

Is asexual reproduction more important at geographical limits? A genetic study of the seagrass *Zostera marina* in the Ria Formosa, Portugal

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ABSTRACT: In populations of species that are able to reproduce both sexually and asexually, there may be local differences with regard to the relative importance of the 2 modes of reproduction. Studies of plant species with such a life history have shown that the contribution of sexual reproduction to population maintenance may be lower at the geographical margins, with genotypic diversity often used as an indicator of the relative importance of vegetative and sexual reproduction. This hypothesis was examined in a collection of samples of eelgrass *Zostera marina* (a marine flowering plant) from its eastern Atlantic southern limit (Ria Formosa, Portugal). Samples from 12 sites were genotyped using 9 microsatellite loci to compare within-site clonal and genetic diversity, and among-site distribution of genetic diversity, with previously published values from central sites. Sites within the Ria Formosa had lower clonal diversities (mean = 0.29, range = 0.07 to 0.68) than the central sites (mean = 0.86, range = 0.33 to 1.00), lower levels of expected heterozygosity ($H_e = 0.423$ vs 0.486) and exhibited heterozygote excesses rather than deficits. Similarly, genetic differentiation was found to be much greater in the Ria Formosa, with the F_{ST} of 0.233 being over 10-fold greater than that reported for populations in the Baltic Sea. Results from this study were consistent with previous findings of reduced sexual reproduction, genotypic diversity and among-population gene flow at species limits.

KEY WORDS: Genetic diversity · Species limit · Asexual reproduction · Clonal plant · Seagrass · *Zostera marina*

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INTRODUCTION

Species with a mixed mode of reproduction may exhibit wide variation among locations in the relative contribution of sexual and asexual reproduction within populations (Ashton & Mitchell 1989, Eckert & Barrett 1992, Eriksson 1996). Furthermore, asexual reproduction has been shown to be more important at a species geographical margin when compared to central populations (reviewed in Eckert 2002) and to be related to lower sexual reproduction via flowers, fruits and seedling recruitment (Eriksson 1989, 1996). For example, populations of *Decodon verticillatus* (an aquatic plant) in the central portion of its range exhibited prodigious seed production, whilst those at the northern geo-

graphical limit showed severely reduced seed production (Eckert & Barrett 1995, Dorken & Eckert 2001).

Levels of sexual and asexual reproduction within populations can be inferred from estimates of population genotypic diversity, and reduced sexual reproduction at species limits has been found to correlate with reduced genotypic diversity (Eckert 2002). For example, northern populations of *Decodon verticillatus* were also shown to be genetically monomorphic (using allozymes), whilst southern populations were genetically diverse and in Hardy-Weinberg equilibrium (Eckert & Barrett 1993, Dorken & Eckert 2001). Apart from influencing genetic variation within populations, increased levels of asexual reproduction at species' limits will also affect among-population differentiation.

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Among-population distribution of genetic variability is dependent upon the relative strengths of gene flow, genetic drift and natural selection; therefore, relatively high contributions of asexual reproduction at a species limit should increase genetic differentiation as gene flow is restricted due to reduced seed production and dispersal (Slatkin 1985). Similarly, populations with individuals that reproduce asexually have a reduced effective population size, which increases the likelihood of genetic drift and population differentiation (Silander 1985, Muirhead & Lande 1997). However, when sexual and predominantly clonal species were compared, little difference in genetic variability among populations was observed (Hamrick & Godt 1990, McLellen et al. 1997); this has been attributed to sampling scales (Eckert 2002). Nevertheless, few studies have examined how the reproductive mode and distribution of genetic diversity vary in species with mixed modes of reproduction (Eckert 2002).

In seagrasses (marine angiosperms), it has been hypothesised that extensive clonal spread may limit the genetic diversity of populations while amplifying the genetic substructure among populations (Les 1988). *Zostera marina* is a seagrass that reproduces both vegetatively (via the clonal lateral spread of rhizomes) and sexually through seeds (den Hartog 1970, Phillips & Meñez 1988). The numerous spathe (inflorescences) along the flowering shoots each contain 1 row of alternating male and female flowers; however, their flowering is asynchronous (de Cock 1980), which is likely to reduce selfing. Pollination is subaqueous, with the filiform pollen released underwater and carried to stigmata through water movement (de Cock 1980, Cox et al. 1992). *Z. marina* is widely distributed in shallow coastal waters of the northern temperate zone (den Hartog 1970, Phillips & Meñez 1988). In Europe, *Z. marina* is the only seagrass to extend into the Arctic Circle; its southern limit (along the eastern Atlantic coast) occurs in the Ria Formosa, Portugal (den Hartog 1970).

Z. marina also appears on both the Atlantic and Pacific coasts of the Americas, extending from Greenland to North Carolina in the east and from Alaska to Baja California, Mexico, in the west (den Hartog 1970).

Microsatellite-based studies of perennial *Zostera marina* populations, including populations from both the east and west coasts of North America and Europe, have shown genetic diversity to be generally high (populations: 2 from Nova Scotia, 44° N; 1 from California, 33° N; 1 from the Finnish Baltic, 60° N; 5 from the German Baltic, 54° N; 2 from the North Sea, 53° N; and 1 from France, 48° N) (Reusch et al. 2000, Reusch 2002). Differences in clonal diversity were apparent, ranging from monoclonal populations to populations in which every sample collected was genetically distinct (Reusch et al. 2000, Reusch 2002). Expected heterozygosities ranged from 0.324 to 0.575 (mean = 0.486) and only 1 population deviated significantly from Hardy-Weinberg expectations (a heterozygote deficit) when unique multi-locus genotypes (clones) were analyzed in the 10 populations that contained more than 1 clone (Reusch et al. 2000, Reusch 2002). Genetic differentiation over scales of 100 to 1500 km was high ($F_{ST} = 0.29$), with phylogenetic analyses showing populations to be grouped on a regional basis (<50 km, i.e. North Sea, Baltic Sea and Nova Scotia). However, relatively low levels of genetic differentiation were found among populations within regions ($F_{ST} = 0.018$; Reusch et al. 2000, Reusch 2002).

In this study we hypothesized that *Zostera marina*, at its southern eastern Atlantic distributional limit in the Ria Formosa, Portugal (~37° N), would exhibit relatively high levels of asexual reproduction, which would be reflected in the population genetic structure. In particular, we used microsatellite markers to assess whether these marginal sites exhibited (1) lower levels of clonal and genetic diversity within sites, and (2) higher levels of genetic differentiation among sites, when compared to more central regions.

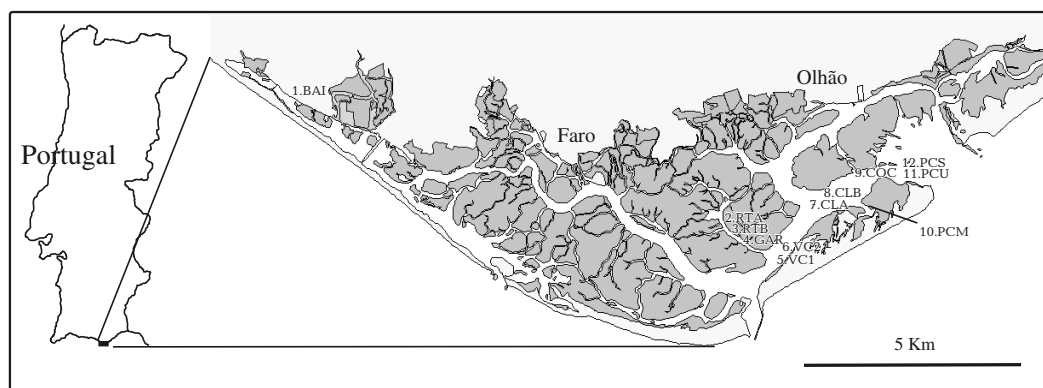


Fig. 1. Map of the Ria Formosa coastal lagoon system, southern Portugal, showing the 12 sites at which *Zostera marina* was found and collected for genetic analysis. See Table 1 for definition of site abbreviations

MATERIALS AND METHODS

Study region. The Ria Formosa coastal lagoon system, southern Portugal (Fig. 1), is a tidal system of channels that extends 55 km and is up to 6 km wide. The Ria Formosa covers an estimated 17 000 ha (11 000 ha of which is inundated at high tide) and consists of a mosaic of saltmarsh, beach, silt-sand tidal flats and essentially sandy channels which allow water circulation (Abreu & Machado 2000). The depth at high tide of the tidal flats is variable, averaging 2 m, whilst the channels are up to 6 m deep (Abreu & Machado 2000).

Within the Ria Formosa, 3 species of seagrass occur: *Zostera marina*, *Zostera noltii* and *Cymodocea nodosa*. *Z. noltii* is limited to the tidal flats. In contrast, *Z. marina* and *C. nodosa* are subtidal, although they may co-occur with *Z. noltii* over a narrow region in the extreme low intertidal. The subtidal seagrass meadows may be monospecific, (containing either *Z. marina* or *C. nodosa*), or meadows may be mixed (containing both *Z. marina* and *C. nodosa*).

Sampling and analyses. The Ria Formosa was extensively searched for *Zostera marina* over a distance of 25 km, from Praia de Faro to Fuzeta. From the 12 sites where *Z. marina* was identified, a total of 270 samples were collected within the 15 km separating the 2 most distant sites (Fig. 1). At each site (except Site 11), the sample position was recorded using 2 measuring tapes separated by a fixed distance (along the 'base-line'), so that x-y co-ordinates could be determined within each site. The area over which sampling was carried out varied depending on the size of the *Z. marina* site. At the larger sites (>300 m², see Table 1), where only a portion of the meadow was sampled, samples were collected at regular intervals along the base-line as well as at haphazard distances from the base-line. At the smaller sites (≤300 m²), where collecting could encompass the whole stand, samples were collected haphazardly both within and around the periphery of the stand. Sampling involved collecting vegetative shoots and dissecting out the basal 2 to 3 cm of meristematic tissue. Samples were subsequently freeze-dried pending microsatellite analysis.

DNA extraction, microsatellite characterization and locus scoring followed the procedures outlined in Reusch et al. (1999, 2000). In brief, DNA was extracted from 0.02 to 0.05 g of dried plant tissue using Qiagen plant extraction kits (Dneasy, Qiagen). Individuals were genotyped, using fluorescently labelled PCR products on an Applied Biosystems 3100 automated sequencer, at 9 microsatellite loci which had previously been shown to be highly polymorphic (GenBank accession numbers: AJ009899, AJ009901, AJ009902, AJ009905 and AJ249303 to AJ249307; Reusch et al.

1999, 2000, Reusch 2000). Of the 270 samples, 245 were assayed for these 9 microsatellite loci.

Clonal diversity within sites was calculated as P_d , the proportion of unique multi-locus genotypes (clones) identified within the samples (Ellstrand & Roose 1987). Observed (H_o) and non-biased expected (H_e) heterozygosities were determined for each site, after excluding the multiply sampled clones, using Genetix 4.02 (Belkhir et al. 2002). The statistical significance of heterozygote excesses at sites with more than 1 clone was tested using the Global Test in Genepop 3.1 (Raymond & Rousset 1995). The multi-locus F_{IS} value was determined using Genetix 4.02 (Belkhir et al. 2002).

Two tests were carried out to examine whether individual clone heterozygosity was related to clone size (estimated as the largest distance between samples of the same clone, and used as a measure of fitness). First, linear and quadratic regressions were carried out (SigmaPlot Ver. 6.00, SPSS). Second, in order to avoid the over-representation of small size classes, a randomization test to generate 95% confidence intervals was performed using MATLAB 6.12 codes (Math Works I) for clone assignment and GENSTAT 5 (Payne 1997) for the statistical analysis (Hämmerli & Reusch 2003).

Allele frequencies within sites, and the genetic differentiation among sites as estimated by F_{ST} , were determined using Genetix 4.02 after excluding multiply sampled clones. The genetic structuring of clones within the whole study area was examined using factorial cor-

Table 1. *Zostera marina*. Sites at the Ria Formosa, southern Portugal. The entire area was sampled, unless otherwise indicated. N_s : number of shoots sampled; N_g : number of genotypes; P_d = clonal diversity (N_g/N_s). -: not determined as only 1 shoot was collected

Site	Abbreviation	Area (m ²)	N_s	N_g	P_d
1, Esteiro do Baião	BAI	300	39	19	0.49
2, Retorta A	RTA	20	12	1	0.08
3, Retorta B	RTB	15	11	1	0.09
4, Garganta	GAR	50	15	1	0.07
5, Viveiro da Culatra 1	VC1	350 ^a	29	5	0.17
6, Viveiro da Culatra 2	VC2	10	5	1	0.20
7, Culatra A	CLA	4	3	1	0.33
8, Culatra B	CLB	1	1	1	-
9, Ilha do Coco	COC	2000	41	7	0.17
10, Ponta da Culatra (mixed bed)	PCM	1500 ^b	20	7	0.35
11, Ponta da Culatra (random sample)	PCU	1200 ^b	22	12	0.55
12, Ponta da Culatra (single species meadow)	PCS	4000 ^c	47	32	0.68
Area sampled (m ²): ^a 120; ^b 1000; ^c 2000					

Table 2. *Zostera marina*. Expected (H_e) and observed (H_o) heterozygosities at Ria Formosa sites with >1 clone (Genetix 4.02). p: significance of heterozygote excesses determined by the global test (Genepop 3.1)

Site	H_e	H_o	p
1	0.344	0.427	<0.01
5	0.484	0.644	<0.005
9	0.497	0.556	>0.15
10	0.458	0.540	<0.05
11	0.393	0.444	>0.15
12	0.361	0.416	<0.001

respondence analysis (FCA: Genetix 4.02). The phylogenetic relationship among sites was examined by producing an unrooted neighbor-joining (NJ) tree using Cavalli-Sforza's and Edwards genetic distance using 500 bootstrap runs (following Reusch et al. 2000).

RESULTS

P_d was found to average 0.29 and vary among sites, ranging from 0.07 to 0.68, with samples from the sites consisting of 1 to 32 clones (Table 1). For example, the 15 samples from Site 4 (which covered an area of approximately 50 m²) represented a single clone (P_d = 0.07), whilst the 39 samples from Site 1 (area approximately 300 m²) contained 19 clones (P_d = 0.49; Table 1). The site with the highest sample P_d was Site 12 (P_d = 0.68), with 32 clones identified in the 47 samples (Table 1).

H_o was higher than H_e within all the 6 sites that contained more than 1 clone (Table 2). At 4 of the 6 sites, this heterozygote excess was statistically significant (p < 0.05; Table 2). H_o ranged from 0.416 to 0.644

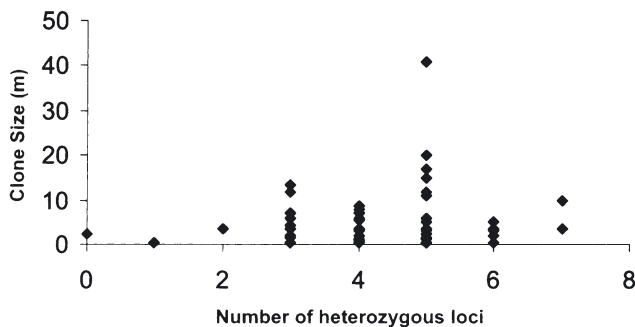


Fig. 2. *Zostera marina*. Plot of the number of heterozygous loci identified for each clone versus the size of the clone as measured by the maximum distance between samples sharing the same multi-locus genotype

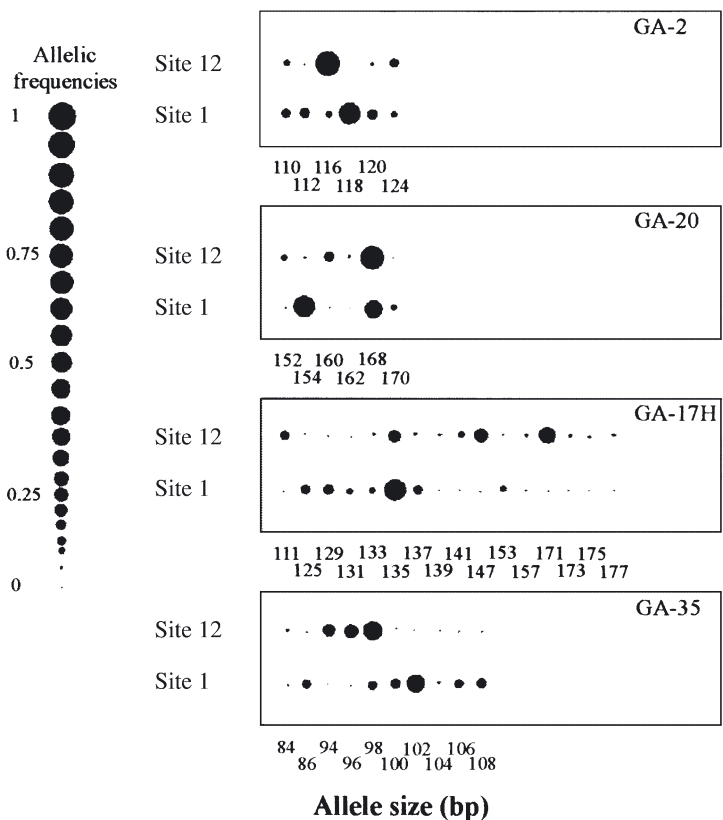


Fig. 3. *Zostera marina*. Allele frequencies for the 2 most distant sites (Sites 1 and 12) at 4 microsatellite loci (GA-2, GA-20, GA-17H and GA-35) which show the differences between sites. Allele frequencies are represented by the size of the dots

(mean = 0.505), whilst H_e ranged from 0.344 to 0.497 (mean = 0.423). In addition, the multilocus F_{IS} value of -0.195 supported the existence of heterozygote excesses. No relationship between clone heterozygosity and size was evident using both linear and second order models (p > 0.35 and 0.40, respectively) and the randomization test (only 2 mean values deviating from random expectations), although the shape of the plot suggests that the largest clones may have intermediate levels of heterozygosity (Fig. 2).

Allele frequencies (available on request from Corresponding author) indicated high levels of genetic differentiation among sites, as exemplified by the 2 most distant sites, Sites 1 and 12 (Fig. 3). For example, at Site 1, the frequency of allele 118 at the GA-2 locus was 0.58, while this allele was not detected at Site 12, where the most common allele was 116, which occurred at a frequency of 0.86 (Fig. 3). The low levels of gene flow among sites were also reflected in the multi-locus F_{ST} value of 0.223 (F_{ST} = 0.218, with only the 6 multi-clonal sites included). Sites which were close geographically were also found to be more similar genetically when compared to more distant sites.

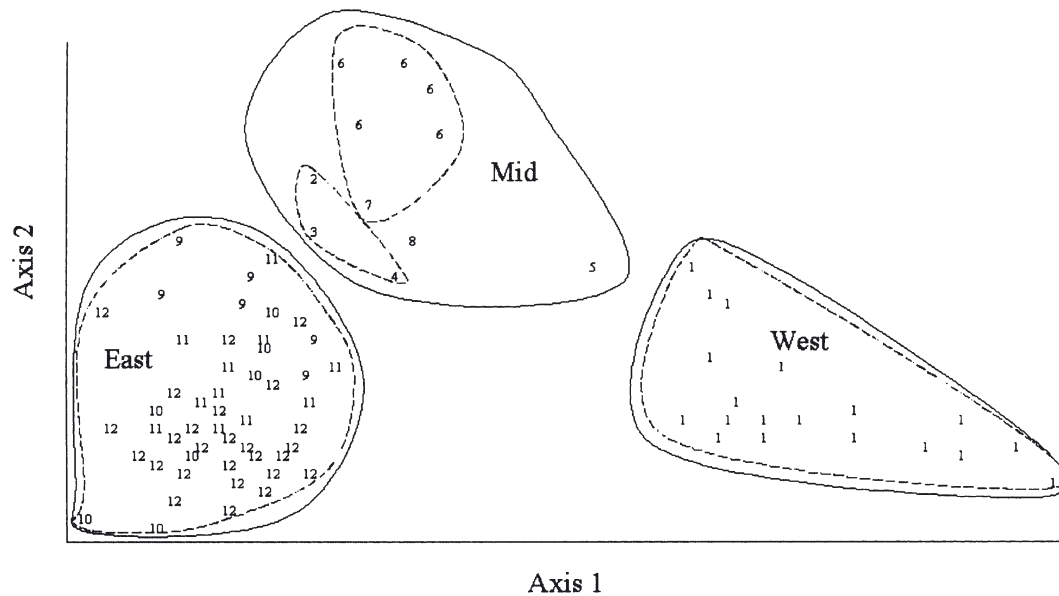


Fig. 4. *Zostera marina*. Plot of the factorial correspondence analysis (Genetix 4.02) for clones identified in the Ria Formosa. Numbers indicate the sites at which the clones were identified (see Table 1). Solid lines enclose individuals located in the east, middle and west of the study area. Dashed lines enclose sites that were determined to be phylogenetically similar with bootstrap values $>65\%$ on the unrooted neighbor-joining tree (using Cavalli-Sforza's and Edwards' genetic distance with 500 bootstraps)

Individual clones from the east, middle and west of the Ria Formosa all grouped near each other in the FCA and in the same branches of the NJ tree, often with bootstrap values $>65\%$ (Fig. 4).

DISCUSSION

Results from this study of *Zostera marina* were consistent with findings of reduced P_d at geographical limits in species that are able to reproduce both sexually and asexually. Sites within the Ria Formosa were found to have an average P_d of 0.29 (range 0.07 to 0.68), which is lower than the average of 0.86 (range 0.33 to 1.00) observed in the 10 perennial populations from ca. 44 to 54°N (Reusch et al. 2000, Reusch 2002). This result indicates that clonal growth was more important for maintaining sites at the *Z. marina* geographical limit when compared to more central sites. Furthermore, reduced clonal diversity at the species limit is supported by the geographical survey of Reusch et al. (2000), as the 2 monoclonal populations identified in that study were the most southern and most northern sampled ones (i.e. California and Finland). Reduced levels of genetic diversity in the Ria Formosa sites compared to the central sites were also detected. The levels of genetic diversity, as estimated by H_e , averaged 0.423 in the 6 non-monoclonal Ria Formosa sites compared to 0.486 in the 10 central populations (Reusch et al. 2000, Reusch 2002).

The finding of reduced clonal and genetic diversity in the Ria Formosa, when compared to northern sites, still remains valid when the different sampling strategies are taken into account, a factor that is important when estimating genetic diversity of clonal plants (Eckert 2002). When the 4 sites in the Ria Formosa which covered sufficient area to sample 1000 to 2000 m² were included in the analyses (to approximate the sampling of the central sites over areas of 1600 m²), the average P_d (0.43) and H_e (0.438) were still lower than the central sites. (As a further comparison, the 5 Baltic Sea populations had $P_d = 0.76$ and $H_e = 0.504$; Reusch 2002.)

Differences between the Ria Formosa sites and the Baltic Sea (the only central region with sufficient populations sampled to enable comparison) were also reflected in the levels of genetic differentiation among sites. Genetic differentiation was found to be much greater in the Ria Formosa than the Baltic, indicating much lower levels of gene flow among sites at the species limit. However, the clustering of sites in the FCA analysis (Fig. 4) indicates that genetic isolation by distance was present, which was also found in the Baltic Sea populations (Reusch 2002). In the present study, the F_{ST} value of 0.233 was over 10-fold greater than that for the Baltic Sea ($F_{ST} = 0.018$; Reusch 2002) even though the Ria Formosa sites were only separated by 15 km, compared to 54 km in the Baltic Sea. Furthermore, when the most distant site (Site 1) was excluded from the analysis, resulting in a maximum site separation of only 5.4 km, the F_{ST} value remained high at

0.156 (0.127 when monoclonal sites were excluded), representing an F_{ST} over 8 times greater than that for the Baltic, even though sites were separated by one-tenth of the distance.

Contrasting patterns between the Ria Formosa and central sites were also apparent in the relationship of sites to Hardy-Weinberg expectations. In the Ria Formosa, all 6 sites that we were able to test showed more heterozygotes than expected under Hardy-Weinberg equilibrium (4 being statistically significantly). In contrast, 4 of the 5 populations from the Baltic showed more homozygotes than expected under Hardy-Weinberg conditions (significance levels not tested; Reusch 2002). In the Ria Formosa the heterozygote excesses may be due to the selective advantage of individual clones, which are heterozygous at many loci and which out-compete less heterozygotic individuals in what may be relatively harsh conditions at the species geographical limit. This may be the case in the present study, as individuals with intermediate levels of heterozygosity covered the largest distances (Fig. 2); however, this was not statistically significant, possibly due to the relatively few genotypes identified, which results in low statistical power. Selection for high levels of heterozygosity has been shown in *Zostera marina* (Hämmerli & Reusch 2003), and common garden experiments and reciprocal transplant experiments have shown a heritable component to plant performance and localized adaptation (Backman 1991, Hämmerli & Reusch 2002).

Underlying mechanisms that may increase asexual reproduction at species' limits include both biotic and abiotic factors limiting seed production and sexual recruitment. For example, deep water (Hutchinson 1975) and cold temperature (Pigott & Huntley 1981) have both been shown to be key factors affecting reproduction in other plant species. In addition, sexual reproduction may be affected by ploidy levels, sterility mutations or the interaction of genetic and ecological factors over evolutionary time, through environmental selection against traits involved with sexual reproduction (Eckert 2002). Furthermore, reproduction and genetic diversity may also be affected as extinction and recolonization rates may be higher at species limits (Coyer et al. 2003). Factors that may contribute to the reduced genetic diversity of *Zostera marina* in the Ria Formosa are currently under study.

Regardless of the causes, the low genetic diversity and high genetic differentiation identified at the southern limit of *Zostera marina* in the eastern Atlantic may be representative of species that have the capacity to reproduce both sexually and asexually. Although regions were not replicated in the present study, the results support previous findings that populations at species geographical limits may have reduced sexual reproduction when compared to central populations.

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