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ORIGINAL ARTICLE

Genetic differentiation and secondary contact zone in the seagrass *Cymodocea nodosa* across the Mediterranean–Atlantic transition region

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ABSTRACT

Aim A central question in evolutionary ecology is the nature of environmental barriers that can limit gene flow and induce population genetic divergence, a first step towards speciation. Here we study the geographical barrier constituted by the transition zone between the Atlantic Ocean and the Mediterranean Sea, using as our model *Cymodocea nodosa*, a seagrass distributed throughout the Mediterranean and in the Atlantic, from central Portugal to Mauritania. We also test predictions about the genetic footprints of Pleistocene glaciations.

Location The Atlantic–Mediterranean transition region and adjacent areas in the Atlantic (Mauritania to south-west Portugal) and the Mediterranean.

Methods We used eight microsatellite markers to compare 20 seagrass meadows in the Atlantic and 27 meadows in the Mediterranean, focusing on the transition between these basins.

Results Populations from these two regions form coherent groups containing several unique, high-frequency alleles for the Atlantic and for the Mediterranean, with some admixture west of the Almeria–Oran Front (Portugal, south-west Spain and Morocco). These are populations where only one or a few genotypes were found, for all but Cadiz, but remarkably still show the footprint of a contact zone. This extremely low genotypic richness at the Atlantic northern edge contrasts with the high values (low clonality) at the Atlantic southern edge and in most of the Mediterranean. The most divergent populations are those at the higher temperature range limits: the southernmost Atlantic populations and the easternmost Mediterranean, both potential footprints of vicariance.

Main conclusions A biogeographical transition region occurs close to the Almeria–Oran front. A secondary contact zone in Atlantic Iberia and Morocco results from two distinct dispersal sources: the Mediterranean and southernmost Atlantic populations, possibly during warmer interglacial or post-glacial periods. The presence of high-frequency diagnostic alleles in present-day disjunct populations from the southernmost Atlantic region indicates that their separation from all remaining populations is ancient, and suggests an old, stable rear edge.

Keywords
Biogeographical barrier, clonal plant, *Cymodocea nodosa,* genetic structure, marine connectivity, microsatellites, Pleistocene glacial refugia, range edge, seagrass.

INTRODUCTION

The evolutionary processes that lead to population isolation, the first step towards allopatric speciation, are determined by a variety of factors including species reproductive systems, population history, geographical distance, dispersal means and past geological events. In marine environments, biogeographical barriers, although less apparent (Vermeij, 1987; Avise, 1994; Palumbi, 1994), have been highlighted by the use of molecular markers for marine populations (Karl & Avise, 1992; Grosberg & Cunningham, 2001). One approach to identifying marine biogeographical discontinuities, and evaluating their importance as barriers to gene flow, is to determine population divergence for species with ranges expanding across candidate regions.

The transition zone between the Atlantic Ocean and the Mediterranean Sea, at the Strait of Gibraltar, constitutes one of the best documented biogeographical transitions in the marine environment, due to both the present and past physical properties of this marine area. During the last 6 Myr, this region has been affected by dramatic geological events and climatic fluctuations, the most important being the closure of the Rifean and Baetic gateways between the Atlantic Ocean and the Mediterranean Sea some 5.9 Ma, transforming the Mediterranean Sea into a series of hypersaline lakes, a period called the Messinian salinity crisis (Maldonado, 1985; Krijgsman *et al.*, 1999, 2004; Duggen *et al.*, 2003, 2004). More recently, during the last glacial maximum (LGM 18–10 kyr BP), oscillations of sea level led to periods of reduced connectivity between the Eastern and Western basins, which stabilized at about 11 kyr BP (Collina-Girard, 2001). Nowadays, two factors have been proposed to account for the maintenance of a biogeographical barrier in the area: the one-way surface current of Atlantic water flowing through the Strait of Gibraltar into the Mediterranean (Parilla & Kinder, 1992; Bryden *et al.*, 1994); and the presence of gyres forming a well-defined hydrogeographical boundary of surface waters between Almeria in south-east Spain and Oran in Algeria, the so-called Almeria–Oran oceanographic front (AOF) (Tintore *et al.*, 1988).

Recent molecular studies suggested that the AOF front acts as a barrier for dispersal of oysters (Saavedra *et al.*, 1993), mussels (Quesada *et al.*, 1995), fish (Borsa *et al.*, 1997; Naciri *et al.*, 1999; Lemaire *et al.*, 2000; Lo Brutto *et al.*, 2004; Cimmaruta *et al.*, 2005), barnacles (Pannacciulli *et al.*, 1997), northern krill (Zane *et al.*, 2000), scallops (Rios *et al.*, 2002) and cuttlefish (Perez-Losada *et al.*, 2002). Many other studies detected a strong differentiation between Atlantic and Mediterranean populations, although sampling was not designed to test for a specific effect of the AOF (Kotoulas *et al.*, 1995; Chikhi *et al.*, 1997; Roldan *et al.*, 1998; García-Martínez *et al.*, 1999; McFadden, 1999; Aurelle *et al.*, 2003; Bargelloni *et al.*, 2003; Duran *et al.*, 2004a,b; Viñas *et al.*, 2004; Baus *et al.*, 2005). However, this genetic discontinuity was absent in other studies (Launey *et al.*, 2002; Bargelloni *et al.*, 2003; Stamatis *et al.*, 2004; Zardoya *et al.*, 2004), which focused mostly on fish and marine invertebrate taxa. For marine flowering plants (seagrasses), the two published studies including samples from the Atlantic and Mediterranean basins (Coyer *et al.*, 2004; Olsen *et al.*, 2004) did not specifically detail the patterns of gene flow across this contact zone, which also represents the distributional boundary of the Mediterranean endemic seagrass *Posidonia oceanica* (Duarte, 2001). Species with low dispersal potential are expected to reflect more strongly the effects of biogeographical barriers and to show clear patterns of genetic structure.

Populations at the margins of geographical range distributions are particularly affected by climate variations, affecting their genetic diversity and structure (Petit *et al.*, 2003, 2004; Vucetich & Waite, 2003). Many studies tend to focus on high-latitude range edges, yet populations at the 'rear edge' of post-glaciation colonization, those that may have survived recent glaciations, may be of extreme importance for the long-term conservation of genetic diversity (Hampe & Petit, 2005). The classic centre–periphery hypothesis assumes that edge populations should be smaller and have lower genetic variability and higher extinction probability, and although supported by some empirical studies (e.g. Arnaud-Haond *et al.*, 2006), this view has been challenged by other empirical work (Channell & Lomolino, 2000; Sagarin & Gaines, 2002; Vucetich & Waite, 2003). For organisms with mixed sexual and clonal reproductive modes, a leading biogeographical hypothesis is that clonal reproduction becomes predominant at distributional edges (e.g. Eckert, 2001), although this pattern is often associated with physically stressed edge populations, such as in high-latitude habitats affected by Pleistocene climate changes (Kearney, 2005). Genotypic richness, an indicator of the relative importance of sexual vs. clonal growth for population dynamics, is thus expected to correlate, across geographical distributional ranges, with climate oscillations and centre–periphery distribution patterns. Yet we still understand only poorly the distribution patterns of genotypic richness for marine clonal organisms with mixed sexual and asexual reproduction modes (but cf. Billingham *et al.*, 2003; Coyer *et al.*, 2004; Olsen *et al.*, 2004; Tatarenkov *et al.*, 2005; Alberto *et al.*, 2006).

In this study, we explored the importance of the Atlantic–Mediterranean transition zone as an oceanographic barrier to past and current gene flow, using as our model species the seagrass *Cymodocea nodosa* (Ucria) Ascherson. The combination, in the same species, of an Atlantic–Mediterranean distribution range with extremely reduced dispersal potential (Buia & Mazzella, 1991; Alberto *et al.*, 2005; Ruggiero *et al.*, 2005) renders *C. nodosa* a suitable model for studying the biogeographical influence of the transition zone between these two distinct water masses. Eight microsatellite loci were used to estimate genetic diversity within and among populations from the Atlantic and the Mediterranean. The two hypotheses we aimed to test were that, over a long time scale: (1) the transition zone acted as a major barrier to gene flow between the Atlantic and Mediterranean, and (2) over the north–south

axis of distribution in the Atlantic there is a gradual decline of genotypic richness with increasing latitude, because rear-edge populations are less affected by climate oscillations.

MATERIALS AND METHODS

Model species

Seagrasses are marine flowering plants capable of occupying space through clonal reiteration of shoots, as a result of rhizome extension (Marbà & Duarte, 1998; Hemminga & Duarte, 2000). The dioecious seagrass *C. nodosa* is found throughout the Mediterranean basin and in the North Atlantic from central Portugal to its southern limit in Banc d'Arguin in Mauritania, as well as in the Canary Islands archipelago and at Madeira (Fig. 1). The occurrence of congeneric species in the Red Sea and in the Indian Ocean suggests that *C. nodosa* may have a vicariant origin associated with the closure of their connection to the Mediterranean. *Cymodocea nodosa* can occur from the extreme low intertidal down to depths of 30 m, depending on light penetration. Fruits are developed at the base of shoots, thus suggesting limited seed dispersal (Marbà & Duarte, 1995), which was confirmed by spatial autocorrelation analyses (Alberto *et al.*, 2005; Ruggiero *et al.*, 2005). Yet *C. nodosa* is present in areas separated from its closest sources by hundreds of kilometres, such as some Atlantic islands and the range-edge populations of Mauritania. Long-distance dispersal may be the result of rare jump-dispersal events, perhaps through transport of fruits on rafts made of tangled sections of broken rhizome during winter storms.

Sampling

Sampling aimed to cover the Western Mediterranean and Atlantic distribution of *C. nodosa* (Fig. 1; Table 1). The Atlantic group consisted of 20 sites distributed from Mauritania (3), the Canary Islands (10), Madeira (1), Morocco (1), Portugal (3) and Spain (2), including all sites where *C. nodosa* is known to occur along the Atlantic coasts of Iberia, up to its northern limit at Tróia. In the Western Mediterranean area, 21 sites were sampled, in Iberia (8) and the Balearic Islands (13). East of the region that is the focus of this study, we also sampled six sites: Sicily (2), Croatia (1), Greece (2) and Cyprus (1). These were included as a reference to understand the pattern of colonization along the geographical distributional range, as the occurrence of the genus *Cymodocea* in the Red Sea suggests that the Eastern Mediterranean is the geographical origin of this species.

At each site, 40 ramets, each consisting of three to five shoots from the same horizontal rhizome, were randomly collected inside a 60 × 14-m area by scuba divers. At the sites where this sampling design was not feasible due to meadow fragmentation and/or size, the sample size was smaller than 40 sample units and was taken haphazardly (Table 1). For one site, the Ria Formosa coastal lagoon, a larger sample size was available.

Genet assignment

After DNA extraction (Doyle & Doyle, 1988), samples were genotyped for eight microsatellite loci (Alberto *et al.*, 2003). Each reaction contained one of the following multiplexes of

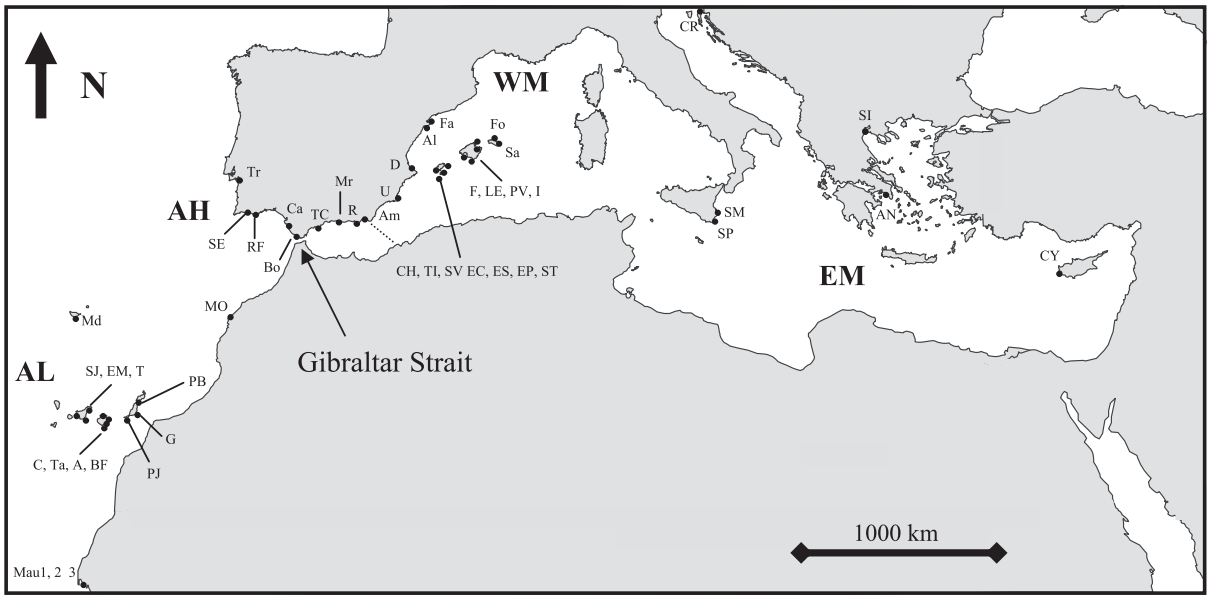

Figure 1 Locations of *Cymodocea nodosa* sites sampled. Site codes are given in Table 1. The text refers to geographical groups of sites: close to low-latitude Atlantic range edge (AL); close to high-latitude Atlantic range edge (AH); Western Mediterranean (WM); Eastern Mediterranean (EM). The Almeria–Oran front is indicated by the dashed line east of the Strait of Gibraltar.

Table 1 *Cymodocea nodosa* genetic variability statistics for all sites studied in the Atlantic Ocean and the Mediterranean Sea.

Site	Code	Group	*n*	*G*	*R*	$\hat{A}_{(G = 10)}$	*He*	*Ho*	*f*
Atlantic Ocean									
Mauritania									
1. Banc d'Arguin I	M1	AL	43	18	0.40	2.44 ± 0.17	0.372	0.333	0.108
2. Banc d'Arguin II	M2	AL	44	30	0.67	2.45 ± 0.21	0.383	0.430	−0.099
3. Banc d'Arguin III	M3	AL	40	10	0.25	2.5	0.375	0.431	−0.081
Canary Islands									
4. Playa Blanca	PB	AL	38	30	0.78	2.40 ± 0.12	0.391	0.371	0.051
5. Punta Jandia	PJ	AL	35	19	0.53	2.92 ± 0.19	0.467	0.434	0.072
6. Gran Tarajal	G	AL	39	33	0.84	2.45 ± 0.20	0.411	0.345	0.162**
7. Playa San Juan	SJ	AL	39	27	0.68	2.32 ± 0.17	0.286	0.296	−0.016
8. El Medano	EM	AL	36	34	0.94	2.90 ± 0.16	0.438	0.360	0.178**
9. Teresitas	T	AL	35	21	0.59	2.20 ± 0.10	0.381	0.388	−0.019
10. Canteras	C	AL	39	13	0.32	2.16 ± 0.09	0.285	0.317	−0.119
11. Taliarte	Ta	AL	38	15	0.38	2.87 ± 0.15	0.469	0.458	0.023
12. Arinaga	A	AL	35	30	0.85	2.51 ± 0.12	0.480	0.558	−0.166
13. Bahia Feliz	BF	AL	39	32	0.82	2.61 ± 0.15	0.449	0.398	0.114
Madeira Island									
14. Lido	Md	AL	38	1	0.00	1.62§	0.625	0.625	–
Morocco									
15. Oualidia	MO	AH	35	1	0.00	1.38§	0.375	0.375	–
Portugal (Iberia)									
16. Tróia	Tr	AH	40	1	0.00	1.38§	0.375	0.375	–
17. Ria Formosa	RF	AH	220	5	0.03†	2.50§	0.425	0.475	−0.134
18. Stª Eulália	SE	AH	30	3	0.07	2.38§	0.550	0.542	0.019
Spain (Iberia)									
19. Cadiz	Ca	AH	40	24	0.59	3.44 ± 0.16	0.579	0.672	−0.164***
20. Bolonia	Bo	AH	28	3	0.07	2.50§	0.567	0.750	−0.440
Mediterranean Sea									
Spain (Iberia)									
21. Cala Honda	TC	WM	35	2	0.03	2.00§	0.583	0.625	−0.111
22. Maro	Mr	WM	30	1	0.00	1.62§	0.625	0.625	–
23. Roquetas	R	WM	39	9	0.21	3.12§	0.450	0.472	−0.052
24. Almerimar	Am	WM	30	26	0.86	3.78 ± 0.15	0.561	0.520	0.075
25. Urrutias	U	WM	30	24	0.79	3.22 ± 0.14	0.578	0.573	0.003**
26. Denia	D	WM	32	17	0.52	3.46 ± 0.15	0.576	0.493	0.148
27. Alfacs	Al	WM	40	19	0.46	2.85 ± 0.12	0.531	0.566	−0.068
28. Fangar	Fa	WM	34	28	0.82	3.26 ± 0.20	0.571	0.670	−0.176***
Balearic Islands‡									
29. Calla d'Hort, IB	CH	WM	38	29	0.76	2.94 ± 0.17	0.496	0.517	−0.044
30. Talamanca, IB	Tl	WM	39	25	0.63	3.60 ± 0.17	0.530	0.508	0.042
31. Calla St Vicent, IB	SV	WM	40	13	0.31	3.42 ± 0.16	0.509	0.519	−0.022**
32. Es Cabalet, IB	EC	WM	28	26	0.93	3.00 ± 0.19	0.464	0.481	−0.036**
33. Es Banc, FO	ES	WM	31	15	0.47	2.90 ± 0.15	0.516	0.500	0.032
34. Es Pujols, FO	EP	WM	40	8	0.18	2.88§	0.543	0.563	−0.039
35. Sa Torreta, FO	ST	WM	32	14	0.42	2.49 ± 0.03	0.366	0.429	−0.180
36. Calla Formentor, MA	F	WM	36	23	0.63	3.24 ± 0.21	0.556	0.620	−0.117
37. Lago Esperanza, MA	LE	WM	35	7	0.18	2.38§	0.338	0.321	0.053*
38. Pt Vells, MA	PV	WM	28	13	0.44	3.17 ± 0.16	0.432	0.452	−0.048
39. Ses Illetes, MA	I	WM	13	3	0.17	2.00§	0.383	0.458	−0.257
40. Fornels, ME	Fo	WM	38	27	0.70	3.30 ± 0.14	0.538	0.588	−0.095
41. Sanitja, ME	Sa	WM	32	14	0.42	2.50 ± 0.10	0.401	0.491	−0.234*
Sicily									
42. Marzamemi	SM	EM	40	15	0.36	3.44 ± 0.19	0.578	0.574	0.007
43. Porto Palo	SP	EM	36	29	0.80	4.46 ± 0.30	0.647	0.612	0.054***
Croatia									
44. Rovnij	CR	EM	37	20	0.53	2.69 ± 0.17	0.396	0.356	0.103*

Table 1 Continued

Site	Code	Group	*n*	*G*	*R*	$\hat{A}_{(G = 10)}$	*He*	*Ho*	*f*
Greece									
45. Thessaloniki	Sl	EM	13	8	0.58	2.88§	0.487	0.511	−0.053
46. Agios Nicolaos	AN	EM	27	13	0.46	3.30 ± 0.09	0.521	0.462	0.119***
Cyprus									
47. Amathous	CY	EM	11	11	1.00	4.56 ± 0.10	0.690	0.735	−0.071*

Group, sample geographical group (see text); *n*, number of ramets analysed; *G*, number of different genotypes; *R*, genotypic richness; $\hat{A}$, allelic richness (±SE) estimated after standardizing *G* to 10 except where $G < 10$; *He*, *Ho*, expected and observed heterozygosities, respectively; *f*, inbreeding coefficient. Significant *f* values are given (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

†Only five genets discovered in 220 ramets distributed from different sites over *c.* 50 km of coastal lagoon.

‡Balearic Islands: Ibiza (IB), Formentera (FO), Mallorca (MA), Menorca (ME).

§Observed allelic richness is shown as it cannot be standardized because $G < 10$ (see text).

fluorescently labelled *C. 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; MC, Cn4-19/NED and Cn2-24/NED. PCR and automated sequencer methods are reported by Alberto *et al.* (2003).

When working with clonal organisms it is important to discriminate ramets (number of ramets = *N*, modular units of the same genetic individual) from genets (number of genets = *G*, the genetic individuals originating from distinct sexual recombination events, which can be composed of several ramets). This can be done by discriminating the genets on the basis of their multi-locus genotypes (MLG). Yet a problem that must be addressed is that identical MLGs observed in two sampled ramets can correspond either to two ramets belonging to the same genet, or to two different genets if, by chance, the sampled alleles are all identical between the two genets. The probability of encountering the latter depends on the population frequencies of the 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 MLG occurring *n* times, repeated as a consequence of different recombination events (P_{sex}) (Parks & Werth, 1993) using the GenClone software (Arnaud-Haond & Belkhir, 2007). Arnaud-Haond *et al.* (2005) provide a detailed description of P_{sex} estimation and genet assignment for *C. nodosa*. Genotypic richness, the proportion of different genets in each sample, was estimated as by Dorken & Eckert (2001): $R = (G - 1)/(N - 1)$.

Data analyses

After removal of clonal replicates from the data set, the population level observed heterozygosity (*Ho*) and unbiased (*He*) gene diversity (Nei, 1978) were estimated using genetix 4.05.2 software (Belkhir *et al.*, 2001). The number of genets found at each site was standardized to 10 by multiple random reduction (Leberg, 2002), for interpopulation comparisons of estimates of allelic richness ($\hat{A}$), the average number of alleles per locus in each population. This approach prevents comparisons of allelic richness from being influenced by the variable number of genets detected in each population sample (Alberto *et al.*, 2006). A second standardization to $G = 3$ was applied for comparisons with samples that had a very low genet number.

The F_{IS} estimator of departure from Hardy–Weinberg expectation, *f* (Weir & Cockerham, 1984), was calculated with the genepop software (Raymond & Rousset, 1995a). The null hypothesis of random union of gametes was also tested with the exact Hardy–Weinberg test using the probability test (Raymond & Rousset, 1995a) available in genepop.

Levels of differentiation between sites were described by the F_{ST} estimator θ (Weir & Cockerham, 1984), and the null hypothesis of no differentiation was tested using Fisher's exact test (Raymond & Rousset, 1995b). Biogeographical relatedness among sites was analysed with a neighbour-joining (NJ) tree based on Cavalli–Sforza distances estimated from microsatellite allelic frequencies, with 1000 bootstrap replicates (phylip software, Felsenstein, 1994).

In order to quantify the effect on genetic differentiation caused by the Atlantic–Mediterranean transition zone, we employed a hierarchical *F*-statistics analysis (hierfstat software, Goudet, 2005; Trouve *et al.*, 2005). This was used to detect structure at two different levels: region and population. We grouped populations into two regions, Atlantic vs. Mediterranean locations. These were later subdivided in order to quantify the genetic differentiation between the main groups of populations: (1) between an 'Atlantic lower latitude' group of samples from Madeira, the Canary Islands and Mauritania (hereafter referred to as the AL group) and an 'Atlantic higher latitude' group consisting of the remaining Atlantic sites (AH group); (2) between the AH and the Western Mediterranean (WM) groups; and finally (3) between the western (WM) and Eastern Mediterranean (EM) groups. The variance components were estimated with the algorithm described by Yang (1998). The deviations from zero of all *F* values were tested with permutation procedures described by Trouve *et al.* (2005).

In order to detect the location of a putative genetic discontinuity region between Atlantic and Mediterranean populations, we used a Bayesian method implemented in the geneland software (Guillot *et al.*, 2005). This approach uses the genetic and geographical information from each sample unit, with no prior assumptions about population groups or

boundaries, to infer the number k of populations in the data. We used a strategy proposed by Coulon *et al.* (2006): first we ran the MCMC (Markov chain Monte Carlo) computations 10 times allowing k to vary followed by 100 additional runs, to check for consistency, with k fixed to the modal k found on the initial 10 runs. The mcmcFmodel function was set with the following parameters: 100,000 iterations, maximum rate of Poisson process to 650, uncertainty attached to spatial coordinates to 0.4°, minimum k fixed to 1, maximum k fixed to 25, maximum number of nuclei in the Poisson–Voronoi tessellation fixed to 300, and the Dirichlet model for allelic frequencies. From these 100 runs, we kept the 20 with the highest mean logarithm of posterior probability. Posterior probabilities of population membership for each pixel of the spatial domain were then computed using a burn-in of 50,000 iterations. We used the modal population assigned to individuals and group populations to detect putative genetic discontinuities.

Because GENELAND does not incorporate an admixture model, we used a maximum likelihood algorithm to analyse the admixture at the transition region. For this, we chose to use the R package PSMIX (Wu *et al.*, 2006), which has been shown to produce similar estimates to the more computationally demanding Bayesian MCMC methods of STRUCTURE (Pritchard *et al.*, 2000). The methods implemented by PSMIX are described by Tang *et al.* (2005). The k parameter on PSMIX was set by the previously described GENELAND estimate.

RESULTS

A total of 1784 ramets of *C. nodosa*, distributed among 45 sites along the Atlantic and Mediterranean coasts, were genotyped with eight microsatellite loci, revealing 117 distinct alleles and 789 distinct MLGs. Excluding monoclonal samples, all the estimated probabilities of identical MLG having been derived from independent reproductive events were < 0.05, leading to the recognition of 789 individual genets.

All Atlantic sites north of the Canary Islands, with the exception of Cadiz Bay, and also the two westernmost sites in the Mediterranean, were characterized by a much reduced genotypic richness (mean $R \pm$ SD: 0.11 ± 0.03 with Cadiz and 0.03 ± 0.023 without Cadiz), where a single genet (or two to five genets in some) was detected from all shoots sampled (Table 1). In contrast, all other Atlantic and all Mediterranean meadows (with the exception of Maro, close to the AOF front) were composed of multiple genets (mean $R \pm$ SD: 0.5 ± 0.27), with a minimum R 0.17–0.18 from two meadows in Mallorca, and a maximum 1.0 in Amathous, Cyprus (Table 1).

Comparisons of allelic richness ($\hat{A}$) among sites (Fig. 2) were standardized to a minimum common genet number by resampling to a sample size of 10 genets, except for sites where < 10 genets were found, that is, all populations from the Atlantic North of the Canary Islands and some Mediterranean ones ($\hat{A}$ values for these are given in Table 1 and indicated in Fig. 2 by open symbols). The maximum allelic richness ($\hat{A} = 4.56$) was found in the Amathous sample from Cyprus, the easternmost site analysed; this extreme value was three SEs higher than the Mediterranean mean. Mediterranean populations show higher (t-test, $P < 0.001$) allelic richness (mean $\hat{A} \pm$ SD: 3.24 ± 0.53) than Atlantic populations (mean $\hat{A} \pm$ SD: 2.59 ± 0.36), even when the Cyprus site was excluded from the analysis.

Allelic richness in Fig. 2 is not comparable between high (AH) and low (AL) latitude sites in the Atlantic because all AH sites, with the exception of Cadiz, had fewer than 10 genets. As estimates of $\hat{A}$ vary with sample size (genet number in the sample), they should thus be compared for standardized genet numbers. Standardized to $G = 10$, AL sites and non-standardized AH sites (excluding Cadiz) had comparable mean $\hat{A}$ values: 2.52 and 2.46, respectively (t-test, $P = 0.663$). When we applied a standardization to $G = 3$, allowing the inclusion of Santa Eulália, Ria Formosa and Bolonia AH sites in the comparison, we obtained a significantly higher value of 2.40 ± 0.19 for AH and 2.00 ± 0.15 for AL (t-test, $P < 0.001$). Thus, when comparisons were based on an equal number of genotypes, there was a higher allelic richness in Atlantic Iberian–Moroccan (AH) populations than at lower

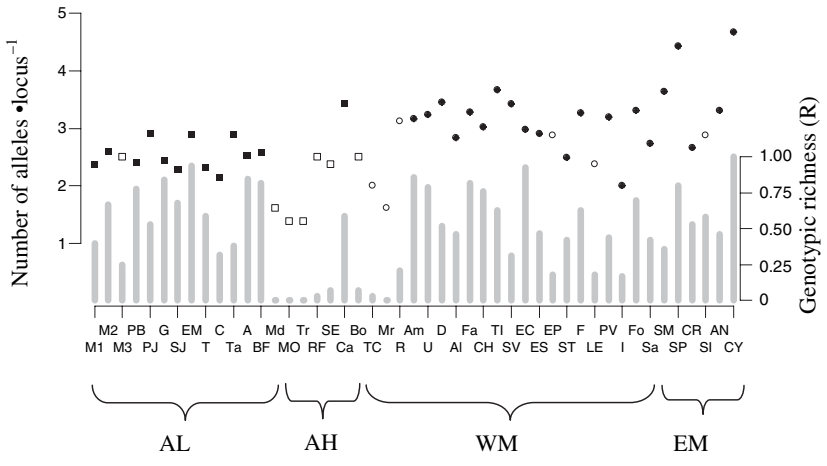

Figure 2 Allelic richness, shown as points ($\hat{A}$ is the mean number of alleles per locus standardized for number of genets $G = 10$) and genotypic richness (R), shown as grey bars, found for all sampled populations of *Cymodocea nodosa* arranged from the lower- to higher-latitude edge in the Atlantic followed by the West to East Mediterranean (squares, Atlantic; circles, Mediterranean). Samples with < 10 genets are shown with open symbols to indicate that $\hat{A}$ are the observed values in the sample, as these could not be standardized to $G = 10$ (see text).

latitudes in the Atlantic, despite the extremely low number of genets found in each of these AH populations (Fig. 2).

It is remarkable that, although only one or a few genotypes were detected at AH sites (except Cadiz), these still contained a mixture of alleles typical of the Atlantic southern edge and alleles typical of the Mediterranean (Fig. 3). Several unique alleles were present for the Atlantic and Mediterranean regions (Fig. 3). Some were present in almost every population from one region, often with high frequency, and completely absent in the other (e.g. allele 120 at locus Cn2-18, 114 at Cn2-16, 175 and 177 at Cn2-24 and 224 at Cn2-14, all specific for the Atlantic, although some occur in low frequency in the Eastern Mediterranean; Fig. 3). Conversely, several alleles were present throughout the distribution but absent at the Atlantic southern edge of the species (Fig. 3). With the exception of locus Cn2-45, all loci revealed diagnostic alleles for Atlantic vs. Mediterranean populations (Fig. 3). In the Atlantic higher latitude sites (Iberian–Moroccan), alleles typical of these two regions occurred together.

When the hypothesis of random union of gametes was tested, seven locations exhibited significant positive f values (heterozygote deficit) while six showed significant negative values (heterozygote excess) (Table 1). There was no geographical pattern of heterozygote deficiency and excess. All pairwise differentiation values (Table S1 in Supplementary Material) were significantly different from zero, revealing that allelic distributions differ across all populations with the exception of Ria Formosa and the neighbouring Sta Eulália.

The NJ tree and MDS analysis (Figs 4 & 5) reveal concordant representations of genetic structure according to site geographical location. Along the whole distributional range, the most genetically divergent cluster (90% bootstrap support; Fig. 4) groups the Atlantic southern edge sites (the AL group: Canary Islands, Madeira and Mauritania). Inside this cluster there is still a significant substructure, with the southernmost samples from Mauritania branching with 97% bootstrap support (Fig. 4). The Eastern Mediterranean is another well supported (63% bootstrap) differentiated cluster of *C. nodosa* populations, distinct from the Western Mediterranean, as expected from the allelic distributions (Fig. 3).

Genetic differentiation among sites closer to the transition region was more complex. The NJ analysis did not contradict the hypothesis that the AOF is important as a biogeographical boundary between the Atlantic and the Mediterranean, but the genetic structure is weaker and a pattern typical of a contact zone is present west of the AOF, rather than a sharp transition. The NJ bootstrap configurations place Cadiz (the only site in the region with high genotypic richness) about half of the time on the Atlantic side and equally frequently on the Mediterranean side, a pattern suggested by the shared presence of typically Mediterranean and Atlantic alleles (see above, Fig. 3). GENELAND probabilities of assignment of individuals to populations (see details below) confirmed an effect of the transition zone with all AH sites grouped together, including Cala Honda, the first Mediterranean site after Gibraltar, still west of the AOF.

GENELAND gave a modal number of clusters (k) of 13 and 14 on eight and two of the 10 runs, respectively, thus we fixed k for the minimum modal k at 13 for the 100 subsequent runs. The 20 selected runs of the 100 processed, assigned individuals on average to nine clusters (min: 7, max: 11). Three groups: (1) Mauritania, (2) the Canary Islands and Madeira, and (3) the AH group of Atlantic Iberia and Morocco, including Cala Honda, were consistent in 17, 19 and 16, respectively, of the 20 selected runs. The patterns for the WM sites were less consistent, confirming the NJ tree topology for this region. The PSMIX individual admixture analysis confirmed AL as the most divergent group and Mauritania as a separate cluster within it. In Fig. 6, we can see that the genetic ancestry for individuals within these clusters is dominated by a single cluster (yellow for Mauritania and dark blue for Macaronesia). The more complex genetic ancestry pattern in the AH region again reveals admixture from both Mediterranean and Atlantic sources. The separation of genetic admixture sources for WM vs. EM also confirms the previous analyses.

Hierarchical F-statistic analysis (Table 2) shows that the multilocus estimate of spatial differentiation among populations relative to the whole sampled distribution was large ($F_{\mathrm{PopTotal}} = 0.426$). Genetic variation in *C. nodosa* does not have a clear hierarchical structure, given that at a smaller spatial scale, of populations inside regions ($F_{\mathrm{PopReg}} = 0.255$), differentiation is comparable with that at a regional scale ($F_{\mathrm{RegTotal}} = 0.230$). The separate analyses of subsets of the data confirm that most of the variation among regions results from the genetic differentiation between the Atlantic lower latitude sites vs. all others.

DISCUSSION

We investigated the hypothesis of a genetic discontinuity in the distributional range of *C. nodosa* induced by the transition between the Atlantic Ocean and Mediterranean Sea, on the Almeria–Oran water front (AOF). Our results show that the populations from these two regions form coherent (topologically separated) groups, as supported by the occurrence of diagnostic alleles for each group but also revealing admixture from both regions along a contact zone at the higher latitude range in the Atlantic (Atlantic Iberia and Morocco). The most significantly differentiated groups are, however, located at the range limits: the lower latitude range in the Atlantic (Macaronesian/Mauritania group) and the easternmost Mediterranean. Along the disjunct Atlantic distribution, we found strong genetic structure and a contrasting genotypic diversity between the more clonal higher-latitude edge and the genotypically richer lower-latitude populations.

The Almeria–Oran front, a barrier to entering but not exiting the Mediterranean

The allelic patterns found for the northern edge in the Atlantic show evidence of a secondary contact zone west of the AOF, containing a mixture of alleles that are otherwise exclusive to

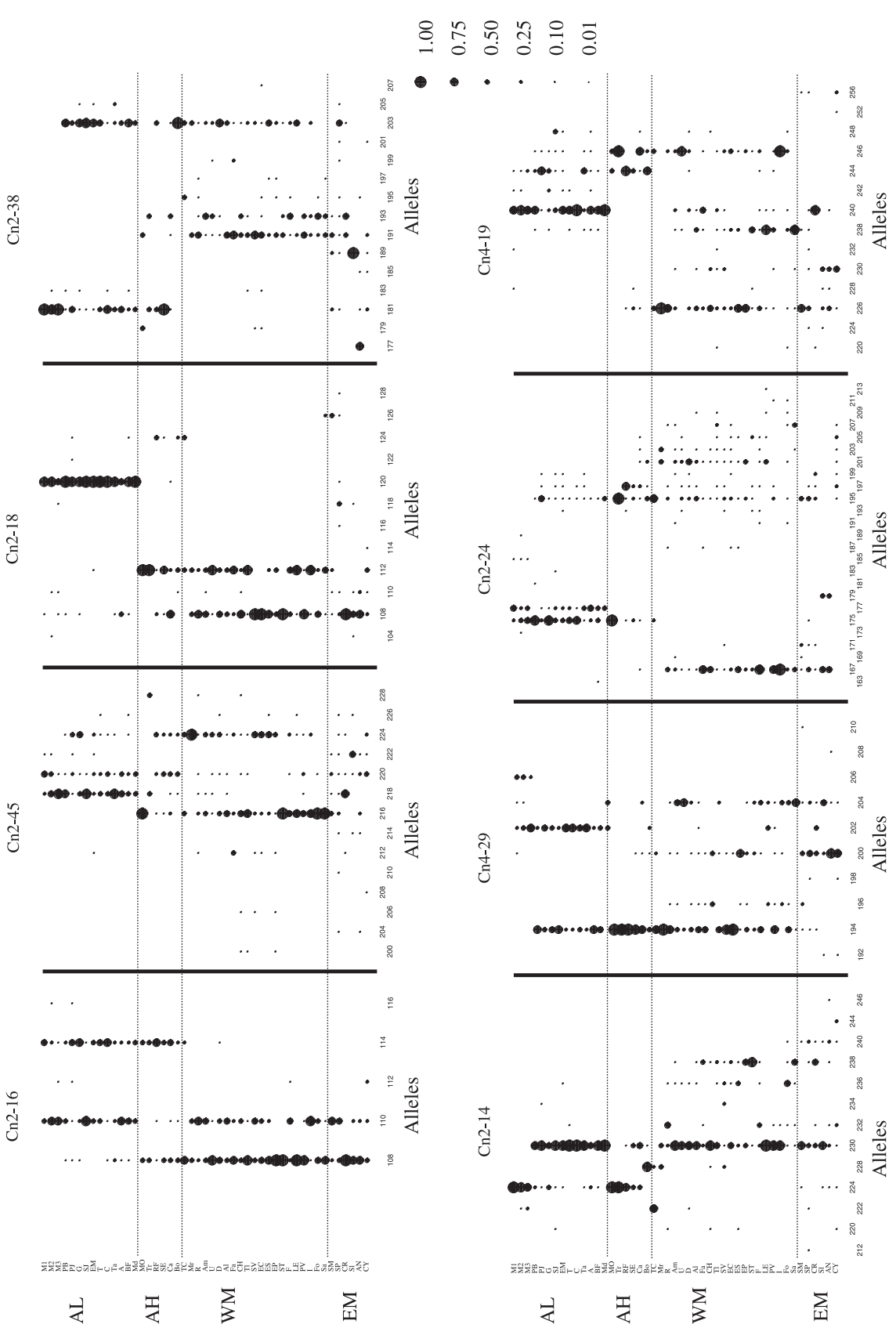

Figure 3 Allele presence and frequency along the distribution of *Cymodocea nodosa* samples analysed here. Sites are arranged from lower latitude in the Atlantic (AL), followed by the higher-latitude Atlantic edge at the contact zone (AH), Western Mediterranean (WM) and Eastern Mediterranean (EM). For each site, a circle indicates that the corresponding allele was present; its diameter represents the frequency of that allele in the sample.

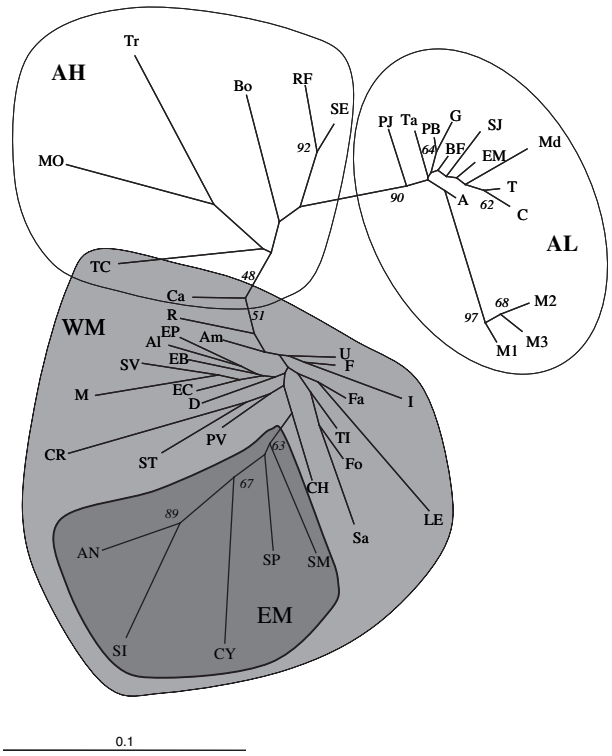

Figure 4 Neighbour-joining tree based on Cavali–Sforza distance derived from *Cymodocea nodosa* microsatellite allele frequencies at Atlantic and Mediterranean sites.

the Mediterranean or the Atlantic. It is remarkable that even populations where only one or a few genotypes were found still exhibited these shared allele patterns. Cadiz showed higher allelic richness than elsewhere in the Atlantic (as did other sites in this AH region if comparisons were standardized for the fewer genets in the samples). Higher allelic richness outside putative refugia can be the result of admixture of divergent lineages colonizing from separate southern refugia during range extension (see below; Petit *et al.*, 2003).

The occurrence of this contact zone west of the Almeria–Oran front indicates that gene flow has occurred from the Mediterranean to the Atlantic but not the opposite, for Mediterranean alleles were found in these northern range Atlantic populations, but Atlantic private alleles were not found in the Mediterranean. This contradicts expectations based on the predominant surface currents (dispersal from the Atlantic to the Mediterranean; Cramp & O'Sullivan, 1999). In addition, most of the Mediterranean animal populations are believed to be of Atlantic origin, entering this basin after the opening of the Gibraltar Strait (Borsa *et al.*, 1997). However the gene-flow patterns reported here, in the opposite direction, could be accounted for by near-shore counter-currents (Relvas & Barton, 2002) and surface wind patterns, particularly during storms. It is rarely acknowledged that dispersal direction might be more likely to be determined by rare storm occasions rather than yearly averages of oceanographic conditions. *Cymodocea nodosa* meadows are severely affected by winter storms, releasing large quantities of biomass in the form of complete sections of living rhizome network, therefore it is during such stormy periods that the dispersal potential is maximized, particularly as seeds have very low dispersal potential (Alberto *et al.*, 2005; Ruggiero *et al.*, 2005).

Colonization–recolonization pathways in the Atlantic and Western Mediterranean

In general, the Atlantic sites revealed reduced allelic richness when compared with populations from the Mediterranean. This could be attributed to present-day low effective population size or to founder events during the past colonization of the Atlantic from the Mediterranean. The first hypothesis is unlikely because: (1) sexual reproduction seems to be an important component of population growth in the Canary Islands and Mauritania, as seen by the levels of genotypic richness reported here; and (2) the occurrence in this region of the largest continuous meadows of *C. nodosa* that we are aware of (particularly in Mauritania) indicate that effective population size is unlikely to be smaller than elsewhere in the species range. The second hypothesis suggests that only a small proportion of the alleles from the source habitat were retained, due to the reduction in effective population size of migrant

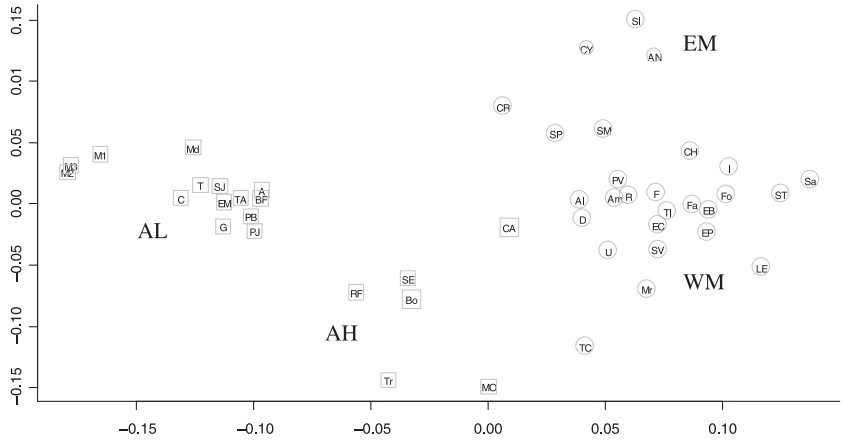

Figure 5 Multidimensional scaling of genetic differentiation among *Cymodocea nodosa* sites. Squares, Atlantic sites (AL, lower-latitude Atlantic; AH, higher-latitude Atlantic edge); circles, Mediterranean sites (WM, Western Mediterranean; EM, Eastern Mediterranean).

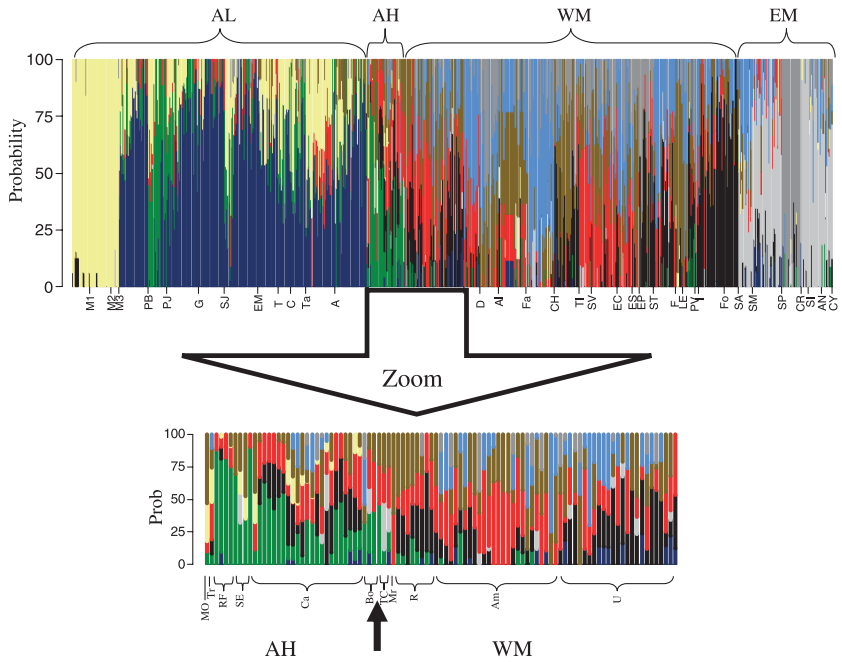

Figure 6 PSMIX assignment of *Cymodocea nodosa* individuals to $k = 9$ inferred clusters (k estimated under GENELAND). Each individual is represented by a column; different colours within columns indicate the maximum likelihood probability of belonging to different clusters, a measure of gene ancestry and admixture (AL, lower latitude Atlantic; AH, higher latitude Atlantic edge; WM, Western Mediterranean; EM, Eastern Mediterranean). Higher panel, total area studied; lower panel, enlargement of transition area between AH and WM, indicated by a dark arrow.

Table 2 Hierarchical *F* statistics for *Cymodocea nodosa*.

Regions	F_{IndPop}	F_{PopReg}	$F_{RegTotal}$	$F_{PopTotal}$
Atlantic and Mediterranean	0.002	0.255	0.230	0.426
AL and AH	0.021	0.211	0.252	0.410
AH and WM	–0.041	0.220	0.089	0.289
WM and EM	–0.010	0.237	0.098	0.312

Four different analyses were carried out: Atlantic and Mediterranean, and three subsets with regions defined by the groups indicated in Fig. 1. All *F* estimates are significant ($P < 0.001$).

populations; thus recently colonized populations should exhibit a subset of the allele composition of source populations (Hewitt, 1996) with drastically rearranged frequencies. Studies on other taxa have inferred colonization from the Mediterranean to the Atlantic based on lower allelic richness in the Atlantic (Saavedra *et al.*, 1993; Pannacciulli *et al.*, 1997; Duran *et al.*, 2004b). In the case of *C. nodosa* in the Atlantic, the pattern is not that simple. Rather, several alleles were present at a high frequency in the Atlantic, and were absent in the Western Mediterranean (potential source or at least stepping-stone populations), but some of these were detected at a low frequency in the Eastern Mediterranean (Fig. 3), and close to the transition region there is evidence of admixture on the Atlantic side. These results suggest that the separation between the low-latitude Atlantic and Mediterranean groups is thought to be ancient, based on the evolutionary time required to accumulate so many private alleles. The number of such alleles could actually be higher if the alleles shared between AL and EM, the most distant regions, are in fact homoplasious due to the constraints on microsatellite allele sizes (Garza *et al.*, 1995; Nauta & Weissing, 1996) and the long coalescent time separating these groups.

The high genetic differentiation levels reported here suggest that colonization of the present-day Atlantic range is not recent, and might have taken place before the Pleistocene glaciations. During the glaciation cycles over the past 1.8 Myr (Croll–Milankovitch cycles; Hays *et al.*, 1976), *C. nodosa* should have been locally excluded from Western Mediterranean and Atlantic high-latitude edge sites, considering the present-day sea-surface temperature throughout the species range. During the LGM (18–10 kyr BP), the Western Mediterranean had colder water temperature and higher thermal amplitude when compared with the more stable Levantine Sea (Thunell, 1979; Hayes *et al.*, 2005) and the Atlantic in the Canaries–Mauritania region (Dynesius & Jansson, 2000). This contraction of the species range during the glacial to areas today representing range edges (the Eastern Mediterranean and the lower-latitude Atlantic) may explain the high genetic differentiation by a vicariance process set by the glacial cycles. The post-glacial range extension from Mediterranean and Atlantic refugia would have given rise at the AH secondary contact zone to the admixture of allelic forms typical of each side. This scenario is supported by the extremely high allelic richness found here for the Amathous sample from Cyprus, and the higher allelic richness found for AH as compared with AL (after standardizing the number of genets), which may be explained by the admixture at AH sites from both AL and WM extending range edges following glacial maxima.

Strong genetic divergence between eastern and western basins was revealed here, and has also been detected recently for the endemic Mediterranean seagrass *P. oceanica*, with admixture allele patterns at an intermediate region corresponding to the Sicily–Tunisia Strait (Arnaud-Haond *et al.*, 2007). Sampling throughout the Eastern Mediterranean should be extended to characterize fully what seems to be an important hotspot of genetic diversity for *C. nodosa*.

Likewise, sampling along the Western Mediterranean North African coast and in the Sicily–Tunisia Strait is important to understand if the species might have had glacial refugia in the south-western Mediterranean. The lack of geographical genetic structure for the Western Mediterranean and the relatively shallower tree configuration may be signals of range expansion from Mediterranean refugia following the LGM.

Stability and age of Atlantic rear edge populations

Patterns of allelic diversity have been studied across gradients of latitude of cold-adapted seagrass and algal species (Coyer *et al.*, 2003; Olsen *et al.*, 2004), and inferences have been made about the effects of Pleistocene glaciations, whereby species tend to be consistent with a 'leading edge–trailing edge' paradigm with both rear and leading range edges having impoverished diversity (Hewitt, 1996, 2000; Hampe & Petit, 2005) with low abundance, high extinction risk, low effective size and strong genetic structure among remaining pockets of habitat. What we found for *C. nodosa*, with populations from putative warmer refugia (Atlantic southern edge and Eastern Mediterranean) being genetically more diverse, is more consistent with the case where the species has stayed *in situ*, either surviving at suitable sites or adapting, that is, a 'stable' rear edge (Channell & Lomolino, 2000). Such populations are expected to be two to three orders of magnitude older than those on the expanding range, and are considered of great importance for the conservation of intraspecific biodiversity (Hampe & Petit, 2005). It is remarkable that Atlantic rear-edge populations in Mauritania form the largest continuous meadows throughout the species range.

The different temperature tolerances of the above-mentioned species may explain the different outcomes of past climate-driven changes in range dynamics. Temperate species (Coyer *et al.*, 2003; Olsen *et al.*, 2004) were likely to have southern refugia further north than *C. nodosa* and *Zostera noltii* (Coyer *et al.*, 2004), the southern edge of which occurs in sub-tropical zones. Higher latitudes were more likely to experience larger temperature variation during glacial–interglacial oscillations, which means a lower chance of species finding favourable sites or adapting to changing conditions, resulting in latitudinal range displacement and trailing edge patterns. In contrast, the lower temperature variance experienced further south might have allowed *C. nodosa* and *Z. nolti* (Coyer *et al.*, 2004) to persist, adapt and form large populations. Therefore both these seagrass species constitute excellent models to study the roles of adaptation vs. plasticity and speciation at lower-latitude range margins.

Patterns of genotypic richness

Genotypic richness (R) is an important component of genetic variation for organisms with mixed modes of reproduction, sexual and clonal, because it estimates the balance between effective sexual and asexual reproduction over several generations (Dorken & Eckert, 2001; Reusch, 2001). Several hypotheses have been advanced to try to explain the patterns of genotypic richness found in natural populations. One is the intermediate disturbance theory (Connell, 1978; Weider, 1992), which states that the highest level of diversity will be maintained at intermediate scales of disturbance, sufficient to clear space and enhance sexual recolonization (Edwards *et al.*, 2005). Previous studies have used this idea to explain patterns of genotypic richness found in some seagrass meadows (Hammerli & Reusch, 2003; Coyer *et al.*, 2004). Under this hypothesis, in lower disturbance regimes competitive exclusion between clones is not precluded and population structure would become more clonal. This pattern was not observed in the Cadiz sample, collected inside a bay with a relatively stable environment where both allele and genotypic richness were relatively high, in contrast with all other sites in the contact region, including other large coastal lagoons, such as Ria Formosa, with extremely low genotypic richness.

The low genotypic richness in the northern-edge Atlantic populations is therefore more probably explained by historical contingency such as geographical isolation and climate oscillations, rather than by present-day disturbance regimes. South of the Strait of Gibraltar, Oualidia (Morocco) and Madeira (Fig. 1) are the first sites where the species was found, spanning around 500 km of separation; extensive populations are known to occur further south only in the Canary Islands and Mauritania. North-west of Cadiz, the Ria Formosa in southern Portugal is the first place where the species is present (at 160 km distance). Tróia, the northern Atlantic range limit of the species, is located more than 270 km from the nearest meadow and beyond the important geographical barrier of Cape São Vicente. The colonization of these sites might have been characterized by strong founder effects (Alberto *et al.*, 2001), whereby only a small number of individuals reached the outer sites. Throughout the Mediterranean, genotypic richness was highly variable but ranged from very low to a maximum of one at the easternmost sample, where every ramet sampled was from a unique genet. These differences could not be accounted for by any evident relationship with disturbance/stability or any other environmental factor.

An alternative hypothesis that may explain some of the low clonal richness values reported here is the global trend of seagrass decline associated with human development (Duarte, 2002; Orth *et al.*, 2006), which may cause population bottlenecks followed by recolonization by clonal growth of the few remaining genotypes. This could be the case in southern Portugal, where there is historical evidence (20th century) of large seagrass meadows having occurred on the open coast (Luis C. da Fonseca, pers. comm.) at sites where they have since disappeared. If the current populations are the result of recolonization from a few surviving genets, clonal propagation may be predominant given that the species is dioecious, thus requiring co-occurrence of different male and female genets for successful sexual propagation. Monoclonal meadows are thus unable to produce seeds even if flowering investment is high.

CONCLUSIONS

We have shown a strong genetic structure among populations of *C. nodosa* along their geographical distribution. Populations at the southernmost limit (Atlantic) are extremely well differentiated from all remaining populations, and those in the Eastern Mediterranean are also significantly distinct from all others. There appears to be a trend for decreasing allelic richness from the Eastern Mediterranean towards the Atlantic, but diagnostic (private) alleles were found for the Mediterranean and for the Atlantic.

The southern edge Atlantic populations appear to form a stable rear edge, contrasting with less diverse and smaller Atlantic northern edge populations. These populations and a population immediately east of Gibraltar revealed a secondary contact zone, where a mixture of alleles otherwise exclusive to the Mediterranean or to the Atlantic can be found. The latter is remarkable given that all these populations, with the exception of Cadiz, are composed of one or very few genotypes (clones).

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online from http://www.blackwell-synergy.com:

Table S1 F_{ST} table: pairwise genetic distance values (F_{ST} estimator, θ) between *Cymodocea nodosa* sites. Site codes are given in Table 1.

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BIOSKETCHES

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