

Genetic sub-structure and intermediate optimal outcrossing distance in the marine angiosperm *Zostera marina*

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Abstract The spatial distribution of genetic variability depends on the spatial patterns of clonal and sexual reproduction, gene flow, genetic drift and natural selection. Species with restricted dispersal may exhibit genetic structuring within populations with immediate neighbours being close relatives, and may show differentiation among populations. Genetic structuring of a species may have important genetic, evolutionary and ecological consequences including distance-dependent mating success. In this study we used microsatellite markers to show that clones of *Zostera marina* in a population in the Ria Formosa, Portugal, were aggregated and covered distances of up to 3–4 m. Clones

within 4 m of each other exhibited significant and positive coancestry values, reflecting the limited seed dispersal of this species. Hand-pollinations between near (0–10.9 m), intermediate (11–32 m) and far (15 km) individuals resulted in similar levels of seed set, although the near pollinations had higher, although not statistically significant, levels of seed abortion during maturation. Seeds from intermediate-distance pollinations had a significantly higher proportion of seeds germinate and shorter germination time than both the near and far seeds. Similarly, the average number of seedlings produced per pollination, used as an overall estimate of fitness, was significantly greater for the intermediate distance when compared to both near and far pollinations. These results suggest that the genetic structuring observed may result in both inbreeding and outbreeding depression, which gives rise to an intermediate optimal outcrossing distance.

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Introduction

The spatial distribution of genetic variability, both within and among populations of a species, is dependent upon the spatial pattern and strengths of gene flow, genetic drift and natural selection (Slatkin 1985, 1987). High levels of gene flow will tend to have a genetic homogenizing effect, whilst restricted gene flow will increase the likelihood of genetic structuring due to genetic drift, especially when population sizes are small (Wright 1978). When selection regimes vary between locations, individuals may exhibit adaptive differentiation (i.e. be locally adapted), which will also result in genetic heterogeneity (Shields 1982; Slatkin 1985, 1987). Over evolutionary time, spatial structure will be determined by the relative scales over which gene flow and environmental heterogeneity occur and the

intensity at which selection is operating (Jain and Bradshaw 1966; Endler 1977).

Genetic differentiation within and among plant populations has been shown to be dependent upon the dispersal capacity of the species (e.g. Hamrick and Godt 1996), although ecological factors such as breeding system, phenology and floral morphology are also likely to be important (Loveless and Hamrick 1984; Bush and Smouse 1992). Species ranging from aquatic plants, to ferns and forest trees may exhibit within-population genetic structure, with the extent often depending upon their dispersal mechanisms (e.g. Levin and Kerster 1974; Nason et al. 1997). However, genetic structuring within plant populations is not ubiquitous (Kudoh and Whigman 1997) and in some cases this may be directly attributable to high levels of gene flow (e.g. Leonardi et al. 1996). Furthermore, levels of among-population differentiation have been shown to be dependent upon seed dispersal distances, where species with restricted seed dispersal typically exhibit higher population differentiation (Loveless and Hamrick 1984).

Genetic structuring of a species can have important evolutionary consequences. In structured populations restricted pollen dispersal will result in self or genetically related pollen reaching a stigma (Levin and Kerster 1974). In many plant species this related pollen may then be rejected due to a self-incompatibility (SI) system, in which pollen growth is arrested on the stigma, in the style or during early embryo development (de Nettancourt 1977; Charlesworth and Charlesworth 1987). In addition, progeny from selfed or near neighbour pollinations may exhibit relatively low fitness, or so-called inbreeding depression (Price and Waser 1979; Falconer 1981; Charlesworth and Charlesworth 1987; Busch 2005). Inbreeding depression may be manifested as smaller or fewer seed per pollination (or even embryo abortion, which makes it difficult to distinguish between late acting SI and early acting inbreeding depression; Seavey and Bawa 1986; Waser 1993) poor seed germination, and reduced adult size, survival and fertility (Charlesworth and Charlesworth 1987).

At the other end of the scale, reduced seed set and offspring fitness may also result when pollen moves over relatively large distances so that genetically dissimilar pollen is deposited on stigmas, although this phenomenon has been the focus of much less attention than near neighbour pollinations (Waser 1993). Reduced fertilization of long distance pollen is possible if fertilization is under multilocus control, as suggested by the presence of pseudoincompatibility (in which successful selfed pollinations are relatively few, although not zero) and continuous variation in responses of individuals (e.g. seed production; Waser 1993). Similarly, long distance pollinations may give rise to outbreeding depression, where progeny from crosses between distant individuals have reduced fitness (Price and

Waser 1979; Shields 1982). Outbreeding depression may result when selection has led to localized adaptation and therefore spatial differentiation of genotypes. Crosses between environments therefore lead to the dilution of advantageous alleles (Endler 1977; Price and Waser 1979; Shields 1982). Alternatively, outbreeding depression may result from the disruption, by recombination, of co-adapted gene complexes or other genetic mechanisms such as epistasis (Price and Waser 1979; Shields 1982; Waser 1993).

If seed set or relative fitness are reduced for short and long distance pollinations, intermediate distance pollinations will result in the best performance. In these cases an intermediate optimal outcrossing distance results for gamete receptivity and/or offspring fitness (Waser 1993). Intermediate optimal outcrossing distances have been observed in a number of plant species (e.g. Price and Waser 1979; Waser and Price 1989, 1994; Stacy 2001; Willi and van Buskirk 2005). However, in some cases an intermediate optimum has not been observed (e.g. Fenster 1991; Hauser and Loeschcke 1994) even though outbreeding depression has been identified (Waser and Price 1994; Heiser and Shaw 2006).

Zostera marina L. is a seagrass (marine angiosperm) that reproduces both vegetatively (via the clonal lateral spread of rhizomes) and sexually through seeds (den Hartog 1970; Phillips and Meñez 1988). Reproductive shoots contain branches (rhipidia) which produce numerous inflorescences (spathe) in which male and female flowering is asynchronous (protogynous) and therefore likely to reduce within spathe selfing (de Cock 1980). Controlled pollinations have shown *Z. marina* to be self-compatible (Ruckelhaus 1995; Hämmerli and Reusch 2003a). Pollination is subaqueous (de Cock 1980; Cox et al. 1992) and 75% of the pollen has been shown to travel less than 6 m (Ruckelhaus 1996). Seed maturation takes 4–5 weeks (de Cock 1980) with field observations showing seed dispersal to be restricted to mostly less than 2 m (Orth et al. 1994; Ruckelhaus 1996). In the Ria Formosa, Portugal (near the species southern European limit) meadows of *Z. marina* occupy continuous patches, which grow in muddy substrates and extend from the low intertidal to depths of 6 m in this tidal lagoon system (Billingham et al. 2003).

Genetic analyses have shown that clonal growth and restricted dispersal of both pollen and seed in *Z. marina* results in genetic structuring within populations (e.g. Ruckelhaus 1998; Hämmerli and Reusch 2003b; Rhode and Duffy 2004). For example, Hämmerli and Reusch (2003b) used microsatellite markers to show that for two Baltic Sea populations clones generally covered distances of 2–4 m, with some results suggesting significant biparental genetic relatedness (coancestry) between samples collected from 6–10 m. Restricted dispersal in *Z. marina* has also led to high levels of among population genetic differentiation (Reusch et al. 2000; Reusch 2002), with the highest levels

reported being an F_{ST} of 0.233 over 15 km, near the species southern distributional limit in the eastern Atlantic, in the Ria Formosa, Portugal (Billingham et al. 2003).

The aims of this study of *Z. marina* in the Ria Formosa, Portugal were to examine; (1) the genetic structure within a population using microsatellite markers and (2) the effect of pollination distance (up to 15 km) on fertilization success and seed germination.

Materials and methods

Genetic structure

Sample collection was conducted in the Ria Formosa, Portugal to examine gene flow among sites as described in detail in Billingham et al. (2003). As part of that study, 39 samples were collected from the Esteiro Baião site, an almost continuous patch of *Z. marina*, which covered an area of $\sim 12 \times 35 \text{ m}^2$ from the low intertidal to 2 m depth. When samples were collected the sample position was recorded, using two measuring tapes separated by a fixed distance (along the “base-line”), so that XY co-ordinates could be determined. Sampling involved collecting vegetative shoots from the seagrass “carpet” and dissecting the basal 2–3 cm of meristematic tissue. Samples were subsequently freeze-dried pending microsatellite analysis.

The DNA extraction, microsatellite characterization and locus scoring followed the procedures of 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, Hilden, Germany). Individuals were genotyped, using fluorescently labelled PCR products on a Applied Biosystems 3100 automated sequencer, at nine microsatellite loci, which had previously been shown to be highly polymorphic (GenBank accession numbers: AJ009899, -901, 902, 905 and AJ249303-307; Reusch et al. 1999, 2000; Reusch 2000).

To test for the likelihood of shared multi-locus genotypes arising from sexual reproduction we used the method of Arnaud-Haond and Belkhir (2007). Multilocus autocorrelation analyses (SPAGeDi; Hardy and Vekemans 2002; using the coancestry of Loiselle et al. 1995) were employed, using the genotypes of all samples as well as only the unique genotypes (Reusch et al. 1999). To distinguish between genetic structuring due to clonal spread and restricted seed dispersal, all sampled genotypes were analysed using pair-wise comparisons which were divided into two distance categories, less than 11 m and greater than 11 m. Eleven metres was chosen as the cut off point as previous studies have indicated high levels of genetic relatedness over similar scales in *Z. marina* (Ruckelhaus 1996; Hämmerli and Reusch 2003b, c) and because in this study

approximately half of the hand-pollinations from the Esteiro Baião site were within this distance (see next section).

In order to examine genetic structuring due only to restricted seed dispersal, the unique multi-locus genotypes were analysed using the centre of a clone as the coordinate in the autocorrelation analysis (Hämmerli and Reusch 2003b; Alberto et al. 2005). As seed dispersal in *Z. marina* has been shown to be generally less than 2 m (Orth et al. 1994; Ruckelhaus 1996), autocorrelation analysis was conducted using five distance categories chosen to have similar numbers of pair-wise comparisons. For all autocorrelation analyses, 95% confidence intervals around the coancestries were determined using permutation tests re-sampling 1,000 times (Hardy and Vekemans 2002).

Pollinations

Hand-pollinations were carried out in filtered seawater in flow-through tanks at the Universidade do Algarve, Ramalhete field station situated near the Ria Formosa, Portugal. Six flow-through tanks were used in the experiments. For each plant to be used in the experiment, distance measurements were taken from two fixed points in order to map the collected plants and enable distances between plants to be determined. All pollen recipient plants were collected using SCUBA or snorkel from the Esteiro Baião population (37°01'N, 7°59'W; see Billingham et al. 2003 for a map) between February and April, 2003 with pollinations taking place between 4 March and 20 May, which covered most of the flowering season. Pollen recipients were maintained in individual plastic pots of 17 cm diameter and 17 cm depth within three of the flow-through tanks. When collecting pollen recipients, as much rhizome as possible was extracted before being placed in the pot and covered with sediment and a thin layer of sand. In total, 36 plants were collected for use as pollen recipients of which 27 produced flowers able to be pollinated. The number of pollinations per pollen recipient ranged from 1 to 13 (mean $6.48 \pm 0.80 \text{ SE}$).

Pollen donor plants were also collected throughout the course of the experiment. Pollen donors from the Esteiro Baião population were mapped in the same fashion as pollen recipient plants in order to determine the pollination distances. Long-distance pollen donors, all regarded as coming from 15 km away, were collected by SCUBA diving at Ponta da Culatra (PCU and PCM sites in Billingham et al. 2003) located at 37°00'N, 7°49'W. Relatively large distances (10–20 m), or sand, separated all collected individuals in order to maximize the number of genotypes included in the experiment (i.e. to reduce re-sampling of clones). For the pollen donors only as much rhizome as would easily come loose was collected. Donors were maintained with five plants per pot, and those from Esteiro Baião and Ponta da Culatra were kept in different flow-through

tanks. In total, 49 pollen donors were collected from the mapped Esteiro Baião site and 55 from the Ponta da Culatra. Twenty-four pollen donors were used from Esteiro Baião, with the number of pollinations ranging from 1 to 13 (mean 4.38 ± 0.69 SE), whilst 21 Ponta da Culatra pollen donors were used with the number of pollinations ranging from 1 to 13 (mean 3.33 ± 0.63 SE).

Pollen recipients were monitored daily to assess individual inflorescence maturity. One or two days before the inflorescences were mature (i.e. ready to open), 0.3 mm mesh bags were gently tied around them to prevent pollen from other pollen recipients in the same tank from fertilizing the female flowers when they opened (Ruckelhaus 1995; Rhode and Duffy 2004). The pollen to be used (from a pollen donor with mature male flowers releasing pollen) was collected using a pasteur pipette and transferred to a clean glass Petri dish containing filtered seawater. The inflorescence to be pollinated (still attached to the plant) was then removed from the tank and the mesh bag removed. The inflorescence was then immersed in the Petri dish for about 30 s and moved through the filiform pollen so that the pollen contacted and attached to the stigmas. At this time the number of female flowers in the inflorescence with extended stigmas was counted and recorded. Finally, the mesh bag was once again placed on the inflorescence and the plant returned to the tank. Each pollination was labelled with a marked piece of masking tape.

After the male phase of flowering had finished within each hand-pollinated inflorescence, the mesh bag was removed. (Note that due to the asynchrony of the female and male flowers, it was very unlikely that males from within an inflorescence could pollinate female flowers of the same inflorescence.) During the following 3 weeks each hand-pollinated inflorescence was monitored to determine how many seeds were developing. When seeds were nearly mature and ready for release (5–6 weeks) 0.3 mm mesh bags were once again placed on the inflorescences to capture the released seeds. Following seed release the mesh bags containing the seeds were stored in one of the flow-through tanks (Phillips et al. 1983; Moore et al. 1993). In total, 181 inflorescences were hand-pollinated, although six were accidentally broken and were not included in the analyses.

Pollinations from the three different distance categories of 0–10.9 m, 11–32 m and 15 km (i.e. “near”, “intermediate” and “far”, respectively) continued throughout the experiment to minimize any temporal effects. When pollinations were analysed as four approximately equal time periods during the experiment (i.e. early late), no statistical difference was found among the number of pollinations from the different distance categories (χ^2 test, $P > 0.15$) indicating that temporal effects could be ignored. Similarly, no significant correlation between the number of female flowers pollinated in each inflorescence and the number of

seeds forming was found ($r^2 = 0.023$, $P > 0.90$), so the number of female flowers pollinated was not included in the analyses. In addition, there was no statistical difference between the numbers of stigmas per pollination among the distance classes ($P > 0.80$).

After all seeds had matured the mass of each seed was measured. Then, in order to assess germination, seeds were placed in 10‰ seawater and kept at 15°C; conditions thought to induce germination in this species (Phillips et al. 1983; Hootsmans et al. 1987; Moore et al. 1993). Individual seeds were followed by placing them in wells of 96 well PCR micro-plates, which were then kept in a plastic container with the aerated seawater. The plastic container was maintained in a 700 EDTU Fitoclima (ARALAB, Oeiras, Portugal) with $80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ light intensity and a 14 h:10 h day/night photoperiod. The filtered seawater was changed weekly. The occurrence of germination, and number of days to germination, were recorded for 1 month.

For statistical analyses the data were analysed as three distance categories; near (0–10.9 m), intermediate (11–32 m) and far (15 km). Comparisons of proportions (i.e. pollination and germination success as well as the average number of seedlings produced per pollination—used as a measure of overall fitness) were tested using the normal approximation to the binomial test (Zar 1999). Comparisons of measures (i.e. seed mass and time to germination) were analysed with ANOVA's and post hoc pair-wise *t*-tests using SigmaPlot, Version 6.00, SPSS, Chicago, IL, USA.

Results

All repetitions of multilocus genotypes had $P < 0.05$ of being the result of sexual reproduction and were therefore considered to result from repeated sampling of the same clone. Samples of the same clone within the Esteiro Baião site were generally aggregated (Fig. 1). For example, clones A, B, F and G consisted of 4–5 adjacent samples stretching distances of 3–4 m (and covering areas of ~ 4 – 20 m^2) with no different clones identified within each aggregation (Fig. 1). However, samples of two clones (I and N) were separated by ~ 20 m and different clones grew between these samples (Fig. 1).

Neighbouring individuals (0–10.9 m) had significantly higher coancestry ($P < 0.005$) than individuals separated by intermediate distances (11–32 m; Fig. 2). Evidence for restricted seed dispersal was found when replicate samples of the same clone (and the “non-clustered” clone samples I and N which would contradict the centroid analysis) were omitted from the analysis, with a significant negative overall slope ($P < 0.01$; Fig. 3). Comparisons within distance categories showed coancestry of clones from 0 to 3.5 m to be more genetically similar than expected by random

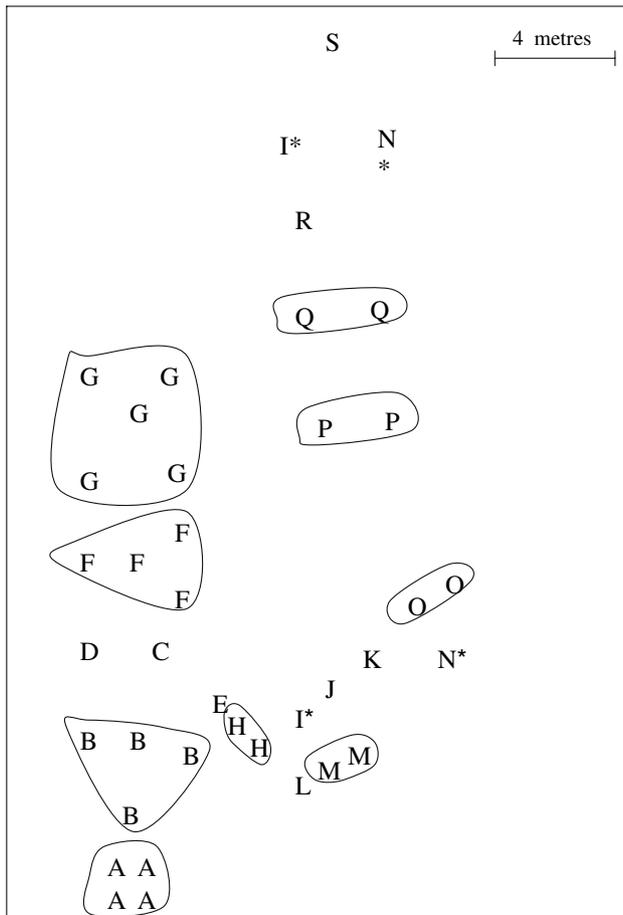


Fig. 1 Map showing the within site clonal structuring at Esteiro Baião. Letters indicate the different clones that were detected. Letters enclosed within a shape indicate those clones that were represented by more than one sample. Letters marked with an asterisk indicate samples of the same clone that were not adjacent (I and N)

chance indicating that seeds are typically dispersed over a distance less than 4 m ($F_{ij} = +0.051$, $P < 0.01$). At distances greater than 3.5 m no statistical differences from random expectations were observed (Fig. 3).

Near pollinations had the highest incidence of seed forming at 3 weeks, although by 6 weeks the far pollinations had the highest proportion of set seed and intermediate pollinations the lowest (Fig. 4). This change was due to the higher rate of abortion of seeds in the near pollinations compared to the intermediate and far pollinations. However, none of these trends were statistically significant ($P < 0.1$), which could be due to insufficient sample sizes to detect such small differences.

Seed mass varied significantly among distances ($F = 3.41$, $df = 2$, $P < 0.05$, Fig. 5). Seeds from intermediate distances were on average heavier than both the near and far distance seeds, although this was only significant at the $P < 0.1$ level ($t = 1.85$, $df = 1$, $P < 0.08$ and $t = 1.93$, $df = 1$, $P < 0.07$, respectively, Fig. 5). Similarly, the time to germination of

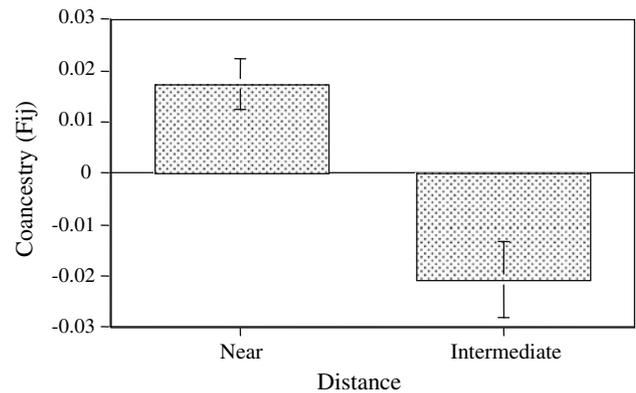


Fig. 2 Plot of the coancestry for both near and intermediate distances for all samples within the Esteiro Baião site. Coancestry was determined using the method of Loiselle et al. (1995) (using SPAGeDi; Hardy and Vekemans 2002), with 1,000 random permutations carried out to perform numerical re-sampling tests. Significance levels indicate $P < 0.05$

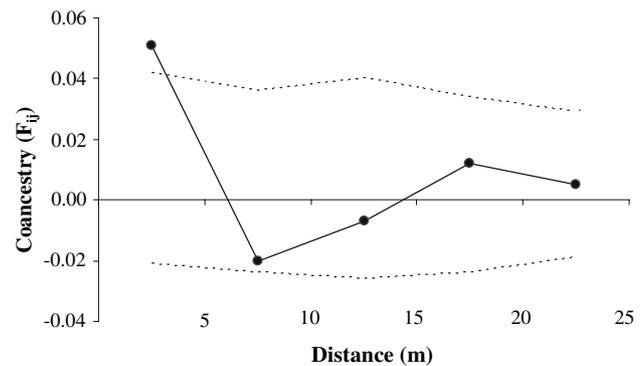


Fig. 3 Plot of coancestry versus distance, using unique multi-locus genotypes only. Coancestry was determined using the method of Loiselle et al. (1995) (using SPAGeDi; Hardy and Vekemans 2002), with 1,000 random permutations carried out to perform numerical re-sampling tests. Ninety-five per cent confidence intervals are shown

seeds varied among distance categories ($F = 3.21$, $df = 2$, $P \leq 0.05$, Fig. 6). Intermediate distance seeds required approximately half the time to germinate compared to both the near and far seeds ($t = 2.48$, $df = 1$, $P < 0.05$ and $t = 2.99$, $df = 1$, $P < 0.01$, respectively, Fig. 6).

Seeds from the intermediate distance had a higher probability of germination than both the near and far distance categories ($z = 9.3$ and 3.49 , respectively, $P < 0.001$), which were not statistically different from each other ($z = 0.76$, $P > 0.50$; Fig. 7). Intermediate distance pollinations produced nearly twice as many seedlings per pollination as either the near and far pollinations ($z = 2.296$ and 2.756 , respectively, $P < 0.05$, Fig. 8).

Discussion

The Esteiro Baião population of *Z. marina* was structured genetically due to clonal expansion of individuals and the

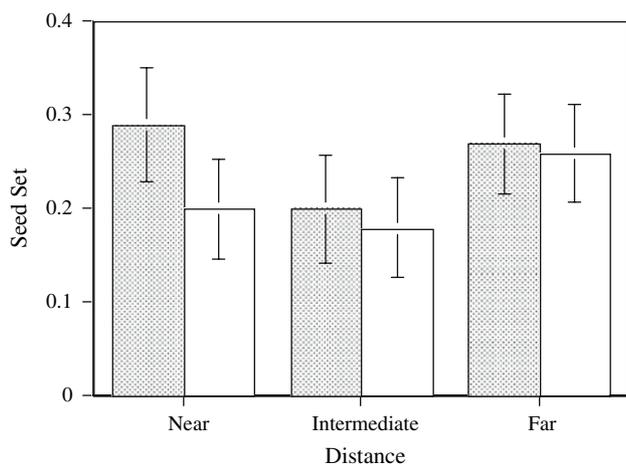


Fig. 4 Proportion of pollinations that formed seed versus pollination distance. *Hatched bars* indicate the proportion forming seed after 3 weeks, *white bars* indicate the proportion that produced mature seed at ~6 weeks. *Error bars* indicate one SE. No statistical differences were detected ($P > 0.05$)

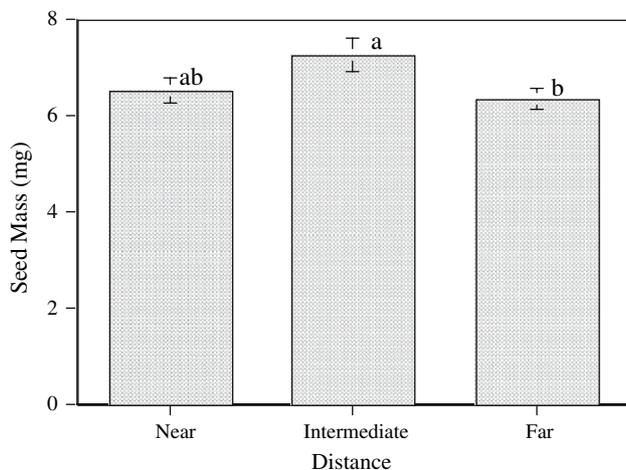


Fig. 5 Mass of individual seeds versus pollination distance. *Error bars* indicate one SE. *Different letters* indicate statistical differences at $P < 0.1$ (i.e. not significant at $P < 0.05$ level)

limited dispersal of seeds. Multiply sampled clones covered distances of 3–4 m (4–20 m²), similar to clone sizes identified in other populations of *Z. marina*. For example, two populations from the Baltic Sea had clones generally smaller than 7 m² (Reusch et al. 1999; Hämmerli and Reusch 2003b, c), although some clones up to 32 m² were identified (Hämmerli and Reusch 2003c). Furthermore, our inference that most seeds disperse less than 4 m agrees with the 2–4 m found by Hämmerli and Reusch (2003b), and with direct observations (Orth et al. 1994; Ruckelhaus 1996). Clonal structure at the Esteiro Baião site was typical of the Ria Formosa, in which analyses showed clones to be aggregated (with no intermingling of clones) with the largest clone occupying ~500 m² (M. R. Billingham, unpublished data).

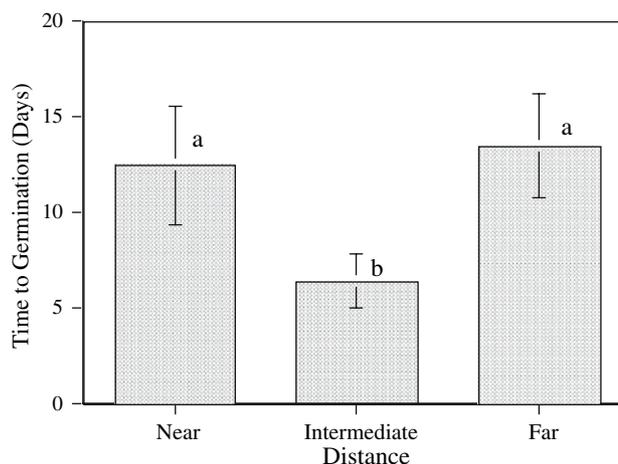


Fig. 6 Time to germination of the seeds versus pollination distance. *Error bars* indicate one SE. *Different letters* indicate statistical differences at $P < 0.06$

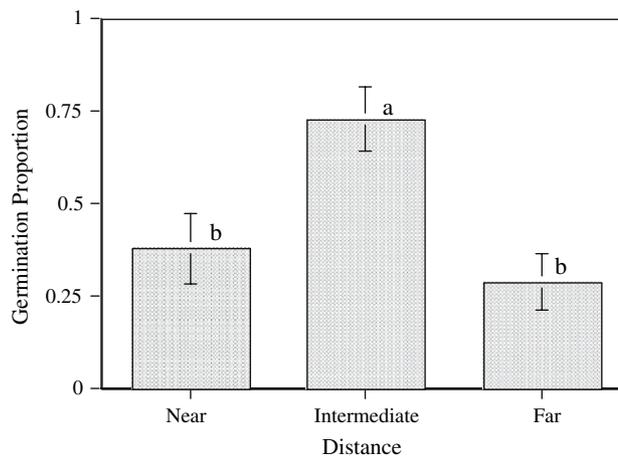


Fig. 7 Germination proportion of seeds versus pollination distance. *Error bars* indicate one SE. *Different letters* indicate statistical differences at $P < 0.001$

Near pollinations produced similar levels of seed at 3 weeks compared to both the intermediate and far pollinations. Given the genetic structure of the population, these near pollinations would have included both self-matings and matings among related individuals (especially as $F_{IS} = -0.304$, $P < 0.001$, M. R. Billingham, unpublished data). Thus the Esteiro Baião population exhibited no early acting self-incompatibility (de Nettancourt 1977; Charlesworth and Charlesworth 1987), a finding that reflects previous work on other populations of this species (Ruckelhaus 1995; Hämmerli and Reusch 2003a). However, the highest rates of abortion for the near seeds between 3 weeks and maturity suggest that either late-acting self-incompatibility or early acting inbreeding depression may have occurred.

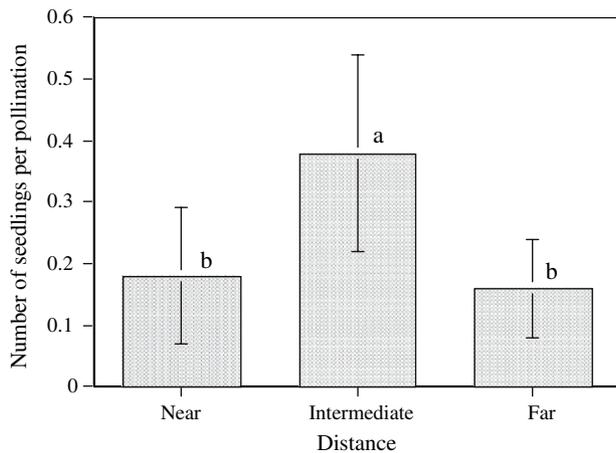


Fig. 8 Number of seedlings produced per pollination (used as an overall measure of fitness) versus pollination distance. Error bars indicate one SE. Different letters indicate statistical differences at $P < 0.05$

Progeny from the near pollinations performed more poorly in all fitness measurements than those of intermediate pollinations, indicating that inbreeding depression occurred. These results support most previous studies of *Z. marina*. For example, Ruckelhaus (1995) used hand-pollinations to show that seed set and seed survival were lower for self-pollinations than outcross pollinations and Reusch (2001), inferred selection against inbred offspring over the whole life cycle using molecular markers. Conversely, Rhode and Duffy (2004) found highest seed set for “selfed”, compared with “inbred” and “outbred”, pollinations in Chesapeake Bay, USA, although later life-history stages such as germination were not examined. However, demographic factors such as unstable patches, which suffer from summer defoliation and dieback, may have lead to increased inbreeding in Chesapeake Bay and the purging of deleterious alleles (Rhode and Duffy 2004).

Seavey and Bawa (1986) suggested that late-acting SI (due to post-zygotic rejection of the embryo) could be distinguished from early acting inbreeding depression by a relatively uniform timing of seed abortion rather than a more gradual loss of individuals throughout the life cycle. As near seeds had a higher rate of abortion, were lighter, required longer germination times and had relatively poor germination we conclude that they suffered inbreeding depression. Furthermore, as near seeds continued to express genetic load from seed formation to germination, this inbreeding depression might be expected to continue throughout the life cycle.

Selection against inbred progeny at Esteiro Baião (and all populations in the Ria Formosa) is also consistent with a significant heterozygote excess in the established population (Billingham et al. 2003). Given the genetic structuring and self-compatibility, along with restricted pollen dispersal in this species (Ruckelhaus 1995), much of the natu-

rally produced seed would be expected to be from selfed or related matings and to exhibit heterozygote deficits. Therefore any change from a heterozygote deficiency to excess suggests selection against inbred progeny. In addition, clone size is related to heterozygosity, which suggests that the more outbred and heterozygous individuals outcompete their inbred and relatively homozygous neighbours (Billingham et al. 2003; Hämmerli and Reusch 2003c) and that inbreeding depression continues to be expressed into the adult stage.

At the other end of the scale, far pollinations ranked highest in seed set at maturity indicating that no early acting genetic interactions took place to reduce fecundity (Waser 1993). However, as with the near pollinations, far pollinations were outperformed by intermediate pollinations in seed mass, time to germination, proportion of seed that germinated and average final number of seedlings per pollination. These results indicate that outbreeding depression occurred in the Esteiro Baião population. Ruckelhaus (1995), comparing self and outcross hand pollinations of *Z. marina*, also found evidence of outbreeding depression as the relative performance of the outcrossed seed decreased gradually during the period from initial seed set to survival at 7 months. In contrast, Rhode and Duffy (2004) found no evidence of outbreeding depression in Chesapeake Bay. The observed outbreeding depression in the Ria Formosa population may be due to higher levels of genetic differentiation ($F_{ST} = 0.233$ using microsatellites) compared with Chesapeake Bay ($F_{ST} = 0.198$ using allozymes) over similar distances, although these markers are not readily comparable.

Although localized adaptation has been identified in this species (Ruckelhaus 1994; Hämmerli and Reusch 2002) it is unlikely that the dilution of locally adapted alleles was responsible for the observed outbreeding depression. This is because the outbreeding depression was observed under controlled laboratory conditions where the local environment has no effect. It is therefore most likely that the observed outbreeding depression was due to the disruption, by recombination, of co-adapted gene complexes or other genetic mechanisms (Shields 1982; Waser 1993).

As both inbreeding and outbreeding depression were identified, it indicates that an intermediate optimal outcrossing distance exists in the Esteiro Baião population. Most studies of optimal outcrossing distance have been carried out without assessment of the genetic relatedness of individuals, with geographic distance being used as a correlate for genetic distance (Waser 1993). However, studies that have examined genetic structuring have found that pollinations between related individuals have reduced seed production and/or offspring fitness (Waser and Price 1989, 1994; Hardner et al. 1998). For example, populations of *Delphinium nelsonii* were genetically structured up to 5 m,

and 3 and 10 m pollinations had higher overall fitnesses than 1 and 30 m pollinations (Waser 1987; Waser and Price 1989, 1994). In addition to inbreeding depression, several studies relating genetic structure to outcrossing distance have found reduced fitness for the longest outcrossing distances for at least some fitness measures (e.g. Waser and Price 1989, 1994; and reviewed in Waser 1993).

Inbreeding and outbreeding depression in this study indicate that strong genetic effects operate in the Ria Formosa *Z. marina*. These differences were detectable in experiments conducted over one flowering season, due to the large magnitude of fitness differentials between intermediate pollinations and the near and far pollinations. Such differences most likely reflected the high level of genetic differentiation within and among *Z. marina* populations of the Ria Formosa. Consequently, these fitness differences may represent the extreme as the study population was at the species geographical limit. Studying the effects of pollination distance on fitness parameters in populations and species with lower levels of genetic differentiation might give different results.

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