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Genetic diversity of a clonal angiosperm near its range limit: the case of *Cymodocea nodosa* at the Canary Islands

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ABSTRACT: The seagrass *Cymodocea nodosa* forms a unique community in the Canary Islands, where it is classified as an endangered species. Biogeographic theory predicts that clonal species on islands near their distributional limits might show lower proportions of sexual (versus clonal) reproduction, lower genetic diversity, and higher differentiation. We addressed these hypotheses by comparing the genetic structure of *C. nodosa* from 10 meadows in the 4 main Canary Islands with 2 Iberian sites (Atlantic and Mediterranean) using microsatellites. A resampling method was proposed to standardize, among samples, genetic variability statistics estimating genotypic richness (R) and allelic richness ($\hat{A}$). A high degree of genotypic richness at the Canary Islands ($R = 0.30 – 0.94$, mean = 0.67) relative to Iberian sites revealed that *C. nodosa* performs effective sexual reproduction here. In contrast, lower $\hat{A}$ suggested a founder effect during the colonization of the archipelago, and similar allelic composition across all islands indicated colonization from a single source. A hotspot of genetic diversity was observed in El Medano (Tenerife), probably associated with lower drift in this meadow, the largest of the archipelago. Predominant north-south surface currents and a greater distance to the mainland could explain lower allelic richness of 2 northwestern sites on different islands and greater similarity between them. All meadows were differentiated from each other and there was no correlation between genetic and geographic distances. This non-equilibrium migration-mutation system was therefore mostly influenced by diversity resulting from genetic drift, and less by the homogenizing effects of gene flow.

KEY WORDS: Seagrass · *Cymodocea nodosa* · Clonal plant · Island biogeography · Microsatellites · Genetic differentiation · Marine dispersal · Population gene flow

INTRODUCTION

Island biogeography and evolutionary ecology of species' ranges predict that island colonizers would be composed of species or individuals with higher dispersal capacity (Holt 2003). The colonization of systems of isolated islands by species with weak dispersal potential therefore remains a fascinating mystery. The dioecious seagrass *Cymodocea nodosa* in the Canary Islands is a particularly interesting model to address as it is one of the dominant primary producers of the islands. However its very weak dispersal potential (Alberto et al. 2005) led us to question the mechanisms behind colonisation and population maintenance along this archipelago.

Population bottlenecks are severe reductions in effective population size caused by habitat fragmentation and isolation, which are generally conducive to a loss of genetic diversity (Leberg 1992), which is particularly noticeable at the level of allelic richness. Founder effects are population bottlenecks generated during a colonization process, as generally occurs when islands are colonized by jump dispersal, as opposed to short-range diffusion dispersal across reasonably favourable habitat. Furthermore, if populations remain isolated, the effects of genetic drift become

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more pronounced with smaller population size and will further decrease allelic richness. Gene flow can act as an evolutionary force that counterbalances the effects of genetic drift, and this may be a simple function of geographic separation between populations or may be influenced by other factors such as dispersal vectors and barriers. *Cymodocea nodosa* has low dispersal potential, at the scales of tens of meters, as shown by indirect methods using spatial autocorrelation statistics of microsatellite markers (Alberto et al. 2005, Ruggiero et al. 2005); however, the dispersal potential for this species in the Canary Islands remains unstudied. Low dispersal potential leads to the prediction that genetic drift will play the most predominant role in shaping genetic structure of *C. nodosa* in this archipelago.

Due to strong drift effects on peripheral populations, high population differentiation is expected in island habitats if stronger environmental stochasticity results in demographic bottlenecks, provided that gene flow from the mainland or central distribution ranges is not strong enough to balance drift. Levels of genetic differentiation are highly variable among individual seagrass meadows; even so, a trend for higher differentiation at species distribution limits has been detected (e.g. Billingham et al. 2003). The population of *Cymodocea nodosa* in the Canary Islands is peripheral to the general range of the species: the Canary Islands represent the western distributional limit, and are close to the southern distributional limit (Green & Short 2003). Therefore, high population differentiation and low gene flow are predicted at this location.

The species' range comprises the whole Mediterranean, but in the Atlantic is restricted to southwest Iberia, northwest Africa, and the Canary and Madeira Islands. In the Canary Islands, hydrodynamic conditions appear to limit its dispersal capabilities: it is only present along protected southern coasts of the islands, where it contributes to highly productive ecosystems (Carrillo & Rodriguez 1980, Reyes et al. 1995, Pavón-Salas et al. 2000, Barberá 2005). Although present at all islands in the Canary archipelago, only at Furteventura, Tenerife, and Gran Canaria does it develop into continuous meadows (Pavón-Salas et al. 2000), and the global species distribution in the archipelago is best defined as patchy and discontinuous. Recent losses of seagrass habitat in the Canary Islands have been associated with increases in coastal construction and implementation of fish cage farms (Tuya et al. 2002). Growing awareness of threats to the maintenance of these seagrass meadows has increased efforts to assess its extent and conservation status in the Canary Islands.

Seagrasses are capable of both clonal and sexual reproduction (Hemminga & Duarte 2000). The extent of asexual versus sexual reproduction can influence ecological and evolutionary processes in populations (de Kroon & Groenendael 1997). The leading hypothesis for marginal edge habitats, such as isolated island systems, predicts lower contributions of sexual reproduction and, consequently, higher environmental vulnerability of clonal plants due to a lower capacity for reliance on seeds for colonization and persistence. Genotypic richness — the proportion of individual units that are distinct genotypes in a population—is a good indication of whether asexual or sexual propagation is the prevalent mode of population growth (Eckert & Barrett 1993, Dorken & Eckert 2001).

The genotypic richness of clonal organisms can be examined provided that an appropriate set of high-resolution markers is available (Arnaud-Haond et al. 2005). Such analyses have revealed a wide range of genotypic richness across seagrass populations, from monoclonal stands (Reusch et al. 1999, Billingham et al. 2003) to highly diverse stands (Reusch 2001, Coyer et al. 2004, Diekmann et al. 2005, Arnaud-Haond et al. 2005). Seagrass meadows with extremely low levels of genotypic richness, which are highly dependent on vegetative growth, have been observed mainly at distributional edges (e.g. Reusch et al. 1999, Alberto et al. 2001, Billingham et al. 2003). This effect is even more pronounced in dioecious species like *Cymodocea nodosa*, because both male and female genotypes are needed to produce sexual propagules. Recent experimental manipulations of seagrass genotypic richness have suggested short-term advantages of clonal diversity: both the resistance to disturbance (Hughes & Stachowicz 2004) and the resilience (Reusch et al. 2005) of experimental *Zostera marina* patches were improved with greater genotype richness. On the other hand, successful sexual reproduction increases genetic variation, which may provide the local adaptive potential required for long-term persistence of a population (Frankel & Soulé 1981).

In this study we used microsatellite loci (Alberto et al. 2003) to analyse genetic diversity patterns in *Cymodocea nodosa* across the Canary Islands, in order to address the following questions: (1) What is the relative importance of clonal versus sexual reproduction? (2) What is the range of allelic richness across the archipelago? (3) How does the dispersal potential of *C. nodosa* in the Canary Islands compare to the potential of other populations? (4) To what extent are the Canary Island populations isolated from each other and from others outside the archipelago? (5) Is there evidence for multiple colonization events?

MATERIALS AND METHODS

Sample collection. Ten *Cymodocea nodosa* meadows were sampled during June 2003 at the islands of

Tenerife, Gran Canaria, Fuerteventura and Lanzarote, in the Canary archipelago (Fig. 1). Collections at each site were performed by SCUBA diving and consisted of 40 sample units (N = 40) from a continuous meadow surface of 60 × 14 m. Random *x* and *y* coordinates for each sample unit were pre-determined and written onto a diving slate. Divers used 2 transect lines to swim to each intersection of *x* and *y* coordinates and collect pieces of horizontal rhizome with 3 to 5 vertical shoots attached. For 5 populations (Teresitas, El Medano, San Juan, Bahía Feliz and Las Canteras) this design was not possible due to depth or fragmentation of the seagrass meadow, and sampling was performed haphazardly, along a comparable area, without taking spatial coordinates. For some analyses presented here we compared results from the Canary Islands with 2 previously analysed meadows from Cadiz Bay (N ramets = 214) on the Atlantic southern Spanish Coast, and Alfacs Bay (N ramets = 203) on the Mediterranean Spanish coast (Alberto et al. 2005). In order to do so, adequate standardizations for sample size were employed as described below.

Microsatellite genotyping. After CTAB extraction (Doyle & Doyle 1988), samples were genotyped for 8 microsatellite loci (Alberto et al. 2003). Three PCR multiplexes with fluorescently labelled primers were used to analyse all loci on an ABI 377 automated sequencer using GENESCAN software (Applied Biosystems). Approximately 10 ng of DNA was amplified in a 15 µl volume containing: 60 µM each of dCTP, dGTP, dATP and dTTP; 2 mM of $MgCl_2$; 200 mM Tris-HCl (pH 8.4); 500 mM KCl; and 1U *Taq* DNA polymerase (Invitrogen, Life Technologies). Each reaction contained one of the following multiplexes of fluorescently labelled *Cymodocea nodosa* microsatellite primers: MA: Cn2-38/HEX and Cn2-14/6FAM; MB: Cn2-16/HEX, Cn2-18/6FAM, Cn4-29/NED and Cn2-45/6FAM; and, MC: Cn4-19/NED and Cn2-24/NED. Individual primer concentrations ranged from 0.06 to 0.23 µM. Cycling conditions consisted of an initial denaturing step of 4 min at 94°C, followed by 24 cycles of 'touchdown' PCR consisting of 30 s at 94°C, 30 s at 55°C (reduced by 0.2°C in each subsequent cycle), and 30 s at 72°C, then 10 additional cycles consisting of 30 s at 94°C, 30 s at 50°C and 40 s at 72°C, and a final elongation step at 72°C for 10 min.

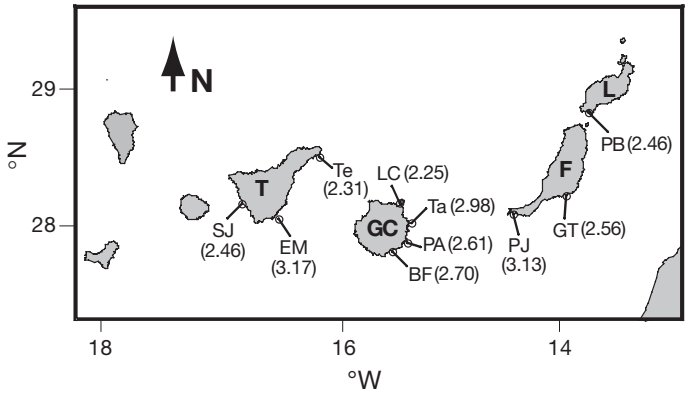

Fig. 1. Sampling sites in the Canary Islands. Tenerife (**T**) sites: (Te) Teresitas, (EM) El Medano and (SJ) San Juan; Gran Canaria (**GC**) sites: (LC) Las Canteras, (Ta) Taliarte, (PA) Playa de Arinaga and (BF) Bahía Feliz; Fuerteventura (**F**) sites: (PJ) Punta Jandia and (GT) Gran Tarajal; Lanzarote (**L**) site: (PB) Playa Blanca. Parenthesis: allelic richness ($\hat{A}$)

Genet assignment. When working with clonal organisms, it is important to discriminate ramets (modular units of the same genetic individual) from genets (genetic individuals originating from distinct sexual recombination events, which can be composed of several ramets). The problem arises because identical multilocus genotypes observed in 2 sampled ramets can either correspond to 2 ramets belonging to the same genet, or to 2 different genets if (by chance) the sampled alleles are all identical between the 2 genets. The probability of encountering the latter depends on the population frequencies of alleles observed in that genet and on the number of loci used to fingerprint samples. To address this issue, we calculated the probability of a given multi-locus genotype occurring n times, having been repeated as a consequence of different recombination events (P_{sex}) (Parks & Werth 1993). Arnaud-Haond et al. (2005) provide a detailed description of P_{sex} estimation and genet assignment for *Cymodocea nodosa*.

Genotypic richness. Genotypic richness *R* was estimated for each site according to Dorken & Eckert (2001) as:

$$R = \frac{(G-1)}{(N-1)}$$

where *G* is the number of different multilocus genotypes observed, and N is the number of sample units (ramets) analyzed. In order to compare *R* estimates from the Canary Islands with those from Cadiz and Alfacs, where sampling was conducted at a higher density, we standardized the data sets from the latter meadows to minimum N (40) by randomly resampling ramets. We replicated the resampling scheme 100 times in order to obtain SEs of *R* estimates. Resampling and genet identification from resampled data sets was performed using an R statistics package routine (R Development Core Team).

Allele and gene richness. After removal of clonal replicates from the data set, allele frequencies and the inbreeding coefficient F_{IS} (Weir & Cokerham 1984) were estimated. The H_0 of random union of gametes was also tested with the exact Hardy-Weinberg test using a probability test (Raymond & Rousset 1995a).

Genotypic linkage disequilibrium was tested using a Fisher exact test. All the above mentioned analyses were performed with GENEPOP (Raymond & Rousset 1995b). Gene diversity H_{exp}, unbiased for sampling size, was estimated using GENETIX 4.05.2 (Belkhir et al. 2001).

For each population, allelic richness $\hat{A}$ (average number of alleles per locus; Leberg 2002b) was also estimated. Because clonality leads to unpredictability of the number of genets G found in each sample, and because $\hat{A}$ depends on the size of G, a standardization of G was employed before comparing $\hat{A}$ across populations. The standardization was performed using a multiple random reduction of G (Leberg 2002b). An R routine was programmed to standardize G to the minimum genet number found in all populations (G = 14, genes = 28). We did so by computing $\hat{A}$ after reducing G in increments of 1 until all populations had a similar G. For each decreasing step of G, $\hat{A}$ was estimated for each of 100 replicates, allowing us to plot the decrease of $\hat{A}$ with decreasing G and to estimate the SD of $\hat{A}$. This method produces estimates of $\hat{A}$ similar to those delivered by the rarefaction method (Petit et al. 1998, Leberg 2002b).

Spatial analysis of genetic and clonal structure. The spatial genetic structure (SGS) of a population can be characterized by spatial autocorrelation, the relationship of a kinship coefficient between pairs of individuals with the spatial distance separating them. Analysis of SGS by means of spatial autocorrelation analysis was possible for 5 sites where spatial coordinates were available. We estimated coancestry using the kinship coefficient F_{ij} of Loiselle et al. (1995), between all pairs of genets (genet level analysis) and ramets (ramet level analysis). For the genet level analysis, the central coordinates (average x and y coordinates of genets sampled more than once) were used to represent genet location. If populations are at drift-dispersal equilibrium, average F_{ij} at the genet level estimated for a series of spatial distance classes (here defined as $F_{ij[\text{spatial limit of class m}]}$), is expected to decrease linearly with the logarithm of spatial distance for 2-dimensional populations (Rousset 1997, Hardy & Vekemans 1999). The extent of SGS can be quantified by the regression slope of this relationship (hereafter defined as *blog*) (Vekemans & Hardy 2004).

The SGS analysis was also performed at the ramet level, and an additional analysis was performed that considered only ramet pairs that corresponded to different genets (option 'only pairs among categories' in SPAGeDi software). The spatial distance at which these 2 analyses show the same mean kinship values defines the spatial limits of the clonal subrange (Alberto et al. 2005), the spatial scale beyond which the effects of clonal structure on SGS becomes negligible. The clonal subrange estimates the distance range where pairs between clonemates can be found.

In order to have a uniform distribution of pairs across spatial distance classes, we had to use wider class limits at the genet level since fewer pairs (depending on R) were used in the analysis. Thus, the spatial limits of classes for ramet and genet levels were <8, 8–15, 15–20, 20–25, 25–30, 30–60 m, and <8, 8–15, 15–20, 20–60 m, respectively. Spatial coordinates were randomly permuted among individuals 10 000 times, in order to test for each spatial distance class whether the observed mean kinship values were different from those expected under a random distribution of genotypes. A distribution of permuted regression slopes was also constructed: the test p-values for the observed regression were estimated as the fraction of this distribution greater than the observed slope. The calculations were performed using SPAGeDi (Hardy & Vekemans 2002).

The ability of autocorrelation analysis to detect significant SGS depends on the size of the sample (Epperson & Li 1997). We used resampling techniques on larger samples from a previous study (N = ca. 220 for 2 Iberian sites, Cadiz and Alfacs, in Alberto et al. 2005) that evidenced spatial autocorrelation in order to test whether the smaller samples used here (N = 40) were large enough to detect a SGS pattern at least as large as detected before with larger sample size. To achieve this, larger data sets were randomly resampled so that they were identical in size to Canary Islands samples (N = 40), and the above-mentioned genet level SGS parameters were estimated. Finally, we repeated this simulation 100 times to determine the ability of an N = 40 sampling scheme to detect significant SGS patterns at the 2 levels observed at Cadiz and Alfacs, respectively. The power of tests for the shortest distance ($F_{ij[8\ m]}$) and *blog* were calculated as the proportion of data sets that resulted in significant tests.

Population differentiation. Genetic differentiation among sites was assessed using the data set without clonal replicates. Levels of differentiation were described by the F_{ST} estimator θ (Weir & Cokerham 1984), and significant departures from H_0 'no differentiation' were tested for with an appropriate Fisher exact test using GENEPOP (Raymond & Rousset 1995a,b). The hypothesis of isolation-by-distance (IBD) (Wright 1943, Rousset 1997) was tested at 2 spatial scales of geographic separation: among the Canary islands populations, and among a larger set of data including the distant Cadiz Bay and Alfacs Bay populations. In order to test for IBD, the H_0 'no correlation between pairwise estimates of $\theta(1-\theta)^{-1}$' (Rousset 1997) and the logarithm of geographic distance between populations was assessed using Mantel's test (Mantel 1967) with ISOLDE, part of GENEPOP.

In order to analyse biogeographical relatedness among Canary archipelago meadows, we computed Cavalli-Sforza distances from microsatellite allelic frequencies, and also included as an 'outgroup' the datasets from Cadiz (south Iberian Atlantic) and Alfacs (Iberian Mediterranean) from Alberto et al. (2005). A neighbor-joining (NJ) tree was constructed and bootstrap resampling using 1000 replicates was performed. All of these analyses used PHYLIP (Felsenstein 1994).

RESULTS

Genetic diversity

A total of 373 *Cymodocea nodosa* ramets from meadows of the Canary islands were genotyped, revealing a total of 45 alleles from 8 loci and 255 distinct multilocus genotypes. All multiple copies of each multi-locus genotype had $P_{sex} < 0.05$, and were thus considered as different ramets of the same genet. The spatial distribution of ramets and genets in space at 5 sites where spatial coordinates were available is presented in Fig. 2. Intrapopulation genetic diversity statistics are summarized in Table 1. For our sample size (N = ca. 40), the number of genets *G* found per meadow ranged from 14 in Las Canteras to 34 in El Medano. Genotypic richness *R*, which describes the relative importance of clonal versus sexual propagation, was estimated after the number of ramets sampled n in the different meadows was standardized to equal the minimum used over all meadows (N = 35). Las Canteras had the lowest *R* (mean *R* over standardization replicates ± SD = 0.30 ± 0.02). The highest *R* was found in El Medano (*R* = 0.94 ± 0.01), where nearly every ramet originated from a different seed. The average *R* value for the Canary Islands was 0.67, which was similar to that found for Cadiz Bay and Alfacs Bay after standardization of N was applied (both *R* = 0.62).

Several low frequency alleles found in Canary Island samples had not been reported for specimens collected from the Iberian Peninsula (Cadiz and Alfacs; Alberto et al. 2005) (Fig. 3); some were exclusive to specific populations, while others were identified in several meadows from different islands. These exclusive alleles became rarer in the more northwesterly located populations, and this was correlated with a similar trend in allelic richness (see below). On the other hand, many alleles identified in Iberian coast populations were not detected in the Canary Islands (Fig. 3); therefore, neither of these regions appeared to be a genetic subset of the other.

We used a multiple random reduction method to standardize *G* before estimating and comparing allelic

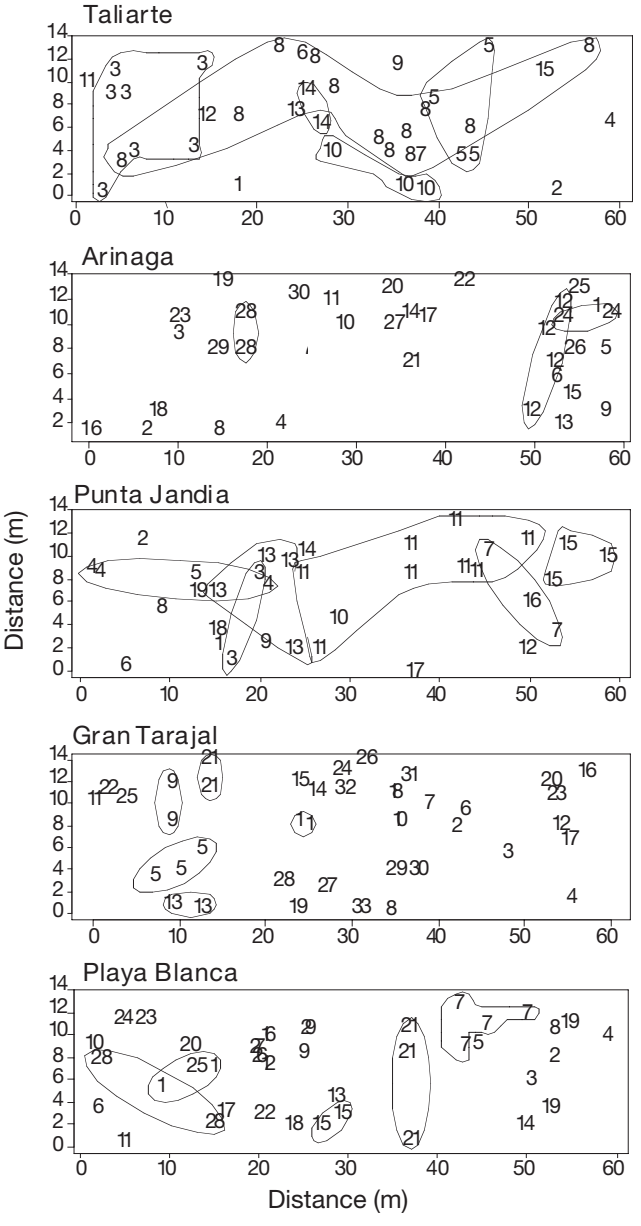

Fig. 2. *Cymodocea nodosa*. Spatial representation of clones (genets) for 5 meadows at Canary Islands. Numbers: genets at sampling coordinates. Clones with >1 ramet identified per sample are grouped to facilitate identification

richness $\hat{A}$ among populations. This procedure allowed us to plot $\hat{A}$ as a function of *G* (Fig. 4). Allelic richness was lower at the Canary Islands (mean $\hat{A}$ over standardization replicates = 2.67, *G* = 14) than at Cadiz (3.53) and Alfacs (3.18); however, $\hat{A}$ at El Medano was comparable to Alfacs. The lowest $\hat{A}$ value (2.25) was found at Las Canteras. Gene diversities H_{exp} were lower at the Canary Islands (mean = 0.406) than at Cadiz (0.582) and Alfacs (0.534), with lowest values

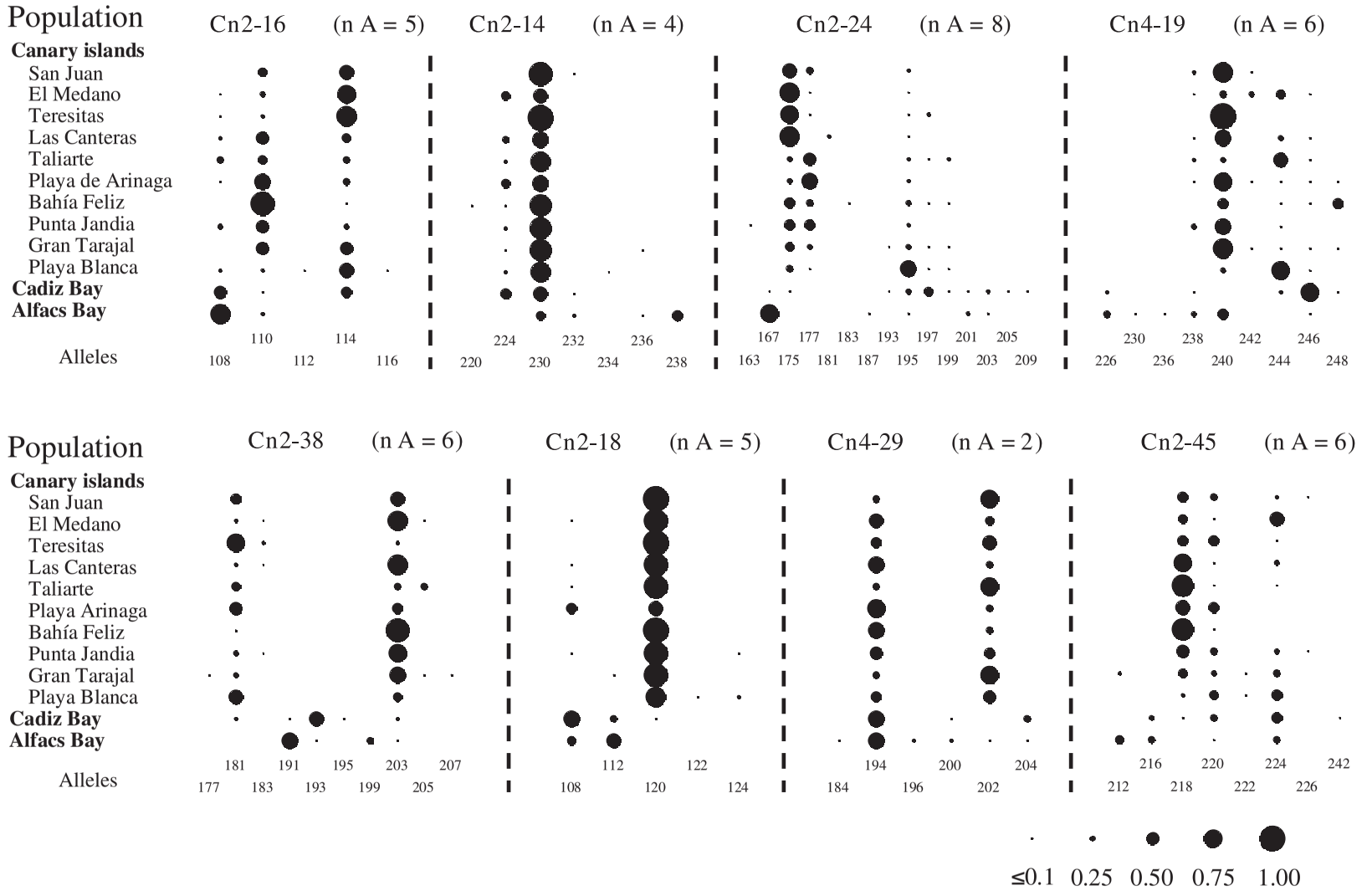

Fig. 3. *Cymodocea nodosa*. Microsatellite allele frequencies from Canary Islands, Cadiz Bay (Atlantic), and Alfacs Bay (Mediterranean). Circles: alleles; diameter of circles: allele frequency. Number of alleles (A) indicated only for Canary Island populations

Table 1. *Cymodocea nodosa*. Analysis of clonal and genetic diversity of meadows sampled at Canary Islands. T: Tenerife; GC: Gran Canaria; F: Fuerteventura; L: Lanzarote; N: number of sampled ramets; *G*: number of genets; *R*: genotypic richness standardized to minimum N (35); $\hat{A}$: allelic richness after standardization to minimum *G* (14); H_{exp}: Nei's gene diversity; F_{IS}: inbreeding coefficient. Significant departure from Hardy-Weinburg equilibrium: ***p < 0.001, **p < 0.05. Estimates for F_{ij}, H_{exp} and F_{IS} produced after removal of clonal replicates. Arinaga: Playa de Arinaga

Population	N	*G*	*R*	$\hat{A}$	H_{exp}	F_{IS}
Canary Islands						
San Juan (T)	39	27	0.70 ± 0.03	2.46 ± 0.14	0.286 ± 0.267	−0.037
El Medano (T)	36	34	0.94 ± 0.01	3.17 ± 0.19	0.438 ± 0.247	0.180***
Teresitas (T)	35	21	0.53	2.31 ± 0.11	0.381 ± 0.243	−0.018
Las Canteras (GC)	39	14	0.30 ± 0.02	2.25	0.293 ± 0.254	−0.010**
Taliarte (GC)	38	15	0.39 ± 0.02	2.98 ± 0.10	0.469 ± 0.230	0.023
Arinaga (GC)	35	30	0.85	2.61 ± 0.07	0.480 ± 0.035	−0.166
Bahia Feliz (GC)	39	32	0.83 ± 0.03	2.70 ± 0.13	0.449 ± 0.199	0.114
Punta Jandia (F)	35	19	0.53	3.13 ± 0.15	0.467 ± 0.134	0.072
Gran Tarajal (F)	39	33	0.86 ± 0.02	2.56 ± 0.18	0.410 ± 0.178	0.162
Playa Blanca (L)	38	29	0.76 ± 0.02	2.55 ± 0.12	0.391 ± 0.16	0.052
Other						
Cadiz bay	40[a]	26 ± 2.3[a]	0.62 ± 0.06	3.53 ± 0.20	0.564 ± 0.120	−0.129***
Alfacs bay	40[a]	24 ± 2.0[a]	0.62 ± 0.07	3.18 ± 0.35	0.510 ± 0.140	−0.064***

[a]Values after standardization to N = 40

occurring at San Juan (0.281) and Las Canteras (0.282) (Table 1). Thus, heterozygosity followed the same trend as allelic richness, and was also lower at the Canary Islands (mean H_{exp} = 0.406) than at Cadiz (0.564) or Alfacs (0.510). All meadows were in Hardy-Weinberg equilibrium, except for a significant heterozygote deficiency detected at El Medano (F_{IS} = 0.180) and significant weak heterozygote excess (F_{IS} = −0.01) at Las Canteras (Table 1). When testing for genotypic linkage disequilibrium in each population, and after applying Bonferroni's correction, only a few pairs of loci rejected the H_0 that genotypes at one locus were independent of genotypes at the other locus. The pairs of loci that yielded signifficant tests were different across the populations.

Small-scale spatial genetic structure (SGS)

We determined the power of our sample size from the Canary Islands to identify SGS patterns of the magnitude detected when larger samples were collected (Alberto et al. 2005). One such sample of *Cymodocea nodosa*, from Cadiz Bay, was found to have very restricted gene dispersal as estimated from the high degree of SGS; both the smaller distance class ($F_{[8\ m]}$ = 0.0407, p < 0.05) and the *blog* (−0.0434, p < 0.05) were significantly different from that expected under the hypothesis of random distribution of genotypes (Alberto et al. 2005). With our simulation, we found that all of the 100 resampled data sets (n = 40) from Cadiz resulted in significant tests for the smaller distance class ($F_{[8\ m]}$) and the *blog*. Therefore, the power of the sampling scheme used in the Canary Islands to detect such SGS patterns was 1.00. For weaker but significant SGS values, like the one observed at Alfacs Bay ($F_{[8\ m]}$ = 0.0074, p < 0.05; *blog* = −0.0122, p < 0.05), the power was only 0.21 for the test of the first distance class ($F_{[8\ m]}$) and 0.33 for the slope (*blog*) test. This approach was only valid for sites with similar genotypic richness.

For 5 *Cymodocea nodosa* meadows at the Canary Islands, it was possible to study genetic structure using spatial autocorrelation analysis because spatial coordinates were available for all sampled ramets. The potential inflating effects of clonality on the levels of coancestry between sampled ramets were evaluated using separate genet (only 1 copy of each genet) and ramet (complete data) level analyses, respectively. The genet level analysis, with the exception of the Gran Tarajal meadow, did not detect any significant SGS pattern (Table 2). Significant genetic structure was found at Gran Tarajal, as the first distance class (<8 m) had a mean F_{ij} higher than expected under a H_0 of random distribution of

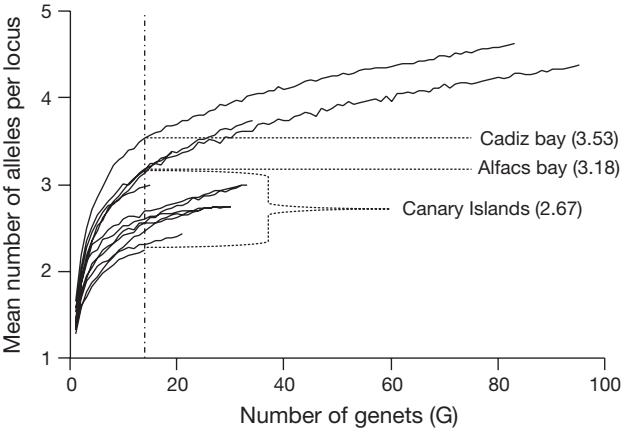

Fig. 4. *Cymodocea nodosa*. Mean number of alleles per locus as a function of number of different genets *G* resampled for the Canary Islands, Cadiz Bay (Atlantic), and Alfacs Bay (Mediterranean). -·-·-: minimum number of genets found at any meadow (observed at Las Canteras); this value was used to standardize *G* across populations in order to estimate allelic diversity ($\hat{A}$; see Table 1)

Table 2. *Cymodocea nodosa.* SGS results for the Canary Islands. $F_{(<8\ m)}$: mean F_{ij} kinship values found for shortest distance intervals used in autocorrelation analysis; *blog*: slope of regression of mean kinship with logarithm of spatial distance; clonal sub range: linear distance under which clonality has non negligible effects on genetic structure. Arinaga: Playa de Arinaga; see Table 1 for island abbreviations

Population	Genet level		Ramet level	
	$F_{(<8\ m)}$	*blog*	$F_{(<8\ m)}$	Clonal sub-range
Taliarte (GC)	−0.0084	−0.0133	0.0735	>50 m
Arinaga (GC)	−0.0301	0.0139	−0.0181	<15 m
Punta Jandia (F)	−0.0206	−0.0244	0.0652***	<40 m
Gran Tarajal (F)	0.0424***	−0.0419***	0.0440***	<15 m
Playa Blanca (L)	−0.0117	−0.0112	0.0360***	<25 m

genets in space ($F_{[8\ m]}$ = 0.0424, $p < 0.05$; Table 2). The *blog* between the mean pair-wise kinship estimates and spatial distance was also significantly more negative than expected under the same H_0 (*blog* = −0.0419, $p < 0.05$). The SGS estimates for Gran Tarajal were similar to those reported for Cadiz Bay (Alberto et al. 2005).

The ramet-level analysis revealed that the effect of clonality on SGS varied widely among meadows (Table 2). The distance class at which the kinship coefficient for the ramet level equals the among-genet level defines the clonal subrange: an estimate of the spatial range at which clonality has non-negligible effects on the SGS. The clonal subrange (Table 2) varied from <12 m at Playa de Arinaga and Gran Tarajal to an undefined maximum at Taliarte, where it probably extended further than the sampling scale used in this study (60 m).

Patterns of population differentiation

All sites sampled at the Canary Islands were genetically differentiated: all pair-wise population comparisons rejected the H_0 of identical allelic distribution across populations (all p values < 0.001). Across larger scale comparison between the Canary Islands and Cadiz and Alfacs Bays, genetic differentiation was also significant but the differentiation level was approximately twice as large. The mean pair-wise θ for *Cymodocea nodosa* meadows at the Canary Islands was 0.177, with a minimum value of 0.040 between Bahia Feliz and El Medano, and a maximum value of 0.425 (only pairs within populations at the Canary Islands were considered) between Las Canteras and Sanjuan (Table 3). Both maximum and minimum pair-wise θ values corresponded to sites situated on different islands, as differentiation within and among islands was comparable. Across the archipelago there was no significant IBD (Fig. 5, closed circles). Genetic differentiation was, as expected, higher (twice as high) for larger scales of geographic separation, i.e. when the Canary Islands were compared with the Iberian Peninsula sites of Cadiz (mean θ = 0.368) and Alfacs (mean θ = 0.445).

The NJ analysis (Fig. 6) revealed a distinct Canary Island cluster, with 100% bootstrap resolution separating long branches of all Canary Island meadows from the Iberian Peninsula at Cadiz (Atlantic) and Alfacs (Mediterranean). The relative position and length of branches connecting this cluster with Cadiz and Alfacs was consistent with the geographic position of the sites. Inside the Canary Islands cluster there was no further significant grouping, and populations paired with each other independent of the island from where they were sampled. For example, the meadows at Las

Table 3. *Cymodocea nodosa.* Pair-wise genetic differentiation (F_{ST}) among populations at the Canary Islands estimated by θ (Weir & Cokerham 1984)

	San Juan	El Medano	Teresitas	Las Canteras	Taliarte	Playa de Arinaga	Bahia Feliz	Punta Jandia	Gran Tarajal	Playa Blanca	Cadiz Bay
Canary Islands											
San Juan											
El Medano	0.192										
Teresitas	0.255	0.015									
Las Canteras	0.425	0.141	0.074								
Taliarte	0.269	0.151	0.160	0.263							
Playa de Arinaga	0.216	0.156	0.166	0.229	0.172						
Bahia Feliz	0.127	0.040	0.049	0.180	0.116	0.092					
Punta Jandia	0.380	0.173	0.199	0.252	0.167	0.253	0.194				
Gran Tarajal	0.291	0.151	0.180	0.262	0.231	0.249	0.153	0.181			
Playa Blanca	0.164	0.128	0.145	0.237	0.206	0.177	0.083	0.256	0.090		
Other											
Cadiz Bay	0.444	0.369	0.398	0.407	0.359	0.313	0.359	0.304	0.348	0.376	
Alfacs Bay	0.496	0.438	0.460	0.480	0.434	0.397	0.412	0.430	0.456	0.443	0.287

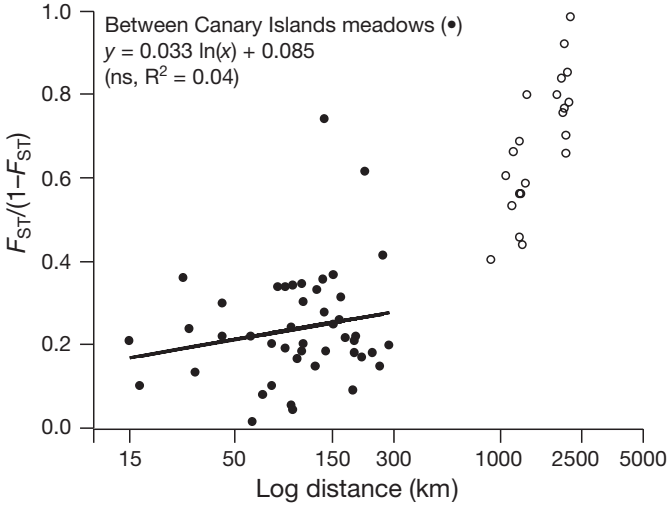

Fig. 5. *Cymodocea nodosa.* Isolation by distance (IBD) for Canary Islands. ●: pair-wise values of genetic and log geographic distance for comparisons among meadows within the archipelago; ○: pair-wise values for comparisons between Canary Islands and Iberian Cadiz and Alfacs bays. Regression estimates of slope, intercept, and R^2 were calculated for Canary Islands only

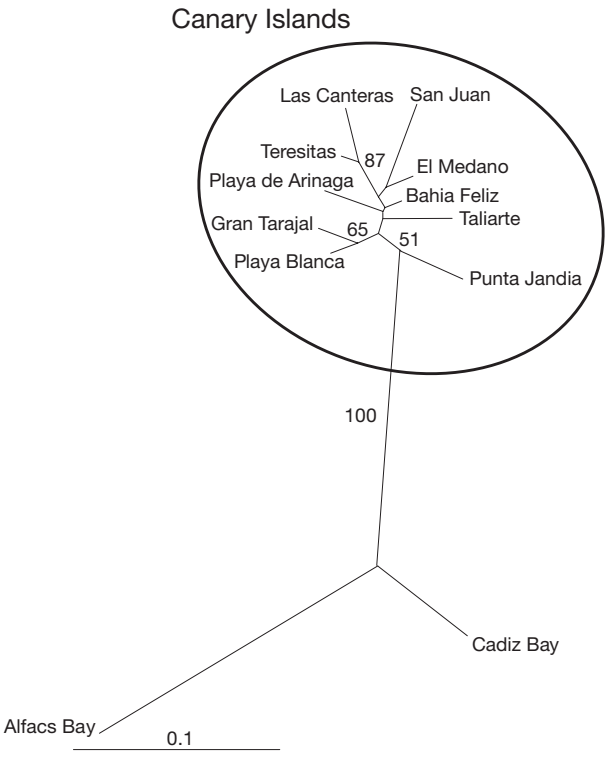

Fig. 6. *Cymodocea nodosa.* Neighbor joining (NJ) network representing relationship among meadows from Canary Islands, Cadiz bay (Atlantic), and Alfacs Bay (Mediterranean). Bootstrap values derived from 1000 permutations of allele frequencies; only values >50 shown

Canteras (Tenerife) and Teresitas (Gran Canaria) clustered together in 87% of bootstrap configurations (Fig. 6).

DISCUSSION

Genotypic richness

Sexual allocation in clonal plants may become reduced due to environmental (Kanno & Seiwa 2004, Honnay & Bossuyt 2005) and genetic factors (Klekowski 1988, Barret et al. 1993, Eckert 2002), which leaves asexual reproduction as the only means of population growth and colonization and may even set the stage for genetic sterility (Eckert 2002). Several clonal plant species show reduced sexual capacity at the margins of their distribution (Erikson 1996, Reusch et al. 1999, Eckert 2002, Billingham et al. 2003). At the range limit of *Cymodocea nodosa* in the northern Atlantic (south Portugal), a study that used RAPD markers identified extremely limited genetic diversity (Alberto et al. 2001). A general hypothesis in biogeographical theory is that sexual reproduction contributes less to population maintenance near the range limit of clonal species (Eckert 2002). By screening microsatellite multi-locus genotypes, we were able to reject this hypothesis and demonstrate that at the Canary Islands, this species is characterized by high genotypic richness (mean R = 0.67), even though R varies markedly from meadow to meadow. Thus, at these islands, sexual reproduction is an important component of population maintenance, which is in agreement with the high reproductive output reported by Reyes et al. (1995). Indeed, during the sampling campaign, several seedlings were observed developing at the margins of meadows. High flowering rates by this species have been observed elsewhere in the Mediterranean (Buia & Mazzella 1991, Terrados 1993, Duarte & Sand-Jensen 1996), but cases have been reported where seedlings were not detected even though flowering occurred (Caye & Meinesz 1985).

A threshold of genotypic richness below 0.7, which was evident at Teresitas, Las Canteras, Taliarte and Punta Jandia, coincided with both higher fragmentation of the sampled meadows and smaller meadow size (Espino 2004). These samples corresponded either to patchy meadows or to shallower borders of continuous seagrass beds fragmented by wave action. It is possible that in some cases, the disturbance affecting these meadows reduces their genotypic richness by promoting recovery through clonal growth. In contrast, specimens from Bahia Feliz, which were characterised by high genotypic richness (R = 0.83), were also sampled

from a group of very small (1 to 2 m long) scattered patches; this may either have resulted from the recent fragmentation of a continuous meadow with high genotypic richness, or from a recent colonization through the establishment of seedlings. Disturbance may result in higher or lower genotypic richness, depending on whether recovery takes place by clonal growth from a few remaining fragments, or by seedling establishment.

The average level of genotypic richness was similar to that found at Cadiz Bay and Alfacs Bay, and therefore the relative importance of sexual versus clonal propagation at the Canary Islands appeared to be equivalent to the mainland. However, it is important to bear in mind that estimates of genotypic richness such as *R* are also affected by sampling design (Widén et al. 1994): meaningful comparisons require similar sampling density. In order to reduce this bias, we proposed here a randomization method to standardize sample size before comparing estimates of parameters such as *R*, and we demonstrated that this approach is crucial if realistic comparisons are to be drawn. For example, at Cadiz and Alfacs bays, *R* was 0.38 and 0.48 before standardization (N = 220), respectively (Alberto et al. 2005); after standardization (N = 40), *R* increased to 0.62 at both sites.

Genetic diversity

Our results supported the hypothesis that founder events during island colonization resulted in low genetic diversity. Despite similar genotypic richness, the Canary Islands meadows showed on average lower allelic richness (mean $\hat{A}$ = 2.67) than Iberian populations from Cadiz bay (3.53) and Alfacs (3.18), once appropriate *G* standardization was completed (Leberg 2002b). A similar trend was also observed for gene diversity. It is possible that lower allelic richness and gene diversity at the Canary Islands is the result of a founder effect established during the colonization of these Islands. A similar conclusion was drawn for the sponge *Crambe crambe* at the Canary islands, where the levels of allelic richness were lower than in the Mediterranean (Duran et al. 2004). Generally, it is hypothesized that a species should present higher allelic richness near its place of origin; a longer evolutionary history should be reflected by the accumulation of additional mutations (Comps et al. 2001). However, change in genetic diversity as measured by gene diversity may not always correlate with that measured by allelic richness (Comps et al. 2001, Widmer & Lexer 2001). Reduced allele diversity at the Canary Islands is best explained by a founder effect resulting from colonization of the archipelago by a small number of migrants. The allelic composition at all sites was similar, which suggested a single colonization event or several colonizations from a single source.

Results obtained from 2 distinct meadows deserve particular attention: Las Canteras, situated near the city of Las Palmas at the island of Gran Canaria, and El Medano at Tenerife, one of the most well-studied meadows in the archipelago (Reyes & Sansón 1991, 1997, 2001, Reyes et al. 1995). The meadow of Las Canteras has a well-described history of anthropogenic disturbance that has resulted in severe seagrass decline (González 1986, Pavón-Salas et al. 1998). Our data supports the hypothesis that this loss resulted in a population bottleneck and consequent reduction in genetic diversity; Las Canteras showed the lowest values of both gene diversity ($\hat{A}$ = 2.25) and genotypic richness (*R* = 0.30). In contrast, the levels of gene diversity ($\hat{A}$ = 3.17) and genotypic richness (*R* = 0.94) in the meadow of El Medano were by far the highest observed, which corresponded to a hotspot of genetic diversity for the species at these islands. These results may be explained by the large size of the El Medano meadow; in this region, one can find the most extensive and continuous *Cymodocea nodosa* beds in the archipelago (Wildpret et al. 1987, Pavón-Sallas et al. 2000, Espino 2004). Thus, our results supported the hypothesis that *C. nodosa* at El Medano was subjected to less genetic drift, which explains the high allelic richness observed there.

Small-scale SGS

SGS was identified by ramet level analysis at Punta Jandia, Gran Tarajal, and Playa Blanca, and resulted from the spatial aggregation of clone mates as has been previously observed at Cadiz and Alfacs (Alberto et al. 2005). At Taliarte there was no significant coancestry for the first distance class, even though genotypic richness was very low. This was because clonality in this meadow was largely affected by a large clone elongated across a 60 m dimension of the sampling plot, which also probably extended in size beyond the sampling area as the clonal subrange was larger than 50 m.

After clonal replicates were removed, SGS analysis revealed a random distribution of genotypes in space. The only exception was the meadow at Gran Tarajal that showed a steep decrease of pair-wise kinship with spatial distance. Several hypotheses might explain the predominance in our results of a random distribution of genotypes at the Canary Islands. First, the low sample size used reduces the power for detecting moderate SGS patterns. The simulation performed in this work showed that only severe cases of limited dispersal (like

at Gran Tarajal or Cadiz) could be inferred with confidence given our sample size. Second, it is also possible that *Cymodosa nodosa* may actually have a high dispersal potential in the region, which is supported by the (surprising) ease with which developing seedlings were found around meadows. It is likely that colonization of the Canary Islands, at the limits of the species' range, was achieved through rare jump dispersal events. In such cases, given genetic variation in dispersal rates, individuals with higher dispersal rates are likely to occur with increasing frequency near the range limits (Holt 2003). Finally, it is possible that the analyzed meadows were not in drift-dispersal equilibrium, thus hindering the development of SGS (Vekemans & Hardy 2004).

Patterns of population differentiation

Our study supported the prediction of high population differentiation due to high drift and low migration capacity. All populations at the Canary Islands were genetically differentiated. This suggests genetic isolation from very restricted current gene flow, causing population allele frequencies to be altered mainly by the diversifying effects of genetic drift (but see Hedrick 1999). Given the species' reduced dispersal abilities, an IBD pattern expected under the assumption of migration-drift equilibrium might be restricted to much smaller spatial scales (Hutchinson & Templeton 1999) than the minimum geographic separation of our sampled sites (15 km between Playa de Arinaga and Taliarte). It is also possible that we did not observe IBD within the Canary archipelago because populations, independent of their scale of separation, were not in migration-drift equilibrium (Grosberg & Cunningham 2001). Differentiation levels were much higher between the Canary Islands and the Iberian peninsula at Cadiz or Alfacs than among the Canary Islands, as would be expected from the longer temporal separation between these groups.

All Canary Island populations grouped in a distinct clade (NJ analysis) that connected first with Cadiz and then with Alfacs by long branches (Fig. 6), again in accordance with the geographic separation among groups. Although not supported by strong bootstrap values, the meadows sampled from the eastern islands of Fuerteventura and Lanzarote (Punta Jandia, Gran Tarajal, and Playa Blanca) appeared predominantly at the base of the consensus network. This group is closer to the North African coast and it is therefore likely that initial colonization would have taken place here. The meadows at Teresitas and Las Canteras formed a distinct subgroup despite being located on different islands (Tenerife and Gran Canaria, respectively). Las Canteras, the only population on the north coast of Gran Canaria, may be isolated from other southern meadows due to the predominant Alisios trade winds that generate a southwards surface water transport. Therefore, it is likely that the Las Canteras population only receives gene flow from North Tenerife. Its isolation from the nearest meadows at Gran Canaria further hinders conservation of this meadow, which is the most disturbed by anthropogenic activities in the whole archipelago.

The colonization of Atlantic waters by *Cymodocea nodosa* from the Mediterranean may be partially linked with the Canary current water transport. This current, produced by prevalent trade winds, flows north to south along the African coast between 30°N and 10°N, and offshore to 20°W (Barton et al. 1998). The range and effect of this current overlap remarkably with the range of *C. nodosa* from south of the Gulf of Gibraltar to Morocco, Madeira, the Canary Islands, and finally to the range limit at Mauritania, where the influence of the current ends. Although the species distribution along the Moroccan coast is now very fragmented, it is possible that the available lagoons and estuaries along this coast provided a stepping stone habitat and offered a southern colonization route from the Mediterranean, supported by the Canary current. Current-mediated seagrass transport has been observed for *Zostera marina* (Reusch 2002) up to distances of 54 km, and Olsen et al. (2004) suggested the existence of a very recent and still current connectivity between Pacific and North Atlantic *Z. marina* populations. The Cadiz current that flows polewards along southwestern Iberia has also been cited to account for genetic differentiation among *Zostera noltii* populations in southwest Iberia (Diekmann et al. 2005). Although dispersal of *C. nodosa* seeds is likely to be very limited compared to *Zostera* spp. (due to negative seed floatability and basicarpy), sufficient time and an appropriate dispersal vector may have facilitated rare dispersal events over long distances. For example, seeds attached to floating shoots have been observed (F. Alberto pers. obs.) and may constitute such means of dispersal by currents.

CONCLUSIONS

Contrary to the leading biogeographical hypothesis (Eckert 2002) we observed that sexual reproduction makes an important contribution to population maintenance of *Cymodocea nodosa* in the Canary archipelago, as revealed by a high genotypic richness comparable to that found in populations closer to the center of the distribution range. We proposed here a randomization method to standardize sample size before compar-

ing estimates of parameters such as genotypic richness, and we demonstrated that this approach is crucial to reduce the bias of comparisons across studies with different sampling sizes.

The allelic richness observed at the Canary Islands was low in comparison to populations closer to the centre of the species' range, which suggested the occurrence of a founder effect during colonization of the archipelago. Similar allelic composition observed across sites suggested that the whole archipelago has been colonized from a single source, which probably began at the eastern islands. Founder effects and non-equilibrium migration-mutation, with a greater influence of genetic drift compared to gene flow, were the most likely justifications for the lack of SGS within populations of *Cymodocea nodosa*, and for the lack of IBD between populations.

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