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CHAPTER 6

PHYLOGEOGRAPHY OF THE HAPLOSCLERID SPONGE
Petrosia ficiformis (Porifera, Petrosiidae)
REVEALS A DEEP-WATER AFFINITY OF SOME
SHALLOW-WATER POPULATIONS

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Manuscript in preparation
ABSTRACT

In this study we assessed sequence variation of two mitochondrial DNA gene fragments (COI and Atp8) and a newly developed nuclear ribosomal marker (the 3′ end of 28S and part of the 28S-18S intergenic spacer) in nine shallow-water populations of the haplosclerid sponge *Petrosia ficiformis*. The newly developed marker (rDNA 28S-IGS) showed much higher rates of sequence variation (\(\pi_{28S-IGS}=0.01477\)) than the values observed for the mitochondrial genes (\(\pi_{COI}=0.00060\) and \(\pi_{Atp8}=0.00046\)) thus exhibiting higher phylogeographic resolution. We used a multiple approach (diversity measures, phylogenetic inference, and nested clade phylogeographic analysis) to assess the genetic structure and elucidate the evolutionary history of this species throughout its Atlanto-Mediterranean distribution range, spanning over 5,000 km. Phylogenetic and phylogeographic reconstructions revealed the existence of two divergent lineages with different biotic affinities: a shallow-water lineage widely distributed in the Mediterranean and at the Macaronesian islands of Madeira and Canaries, and a putative deep-water lineage occurring in the northwest Mediterranean (in sympatry with the shallow-water lineage) and in the Azores archipelago. This study further revealed highly structured populations affected by restricted gene flow with isolation by distance within each basin as well as allopatric fragmentation between Mediterranean (mainland) and Atlantic (island) populations. A contiguous range expansion across the species geographical range was inferred. The high genetic diversity and the presence of ancestral sequence-types in the eastern Mediterranean suggest this basin as the origin of the species. The genetic diversity patterns observed are consistent with the effects of the Pleistocene glaciations and are likely the result of the climatic changes at the Last Glacial Maximum. The results found for *P. ficiformis* are a good example of the interplay between historical and contemporary factors in shaping the genetic structure of marine benthic species.

**KEYWORDS:** Atlanto-Mediterranean, phylogeography, mtDNA, rDNA, intergenic spacer, nested clade analysis
INTRODUCTION

Understanding the spatial patterns of genetic diversity and both the historical and contemporary factors that have shaped such genetic structure is crucial for the development of effective conservation strategies in the increasingly threatened marine realm. The Northeast Atlantic and Mediterranean region provides an interesting as well as challenging model area to study these topics for several reasons: it presents an extremely diverse and relatively well studied biota (e.g. Bianchi & Morri, 2000); it encompasses a wide range of subtropical, temperate, and subarctic climatic conditions; it possesses some distinguishable 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 (e.g. dispersal ability, trophic niche), 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). Besides providing important insights into the tempo and mode of marine speciation (e.g. Domingues et al., 2005, 2007c), the results of such studies may have profound implications for the design of marine reserves (Palumbi, 2003; Pérez-Ruzafa et al., 2006), the protection of threatened species (Gilles et al., 2000), or the management of commercially exploited ones (Lundy et al., 1999; Magoulas et al., 2006; Pérez-Losada et al., 2007).

Despite the remarkable sponge diversity of the northeast Atlantic and Mediterranean (700+ shallow-water species; Xavier & Van Soest, submitted; Chapter 2) information on the phylogeography and population structure is available for only three sponge species, viz. the poecilosclerids *Crambe crambe* (Duran et al., 2004a, b, c; Calderón et al., 2007) and *Phorbas fictitius* (Xavier et al., submitted; Chapter 5), and the halichondriid *Scopalina lophyropoda* (Blanquer, 2007; Blanquer et al., 2009). In a previous study on the intraspecific variability levels of *C. crambe*, based on sequences of the rDNA internal transcribed spacers (ITS-1 and ITS-2), Duran and co-workers found highly structured populations
due to restricted gene-flow with isolation by distance, as well as evidences of a range expansion of the species as a consequence of human-mediated colonization of the Macaronesian islands (Duran et al., 2004b). Microsatellite genotyping of the same populations confirmed these patterns and further showed that clonality, although present, is not an important structuring factor in this species at the considered scale (Duran et al., 2004c). Similar patterns of structured populations, isolation by distance, and negligible effects of clonality emerged from a microsatellite analysis in *S. lophyropoda* populations along the NW Mediterranean coasts of Spain and France (Blanquer, 2007).

The present study aims to examine the phylogeography and population structure of another species from a different group of sponges (order Haplosclerida, family Petrosiidae) which exhibits a geographical distribution very similar to that of *C. crambe*. *Petrosia ficiformis* (Poiret, 1789) is a shallow-water species with a subtropical affinity, widely distributed throughout the Mediterranean and the adjacent Atlantic Ocean at the Macaronesian islands. It is an oviparous species in which the oocytes are spawned in a very short period of 1-2 days. Once fertilized externally, the eggs develop into a non-swimming larval stage (ciliated embryos) that promptly settles on the substrate (Ana Riesgo, pers. commun. 2008; Maldonado & Riesgo, in press). These characteristics suggest that *P. ficiformis* larvae have low dispersal capability and therefore highly structured populations are expected to occur in this species. We tested two mitochondrial [cytochrome oxidase subunit I (COI) and ATP synthase subunit 8 (Atp8)] gene fragments and developed a new nuclear ribosomal marker (the 3’ end of 28S and part of the 28S-18S intergenic spacer, abbreviated as 28S-IGS) and combined it with a multi-analytical approach (genetic diversity measures, phylogenetic inference, and nested clade phylogeographic analysis) to investigate the role of contemporary and historical processes in shaping the relationships between *P. ficiformis* populations across its Atlanto-Mediterranean geographical range spanning over 5,000 km from the eastern Mediterranean (Crete) to the Macaronesian islands of the Azores.

**MATERIAL AND METHODS**

*Sampling*

Specimens of *P. ficiformis* (N=118) were collected by scuba diving at depths of 5-25 m at nine locations throughout the species distribution range in the
Mediterranean, Adriatic, and Aegean Seas as well as the adjacent Atlantic (Macaronesian islands) (Fig. 1). Two specimens of the congener deep-sea species Petrosia crassa (Carter, 1876) were collected by dredge at depths between 200 and 400 m in the Norwegian Korsfjord, and used as outgroup. Voucher samples were preserved in 96% ethanol and deposited in the Porifera collection of the Zoological Museum of Amsterdam (ZMAPOR). Sample fragments taken for genetic analyses were kept at -10°C until further processing.

**Figure 1.** Geographical distribution and relative frequencies of 28S-IGS rDNA sequence-types. For location codes see Table 1.

**DNA extraction, amplification, and sequencing**

Total DNA was extracted using DNeasy® Tissue kit (QIAGEN) following the manufacturer’s instructions. Two mitochondrial (COI, Atp8) and one nuclear ribosomal (28S-IGS) gene fragments were amplified and sequenced. The alternative partition of the COI gene that overlaps approximately 60 base pairs with Folmer’s 3’ partition (Folmer et al., 1994) and includes Erpenbeck’s ‘I3-
M11’ (Erpenbeck et al., 2006), was amplified and sequenced using the sponge-specific primers PorCOI2fwd (5’–AATATGNGGGCNCNCNGGNATNAC–3’) and PorCOI2rev (5’– ACTGCCCCCATNGATAAAAACAT–3’) (Xavier et al., submitted b; Chapter 4). The Atp8 gene was amplified using primers ds-cox2-f1 (5’-TGGNGCAAATCATTCNTTTATGC–3’) and ds-atp6-r1 (5’–CTACATTAAATTGATCAAAATANGC–3’) (Lavrov et al., pers. comm.).

Because mitochondrial genes in Porifera are known to exhibit extremely low variability (Duran et al., 2004a; Wörheide, 2006), we additionally searched for a new marker that could address the genetic structure at the population level. The ribosomal DNA array of a eukaryote nuclear genome is typically composed of several hundreds of tandemly repeated copies of the transcription unit that comprise the 18S coding region, the internal transcribed spacer 1 (ITS-1), the 5.8S coding region, internal transcribed spacer 2 (ITS-2), and the 28S coding region. These units are separated by the intergenic spacer (IGS) and the external transcribed spacer (ETS) (Fig. 2). The intergenic spacer, also known as non-transcribed spacer, is the most rapidly evolving region of the rDNA (Weider et al., 2005). Therefore, GenBank sequences of P. ficiformis 28S (AF441347) and Petrosia sp. 18S (DQ927321) were used to develop a primer pair to amplify a region spanning the 3’ end of the 28S gene and the complete IGS region (Pfici28Sfwd 5’–CACACTTGTCGTCGGGTTCGCTGC–3’ and Pfici18Srev 5’-GCATGGCTTAGTCTTTGAGACAAGC–3’). The 5’ and 3’ ends of the amplified product were sequenced and used to design a new internal primer set (PficiIGSfwdB 5’–TGTCAGAAAAGTTACCACAGG-3’ and PficiIGSrevB 5’–TTGCACAACATCTGCTATTTTC-3’) and this primer set was then used for subsequent amplification and sequencing of all studied individuals.

![Figure 2. Organization of the rDNA repeat in eukaryotic organisms. Boxes indicate coding regions. The grey line represents the 28S-IGS fragment studied.](image-url)
Amplifications were carried out in 20 μl reaction volume containing 2 μl of 10x buffer (Sphaero Q), 4 μl dNTPs (1 mM), 0.5 μl BSA (20 mg/ml), 0.5 μl MgCl₂ (25 mM), 0.3 μl (5 U/μl) of Taq polymerase (Sphaero Q), 0.6 μl of each primer (10 μM), and 2 μl of DNA. The amplification profile for COI and Atp8 followed an initial denaturing step of 95 °C for 3 min, 36 cycles [94 °C for 30 s, 57 °C (COI) / 45 °C (Atp8) for 45 s, and 70 °C for 90 s], and a final extension of 72 °C for 10 min. The amplification profile for the 28S-IGS fragment was as follows: initial denaturing step of 94 °C for 3 min, 34 cycles (94 °C for 30 s, 60 °C for 40 s, and 72 °C for 2 min) 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 (Applied Biosystems), and purified products were directly sequenced in both directions on an ABI 3700 automated sequencer at the Amsterdam Academic Medical Centre.

**Genetic diversity and structure**

Pairwise and multiple alignments were performed and checked in BioEdit (Version 7.0.0; Hall 1999). Sequence-type and nucleotide diversity were calculated for each population in DnaSP (version 4.0; Rozas et al., 2003). In order to assess hierarchical population structure, analysis of molecular variance (AMOVA) was performed by both pooling populations into Atlantic and Mediterranean, and without population pooling. A Mantel test was applied on the pairwise genetic and geographical distance matrices in order to test for isolation by distance. All analyses were implemented in Arlequin v3.1 (Excoffier et al., 2005).

**Phylogenetic and phylogeographic relationships**

Phylogenetic reconstruction of the 28S-IGS sequence types was performed under maximum likelihood (ML) on the nucleotide data set implemented in PAUP* v. 4.0b10 (Swofford, 2002). The likelihood settings for ML analysis were derived from Modeltest 3.06 (Posada & Crandall, 1998) under the Akaike Information Criterion (AIC) as the best-fitting model. Topological confidence of the trees was assessed by heuristic search with 500 bootstrap replicates of stepwise addition with tree bisection and reconnection branch swapping (TBR). The MEGA software (Kumar et al., 2004) was used to calculate p-distances between sequence-types.
CHAPTER 6

To infer the pattern of historical processes that may have shaped the species’ current distribution range, we performed nested clade phylogeographic analyses (NCPA; Templeton et al., 1995, 1998; Templeton, 2004) which has proved to be a useful technique to resolve phylogeographic relationships at large spatial scales in two other sponge species (Duran et al., 2004b; Wörheide et al., 2002). 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 and errors during the phylogeographic inference, the NCPA analysis was performed in ANeCA v.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 v2.5. which tests for the geographical association of haplotypes through calculation of nested clade statistics and their significance (Posada et al., 2000; Posada et al., 2006).

RESULTS

Genetic diversity and population structure

We obtained sequences of COI (574 bp) and 28S-IGS (553 bp) for all 118 ingroup and two outgroup specimens plus 75 sequences of Atp8 (414 bp). The poriferan origin of our sequences was confirmed through BLAST searches in GenBank which revealed best matches of our COI, Atp8, and 28S-IGS data with sequences of three other haplosclerid species, viz. *Xestospongia muta* (EU237490), *Callyspongia plicifera* (EU237477), and *Amphimedon queenslandica* (EF654518).

The region comprising the mitochondrial ATP synthase subunit 8 gene (Atp8), although easily amplified and sequenced, exhibited almost no sequence variation (only one variable site was observed). The two haplotypes result from an A-C transversion at position 278 of the fragment. This silent mutation (both TCA and TCC encode the amino acid serine) separates haplotype A2, comprising some specimens from Catalunya in the NW Mediterranean, from the remaining Atlanto-Mediterranean specimens (A1). The two specimens of the deep-sea *P. crassa* resulted in haplotype A3 which is 3 bp different from A2 and 2 bp different from A1. Similarly, sequences of the ‘I3-M11’ COI partition of *P. ficiformis* translated in only two haplotypes that result from a transition of cytosine to
thymine (position 145 of the alignment) in all Azorean specimens. This is again a silent mutation as both triplets (CTA and TTA) encode for leucine. Haplotype C1 comprised all Azorean specimens while haplotype C2 comprised the remaining Atlanto-Mediterranean specimens. The outgroup *P. crassa* was represented by a single haplotype (C3) that is 4 bp different from the Azorean haplotype and 3 bp different from the Atlanto-Mediterranean haplotype (Table 1). Given the low sequence variation observed at both mitochondrial genes we restricted our population analyses to the rDNA 28S-IGS fragment.

Table 1. *Petrosia ficiformis* and *Petrosia crassa* mitochondrial DNA haplotypes and their polymorphic sites.

<table>
<thead>
<tr>
<th>Polymorphic positions</th>
<th>COI</th>
<th>88</th>
<th>145</th>
<th>339</th>
<th>417</th>
<th>Atp8</th>
<th>213</th>
<th>218</th>
<th>278</th>
<th>391</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
<td>C</td>
<td>T</td>
<td>A</td>
<td>A</td>
<td>A1</td>
<td>T</td>
<td>A</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>A</td>
<td>A2</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>C3 - <em>P. crassa</em></td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A3 - <em>P. crassa</em></td>
<td>A</td>
<td>T</td>
<td>A</td>
<td>C</td>
</tr>
</tbody>
</table>

Sequences of the 28S-IGS region resulted in 12 sequence-types defined by 22 variable sites (Table 2). The geographical distribution of the 28S-IGS sequence-types and their frequency across the studied area are shown in Fig. 1 and Table 3. Overall sequence-type and nucleotide diversities found for *P. ficiformis* were 0.842 and 0.0148, respectively. The highest values of sequence-type diversity were observed in the populations of the Adriatic Sea (SLO, CRO) as well as the population from Madeira island (MAD). The northwestern Mediterranean populations of Catalunya (CAT) and Liguria (LIG), despite low sequence-type diversity, exhibited the highest nucleotide diversity (Table 4).

Analysis of molecular variance (AMOVA) detected significant structure at all hierarchical levels. When populations were divided into Atlantic and Mediterranean, variation among groups accounted for 21%, whereas variation among populations and within populations accounted for 42 and 37% of the total variance, respectively. When the same analysis was performed without grouping of populations, variation among populations explained 59% of the total variance. All fixation indices were statistically significant at P<0.001 or P<0.05 (Table 5). There was a significant correlation between genetic and geographical distances (Mantel test: r=0.494, P=0.002).
Phylogenetic and phylogeographic relationships

Phylogenetic reconstructions of the rDNA sequence-types revealed a strongly supported clade comprising S1-S9 (bootstrap 100%) with a sister group relationship to S10. Some further sub-structuring was observed with two sub-clades containing S2-3 and S5-7 but these were only moderately supported (bootstrap values below 65%). Sequence-types S10 and S11 appear in the ML tree much closer to the outgroup P. crassa (S12) than to the other P. ficiformis sequence-types (Fig. 3). The statistical parsimony analysis revealed a network with three
Table 4. Diversity measures (28S-IGS rDNA) for *Petrosia ficiformis* populations. Population code (Pc), sample size (N), number of sequence-types (Ng), sequence-type diversity (Gd), and nucleotide diversity (π) are presented. Standard deviations for Gd and π are given in parenthesis.

<table>
<thead>
<tr>
<th>Population</th>
<th>Sampling location</th>
<th>Pc</th>
<th>N</th>
<th>Ng</th>
<th>Gd</th>
<th>π</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azores</td>
<td>Flores and S. Miguel Island</td>
<td>AZO</td>
<td>25</td>
<td>1</td>
<td>0.000 (0.000)</td>
<td>0.00000 (0.00000)</td>
</tr>
<tr>
<td>Canaries</td>
<td>Tenerife Island</td>
<td>CAN</td>
<td>8</td>
<td>1</td>
<td>0.000 (0.000)</td>
<td>0.00000 (0.00000)</td>
</tr>
<tr>
<td>Madeira</td>
<td>Madeira Island</td>
<td>MAD</td>
<td>13</td>
<td>3</td>
<td>0.692 (0.075)</td>
<td>0.00264 (0.00031)</td>
</tr>
<tr>
<td>Catalunya</td>
<td>Blanes, SE Spain</td>
<td>CAT</td>
<td>12</td>
<td>2</td>
<td>0.485 (0.106)</td>
<td>0.01315 (0.00287)</td>
</tr>
<tr>
<td>Marseille</td>
<td>Grote a coral</td>
<td>MAR</td>
<td>12</td>
<td>1</td>
<td>0.000 (0.000)</td>
<td>0.00000 (0.00000)</td>
</tr>
<tr>
<td>Ligurian Sea</td>
<td>Portofino</td>
<td>LIG</td>
<td>15</td>
<td>2</td>
<td>0.419 (0.113)</td>
<td>0.01137 (0.00307)</td>
</tr>
<tr>
<td>nAdriatic</td>
<td>Slovenian coast</td>
<td>SLO</td>
<td>13</td>
<td>4</td>
<td>0.795 (0.059)</td>
<td>0.00264 (0.00029)</td>
</tr>
<tr>
<td>sAdriatic</td>
<td>Croatian coast</td>
<td>CRO</td>
<td>13</td>
<td>3</td>
<td>0.667 (0.078)</td>
<td>0.00190 (0.00020)</td>
</tr>
<tr>
<td>Aegean Sea</td>
<td>Crete</td>
<td>CRE</td>
<td>7</td>
<td>3</td>
<td>0.524 (0.209)</td>
<td>0.00138 (0.00059)</td>
</tr>
<tr>
<td>Total</td>
<td>All populations</td>
<td></td>
<td>118</td>
<td>11</td>
<td>0.842 (0.016)</td>
<td>0.01477 (0.00034)</td>
</tr>
</tbody>
</table>

Table 5. Analysis of molecular variance (AMOVA) for 28S-IGS sequences of *Petrosia ficiformis*. Groups correspond to Atlantic and Mediterranean populations. Va, Vb, and Vc represent the associated covariance components.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>% of variation</th>
<th>Fixation indices1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMOVA with 2 groups (Atl/Med)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among groups</td>
<td>1</td>
<td>9.563</td>
<td>0.10613 Va</td>
<td>21.19</td>
<td>F Cait= 0.21185*</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>7</td>
<td>19.571</td>
<td>0.21049 Vb</td>
<td>42.02</td>
<td>F Scai= 0.53310**</td>
</tr>
<tr>
<td>Within populations</td>
<td>109</td>
<td>20.095</td>
<td>0.18435 Vc</td>
<td>36.8</td>
<td>F Stai= 0.63201**</td>
</tr>
<tr>
<td>AMOVA without groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among populations</td>
<td>8</td>
<td>29.134</td>
<td>0.26827 Va</td>
<td>59.27</td>
<td>F Stai= 0.59270**</td>
</tr>
<tr>
<td>Within populations</td>
<td>109</td>
<td>20.095</td>
<td>0.18435 Vb</td>
<td>40.73</td>
<td></td>
</tr>
</tbody>
</table>

1Significance: *P<0.05, **P<0.001.

major branches diverging from S4 which has a central position in the network and is widely distributed in the Mediterranean. The remaining eastern Mediterranean sequence-types (S5-S9) are connected by one or two mutational steps to S4. Sequence-type 6 connects the Mediterranean with those present in the adjacent Macaronesian islands of Madeira and Canaries (S1-S3). This group (S1-S9), hereafter referred to as the shallow-water lineage, is then connected by a long branch (9 to 14 mutational steps) to another group comprising sequence-type 10
(exclusively present on the Azorean islands) and sequence-type 11 that occurs in the NW Mediterranean (CAT, MAR, LIG). Sequence-types 10 and 11 are, respectively, five and four mutational steps away from the outgroup *P. crassa* sequences, and compose a lineage that is hereafter called the deep-sea lineage (Fig. 4a).

The nested clade design estimated twelve 1-step clades, six 2-step clades, and three 3-step clades (Fig 4b). Significant associations between sequence-types and geographical distribution were found at 2- and 3-step levels as well as for the entire cladogram. Restricted gene flow with isolation by distance were inferred in two 2-step clades (clades 2-1 and 2-2) involving the populations in the eastern Mediterranean and the Madeira/Canary islands, respectively, while allopatric fragmentation was shown to be the process involved in higher 3-step clades (clades 3-1 and 3-2). A contiguous range expansion of the species was the process inferred for the total cladogram (Table 6).

**Figure 3.** Majority-rule consensus tree based on maximum likelihood (ML) reconstruction of the 11 rDNA sequence types of *P. ficiformis* (rooted with sequences of *P. crassa* S12). Bootstrap values above 50% are given above or below branches. For sequence-type labels see Table 2.
**Figure 4.** Statistical parsimony network of the 28S-IGS rDNA sequence-types of *Petrosia ficiformis*: (a) topology of the network and relative frequency (size of the polygons) of each sequence-type (see also Table 3); (b) nested clade design. For sequence-type labels see Table 2. The sequence-type S12 corresponds to the outgroup *P. crassa*.

**Table 6.** 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>Prob</th>
<th>Chain of inference</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-6</td>
<td>14.0000</td>
<td>0.0028</td>
<td>1-2 IO</td>
<td>I-T Status Undetermined: Inconclusive outcome</td>
</tr>
<tr>
<td>1-10</td>
<td>0.4762</td>
<td>1.0000</td>
<td>Null hypothesis cannot be rejected</td>
<td>Moving on to next clade</td>
</tr>
<tr>
<td>2-1</td>
<td>12.5192</td>
<td>0.0098</td>
<td>1-2-3-4 NO</td>
<td>Restricted gene flow with isolation by distance</td>
</tr>
<tr>
<td>2-2</td>
<td>6.4615</td>
<td>0.0184</td>
<td>1-2-3-4 NO</td>
<td>Restricted gene flow with isolation by distance</td>
</tr>
<tr>
<td>3-1</td>
<td>52.0000</td>
<td>0.0000</td>
<td>1-19 NO</td>
<td>Allopatric fragmentation</td>
</tr>
<tr>
<td>3-2</td>
<td>56.0000</td>
<td>0.0000</td>
<td>1-19 NO</td>
<td>Allopatric fragmentation</td>
</tr>
<tr>
<td>Total cladogram</td>
<td>148.1035</td>
<td>0.0000</td>
<td>1-2-11-12 NO</td>
<td>Contiguous range expansion</td>
</tr>
</tbody>
</table>
DISCUSSION

Sequence variation in mtDNA and rDNA

The sequence variation found in the I3-M11 partition of COI of *P. ficiformis* ($\pi = 0.00060$) was smaller than that observed in the same partition at the intraspecific level in other sponges: 0.00386 in *Xestospongia muta* (López-Legentil & Pawlik, 2009), 0.0042 in *Phorbas fictitus* (Xavier et al., submitted a; Chapter 4), and between 0.00084 and 0.00464 in species of the *Cliona aff. celata* complex (Xavier et al., submitted b; Chapter 3). It was similar though to variation levels observed in Folmer’s COI partition of *C. crambe* ($\pi = 0.0006$; Duran et al., 2004), *Astrosclera willeyana* (0.00049; Wörheide, 2006) and *Xestospongia muta* (0.00058; López-Legentil & Pawlik, 2009). Variation found in the fragment containing the Atp8 gene ($\pi = 0.00046$) was similar to that found in COI and again lower than the values found in species of the *C. aff. celata* complex ($0< \pi<0.00465$; Xavier et al., submitted b).

Genetic divergence (p-distances) between A1 and A2 was 0.2%, while between these and the outgroup *P. crassa* divergence varied between 0.7 and 1.0%. Divergence among C1 and C2 was 0.2%, whereas between these and the outgroup *P. crassa* varied between 0.5 and 0.7%. These intraspecific divergence values are similar to those found for other sponge species (0.16% in *Tedania ignis*, Wullf, 2006; 0.41% in *Astrosclera willeyana*, Wörheide, 2006; 0.1% to 0.5% in *C. aff. celata* species, Xavier et al., submitted b; Chapter 3). The interspecific divergence (0.5-0.7%) between *P. ficiformis* and *P. crassa* is moderate but conforms to that found among two other congeneric petrosiids, *Xestospongia muta* and *X. proxima* whose COI divergence is 0.9% (calculated from EU237490 and AM076980 available from GenBank).

Contrastingly, sequence variation observed in the newly developed 28S-IGS marker ($\pi = 0.01477$) was much higher than the value observed for the mitochondrial genes and is of the same order of magnitude as the variation found in the ITS spacers in other sponge species (e.g. *Leucetta chagosensis* ITS, $\pi = 0.00863$; Wörheide et al. 2008). This marker proves therefore more suitable to examine population level relationships than COI or Atp8. Considering the above, it can be concluded that clear differences in the evolutionary rates exist, not only between different markers but also between different species for the same marker (Ho et al., 2005; Erpenbeck et al., 2008).
We found no evidence of intragenomic variation in the 28S-IGS marker in *P. ficiformis* but this will have to be further investigated with cloning of amplified products. The different regions of the rDNA are under different levels of selective constraint and this makes them to vary considerably in their rates of divergence among species and in their levels of intraspecific and intragenomic polymorphism (Weider et al., 2005; Wörheide et al., 2004). The primers developed in this study, along with the existent primers for the region spanning from the 18S to the 28S gene including the internal transcribed spacers 1 and 2 (e.g. primers RA2 and ITS2.2, Wörheide et al., 2002), now provide an excellent toolkit to assess the level of intragenomic, intraspecific, and interspecific variation across the entire ribosomal array in Petrosiidae.

*Phylogenetic relationships among sequence-types*

One of the most remarkable results of this study was the discovery of a putative deep-water affinity of some specimens occurring in the northwest Mediterranean and Azores shallow-water populations. Two *P. ficiformis* sequence-types, namely S10 exclusive to the Azorean specimens and S11 occurring in the NW Mediterranean, seem to be closer related to the deep-sea *P. crassa* than to the other shallow-water specimens. This affiliation is further corroborated by the fact that specimens from Marseille (S11) were collected in a cave, an environment with characteristically deep-sea conditions and fauna (Vacelet et al., 1994; Vacelet, 1996; Harmelin & Vacelet, 1997). Particularly for the Azores, these findings provide new insights into the biogeographical affinities of its marine biota, the origin of which remains rather enigmatic (Morton & Britton, 2000). Indeed the study of the phylogenetic affinities of shallow-water species may reveal a so far unacknowledged colonization of shallow-water ecosystems in this remote archipelago by bathymetric expansion of deep-water species. Confirmation of such a scenario will require further sequencing of other petrosiids known to occur in this region’s deep-water (Topsent, 1904, 1928).

Intriguingly, the specimens of the deep-water lineage (S10, S11) share their mtDNA haplotypes with the remaining shallow-water *P. ficiformis* and are differentiated (even if by few mutations) from the deep-sea species *P. crassa*, conferring some uncertainty to their phylogenetic affinity. The presence of this divergent genotype in the rDNA, with a putative deep-sea origin, in some of the investigated shallow-water populations could be the result of several evolutionary processes, including i) a gene duplication event that gave rise to a
paralog retained in some individuals/populations, ii) recent speciation within the genus with maintenance of ancestral polymorphism and incomplete lineage sorting, or iii) interspecific hybridization (between a shallow and a deep-water species) (see Van Oppen et al., 2002; Alvarez and Wendel, 2003; Marquez et al., 2003; Wörheide et al., 2004). Any of the above-mentioned scenarios should be regarded cautiously and verified with a more thorough investigation of the characteristics of 28S-IGS and the use of additional unlinked markers such as nuclear introns, which were successfully employed in this taxonomic group by Wörheide and co-workers (Bentlage & Wörheide, 2007; Wörheide et al., 2008). Interspecific hybridization is known to occur in several marine groups (see Gardner, 1997) and it has been argued that it plays an important role in the evolution of other lower metazoans such as corals (see review in Willis et al., 2006). In Porifera, interspecific hybridization has never been confirmed, although it has been proposed as an explanation for the genetic patterns that were observed in the rDNA internal transcribed spacers of *Prosuberites laughlini* (Wörheide et al., 2004), *Aixinella aruensis* (Alvarez et al., 2007), and *Leucetta chagosensis* (Wörheide et al., 2008). Similarly, the intermediate morphologies that were observed in a Mediterranean *Aixinella* species (Solé-Cava et al., 1991) and in the freshwater sponge *Saturnospongilla carvalhoi* (Volkemer-Ribeiro, 1976) could be the result of hybridization.

In a previous study, Bavestrello and colleagues examined several *Petrosia* specimens collected along the coast of Italy in both open (cliffs) and closed (caves) habitats by means of allozyme electrophoresis. Their analysis revealed genetic differences between populations to such an extent that they concluded that more than one *Petrosia* species occurred in the area (Bavestrello & Sarà, 1992; Bavestrello et al., 1994). However, their results were not entirely conclusive as to the status of each *Petrosia* morphological type. In fact, there seemed to be a morphological gradient among their type-A and type-C (putative *P. clavata*, and *P. pulitzeri*) in caves and their type-B (putative *P. ficiformis*) inhabiting open habitats (see Fig. 5 in Bavestrello et al., 1994 and Pansini, 1996). This further makes us suspect that their and our cave specimens may represent hybrids between two *Petrosia* spp. Thus, given the uncertain specific status of the specimens in our study that hold genotypes S10 and S11 they will continue to be referred to as the deep-sea lineage.
Genetic diversity, structure, and population history

Genetic diversity of *P. ficiformis* populations varied substantially throughout the study area. The low genetic diversity observed in the NW Mediterranean may be the result of the climatic oscillations of the Pleistocene and particularly of the Last Glacial Maximum (LGM). Indeed, at the glacial peak this coast endured the greatest sea surface temperature (SST) anomaly of the entire Mediterranean basin with temperatures dropping to 11 °C below current temperatures (Hayes et al., 2005). This decrease in temperature may have caused a severe reduction in *P. ficiformis* numbers, or even have resulted in its local disappearance, due to disruption of the reproductive cycle. Gametogenesis (oogenesis) in *P. ficiformis* is closely regulated by water temperature and ceases in the cold winter months when temperatures fall below 13 °C (Ana Riesgo, pers. commun. 2008; Maldonado & Riesgo, in press). A temperature of 13 °C was exactly the thermal condition that was observed in the glacial summer along this coast having reached its minimum of 10 °C in the Gulf of Lions. During the glacial winter, temperatures could even get as low as 7 °C. Contrarily, SST conditions along the eastern Mediterranean basin were similar to today’s temperatures and only suffered a maximum cooling of 3 °C (Hayes et al., 2005). These conditions make it therefore likely that shallow-water *P. ficiformis* populations in the north-western Mediterranean have suffered a severe bottleneck or local disappearance, whereas those along its eastern parts survived. The deep-water lineage (S11), capable of thriving under colder conditions, could have expanded its bathymetric range to shallower-water during this period. Upon restoration of interglacial conditions, the western basin would then have been recolonized from the adjacent eastern shallow-water populations (S4) thus creating a contact zone between the two divergent lineages. In the Azores, the lack of genetic diversity in island populations that are approximately 500 km apart suggests that this expansion (S10) must have occurred not only recently but also extensively. The lack of genetic diversity in the Canary island populations suggests either a recent colonization via a founder event (from Madeira) or a bottleneck of its populations. Indeed, due to the shorter distance to the mainland, the islands of this archipelago have experienced a stronger SST anomaly than Madeira (see Fig. 9 in Xavier and Van Soest, submitted; Chapter 2).

In Porifera, the best examples of bathymetric range expansion from deeper to shallower waters come from species that occur at shallow-depths in Mediterranean caves that exhibit a clear deep-sea affinity, such as the
hexactinellid *Oopsacas minuta* or the cladorhizid *Asbestopluma hypogea* (Vacelet et al., 1994; Vacelet, 1996; Harmelin & Vacelet, 1997; Bakran-Petricioli et al., 2007). Given the LGM-associated sea level fluctuations (130 m below current level) these caves were at that time emerged and therefore expansions must have occurred post-LGM which is in line with our own findings.

To determine probable directions of range expansion one must first infer the relative ages of the sequence-types in the network by finding its root. Castelloe and Templeton (1994) have shown that for intraspecific gene trees methods employing coalescent theory (outgroup weights) are better suited than the traditional techniques of outgroup rooting that are commonly employed in interspecific trees. They also showed that in general the oldest haplotype of the tree is usually one of the four haplotypes with the highest outgroup weights. In our data, the four 18S-IGS sequence-types that exhibited the highest outgroup probabilities were all exclusive to the Mediterranean Sea (outgroup weights: $S_{4ow}=0.180$, $S_{9ow}=0.180$, $S_{7ow}=0.162$, and $S_{6ow}=0.144$) which strongly suggest that this species originated in this basin and subsequently expanded into the Atlantic. This scenario is further corroborated by the distribution of *P. ficiformis* in the Atlantic which is limited to the Macaronesian islands and the species does not occur on the Portuguese coast nor further north. Yet, the high genetic diversity and the private sequence-types observed in the Madeira population indicate that this expansion is likely to pre-date the LGM and that these island populations persisted throughout this period. This conforms to the island refugia pattern that was identified for several marine organisms such as the pomacentrid *Chromis limbata* (Domingues et al., 2006), the white seabream *Diplodus sargus* (Domingues et al., 2007a), the blennids *Coryphoblennius galerita* (Domingues et al., 2007b) and *Parablennius parvicornis* (Domingues et al., 2008), the thornback ray *Raja clavata* (Chevolot et al., 2006), and the poecilosclerid sponge *Phorbas ficitius* (Xavier et al., submitted a; Chapter 5). Our results contrast, however, with the pattern of *C. crambe* in which a low genetic diversity and evidence of multiple and recent human-mediated colonization of the Macaronesian islands was found (Duran et al., 2004b).

The NCPA revealed a pattern of restricted gene flow with isolation by distance of the shallow-water lineage in both the Atlantic (clade 2-2) and the Mediterranean Sea (clade 2-1). This pattern was confirmed by the results of the Mantel test and is consistent with the restricted dispersal abilities of a species that lacks a dispersing larval stage. At higher step clades, the same analysis inferred a
pattern of allopatric fragmentation of the Atlanto-Mediterranean shallow-water lineage (clade 3-1) involving the Macaronesian islands of Madeira and Canaries as well as of the deep-water lineage between the western Mediterranean and the Azores archipelago (clade 3-2). This finding, along with the significant structure that is observed between the two basins in the AMOVA, corroborates the role played by the Strait of Gibraltar (or the Almeria Oran front) as a phylogeographic break for marine populations (see the many examples in Patarnello et al., 2007).

Overall, the results of the NCPA procedure are very similar between P. ficiformis and C. crambe, a species that occurs in the same area. Indeed, in both cases restricted gene flow with isolation by distance for 2-step clades and a contiguous range expansion, i.e. an expanding population front via individual short distance dispersal, in the entire distribution area of both species were inferred. With similar results found in S. lophyropoda, although at a smaller scale, our findings reveal the emergence of a phylogeographic pattern for the Atlanto-Mediterranean sponge fauna: highly structured populations as a combined result of restricted gene flow and isolation by distance with, in some cases, signatures of allopatric fragmentation and refugia.

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