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Population genetics of dwarf eelgrass *Zostera noltii* throughout its biogeographic range

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ABSTRACT: The marine angiosperm *Zostera noltii* (dwarf eelgrass), an important facilitator species and food source for invertebrates and waterfowl, predominantly inhabits intertidal habitats along eastern Atlantic shores from Mauritania to southern Norway/Kattegat Sea and throughout the Mediterranean, Black and Azov seas. We used 9 microsatellite loci to characterize population structure at a variety of spatial scales among 33 populations from 11 localities throughout the entire biogeographic range. Isolation by distance analysis suggested a panmictic genetic neighborhood of 100 to 150 km. At the global scale, a neighbor-joining tree based on Reynolds distances revealed strongly-supported groups corresponding to northern Europe, Mauritania and the Black/Azov Sea; separate Mediterranean and Atlantic-Iberian groups were poorly supported. Clones (genets with multiple ramets) were present in most populations but were generally small (ca. <3 m^2). Exceptions were found in Mauritania (ca. 29 m in length), the Azov Sea (ca. 40 m in length) and the Black Sea (ca. 50 m in length). Although genetic diversity and allelic richness generally decreased from Mauritania to Denmark, the putative post-glacial recolonization route, both were unexpectedly high among populations from the German Wadden Sea.

KEY WORDS: Seagrasses · *Zostera noltii* · Genetic structure · Clonality · Genets · Ramets · Ice age · Microsatellites · Phylogeography

INTRODUCTION

Seagrasses are recognized as important habitat-modifying or facilitator species because they create spatially complex structure, enhance supply and retention of propagules, and increase resource supply for a variety of associated species (Bruno & Bertness 2001, Williams & Heck 2001). Their facilitator status is tempered, however, by the fact that seagrass meadows have suffered dramatic declines worldwide during the past 25 years through a combination of natural and anthropogenic deterioration (Short & Wyllie-Echeverria 1996, Hemminga & Duarte 2000, Green & Short 2003). Effective management of threatened seagrass populations requires population genetic information with regard to dispersal, genetic neighborhood size and diversity (Reusch 2001a).

All seagrass species reproduce sexually through seeds and vegetatively through production of horizontal rhizomes and leaf shoots (Den Hartog 1970). Sexual reproduction, however, is thought to be constrained because of hydrophilous pollination of flowers, limited dispersal of pollen and seeds, limited outcrossing rates associated with extensive clonal growth, and low survivorship of seeds and seedlings (Les 1988, Laushman 1993, Hemminga & Duarte 2000, Reusch 2001b). This life-history bottleneck, in combination with extensive clonal (vegetative) growth, led to the prediction that

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seagrass meadows would harbor low clonal diversity and be strongly structured with little gene flow (Les 1988, Barrett et al. 1993, Hemminga & Duarte 2000).

Recent findings of DNA-based population genetic studies, however, reveal that subtidal seagrass populations can vary widely in these population parameters. For example, genet size ranged from single shoots to large patches (10s of m) to entire meadows (100 to 1000 m) (Waycott 1995, Kirsten et al. 1998, Procaccini & Mazzella 1998, Reusch et al. 1999a, 2000, Ruggiero et al. 2002, Billingham et al. 2003) and populations displayed weak to excessively positive (heterozygous deficiency) or negative (heterozygous excess) deviations from Hardy-Weinberg Equilibrium (HWE) (Procaccini & Mazzella 1998, Reusch et al. 2000, Procaccini et al. 2002, Billingham et al. 2003). Furthermore, some populations of the subtidal eelgrass *Zostera marina* separated by 33 to 54 km displayed non-significant differentiation, presumably because of connectivity due to rafting reproductive shoots (Reusch 2002). Variable population subdivision and isolation by distance have been detected among populations of several seagrass species (see review by Reusch 2001a) and in *Z. marina* (Olsen et al. 2004).

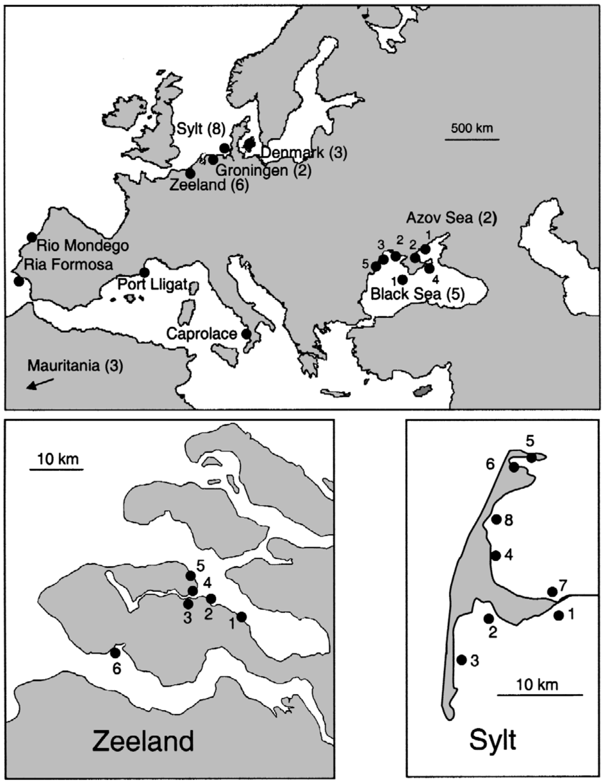

Fig. 1. *Zostera noltii*. Localities and populations sampled. Parenthetical values indicate the number of populations sampled within each locality. Sylt is an island connected by a narrow causeway to mainland Germany (on the map, thin line to right) near the German-Danish border. For reference, the locations of population 4 at Zeeland and Sylt are 51° 33′ N, 3° 52′ E and 54° 55′ N, 8° 20′ E, respectively

The dwarf eelgrass *Zostera noltii* is widely distributed along the Atlantic shores of Europe from temperate southern Norway to tropical Mauritania, as well as throughout the Kattegat, Mediterranean, Black, Azov, Caspian and Aral Seas (Green & Short 2003). It is one of the few seagrass species that is predominantly intertidal, although it also occurs subtidally in brackish waters of the Kattegat Sea and along the Mediterranean coast (Den Hartog 1970), often co-occurring with *Z. marina* and *Cymodocea nodosa*, respectively.

Several studies of *Zostera noltii* along the coasts of Italy and The Netherlands concluded that sexual reproduction was unimportant relative to asexual reproduction (Hootsmans et al. 1987, Harrison 1993, Philippart 1995, Curiel et al. 1996) and high seed production (9000 seeds m^{-2}) did not correspond to the very low number of seeds (0 to 150 m^{-2}) actually observed in the sediments (Hootsmans et al. 1987). Additionally, the specific requirements of low salinity and tegument incision for seed germination, combined with the high seed and seedling mortality, and rare seedling occurrence observed at sites in the Mediterranean and The Netherlands, further suggested that seeds and sexual reproduction were of minor importance in propagating *Z. noltii* (Den Hartog 1970, Jacobs et al. 1983, Hootsmans et al. 1987, Vermaat et al. 1987, Loques et al. 1990, Philippart 1995, Vermaat & Verhagen 1996). More recently, however, up to 1000 seeds m^{-2} have been found in sediments off the German coast (Sylt) in October (Ehlers 2002), thereby questioning the generality of earlier findings.

In the present study, we ask: (1) How does geographic scale influence levels of diversity and connectivity of *Zostera noltii* populations? (2) Do latitudinal and longitudinal patterns of genetic and allelic diversity reflect recolonization pathways from southern refugia since the Last Glacial Maximum (LGM)? (3) To what extent does an intertidal existence influence genetic structure and isolation by distance? We approach the questions using microsatellite DNA polymorphisms to first assess population-level diversity at the meter to thousands-of-kilometers scale, then determine population disjunctions and putative gene flow between populations using neighbor-joining (NJ) analysis of pairwise genetic distances and a stepping-stone model of gene flow (isolation by distance).

MATERIALS AND METHODS

Sampling sites and collections. 1 to 8 populations were sampled at each locality (Fig. 1, Table 1) by walking along a linear transect and collecting 45 to 50 indi-

Table 1. *Zostera noltii.* Genetic variability and F_{IS} (f) estimates for all 9 microsatellite loci. The total number of individuals collected is presented in column 1; H_{exp}, H_{obs} and the average number of alleles per locus (α) were calculated for all genets (column 5), as well as for a standardized genet sample size of 28. $H_{exp\,=}$ Nei's gene diversity and * indicates significant departure from 0 (HWE) after sequential Bonferroni adjustment of Type I error level (Rice 1989); G/N = number of genets divided by total number of shoots

Population (n)	All genets								Genets = 28			
	H_{exp}	H_{obs}	α	No. genets	G/N	Genets with >1 ramet in sample	Ramets sampled genet^{-1}	F_{IS}	H_{exp}	H_{obs}	α	F_{IS}
DENMARK												
Myrbjerg (40)	0.376	0.325	5.2	39	0.97	1	2	0.150*	0.369	0.325	4.6	0.135
Ellinge (37)	0.415	0.420	5.1	37	1.00	–	–	0.001	0.409	0.417	4.7	−0.001
Ølstad (39)	0.339	0.297	4.2	37	0.95	2	2	0.136	0.356	0.329	3.8	0.092
GERMANY												
Sylt-1 (40)	0.623	0.642	7.0	36	0.90	4	2	−0.017	0.628	0.647	6.7	−0.011
Sylt-2 (50)	0.615	0.620	7.0	26	0.52	11	2–5	0.012	–	–	–	–
Sylt-3 (46)	0.630	0.633	7.7	40	0.87	6	2	0.007	0.630	0.647	7.2	−0.008
Sylt-4 (50)	0.614	0.634	7.2	31	0.62	13	2–4	−0.017	0.615	0.635	7.2	−0.015
Sylt-5 (49)	0.630	0.596	7.1	30	0.61	9	2–9	0.070	0.635	0.595	6.9	0.081
Sylt-6 (50)	0.675	0.667	8.7	39	0.78	8	2–4	0.026	0.651	0.627	7.8	0.056
Sylt-7 (50)	0.642	0.616	8.3	46	0.92	4	2	0.051	0.625	0.611	7.4	0.040
Sylt-8 (49)	0.651	0.643	8.4	43	0.88	6	2	0.024	0.628	0.627	7.3	0.020
THE NETHERLANDS												
Groningen-1 (227)[a]	0.526	0.490	4.8	22	0.10	13	2–20	0.091	–	–	–	–
Groningen-2 (44)[b]	0.592	0.584	6.6	43	0.97	1	2	0.025	0.603	0.579	6.0	0.057
Zeeland-1 (50)[c]	0.491	0.490	5.6	46	0.92	4	2	0.012	0.490	0.460	4.7	0.078
Zeeland-2 (47)[c]	0.437	0.465	4.4	44	0.94	3	2	−0.052	0.444	0.488	3.9	−0.082
Zeeland-3 (49)[c]	0.463	0.500	4.6	44	0.90	5	2	−0.070	0.457	0.528	4.0	−0.137*
Zeeland-4 (43)[c]	0.450	0.450	4.7	43	1.00	0	–	0.013	0.442	0.432	4.4	0.039
Zeeland-5 (50)[c]	0.474	0.472	5.3	48	0.96	2	2	0.015	0.447	0.429	4.4	0.060
Zeeland-6 (50)[d]	0.167	0.163	1.6	17	0.34	11	2–11	0.050	–	–	–	–
PORTUGAL												
Rio Mondego (48)	0.579	0.556	5.7	35	0.73	7	2–6	0.054	0.591	0.560	5.6	0.071
Ria Formosa (48)	0.632	0.635	5.9	28	0.58	7	2–12	0.015	0.632	0.635	5.9	0.015
MAURITANIA												
Mauritania-1 (49)[e]	0.639	0.722	4.7	10	0.20	4	2, 2, 10, 29	−0.078	–	–	–	–
Mauritania-2 (45)[e]	0.597	0.566	7.2	42	0.93	3	2	0.063	0.602	0.568	6.3	0.075
Mauritania-3 (50)[f]	0.574	0.648	6.3	36	0.72	8	2–4	−0.115*	0.583	0.659	6.2	−0.113*
SPAIN												
Port Lligat (36)	0.475	0.457	4.4	35	0.97	1	2	0.053	0.467	0.464	4.3	0.024
ITALY												
Caprolace (31)	0.533	0.614	3.6	17	0.55	5	2–5	−0.124	–	–	–	–
UKRAINE												
Black Sea-1 (49)[g]	0.526	0.545	4.8	43	6	2–3	−0.026	0.501	0.524	4.2	−0.027	
Black Sea-2 (49)[h]	0.511	0.483	4.9	49	1.00	0	–	0.065	0.522	0.476	4.3	0.106
Black Sea-3 (47)[i]	0.279	0.556	1.6	1	0.02	1	47	0.000	–	–	–	–
Black Sea-4 (50)[j]	0.578	0.622	5.7	35	0.70	11	2–6	−0.063	0.573	0.623	5.6	−0.069
Black Sea-5 (49)[k]	0.387	0.347	4.2	42	0.86	3	2, 3, 5	0.116	0.368	0.333	3.9	0.113
Azov Sea-1 (46)[l]	0.624	0.502	6.6	31	0.67	7	2–6	0.218*	0.626	0.508	6.1	0.206*
Azov Sea-2 (49)[m]	0.506	0.667	2.9	3	0.06	2	8, 40	−0.125	–	–	–	–

[a] Inner Emmapolder; [b] outer Emmapolder; [c] Oosterschelde; [d] Westerschelde; [e] inner Banc d'Arguin; [f] outer Banc d'Arguin; [g] Kazachya Bay, Sevastopol; [h] Tiligul coastal lake; [i] Odessa City Beach; [j] Fonar Cape, Kerch Strait, eastern Crimea; [k] Sukhoy Liman; [l] Molochny Liman; [m] Utlyuk Liman

viduals at ca. 1 m intervals or randomly along a 60 × 20 m transect (for the Iberian populations). The populations in Denmark and Italy were collected in the subtidal. Separately, 80 randomly selected individuals were mapped (using triangulation techniques) and collected from two 10 × 25 m^2 plots at Sylt (separated by 800 m near population S-5) and one 10 × 15 m^2 plot near Zeeland-4. In all cases, collection entailed the removal of 2 to 3 leaves from a shoot. Leaves were blotted dry, cut into smaller pieces and placed into 1.7 ml plastic tubes filled with silica crystals for rapid dehydration and subsequent storage.

DNA extraction and microsatellite development. Template DNA for PCR reactions utilizing 9 microsatellite primers (Coyer et al. 2004) was obtained from 2 to 3 pieces (5 to 10 mm in size) of silica-dried leaves. Procedures for DNA extraction, PCR amplification and determination of genotypes with an automated sequencer are described in Coyer et al. (2004).

Data analysis. As for all seagrasses, an individual plant or genet (genetic individual) consists of several shoots or ramets (morphological individuals arising from vegetative reproduction) (Harper 1977). The distinction is important, as inclusion of several ramets into population analyses is equivalent to resampling genets, leading to spurious positive deviations from HWE and downwardly-biased estimates of genetic diversity (Reusch et al. 1999b, Reusch 2001a). As our microsatellite analysis (9 loci) could distinguish genets (unique multilocus genotype) from ramets (identical multilocus genotypes), all analyses were conducted on genets only.

Descriptive statistics of the within-locality and among-locality genetic diversity (number of alleles; observed heterozygosity, H_{obs}; Nei's gene diversity, H_{exp}) (Nei 1978), as well as estimators of F_{IS} and F_{ST} (Wright 1969) as f and θ, (Weir & Cockerham 1984) and linkage disequilibrium were calculated using the GENETIX 4.02 program (http://www.univ-montp2.fr/~genetix/genetix/genetix.htm) (Belkhir et al. 2001). The significance of all f, θ and linkage disequilibrium estimates was tested using permutations (N = 2000, 2000 and 10 000, respectively) and sequential Bonferroni corrections (Rice 1989).

Populations that have experienced a recent reduction in effective population size (bottleneck) exhibit a faster decrease in allele numbers than in gene diversity at polymorphic loci (Cornuet & Luikart 1996). Thus, in recently bottlenecked populations, the observed gene diversity is higher than the expected gene diversity. The probability of a given population resulting from a recent bottleneck was calculated for *Zostera noltii* using the BOTTLENECK 1.2 program (http://www.montpellier.inra.fr/URLB/bottleneck/bottleneck.html) (Cornuet & Luikart 1996). The Two-phased Model (TPM) of mutation was used in the program as it is more appropriate for microsatellite data sets which are intermediate between the Infinite Alleles Model and Stepwise Mutation Model (Di Rienzo et al. 1994). Default values of TPM variance and probability for 9 microsatellite loci were applied and tested with a Wilcoxon Sign-Rank Test, which provides higher statistical power for small sample sizes (Cornuet & Luikart 1996).

Estimates of allelic richness after rarefaction were obtained with the CONTRIB program (http://www.pierroton.inra.fr/genetics/labo/Software/Contrib) (Petit et al. 1998). The program requires input of haplotypes; consequently, sample sizes for all populations (Mauritania-1, Black Sea-3 and Azov Sea-2 were not used as all had small genet sizes because of very large clones) were doubled (haplotype = allele) and the rarefaction size was selected to be smaller than the smallest haplotype or population size (Petit et al. 1998).

To test for isolation by distance (IBD) (Wright 1943, Slatkin 1993), estimates of $F_{ST}/1–F_{ST}$ (Rousset 1997) using the θ estimator (Weir & Cockerham 1984) in GENETIX 4.02 were correlated with the log of geographical distance (1-dimensional stepping stone model) using matrix correlation methods based on the Mantel test (Manly 1994) in the IBD 1.2 program with 10 000 randomizations (http://www.bio.sdsu.edu/pub/andy/IBD.html) (Bohonak 2002). The strength of the isolation by distance relationship was determined with reduced major axis (RMA) regression, which is more appropriate than standard ordinary least squares regression when the independent axis (geographic distance) is measured with error (Sokal & Rohlf 1981, Hellberg 1994), and calculated with IBD 1.2 (Bohonak 2002).

Pairwise distances among all population pairs were calculated from allele frequency data using Reynolds distances, which is a direct function of F_{ST} estimates, (Reynolds et al. 1983) in GENDIST. Neighbor-joining was used to construct the tree in NEIGHBOR, whereas bootstrap resampling (2000 replications) was performed using SEQBOOT and CONSENS. All programs are part of PHYLIP 3.5 (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein 1994).

RESULTS

Genetic diversity

We identified 195 alleles from the genotypes of 1118 genets obtained with 9 microsatellite loci. A sample size of ca. 30 genets was adequate to assess allelic diversity, based on a general flattening of the curve when plotting mean number of alleles $locus^{-1}$ versus

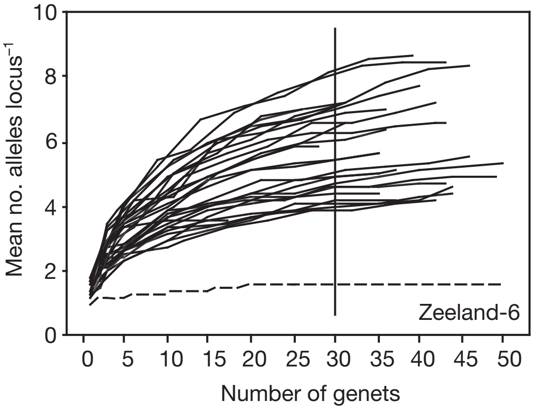

Fig. 2. *Zostera noltii.* Mean number of alleles locus^{-1} and number of genetic individuals (genets). Vertical line indicates minimum number of genets necessary (30) to capture allelic diversity using the 9 microsatellite loci. The dashed line represents the relationship for an anomalous population at Zeeland (see text)

number of genets (Fig. 2). Using a standardized sample of 28 genets (Sylt-2, Groningen-1, Zeeland-6, Mauritania-1, Caprolace and Black Sea-3 were not included in this analysis because genet size was <28), the 7 populations from the island of Sylt were most diverse, collectively over 60% higher (mean number of alleles locus^{-1}, SD: 7.21, 0.35) than the least diverse groups consisting of 5 Zeeland (4.28, 0.33) and 3 Danish populations (4.37, 0.49) (Table 1). When all genets and populations were used, the sample-size-independent, non-biased estimate of H_{exp} (mean, SD) for Sylt (0.645, 0.021) was 54 and 69% higher than for the Zeeland (0.419, 0.122) and Denmark (0.381, 0.038), respectively. The lowest diversity values were recorded at the Westerschelde site in the Netherlands (Zeeland-6; 1.56, 0.167) and Odessa City Beach (Black Sea-3; 1.56, 0.279), the latter being a single large genet or clone consisting of a single genotype for all 47 ramets sampled.

Genetic diversity and allelic richness of *Zostera noltii* populations (rarefaction = 40) generally declined from low to high latitudes along the eastern Atlantic coastline from Mauritania (putative glacial refugium) to Denmark (Fig. 3). The Sylt and Groningen populations, however, revealed values of H_{exp} and mean number of alleles locus^{-1} that were higher than the general trend and allelic richness for the Sylt populations also was higher than the general trend. No pattern in genetic diversity and allelic richness (rarefaction = 30) was observed as a function of longitude from the southern Atlantic (Mauritania, Ria Formosa) into the Mediterranean (Port Lligat, Caprolace) and the Black Sea/Azov Sea regions (Fig 3).

The Black/Azov Sea and Port Lligat localities were characterized by high proportions of unique alleles (30 and 28%, respectively), followed by Mauritania (20%) and Sylt (19%) (Table 2). Other localities had 3- to 10-fold fewer unique alleles.

Deviations from HWE and linkage disequilibrium

Significant heterozygote deficiencies (F_{IS}) were noted in 2 populations, Denmark-M (0.150) and Azov Sea-1 (0.218), whereas a heterozygote excess was recorded in Mauritania-3 (–0.115) (Table 1). Significant linkage disequilibrium was not observed among the 9 loci for any population (10 000 permutations; all $p \geq 0.12$).

Clone architecture

Genotyping of the mapped individuals within two 250 m^2 plots at Sylt and one 150 m^2 plot at Zeeland re-

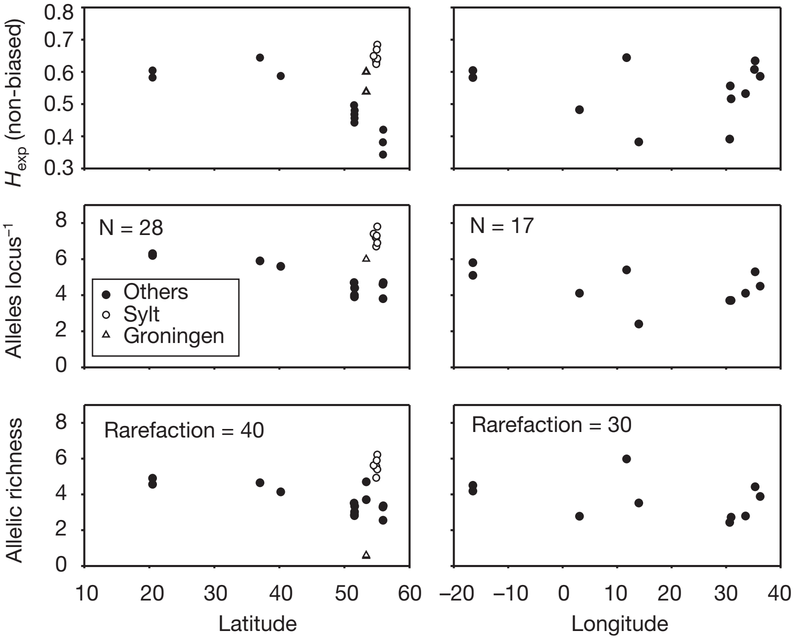

Fig. 3. *Zostera noltii.* Genetic diversity estimators as a function of latitude and longitude. The analysis using H_{exp} incorporated all genets (excluding clones) for each population, whereas the analysis using the number of alleles locus^{-1} employed a standardized sample of 28 (latitude) or 17 (longitude). Allelic richness was computed using rarefaction samples of 40 (latitude) or 30 (longitude) (Petit et al. 1998)

Table 2. *Zostera noltii.* Locality and unique alleles. Parenthetical values for locality indicate the number of genets; for loci, the total number of alleles observed. Listed for each locality are the number of unique alleles/total number of alleles for each locus and the percentage of unique alleles (all loci) relative to the total number of alleles (all loci)

Locality (genets)	Locus ZnB1	 ZnB3	 ZnB8	 ZnD6	 ZnE7	 ZnF8	 Znf11	 ZnH8	 ZnH10	Alleles Unique	 Total	%
	(19)	(22)	(23)	(27	(26)	(17)	(22)	(20)	(21)	83	197	42.1
Denmark (113)	–/10	–/10	–/4	–/5	1/6	–/5	–/5	–/5	–/4	1	54	1.8
Sylt (291)	4/16	–/16	5/11	–/6	6/14	–/10	3/12	2/11	–/10	20	106	18.9
Groningen (65)	–/11	1/10	–/5	–	–/5	–/6	–/4	2/8	–/7	3	56	5.3
Zeeland (242)	–/7	–/12	–/2	–/5	–/9	1/8	–/4	–/5	–/7	1	59	1.7
Rio Mondego (35)	–/8	–/7	–/5	–/4	–/1	1/5	–/5	–/4	1/9	2	48	4.2
Ria Formosa (28)	–/6	–/8	1/4	–/6	–/2	–/5	–/6	–/6	1/8	2	51	3.9
Mauritania (88)	1/11	1/11	2/6	5/11	3/13	1/7	1/7	–/6	2/6	16	78	20.5
Port Lligat (35)	–/2	–/5	4/7	4/9	–/1	–/5	–/4	3/4	–/2	11	39	28.2
Caprolace (16)	–/3	–/3	1/3	–/2	1/2	–/3	–/3	–/1	–/2	2	22	9.1
Black Sea/Azov Sea (204)	1/10	1/9	1/8	6/14	2/4	3/11	6/13	3/8	2/6	25	83	30.1

vealed the presence of 9 to 15 clones, most projected to be ≤3 m^2 in size (Fig. 4). Although many unique genotypes were recorded in the plots, it should not be inferred that these shoots represented single-ramet genets as it was impossible to genotype all individual shoots in the plots. Consequently, the number of clones we measured is likely to be an underestimate, but the lack of spatial domination by a single clone remains a valid observation. Our clone map also revealed that most clones at Sylt and Zeeland were contiguous. Fragmentation and/or possible reattachment was suggested by only 1 clone at Sylt-1 (symbol ▼; in Fig. 4), with ramets separated by ca. 6 m. No clones were common to both plots at Sylt, which were separated by ca. 800 m.

Analysis of individuals at 1 m intervals along a ca. 50 m linear transect line revealed that clones were present in all populations except Denmark-E, Zeeland-4, and Black Sea-2 (Table 1). The majority of clones were small, represented in the sample by 2 to 5 ramets, but large clones were observed at Groningen-1 (20 ramets sampled), Mauritania-1 (29 ramets sampled), Azov Sea-2 (40 ramets sampled) and Odessa City Beach (Black Sea-3), where all 47 ramets collected along the transect line were from a single genet.

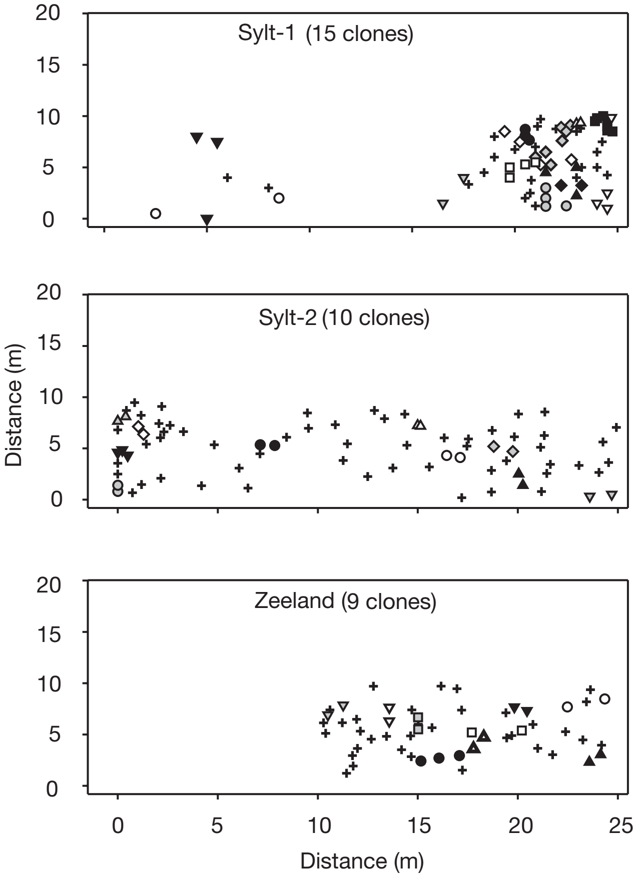

Fig. 4. *Zostera noltii.* Size and spatial arrangement of genets. Each individual was mapped and individuals with identical multilocus genotypes (9 microsatellite loci) were considered ramets (morphological individuals) of the same genet (genetic individual). Unique genotypes (single genets) are indicated by +; clones (multiple ramets) are identified by various symbols. A symbol in one panel does not imply relatedness to the same symbol used in another panel and no clones were common to Sylt-1 and -2. The open area in the center of the upper panel was an area of very few shoots

Population differentiation and isolation by distance

Pairwise estimates of θ were used to estimate genetic differentiation among the 33 populations (matrix available from authors on request). No significant differentiation (θ) ($p \leq 0.05$) was detected among: the 8 Sylt populations (0.0004 to 0.0142; 2 to 78 km); Zeeland-2, -3,-4,-5 (0.002 to 0.010; 2 to 7 km); Groningen-1 and -2 (0.006; 0.2 km); Mauritania-1 and -3 (0.055; 9 km); Black Sea-3 (clone) with Black Sea-1,-2,-4,-5 (–0.007 to 0.126; 18 to 550 km), Azov Sea-2 with Azov Sea-1 and

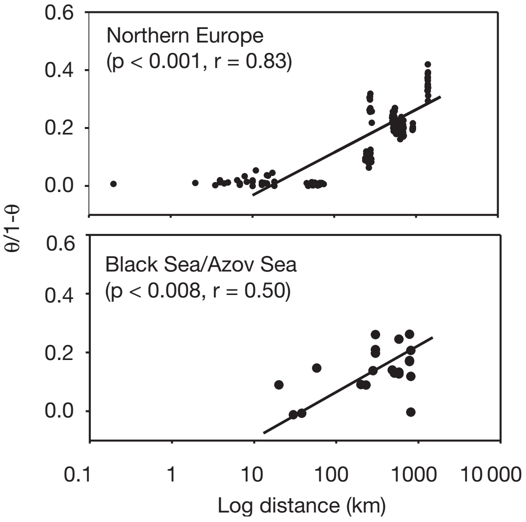

Fig. 5. Isolation by distance. Pairwise comparisons of genetic and log geographic distance among northern European (N = 18) and Black Sea/Azov Sea (N = 7) populations. The genetic and log geographic distance matrices were compared with the Mantel test and 10 000 randomizations (Bohonak 2002); p-values and r (correlation between 2 matrices, ranging from −1 to +1) are given in the figure. Estimates of slope, intercept and r^2 of the relationships were calculated using reduced major axis (RMA) regression (Bohonak 2002): northern Europe: $y = 0.148x - 0.180$, $r^2 = 0.68$; Black/Azov Seas: $y = 0.158x - 0.251$, $r^2 = 0.25$

Black Sea-4 (−0.012, 0.090; 30, 160 km), and Denmark M with Ø/E (0.012, 0.018; 4, 10 km). All other population pairs exhibited significant differentiation, with θ ranging from 0.033 (7 km, Denmark Ø and M) to 0.800 (ca. 8000 km, Zeeland-6 and Black Sea-3).

Significant (Mantel test) IBD was observed in northern Europe ($p \leq 0.001$) and the Black Sea/Azov Sea region ($p \leq 0.008$) (Fig. 5), each of which formed a distinct biogeographic cluster (see Fig. 6 and below). In northern Europe, pairwise separation distances ranged from 0.2 to 1400 km and genetic differentiation increased markedly only at distances ≥100 to 150 km. As no IBD was detected ≤100 km, the panmictic unit was estimated to be 100 to 150 km. No IBD was observed among 5 populations at Zeeland (population 6 was ignored, see Table 1, Fig. 2) (1 to 18 km, $p = 0.06$) and 8 populations at Sylt (2 to 74 km, $p = 0.77$). The near significant relationship among Zeeland populations, however, might become significant with additional samples (see also Fig. 6).

Pairwise separation distances for Black Sea/Azov Sea populations were less comprehensive (20 to 800 km) than for northern Europe. Nevertheless, the panmictic unit may be similar in size, as the slope of the relationship for the Black Sea/Azov Sea region (0.158) was nearly identical to that of northern Europe (0.148).

Global relationships among geographically distinct populations

A neighbor-joining analysis based on Reynolds distance revealed 3 highly resolved clusters tightly coupled to geographic areas: northern Europe (bootstrap = 91), Mauritania (bootstrap = 100) and the Black/Azov Seas (bootstrap = 98) (Fig. 6). The relative position of the major clusters in the unrooted tree also corresponded to geographic orientation, as the 2 Mediterranean localities were intermediate to northern Europe and the Black/Azov Seas, and the 2 Portuguese localities were intermediate between northern Europe and the Mauritania/Mediterranean sites.

All of the northern European localities were highly resolved (bootstrap = 82 to 100), as were some populations within Zeeland (bootstrap = 84) and Denmark (bootstrap = 96) (Fig. 6). The Zeeland-6 population was more differentiated from other Zeeland populations (bootstrap = 86) than all Sylt populations were from each other, and from the Azov Sea/Black Sea-1 and 4 populations (all separated by equivalent distances). The distinction of the Zeeland-6 population, however, was not due to a recent reduction in effective population size or bottleneck (2-phased model of mutation, Wilcoxon sign-rank test, $p = 0.41$).

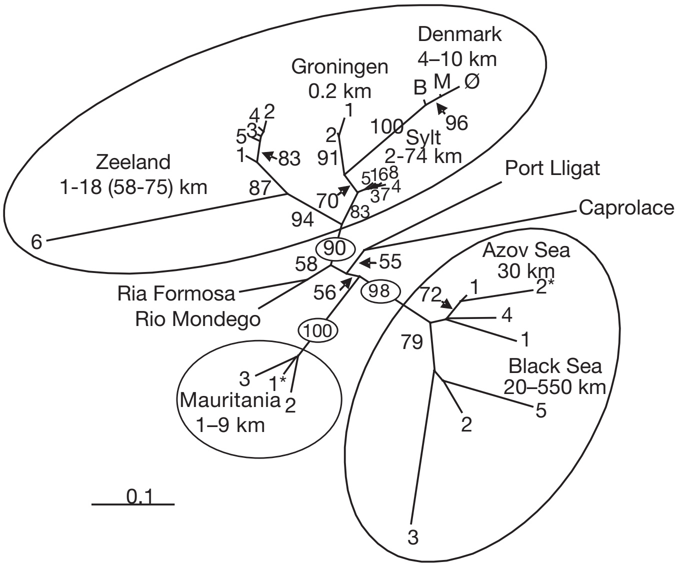

Fig. 6. *Zostera noltii*. Neighbor-joining tree illustrating the relationships among 33 populations. The tree was based on pairwise Reynolds distances (Reynolds et al. 1983) between genets only; bootstrap values were derived by 2000 permutations of allele frequencies. Minimum and maximum geographic distances among populations within a given locality are indicated. *identifies populations with a small number of genets because large clones were present: Mauritania-1, genets = 10; Black Sea-3, genets = 1; Azov Sea-2, genets = 3

DISCUSSION

The regional neighborhood

Degradation and loss of seagrass meadows has led to the general notion of low genetic diversity, extensive clonality and minimal gene flow among populations because of constrained sexual reproduction. At the landscape scale, significant IBD became apparent at ≥100 to 150 km in *Zostera noltii*. Significant IBD also has been documented for *Z. noltii* populations in Portugal at distances of ca. 100 km (O. E. Diekmann unpubl. data) and for predominantly subtidal *Z. marina* among northern European, western Atlantic and eastern Pacific populations at distances ≥100 to 150 km (Reusch et al. 2000, Olsen et al. 2004). Thus, substantial gene flow among intertidal and subtidal populations of *Zostera* spp. occurs at the level of 10s of km, with differentiation and genetic substructuring occurring primarily at distances of 100s of km. The similarity of IBD relationships for the small (≤100 cm length) intertidal *Z. noltii* and large (≥1 m length) subtidal *Z. marina* further suggests that habitat and/or size does not significantly affect dispersal. What may be most important in dispersal of *Zostera* species is the ability of intact plants (or reproductively mature portions thereof) to raft from one area to another (see Ruckelshaus 1998, Reusch 2002).

Global scale differentiation and history

During the LGM (18 000 to 20 000 yr BP), ice sheets covered most of Scandinavia and the British Isles (Dawson 1992). Seas north of the polar front, believed to be located near the present day northern Iberian coast, were covered with sea ice during the winter (Frenzel et al. 1992), thus glacial refugia for *Zostera noltii* most likely occurred south of the polar front along the coasts of the southern Iberian Peninsula, NW Africa (Mauritania) and throughout the Mediterranean. As the ice sheets retreated, *Z. noltii* populations expanded into suitable habitats according to the 'leading-edge' hypothesis, as has been demonstrated for algal, plant and animal species in Europe (van Oppen et al. 1995, Hewitt 1996, 2000, Röhner et al. 1997, Nilsson et al. 2001, Gabrielsen et al. 2002, Reusch 2002, Coyer et al. 2003).

Genetic studies of post-glacial recolonization usually compare genetic variation within and among extant populations inhabiting areas formerly covered by ice, to extant populations in putative glacial refugia. In making these comparisons, it is widely accepted that the founding populations colonizing new areas of suitable habitat should represent subsets of the source populations, with fewer alleles and increased levels of homozygosity (=lower genetic diversity) (Hewitt 1996, 2000, Johnson et al. 2000, Zink et al. 2000). The expectation of decreased genetic diversity in colonizing populations, however, may be too simplistic and a recent study has demonstrated that genetic diversity in founding populations can be either higher or lower depending on the estimator (Comps et al. 2001). Genetic diversity of *Zostera noltii*, as measured with 3 estimators, decreased northward along the Atlantic coast from a putative southern refugium in Mauritania. However, the highest diversity was not found in the middle-southern populations, but in the German Wadden Sea (see below). With respect to longitudinal patterns, the lack of correlation between genetic diversity/allelic richness and longitude from Mauritania through the Mediterranean may stem from the fact that both areas are putative refugia.

Northern Europe

Significant differentiation was observed among the 4 localities of the northern European cluster (200 to 1400 km, Fig. 6). It is unlikely that a hypothesis of multiple invasions of northern Europe from different southern refugia can explain the patterns of differentiation because all northern European areas comprise 1 strongly-resolved cluster. It is more likely that following a massive colonization sweep from southern refugia, each northern European locality subsequently evolved in relative isolation. The high levels of genetic diversity and allelic richness observed at Sylt were unexpected, but mirror results of a parallel study for *Zostera marina* (Olsen et al. 2004). Given that the area has become a marine habitat only within the past 7500 yr, the observed high diversity must have another explanation, perhaps disturbance.

Disturbances due to waterfowl grazing, lugworm interactions and epiphytic overgrowth are common at Sylt, and elsewhere in the Wadden Sea (Jacobs et al. 1981, Reise 1985, Philippart 1994, Nacken & Reise 2000, Schanz et al. 2002). It is conceivable, for example, that high levels of disturbance by grazing waterfowl in autumn will elevate the relative importance of sexual reproduction (and genetic diversity-producing meiotic recombination) by creating open space in which seeds can germinate and seedlings can survive during the following spring. Ice-scour in winter also may create open space in the spring and disturbance events during the summer may uproot proportionally more segments of reproductively mature plants, which are then rafted to other locations by tidal and/or wind-driven currents.

The importance of disturbance events on seagrass genetic diversity is largely unknown, but a relationship similar to that predicted by the Intermediate Disturbance Hypothesis for species diversity (Connell 1978) has been suggested (Hemminga & Duarte 2000): highest levels of genetic diversity in areas with intermediate levels of disturbance. Two studies of *Zostera marina* may support the relationship. First, higher genetic diversity was documented in a population at a Baltic Sea site characterized by extensive grazing by swans (Hämmerli & Reusch 2003). Secondly, seed germination (which should increase local genetic diversity) was greater in areas where winter ice had removed whole plants, than among mature ramets that had survived the ice disturbance (Robertson & Mann 1984). Additionally, a recent experimental study has demonstrated that autumn grazing by brent geese (*Branta bernicla bernicla*) was necessary for persistence of *Z. noltii* in the following spring, probably by reducing the amount of sediment accumulation (Nacken & Reise 2000). The relationship between disturbance and genetic diversity and/or seasonal persistence has important ramifications for conservation and management of seagrass populations and needs to be more widely examined.

Alternatively, the high levels of genetic diversity and allelic richness (and lack of IBD) at Sylt may be due to the importance of the area to migratory waterfowl. Migrating birds may carry individual seeds and/or spathes of *Zostera noltii* from one meadow to another, thereby increasing genetic diversity and gene flow. Although *Z. noltii* seeds collected from fecal droppings of brent geese were not viable (Ehlers 2002), the viability of seeds that have passed through the alimentary tract of other waterfowl species such as ducks (*Anas penelope*, *A. acuta*, *A. platyrhynchos*), as well as the potential for any waterfowl species to directly or indirectly transport reproductively mature spathes externally, remain to be tested.

Black and Azov seas

The Black and Azov seas also became marine and, therefore, suitable for *Zostera noltii* ca. 7500 yr BP; in this case, after the Bosphorous Strait opened (Ryan et al. 1997, Aksu et al. 2002). Similarly, high levels of differentiation were observed among most populations in these areas, which corresponded to significant IBD (Fig. 5). High allelic diversity, as well as the highest proportion of unique alleles (30 %), was recorded in the Black/Azov Sea regions. Although some of the populations sampled were dominated by a few large clones, the overall genetic diversity in these areas was high.

Both the Black and Azov seas probably were recolonized from Mauritania and presently represent high diversity relict populations. In the absence of haplotype data, we cannot establish that these populations are the oldest, but judging from their position in the NJ tree (Fig. 6) relative to Mauritania and the Mediterranean, it is reasonable to assume that these populations are modern day outposts. The residual diversity is unexpected, however, based on the marginal state of many seagrass beds along the Black Sea rim at present.

Mauritania as ice-age refuge and contemporary haven

During the LGM, Mauritania undoubtedly served as a refuge for *Zostera noltii* with subsequent intrusions into the Mediterranean and Black seas. Even today, both the Black Sea and Mauritania coasts have large coastal populations characterized by high allelic diversity. The coasts of Portugal, and Atlantic and Mediterranean Spain, may now represent marginal habitats for *Z. noltii*, because populations are restricted to isolated patches of suitable habitat (i.e. estuaries and coastal lagoons) and are thus likely to have small population sizes, low clonal diversity and significant departures from HWE (O. E. Diekmann unpubl. data). Though only weakly supported, populations from Spain and Italy (Mediterranean) group together as do Ria Formosa and Rio Mondego (Iberian-Atlantic); both groups are linked to Mauritania.

A recently completed survey of *Zostera noltii* along the Portuguese coast detected a sharp biogeographic break on either side of the Tagus River (O. E. Diekmann unpubl. data). Whether the discontinuity is due to rapid genetic drift associated with small and isolated populations, or represents isolation due to large distances between adjacent populations, is not clear. The Brittany peninsula to the north is characterized by high genetic diversity in the fucoid seaweed *Fucus serratus* (Coyer et al. 2003), but not for *Z. marina* (Olsen et al. 2004). Thus, the Black, Azov and Wadden Seas may be considered as diversity hotspots.

Large clones and the concept of diversity

Clonality generally is correlated with frequency of localized disturbances, with low disturbance favoring maximum clonal growth. Large clones, therefore, tend to be older and may provide insights into the population's stability. A single large clone of *Zostera noltii* was present at Odessa City Beach and a parallel sample of 50 *Z. marina* shoots collected on the same day and in the same manner also revealed the presence of a single large clone (Olsen et al. 2004). Using mean rhizome elongation rates of 26 and 68 cm yr^{-1} for *Z. marina* and *Z. noltii*, respectively (Marbà & Duarte 1998), we estimated the ages of the genets to be 96 to 192 yr for *Z. marina* (data from Olsen et al. 2004) and

34 to 69 yr for *Z. noltii.* As the beach is very near one of the largest harbors in the Black Sea, the most parsimonious explanation for the extensive clonality of both species is the survival and expansion of a single genet following anthropogenic disturbance. Although the growth data do not allow an unambiguous determination of whether a putative disturbance event affected each species simultaneously or sequentially, the spatial and temporal aspects of the clones suggest relatively undisturbed conditions since the initiating event. The ages of large *Z. noltii* clones in the Azov Sea and Mauritania were estimated to be 29 to 59 yr and 21 to 42 yr, respectively. While the ages of *Z. noltii* are not as dramatic compared with estimates for *Z. marina* in the eastern Baltic (Reusch et al. 1999a) or *Posidonia oceanica* in the northern Adriatic (Ruggiero et al. 2002), both of which covered thousands of m^2 and were estimated to be ≥1000 yr old, they do indicate that population turnover times for *Z. noltii* are of the order of 2 to 6 decades in widely separated areas.

Whereas a few clones dominating an area have a small effective population size and implicitly low genotypic diversity (e.g. low multi-locus heterozygosity), this does not necessarily mean low fitness or low persistence. For example, the Zeeland-6 clone was homozygous for 4 loci and heterozygous with only 2 alleles at each of the remaining 5 loci. Although the source and history of the Zeeland-6 clonal population remain unknown, the population spanned 1000s of m^2 in size, strongly indicating a high degree of success in the area. The trade-off in growth/persistence versus reproduction (flowering frequency, degree of outcrossing, seed production) in successful, but low diversity populations is currently under investigation. The main point is that diversity in seagrasses is present at the levels of both clonality and multi-locus heterozygosity, each of which need to be considered separately.

Concluding remarks

The large and multi-year-old clones present in some populations of *Zostera noltii* suggest local adaptation in areas with low disturbance, as is also the case for populations characterized by very low levels of genetic diversity and large size. Although local adaptation can be expected to increase differentiation between adjacent populations, the differentiation may be ameliorated by disturbance events, which provide new space for seed germination and/or promote rafting of reproductively mature plants for 100s of km.

We also hypothesized that the predominantly intertidal habitat of *Zostera noltii* would lead to greater connectivity and hence, a larger spatial scale of the panmictic unit than that found in the predominantly subtidal habitat of *Z. marina*, in which buffering would lead to greater differentiation at the same scales. Patterns of differentiation and connectivity were similar over regions in which the 2 species co-occurred, however, suggesting that macro-features of the landscape-shoreline may play a more important role in delimiting population connectivity than dispersal, life history, or distinctions based on intertidal versus subtidal habitat, at least throughout northern Europe and at the regional neighborhood scale.

We conclude that despite the ongoing degradation of seagrass populations, mainly because of habitat destruction and fragmentation, the genetic potential for recovery is present in many locations. Future considerations of seagrass conservation and management should continue to insure the preservation of habitat and water quality, but also should consider the importance of disturbance (e.g. in a conservation context, not in a broad sense that includes habitat destruction) as a means to promote connectivity (and stability) between populations.

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