Some genetic variation between bays and lake was detected ($P = 0.002$; Table 4). Significant correlation between genetic and geographical distances was not detected among all sites (Mantel test, $P > 0.05$), whereas significant correlation was detected among the six sites in Otsuchi Bay and Yamada Bay ($P < 0.01$; Fig. 3).

Discussion

Genetic diversity (H_E) of *Z. marina* is reported to range from 0.15 to 0.69 in North America and Europe (Olsen *et al.*, 2004), 0.491 to 0.563 (average = 0.539) in San Quintin Bay, Mexico (Muñiz-Salazar *et al.*, 2006), 0.312 to 0.541 in the Southern California Bight, Mexico (Coyer *et al.*, 2008), 0.464 to 0.708 (average = 0.620) in Tokyo Bay, Japan (Tanaka *et al.*, 2011), and 0.364 to 0.690 (average = 0.567) in Sagami Bay, Japan (Tanaka and Lim, 2011). Coyer *et al.* (2004) showed the diversity of *Z. noltii* to lie between 0.297 and 0.667. The genetic diversity of *Z. caespitosa* in this study (0.343 to 0.544) fell within these ranges (Table 2). However, the average value for *Z. caespitosa* is 0.431, which is lower than that of *Z. marina* reported in the previous studies. Because the rhizomes of *Z. caespitosa* sub-erect and do not creep unlike other *Zostera* species, we believe that this species increases mainly by sexual propagation. Accordingly, we predicted a higher genetic diversity in the experimental populations than in other *Zostera* species. However, the genetic diversity in this study was low. Genetic variation of *Z. caespitosa* based on RAPD analysis was also characteristic of inbreeding plant species (Tanaka *et al.*, 2002). Although we observed that *Z. caespitosa* is protogynous similar to *Z. marina* (De Cock, 1980), it may be geitonogamous, given that an individual generates several flowering shoots simultaneously. However, because the clonal diversity was lower than that of *Z. marina* in Tokyo Bay (0.9) and Sagami Bay (0.8), and all sites in this study were in HWE, the low genetic diversity could result from vegetative reproduction rather than from geitonogamy. Further studies are needed to analyze other sites and regions using more loci.

Genetic differentiation was detected among most sites, though not between Akahama and Murohama, Namiitasaki and Ohshima, and Asamushi and Noheji (Table 3). In a NJ tree based on F_{ST} values, all sites were grouped into four clusters, which were consistent with the geographical distribution (Fig. 2), although AMOVA did not show significant genetic differentiation among bays and lake (Table 4). By RAPD analy-

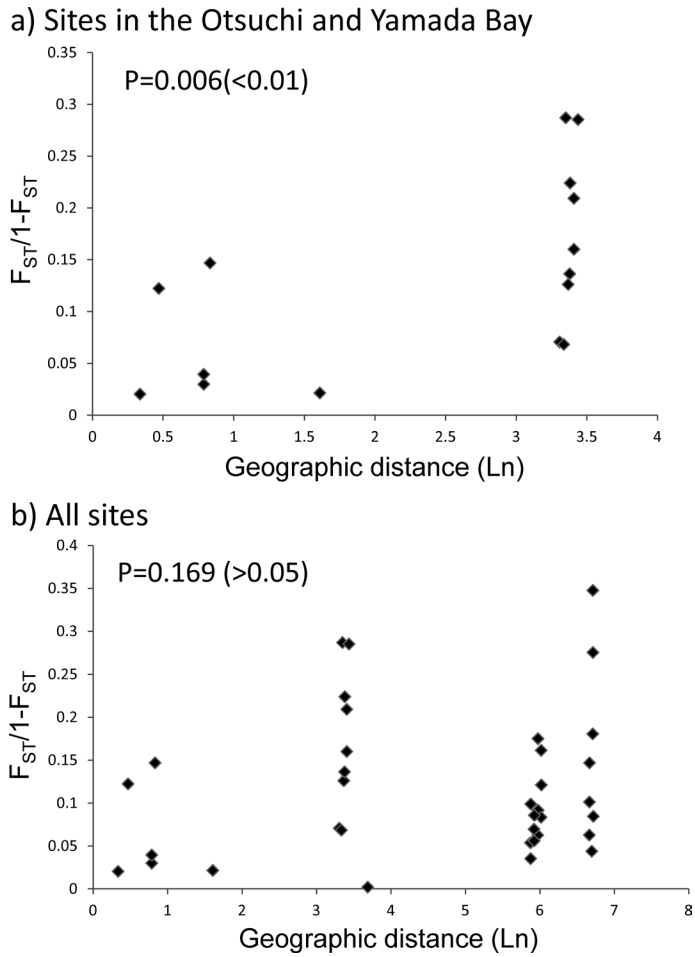

Fig. 3. Isolation-by-distance (IBD) used Mantel tests for *Zostera caespitosa* sites in this study based on F_{ST} as genetic distance. a) Six sites within the Otsuchi Bay and Yamada Bay: b) All *Z. caespitosa* sites. Pairwise genetic differentiation between populations were linearized and plotted against the geographic distance.

sis, in contrast, sites of Otsuchi Bay were not grouped into a single cluster (Tanaka *et al.*, 2002). Lougheed *et al.* (2000) asserted that microsatellites were superior to RAPD markers for discerning fine-scale genetic differentiation between subpopulations separated by tens of kilometers. Their assertion favors acceptance of the genetic relationships among sites of Otsuchi Bay and Yamada Bay observed in the present study.

Seeds of *Z. caespitosa* are probably dispersed by means of detached generative shoots that drift on the surface of water similar to those of *Z. marina*. Often no IBD is observed when strong gene flow occurs among populations within a slightly closed region such as a gulf or a bay (Muñiz-Salazar *et al.*, 2005, 2006; Tanaka *et al.*, 2011). This could be due to nearly saturated gene flow in a closed environment. IBD was detected among the six populations of Otsuchi Bay and Yamada Bay (Fig. 3b), but no IBD was detected among the sites within each bay ($P > 0.05$; result not shown). There was also no genetic differentiation between the two sites of Mutsu Bay. We conclude that strong gene flow occurs in *Z. caespitosa*, at least in nearly closed environments such as bays. IBD was also not detected among all sites (Fig. 3a). Besides the present study sites,

Z. caespitosa sites occur in many other regions of Japan (Tanaka *et al.*, 2009). To evaluate large-scale genetic structure such as that among bays and regions, it will be necessary to sample many sites in other regions using not only microsatellite analysis but also haplotype analysis based on DNA sequence.

The present situation of *Z. caespitosa* sites in Otsuchi Bay and Yamada Bay is unknown after the tsunami of March 11, 2011. The results of our study may be useful for evaluating the influence of the tsunami on the genetic structure of the species, owing to its disappearance or decline and subsequent regeneration by growth and/or reintroduction.

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