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SMALL-SCALE GENETIC STRUCTURE OF *CERASTODERMA GLAUCUM* IN A LAGOONAL ENVIRONMENT: POTENTIAL SIGNIFICANCE OF HABITAT DISCONTINUITY AND UNSTABLE POPULATION DYNAMICS

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ABSTRACT

Environmental heterogeneity in coastal lagoons is expected to facilitate local adaptation in response to different ecological conditions, causing significant genetic structuring within lagoon populations at a small scale and also differentiation between lagoons. However, these patterns and processes of genetic structuring are still poorly understood. The aims of our study were (1) to seek genetic structure at a small scale in *Cerastoderma glaucum* inside the Mar Menor coastal lagoon using a mitochondrial DNA marker (COI) that has previously detected genetic differentiation inside the lagoon in other species and (2) to evaluate the influence of extreme environmental conditions and habitat discontinuity on its genetic composition. The results indicate high levels of haplotype diversity and low values of nucleotide diversity. COI data provide evidence of significant population differentiation among some localities within the lagoon. Limited gene flow and unstable population dynamics (i.e. fluctuations in population size caused by local extinction and recolonization), probably due to the high environmental heterogeneity, could generate the small-scale genetic divergence detected between populations within the lagoon.

INTRODUCTION

Coastal lagoons are shallow water bodies separated from the ocean by a barrier and connected with it by one or more restricted inlets. They are well known to harbour a range of distinctive lagoonal morphotypes of invertebrates, which differ from the typical oceanic forms in relatively minor morphological, biological and ecological features (Barnes, 1980; Porter *et al.*, 2001; Ladhar-Chaabouni *et al.*, 2010). These features include shell shape, habitat preference, burying ability, adaptation to air exposure, salinity and temperature tolerance, egg type, larval dispersal and juvenile mobility (Reise, 2003). The existence of these morphotypes suggests that physical isolation and ecological discontinuities may exert strong selective pressures on marine organisms (Porter *et al.*, 2001; Bilton, Paula & Bishop, 2002; Iannotta, Toscano & Patti, 2009; Richards, Wares & Mackie, 2010; Sanford & Morgan, 2011). By extension, environmental components within coastal lagoons could drive genetic structure on a small spatial scale. However, these patterns and processes of genetic structuring are still poorly understood.

A typical lagoonal species particularly common within the Mar Menor in southeastern Spain is the lagoon cockle, *Cerastoderma glaucum* (Poiret, 1789), an ideal species to study small-scale genetic structure and adaptation to extreme conditions. *Cerastoderma glaucum* has a wide distribution around European coasts, ranging from the northern Baltic Sea to the Black Sea, the Caspian Sea and even the Aral Sea (Russell & Petersen, 1973; Brock, 1979). In the Atlantic and Mediterranean, *C. glaucum* typically inhabits closed brackish-water lagoons and estuaries. The distribution of this species is limited by exposure to waves, so it is never found in loose sediments (Boyden & Russell, 1972; Brock, 1979; David & Tigan, 2011). Any autonomous long-distance dispersal of this gonochoristic species is restricted to the 1-week pelagic larval stage (Barnes, 1980). The species is euryhaline and eurythermal, but intolerant of exposure to air (Russell, 1971). Such ecological features make *C. glaucum* an interesting subject for the study of genetic variation.

The existing information about population genetics of *C. glaucum* indicates that this species has undergone population growth in the Mediterranean Sea. This was perhaps caused by historical climatic events that may still play a role in determining the pattern of spatial genetic variation (Mariani, Ketmaier & De Matthaeis, 2002) and was also likely linked with hydrographical changes during the Pleistocene, particularly sea-level fluctuations (Nikula & Väinölä, 2003; Ladhar-Chaabouni *et al.*, 2010; Tarnowska *et al.*, 2010, 2012). The Quaternary glaciations greatly affected the distributions and population sizes of temperate marine species, which retreated southward as climate cooled (Maggs *et al.*, 2008). As a consequence of climate cycles during the late Pliocene and throughout the Pleistocene, the levels of genetic differentiation among cockle populations are significant and we can discriminate subpopulations throughout its geographic range (Maggs *et al.*, 2008). The genetic signatures of glacial refugia have been detected in *C. edule* on northeastern Atlantic shores using the COI gene (Krakau *et al.*, 2012). Recent studies of *C. glaucum* populations from the Mediterranean and Atlantic using ITS and COI markers have shown significant genetic differentiation among basins, which could result from the geographical isolation brought about by climatic cycles during the late Pliocene and throughout the Pleistocene (Ladhar-Chaabouni *et al.*, 2010). Also, these same authors detected two phenotypes in the Mediterranean population of *C. glaucum*, which seem to be related to genetic variability in ITS1 and COI genes. Using allozymes, other studies have demonstrated that adaptation to different salinity regimes and wave exposure may have driven genetic divergence in *C. glaucum* populations from Mediterranean, Tyrrhenian and Adriatic coastal lagoons (Ketmaier *et al.*, 1997; González-Wangüemert *et al.*, 2009), although positive selection has not yet been explicitly tested. Despite this theoretical background, no studies have been done to examine the genetic structure of this species at a small geographic scale (1–10 km) inside a coastal lagoon using mtDNA markers and considering the importance of habitat continuity and environmental variables in the persistence of its populations.

Within the Mar Menor lagoon, spatial environmental heterogeneity creates habitat discontinuities and ecological gradients which, together with temporal physical environmental stress *sensu* Sanders (1968), make it an optimal place to examine the influence of environmental conditions on genetic composition of lagoon populations (Pérez-Ruzafa *et al.*, 2007). Therefore, the present study addresses the question of small-scale spatial genetic variation of *C. glaucum* at nine sampling localities inside this lagoon and its relationship with habitat features. These localities were chosen on the basis of previous work on this species in Mar Menor using allozymes, which found interesting genetic patterns linked to the percentage of fine sand in sediments, salinity and currents (González-Wangüemert *et al.*, 2009). Similar patterns have been corroborated in other species inside the Mar Menor lagoon (*Elysia timida*: González-Wangüemert, Giménez-Casalduero & Pérez-Ruzafa, 2006; Giménez-Casalduero *et al.*, 2011; *Holothuria polii*: Vergara-Chen *et al.*, 2010a; *Pomatoschitus marmoratus*: Vergara-Chen *et al.*, 2010b; *Diplodus sargus*: González-Wangüemert & Pérez-Ruzafa, 2012).

The objectives of our study were (1) to seek genetic structure at a small scale in *Cerastoderma glaucum* inside the Mar Menor coastal lagoon using a mitochondrial DNA marker (COI) and (2) to evaluate the influence of extreme environmental conditions and habitat discontinuity on its genetic composition.

MATERIAL AND METHODS

Study area

The Mar Menor is a hypersaline coastal lagoon with a surface area of about 135 km^2 located on the southeastern coast of Spain. The mean depth is 3.5 m with a maximum depth of 6 m. The salinity of the lagoon waters ranges between 38 and 51 psu, due to high evaporation and low exchange rates with the Mediterranean. Water temperature ranges from 10°C in winter to 31°C in summer (Pérez-Ruzafa *et al.*, 2005, 2007). The lagoon is bordered on the seaward side by La Manga sand bar, crossed by three inlets (El Estacio, Las Encañizadas and Marchamalo) that regulate water exchange with the open sea. According to their hydrographical characteristics, three main basins have been differentiated inside the lagoon (Pérez-Ruzafa *et al.*, 2004, 2007). The sampling strategy was based on a previous comparison among these three basins (northern, central and southern; Fig. 1) during previous work on *C. glaucum* (González-Wangüemert *et al.*, 2009). The northern basin has the greatest influence of Mediterranean waters through the inlets in the north of La Manga and El Estacio, and has the lowest mean salinity values. The southern basin is a more confined area and has the most saline water. The central basin has intermediate salinity due to the mixing of Mediterranean and lagoonal waters. In addition to different levels of salinity and temperature among these basins, there are different substrate types (pebbles, sand and mud) and patches of *Caulerpa prolifera* and seagrass beds. The oceanographic information comes from the E065-05 research team at the Universidad de Murcia and is available at: www.indamar.ieo.es and http://www.puertos.es/oceanografia_y_meteorologia/redes_de_medida/index.html.

Sampling and COI gene sequencing

Samples of *C. glaucum* were collected by hand from shallow-water benthic habitats (<1 m depth) at nine sites (Las Encañizadas, El Peduchico, South Pedruchico, La Carrasquilla, Los Nietos, Los Urrutias, Los Alcázares, Los Narejos and Lo Pagán) spanning the full extent of the Mar Menor coastal lagoon and including its three basins (Fig. 1). The sampling was carried out in a single effort during 2008, targeting the same year classes in all localities. We measured the sizes of individuals from three population samples (Los Urrutias = 2.28 ± 0.19 cm; Lo Pagán = 2.10 ± 0.14 cm; South Pedruchico = 2.25 ± 0.11 cm) and confirmed similar sizes and therefore the same year classes. The sample size consisted of 20–31 individuals per collecting site. All samples were stored in ethanol at −20°C for DNA analyses. DNA was extracted from the foot muscle using lysis buffer solution with proteinase K, protein precipitation and final precipitation with ethanol (Sambrook & Russell, 2001). The universal primers HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) and LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) (Folmer *et al.*, 1994) were used to amplify a 658-bp fragment from the 3′ end of the mitochondrial cytochrome oxidase I (COI) gene. However, a 491-bp segment reliably sequenced was used in the analyses to reduce sequence errors. Each PCR reaction mixture (volume 20 µl) contained 30–50 ng/µl of total genomic DNA as template, 4 mM each primer, 200 mM dNTPs, 1X PCR buffer, 3.5 mM $MgCl_2$ and 2 units of EcoTaq polymerase (all reaction chemicals manufactured by Ecogen).

Amplification was performed in a C-1000 thermal cycler (BioRad, USA), programmed for an initial denaturation step at 95°C for 2 min, then 35 cycles of denaturation at 95°C for 50 s, annealing at 49°C for 50 s, and extension at 70°C for 1 min 20 s, followed by a final extension at 72°C for 7 min. A 5-µl sample of each PCR product was run on 2% agarose gel and stained with ethidium bromide before being viewed with UV light. A 2.5 µl volume of each amplified product was purified using ExoSAP-IT (Amersham Pharmacia Biotech) and sequenced in one direction according to the protocols of the University of Murcia Integrative Laboratory for Molecular Biology, using an

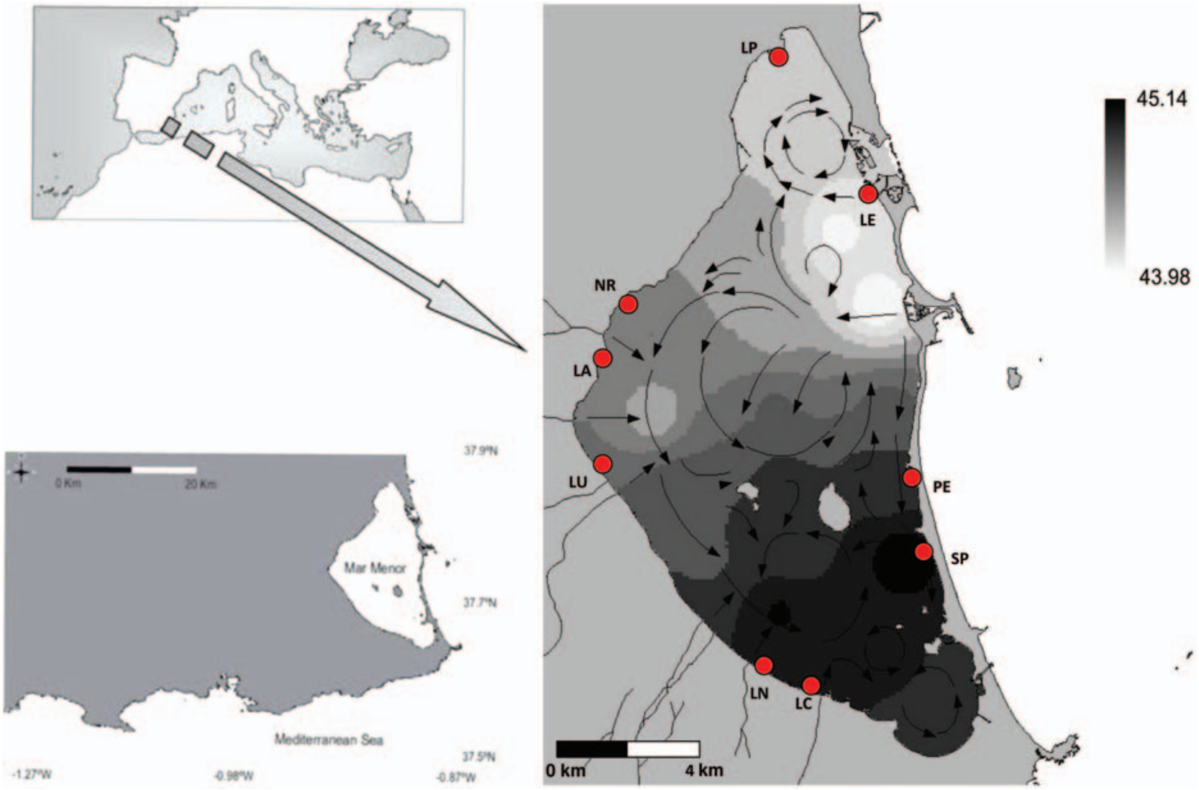

Figure 1. The Mar Menor lagoon (southeastern Spain) showing sampling locations (circles) for *Cerastoderma glaucum*: Lo Pagán (LP), Las Encañizadas (LE), El Pedruchico (PE), South Pedruchico (SP), La Carrasquilla (LC), Los Nietos (LN), Los Urrutias (LU), Los Alcázares (LA), Los Narejos (NR). The arrows indicated the hydrodynamic patterns inside the lagoon and the straight lines the edges of the hydrographical basins, showing the surface gradient of mean salinity (González-Wangüemert *et al*., 2009).

ABI Prism 3130 automated genetic analyser (Applied Biosystems). Sequences of all found haplotypes were deposited in GenBank (HQ432824 to HQ432862).

Data analysis

The sequences were aligned using BioEdit software (Hall, 1999). Genetic diversity within samples was estimated as haplotype and nucleotide diversities (Nei, 1987), haplotype richness, number of polymorphic sites, number of exclusive haplotypes and number of singleton haplotypes. A singleton mtDNA haplotype is a single sequence in the sample (Posada & Crandall, 2001) or a haplotype shown in only one individual (Luzier & Wilson, 2004). Pairwise estimates of F_{ST} and Φ_{ST} between locations (Weir & Cockerham, 1984) were calculated with Arlequin v. 3.11 (Excoffier, Laval & Schneider, 2005). The sequential Bonferroni correction for multiple comparisons (Rice, 1989) was applied to all probability values from F_{ST} estimates to compensate for possible type I errors resulting from multiple pairwise comparisons. Populations were spatially clustered using correspondence analysis implemented in the BiodiversityR package in *R* software (*R* Development Core Team, 2007), which utilizes the haplotype frequencies of populations as variables in order to visualize similarities among locations, without assuming tree-like relationships. The correspondence analysis summarizes all the variation in the study area and accommodates each population as a study unit (Manel *et al*., 2003; González-Wangüemert *et al*., 2010, 2012). A haplotype network was constructed under the statistical parsimony criterion (Templeton, Crandall & Sing, 1992) with TCS v. 1.21 software (Clement, Posada & Crandall, 2000). A hierarchical analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) implemented in Arlequin v. 3.11 was used to test for significant heterogeneity among locations considering three groups: northern basin (Lo Pagán, Las Encañizadas, Los Narejos, Los Alcázares), central basin (Los Urrutias, El Pedruchico and South Pedruchico) and southern basin (Los Nietos and La Carrasquilla). These groups are based on hydrological and sediment characteristics characterized by Pérez-Ruzafa *et al*. (2004, 2005, 2007).

Using Arlequin v. 3.11 software, we tested whether the pattern of observed polymorphism within *C. glaucum* is consistent with a neutral Wright-Fisher model using Tajima's *D* and Fu's F_{S} (Tajima, 1989; Fu, 1997). Positive values indicate a lack of significant recent mutations that may have resulted from balancing selection, population structure or decline in population size. Negative values reflect excesses of recent mutations that may indicate population expansion or selective sweeps. Additionally, mismatch distribution analysis in Arlequin, comparing the frequency distribution of pairwise differences among haplotypes against a model of rapid expansion (Rogers & Harpending, 1992), was carried out on each location. Significance was assessed with 1000 parametric bootstrapping replicates. To estimate the approximate time of expansion, the formula $\tau = 2\ \mu t$ was used. According to Rogers & Harpending (1992) the wave's crest is determined by this equation, where τ is the mode of mismatch distribution, t represents the approximate time of expansion and μ is the mutation rate of the entire region under study, which is the mutation rate per nucleotide multiplied by the number of nucleotides of the analysed fragment.

To consider evidence of selection on COI sequences, the number of synonymous substitutions per synonymous site (dS) and the number of nonsynonymous substitutions per nonsynonymous site (dN) were estimated by bootstrap (10 000 replicates) using the codon-based *Z*-test (Nei & Gojobori, 1986). As the

COI gene is a protein-coding gene, we also estimated selection for each codon to test whether positive selection is operating on codons and to compare the relative abundance of synonymous and nonsynonymous substitutions. These estimates are produced using the joint maximum-likelihood reconstructions of ancestral states implemented with HyPhy software (Kosakovsky Pond, Frost & Muse, 2005). A positive value for the test statistic indicates an overabundance of nonsynonymous substitutions. In this case, the probability of rejecting the null hypothesis of neutral evolution was calculated (Kosakovsky Pond *et al.*, 2005). All tests of selection were conducted using MEGA5 (Tamura *et al.*, 2011).

RESULTS

Analysis of COI sequences 491 bp in length from 231 individuals of *Cerastoderma glaucum* from Mar Menor lagoon revealed high values of haplotype diversity and low nucleotide diversity. Overall, we found 39 different haplotypes, of which 22 were singletons, 7 exclusive haplotypes and 10 shared haplotypes. A mean of 11.44 polymorphic sites (2.33%) in the 491-bp fragment sequenced was observed over all samples. The El Pedruchico (PE) sample showed the highest number of haplotypes (12); however, the Los Nietos (LN) sample showed the highest haplotype diversity (0.8985) and 11 haplotypes. At Las Encañizadas (LE) and Los Narejos (NA), we found the lowest haplotype number (4) and lower haplotype diversity (0.5688 and 0.3333, respectively) (Table 1).

The pattern of population differentiation based on F_{ST} and Φ_{ST} statistics pointed to significant differences between 13 sample comparisons, while we detected no differentiation between 23 sample comparisons (Table 2). There were two discrepancies between the results based on these two indices: only F_{ST} showed differentiation among the comparisons La Carrasquilla (LC)-Los Narejos (NA) and Lo Pagan (LP)-Los Urrutias (LU), while only Φ_{ST} indicated significant differences among Los Alcazares (LA)-Los Urrutias (LU) and Los Urrutias (LU)-South Pedruchico (SP). These significant differences could be caused by frequency differences in common shared haplotypes. After sequential Bonferroni correction, the data showed significant interpopulation differentiation in 12 pairwise comparisons. These significant differences were found between La Carrasquilla-Los Narejos (LC-NR), Las Encañizadas-Los Narejos (LE-NR), Los Urrutias-Los Narejos (LU-NR), Lo Pagán-Los Urrutias (LP-LU), and between Los Nietos (LN) and all remaining locations. The interpopulation differentiation in these cases was sufficient to maintain significance after correction for paired tests. In the correspondence analysis (CA) based on haplotype frequencies, the first two ordination axes explained 51.26% of the total variance in the data, revealing geographic structuring showing three groups (Fig. 2): (1) Los Nietos sample on the positive side of Axis I; (2) Los Urrutias sample on the negative side of Axis II; (3) a group composed of the seven remaining localities on the negative side of Axis I.

Table 1. Molecular diversity indices for populations of *Cerastoderma glaucum* from the Mar Menor lagoon (SE Spain) using 491-bp of mtDNA COI.

Locations	*N*	Haplotypes	*Ps*	*h*	π
Northern Basin					
LP	28	11 (5)	18	0.674 ± 0.098	0.003 ± 0.002
LE	24	4 (1)	3	0.569 ± 0.074	0.001 ± 0.001
LA	20	5 (1)	4	0.600 ± 0.101	0.002 ± 0.001
NR	22	4 (0)	3	0.333 ± 0.124	0.001 ± 0.001
Central Basin					
PE	29	12 (4)	18	0.815 ± 0.066	0.005 ± 0.003
SP	31	10 (4)	16	0.587 ± 0.104	0.004 ± 0.002
LU	24	9 (1)	18	0.891 ± 0.033	0.009 ± 0.005
Southern Basin					
LC	27	9 (1)	14	0.684 ± 0.094	0.004 ± 0.002
LN	26	11 (5)	10	0.898 ± 0.034	0.007 ± 0.004

Sampled locations: Lo Pagán (LP), Las Encañizadas (LE), Los Alcázares (LA), Los Narejos (NR), El Pedruchico (PE), South Pedruchico (SP), Los Urrutias (LU), La Carrasquilla (LC), Los Nietos (LN). *N*, number of individuals; H, number of haplotypes and in brackets number of singletons; Ps, number of polymorphic sites; *h*, haplotype diversity; π, nucleotide diversity.

The statistical parsimony network of the COI haplotypes recovered by TCS revealed 10 shared haplotypes (COI-3, COI-5, COI-9, COI-10, COI-11, COI-12, COI-13, COI-14, COI-15 and COI-24) with two haplotypes (COI-3 and COI-5) shared by all samples and clustered roughly into two main groups, with remaining haplotypes connected to these principal ones (Fig. 3). The most distinct haplotypes were COI-38 (in SP), COI-10 (in PE, LC, LP and LU), and COI-8 (in PE) and differed by 7, 8 and 10 mutational steps, respectively. The rare variants—mainly from Los Nietos (COI-26, CO-27, COI-28, COI-29, COI-30, COI-31, COI-32 and COI-33), Los Urrutias (COI-34, COI-35) and South Pedruchico (COI-36, COI-37, COI-38 and COI-39)—represent more recent mutations according to Posada & Crandall (2001).

The analysis of molecular variance (AMOVA), considering the three previously described basins, pointed to nonsignificant differences among the three groups established (FCT = 0.03385; percentage of variance = 3.13%; $P = 0.07$). However, the analysis revealed significant differences among populations within groups (FSC = 0.07503; percentage of variance = 6.93%; $P < 0.001$) and within populations (FST = 0.97360; percentage of variance = 89.94%; $P < 0.001$).

The mismatch analysis of COI sequences revealed two patterns of population distribution, supporting two types of mismatch distribution according to Patarnello, Volckaert & Castilho (2007). All samples showed skewed distributions, related to a recent bottleneck or sudden population expansion, except Los Nietos and Los Urrutias, which showed a bimodal distribution usually linked to constant population size (Fig. 4). The dataset exhibited negative and significant Tajima's D and Fu's F_S values, corroborating a sudden population expansion in the El Pedruchico, La Carrasquilla, Lo Pagán and South Pedruchico samples. The Los Narejos sample showed a significant F_S value, but the D index was nonsignificant (Table 3). SSD values indicated sudden population expansion in all localities except for the Los Urrutias sample. Considering the sequence length of 491 bp and estimating a mutation rate μ of 2.5% per million years (Nikula & Väinölä, 2003), the start of expansions at these localities was dated as follows: PE = 56 000 years B.P.; LC = 56 000 years B.P.; LP = 46 000 years B.P.; and SP = 127 000 years B.P. However, since the F_{ST} values between several localities are not significant, it does not make sense to consider them as different populations. Thus, when all samples were pooled, the expansion event was calculated at about 62 000 years B.P.

The results from the $\mathcal{Z}$-test indicated significant evidence of purifying selection in the COI sequences of *C. glaucum*. The HyPhy package detected significant positive selection over seven codons.

DISCUSSION

Our mitochondrial DNA survey of *Cerastoderma glaucum* revealed high haplotype diversity, low nucleotide diversity and occurrence of 39 different haplotypes within the Mar Menor coastal lagoon. The analysis showed low nucleotide diversity compared

Table 2. Pairwise fixation indices (F_{ST}) between populations of *Cerastoderma glaucum* from the Mar Menor lagoon (southeastern Spain) based on mtDNA COI sequences.

Locations	PE	SP	LC	LN	LE	LP	LA	NR	LU
Pedruchico (PE)									
South Pedruchico (SP)	−0.004								
La Carrasquilla (LC)	−0.009	−0.023							
Los Nietos (LN)	0.207*	0.221*	0.214*						
Las Encanizadas (LE)	0.023	0.002	0.008	0.242*					
Lo Pagán (LP)	−0.014	−0.014	−0.020	0.225*	0.011				
Los Alcázares (LA)	0.006	−0.014	0.002	0.235*	−0.003	−0.007			
Los Narejos (NR)	0.030	0.019	0.035*	0.303*	0.143*	0.012	0.049		
Los Urrutias (LU)	0.028	0.057	0.038	0.173*	0.085*	0.054*	0.087	0.144*	

P is the probability that any random value obtained after 1000 permutations is > observed value. *Significant F_{ST} values ($P < 0.05$).

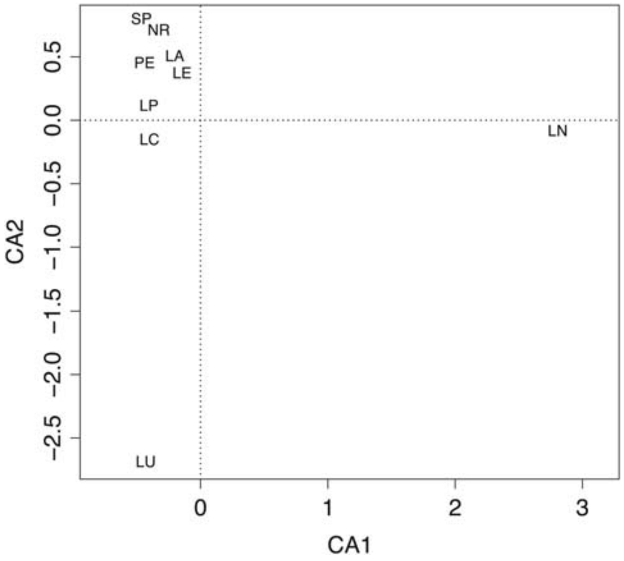

Figure 2. Correspondence analysis of mtDNA COI haplotype frequencies of *Cerastoderma glaucum* populations within the Mar Menor lagoon (southeastern Spain). Populations are abbreviated as follows: Lo Pagán (LP), Las Encañizadas (LE), El Pedruchico (PE), South Pedruchico (SP), La Carrasquilla (LC), Los Nietos (LN), Los Urrutias (LU), Los Alcázares (LA), Los Narejos (NR) (see Fig. 1).

with previous studies of *Cerastoderma* species (Tarnowska *et al.*, 2010, 2012; Krakau *et al.*, 2012), probably because our samples come from a population that has recently spread into the lagoon. The combination of high haplotype diversity and low nucleotide diversity, as observed in our data, can be a signature of a rapid demographic expansion from a small effective population size (Avise, Neigel & Arnold, 1984; Grant & Bowen, 1998; Avise, 2000; Dodson *et al.*, 2007; Winkler *et al.*, 2011). A previous study of this species carried out at the same lagoon using allozymes (González-Wangüemert *et al.*, 2009) also detected high gene diversity. Moreover, Tarnowska *et al.* (2010), using COI sequences for the same species, showed that the highest values of haplotype diversity (H_D) were found in coastal lagoons (Etolikon, Greece, $H_D = 0.911$; Berre lagoon, France, $H_D = 0.814$; Tunis Bay, Tunisia, $H_D = 0.860$; Cabras, Sardinia, Italy, $H_D = 0.776$) and that simultaneously these localities had the highest number of haplotypes (Berre lagoon = 11; Cabras = 10; Tunis Bay = 10). However, it is important to stress that although the work of Tarnowska *et al.* (2010) was carried out in several lagoons, it did not include different localities from each lagoon and that therefore the authors did not consider the analysis of genetic variation in *C. glaucum* at a small spatial scale, instead assuming genetic homogeneity inside each sampled lagoon.

We found nonrandom genetic differentiation among our sampled *C. glaucum* populations across the lagoon. In general, the main genetic differentiation was detected between the Los Nietos population in the south basin and all other localities, and Los Urrutias in the central basin and some localities from the north, central and south basins. The genetic divergence of Los Nietos could be an outcome of its relatively high population stability due to its more favourable local environment, because this locality is characterized by a low hydrodynamics, fine sediment and slight variations in salinity. In Los Nietos the environmental conditions are optimal for development and survival of a local cockle population, resulting in a temporally stable population, which has not undergone demographic expansions or bottlenecks. This environmental and population stability has allowed the Los Nietos population to differentiate genetically from other populations. These environmental factors should promote a constant demographic size as confirmed by our mismatch distribution analysis and should also favour the preservation of some undetected haplotypes in other populations.

On the other hand, changes in habitat availability and other ecological factors might promote local extinction and recolonization at specific localities, so recently founded populations might not have reached mutation-drift equilibrium, explaining the results of Tajima's tests and lack of differentiation in F_{ST}. Cockle populations undergoing critical situations are characterized by a few age classes that undertake repeated or uninterrupted spawning, leading to numerous cohorts. Thus, any subsequent population decline may be due to loss of older individuals, unsuccessful recruitment and/or a high population growth rate, which may lead to population instability (Ducrotoy *et al.*, 1989). Moreover, several spawning events may occur annually, apparently triggered by rising seawater temperature (Derbali, Jarboui & Ghorbel, 2009). In this context, an alternative hypothesis is that this reproductive strategy of cockles plus the highly variable environmental conditions (i.e. temperature) may create the potential for sweepstakes reproductive success (Hedgecock, 1994; Hedgecock *et al.*, 2007). However, our main hypothesis to explain the observed results is the role of environmental heterogeneity in shaping small-scale population structure, most likely caused by restricted gene flow and also unstable population dynamics with large effects of genetic drift (Broquet, Viard & Yearsley, 2012).

Our significant F_{ST} and Φ_{ST} values are similar to those previously obtained from COI sequence data of *C. glaucum* populations, although at a larger spatial scale, between localities

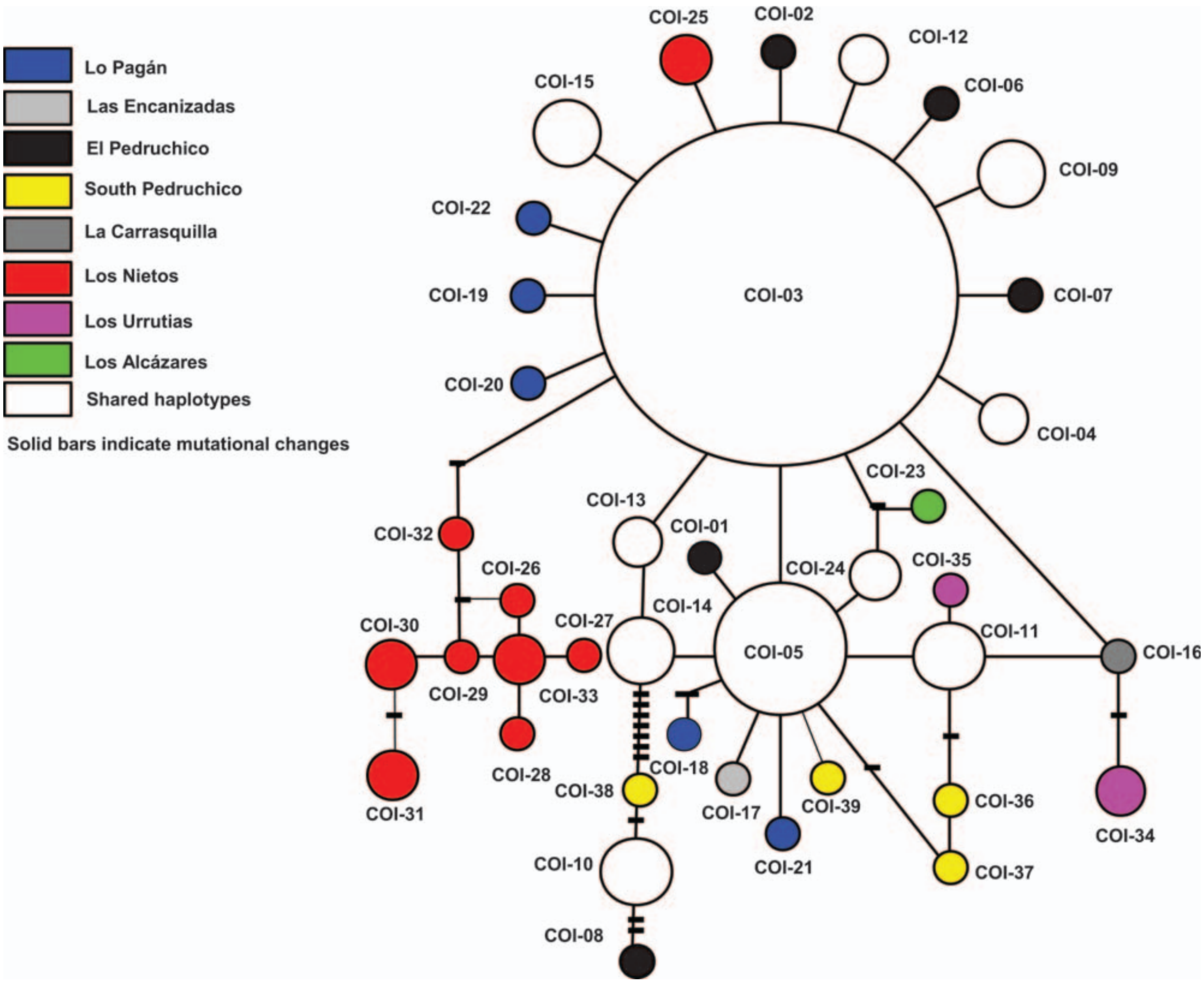

Figure 3. Statistical parsimony network based on mtDNA COI sequence haplotypes of *Cerastoderma glaucum* from the Mar Menor lagoon (southeastern Spain). Each haplotype is defined by its corresponding number. The area of each circle is proportional to the number of individuals.

hundreds of kilometres apart (Tarnowska *et al*., 2010). Although F_{ST} and Φ_{ST} both quantify the loss of diversity due to the absence of random mating in each population of our study, the differences in significance may be caused because Φ_{ST} incorporates Euclidean interhaplotype distances as a component of variance and is relatively unaffected by high variation in detected haplotype diversity (Neigel, 2002; Bird *et al*., 2011). At the interpopulation level, genetic differentiation at small spatial scales is generally accepted to result from temporal variation in the genetic composition of larvae and recruits (Planes & Lenfant, 2002; González-Wangüemert *et al*., 2007; Lee & Boulding, 2007, 2009; Marino *et al*., 2010); therefore, the divergence found in *C. glaucum* could also be a response to stochastic processes linked to reproduction/recruitment and/or to selection on larval populations (Arnaud-Haond *et al*., 2008). The genetic differentiation detected among Mar Menor populations suggests either limited gene flow between some localities due to habitat discontinuity and/or extreme populations dynamics. Several studies on other marine invertebrates have associated similar findings with environmental discontinuities (Pearson, Rogers & Sheader, 2002; Jolly, Rogers & Sheader, 2003; Darling, Reitzel & Finnerty, 2004). Inside the Mar Menor lagoon there are important habitat discontinuities related to spatial distribution of sediments and submerged vegetation (Verdiell Cubedo *et al*., 2008; González-Wangüemert *et al*., 2009; Quintino *et al*., 2010) and to spatio-temporal variability in salinity and temperature of the water column (Pérez-Ruzafa *et al*., 2007). Moreover, as a result of the enlargement and deepening of El Estacio inlet in the early 1970s, the former dominant macrophyte of the Mar Menor, the seagrass *Cymodocea nodosa*, has been replaced by the macroalga *Caulerpa prolifera* that is able to alter the superficial sediments by trapping fine particles in its root system (Quintino *et al*., 2010). Furthermore, pumping and dredging of sands have accelerated the process of sandy-bottom destruction with consequent loss of environmental diversity (Pérez-Ruzafa *et al*., 2006). The combination of these natural and anthropogenic environmental perturbations creates habitats unavailable for bivalves (including *C. glaucum*) and favours extreme population dynamics and probably small-scale genetic differentiation inside the lagoon.

The parsimony network and correspondence analysis of haplotypes revealed that variation in the COI gene is not randomly distributed among locations. Several populations are differentiated by haplotype frequencies and unique mutations. The network analysis shows a tight assemblage of haplotypes, mostly separated by a small number of mutations except for the most divergent haplotypes (COI-08, COI-10 and COI-38). Some haplotypes are common while others are rare, but all interrelated in a genealogical pattern consistent with a scenario of

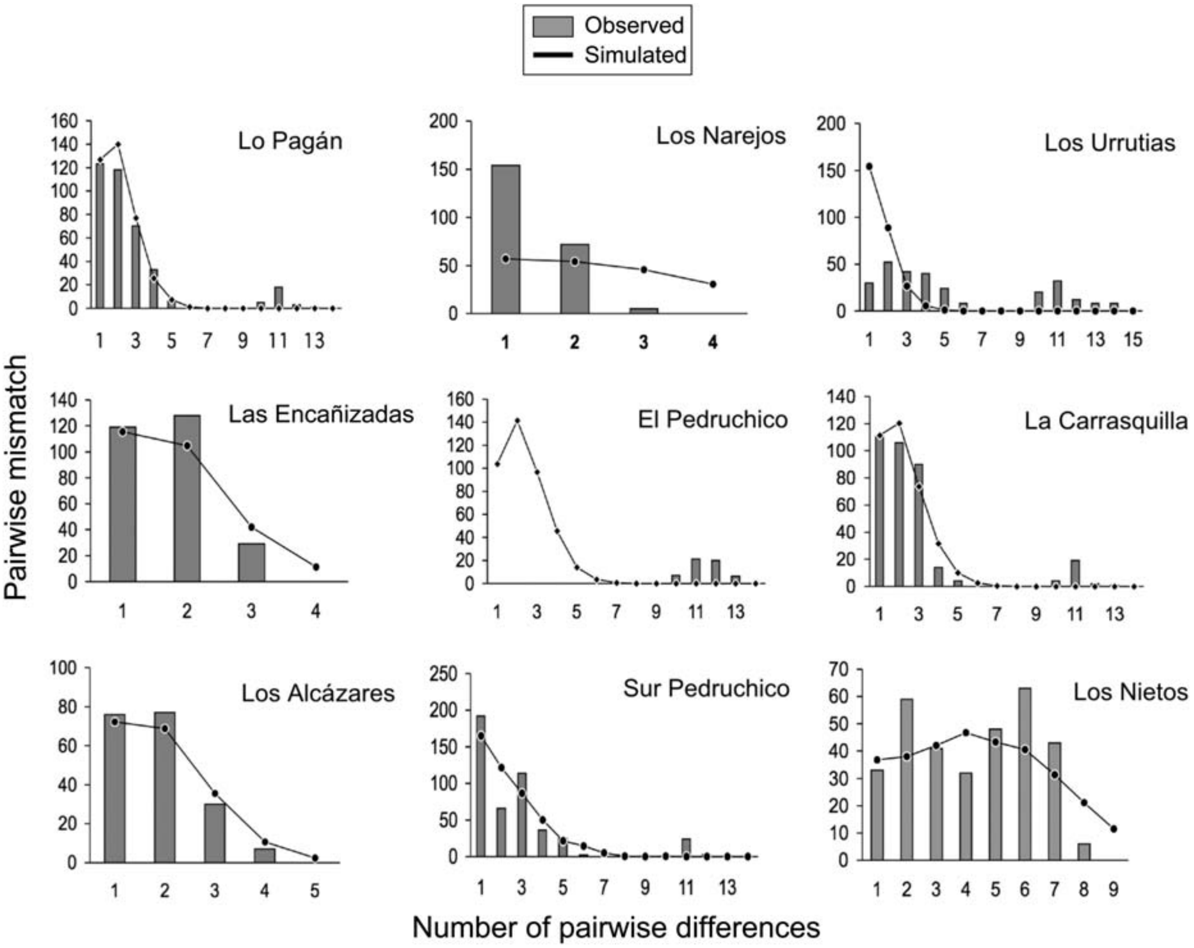

Figure 4. Pairwise mismatch distributions of haplotypes for each sampling location of *Cerastoderma glaucum* within the Mar Menor coastal lagoon (southeastern Spain) based on mtDNA COI sequences.

Table 3. Neutrality tests statistics and demographic expansion parameters of populations of *Cerastoderma glaucum* from the Mar Menor lagoon (southeastern Spain).

Locations	PE	LC	LE	LP	LA	NR	LN	LU	SP
Parameters									
Tajima's *D*	−1.653*	−1.796*	−0.405	−2.093*	−0.759	−1.471	0.739	−0.288	−1.899*
Fu's *Fs*	−4.556*	−3.055*	−0.726	−5.334*	−1.592	−2.262*	−2.469	0.099	−3.694*
τ	1.375	1.369	0.824	1.127	0.906	2.980	4.906	0.576	3.115
θ_0	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000
θ_1	99999.000	5.523	99999.000	15.638	99999.000	3.600	8.008	99999.000	1.587
SSD	0.019	0.009	0.012	0.005	0.002	0.226	0.015	0.274*	0.023

Sampled locations: El Pedruchico (PE), La Carrasquilla (LC), Las Encañizadas (LE), Lo Pagán (LP), Los Alcázares (LA), Los Nietos (LN), Los Narejos (NR), Los Urrutias (LU), South Pedruchico (SP). τ, the expansion parameter; θ_0, population size before expansion; θ_1, population size after expansion; SSD, sum of squared deviations. *Significant values of parameters ($P < 0.05$).

recent population expansion (Crandall & Templeton, 1993; Avise, 2000). The haplotype COI-03 is the most abundant and was likely present in the ancestral lineage, because there is a direct relationship between haplotype frequencies and ages of haplotypes (Posada & Crandall, 2001). The correspondence analysis suggests that the Los Nietos sample is different from all the others; this result is congruent with haplotype groups indicated by parsimony network analysis. This divergence is probably a response to local environmental stability in Los Nietos as explained above; the low wave exposure and fine sand bottoms and low silt-clay contents, together with slight variations in salinity, all promote a constant demographic size, as supported by our mismatch distribution analysis. Moreover, the relatively high genetic diversities observed at this locality might further suggest a comparatively large effective population size.

Recent evidence supports the theory that environmental factors can be important determinants of population differentiation (e.g. Cimmaruta, Bondanelli & Nascetti, 2005; Gaggiotti *et al.*, 2009; Ross *et al.*, 2012). Accordingly, salinity may be only one of the several environmental factors of the water layer surrounding marine bivalves that is causing population differentiation. Remarkably high interpopulation variation has been found between samples from closely situated locations along salinity gradients (e.g. Ridgway, 2001). For example, in the only entrance to the brackish water basin of the Baltic Sea, there is a steep salinity gradient that drops from 30 to 3, shaping the genetic structure of several populations of marine species (Väinölä & Hvilsom, 1991; Johannesson & André, 2006; Luttikhuizen *et al.*, 2012). Furthermore, it has been demonstrated by a quantitative ecological approach (generalized additive models, GAMs) using haplotype frequencies of the 16S rRNA mitochondrial gene as the dependent variable and independent environmental variables such temperature and salinity, that there is a significant relationship between abiotic parameters and genetic structure of the sea cucumber *Holothuria polii* inhabiting the Mar Menor lagoon (Vergara-Chen *et al.*, 2010a).

Previous studies of genetic structure of different species at a small spatial scale inside the Mar Menor lagoon (*C. glaucum* with allozymes; *Holothuria polii* with mtDNA markers; *Diplodus sargus* with microsatellites and mtDNA markers) allowed us to use GAM analysis to demonstrate a significant link between genetic structure and environmental variables such as salinity, temperature and granulometry (González-Wangüemert *et al.*, 2009; Vergara-Chen *et al.*, 2010a; González-Wangüemert & Pérez-Ruzafa, 2012). These GAMs were developed for the first principal component of the correspondence analysis (CA), which usually explains the higher values of variance. We used the coordinates of each locality derived from this analysis to include them in GAMs. However, component I of the CA carried out on the haplotype frequencies of *C. glaucum* only explained 34% of the total variance and this value was not enough to apply the GAMs. Nevertheless, it has been demonstrated by several studies (González-Wangüemert *et al.*, 2009; Knutsen *et al.*, 2009; Ciannelli *et al.*, 2010; Vergara-chen *et al.*, 2010a) that extreme environmental variables can affect the genetic structure and diversity of marine species. Therefore, environmental variables could be shaping the genetic structure and diversity observed in *C. glaucum*. This is also suggested by the results about the existence of selection. The *Z*-test indicated significant evidence of purifying selection on *C. glaucum* and HyPhy software detected significant positive selection over seven codons. These nonsynonymous mutations can directly affect protein function, so are more likely to influence fitness than synonymous mutations that leave the amino acid sequence unchanged (Kosakovsky Pond *et al.*, 2005).

Concerning the unstable population dynamics, the patterns obtained from the number of pairwise differences (Fig. 4) indicate a constant population size only at Los Nietos, while the other populations showed a pattern linked to bottlenecks and sudden population expansion (according Patarnello *et al.*, 2007). Therefore, we can state that most of the populations (except for Los Nietos and Los Urrutias) exhibit unstable population dynamics marked by bottlenecks and expansion. Such events can affect genetic diversity and structure as demonstrated in other species (Luttikhuizen *et al.*, 2003; Saavedra & Peña, 2005; Coykendall *et al.*, 2011; Santos *et al.*, 2012). However, further studies using more molecular markers, GAMs linking genetic and environmental variables, and a genomics approach, will be needed to validate this hypothesis on *C. glaucum* in the Mar Menor coastal lagoon.

In conclusion, the observed genetic differences among localities at a small scale (1–10 km) could be promoted by discontinuous gene flow at least between Los Nietos and some other localities and by extreme population dynamics in response to wide environmental variability related to abiotic and biotic factors inside the lagoon.

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