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ORIGINAL RESEARCH

Genetic diversity and connectivity remain high in *Holothuria polii* (Delle Chiaje 1823) across a coastal lagoon-open sea environmental gradient

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Abstract Coastal lagoons represent habitats with widely heterogeneous environmental conditions, particularly as regards salinity and temperature, which fluctuate in both space and time. These characteristics suggest that physical and ecological factors could contribute to the genetic divergence among populations occurring in coastal lagoon and open-coast environments. This study investigates the genetic structure of *Holothuria polii* at a micro-geographic scale across the Mar Menor coastal lagoon and nearby marine areas, estimating the mitochondrial DNA variation in two gene fragments, cytochrome oxidase I (COI) and 16S rRNA (16S). Dataset of mitochondrial sequences was also used to test the influence of environmental differences between coastal lagoon and marine waters on population genetic structure. All sampled locations exhibited high levels of haplotype diversity and low values of nucleotide diversity. Both genes showed contrasting signals of genetic differentiation (non-significant differences using COI and slight differences using 16S, which could due to different mutation rates or to differential number of exclusive haplotypes. We detected an excess of recent mutations and exclusive haplotypes, which can be generated as a result of population growth. However, selective processes can be also acting on the gene markers used; highly significant generalized additive models have been obtained considering genetic data from 16S gene and independent variables such as temperature and salinity.

Keywords Coastal lagoon · Gene flow · Genetic diversity · *Holothuria polii* · Mitochondrial DNA · Selection · Generalized additive models (GAMs)

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Introduction

Coastal lagoons are habitats characterized by the variability of their physical and chemical parameters, especially salinity and temperature (Cognetti and Maltagliati 2000; Gamito et al. 2005). Therefore, they have been considered as physically stressed environments *sensu* Sanders (1968) (Gamito et al. 2005; Pérez-Ruzafa et al. 2007). Coastal lagoons constitute separate environments from the open sea by geographic and ecological barriers, which could promote the population genetic differentiation. In fact, there are several studies at small-geographic scales, which have established significant genetic differences between populations from coastal lagoons and open sea (Allegruci et al. 1997; Michinina and Rebordinos 1997; Cognetti and Maltagliati 2000; Camilli et al. 2001; González-Wangüemert et al. 2006; Bisol et al. 2007; Marko and Barr 2007; González-Wangüemert et al. 2009). Therefore, coastal lagoons will be a major focus of interest with regard to their potential influence on the genetic structure of marine populations which can stay alive into these lagoons during all or a part of its life cycle.

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In general, echinoderms are considered species with low adaptation to salinity changes (Lawrence 1987, 1990), however some species are able to survive into tidal ponds, estuaries or coastal lagoons. Despite this interesting ecological feature of echinoderms, there are no works published about echinoderm population genetics under such conditions. The sea cucumber *Holothuria polii* (Delle Chiaje 1823) is a typical marine species whose Mar Menor populations have been able to adapt to this coastal lagoon environment with high salinity and temperature fluctuations. This context suggests that *H. polii* could be a good biological model to contribute to the general issue of microevolution across an environmental gradient from coastal lagoon to open sea: one could expect to find that the low ability to tolerate changes in salinity and temperature inside the lagoon might be offset with populations showing high genetic diversity and new haplotypes.

In the present study, two mitochondrial DNA markers were used to examine genetic structure of *H. polii* at a micro-geographic scale across the Mar Menor coastal lagoon and nearby marine areas. Our substantial data set of mitochondrial sequences was also used to test the influence of environmental differences between coastal lagoon and marine waters on population genetic structure, and determinate if gene flow is enough to preclude genetic differentiation of these areas.

Materials and methods

Study sites

The Mar Menor with a surface about 135 km^2 is one of the largest coastal lagoons in Europe and the Mediterranean Basin (Fig 1). It has a coastal environment characterized by high salinity (38–51 psu) and severe temperature changes (from >30°C in summer to 10°C in winter, Pérez-Ruzafa et al. 2005). It is relatively shallow with an average depth of 3.5 m and a maximum depth of 6 m. The lagoon is bounded on the seaward side by the La Manga sand bar of 22 km length, which is crossed by three inlets (El Estacio, Las Encañizadas and Marchamalo) that regulate water exchange. Southwest Mediterranean Sea is characterized by lower extreme values of temperature and salinity than Mar Menor coastal lagoon, oscillating its salinity between 36.84 and 37.41 psu and the temperature between 13.83 and 26.44°C (information available in http://www.noaa.gov).

Three sampling sites were selected inside the Mar Menor coastal lagoon: El Estacio, near the largest inlet of this lagoon, Los Urrutias located on the western shore of the lagoon, and Isla del Ciervo, on the southern side. Two sampling locations were selected in the Mediterranean Sea: Torre de la Horadada (Alicante) and Cabo de Palos (Murcia) located to the North and South of the Mar Menor, respectively (Fig. 1).

Field sampling procedures

Individuals of *Holothuria polii* were collected from shallow benthic habitats by snorkeling in August 2007. The sample size consisted of 26–39 individuals per collecting site. The sea cucumbers were identified a priori on the basis of external characters, transported on ice to the laboratory, relaxed by cooling them to temperatures close to freezing and dissected. Tissue samples of muscle were removed from each specimen and preserved in 100% ethanol. Species identification was confirmed in the laboratory on the basis of criteria from Koehler (1921), Thandar (1988).

DNA extraction, PCR and sequencing

The tissue was dissolved using lysis buffer solution with proteinase K, and total genomic DNA was isolated by protein precipitation and final precipitation with ethanol (Sambrook et al. 1989). A fragment of 484-bp of the cytochrome oxidase I gene was amplified by PCR using primers COeI-F (5′-ATAATGATAGGAGGRTTTGG-3′) and COeI-R (5′-GCTCGTGTRTCTACRTCCAT-3′) (Arndt et al. 1996). In addition, a fragment of 449-bp, which includes a portion of the 16S rRNA gene was PCR amplified using primers 16Sar-L (5′-CGCCTGTTTAT CAAAAACAT-3′) and 16Sbr-H (5′-CCGGTCTGAACT CAGATCACGT-3′) (Palumbi et al. 1991). In both genes, double-stranded DNA was PCR synthesized in 25 µl reactions contained 2.5 µl amplification buffer (10X), 2.5 µl of each of the above primers (10 mM), 1.0 µl $MgCl_2$ (50 mM), 0.2 µl of dNTP mix (25 mM) and 1 U Taq polymerase (Ecogen), 1 µl DNA diluted 1: 400 and purified water to complete the final volume. Amplification proceeded with an initial denaturation at 95°C for 3 min, then 40 cycles of denaturation at 94°C for 20 s, annealing to 45°C for 20 s, and extension at 72°C for 20 s followed by a final extension at 72°C for 10 min. A 5-µl sample of each PCR product was run on 2% agarose gel and stained with ethidium bromide. 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 Secugen, S.L. (Madrid, Spain) and the Servicio de Biología Molecular, Universidad de Murcia (Murcia, Spain), using an ABI Prism 3130 automated genetic analyzer (Applied Biosystems).

Data analysis

We analyzed both COI and 16S rRNA gene fragments as independent genetic markers because current evidence

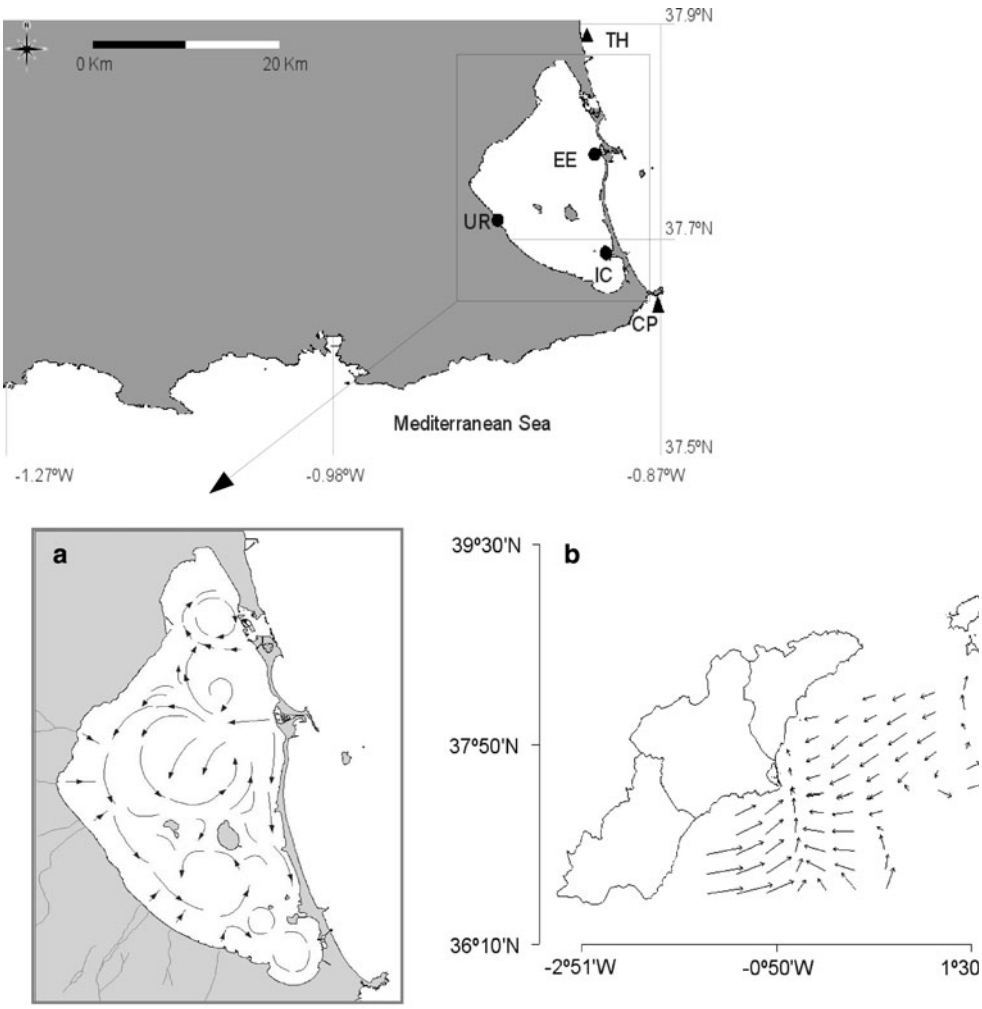


Fig. 1 Study Area. Marine and lagoon localities of *Holothuria polii* sampled for this study. Inside the Mar Menor coastal lagoon, El Estacio (EE), Los Urrutias (UR) and Isla del Ciervo (IC), seaside localities, Torre de la Horadada (TH) and Cabo de Palos (CP). **a** Water circulation pattern in the Mar Menor; **b** Water circulation pattern in Southwest Mediterranean obtained from NOAA data (August month)

suggests that several invertebrate species show mitochondrial DNA recombination (Rokas et al. 2003; Tsaousis et al. 2005). Also, mitochondrial COI gene is a protein-coding region and 16S rRNA gene is non-protein-coding region, with different mutation rates. The sequences were aligned using the BioEdit software (Hall 1999). Genetic diversity within samples was estimated from haplotype (h) and nucleotide (π) diversities (Nei 1987) using DnaSP software (Rozas et al. 2003). For other genetic analyses (F_{ST}, AMOVA, mismatch distribution, Tajima's D and Fu's Fs indices), ARLEQUIN version 2000 (Schneider et al. 2000) was used. The genetic differentiation between pairs of samples was evaluated by the rate of fixation (F_{ST}), while the significance of F_{ST} values was tested using 10,000 random permutations (Weir and Cockerham 1984). The distribution of variation within and between samples was inferred using an analysis of molecular variance (AMOVA; Excoffier et al. 1992) considering two groups: coastal lagoon (El Estacio, Isla del Ciervo and Los Urrutias) and Mediterranean Sea (Cabo de Palos and Torre de la Horadada). Haplotype networks were estimated using the TCS software (Clement et al. 2000), which implements the method of statistical parsimony of Templeton et al. (1992).

Neutrality tests and mismatch distribution analyses were carried out to infer population expansion events and to test the deviations from a strictly neutral model of evolution. We performed the neutrality test of Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997). The significance of Fu's F_s and Tajima's D were tested by random permutation using 1,000 replicates. The observed mismatch distributions were compared with the simulated distributions under the model of demographic expansion. The model assumes that non-subdivided populations suddenly expand in population size and increase the total number of individuals (Rogers and Harpending 1992). To estimate the approximate time of expansion for *H. polii*, the formula $\tau = 2\ \mu t$ was used. According to Rogers and 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 entire region under study, which is the mutation rate per nucleotide multiplied by the number of nucleotides of the analyzed fragment.

Mutation rate of 0.5% per nucleotide per million years was used for 16S, and a rate of 1.5% for COI, both calculated for echinoids (Lessios et al. 2001; Chenuil and Féral 2003).

To test the possible relationships between environmental variables and genetic structure, we developed generalized additive models (GAMs) for the first principal component of the correspondence analysis (CA). Populations were spatially clustered using CA, which utilized the haplotype frequencies of populations as the variables. CA summarizes all the variation in the study area and accommodates each population as a study unit (Manel et al. 2003; González-Wangüemert et al. 2007; González-Wangüemert et al. 2010) We used the coordinates of each locality derived of this analysis to include them in generalized additive models (GAMs). GAMs are a non-parametric extension of generalized linear models (GLM) that fit a wide variety of forms of stochastic variation in the response. GAMs represent the relationship between the response variable and the predictors by smooth functions, which can take virtually any form (Hastie and Tibshirani 1990). These models have already applied on genetic and environmental data (Snäll et al. 2004; Parisod and Bonvin 2008; González-Wangüemert et al. 2009). The GAM was evaluated by examining the proportion of explained deviance and minimizing the generalized cross validation (GCV; Wood 2000; Wood and Augustin 2002) and the Akaike information criterion (AIC; Venables and Ripley 2004) scores. As independent variables we used maximum, minimum and mean values of temperature and salinity (Data from: http://www.noaa.gov; Research Group "Ecología y Ordenación de Ecosistemas Marinos Costeros"), which are expected to influence genetic structuring. CA analyses and GAM models were performed using "ade4" (Chessel 1992) and "mgcv" (Wood 2006) packages from R statistical software (R Development Core Team 2007).

Results

Sequence analysis

Good-quality sequences were obtained for cytochrome oxidase I (COI) and 16S rRNA (16S) mitochondrial genes. A total 484-bp of COI sequences were obtained for 158 individuals and 449-bp of the 16S sequences were resolved for 151 individuals of *Holothuria polii* from the five geographical locations studied. An alignment of all COI and 16S haplotypes obtained was deposited at GenBank (16S haplotype accession numbers: from EU750754 to EU750792, and COI haplotype accession numbers: from EU750793 to EU750824).

The COI sequences were characterized by low nucleotide and high haplotype diversity; the values being higher than those observed in the 16S dataset. Overall, 32 different haplotypes were detected and polymorphisms were observed at 35 of the 484-bp (7.23%) sequenced. The Isla del Ciervo sample showed the highest number of haplotypes (15) and haplotype diversity (0.8992) compared with the other four samples. The Torre de la Horadada location had the lowest number of haplotypes (9) (Table 1). On the other hand, the 16S sequences yielded 39 different haplotypes and 41 polymorphic sites of the 449-bp (9.26%). The highest haplotype diversity was found in Torre de la Horadada (0.8623) and the lowest in Los Urrutias (0.7034) (Table 1). We did not find significant differences among any of these localities.

Table 1 Molecular diversity indices for populations of *Holothuria polii* using 449-bp of 16S rRNA and 484-bp of COI based on Kimura's (1980) 2-parameters substitution model

Locations	Sample size	Haplotypes	Polymorphic sites	Haplotype diversity	Nucleotide diversity
16S					
El Estacio (lagoon)	27	13	16	0.8034 ± 0.0771	0.0053 ± 0.0033
Los Urrutias (lagoon)	30	10	18	0.7034 ± 0.0855	0.0036 ± 0.0025
Isla del Ciervo (lagoon)	26	11	19	0.7846 ± 0.0806	0.0049 ± 0.0031
Cabo de Palos (marine)	29	7	9	0.7670 ± 0.0511	0.0039 ± 0.0026
Torre de la Horadada (marine)	39	15	17	0.8623 ± 0.0327	0.0063 ± 0.0036
Total	151	39	41	0.7841 ± 0.0654	0.0048 ± 0.0030
COI					
El Estacio (lagoon)	32	13	21	0.8831 ± 0.0351	0.0056 ± 0.0034
Los Urrutias (lagoon)	31	11	16	0.8495 ± 0.0390	0.0046 ± 0.0029
Isla del Ciervo (lagoon)	32	15	17	0.8992 ± 0.0379	0.0048 ± 0.0030
Cabo de Palos (marine)	33	12	18	0.8731 ± 0.0363	0.0057 ± 0.0034
Torre de la Horadada (marine)	30	9	9	0.8621 ± 0.0317	0.0042 ± 0.0027
Total	158	32	35	0.8734 ± 0.0360	0.0050 ± 0.0031

Population genetic structure

The exact test of population differentiation based on haplotype frequencies and the F_{ST} values using COI haplotypes pointed to no significant differences $P = 0.2438$ and $P > 0.05$ respectively, between coastal lagoon (El Estacio, Los Urrutias and Isla del Ciervo) and marine samples (Cabo de Palos and Torre de la Horadada). Significant differences among marine locations were not detected either (Table 2). However, using 16S data for exact test ($P = 0.000$) and F_{ST} ($P < 0.05$) pointed to significant differences between the coastal lagoon and marine samples (Table 2). These significant differences could be influenced by the presence of a higher number of exclusive haplotypes in coastal lagoon samples. The F_{ST} values for 16S data between coastal lagoon samples and Torre de la Horadada were higher than those between the Mar Menor samples and Cabo de Palos, which could indicate a prevailing gene flow between Cabo de Palos and Mar Menor such as the current pattern indicates (Fig 1b).

The analysis of molecular variance (AMOVA) using COI did not reveal significant differences among groups (coastal lagoon and marine samples) or among populations within groups. Similar results were obtained from 16S gene data: the highest proportion of the total variance was attributed to differences within sampled locations (95.02%; $P = 0.0059$) and although 5% of the variation was attributed to differences among groups (marine-coastal lagoon), this value was not significant ($P = 0.1144$) (Table 3).

Haplotype network analysis

The statistical parsimony procedure of the COI data (Fig. 2) showed several ambiguous connections and pointed to four haplotypes shared between all sampling locations (COI-2, COI-5, COI-7 and COI-10). The haplotype COI-11 was present in three locations (El Estacio, Torre de la Horadada and Cabo de Palos). This haplotype could be a marine haplotype that is retained in the inlet mouth (El Estacio) but has not been introduced into the coastal lagoon. Samples showed some exclusive haplotypes: El Estacio (5), Los Urrutias (3), Isla del Ciervo (6), Cabo de Palos (5) and Torre de la Horadada (2). Seventeen (17) exclusive haplotypes characterized the coastal lagoon samples, while, Mediterranean samples (Cabo de Palos and Torre de la Horadada) showed seven (7) exclusive haplotypes. The haplotypes COI-3, COI-11, COI-15 and COI-17 were present in both Mar Menor and Mediterranean samples.

Table 2 Pairwise fixation indices between four *Holothuria polii* populations based on 16S rRNA sequences (F_{ST}, below diagonal) and on COI sequences (F_{ST}, above diagonal)

	El Estacio	Los Urrutias	Isla del Ciervo	Cabo de Palos	Torre de la Horadada
El Estacio (lagoon)		0.0212	−0.0017	−0.0079	0.0110
Los Urrutias (lagoon)	−0.0035		−0.0097	0.0033	−0.0177
Isla del Ciervo (lagoon)	−0.0098	−0.0108		0.0024	−0.0017
Cabo de Palos (marine)	0.0430*	0.0446*	0.0449*		−0.0081
Torre de la Horadada (marine)	0.0552*	0.0741*	0.0659*	0.0207	

P is the probability that any random value obtained after 1,000 permutations is > observed value

* Significant F_{ST} values ($P < 0.05$)

Table 3 Analysis of molecular variance (AMOVA) among *Holothuria polii* 16S rRNA and COI haplotypes based on Φ_{ST}

Molecular marker	Hierarchical level	% Variation	Fixation indices	*P*
16S rRNA	Among groups	5.00	$F_{CT} = 0.0499$	0.1144 ns
	Among populations within groups	−0.02	$F_{SC} = 0.0002$	ns[a]
	Within populations	95.02	$F_{ST} = 0.0498$	0.0059*
COI	Among groups	−0.05	$F_{C\ T} = -0.0000$	0.3851 ns
	Among populations within groups	−0.04	$F_{SC} = -0.0004$	0.4457 ns
	Within populations	100.9	$F_{ST} = -0.0093$	0.4946 ns

ns Non-significant

* Significant values ($P < 0.05$)

[a] *P* (random value > observed value) = 0.0156

P (random value = observed value) = 0.0000

P (random value ≥ observed value) = 0.0156 ± 0.0037

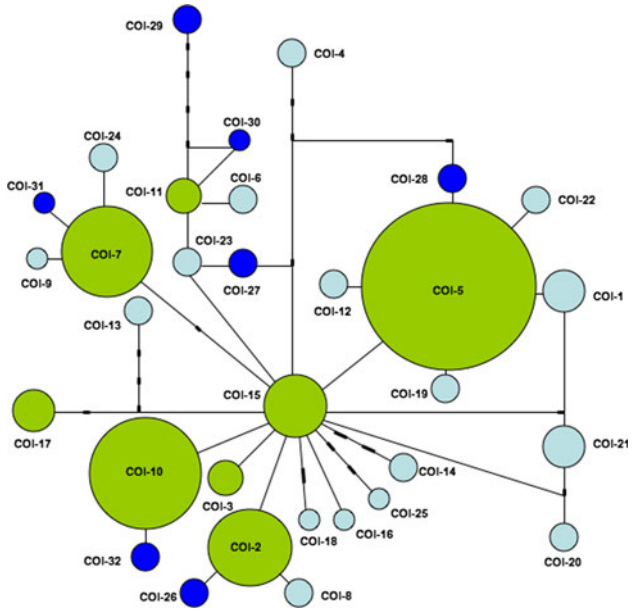
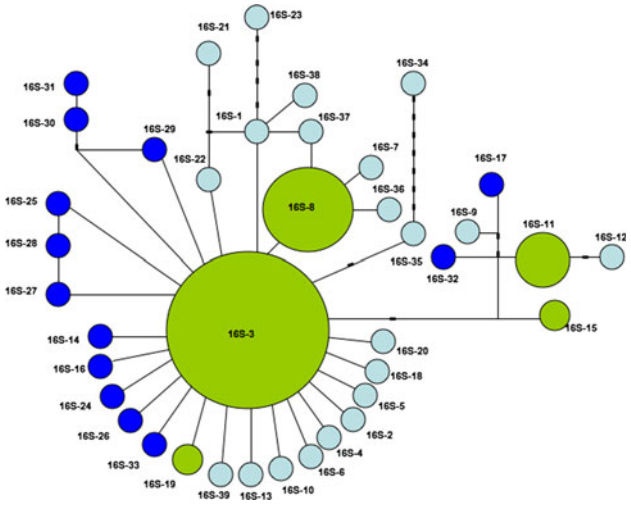


Fig. 2 Statistical parsimony network based on COI sequence haplotypes of *Holothuria polii*. Each haplotype is defined by its corresponding number. The area of each *circle* is proportional to the number of individuals. *Gray circles*: shared haplotypes; *black circles*: marine haplotypes; *light gray circles*: lagoon haplotypes. *Solid bars* indicate mutational changes



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The haplotype network obtained for 16S rRNA data (Fig. 3) revealed that the most frequent haplotype (16S-3) had a central position in the network and the remaining ones were, in general, closely connected to the common haplotypes, showing a star phylogeny. The more distinct haplotype (16S-34) differed by eight (8) mutational steps. Rare variants (16S-34, 16S-23 and 16S-21), which represent more recent mutations according to Posada and Crandall (2001), belong to Isla del Ciervo and Los Urrutias localities. Two 16S haplotypes (16S-3 and 16S-8) were shared by all the samples. All five locations showed exclusive haplotypes: El Estacio (7), Los Urrutias (4), Cabo de Palos (3), Torre de la Horadada (10), and Isla del Ciervo (6). Twenty-one exclusive haplotypes were only recorded in coastal lagoon samples, while the Mediterranean samples exhibited 13 marine haplotypes, which were not present in the Mar Menor. The remaining haplotypes were present in both Mar Menor and Mediterranean samples.

Mismatch distributions

Given the absence of significant population differentiation, we pooled all COI sequences in one group and the 16S sequences into two groups, coastal lagoon (83 sequences) and marine (68 sequences), to construct mismatch distribution diagrams (Fig. 4). The parameters of the mismatch distribution for each gene showed similar patterns of population distribution supporting one type of mismatch distribution according Patarnello et al. (2007). The 16S and COI data showed a skewed unimodal distribution related to recent bottlenecks or sudden population expansion. The parameters and the goodness-of-fit tests of the model of sudden expansion are given Table 4. Mismatch distributions were significantly different from the sudden expansion model for the coastal lagoon group (16S data), but the SSD could no reject the expansion hypothesis based on the 16S data (marine group). The 16S and COI dataset exhibited negative and significant *D* (Tajima 1989) and *Fs* (Fu 1997) values corroborating the population expansion.

We estimated an approximate expansion time around 62,259–291,322 years ago considering the minimum and maximum values from τ (COI). For the 16S gene, assuming only one demographic event, the expansion would have taken place around 93,388–436,983 years ago.

Generalized additive models (GAMs)

When the data from the 16S gene are considered as dependent variables, a linear response was observed to mean salinity and a non-linear response to mean salinity and mean temperature. The deviance explained by the GAMs was high ($Dev_{salinity} = 89.4\%$, Pr salinity $= 0.015$ and $Dev_{salinity+temperature} = 99.9\%$, Pr salinity $= 0.0011$, Pr temperature $= 0.004$). The GCV and AIC scores were significant in both cases ($GVC_{salinity} = 0.289$, $AIC_{salinity} = -8.886$ and $GVC_{salinity+temperature} = 0.005$, $AIC_{salinity+temperature} = -13.710$). Non significant model was obtained using COI data and environmental variables.

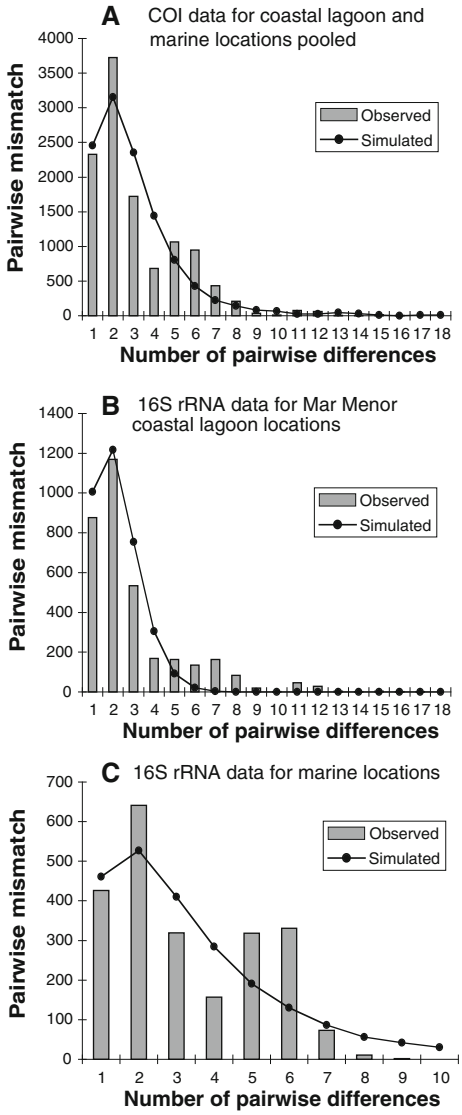

Fig. 4 Pairwise mismatch distributions of haplotypes of *Holothuria polii* for Mar Menor and Mediterranean Sea. **a** COI data for coastal lagoon and marine locations pooled; **b** 16S rRNA data for Mar Menor coastal lagoon locations; C: 16S rRNA data for marine locations

Discussion

High levels of haplotype diversity and low levels of nucleotide diversity characterized the sea cucumber local samples. The values of nucleotide diversity within sampled locations were comparable to those observed in others echinoderms (Uthicke and Benzie 2003; Durán et al. 2004; Iuri et al. 2007; Calderón et al. 2008). This pattern of genetic diversity could be promoting occurrence of exclusive haplotypes in *H. polii* that has been frequently attributed to expansion after a period of low effective population size, retaining the new mutations (Grant and Bowen 1998; Avise 2000).

This study shows non-significant genetic differentiation between samples of *H. polii* collected at the micro-geographic scale of the Mar Menor coastal lagoon and adjacent marine locations (COI and 16S for AMOVA; COI for F_{ST} values and exact test). According to our results, we suggested that low population differentiation between coastal lagoon and marine samples of *H. polii* is consequence of unrestricted gene flow that are shaping the population genetic composition of this species at shorter spatial scale analyzed.

Several studies on tropical Western Pacific holothurians reported some significant F_{ST} values as a consequence of restrictions in gene flow among populations (Uthicke and Benzie 2001, 2003; Uthicke and Purcell 2004). Considering the low genetic structure between Mar Menor lagoon and neighboring marine samples, gene flow, as estimated by F_{ST} values, does not seem to be limited. Uthicke and Benzie (2001) studied genetic structure of *Holothuria scabra* at eight localities in Queensland coast, Torres Strait and the Solomon Islands. The two locations from Solomon Islands are separated by about 16 km, one associated to lagoon system, which is nearly closed to the ocean, whereas the other location is open towards the ocean. Lagoon population of *H. scabra* is genetically isolated from others populations and the open coastal population may have nearly unrestricted gene flow with most geographically distant populations, showing no significant genetic distance with samples located about 1,500-km away. Gene flow barriers at scale micro-geographic maybe explain genetic differentiation in *H. scabra* between both locations (Uthicke and Benzie 2001). Notwithstanding, we suggest that an ongoing gene exchange in *H. polii* samples across lagoon and open sea could prevent the genetic differentiation but increase genetic variation and thus enhance the adaptive potential to lagoon environmental fluctuations.

In general, marine invertebrates with high dispersal capabilities and life histories that include pelagic phases and large population sizes are expected to show high levels of gene flow and a low population genetic structure over small spatial scales (Ball and Chapman 2003; Garoia et al. 2004; Stamatis et al. 2004). Holothurians have pelagic larvae with duration of about 13–26 days (Asha and Muthiah 2002; Hamel et al. 2003; Ivy and Giraspy 2006), so that they probably have high genetic connectivity, such as our results have demonstrated. In fact, the COI data not provided significant evidence of population differentiation (F_{ST} values, exact test and AMOVA) and the 16S rRNA data only indicated a moderate level of differentiation between coastal lagoon and marine sites (F_{ST} and exact test) and non-significant genetic discrimination using analysis of molecular variance. Nevertheless, the lack of significance in the AMOVA results could be probably due to low power associate with a low number of populations

Table 4 Mismatch distribution for *Holothuria polii* populations

	All COI samples pooled	16S lagoon samples	16S marine samples
Sudden expansion parameters			
S	57	36	19
θ_0	0.000	0.000	2.184
θ_1	37.266	95.625	452.812
τ	2.747	1.234	0.472
Goodness-of-fit test			
SSD	0.015	0.012	0.019
P	0.000*	0.016*	0.304 (ns)
Tajima's *D* test	−2.245	−2.337	−1.569
P	0.003*	0.002*	0.049*
Fu's *Fs* test	−22.553	−18.622	−7.909
P	0.000*	0.000*	0.000*

Sudden expansion model parameters and goodness-of-fitness test to the expansion model with the respective significance. The analysis was conducted with all samples pooled for COI, and with lagoon and open sea samples separated for 16S

S number of polymorphic sites, θ_0 population size before expansion, θ_1 population size after expansion, τ the expansion parameter, *SSD* sum of squared deviations

* Significant values ($P < 0.05$)

Non-significant values (ns)

per group (Fitzpatrick 2009). Small differences observed in the patterns inferred from these two genes may be due to differences in mutation rates [1.6–3.5% for COI (Lessios et al. 1999; McCartney et al. 2000 in sea urchins), and 0.5% for 16S rRNA (Chenuil and Féral 2003; Calderón et al. 2008)]. A similar pattern has been recorded to *Paracentrotus lividus* from the Mediterranean Sea and Atlantic Ocean (Calderón et al. 2008): 16s rRNA provided a stronger signal of population differentiation between both areas than that observed for COI by Durán et al. (2004) at a similar geographic scale. Differences obtained from the two genes were also explained by mutation rates and ultimately by their functional constraints (Calderón et al. 2008).

On the other hand, we cannot rule out selection as an explanation for these observed differences. Selection alone on the mitochondrial genome caused by environmental constraints in the lagoon and marine habitats, would require strong forces that could be easily recognized over both habitat types. The differences in water salinity and temperature between coastal lagoon and marine environments are developed abruptly (such as it is showed in Mar Menor and Mediterranean Sea), and these differences are higher than environmental fluctuations experienced by marine invertebrates in the open sea (González-Wangüemert et al. 2006; Véliz et al. 2006; Andrade and Solferini 2007). These features could have an influence on genetic structure; in fact we have developed highly significant generalized additive models (GAMs) considering genetic data from 16S gene and independent variables such as temperature and salinity. Selection hypothesis is not contradictory with the important gene flow detected such as it is described by Beheregaray and Sunnucks (2001), who suggest that the 'divergence-with-gene-flow' model of speciation may account for the diversification of estuarine populations.

We also detected an excess of recent mutations and exclusive haplotypes in both genes which can be generated as a result a population growth such as was described by Avise et al. (1984), Ramos-Onsins and Rozas (2002). However, selective processes can be also acting on the gene markers used, causing the occurrence of exclusive haplotypes. Given the importance of mitochondrial function (mtDNA is responsible for high percentage of the energy metabolism), changes in the mtDNA sequence may have a strong impact on the fitness of the organelle (within individuals) and on the fitness of the individual. Ballard and Whitlock (2004) consider that the mitochondria may be influenced first by a strong direct selection and second by indirect effects of selection on other parts of the genome. Selection can change allele frequency even at a locus not responsible for fitness differences, such as 16S. Also, the effect of selection on nuclear genes could potentially influence mtDNA haplotype frequencies in populations, because mitochondrial function depends on the coordinated expression of genes encoded in the nucleus and the mitochondria (Ballard and Whitlock 2004).

In protein-coding DNA, most amino-acid-changing mutations are under negative selection (Li 1997), as shown by their substantially reduced substitution rates compared

with synonymous mutations. A small subset of genes, including immune response and sex-related genes, show enhanced rates of non-synonymous substitutions (Haerty et al. 2007), which indicate predominantly positive selection for change. Statistically more sensitive population-genetic tests based on substitutions and polymorphisms provide evidence for amino acid changes under positive selection in most genes (McDonald and Kreitman 1991; Larracuente et al. 2008). At the same time, the functionality of non-coding DNA and the forces shaping its evolution are less clear. Regulatory elements encode biological information in a more fuzzy way than proteins. This can lead to considerable sequence divergence while the regulatory function is maintained, which makes adaptive evolution hard to detect. However, there is evidence for genome-wide positive selection of moderate strength in non-coding DNA (Kohn et al. 2004; Andolfato 2005; Mustonen and Lässig 2005), and complementary methods have identified selective sweeps under strong positive selection (Schlötterer 2003; Glinka et al 2003; Macpherson et al. 2007; Teschke et al. 2008). A sweep is the rapid fixation of a selected mutation, which also reduces the polymorphism of linked polymorphic loci in its neighborhood and, hence, becomes detectable by a contiguous interval of reduced diversity in the genome.

Tests which examine the neutral equilibrium model for mitochondria, Tajima's D (Tajima 1989), Fu and Li's D* (Fu and Li 1993) and Fu's Fs (Fu 1997), have been used to test the deviations from a strictly neutral model of evolution (Nachman 1998; Rand 2001), because the rejection of the null hypothesis probably means that selection and/or population level processes (expansion, contraction, etc) are operating on the region of interest. The Fu's Fs (Fu 1997) test is based on the haplotype distribution in the sample, specifically in the Ewens sample distribution (Ewens 1972), which takes into account the different haplotypes number in the sample (Fu 1997). Fs negative values are expected if there is an excess of mutations with low frequency (i.e. exclusive haplotypes detected in coastal lagoon samples), turning into an efficient test to detect positive selection or expansion population. Using Tajima's D and Fu's Fs tests (Table 4) we have mainly detected deviations from the neutral model in coastal lagoon samples. However, it is very difficult to distinguish between the contributions of natural selection and demographic history, because variation patterns of neutral DNA sequence closely associated with a site that has undergone a recent adaptive substitution or "selective sweep" are similar to those in an expanding population.

The COI network showed a tight assemblage of haplotypes, mainly separated by a small number of mutations, except for three haplotypes (COI-29, COI-13 and COI-4) from Cabo de Palos and El Estacio localities. Some haplotypes shared important features (they are abundant and have given arise a large number of related and rare haplotypes), a genealogical pattern consistent with a scenario of recent population expansion (Crandall and Templeton 1993; Avise 2000). The COI-15 haplotype would be present in the ancestral lineage and could have given rise to the majority of marine and lagoon maternal lineages (TCS data). A similar pattern was detected in *Holothuria nobilis*, which oldest and most abundant haplotypes (COI-22 and COI-4) were the origin of others (Uthicke and Benzie 2003). The 16S network showed a star phylogeny that corroborated the population expansion. The 16S-3 haplotype is the most abundant so it could belong to the ancestral lineage because there is a direct relationship between haplotype frequencies and ages of the haplotypes (Posada and Crandall 2001), although it is important to stress that founder events and selection can drive new haplotypes to high frequencies. This pattern of few frequent haplotypes and many low-frequency haplotypes with few differences has already been observed in other marine invertebrates (Durán et al. 2004; Lejeusne and Chevaldonné 2006).

Genetic homogeneity observed could result from high levels of gene flow that can reduce inbreeding depression and damp the genetic drift promoted by extreme environmental variability of the lagoon at the micro-geographic scale studied. Therefore, it is acceptable to assume that gene flow, may increase the genetic variation and the adaptive potential to changing conditions (Garant et al. 2007). Our results agree with this assumption indicating high values of genetic diversity in coastal lagoon and marine samples. The moderate differentiation level observed in 16S gene fragment between coastal lagoon and marine samples could be a response to selection on larval populations or stochastic processes linked to reproduction and recruitment (Arnaued-Harond et al. 2008). In the case of natural selection, different selective histories of the larval pools might explain differences in the genetic composition of recruits (Planes and Lenfant 2002). The differential selection favoring genetic differentiation is expected to produce clines in genetic polymorphisms correlated with varying environmental conditions such salinity and temperature (González-Wangüemert et al. 2009; Marino et al. 2010). Based on this hypothesis, the larval pool of *H. polii* could be genetically homogeneous in the open sea off the Mar Menor Lagoon, and then selective forces acting during the recruitment inside the lagoon, might produce genetic differences among local samples.

Several similar cases for marine invertebrates (e.g. Véliz et al. 2006; Andrade and Solferini 2007; Untersee and Pechenik 2007; Iannotta et al. 2009; Marino et al. 2010; Ragioneri et al. 2010) suggest the possibility that local populations are able to adapt to heterogeneous environments due to continuous gene flow. In this scenario, *Holothuria*

polii from Mar Menor coastal lagoon shows high values of genetic diversity and presence of exclusive haplotypes, despite that the lagoon environment is very unstable and susceptible to demographic fluctuations. Hence, the population genetic variability promoted by gene flow could increase the sea cucumber potential to adapt to changing conditions of this lagoon environment.

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