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Phylogeography of the Atlanto-Mediterranean sea cucumber *Holothuria (Holothuria) mammata:* the combined effects of historical processes and current oceanographical pattern

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Abstract

We assessed the genetic structure of populations of the widely distributed sea cucumber *Holothuria (Holothuria) mammata* Grube, 1840, and investigated the effects of marine barriers to gene flow and historical processes. Several potential genetic breaks were considered, which would separate the Atlantic and Mediterranean basins, the isolated Macaronesian Islands from the other locations analysed, and the Western Mediterranean and Aegean Sea (Eastern Mediterranean). We analysed mitochondrial 16S and COI gene sequences from 177 individuals from four Atlantic locations and four Mediterranean locations. Haplotype diversity was high (H = 0.9307 for 16S and 0.9203 for COI), and the haplotypes were closely related (π = 0.0058 for 16S and 0.0071 for COI). The lowest genetic diversities were found in the Aegean Sea population. Our results showed that the COI gene was more variable and more useful for the detection of population structure than the 16S gene. The distribution of mtDNA haplotypes, the pairwise F_{ST} values and the results of exact tests and AMOVA revealed: (i) a significant genetic break between the population in the Aegean Sea and those in the other locations, as supported by both mitochondrial genes, and (ii) weak differentiation of the Canary and Azores Islands from the other populations; however, the populations from the Macaronesian Islands, Algarve and West Mediterranean could be considered to be a panmictic metapopulation. Isolation by distance was not identified in *H. (H.) mammata*. Historical events behind the observed findings, together with the current oceanographic patterns, were proposed and discussed as the main factors that determine the population structure and genetic signature of *H. (H.) mammata*.

Keywords: Echinodermata, genetic structure, *Holothuria (Holothuria) mammata*, Macaronesian Islands, Mediterranean Sea, mtDNA

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Introduction

Marine species that spend part of their life cycle as planktonic larvae have high dispersal potential and therefore are expected to display weaker genetic structure and higher gene flow than species with low dispersal potential. Nevertheless, in some cases, this expectation is not met because gene flow in marine species can be constrained by additional factors (biological, physical and ecological) (Hellberg *et al.* 2002).

The genetic variability and population genetic structure of a species are shaped by both current and historical marine barriers, such as dispersal barriers, namely, narrow and shallow water passages between land masses; salinity gradients; different types of currents; and also by palaeoecological history (Patarnello *et al.* 2007; Pérez-Losada *et al.* 2007).

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One important potential barrier to gene flow in the world's oceans is the Atlantic–Mediterranean transition, which occurs at the point where the Strait of Gibraltar separates the Atlantic and Mediterranean basins. However, the Almería–Oran front is currently considered to be the main barrier in this region. This front is formed by the convergence of two distinct water masses in the east of the Alborán Sea, which is situated to the southeast of Spain (Tintoré *et al.* 1988). Many studies have analysed genetic structure in Atlanto-Mediterranean invertebrates and fishes. However, different conclusions regarding the influence of the Atlantic–Mediterranean division on population structure have been reached depending on the species, molecular markers used and the sampling design (Patarnello *et al.* 2007 and references therein, Pérez-Losada *et al.* 2007; Domingues *et al.* 2007a,b; Calderón *et al.* 2008; Chevolot *et al.* 2006; Palero *et al.* 2008; Zulliger *et al.* 2009; Pérez-Portela *et al.* 2010; González-Wangüemert *et al.* 2010, 2011).

On the other hand, the isolation of the Macaronesian Islands in the Atlantic could promote differentiation and speciation (Emerson 2002). Studies on the genetic relationships between invertebrate and fish populations in the Atlantic Ocean, including the Macaronesian Islands, and those in the Mediterranean Sea have been carried out, and differing conclusions regarding the distinctiveness of populations from these islands have been drawn. Some species exhibit little or a total lack of gene flow and significant genetic divergence between populations of the Macaronesian Islands and Atlantic coast/Mediterranean Sea populations (Sá-Pinto *et al.* 2005, 2008; Chevolot *et al.* 2006; Domingues *et al.* 2007a,b; Zulliger *et al.* 2009), whereas others show high levels of gene flow between these two regions (Duran *et al.* 2004; Pérez-Losada *et al.* 2007; Calderón *et al.* 2008).

Other potential barriers to gene flow within the Mediterranean Sea are the Siculo–Tunisian Strait, which divides the Eastern and Western Mediterranean, and the hydrographic isolation of the Aegean, Ionian and Adriatic Seas (Patarnello *et al.* 2007; Pérez-Losada *et al.* 2007). Although the Siculo–Tunisian Strait was identified as a barrier to gene flow in some studies (Stefanni & Thorley 2003; Zardoya *et al.* 2004), few studies showed genetic breaks at both the Siculo–Tunisian Strait and these three seas (Borsa *et al.* 1997). However, for most species, the major genetic break or limitation to larval dispersal between the Eastern and Western Mediterranean is related to the hydrographic isolation of the Adriatic, Aegean and/or Black Sea (Nikula & Väinölä 2003; Costagliola *et al.* 2004; Domingues *et al.* 2005; Peijnenburg *et al.* 2006; Pérez-Losada *et al.* 2007; Zulliger *et al.* 2009).

In addition to these oceanographical barriers, the geological history of the Eastern Atlantic and Mediterranean Sea, which includes the breaking up of the Tethys Sea, which ended in the formation of the Mediterranean Sea, the Messinian salinity crisis (MSC) (Krijgsman *et al.* 1999; Duggen *et al.* 2003) and the Pleistocene glaciations (Lambeck *et al.* 2002), might have left a strong footprint on the genetic structure of populations.

Against this background, we wanted to investigate the influence of isolation, gene flow barriers and historical processes on the genetic structure of *Holothuria (Holothuria) mammata* Grube, 1840. This species is distributed widely throughout the Mediterranean Sea and northeast Atlantic Ocean, including the continental Atlantic coast of Portugal and the Macaronesian Islands of the Azores, Madeira and Canary Islands (Borrero-Pérez *et al.* 2009). No information about reproductive biology or larval duration is available for this species, but we consider it to be a broadcast spawning invertebrate with a planktotrophic larval stage of 13–22 days, as has been recorded for other *Holothuria* species (Ramofafia *et al.* 1995; McEdward & Miner 2001; Asha & Muthiah 2002; Despalotovic *et al.* 2004; Ivy & Giraspy 2006). Currently, this species is one of the most valuable targets of the sea cucumber fisheries that are expanding in the Mediterranean Sea (Aydin 2008).

Although holothurians in the Indo-Pacific region have been the subject of several population genetic studies using mitochondrial and/or allozyme markers (Uthicke *et al.* 1998, 2001, 2004b; Uthicke & Benzie 2000, 2001, 2003; Uthicke & Purcell 2004), no studies have been conducted in the Atlanto-Mediterranean region. However, other echinoderms have been studied and different patterns of population structure were observed (Féral *et al.* 1995; Duran *et al.* 2004; Baus *et al.* 2005; Calderón *et al.* 2008; Zulliger *et al.* 2009; Pérez-Portela *et al.* 2010).

In the study reported herein, we assess the population genetic structure of the widely distributed Atlanto-Mediterranean sea cucumber *H. (H.) mammata*, considering the role of current and historical barriers to gene flow in several potential genetic breaks: the Atlantic and Mediterranean basins, Western and Eastern Mediterranean Sea in relation to the Aegean Sea, specifically, and the geographical isolation of the Macaronesian Islands.

Material and methods

Sampling

Samples of *H. (H.) mammata* were collected from eight localities that covered a wide range of the species distribution. Four localities were in the Atlantic Ocean, and

corresponded to three Macaronesian Islands (Azores-AZ, Canary Islands-CN and Madeira-MD) and one locality from continental Portugal (Algarve-AL). The remaining four localities were in the Mediterranean area, three in the Western Mediterranean Sea (Cabo de Palos-CP, Gerona-GE and Mallorca-ML) and one in the Aegean Sea, Eastern Mediterranean (Foça-FO) (Table 1, Fig. 1). Specimens were collected by scuba diving or snorkelling. A sample of longitudinal muscle or body wall was removed from each individual and preserved in absolute ethanol.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted in accordance with the procedure of Sambrook *et al.* (1989), with some modifications. Fragments of two mitochondrial genes, the 16S rRNA gene and subunit I of cytochrome oxidase (COI), were amplified by the polymerase chain reaction using the same conditions as Clouse *et al.* (2005), Kerr *et al.* (2005) and Uthicke *et al.* (2005) and the primers 16SA 5′CGCCTGTTTATCAAAAACAT3′ and 16SB 5′CTCCGGTTTGAACTCAGATCA3′ for the former gene and CO1eF 5′ATAATGATAGGAGGRTTTGG3′ and CO1eR 5′GCTCGTGTRTCTACRTCCAT3′ for the latter (Arndt *et al.* 1996; Palumbi 1996). PCR products were purified by dilution in water and sequenced using BIGDYE 3.1 technology (Applied Biosystems).

Genetic diversity

Sequences for both the 16S and COI genes were aligned using ClustalX with the default alignment parameters as implemented in BIOEDIT V7.0.5.3 (Hall 1999) and revised manually. MtDNA haplotype diversity (*H*) and nucleotide diversity (π) were calculated for each location using the software ARLEQUIN version 2.001 (Schneider *et al.* 2000).

Population differentiation

Genetic divergence was estimated using population pairwise F_{ST} based on haplotype frequencies and their significances were calculated by performing 10 000 permutations in ARLEQUIN. For this and all multiple tests performed in this study, the level of significance was corrected by applying a modified false discovery rate procedure developed by Benjamini & Yekutieli (2001), B-Y FDR, as suggested by Narum (2006). An exact test of population differentiation was performed to test the null hypothesis that the observed haplotype distribution is random with respect to sampling location (10 000 random permutations). In order to test the isolation-by-distance model, we calculated the correlation

Table 1 Diversity measures for populations of *Holothuria (Holothuria) mammata*

			16S				COI			
Region	Population	Code	N	*H*	π	Nh	N	*H*	π	Nh
Atlantic Ocean			94	0.9405 ± 0.0131	0.0060 ± 0.0035	39 (28/21)	92	0.8844 ± 0.0276	0.0072 ± 0.0041	35 (25/22)
Macaronesian Islands	Azores	AZ	22	0.9740 ± 0.0217	0.0063 ± 0.0038	17 (9)	22	0.8745 ± 0.0535	0.0063 ± 0.0038	11 (5)
	Canary Islands	CN	26	0.9538 ± 0.0245	0.0066 ± 0.0039	17 (7)	26	0.8185 ± 0.0733	0.0063 ± 0.0038	13 (6)
	Madeira	MD	11	0.8909 ± 0.0740	0.0056 ± 0.0038	7 (0)	9	1.0000 ± 0.0524	0.0098 ± 0.0060	9 (5)
Continental Portugal	Algarve	AL	35	0.9193 ± 0.0335	0.0056 ± 0.0034	20 (9)	35	0.8857 ± 0.0399	0.0075 ± 0.0043	18 (6)
Mediterranean Sea			83	0.8980 ± 0.0208	0.0054 ± 0.0032	26 (15/13)	82	0.9027 ± 0.0241	0.0069 ± 0.0039	39 (29/26)
West Mediterranean	Cabo de Palos	CP	22	0.9221 ± 0.0462	0.0053 ± 0.0033	15 (8)	21	0.9524 ± 0.0399	0.0074 ± 0.0043	17 (10)
	Gerona	GE	15	0.7905 ± 0.1049	0.0043 ± 0.0029	8 (3)	15	0.9143 ± 0.0559	0.0065 ± 0.0040	10 (6)
	Mallorca	ML	25	0.8767 ± 0.0499	0.0050 ± 0.0032	12 (3)	25	0.9667 ± 0.0236	0.0076 ± 0.0044	19 (11)
Aegean Sea, East Med	Foça	FO	21	0.5143 ± 0.0458	0.0044 ± 0.0028	2 (2)	21	0.5143 ± 0.0458	0.0051 ± 0.0032	2 (1)
Total			177	0.9307 ± 0.0116	0.0058 ± 0.0034	54 (–/34)	174	0.9204 ± 0.0128	0.0071 ± 0.0040	64 (–/48)

N, Number of individuals; *H*, Haplotype diversity; π, Nucleotide diversity; Nh, Number of haplotypes (private/singletons).

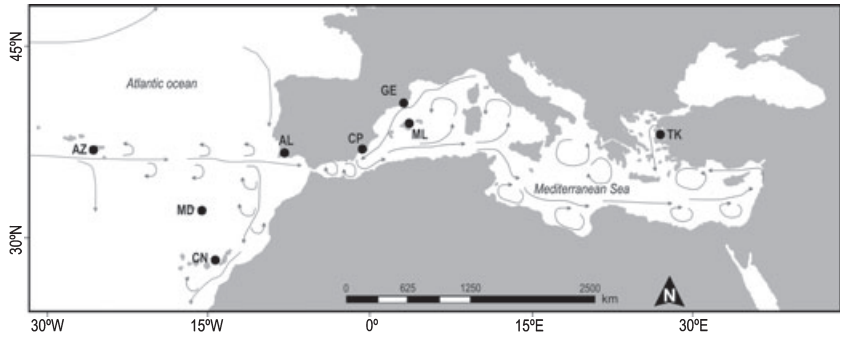

Fig. 1 Sampling sites for *Holothuria (Holothuria) mammata*. AZ, Azores; CN, Canary Islands; MD, Madeira; AL, Algarve; CP, Cabo de Palos; GE, Gerona; ML, Mallorca; and FO, Foça. Dominant surface currents (grey arrows) are indicated (Malanotte-Rizzoli & Bergamasco 1989, Johnson & Stevens 2000, Millot 2005; Juliano & Alves 2007).

between genetic differentiation values (F_{ST}) and geographical distances for all pairs of populations using the Mantel permutation procedures implemented in the software ARLEQUIN. Analysis of molecular variance (AMOVA) using haplotype frequencies was carried out to examine hierarchical population structure, by pooling the samples into various geographical groups: (i) Atlantic (AZ, CN, MD, AL) and Mediterranean (CP, GE, ML, FO) basins; (ii) Atlantic, Western Mediterranean (CP, GE, ML) and Aegean Sea (FO); (iii) Macaronesian Islands (AZ, CN, MD), Western Mediterranean plus AL and Aegean Sea; (iv) Atlantic and Western Mediterranean; and (v) Macaronesian Islands and Western Mediterranean plus AL. The third and fifth groups are justified by the geographically intermediate position of AL between the Macaronesian and Mediterranean localities.

Relationships between and the geographical distribution of mtDNA haplotypes were analysed in a network constructed with the software TCS version 1.21 (Clement *et al.* 2000), which implements the statistical parsimony procedure, with gaps coded as a fifth character state and a 95% connection limit.

Historical demography

The historical demography of the populations was examined using mismatch distribution analysis (Rogers & Harpending 1992) to evaluate possible rapid population expansion or bottleneck events. These analyses were performed in ARLEQUIN. We assessed the fit of the mismatch distribution to the theoretical distribution by Monte Carlo simulations of 1000 random samples. The sum of squared deviations (SSD) between observed and expected mismatch distributions was used as a statistical test and its *P*-value represented the probability of obtaining a simulated SSD that was larger than or equal to the observed one. In order to have a broader view, we also assessed the history of effective population size by means of other statistics such as Tajima's *D* and Fu's F_s.

To estimate the approximate time of expansion of *H. (H.) mammata* populations, the relationship $\tau = 2ut$ was used (Rogers & Harpending 1992). A mutation rate (MR) of 0.5% per nucleotide per million years was used for the 16S gene, and a rate of 1.5% was used for the COI gene, both of which were calculated previously for echinoids (Lessios *et al.* 2001; Chenuil & Féral 2003). In addition, we used the MR of 0.84% per nucleotide per million years, which was calculated previously for the Holothuriidae family, for both the 16S and COI genes (Borrero-Pérez *et al.* 2010). All analyses were performed for the total sample, excluding the Aegean Sea location (FO), in accordance with the population differentiation detected in previous analysis.

Results

Genetic diversity

A total of 54 haplotypes for the 16S gene were detected among the 177 individuals of *H. (H.) mammata* that were analysed and polymorphisms were observed at 42 of the 465 bp (9.0%) sequenced (Table 1; GenBank Accession numbers GU797562–GU797604; JF697304–JF697311). On the other hand, 64 haplotypes were detected for the COI gene among 174 individuals and polymorphisms were identified at 63 sites among the 502 bp (12.5%) sequenced (Table 1; GenBank Accession numbers GU797651–GU797704; JF697312–JF697317). The presence of a dominant haplotype and many closely related haplotypes at low frequency is reflected in both the low nucleotide diversity (16S: $\pi = 0.0058$; COI: $\pi = 0.0071$) and the high haplotype diversity (16S: $H = 0.9307$; COI: $H = 0.9204$) for both genes in the overall sample, as well as in the individual samples from most of the localities (Table 1).

Population differentiation

The analysis revealed restricted gene flow between FO and the locations in the Western Mediterranean Sea and the Atlantic Ocean. F_{ST} values ranged from 0.25 to 0.35 ($P < 0.01$) for the 16S gene and from 0.18 to 0.32 ($P < 0.01$) for COI (Table 2). A second restriction of gene flow was detected, mainly for the COI gene, between the AZ and CN localities and some localities in the Western Mediterranean Sea. Pairwise F_{ST} values for COI between AZ and CN vs. CP and CN vs. ML were significant. When we considered the 16S data, only the comparison between CN and GE was significant (Table 2). For both genes, pairwise F_{ST} showed a high level of gene flow within the Macaronesian Islands and within the Western Mediterranean. In the cases of MD and AL, the pairwise F_{ST} values with all the other localities were not significant (Table 2).

Similarly, the exact tests of differentiation among the samples for the COI and 16S genes showed significant differences ($P < 0.01$) for FO vs. all other localities (data not shown). In the case of COI, the test was also significant for AZ vs. CP/GE and CN vs. CP/GE/ML, whereas, for the 16S gene, no other pair of populations showed a significant difference ($P > 0.05$).

When the pairwise F_{ST} values between samples were correlated with the geographical distances using the Mantel test, a high and significant correlation was detected for COI ($r = 0.87$, $P = 0.00$) and a high and less significant value was found for the 16S gene ($r = 0.73$, $P = 0.03$). Given the high genetic differentiation that was detected between FO and the rest of the localities, we also performed a second analysis with this location excluded to test whether the correlations obtained were an artefact. The determined correlations were significant but low (COI: $r = 0.51$, $P = 0.026$; 16S: $r = 0.49$, $P = 0.031$). On the basis of these results, we conclude that a pattern of isolation by distance does not exist for *H. (H.) mammata*.

The results from AMOVA revealed a high and significant amount of variance that could be explained by the differences among groups when the Atlantic (AZ, CN, MD, AL), Western Mediterranean (CP, GE, ML) and Aegean Sea (FO) groups were considered for both genes (16S: 11.06%, $P = 0.01$; COI: 10.07%, $P < 0.01$). The percentage variation was found to be similar in both genes when AL was included in the Western Mediterranean group and the Macaronesian Islands were considered as a separate group (16S: 10.94%, $P = 0.01$; COI: 10.68%, $P < 0.01$). The percentage variation among groups in the other AMOVA analyses was not significant.

Geographical distribution of the mtDNA haplotypes

The 16S statistical parsimony network revealed that the ancestral and most common haplotype (16S-1) was found at all localities except FO (Fig. 2a). Most of the shared haplotypes (16S-2, 16S-3, 16S-9, 16S-19, 16S-15, 16S-25, 16S-32 and 16S-37) were distributed in Mediterranean, Algarve and Macaronesian localities. Two haplotypes with high frequency (16S-45, 16S-46) were private to the FO location.

For the COI gene, the statistical parsimony network displayed a genealogy with two main haplotypes (Fig. 2b). The most common and ancestral haplotype (COI-4) was detected at all localities sampled except FO, whereas the second most common haplotype (COI-3) was found in every Mediterranean locality and AL. Most of the other shared haplotypes were distributed

Table 2 Pairwise estimates of F_{ST} between samples of *Holothuria (Holothuria) mammata* based on mtDNA 16S (below diagonal) and COI (above diagonal)

	Atlantic				Mediterranean			
	Macaronesian Islands			Continental Portugal	West Mediterranean			Aegean Sea East Med
Localities	Azores	Canary Islands	Madeira	Algarve	Cabo de Palos	Gerona	Mallorca	Foça
Azores		−0.0064	0.0124	0.0163	**0.0644****	0.0455*	0.0297*	**0.3040****
Canary Islands	−0.0024		0.0509	0.0205	**0.0926****	0.0558*	**0.0491****	**0.3271****
Madeira	0.0081	0.0173		0.0156	0.0204	0.0158	0.0001	**0.2838****
Algarve	−0.0008	0.1032	−0.0139		0.0176	−0.0156	0.0012	**0.2198****
Cabo de Palos	0.0025	0.0244	0.0109	0.0001		−0.0107	−0.0096	**0.1833****
Gerona	0.0456*	**0.0716****	−0.0186	0.0083	0.0033		−0.0084	**0.2052****
Mallorca	0.0159	0.0166	−0.0081	0.0026	−0.0032	−0.0097		**0.1991****
Foça	**0.2540****	**0.2581****	**0.3234****	**0.2663****	**0.2801****	**0.3581****	**0.2986****	

Significant P values are indicated by *$P < 0.05$; ** and bold text: significance after false discovery rate correction ($P < 0.02$).

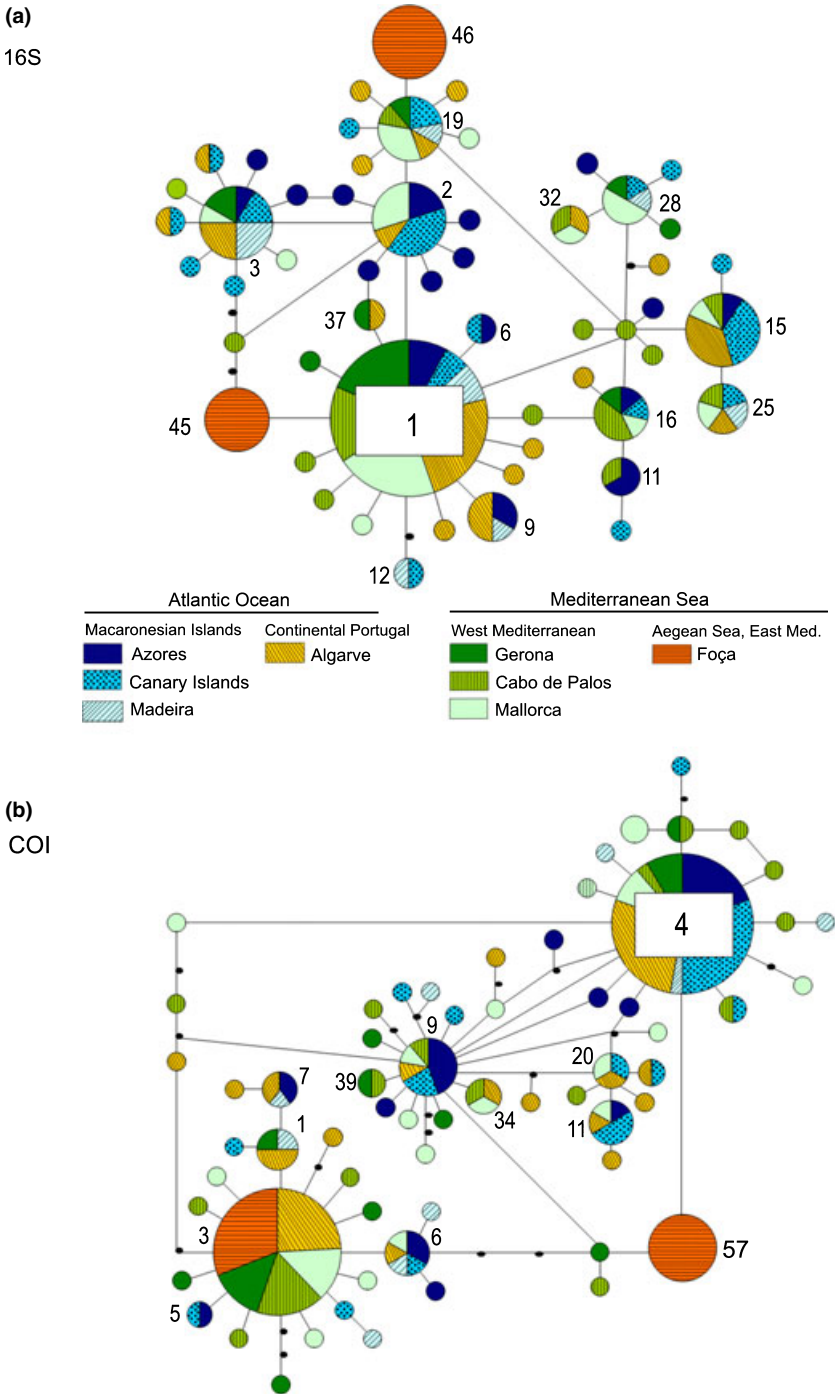

Fig. 2 Statistical parsimony network based on 16S (a) and COI (b) sequences from *Holothuria (Holothuria) mammata*. Circle size is proportional to the number of sampled individuals, and the partitions inside the circles represent the frequency of the haplotype for each population. Each line in the network represents a single mutational change. Small black circles represent missing, probably unsampled or extinct, haplotypes. White squares indicate the ancestral haplotype.

widely. One haplotype with high frequency (COI-57) was private to the FO location.

Historical demography

We performed demographic analyses that considered the Aegean Sea and the Macaronesian Islands/Algarve/West Mediterranean as two different panmictic metapopulations. The mismatch distributions for both the 16S and COI gene showed a unimodal distribution that is characteristic of a sudden expansion model for the Macaronesian Islands/Algarve/Western Mediterranean group (Fig. 3). Neutrality tests for both genes detected a population expansion for this group with significant values for both Tajima's D and Fu's F_s. In the FO population, the mismatch distributions were significantly different from the sudden expansion model and neither neutrality test for neither gene was significant.

We estimated the approximate time of expansion (t) for the population from the Macaronesian Islands/Algarve/Western Mediterranean. For the 16S gene, t was estimated to be around 603 thousand years ago (kya) using a mutation rate (MR) of 0.5%/million year (my) and 359 kya using a MR of 0.84%. In the case of the COI gene, t was calculated to be around 309 kya using a MR of 1.5%/my and 552 kya using a MR of 0.84%. When the same MR of 0.84% was used, the results suggested that COI evolved 1.54 times faster than the 16S gene.

Discussion

Recently, we analysed the phylogenetic relationships within the Holothuriidae family in the northeastern Atlantic and Mediterranean Sea. The analysis included both COI and 16S DNA sequences and suggested that the lineages, including *H. (H.) mammata*, were separated during the Miocene (10–23 Myr), when the African plate moved north (Borrero-Pérez *et al.* 2010); this event ended with the closing of the Tethys Seaway. In agreement with this finding, *H. (H.) mammata* was shown to have existed before the MSC and thus it is possible to hypothesize about its historic distribution, colonization and survival during the MSC. Considering the current Atlantic distribution of *H. (H.) mammata*, it is likely that this species colonized or recolonized the Mediterranean after the MSC, when the Mediterranean Sea was refilled with water from the Atlantic, with the concomitant entry of biota. This progressive colonization or recolonization, as well as other more recent his-

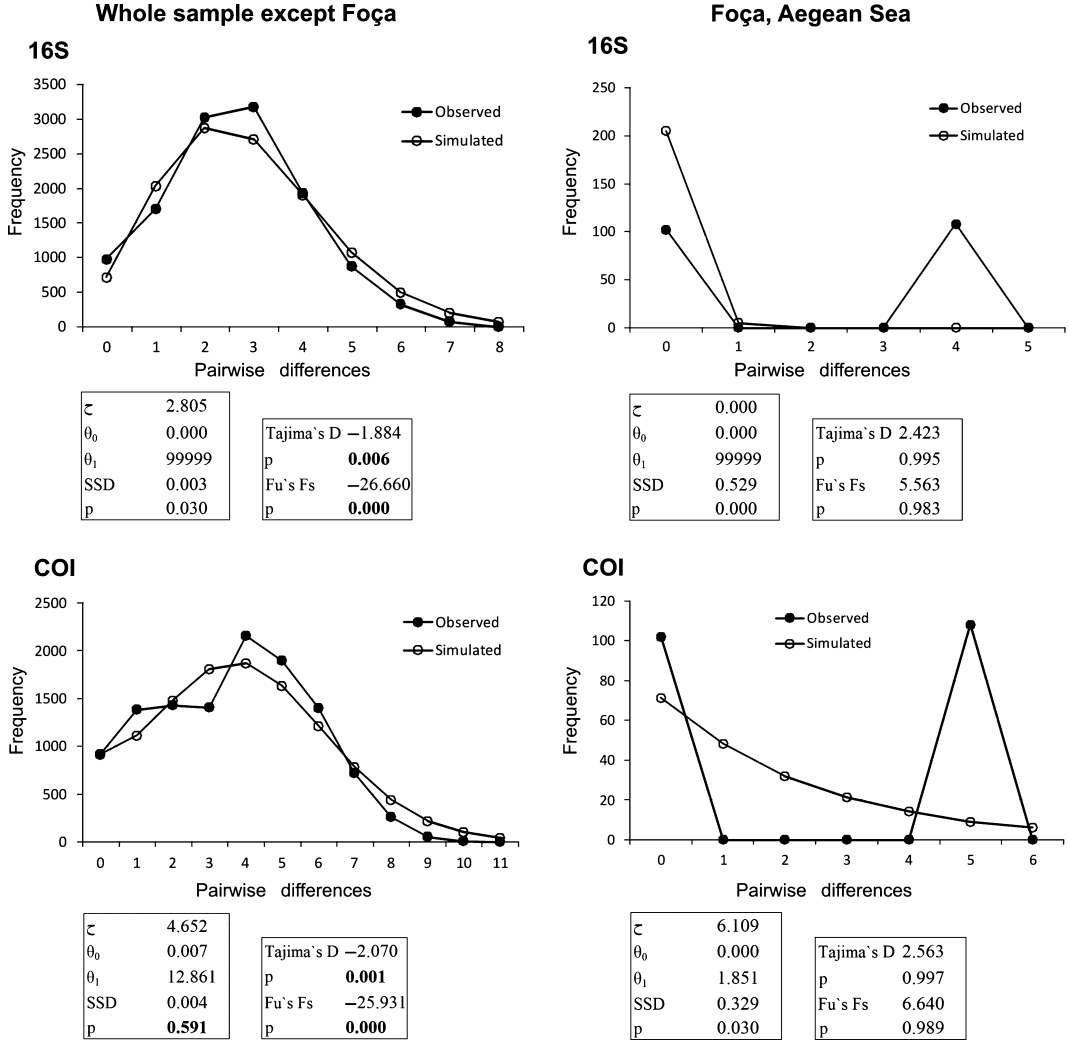

Fig. 3 Mismatch distribution for the 16S and COI genes for defined groups of *Holothuria (Holothuria) mammata*. The lines with empty circles represent the expected distribution under a sudden expansion model; the lines with black circles represent the observed distribution. In the boxes below each graphic are included the parameters of population expansion and test of neutrality. Significant values are indicated with bold letters.

torical processes, such as the Pleistocene glaciations, and the current oceanographic pattern could explain the genetic structure and diversity of *H. (H.) mammata* in the present day.

Our study has revealed two important findings: (i) the existence of a genetic break between the population in the Aegean Sea and those further west that is supported by the data for both mitochondrial genes and (ii) the weak differentiation of populations from the Canary and Azores Islands, although those of the Macaronesian Islands, Algarve and West Mediterranean could be considered to be a panmictic metapopulation.

The major genetic break for *H. (H.) mammata*, which was supported by data for both mitochondrial genes, was identified between the population in the Aegean Sea (FO) and the populations at the other localities. Haplotype diversity was lower in the FO population than in all the other samples: the former displayed only two haplotypes each for the 16S and COI genes, with three of the haplotypes being exclusively located in this area. In addition, the genetic differentiation of this Aegean Sea population was also supported by low but significant rates of gene flow, as shown by the F_{ST} values and AMOVA analysis. Demographic analysis of this population did not detect expansion. The Aegean Sea, which is located in the Eastern Mediterranean basin, might be one of the last places colonized by *H. (H.) mammata* after the MSC. This could explain the low diversity and the presence of private haplotypes in this area. However, it is necessary to analyse samples from more localities in the Eastern Mediterranean to corroborate this hypothesis. These genetic characteristics could also reflect strong bottlenecks because of past and current isolation. Such isolation events might be linked to the radical hydrographical changes that occurred during the course of the Pleistocene climatic cycles, in particular the fluctuations in water level that repeatedly led to the isolation or partial isolation of the Black Sea, Aegean Sea and Eastern Mediterranean (Svitoch *et al.* 2000). Subsequently, the isolation of this population until the present might have been maintained by a quasi-circular anticyclonic front located to the southwest of the Peloponnese peninsula (Malanotte-Rizzoli & Bergamasco 1989; Roussenov *et al.* 1995; Millot 2005), which would have prevented gene flow between the population in the Aegean Sea and other Mediterranean populations. In addition, the hydrography of the Bosporus-Aegean Sea area should impede the spread of larvae from the Mediterranean Sea into the Aegean Sea, but allow the entrance of water from the Black Sea (Nikula & Väinölä 2003; and references therein). Therefore, studies of further samples of *H. (H.) mammata* from various locations throughout the Eastern Mediterranean Sea are necessary to clarify whether there is genetic differentiation between the Eastern and Western Mediterranean basins overall or whether the observed differentiation is limited to the Aegean Sea. Previous genetic studies on populations of fish and invertebrates identified a major genetic break that was related to the hydrographic isolation of the Aegean Sea, which was more marked than that of the Siculo–Tunisian Strait (Nikula & Väinölä 2003; Costagliola *et al.* 2004; Domingues *et al.* 2005; Chevolot *et al.* 2006; Peijnenburg *et al.* 2006; Pérez-Losada *et al.* 2007; Zulliger *et al.* 2009).

The second result, the genetic differentiation of the populations of the Azores and Canary Islands relative to the other populations, could be explained by the isolated location of these oceanic islands and other oceanographic characteristics. The Azores Current dominates the present-day surface current pattern in the Northeast Atlantic and makes larval dispersion in an easterly direction most likely. However, the effect of the countercurrents that exist in the Azores and Canary Currents (Alves & Verdière 1999; Johnson & Stevens 2000; Alves *et al.* 2002; Juliano & Alves 2007) and the isolated location of these islands, in particular the Azores (1800 km from the continental coast and waters deeper than 4000 m), might make larval dispersion to the Mediterranean Sea difficult because of the absence of continuity of habitat and short duration of the larval stage of the species. It is likely that the Madeira Islands differ from the other Macaronesian Islands because of their geographical position (they are located nearer to the Strait of Gibraltar than the other Macaronesian Islands and have an intermediate geographical position between the Azores and Canary Islands) and the highly variable currents around them (Johnson & Stevens 2000). These factors could facilitate larval dispersion between these islands and other localities, which would explain their lack of genetic differentiation. The isolation of the Macaronesian Islands has already been reported for other marine invertebrates with high dispersal potential. For example, certain species of the genus *Patella* and the sea star *Marthasterias glacialis* have shown not to exhibit gene flow between Macaronesian and continental populations (Sá-Pinto *et al.* 2008; Pérez-Portela *et al.* 2010). In addition, a strong and significant genetic break has been detected between the Azores and continental populations of *Diplodus sargus,* a fish species with mobile adults and pelagic larvae (González-Wangüemert *et al.* 2010, 2011).

In addition, the genetic relationship of the populations of the Azores and Canary Islands with those of the Mediterranean locations could be related to mesoscale processes derived from the incoming Atlantic waters and their role in the genetic differentiation of populations. In the case of the Azores, the stronger genetic relationship of its populations with those of

Mallorca than with those of other Mediterranean locations might be explained by the fact that Mallorca is influenced more strongly by Atlantic waters than the other Mediterranean locations (Fig. 1). Similarly, the Mediterranean population that is most different from that of the Azores (on the basis of F_{ST} values and the exact test, mainly for COI) is from Cabo de Palos, which is located in the southwestern basin, and is barely influenced by incoming Atlantic waters. The same pattern has been described for other marine invertebrates and fishes such as the spiny lobster *Palinurus elephas* (Palero *et al.* 2008) and the fish *Diplodus sargus*, for which the F_{ST} values are higher between the Azores and southwest Mediterranean than between the Azores Islands and Mallorca/Banyuls (González-Wangüemert *et al.* 2010).

An interesting result was the geographical distribution of the haplotype COI-3, which is restricted to the Mediterranean localities and Algarve. As described by Horne *et al.* (2008), the existence of haplotypes that are shared across vast distances can be interpreted in two different ways. The most common haplotypes, as well as those that are most widely spread, can be interpreted as being the oldest. Moreover, this is supported further if these haplotypes are in a central position (Posada & Crandall 2001), as was observed in *H. (H.) mammata* for both genes. Alternatively, the presence of shared haplotypes might indicate that gene flow between distant populations has occurred, or has been restricted to a relatively recent evolutionary timescale. Such gene flow would be easier to detect for the COI gene because it evolves faster and is more variable than the 16S gene. The distribution of the COI-3 haplotype could be explained by the current pattern of circulation in the study area (Fig. 1), which was established between 4 and 5 million years ago, during the closure of the Panama Isthmus (Haug & Tiedemann 1998; Haug *et al.* 2001). This pattern of circulation might have impeded the dispersal of larvae from the Mediterranean to the Macaronesian Islands because the Mediterranean outflow tends to stratify in the Atlantic Ocean at a depth of 600–1400 m owing to its greater density. In addition, the surface water in the Northeast Atlantic flows mostly in an eastward direction, with the Azores, Madeira and the Canary Islands being upstream from continental Europe (Jia 2000; Johnson & Stevens 2000).

Unlike the Aegean Sea population, the populations at the other locations were compatible with a model of fast population expansion in *H. (H.) mammata*. This is because of the high haplotype diversity and low nucleotide diversity, the results of the neutrality tests and the mismatch distribution. Haplotype diversity in *H. (H.) mammata* was found to be high (16S: $H = 0.9307$; COI: $H = 0.9204$), and haplotypes were closely related (mean nucleotide diversity: 16S, 0.0055; COI, 0.0070). These values are similar to values determined using COI for other echinoderm species, such as *Holothuria nobilis* (Uthicke & Benzie 2003) and *Paracentrotus lividus* (Duran *et al.* 2004), and for other invertebrates such as *Palinurus mauritanicus* (Pérez-Losada *et al.* 2007), for which fast population expansions have also been detected.

The historical population expansion of *H. (H.) mammata* predates the Last Glacial Maximum (LGM), dating back to 603 or 359 kya (16S) and 552 or 309 kya (COI) using the different mutation rates. The estimated dates of the expansion include two interglacial periods, the Günz–Mindel Interglacial (Cromerian complex) and the Mindel–Riss Interglacial, which was one of the longest warm periods of the Quaternary (Kukla 2005). These dates coincide with those calculated for *Raja clavata* and other marine species from Northwest Europe, such as fishes, invertebrates and algae, which clearly predate the LGM (Chevolot *et al.* 2006 and references herein).

Analysis of the 16S and COI genes has led to different patterns of genetic population structure and times of expansion for the sea urchin *Paracentrotus lividus* (Duran *et al.* 2004; Calderón *et al.* 2008). These differences were explained as the result of different mutation rates, which are nearly three times higher for COI than for 16S (Lessios *et al.* 2001; Chenuil & Féral 2003), despite the fact that mtDNA genes are linked, which would imply a shared evolutionary history. In addition, contradictory results about the efficiency of detection of population structure have been obtained for these two genes in the same sea urchin species (Iuri *et al.* 2007). Against this background, the comparison of these two mitochondrial markers allows us to determine the population structure of *H. (H.) mammata*, because the two genes show congruent patterns for genetic population structure but also display differences that can be analysed in the light of their different mutation rates.

In conclusion, the results of the present study show that palaeoecological history (e.g., glaciations) and present oceanographic processes have a role in shaping the genetic variability and population structure of the sea cucumber *H. (H.) mammata*. Despite the apparently long duration of its planktonic larval stage, restricted gene flow was found between *H. (H.) mammata* populations, which caused genetic differentiation between the population in the Aegean Sea and those in the Western Mediterranean/Algarve/Macaronesian Islands. In order to corroborate the patterns described in this study, it is necessary to undertake studies of samples from across the distribution area of this species, including additional samples from along the coast of Portugal to the Bay of Biscay (which is the most northerly extent of the distribution) and along the coast of the Eastern Mediterranean and North Africa.

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