Genetic characteristics of three Baltic *Zostera marina* populations*

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Abstract

We performed genetic analyses of three Baltic eelgrass (*Zostera marina*) populations in Puck Bay (PB), Cudema Bay (CB) and Greifswalder Bodden (GB). The aim of this study was to identify the eelgrass population genetically closest to that from the PB, which could potentially serve as a reservoir for the restoration of the underwater meadows in this bay, seriously degraded in the past. We applied a 12-microsatellite assay to test the genetic distance between the target eelgrass populations. We found that the allelic richness values of the GB, PB and CB populations were 2.25, 3.77 and 3.50 respectively. The genetic diversity found in GB was low and could be explained by the population's history, whereas the diversity of CB was higher than expected in a population located at the edge of the species' range. Analyses of genetic differentiation and structure showed that of the three populations studied, PB and CB were closer to each other than to the GB population. The reasons for this differentiation in eelgrass populations and the implications of the results of their genetic analysis on the planned restoration of the PB populations are discussed.

1. Introduction

Underwater meadows are considered valuable though very vulnerable coastal habitats (Waycott et al. 2009). Their extinction could have serious consequences, as they provide an indispensable environment for many fish species as a spawning and hatching ground. They are also an important aspect of protection against coastal erosion (Orth et al. 2006, Tanner et al. 2010). According to Short et al. (2011), nearly 25% of all seagrass species are threatened. The main reasons for the deterioration of underwater meadows are human activities, water pollution, diseases and rising water temperatures.

Eelgrass (*Zostera marina*) is a seagrass species, common along the shallow sedimentary coasts of the Northern Hemisphere (Olsen et al. 2004), forming dense meadows, both perennial and annual (Hammerli & Reusch 2003, Muniz-Salazar et al. 2005). Eelgrass reproduces sexually by hydrophilous pollination and also vegetatively (clonally) by rhizomes (Diekmann & Serrao 2012). Eelgrass populations usually consist of several clones, varying greatly in size. The size of the clones was shown to correlate with their fitness (Hammerli & Reusch 2003). During the last 50 years, the number and size of eelgrass meadows has declined dramatically (Baden et al. 2003, Frederiksen et al. 2004) and they have become the target of many aquatic restoration projects (Fonseca et al. 1998, Hizon-Fradejas et al. 2009, van Katwijk et al. 2009, Busch et al. 2010, Campanella et al. 2010, Tanner et al. 2010).

Eelgrass losses caused by several factors (harvesting for agar production, motor boating, water pollution and intensive algal blooms) are particularly

heavy along the Polish Baltic coast (Andrulewicz 1997, Węsławski et al. 2009, Węsławski et al. 2013). Since 2006, eelgrass has been on the *Polish red list of threatened plant and fungi species* (http://water.iopan.gda.pl/projects/Zostera/planting.html). The degradation of eelgrass meadows, together with overfishing, has seriously affected fish populations in Puck Bay. Adapted to brackish waters, the populations of two fish species there – northern pike (*Esox lucius*) and pike-perch (*Sander lucioperca*) – are close to extinction. On the initiative of local fishermen's communities, a project to restore these two fish species in Puck Bay was started in 2010. To improve the chances of success of the fish-restocking programme, the parallel restoration of the eelgrass meadows was envisaged.

The genetic structure of various eelgrass populations was studied by Olsen et al. (2004), subsequently followed by several other authors (Campanella et al. 2010, Campanella et al. 2012, Diekmann & Serrao 2012, Kamel et al. 2012, Ort et al. 2012, Reynolds et al. 2012, Peterson et al. 2013 and references therein). Before 2010, however, nothing was known about the genetic and clonal structure of eelgrass populations from Puck Bay and its other populations in the southern and eastern Baltic. The clonal structure reflects the rate of the species' vegetative and sexual reproduction and has a crucial impact on the demographic and genetic structure of populations (Halkett et al. 2005). According to guidelines formulated in the literature (van Katwijk et al. 2009 and references therein), knowledge of the genetic properties of eelgrass populations is one of the most important factors determining the strategy for its restoration and the selection of an appropriate donor population.

In this paper, we present the results of genetic analyses of the eelgrass population from Puck Bay and two other populations – from Cudema Bay and Greifswalder Bodden, which are potential sources of planting material for the restoration of the Puck Bay underwater meadows. We developed two multiplex PCR assays for screening 12 highly polymorphic microsatellites (msDNA) arranged in two sets and loaded on two sequencing panels. The genetic polymorphism indices of the three populations that we studied were compared with those obtained by other authors (Olsen et al. 2004, Diekmann & Serrao 2012).

2. Material and methods

2.1. Sample collection

Eelgrass specimens (floating shoot fragments) were collected in Puck Bay (PB), Poland ($N = 23$), Cudema Bay (CB), Sarema Island, Estonia, ($N = 24$) and Greifswalder Bodden (GB), Rügen Island, Germany ($N = 23$)

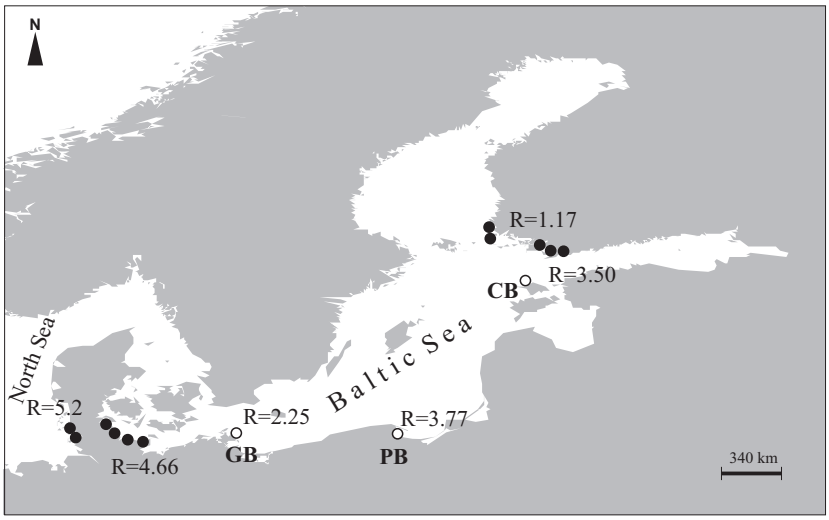

Figure 1. Locations of the eelgrass populations: GB – Greifswalder Bodden; PB – Puck Bay; CB – Cudema Bay. Populations studied by Olsen et al. (2004) and/or Diekmann & Serrao (2012) are represented by black dots. Mean values of allelic richness (R) are given

(Figure 1). At each location shoots were collected at 1 m intervals at least. Care was taken to collect samples from various parts of each of the three bays. After collection, shoot fragments were cryopreserved in liquid nitrogen and stored at −70°C for further analysis.

2.2. DNA extraction and microsatellite analysis

DNA was extracted using the modified phenol:chloroform protocol (Sambrook & Russell 2006). Shoot fragments were homogenised in Fast Prep-24 Instrument (MP Biomedicals) in 1 ml of extraction buffer (0.2 M TRIS, 1% SDS, 1 mM EDTA, pH = 8) using lysing matrix tubes A (MP Biomedicals); for better precipitation of the DNA, 100 μl of 3 M NaAC (pH = 5), 100 μl of LINEAR ACRYLAMIDE (Invitrogen) and 6 μl of PINK (EMD Millipore) were added. To prevent DNA degradation all manipulations were performed on ice. DNA was resuspended in 100 μl of water (Sigma-Aldrich).

12 msDNA loci (Table 1) developed for eelgrass by Reusch et al. (1999) and Reusch (2000a) were assigned to the two multiplexes according to the published allele length. Each multiplex was checked *in silico* using FASTPCR v.3.8.41 software (Kalendar et al. 2011) and each primer pair was tested for potential primer dimerisation. PCR reactions for microsatellite amplification were performed with forward primers labelled with either

Table 1. Characteristics of the two multiplex PCR assays for the amplification of 12 microsatellite loci in eelgrass

Multiplex set	Fluorescent label	Locus name	Primer concentration [μM]	Allele size range	Number of alleles	Allelic richness	Expected heterozygosity
1	6-FAM	CT35	0.1	78–114	14	8.24	0.77
		CT19	0.1	134–146	4	2.31	0.43
	HEX	CT12	0.4	108–140	5	2.37	0.16
		GA6	0.4	160–230	5	3.42	0.46
	TAMRA	CT3	0.2	101–219	10	3.85	0.45
	ROX	GA4	0.2	123–239	5	2.97	0.22
2	6-FAM	GA3	0.3	101–111	5	3.09	0.37
		CT20	0.1	147–159	5	2.41	0.18
	HEX	GA5	0.4	145–155	5	4.06	0.53
		GA2	0.1	158–168	5	2.84	0.53
	TAMRA	CT17	0.3	121–151	15	8.59	0.8
	ROX	GA1	0.1	117–139	8	4.03	0.45

6-FAM, HEX, TAMRA or ROX fluorescent dyes (Applied Biosystems). The optimised reaction mixture (10 μl) contained approximately 100 ng DNA, 1 x MasterMix (Qiagen) and primers at the concentrations given in Table 1. The reactions included an initial denaturation step (15 min at 95°C) followed by 35 cycles of denaturation (30 s at 94°C), annealing (90 s at 60.5°C), extension (40 s at 72°C) and a final extension step of 30 min at 60°C. PCR products were loaded separately on a Genetic Analyser ABI 3730 xl (Applied Biosystems). Allele sizes were scored against the GeneScan LIZ 600 size standard (Applied Biosystems) using PeakScanner v.1.0 (Applied Biosystems).

2.3. Statistical analysis

We used GENCLONE 2.0 software (Dorken & Eckert 2001, Arnaud-Haond & Belkhir 2007) to identify the number of clones, to characterise the clonal diversity and to calculate the statistical power of the marker set for discrimination among clones.

Potential genotyping errors, which might result from stuttering, allelic dropouts or null allele appearance, were checked with MICRO-CHECKER v.2.2.3 freeware (Van Oosterhout et al. 2004) for each of the studied populations using 1000 iterations and 95% CI. The frequency of null alleles was estimated using the same software by applying the Brookfield (1996) method. We used ARLEQUIN v.3.5.1.2 (Excoffier et al. 2005) to test for

linkage disequilibrium (LD) using 1000000 steps in the Markov chain and 100000 dememorisation steps.

The number of alleles (N_A), observed (H_O) and expected heterozygosity (H_E) per locus and population, probability of identity value (PI) and principal coordinate analysis (PCoA) of genetic distance were assessed using GENEALEX v.6.5. (Peakall et al. 2012). Allelic richness (R) and genetic differentiation between populations (F_{ST}) were obtained with FSTAT v.2.9.3.2 software (Goudet 1995). The statistical significance of the F_{ST} values was calculated using a permutational test (Excoffier et al. 1992) with 10000 permutations over all loci as implemented in FSTAT v.2.9.3.2. The inbreeding coefficient (F_{IS}) was calculated using the GENEPOP v.4.0 program (Rousset 2008). The statistical significance of F_{IS} values was estimated using the Hardy-Weinberg exact test (Guo & Thompson 1992) with the Markov Chain Monte Carlo (MCMC) method (1000 dememorisation steps, 1000 batches, 1000 iterations per batch) as applied in GENEPOP v.4.0.

To detect a recent reduction in the population size the BOTTLENECK program (Cornuet & Luikart 1996) was used. The two-phase mutation model (T.P.M.) with 95% of S.M.M. and 12% variance was applied. The significance of heterozygosity excess was tested by the Wilcoxon sign-rank test and the mode-shift test, which evaluates the allele frequency distribution.

To infer population structure the STRUCTURE v.2.3.3 program (Pritchard et al. 2000) was run. The admixture model and the correlated allele frequencies were used with no prior population information. 10 independent runs for the number of genetically different clusters (K) ranging from 1 to 10 were performed using the Markov-chain method with 100000 length of burn in steps followed by 1000000 Markov Chain Monte Carlo steps. Individuals were assigned to genetic clusters based on probability of membership. The most probable number of clusters was determined using the ΔK method (likelihood probability, Evanno et al. 2005) in the STRUCTURE HARVESTER v.0.6.8 program (Earl & von Holdt 2012). Average coefficients of membership across the 71 replicates for the optimal ΔK were computed using the CLUMPP program (Jakobsson & Rosenberg 2007). DISTRUCT software (Rosenberg 2004) was used to graphically display the membership coefficient of an individual to separate clusters.

3. Results

Three eelgrass populations – Puck Bay (PB), Cudema Bay (CB) and Greifswalder Bodden (GB) – were characterised genetically. Their locations are shown on the map (Figure 1) together with those of some Baltic

Table 2. Within-population genetic diversity of 12 microsatellite loci in the three eelgrass populations from Puck Bay, Cudema Bay and Greifswalder Bodden

Population	Sample size	Number of different genotypes	Number of alleles	Number of private alleles	Allelic richness	Inbreeding coefficient	Observed heterozygosity	Expected heterozygosity
Puck Bay	23	20	4.92	1.92	3.77	0.13*	0.45	0.52
Cudema Bay	24	24	4.58	1.75	3.50	0.17*	0.34	0.42
Greifswalder Bodden	23	8	2.25	0.42	2.25	−0.41*	0.55	0.40

*$p < 0.05$.

and North Sea populations studied by other authors (Olsen et al. 2004, Diekmann & Serrao 2012).

Two multiplexes, 6 microsatellites each (Table 1), were developed to estimate clonal diversity and genetic polymorphism within the target populations. The amplification effectiveness of all loci was very high (99.09–100%). The PI value of the marker set we used was 3.9×10^{-8}, indicating a high power of identification of unique genotypes. Genetic profiles for 23, 24 and 23 eelgrass shoots from the PB, CB and GB populations respectively were obtained. We distinguished 20 multilocus genotypes in the PB population and eight in the one from GB (Table 2). The CB population consists of individuals with a different genotype. Thus, clonal diversity in the three populations was 0.86 (PB), 0.32 (GB) and 1.00 (CB).

There was no significant LD for any pair of loci. Similarly, no evidence of significant scoring errors resulting from stuttering, large allele dropout or null alleles presence was recorded. All microsatellite loci were therefore included in further analyses. Altogether, 86 alleles were scored (Table 1), on average 7.17 per locus, ranging from 4 alleles at locus CT19 to 15 at CT17. All three populations shared only 18 of them. Out of 47 private alleles 23, 20 and 4 belonged to the PB, CB and GB populations respectively.

The genetic polymorphism indices of the three populations are shown in Table 2. The average observed heterozygosity (H_O) of the three populations was 0.46 ($SE = 0.08$). The mean expected heterozygosity in the PB, CB and GB collections was 0.45 ($SE = 0.04$). All three populations showed relatively low allelic richness values (mean $R = 3.17$), but the GB population appeared to be much less polymorphic than the other two. This was especially evident when the values of expected heterozygosity (H_E) and allelic richness (R) were compared. The GB population also had the lowest number of private alleles (Table 2). Generally, the genetic diversities of the PB and CB populations were similar to one another but different from that of GB. All the populations showed statistically significant deviations from HWE equilibrium with either significant positive (PB and CB) or negative (GB) F_{IS} values (Table 2). We had checked whether the negative F_{IS} value was due to a genetic bottleneck in the history of this population but we found no evidence for it.

Table 3. Pairwise F_{ST} estimates for the three eelgrass populations based on 12 microsatellite loci

	Cudema Bay	Greifswalder Bodden
Puck Bay	0.06*	0.11*
Cudema Bay		0.19*

*$p < 0.05$.

The results of the genetic differentiation analyses (F_{ST}) are shown in Table 3, which indicates a significant level of genetic distance among the three populations. Following the interpretation of Balloux & Lugon-Moulin (2002), the differentiation between CB and GB should be regarded as large, while that between PB and GB and that between PB and CB as moderate. It is worth noting that the genetic distance between PB and CB was less

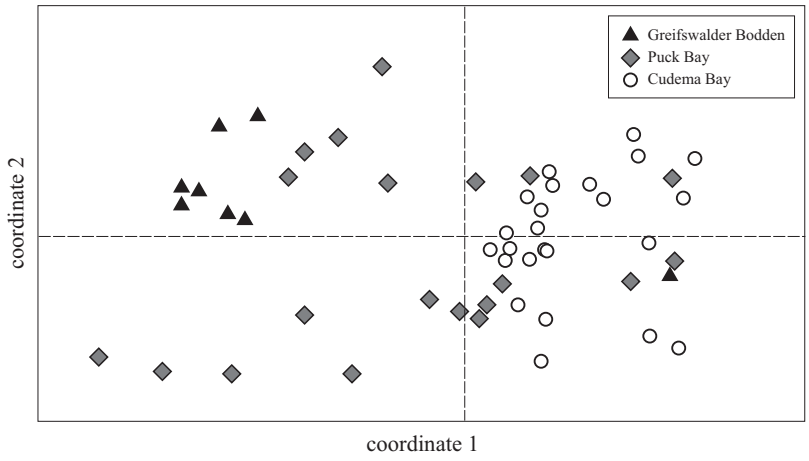

Figure 2. Principal coordinate analysis (PCoA) based on individual pairwise genetic distances estimates, showing associations between different eelgrass genotypes found in the three populations

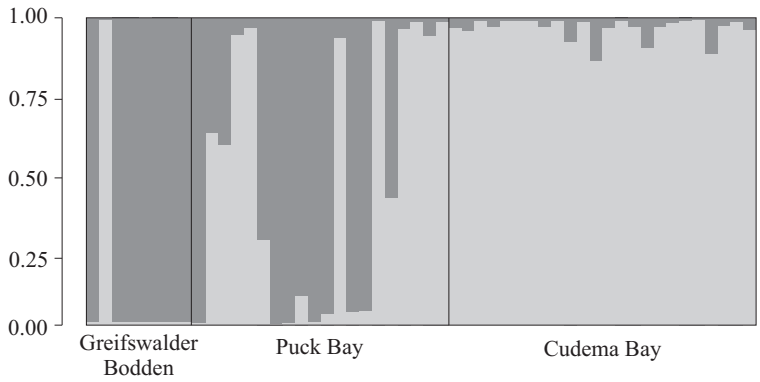

Figure 3. Bar plot of the probability of assigning each individual of a different genotype (GB $N = 8$; PB $N = 20$; CB $N = 23$) to the two inferred genetic clusters ($K = 2$). Each individual of a different genotype is represented on the x-axis (vertical lines). The y-axis gives the probability of that individual belonging to one of the two genetic clusters (dark grey and light grey)

than between PB and GB (Table 3), whereas the geographic distances are ca 1000 and 400 km respectively. The greatest genetic distance ($F_{ST} = 0.19$) was found between the CB and GB populations, which was clearly visualised by the PCoA analysis (Figure 2), showing that the proportion of individuals with a similar genetic profile in these two populations is very small.

The result of the assignment test performed in STRUCTURE is presented in Figure 3. We tested the assignation of sampled individuals to different numbers of genetic clusters (K), ranging from one to 10; we found that the most probable number of genetic clusters was two ($K = 2$). The ΔK values obtained for all the remaining numbers of clusters ($K = 3 - 10$) appeared to have much lower values than for $K = 2$. The result of the assignment test (Figure 3) is therefore in agreement with the one obtained with the PCoA analysis (Figure 2): the populations in CB and PB are genetically closer to each other than to the one in GB.

4. Discussion

Because of the endangered status of seagrass *Zostera marina* and its importance for coastal water ecosystems, studies of the population genetics of this species are expected to become more and more common. For this reason, we developed a multiplex panel permitting the assay of 12 microsatellite loci in two sets, each with 6 loci. The multiplex is composed of msDNA loci described by other authors and already used in analyses of polymorphism in eelgrass populations (Reusch et al. 1999, Reusch 2000b, Reusch 2002). We believe that the multiplex we optimised should facilitate further analyses of genetic structure of populations of this species, and also substantially lower their cost.

The PB population is of special interest as it has become seriously degraded and is in urgent need of restoration. Eelgrass is a key habitat-forming species and in the case of PB indispensable for the maintenance of fish populations, especially of pike and pike-perch, two species that the local fishery and numerous anglers depend on. It is known that populations of eelgrass and top predatory fish are mutually dependent. The eelgrass meadows provide a convenient spawning ground for fish and a shelter for fry. On the other hand, a reduction in size of top predatory fish results in an increase in the number of intermediate predators and herbivorous fish. There is thus greater pressure on mesograzers and zooplankton, leading to the overgrowth of ephemeral algae and phytoplankton as well as to the eutrophication and degradation of the eelgrass meadows (Moksnes et al. 2008, Baden et al. 2010).

The other two populations analysed are from the Greifswalder Bodden (GB) and Cudema Bay (CB), about 400 km to the west and 1000 km

to the north-east of PB respectively. Our results revealed that the three populations are genetically distinct, differing both in the clonal structure and in the level of genetic polymorphism.

Olsen et al. (2004) claim that the North Sea and western Baltic populations of eelgrass, occupying the central part of its range, should exhibit higher allelic richness than those at the limits of the species' distribution. The situation we found in the Baltic seems to be somewhat different. The GB population, the nearest to the 'differentiation hotspot', has the lowest allelic richness and a much more explicit clonal structure, while in the CB population, situated close to the limits of the eelgrass range in the Baltic, no clones were spotted among 24 individuals and the allelic richness was similar to that observed in the North Sea populations (Figure 1).

The low genetic polymorphism of the GB population is understandable, given that this population dramatically decreased in size in the 1990s as a result of the bay's eutrophication (Munkes 2005). The high level of genetic polymorphism in the CB population is more difficult to explain, however. This population is much more variable than several other populations located further north still, off the coast of Finland (Olsen et al. 2004). These populations are regarded as being at the 'leading edge' of the species range (Olsen et al. 2004). The genetic polymorphism of the CB population could have been higher because of the set of 12 markers we used, as against the nine msDNA loci used by Olsen et al. (2004). However, the additional analysis of genetic polymorphism that we performed by testing the nine markers used by Olsen et al. (2004) (data not shown) showed that it was immaterial whether nine or 12 loci were analysed. One can assume that Cudema Bay, being the southernmost part of the Gulf of Finland, was colonised by eelgrass much earlier than the rest of the gulf.

We did not find any correlation between geographical and genetic distance (data not shown). The pairwise F_{ST} values are lower between the PB and CB than between the PB and GB populations, which are located much closer to each other. The STRUCTURE analysis (Figure 3) showed that the genetic characteristics of the GB and CB populations are quite different, whereas the PB population is intermediate. This may suggest that a small-scale gene flow occurred between the three populations. The Baltic Sea is known for its strong currents, frequently changing direction depending on the strength and direction of winds. The long-distance dispersal of eelgrass shoots over the open water, caused by currents or wind, has already been observed (Reusch 2002, Harwell & Orth 2002).

The differences we observed in the genetic structure of the three populations most probably result from their adaptation to local environmental

conditions and their history. These conditions could influence the means of eelgrass reproduction (sexual versus vegetative), which will lead to differences in the population's clonal structure (Halkett et al. 2005).

According to the guidelines listed by van Katwijk et al. (2009) for the restoration of seagrass meadows, the donor population should be polymorphic and genetically as close as possible to the restored one. Moreover, the donor plants should be recruited from populations existing in comparable environments. The CB population but not the one from GB meets the first criterion. To satisfy the second one, we recommend using native PB plants as donors.

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