An evaluation of small-scale genetic diversity and the mating system in
Zostera noltii on an intertidal sandflat in the Wadden Sea

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**INTRODUCTION**

Dwarf eelgrass, *Zostera noltii*, is one of two dominant seagrass species along Europe’s North Atlantic coastline. It is typically intertidal under marine conditions and subtidal in brackish habitats, and ranges from southern Sweden to Mauritania and parts of the Mediterranean, Black and Azov Seas. In the sandy to muddy intertidal found along the northern European coastlines, *Z. noltii* is perennial, characterized by a high genet turn-over and annual genet replacement from seeds. Intertidal genets studied in the German Wadden Sea are typically <1 m² in size, but can be as extensive as 9 m² (Zipperle et al., 2009a, b). In contrast, subtidal *Z. noltii* in the Mediterranean forms larger clones (several square metres) with minimal recruitment via seeds (Ruggiero et al., 2005). Intertidal hydrodynamics (e.g. tidal and storm-induced wave action) and natural disturbances have been invoked to explain a sliding scale of life-history differences between intertidal and subtidal habitats, as well as for the maintenance of high genetic and genotypic diversity in the Wadden Sea (Coyer et al., 2004; Olsen et al., 2004; Zipperle et al., 2009a).

The balance between sexual reproduction via seeds and vegetative propagation by rhizomat spreading greatly influences the spatial genetic structure of a seagrass meadow, its relative diversity, and potential for gene flow. For example, spathes (specialized leaf shoots) of the monococious *Z. noltii* contain four to six female and four to six male inflorescences. Pollination is hydrophilous (den Hartog, 1970) and seeds are negatively buoyant (Orth et al., 1994; Ruckelshaus, 1996). The consequences of this hermaphroditic mating system are that large clones can lead to pollen dispersal limitation, which translates into reduced outcrossing opportunities and increased probability of self-fertilization/inbreeding (Handel, 1985; Charpentier, 2002). Thus, a centrally located ramet in a large clone is not only less likely to receive outcrossed pollen, but is also more likely to have its own pollen fertilize another ramet of the same genet (geitonogamy).

Inbreeding depression is an important post-pollination mechanism affecting evolution of mating systems, as the purging of recessive and deleterious alleles by selection against inbred offspring favours an outcrossing mating system (Lande and Schemske, 1985; Eckert and Barrett, 1994). Selfing or outcrossing strategies depend upon the magnitude of inbreeding depression (Schemske and Lande, 1985; Jarne and Charlesworth, 1993): pure mating systems (selfing or outcrossing) are predominant under abiotic pollination, while mixed mating (selfing and outcrossing) is common in animal-pollinated plants (Goodwillie et al., 2005). If
outcrossing is under genetic control, however, disruptive selection occurs for either an exclusively outcrossing or selfing mating system (Lande and Schemske, 1985).

Previous studies of Z. noltii meadows on sandflats in the German Wadden Sea examined clonal architecture, the importance of seed banks, and the role of geese pits for seed recruitment (Zipperle et al., 2009a, 2009b, 2010). In the present study of a large Z. noltii meadow on an intertidal sandflat, the mating system and pollen dispersal of Z. noltii were investigated at a small (square centimetre) and large (square metre) spatial scale. The specific aims were to: (a) determine the relationship between mating system and clonal structure; (b) examine geitonogamous selfing vs. biparental inbreeding; (c) measure pollen availability expressed as multiple paternity; and (d) estimate pollen dispersal distance (PDD).

MATERIALS AND METHODS

Study site and sampling

A permanent 10 × 10-m plot (divided into 1-m² quadrats) was established within a large Zostera noltii meadow (approx. 0.125 km²) in Königshafen, a shallow intertidal bay on the Island of Sylt (German Wadden Sea; 55°2'46.5"N; 8°26'2.5"E). Water depth during high tide is approx. 0.5 m and the meadow is exposed 6–8 h twice a day. At each sampling period, stakes were relocated (using photographs, landmarks, nylon line) and connected with 10-m transect tapes (Fig. 1). The location of each sampled shoot was mapped using a 20-cm grid (Fig. 2). When samples were collected in early September 2003, seagrass cover within the plot was 75–100 % and comparable to adjacent areas.

Seed-bearing shoots were sampled from eight randomly chosen 1-m² quadrats within the plot, which were further divided into 25 sub-quadrats (20 × 20 cm). When present, a seed-bearing shoot was collected from the centre of each sub-quadrat. Due to the patchy distribution of seed-bearing shoots, however, the number of samples per quadrat varied. In dense quadrats, random samples were collected, which resulted in more than one sample collected from a sub-quadrat in some cases. Additional shoots were sampled for the entire 100-m² plot by collecting one shoot from the centre of each fixed 1-m² quadrat (n = 100). Thus, a total of 256 adult shoots was sampled and used to determine clone structure and genetic diversity for the larger 100-m² plot (Zipperle et al., 2009a). All plant tissue was dried and stored in silica crystals.

DNA extraction and microsatellite genotyping

Seeds were extracted from a spathe and matched with 5 mg of leaf tissue from the reproductive shoot (= mother) for mating system and paternity analysis. Seeds and leaf tissue of all adult samples were pulverized using a Retsch mixer mill MM 301(Qiagen) and DNA extracted as described by Elphinstone et al. (2003). All samples were genotyped for

Fig. 1. Study site: (A) Island of Sylt (white square) in the northern Wadden Sea; (B) List tidal basin with study site (black square) in Königshafen on the northern tip of the island; (C) Z. noltii meadow in Königshafen 2003 with seagrass bed boundaries outlined and relative location of studied plot (black square); (D) specific location of the study plot (black square).
nine microsatellite loci (Coyer et al., 2004) to obtain multi-locus genotypes (MLG). Three primer triplets (plex-1: ZnB1/ZnH10/ZnB3; plex-2: ZnB8/ZnH8/ZnD6; plex-3: ZnE7/ZnF8/ZnF11) were used in multiplex PCR reactions following the protocol in Zipperle et al. (2009b) The PCR reaction mix was: 2 mM MgCl₂, 0.2 mM dNTPs, 0.5 U Taq (Promega), 0.01% bovine serum albumin, reaction buffer (10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100) in a total volume of 20 μL per reaction. PCR reaction protocol was the same for each primer triplet: initial denaturation of 3 min at 94 °C, 35 cycles consisting of 40 s at 94 °C, 40 s at 57.5 °C and 40 s at 72 °C with a final extension step of 10 min at 72 °C. Each multiplexed primer triplet was visualized by gel electrophoresis on an ABI 377 GeneAnalyzer (Applied Biosystems). Alleles were scored against an internal lane standard (ROX 350; Applied Biosystems).

Genetic, genotypic diversity and clone structure

Genetic and genotypic diversity, as well as clone structure, were determined using GECLONE v. 2.0 (Arnaud-Haond and Belkhir, 2007). Duplicate genotypes of a clone were counted only once in subsequent analyses. Genotypic diversity was separately determined for the 1-m² and the 100-m² scales. Allelic diversity (A) and allelic diversity corrected for sample size (A_C) were determined separately for maternal genets, seeds and all adult genets present in the plot; as were
estimates of the global inbreeding coefficient $F_{IS}$, expected ($H_{exp}$) and observed ($H_{obs}$) heterozygosity.

Mating system

Data were analysed with MLTR v.3-3 (Ritland, 2002) using the Newton–Raphson method with a mixed mating model. The mating system was characterized by estimating multi- and single-locus outcrossing rates ($t_{om}$, $t_{os}$); biparental inbreeding as the difference between outcrossing rates ($t_{om} - t_{os}$); maternal inbreeding as the coefficient $F$; the correlated selving rate as $r_s$; and the multi-locus paternity correlation within [$r_{p(pw)}$] and between [$r_{p(pb)}$] families (genets). When true selving is present, the difference between multi- and single-locus estimates of outcrossing, ($t_{om} - t_{os}$), is often used to characterize the level of biparental inbreeding: single-locus estimates include all apparent selving due to biparental inbreeding, whereas multilocus estimates exclude much of the apparent selving due to biparental inbreeding (see Ritland, 2002).

The effective number of pollen donors ($N_{ep}$) was determined by the reciprocal of $r_p$ ($N_{ep} = r_p^{-1}$) (Smouse et al., 2001; Fernández and Sork, 2005). From 32 mother genets, 71 spathes containing 148 seeds were recovered. All seeds retrieved from a genet were grouped in a family. For the analysis of families, default settings for the estimated main parameters ($t = 0.9$, $r_t = 0.1$, $r_p = 0.1$, $F = 0.1$) were used (Ritland, 2002). Parameters were jointly estimated from 1000 bootstrap iterations from which confidence intervals were derived.

Paternity analysis and pollen dispersal

Paternity analysis, or determining the most likely father for a given offspring, was performed with CERVUS v. 3-0 (Kalinowski et al., 2007), which employs a likelihood-based approach for categorical assignment of paternity with co-dominant markers. Paternity is assigned to a male if the log-likelihood ratio or LOD score (the likelihood of paternity co-dominant markers. Paternity is assigned to a male if the approach for categorical assignment of paternity with

Accuracy of paternity assignment depends upon the simulation parameters used and results are influenced by the genotyping error rate, the number of candidate fathers, and the proportion of parents actually sampled from the population. The genotyping error rate in the data set was low (0.01).

RESULTS

Clone structure and genetic diversity

At the 1-m$^2$ scale, the number of genets varied from 2 to 20 with a mean of 7.2 m$^{-2}$ (Table 1). Not surprisingly, sampling at the scale of 20-cm intervals resulted in repeated sampling of a single genet, i.e. 156 ramets reduced to 58 genets. The very high number of genets (20) found in quadrat C represented closely related individuals, as well as older individuals as judged by their size (Fig. 2). At the 100-m$^2$ scale, where the sampling was at 1-m intervals, more genets were recovered in relation to ramets sampled. Only about 15 % were replicates. Allelic richness was high (Table 1), mirroring levels reported earlier for $Z$. noltii in the Wadden Sea (Coyer et al., 2004). Global $F_{IS}$ values gave no indication of departures from HWE.

Mating system

Outcrossing rates were high ($t_{om} = 0.886$; $t_{os} = 0.871$) but also included a fraction of inbred seeds (Table 2). The majority of inbred seeds resulted from selving (geitonogamy and autogamy) and only 1.5 % of the total inbreeding detected was due to biparental inbreeding (matings between close relatives, e.g. sibs). The coefficient of correlated paternity among outcrossed seeds differed significantly from 0 within $[r_{p(pw)} = 0.155]$ and between $[r_{p(pb)} = 0.118]$ genets. Thus, the majority of seeds within spathes of a genet and between different genets...
had multiple fathers. The number of effective pollen donors \( N_{ep} \) was 6.45 within and 8.47 between genets.

### Paternity and PDD

A father could be assigned to 34% of all seeds within the eight randomly placed quadrats under strict analysis conditions (95%) using the \( D \)-statistic. The mother and assigned father were identical in 22% of all assigned seeds indicating self-fertilization (Table 1) and 2–18% of seed-father pairs could be assigned within the 100-m\(^2\) plot depending on the preset proportion of parents sampled from the population (Table 3).

Average PDD (s.d.) ranged from 1.70 (0.79) m to 3.78 (2.44) m with distances ranging from 0.85 to 9.62 m. Because pollen dispersal is commonly leptokurtic, the median provides more explanatory power than the mean. The median of PDD ranged from 1.84 m to 3.22 m (Table 3).

### DISCUSSION

#### Selfing and outcrossing

In most seagrasses, pollination takes place entirely underwater and early studies hypothesized that reduction of pollen diversity due to extensive clonal growth would result in self-fertilization and inbreeding (Cox, 1983; Les, 1988; Philbrick and Les, 1996). More recent experiments, however, demonstrated high outcrossing in three monococious species, with rates of 0.89 for *Posidonia australis* (allozymes: Waycott and Sampson, 1997), 1.00 for subtidal and 0.90 for intertidal *Z. marina* (allozymes: Ruckelshaus, 1995) and 0.97 for
intertidal Z. marina (microsatellites: Reusch, 2000), effectively contradicting the hypothesis of predominant selfing and inbreeding. However, outcrossing rates might be subject to temporal and spatial variation (Ruckelshaus, 1995), and accuracy depends on the chosen marker. In the present study, the outcrossing rate was 0.88, which is high, but not unity. Closer examination of the mating system revealed a high degree of multiple paternity \( p_{fw} = 0.155; p_{fb} = 0.118 \), both within and between genets of Z. noltii. This demonstrates high pollen availability from multiple sources, which is supported by the large number of effective pollinators (8-47 between genets).

At the same time, however, <50 % of the ovules within a single spathe were fertilized and similar low fertilization rates have been reported for other inter- and subtidal populations of Z. noltii (Hootsmans et al., 1987; Loques et al., 1988, 1990; Curiel et al., 1996; Alexandre et al., 2006). Ackerman (2002) hypothesized that the low fertility rate was due to low levels of pollen released as 1000 to 10 000 pollen grains are necessary per ovule for successful fertilization in Z. marina. In contrast, Hämmerli and Reusch (2003a) demonstrated self-incompatibility in Z. marina, where a high number of aborted seeds were the result of selfing. The authors’ observations support the latter view. The fact that inbred seeds are not reflected in the adult populations suggests strong selection against inbred offspring, either through seed abortion or seedling mortality (Ruckelshaus, 1995; Reusch, 2000). Selection acting against selfed progeny has also been detected in field studies of Z. marina where plant fitness (clone size and number of flowering shoots) was positively correlated with heterozygosity (Reusch, 2001; Hämmerli and Reusch, 2003b).

Clone size can significantly affect outcrossing, especially if the clone is larger than the PDD, in which case selfing may become unavoidable. Indeed, Reusch (2001) detected decreasing outcrossing rates with increasing clone area in subtidal and perennial Z. marina with pronounced vegetative propagation. In the present study, Z. noltii clone size averaged 1.4 m², comparable to other reports for intertidal Z. noltii (Brun et al., 2003; Zipperle et al., 2009a), whereas the median PDDs ranged from 1.8 to 3.2 m. Even so, the presence of a high proportion of selfing among seeds (0.193) highlights the delicate balance between clonal propagation and successful mating.

Selfed seeds result from within (autogamy) and/or between (geitonogamy) ramet selfing. In protogynous Z. noltii, geitonogamy potentially is the most likely selfing mode as dichogamy (temporal separation of male and female sexual function) serves as a mechanism to avoid autogamy. Although dichogamy is never complete and might be impaired by environmental factors (Lloyd and Webb, 1986, Ruckelshaus, 1995), self-incompatibility is an additional means to avoid autogamy present in the sister species Z. marina (Hämmerli and Reusch, 2003a).

PDD estimates are based on few positively assigned seed-father pairs (Table 3). However, the lower the proportion of parents sampled used for analysis, the higher the \( \Delta \) for positive assignment. Under the strict analysis conditions applied the results presented are most conservative especially in the range of proportions of parents sampled considered most realistic (0.15–0.45) (Table 3).

Zostera species also have negatively buoyant seeds (Orth et al., 1994; Ruckelshaus, 1996), which means that seeds settling near the mother plant may be related and form a relatedness cluster (Heywood, 1991). Quadrat C (Fig. 2) contains a large number of different genotypes, which most likely are the result of a single recruitment event. Such recruitment clusters have also been documented in relation to geese pits, which accumulate seeds and facilitate spring recruitment (Zipperle et al., 2010). Kinship structure has been demonstrated at the scale of 1–2 m in Z. noltii (Coyer et al., 2004; A. M. Zipperle, unpub. res.) and is consistent with the low proportion of biparental inbreeding (1.5 %) observed in the present study. PDD and kinship structure for intertidal Z. noltii were equivalent to values reported for subtidal Z. marina despite a nearly 10-fold difference in shoot and genet size (Table 4).

**Influences of the local environment**

The study site was characterized by a comparatively high hydrodynamic regime (Schanz and Asmus, 2003) and subjected to geomorphic forcing by mega-sand ripples on a decadal time scale (Dolch and Reise, 2010). The shift towards smaller genets increased with increased hydrodynamics (Schanz and Asmus, 2003), while larger genets tended to dominate with increasing water depth (Brun et al., 2003) probably reflecting decreased water flow. Burial of Z. noltii by mobile sediments can cause plant death as well as forcing small genet sizes. The mega-ripples observed throughout the study site, however, move on decadal time scales making burial effects negligible for the present study.

At the outset of this study, it was hypothesized that the small size of Z. noltii plants (i.e. leaf width and length characteristics) compared with the larger size of Z. marina plants might account for differences in genet size, pollen dispersal and properties of the mating system. This was not the case as PDD and genet

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**Table 4. Comparison of Z. noltii and Z. marina growth and reproduction characteristics**

| Plant height (m) | 0–1–1 | 0–2–2 | den Hartog, 1970 | Reusch et al., 1999; Coyer et al., 2004; Olsen et al., 2004 |
| Clone size (m²) | <1–1960 | <1–6400 | Ruckelshaus, 1996 |
| Pollen dispersal distance (m) | 0–9–6 | 0–15 | This publication; Ruckelshaus, 1996 |
| Seed dispersal distance | | |
| Seeds (m) | – (inferred to be on a metre scale) | 0–10 m | Orth et al., 1994; Ruckelshaus, 1996 |
| Rafting shoots (km) | Approx. 150 | Approx. 150 | Reusch, 2002; Ferber et al., 2008; Erfemeijer et al., 2008 |
| Kinship structure (m) | 1–2 | 2–4 | Coyer et al., 2004; Hämmerli and Reusch, 2003c |

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size were essentially the same for both species (Table 4). This suggests that local environmental characteristics play a major role for dispersal. In the most extreme dichotomy, dominance of a few large genets may result in reduced seed set and subsequent recruitment as a consequence of pollen limitation and unavoidable selfing. If conditions remain relatively benign, however, a large genet may prosper for decades or even centuries in some species. At present, it is known that intertidal Z. noltii can persist for 4–5 years (Zipperle et al., 2009a) and the giant (up to 60 m²) Mediterranean clones (Ruggiero et al., 2005) of Z. noltii are likely to be much older. At the other extreme, high mortality and annual or bi-annual turnover of genets may actually increase seed set as a consequence of pollen abundance and favourable outcrossing. Recruitment will also be enhanced. This is characteristic of the typically dynamic regimes found in the intertidal of the Wadden Sea. Consequently, intermediate disturbance (Connell, 1978; Hemminga and Duarte, 2000) is likely to create the best of both worlds—a heterogeneous mix of genets and genet sizes along with high outcrossing rates and intermediate mortality. Disturbance may thus enhance genotypic diversity within intertidal Z. noltii meadows by: (a) directly promoting pollen and seed dispersal through vigorous hydrodynamics, thus extending seed and PDD; (b) facilitating sexual recruitment through gap formation in a dense meadow (Zipperle et al., 2010); and (c) indirectly increasing the level of outcrossing due to clone size reduction. There is evidence for all three factors.

In conclusion, the intertidal environment selects for small clone sizes of Z. noltii, which indirectly influences the mating system by increasing outcrossing opportunities and allowing for large PDDs and a high degree of multiple paternity. While biparental inbreeding is nearly eliminated, geitonogamy seems to be intrinsic to the clonal life-history of Z. noltii. The combination of a predominantly outcrossing mating system, large PDD, regular annual recruitment, and a short-term persistent seed bank in Wadden Sea populations of Z. noltii (Zipperle et al., 2009b), collectively maintain high genetic and genotypic diversity on a local scale, thereby enhancing the population’s resilience to the stochastic intertidal environment. In Z. marina, Hämmerli and Reusch (2003b) also found higher genet diversity in a subtidal population in the Baltic Sea grazed by swans, compared with an ungrazed population, and Reusch (2006) determined experimentally that seedling recruitment and genet dynamics in Z. marina were enhanced in response to physical disturbance. A similar relationship exists for grazing by geese which facilitates seed recruitment (Zipperle et al., 2010). Gene flow, at the larger scale of the Wadden Sea, occurs by the transport of seed-bearing rafting shoots by tides and/or storms (Coyer et al., 2004; Erftemeijer et al., 2008; Ferber et al., 2008), thereby linking Z. noltii meadows to a meta-population via the export of genetic and genotypic diversity created at the local scale. The perennial life-history of Z. noltii may explain its better performance compared with annual and semi-annual Z. marina in the intertidal of the Wadden Sea.

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LITERATURE CITED