Phylogeography, genetic diversity and structure of the poecilosclerid sponge Phorbas fictitius at oceanic islands


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Phylogeography, genetic diversity and structure of the poecilosclerid sponge Phorbas fictitius at oceanic islands

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Key words: Azores, genetic diversity, Iberia, Macaronesia, mitochondrial DNA, Porifera

Abstract

In this study we assessed the sequence variation in the I3-M11 partition of the mtDNA cytochrome c oxidase subunit I gene (COI) in ten populations of the Atlanto-Mediterranean demosponge Phorbas fictitius (Porifera: Poecilosclerida) at two spatial scales: a regional scale comparing mainland (Iberian) and insular (Macaronesian) populations, and a local (Archipelagic) scale focusing on different island populations of the Azores archipelago. A multiple approach combining diversity measures, FST estimates, phylogenetic inference and nested clade phylogeographic analysis was used to assess the genetic structure and elucidate the evolutionary history of this species. Genetic differentiation, based of FST estimates, was found among most populations at both scales revealing highly structured populations. This results of a presumably low dispersal potential and bathymetric range of the species, and the geographical isolation of the studied populations. However we found evidence of long distance dispersal events between some populations. Phylogenetic and network analyses indicate a separation of insular (Macaronesian) and mainland (Iberian) clades with only two haplotypes shared between these areas. The high genetic diversity and prevalence of ancestral haplotypes suggest the Macaronesian islands as the likely place of origin of this species with posterior expansion to mainland locations via current-mediated dispersal of larvae or sponge fragments. This study adds to the growing evidence of long distance dispersal events between some populations. Phylogenetic and network analyses indicate a separation of insular (Macaronesian) and mainland (Iberian) clades with only two haplotypes shared between these areas. The high genetic diversity and prevalence of ancestral haplotypes suggest the Macaronesian islands as the likely place of origin of this species with posterior expansion to mainland locations via current-mediated dispersal of larvae or sponge fragments. This study adds to the growing evidence of structured populations in the marine realm and highlights the importance of the Macaronesian islands on the evolutionary history of the Northeast Atlantic marine biota.

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putative physical barriers (e.g. Strait of Gibraltar, English Channel); and it experienced a complex geological and climatological history in both the recent (e.g. Last Glacial Maximum, 30-19 kyr BP) as well as the remote past (e.g. the Messinian Salinity Crisis, 5.9-5.3 Myr BP).

Over the past decade, the phylogeography and population genetics of a great variety of marine organisms throughout the Northeast Atlantic and Mediterranean has been the focus of many investigations. These studies uncovered, among other things, the influence of physical barriers, biological traits, and past climate on the structuring of current patterns of genetic diversity and divergence among populations (see reviews in Patarnello et al., 2007; Maggs et al., 2008). However, most studies have focused on the mainland shores and only few examined populations of the Macaronesian islands (e.g. Domingues et al., 2006, 2007b, c, 2008; Chevolot et al., 2006).

Sponges constitute a dominant group in hard-bottom benthic communities both in terms of biomass and species richness (Sarà and Vacelet, 1973). They are sessile in the adult phase and only disperse by means of lecitotrophic larvae with a life span of a few days to two weeks (Maldonado, 2006). Although passive dispersal by water currents may occur, most sponge larvae appear to remain in the immediate vicinity of the parental location (Mariani et al., 2005, 2006). This low dispersal potential has important consequences for the connectivity and structure of sponge populations. Surprisingly, studies addressing the population genetics and phylogeography of sponges are still scarce and limited to a handful of species worldwide (e.g. Wörheide et al., 2002; Duran et al., 2004a; Wörheide, 2006; Bentlage and Wörheide, 2007; Wörheide et al., 2008; Blanquer et al., 2009; DeBiasse et al., 2010). In the Northeast Atlantic and Mediterranean areas, and despite a remarkable diversity of 700+ shallow-water
species, studies into the phylogeography of sponges are restricted to a single species, the Mediterranean Crambe crambe, that also occurs at some locations in adjacent Atlantic waters. In a first study, Duran and co-workers reported low sequence variation of the cytochrome c oxidase subunit I gene (COI) in sponges (Duran et al., 2004c). In fact, with only two mtDNA haplotypes detected in samples spanning over 3,000 km, Folmer's COI fragment (Folmer et al., 1994) in sponges proved to be amongst the slowest evolving ones reported for marine organisms. Although some genetic structure was found among Atlantic and Mediterranean populations, this gene fragment failed to reveal the phylogeographic history of the species. Soon after, sensitivity was increased by sequencing the nuclear rDNA internal transcribed spacers (ITS-1 and ITS-2) and microsatellite genotyping for the same specimens and a recent origin of the species or, alternatively, a recent bottleneck followed by a range expansion from the Mediterranean to the Macaronesian islands by human-mediated transport was then proposed (Duran et al., 2004a, b).

In the present study, we examined the genetic structure and phylogeographical history of another poecilosclerid sponge, the Atlanto-Mediterranean Phorbas fictitius (Bowerbank, 1866), based on mtDNA sequences of an alternative partition of the COI gene (‘I3-M11’) proposed to be suitable to infer intraspecific relationships in Porifera (Erpenbeck et al., 2006; López-Legentil and Pawlik, 2009). Phorbas fictitius is an encrusting shallow-water sponge typical of the rocky subtidal. It has a wide distribution range in the Northeast Atlantic (from the West coast of Scotland to the Canaries) and Mediterranean (from Alboran to the Aegean Sea). No specific information is available about the reproductive ecology of P. fictitius, but members of the family Hyedesmiidae, to which this species belongs, are known to release brooded larvae to the surrounding water (Maldonado, 2006). The main goal of this study was to assess the extent of genetic differentiation and structure of P. fictitius populations at regional [Iberian (mainland) versus Macaronesian (island) populations] and local (populations of different islands of the Azores archipelago) scales.

**Material and methods**

**Sampling**

Specimens of *P. fictitius* (total n = 94) were collected by scuba-diving at ten locations separated by distances ranging from 55 to 3250 km (Fig. 1, Table 1). Specimens were preserved in 96% ethanol and deposited in the Porifera collection of the Zoological Museum of Amsterdam (ZMAPOR, now Netherlands Centre for Biodiversity Naturalis). Small fragments (3 mm³) to be used for genetic analyses were preserved in absolute ethanol and kept at -10°C until further processing.

**Table 1. Diversity measures for *Phorbas fictitius* populations.** Population code (Pc), sample size (N), number of haplotypes (Nh), haplotype diversity (Hd), and nucleotide diversity (π) are presented. Standard deviations for Hd and π are given in parenthesis. GAL, BER and MED correspond to the Iberian populations while MAD, CAN and AZO constitute the Macaronesian populations.

<table>
<thead>
<tr>
<th>population</th>
<th>sampling location</th>
<th>coordinates</th>
<th>Pc</th>
<th>N</th>
<th>Nh</th>
<th>Hd</th>
<th>π</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galicia</td>
<td>Graña</td>
<td>48º28'03.95&quot;N; 08º16'25.32&quot;W</td>
<td>GAL</td>
<td>8</td>
<td>2</td>
<td>0.250</td>
<td>0.00045 (0.00032)</td>
</tr>
<tr>
<td>Berlengas</td>
<td>Berlenga Grande island</td>
<td>39º24'46.16&quot;N; 09º30'28.75&quot;W</td>
<td>BER</td>
<td>9</td>
<td>3</td>
<td>0.750</td>
<td>0.00181 (0.00028)</td>
</tr>
<tr>
<td>Mediterranean</td>
<td>Blanes</td>
<td>41º40'31.39&quot;N; 02º48'12.96&quot;E</td>
<td>MED</td>
<td>6</td>
<td>2</td>
<td>0.333</td>
<td>0.00120 (0.00078)</td>
</tr>
<tr>
<td>Madeira</td>
<td>Madeira island</td>
<td>32º38'45.33&quot;N; 16º53'18.46&quot;W</td>
<td>MAD</td>
<td>13</td>
<td>4</td>
<td>0.526</td>
<td>0.00250 (0.00096)</td>
</tr>
<tr>
<td>Canaries</td>
<td>Tenerife island</td>
<td>28º26'57.53&quot;N; 16º15'55.94&quot;W</td>
<td>CAN</td>
<td>10</td>
<td>4</td>
<td>0.644</td>
<td>0.00364 (0.00094)</td>
</tr>
<tr>
<td>Azores</td>
<td>Azores archipelago</td>
<td>36º56'23.00&quot;N; 25º08'27.90&quot;W</td>
<td>SMA</td>
<td>8</td>
<td>2</td>
<td>0.250</td>
<td>0.00045 (0.00033)</td>
</tr>
<tr>
<td>Santa Maria</td>
<td>Santa Maria island</td>
<td>37º16'14.26&quot;N; 24º46'50.73&quot;W</td>
<td>FOR</td>
<td>16</td>
<td>2</td>
<td>0.125</td>
<td>0.00023 (0.00019)</td>
</tr>
<tr>
<td>Formigas</td>
<td>Formigas islet &amp; Dollabarit Bank</td>
<td>37º44'37.07&quot;N; 25º38'19.25&quot;W</td>
<td>SMG</td>
<td>11</td>
<td>2</td>
<td>0.509</td>
<td>0.00276 (0.00055)</td>
</tr>
<tr>
<td>São Miguel</td>
<td>São Miguel island</td>
<td>38º31'22.90&quot;N; 28º39'29.18&quot;W</td>
<td>FAI</td>
<td>7</td>
<td>1</td>
<td>0.000</td>
<td>0.00000 (0.00000)</td>
</tr>
<tr>
<td>Flores</td>
<td>Flores island</td>
<td>39º27'45.96&quot;N; 31º07'40.51&quot;W</td>
<td>FLW</td>
<td>6</td>
<td>1</td>
<td>0.000</td>
<td>0.00000 (0.00000)</td>
</tr>
<tr>
<td>total</td>
<td>all populations</td>
<td></td>
<td></td>
<td>94</td>
<td>10</td>
<td>0.747</td>
<td>0.00420 (0.00027)</td>
</tr>
</tbody>
</table>
a fragment of the COI gene was sequenced. Folmer’s COI partition (Folmer et al., 1994) has an extremely slow rate of sequence evolution in sponges (Duran et al., 2004c, Wörheide, 2006) and anthozoans (Shearer et al., 2002; France and Hoover, 2002), therefore exhibiting a low resolution in the assessment of relationships at inter- and intraspecific levels in these groups. However Erpenbeck et al. (2006) showed that COI can be a suitable marker if another partition (‘13-M11’) located downstream of Folmer’s fragment is used. In this study, we amplified and sequenced a partition of the COI gene that overlaps approximately 60bp with Folmer’s 3’ partition and includes Erpenbeck’s ‘13-M11’. For that purpose we designed a new primer set from the alignment of three complete poriferan COI sequences available from Genbank (NC006894, NC006990, NC006991; Lavrov et al., 2005) with our own sequences. These primers, PficCOI f (5’ –AA-CATGAGGCCANTGGGAGTAACT– 3’) and PorCOI r (5’ –ACTGCCCATNGATAAAACAT– 3’), were developed for P. fictitius but also appear to successfully amplify and sequence COI from sponge species belonging to orders different than the Poecilosclerida (e.g. Hadromerida, Astrophorida).

Amplifications were carried out in 25 µl volume reaction containing 2.5 μl of 10X buffer (Sphaero Q), 4 μl dNTPs (1 mM), 1.6 μl BSA (10 mg/ml), 1.6 μl MgCl₂ (25 mM), 0.3 μl (5 U/µl) of Taq polymerase (Sphaero Q), 0.8 μl of each primer (10 μM), and 1.5 μl of DNA. The amplification profile was as follows: initial denaturing step of 95°C for 3 min, 36 cycles (94°C for 30 s, 57°C for 45 s and 70°C for 90 s) and a final extension of 72°C for 10 min. Amplified products were excised from 1% TAE gels and purified with QIAquick Gel Extraction kit (QIAGEN) following the manufacturer’s instructions. The same primers were used for the sequencing reaction with the ABI-Big-Dye Ready-Reaction and purified products sequenced on both directions on an ABI 3700 automated sequencer at the Amsterdam Academic Medical Centre.

**Data analyses**

Multiple alignments were performed using the ClustalW tool in BioEdit (version 7.0.0, Hall 1999).

**Genetic diversity and structure**

Haplotype and nucleotide diversities were calculated for each population in DnaSP (version 4.0; Rozas et al., 2003). Genetic differentiation among populations was assessed from pairwise FST analyses and gene flow (M) estimates. Analysis of molecular variance (AMOVA) was performed in order to assess the hierarchical population structure at the considered spatial scales. At the regional scale, all Azorean populations were pooled and compared with the other Iberian populations. At the archipelagic scale, we performed an AMOVA exclusively for the five Azorean populations to assess the level at which island populations of P. fictitius are structured. In order to test for a model of isolation by distance we applied a Mantel test to the pairwise genetic and geographical distance matrices. All analyses were implemented in ARLEQUIN (version 3.11, Excoffier et al., 2005).

**Phylogenetic and phylogeographic relationships**

Phylogenetic relationships among haplotypes were assessed under Maximum Parsimony (MP) by full heuristic searches of the complete data set of a concatenated alignment of COI and mitochondrial 16S rDNA sequences. The full alignment was also used to perform an unrooted neighbour joining analysis in MEGA version 6.06 (Tamura et al., 2013). In order to evaluate the degree of congruence among gene trees, a consensus tree was built after performing a majority rule consensus of the bootstrap replicates of 1000. Support values > 50% are shown in parentheses. The nested clade analysis implemented in GenElan 3.1.3 (Good et al., 2008) was used to test for isolation by distance, and the Mantel test was performed to assess the degree of correspondence between genetic and geographical distance matrices. All analyses were implemented in ARLEQUIN (version 3.11, Excoffier et al., 2005).
ristic search and the confidence was evaluated with 5,000 replicates in PAUP* (version 4.0, Swofford, 1998). To infer the pattern of historical processes that may have shaped the current distribution range of this species, we performed nested clade phylogeographic analyses (NCPA, Templeton et al., 1995; Templeton, 1998, 2004) which has proved to be a useful technique to assess phylogeographic relationships in two other sponge species (Wörheide et al., 2002; Duran et al., 2004a). We are aware of the recent debate on the effectiveness of this inference method (see Knowles, 2008; Petit, 2008; but also Templeton, 2008, 2009) and for that reason we used NCPA as an additional and not an exclusive analytical approach to our data. In order to avoid subjective interpretations during the phylogeographic inference, the NCPA analysis was performed in ANeCA version 1.2, which is a fully automated implementation of this method (Panchal, 2007). This software uses TCS v1.21 to build a haplotype network through implementation of a statistical parsimony criterion (Clement et al., 2000), and GeoDis version 2.5 that tests for the geographical association of haplotypes through calculation of nested clade statistics and their significance (Posada et al., 2000, 2006).

Results

Sequence variation and genetic diversity

A total of 94 partial COI sequences were obtained for *P. fictitius* with an aligned length of 557 bp. These resulted in ten haplotypes defined by nine variable sites, deposited in GenBank under Accession nos. GQ273482 – GQ273491. Distances between haplotypes ranged from 0.18% to 1.28% and the highest number of substitutions found was seven (between haplotypes I and VI).

Overall haplotype and nucleotide diversities were 0.747 and 0.0042, respectively. At the regional scale, island (Macaronesian) populations revealed higher genetic diversity than their mainland (Iberian) counterparts. A gradient of lower diversity at higher latitude was observed. The northern (Galicia) and easternmost (Mediterranean) populations revealed the lowest haplotype and nucleotide diversities. At the archipelagic scale, haplotypic diversity ranged from 0 at the westernmost islands (Flores and Faial islands) to 0.509 at the eastern island group (São Miguel island) (Table 1).

Table 2. Relative frequencies of ten COI haplotypes in each of six *Phorbas fictitius* populations. Population abbreviations as in Table 1.

<table>
<thead>
<tr>
<th>population</th>
<th>haplotype</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL</td>
<td></td>
<td>0.875</td>
<td>0.125</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BER</td>
<td></td>
<td>0.333</td>
<td>0.333</td>
<td>0.333</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MED</td>
<td></td>
<td></td>
<td>0.167</td>
<td>0.333</td>
<td>0.833</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAD</td>
<td></td>
<td>0.154</td>
<td>0.077</td>
<td>0.077</td>
<td>0.692</td>
<td>0.100</td>
<td>0.100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAN</td>
<td></td>
<td>0.200</td>
<td>0.600</td>
<td></td>
<td></td>
<td>0.100</td>
<td>0.100</td>
<td></td>
<td>0.042</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZO</td>
<td></td>
<td>0.271</td>
<td>0.688</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.042</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Phylogenetic and phylogeographic relationships

The geographical distribution of haplotypes at the two spatial scales is shown in Fig. 1. Of the ten detected haplotypes, two (haplotypes I and II) are confined to the northernmost Iberian populations (Galicia and Berlengas; Fig. 1a). Haplotype III is shared between Berlengas and all the Macaronesian archipelagos (Madeira, Canaries, and Azores) and haplotype IV is shared between the latter and the only Mediterranean population (Blanes). Six private haplotypes were detected, one in the Azores (X), two in Madeira (VI and VII) and the Canaries (VIII and IX), and one in the Mediterranean (V) populations. Several haplotypes are prevalent (relative frequency >0.6) in some populations: haplotype I in Galicia, IV in the Canaries and Azores, V in the Mediterranean, and haplotype VII in Madeira (Table 2). The distribution of haplotypes at the archipelagic scale was characterized by the presence of a single (and different) haplotype on the islands of Faial (IV) and Flores (III), in the westernmost part of the archipelago (Fig. 1b).

Due to the low sequence variation, the analysis of phylogenetic relationships between haplotypes resulted in a moderately supported tree, with bootstrap values just above 60 (Fig. 2a). However, both the phylo-
Table 3. Pairwise $F_{ST}$ values (below diagonal) and gene flow estimates $M$ (above diagonal) between *Phorbas fictitius* populations at regional (Iberian/Macaronesian - top) and local (Archipelagic - bottom) scales. * significant values at $P<0.05$, ns - not significant, inf. - infinite.

### regional scale (Iberian/Macaronesian)

<table>
<thead>
<tr>
<th></th>
<th>GAL</th>
<th>BER</th>
<th>MED</th>
<th>MAD</th>
<th>CAN</th>
<th>AZO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL</td>
<td>0.991*</td>
<td>0.048*</td>
<td>0.109*</td>
<td>0.206*</td>
<td>0.176*</td>
<td></td>
</tr>
<tr>
<td>BER</td>
<td>0.335ns</td>
<td>0.137*</td>
<td>0.192*</td>
<td>0.431*</td>
<td>0.301*</td>
<td></td>
</tr>
<tr>
<td>MED</td>
<td>0.913*</td>
<td>0.785*</td>
<td>0.353*</td>
<td>0.849*</td>
<td>0.591*</td>
<td></td>
</tr>
<tr>
<td>MAD</td>
<td>0.821*</td>
<td>0.722*</td>
<td>0.586*</td>
<td>1.406*</td>
<td>1.152*</td>
<td></td>
</tr>
<tr>
<td>CAN</td>
<td>0.708*</td>
<td>0.537*</td>
<td>0.371*</td>
<td>0.262*</td>
<td>inf.</td>
<td></td>
</tr>
<tr>
<td>AZO</td>
<td>0.740*</td>
<td>0.624*</td>
<td>0.458*</td>
<td>0.303*</td>
<td>-0.017ns</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Analysis of molecular variance (AMOVA) for COI sequences of *Phorbas fictitius* at two spatial scales. At the regional scale (Iberian/Macaronesian) the Azorean populations were pooled and at the local (Archipelagic) scale only Azorean populations are considered. $Va$ and $Vb$ represent the associated covariance components. Significant values of $F_{ST}$ ($P<0.001$) are indicated with an asterisk.

<table>
<thead>
<tr>
<th>spatial scale</th>
<th>source of variation</th>
<th>d.f.</th>
<th>sum of squares</th>
<th>variance components</th>
<th>percentage of variation</th>
<th>fixation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>regional</td>
<td>among populations</td>
<td>5</td>
<td>53.456</td>
<td>0.778 $Va$</td>
<td>55.64</td>
<td>$F_{ST} = 0.556^*$</td>
</tr>
<tr>
<td></td>
<td>within populations</td>
<td>88</td>
<td>54.593</td>
<td>0.620 $Vb$</td>
<td>44.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>93</td>
<td>108.049</td>
<td>1.399</td>
<td></td>
<td></td>
</tr>
<tr>
<td>local</td>
<td>among populations</td>
<td>4</td>
<td>21.024</td>
<td>0.544 $Va$</td>
<td>71.12</td>
<td>$F_{ST} = 0.711^*$</td>
</tr>
<tr>
<td></td>
<td>within populations</td>
<td>43</td>
<td>9.494</td>
<td>0.221 $Vb$</td>
<td>28.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>47</td>
<td>30.518</td>
<td>0.765</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Nested contingency analysis of geographical association of the clades and biological inference from the NCPA. $X^2$ is the observed chi-square statistics and $P$ is the probability of a random $X^2$ being greater than or equal to the observed value after 10,000 resamples.

<table>
<thead>
<tr>
<th>clade</th>
<th>$X^2$-statistic</th>
<th>$P$</th>
<th>chain of inference</th>
<th>inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>2.363</td>
<td>0.238</td>
<td>Null hypothesis cannot be rejected</td>
<td>Moving on to next clade</td>
</tr>
<tr>
<td>1-6</td>
<td>49.632</td>
<td>0.018</td>
<td>1-2 IO</td>
<td>I-T Status Undetermined: Inconclusive outcome</td>
</tr>
<tr>
<td>2-1</td>
<td>25.743</td>
<td>0.000</td>
<td>1-2-3-5-6*-7-8 YES</td>
<td>Restricted gene flow/dispersal but with some long-distance dispersal over intermediate areas not occupied by the species; or past gene flow followed by extinction of intermediate populations.</td>
</tr>
<tr>
<td>2-2</td>
<td>47.421</td>
<td>0.000</td>
<td>1-2-11-12-13-14 NO</td>
<td>Long-distance colonisation and/or past fragmentation (not necessarily mutually exclusive).</td>
</tr>
<tr>
<td>total cladogram</td>
<td>85.903</td>
<td>0.000</td>
<td>null hypothesis cannot be rejected</td>
<td>moving on to next clade</td>
</tr>
</tbody>
</table>
genetic tree and the statistical parsimony network (Fig. 2b) showed the same topology: a clade comprising haplotypes IV, VI, VII, and X with a southern (insular) distribution and another one comprising haplotypes I and II restricted to the northern (mainland) locations. Haplotype IV, occurring in all three Macaronesian archipelagos (AZO, CAN, MAD) and in the Mediterranean population (MED) was found to be the ancestral haplotype it yield the highest outgroup probability (0.230).

The statistical parsimony analysis revealed a network with haplotypes III and IV in a central position and from which all other haplotypes derive by few mutations. The nested clade design estimated seven 1-step clades and three 2-step clades. Significant associations between haplotypes and geographical distribution were found at several levels but inferences were inconclusive except for two 2-step level clades. Restricted gene flow/dispersal but with some long-distance dispersal over intermediate areas not occupied by the species or past gene flow followed by extinction of intermediate populations was inferred between the mainland populations of Galicia and Berlengas and the Macaronesian islands (clade 2-1), whereas long distance colonization and/or past fragmentation was the process inferred between the islands and Mediterranean populations (clade 2-2) (Fig. 2c, Table 5).

**Differentiation and structure at regional and local scales**

The genetic structure of *P. fictitius* populations was first assessed by pairwise F$_{ST}$ and gene flow estimates (Table 3). Significant high levels of genetic differentiation were found between most population pairs at the regional spatial scale. The northernmost population (GAL) showed the highest differentiation to all other populations (F$_{ST}$>0.7) with the exception of Berlengas (500 km to the South), to which it showed no significant differentiation. The lowest differentiation (F$_{ST}$<0.4) was found among the Macaronesian archipelagos (AZO/MAD/CAN) with the Azorean populations showing no significant differentiation to the Canarian ones.

At the archipelagic scale, two contrasting results stand out: high F$_{ST}$ values between populations that are only tens of kilometres apart (F$_{ST}$>0.5 between SMA/FOR and SMG) versus non-significant differentiation between populations that are hundred’s of kilometres apart (for instance, F$_{ST}$ = 0.207 between SMG and FLW that are over 500 km apart). The low sequence variation and limited sampling may cause an overestimation of the F$_{ST}$ values and therefore these have to be regarded cautiously. However, population differentiation is evident from the geographical distribution and frequency of the haplotypes. Structure at both spatial scales was further confirmed by the AMOVA results (highly significant F$_{ST}$ values; Table 4). At the Iberian scale, variation was similar within and among populations (F$_{ST}$≈0.5) while at the archipelagic scale 71% of the total variation was found among islands.

The Mantel test, performed at the regional scale, revealed a non-significant trend (r = 0.287, P = 0.209) of increasing genetic differentiation with increasing geographical distance between populations. The same test was again not significant at the archipelagic scale (r = 0.266, P = 0.150) and therefore the observed genetic patterns could not be explained by a model of isolation by distance.

**Discussion**

**Sequence variation and the use of an alternative partition of COI**

Our results confirm the previously reported low sequence variation for mtDNA in sponges. Nonetheless, the overall nucleotide diversity (π = 0.0042) found in the ‘I3-M11’ partition in our study was higher but of the same magnitude as the value found for the giant barrel sponge in the Caribbean (*Xestospongia muta*, π = 0.0039, López-Legentil and Pawlik, 2009) using this same partition, and much higher than the values found in Folmer’s COI partition in several species at similar but also larger spatial scales (*Crambe crambe*, π = 0.0006, Duran *et al.*, 2004c; *Astrosclera willeyana*, π = 0.00049, Wörheide, 2006; *Xestospongia muta*, π = 0.00058, López-Legentil and Pawlik, 2009).

The intraspecific variation (1.28%) found in our study is similar to the one found in *Xestospongia muta* I3-M11 (0.92%, López-Legentil and Pawlik, 2009) and much higher than the values found in Folmer’s COI partition for both *X. muta* and *C. crambe* (0.18%, López-Legentil and Pawlik, 2009; and 0.19%, Duran *et al.*, 2004c, respectively). Our findings therefore support that this alternative partition of the COI gene, located downstream of Folmer’s partition, is indeed more suitable to infer interspecific relationships in sponges as initially suggested by Erpenbeck *et al.* (2006) and even for intraspecific studies but for species with somewhat deeper phylogeographic histories.
Furthermore, the use of taxon-specific primers has obvious methodological advantages over the use of ‘universal’ primers such as those of Folmer (Folmer et al., 1994), particularly in groups like sponges that are known to host diverse microbial communities (Hentschel et al., 2003). The primers developed by us amplify a large range of sponge species belonging to different orders and therefore we highly recommend its use for lower level phylogenetic studies in this taxonomic group.

Structure of P. fictitius at regional and local scales

Despite the low sequence variation, we found P. fictitius to have highly structured populations at both regional and local spatial scales, as evidenced by the pairwise Fst values and the AMOVA results. This structure is consistent with the low dispersal potential and bathymeric range of the species. Previous studies have shown sponge larvae to be philopatric and to recruit at short distance from the parental locations (Mariani et al., 2005, 2006). Furthermore P. fictitius is a shallow-water species inhabiting rocky habitats down to 50 m depth and therefore oceanic depths may constitute a strong barrier to gene flow and range expansion in this species. Similar evidence of genetically structured populations has been found for the demosponge C. crambe at comparable spatial scales in the same area (Duran et al., 2004a, b). The same pattern was also observed in the giant barrel Xestospongia muta in the Caribbean (López-Legentil and Pawlik, 2009) and the common reef sponge Callyspongia vaginalis along the Florida reef tract (DeBiasse et al., 2010).

At the archipelago scale, we found a very patchy distribution of mtDNA haplotypes. However, the differentiation that we found between most population pairs even at spatial scales of the order of tens of km suggests structured and therefore non-paenmicic archipelagic populations that would otherwise exhibit more homogenous haplotype distribution and frequency. The absence of genetic diversity in the populations from Flores and Faial islands may indicate a recent expansion of the species, via a founder event, to the westernmost part of the archipelago or a population bottleneck. However, sampling of these islands was limited (n = 6 and n = 7) and therefore variation could have been missed. A more intensive sampling in these and the remaining islands will be necessary to confirm whether habitat discontinuity by the deep-sea promotes genetic subdivision in shallow-water species of island ecosystems as proposed by some authors. Such patterns of island subdivision have been found for the antherinid fish Craterocephalus capreolus (Johnson et al., 1994) and the intertidal snail Austrocochlea constricta in the Houtman Abrolhos Islands (Johnson and Black, 2006). Contrastingly, no genetic differentiation was found among island populations of the blackbelly rosefish Helicolenus dactylopterus, in the Azores, given the continuity of its deep-sea habitat (Aboim et al., 2005). These examples emphasize the complex interplay between intrinsic biological and ecological traits (e.g. dispersal potential, geographic and bathymetric range, substrate preference) and extrinsic present and past environmental factors (e.g. habitat continuity, geographical distance, bathymetry, prevailing surface circulation) on the structuring of the populations at diverse spatial scales.
Phylogeography of *P. fictitius*

Although only moderately supported, the phylogenetic reconstruction of haplotypes and the parsimony network reveals the existence of insular (Macaronesian) and mainland (Iberian) clades with only two haplotypes (III and IV) shared between these locations. The level of differentiation that we found in our study reflects the high degree of isolation among island and mainland populations of *P. fictitius*. A comparable isolation between the Macaronesian islands and the continental shores has been previously reported for the perciform triplefin *Tripterygion delaisi* (Domingues et al., 2004a) and for several limpet species of the genus *Patella* (Sá-Pinto et al., 2008).

Some important attributes of haplotype networks, derived from coalescent theory, have shown that haplotypes with an interior position in the network are older than haplotypes on the tips (network age polarity) and that older haplotypes are more widespread than younger haplotypes under a restricted gene flow model (haplotypes geographical range and frequency) (Castelloe and Templeton, 1994; Templeton et al., 1995; Templeton, 1998). Haplotype IV having a central position in the network and being the most geographically spread was found to be the oldest. Haplotype III although having a lower outgroup weight (0.110) is also in a central position and is equally widely distributed. Given the prevalence of these ancestral haplotypes and the highest genetic diversity observed in all island populations it seems plausible to assume these archipelagos as the putative origin of the species with posterior expansion via current mediated dispersal of larvae or sponge fragments to mainland locations (haplotype geographical range and frequency) (Castelloe and Templeton, 1994; Templeton et al., 1995; Templeton, 1998). Haplotype IV having a central position in the network and being the most geographically spread was found to be the oldest. Haplotype III although having a lower outgroup weight (0.110) is also in a central position and is equally widely distributed. Given the prevalence of these ancestral haplotypes and the highest genetic diversity observed in all island populations it seems plausible to assume these archipelagos as the putative origin of the species with posterior expansion via current mediated dispersal of larvae or sponge fragments to mainland locations (haplotype geographical range and frequency) (Castelloe and Templeton, 1994; Templeton et al., 1995; Templeton, 1998). Haplotype IV having a central position in the network and being the most geographically spread was found to be the oldest. Haplotype III although having a lower outgroup weight (0.110) is also in a central position and is equally widely distributed.

The Macaronesian refugium

The Pleistocene glaciations, and in particular the Last Glacial Maximum, are known to have shaped the present-day distribution and genetic structure of both terrestrial and aquatic biota in the Northeast Atlantic and Mediterranean areas. Current models of glacial refugia use genetic diversity estimates, the spatial distribution and relative ages of haplotypes to identify refugial and expansion areas. Refugia are usually characterized by possessing the highest genetic diversity (except in cases of contact zones) and by a mixture of ancestral and private haplotypes, while expansion areas are usually genetically depauperate and composed of a subset of the refugial gene pool (Hewitt, 2000; Maggs et al., 2008).

Based on these premises the Macaronesian islands have been proposed as an offshore refugium for several marine organisms such as the pomacentrid *Chromis limbata* (Domingues et al., 2006), the white seabream *Diplodus sargus* (Domingues et al., 2007c), the blennids *Coryphoblennius galerita* (Domingues et al., 2007b) and *Parablennius parvicornis* (Domingues et al., 2008), the thornback ray *Raja clavata* (Chevolot et al., 2006) as well as for several species of the *Patella* genus (Sá-Pinto et al., 2008). This is the result of largely stable climatic conditions experienced by these archipelagos during the Pleistocene glaciations (see Crowley, 1981; Pflaumann et al., 2003, Hayes et al., 2005).

From the geographical distribution of mtDNA haplotypes of *P. fictitius* two observations stand out: (a) highest genetic diversity at the Macaronesian archipelagos and a latitudinal gradient in diversity (highest diversity at southern locations) and (b) high frequency of ancestral (haplotype IV) and private haplotypes at
all three Macaronesian archipelagos. Combined, these observations suggest the Macaronesian islands may have served as putative offshore refugia for *P. fictitius* populations. However, data from additional Mediterranean and northern European populations would be required to further corroborate this hypothesis.

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