

## Phylogeography, genetic diversity and structure of the poecilosclerid sponge *Phorbast fictitius* at oceanic islands

Joana R. Xavier<sup>1,2,3,4,5</sup>, Rob W.M. van Soest<sup>2</sup>, Johannes A.J. Breeuwer<sup>1</sup>, António M.F. Martins<sup>3</sup>, Steph B.J. Menken<sup>1</sup>

<sup>1</sup> *Evolutionary Biology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands*

<sup>2</sup> *Netherlands Centre for Biodiversity Naturalis, section Zoological Museum of Amsterdam (ZMA), University of Amsterdam, Mauritskade 57, 1092 AD Amsterdam, The Netherlands*

<sup>3</sup> *CIBIO - Research Centre in Biodiversity and Genetic Resources, CIBIO-Azores, Department of Biology, University of the Azores, Rua Mãe de Deus, 9501-801 Ponta Delgada, Portugal (current address J.R. Xavier)*

<sup>4</sup> *CEAB - Centre for Advanced Studies of Blanes (CSIC), Camí d'accés a la Cala S. Francesc, 14, 17300 Blanes (Girona), Spain (current address J.R. Xavier)*

<sup>5</sup> *E-mail: joanarxavier@gmail.com*

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 *Phorbast 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,  $F_{ST}$  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 on  $F_{ST}$  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 structured populations in the marine realm and highlights the importance of the Macaronesian islands on the evolutionary history of the Northeast Atlantic marine biota.

### Contents

Introduction .....	119
Material and methods .....	121

<i>Sampling</i> .....	121
<i>DNA extraction, amplification and sequencing</i> .....	121
<i>Data analyses</i> .....	122
<i>Genetic diversity and structure</i> .....	122
<i>Phylogenetic and phylogeographic relationships</i> .....	122
Results .....	123
<i>Sequence variation and genetic diversity</i> .....	123
<i>Phylogenetic and phylogeographic relationships</i> .....	123
<i>Differentiation and structure at regional and local scales</i> .....	125
Discussion .....	125
<i>Sequence variation and the use of an alternative partition of COI</i> .....	125
<i>Structure of P. fictitius at regional and local scales</i> .....	126
<i>Phylogeography of P. fictitius</i> .....	127
<i>The Macaronesian refugium</i> .....	127
Acknowledgements .....	128
References .....	128

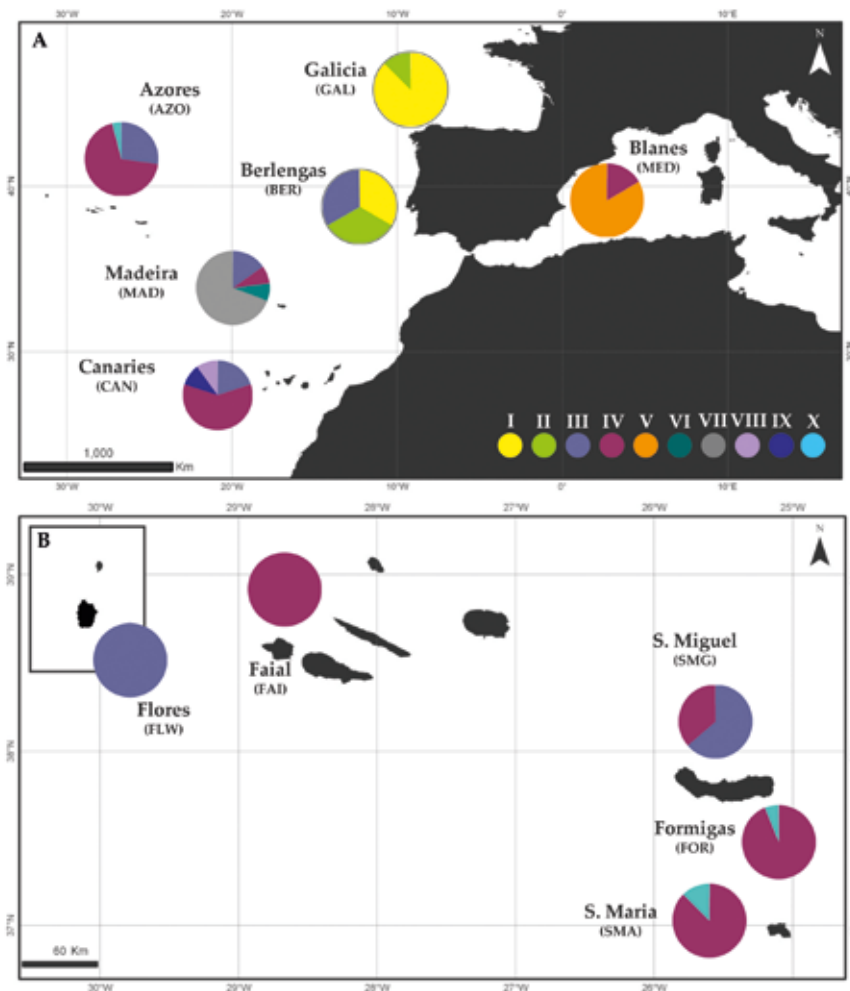
### Introduction

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; 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, and past climate on the structuring of current patterns of genetic diversity and divergence among populations (see reviews in Parnello *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; Chevotat *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 lecithotrophic 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



*Fig. 1.* Geographical distribution of mtDNA haplotypes of *Phorbast fictitius* at (A) regional (Iberian/Macaronesian) and (B) local (Archipelagic) scales. Letters in parentheses refer to population codes (see Table 1).

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 Hymedesmiidae, 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 mm<sup>3</sup>) to be used for genetic analyses were preserved in absolute ethanol and kept at -10°C until further processing.

### DNA extraction, amplification and sequencing

Total DNA was extracted using DNeasy® Tissue kit (QIAGEN), following manufacturer instructions, and

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

population	sampling location	coordinates	Pc	N	Nh	Hd	$\pi$
Galicia	Graña	48°28'03.95"N; 08°16'25.32"W	GAL	8	2	0.250 (0.180)	0.00045 (0.00032)
Berlingas	Berlenga Grande island	39°24'46.16"N; 09°30'28.75"W	BER	9	3	0.750 (0.079)	0.00181 (0.00028)
Mediterranean	Blanes	41°40'31.39"N; 02°48'12.96"E	MED	6	2	0.333 (0.215)	0.00120 (0.00078)
Madeira	Madeira island	32°38'45.33"N; 16°53'18.46"W	MAD	13	4	0.526 (0.153)	0.00250 (0.00096)
Canaries	Tenerife island	28°26'57.53"N; 16°15'55.94"W	CAN	10	4	0.644 (0.023)	0.00364 (0.00094)
Azores	Azores archipelago		AZO	48	3	0.462 (0.062)	0.00234 (0.00033)
	Santa Maria	36°56'23.00"N; 25°08'27.90"W	SMA	8	2	0.250 (0.180)	0.00045 (0.00033)
	Formigas						
	Dollabarat Bank	37°16'14.26"N; 24°46'50.73"W	FOR	16	2	0.125 (0.106)	0.00023 (0.00019)
	São Miguel	37°44'37.07"N; 25°38'19.25"W	SMG	11	2	0.509 (0.101)	0.00276 (0.00055)
	Faial	38°31'22.90"N; 28°39'29.18"W	FAI	7	1	0.000 (0.000)	0.00000 (0.00000)
	Flores	39°27'45.96"N; 31°07'40.51"W	FLW	6	1	0.000 (0.000)	0.00000 (0.00000)
total	all populations			94	10	0.747 (0.034)	0.00420 (0.00027)

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 ('I3-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 'I3-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, PfcCOI<sub>2</sub>f (5' –AA-CATGAGGGCANTGGGAGTAACT– 3') and Por-COI<sub>2</sub>r (5' –ACTGCCCCCATNGATAAAACAT– 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  $\mu$ l volume reaction containing 2.5  $\mu$ l of 10x buffer (Sphaero Q), 4  $\mu$ l dNTPs (1 mM), 1.6  $\mu$ l BSA (10 mg/ml), 1.6  $\mu$ l MgCl<sub>2</sub> (25 mM), 0.3  $\mu$ l (5 U/ $\mu$ l) of *Taq* polymerase (Sphaero Q), 0.8  $\mu$ l of each primer (10  $\mu$ M), and 1.5  $\mu$ l 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 manufac-

turer'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  $F_{ST}$  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 heu-

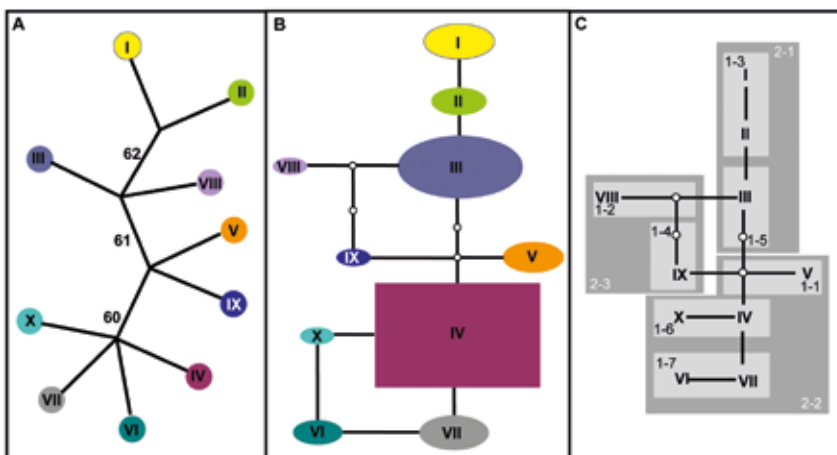


Fig. 2. Phylogenetic relationships of *Phorbast fictitius* haplotypes: (A) unrooted Maximum Parsimony consensus tree. Only bootstrap support values that are >50% are shown; (B) Statistical parsimony network. The area of the polygons is proportional to the frequency of the haplotypes in the total sample; (C) Nested clade design.

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).

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

population	haplotype									
	I	II	III	IV	V	VI	VII	VIII	IX	X
GAL	0.875	0.125								
BER	0.333	0.333	0.333							
MED				0.167	0.833					
MAD			0.154	0.077		0.077	0.692			
CAN			0.200	0.600				0.100	0.100	
AZO			0.271	0.688						0.042

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)						
	GAL	BER	MED	MAD	CAN	AZO
GAL		0.991*	0.048*	0.109*	0.206*	0.176*
BER	0.335ns		0.137*	0.192*	0.431*	0.301*
MED	0.913*	0.785*		0.353*	0.849*	0.591*
MAD	0.821*	0.722*	0.586*		1.406*	1.152*
CAN	0.708*	0.537*	0.371*	0.262*		inf.
AZO	0.740*	0.624*	0.458*	0.303*	-0.017ns	
local scale (archipelagic)						
	SMA	FOR	SMG	FAI	FLW	
SMA	-	inf.	0.446*	inf.	0.024*	
FOR	-0.075ns	-	0.294*	inf.	0.016*	
SMG	0.529*	0.630*	-	0.433*	1.917*	
FAI	-0.018ns	-0.063ns	0.536*	-	0.000*	
FLW	0.954*	0.970*	0.207ns	1.000*	-	

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.  $V_a$  and  $V_b$  represent the associated covariance components. Significant values of  $F_{ST}$  ( $P < 0.001$ ) are indicated with an asterisk.

spatial scale	source of variation	d.f.	sum of squares	variance components	percentage of variation	fixation index
regional (Iberian/Macaronesian)	among populations	5	53.456	0.778 $V_a$	55.64	$F_{ST} = 0.556^*$
	within populations	88	54.593	0.620 $V_b$	44.36	
	total	93	108.049	1.399		
local (Archipelagic)	among populations	4	21.024	0.544 $V_a$	71.12	$F_{ST} = 0.711^*$
	within populations	43	9.494	0.221 $V_b$	28.88	
	total	47	30.518	0.765		

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.

clade	$X^2$ -statistic	$P$	chain of inference	inference
1-3	2.363	0.238	Null hypothesis cannot be rejected	Moving on to next clade
1-6	49.632	0.018	1-2 IO	I-T Status Undetermined: Inconclusive outcome
2-1	25.743	0.000	1-2-3-5-6*-7-8 YES	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.
2-2	47.421	0.000	1-2-11-12-13-14 NO	Long-distance colonisation and/or past fragmentation (not necessarily mutually exclusive).
total cladogram	85.903	0.000	null hypothesis cannot be rejected	moving on to next clade

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 ( $F_{ST}\approx 0.5$ ) 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 ( $\pi = 0.0042$ ) 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*,  $\pi = 0.0039$ , 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*,  $\pi = 0.0006$ , Duran *et al.*, 2004c; *Astrosclera willeyana*,  $\pi = 0.00049$ , Wörheide, 2006; *Xestospongia muta*,  $\pi = 0.00058$ , 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  $F_{ST}$  values and the AMOVA results. This structure is consistent with the low dispersal potential and bathymetric 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). Moreover, a strong fine-scale (from cm to m) genetic structure was also observed in both *Crambe crambe* (Calderón *et al.*, 2007) and *Scopalina lophyropoda* (Blanquer *et al.*, 2009) in the Mediterranean. Together these studies suggest that structured populations are to be expected in most sponge species at various spatial scales as a result of a presumed limited dispersal potential of their lecithotrophic larvae.

However, we observed a non-significant differentiation between Berlengas and Galicia as well as between the Azores and the Canaries, separated by 500 km and 1500 km, respectively. These observations suggest that although *P. fictitius* populations are highly structured as a result of restricted dispersal there may be occasional long-distance dispersal events between some populations. Since very few studies have directly evaluated larval dispersal in sponges this

possibility cannot be discounted. Furthermore, Maldonado and Uriz (1999) have shown that small fragments of reproductive sponges, containing embryos, broken by wave or predatory action can be transported by currents and recruit to a new area. Since *P. fictitius* is a eurytopic species, *i.e.* with a great plasticity in adapting to a wide variety of environmental conditions (Carballo *et al.*, 1996) it is likely that fragments could thrive during such dispersal events. This long distance dispersal, also inferred in the nested clade analysis, would explain why a pattern of isolation by distance was not found at neither scales. Similar evidence of occasional long dispersal events, and lack of isolation by distance, was found in populations of *Callyspongia vaginalis* along the Florida reef tract (DeBiasse *et al.*, 2010).

At the archipelagic 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-panmictic 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 capreoli* (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.*, 2007a) 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 III to the Portuguese mainland and haplotype IV to the Mediterranean) followed by haplotype diversification. The island-to-mainland direction of dispersal is in our opinion more likely given the eastward flowing currents characteristic of this region such as the Azores and the North Atlantic currents (Reverdin *et al.*, 2003). Our findings therefore contrast with those of Duran and colleagues that inferred a recent human-mediated introduction of *C. crambe* in the Macaronesian islands from the Mediterranean (Duran *et al.*, 2004a).

NCPA inferences regarding the population history of *P. fictitius* were only possible for two clades (clade 2-1 and 2-2, Fig. 2c). In the case of clade 2-1 restricted gene-flow but with some long distance dispersal is more likely to occur between the mainland (GAL, BER) and between these and island populations since these are naturally isolated and no intermediate popu-

lations exist. This is further corroborated by the non-significant  $F_{ST}$  values found between Galicia and Berlingas. The inference made for clade 2-2, that comprises island haplotypes and the only Mediterranean haplotype, confirms the occasional long distance dispersal events observed from the non-significant  $F_{ST}$  values between Madeira and the Canaries. It therefore seems that current gene flow patterns more than historical events are the main factors shaping the genetic structure of *Phorbas fictitius* populations (but see next section). Further studies in other species are required if we are to better understand the phylogeographic patterns of the Northeast Atlantic and Mediterranean sponge fauna.

*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 Pleistocenic 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.

## Acknowledgements

The authors wish to thank the following colleagues and respective institutions for their support during the sampling work: the Departments of Biology (A. Costa) and Oceanography and Fisheries (F. Tempera, F. Cardigos, R. Santos), University of the Azores; Marine Biological Station of Funchal (M. Biscoito), Marine Biological Station of Graña (J. Cristobo, P. Rios), and Centre de Estudis Avançats de Blanes (A. Blanquer, M. Uriz); in Tenerife (T. Cruz, L. Moro, J. Bacallado). We thank Rosa Pestana and Andreia Cunha for providing some additional samples from Madeira and São Miguel Islands. We further thank Wil van Ginkel, Betsie Voetdijk, and Peter Kuperus for their valuable help in the molecular lab. We also warmly acknowledge M. Veith for his guidance with the use of some software packages, Paola Rachello-Dolmen for her help in the construction of Fig. 1, and Julie Reveillaud and Nuno Curado for useful comments on an earlier version of the manuscript. This study is part of the PhD-project of J. Xavier, funded by Fundação para a Ciência e Tecnologia (FCT-Portugal, grant no. SFRH/BD/16024/2004).

## References

- Aboim MA, Menezes GM, Schlitt T, Rogers AD. 2005. Genetic structure and history of populations of the deep-sea fish *Helicolenus dactylopterus* (Delaroche, 1809) inferred from mtDNA sequences analysis. *Molecular Ecology* 14: 1343-1354.
- Bentlage B, Wörheide G. 2007. Low genetic structuring among *Pericharax heteroraphis* (Porifera: Calcarea) populations from the Great Barrier Reef (Australia), revealed by analysis of nrDNA and nuclear intron sequences. *Coral Reefs* 26: 807-816.
- Blanquer A, Uriz MJ, Caujapé-Castells J. 2009. Small-scale spatial genetic structure in *Scopalina lophyropoda*, an encrusting sponge with philopatric larval dispersal and frequent fission and fusion events. *Marine Ecology Progress Series* 380: 95-102.
- Bowerbank JS. 1866. A Monograph of the British Spongiadae, volume 2. London: Ray Society.
- Calderón I, Ortega N, Duran S, Becerro M, Pascual M, Turon X. 2007. Finding the relevant scale: clonality and genetic structure in a marine invertebrate (*Crambe crambe*, Porifera). *Molecular Ecology* 16: 1799-1810.
- Carballo J, Naranjo S, Garcia-Gomez J. 1996. Use of marine sponges as stress indicators in marine ecosystems at Algeciras Bay (southern Iberian Peninsula). *Marine Ecology Progress Series* 135: 109-122.
- Castelloe J, Templeton AR. 1994. Root probabilities for intraspecific gene trees under neutral coalescent theory. *Molecular Phylogenetics and Evolution* 3: 102-113.
- Chevolut M, Hoarau G, Rijnsdorp A, Stam W, Olsen J. 2006. Phylogeography and population structure of thornback rays (*Raja clavata* L., Rajidae). *Molecular Ecology* 15: 3693-3705.
- Clement M, Posada D, Crandall K. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657-1660.
- Crowley T. 1981. Temperature and Circulation changes in the Eastern North Atlantic during the last 150,000 years: evidence from the planktonic foraminiferal record. *Marine Micropaleontology* 6: 97-129.
- DeBiaise MB, Richards VP, Shivji MS. 2010. Genetic assessment of connectivity in the common reef sponge, *Callispongia vaginalis* (Demospongiae: Haplosclerida) reveals high population structure along the Florida reef tract. *Coral Reefs* 29: 47-55.
- Domingues VS, Santos RS, Brito A, Almada VC. 2006. Historical population dynamics and demography of the eastern Atlantic pomacentrid *Chromis limbata* (Valenciennes, 1833). *Molecular Phylogenetics and Evolution* 40: 139-147.
- Domingues VS, Almada VC, Santos RS, Brito A, Bernardi G. 2007a. Phylogeography and evolution of the triplefin *Tripterygion delaisi* (Pisces, Blennioidei). *Marine Biology* 150: 509-519.
- Domingues VS, Faria C, Stefanni S, Santos RS, Brito A, Almada VC. 2007b. Genetic divergence in the Atlantic-Mediterranean Montagu's blenny, *Coryphoblennius galerita* (Linnaeus 1758) revealed by molecular and morphological characters. *Molecular Ecology* 16: 3592-3605.
- Domingues VS, Santos RS, Brito A, Alexandrou M, Almada VC. 2007c. Mitochondrial and nuclear markers reveal isolation by distance and effects of Pleistocene glaciations in the north-eastern Atlantic and Mediterranean populations of the white seabream (*Diplodus sargus*, L.). *Journal of Experimental Marine Biology and Ecology* 346: 102-113.
- Domingues VS, Stefanni S, Brito A, Santos RS, Almada VC. 2008. Phylogeography and demography of the Blennioid *Parablennius parvicornis* and its sister species *P. sanguinolentus* from the northeastern Atlantic Ocean and the western Mediterranean Sea. *Molecular Phylogenetics and Evolution* 46: 397-402.
- Duran S, Giribet G, Turon X. 2004a. Phylogeographical history of the sponge *Crambe crambe* (Porifera, Poecilosclerida): range expansion and recent invasion of the Macaronesian islands from the Mediterranean Sea. *Molecular Ecology* 13: 109-122.
- Duran S, Pascual M, Estoup A, Turon X. 2004b. Strong population structure in the marine sponge *Crambe crambe* (Poecilosclerida) as revealed by microsatellite markers. *Molecular Ecology* 13: 511-522.
- Duran S, Pascual M, Turon X. 2004c. Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge *Crambe crambe* (Poecilosclerida). *Marine Biology* 144: 31-35.
- Erpenbeck D, Hooper JNA, Wörheide G. 2006. CO1 phylogenies in diploblasts and the 'Barcoding of Life' – are we sequencing a suboptimal partition? *Molecular Ecology Notes* 6: 550-553.
- Excoffier L, Laval G, Schneider S. 2005. ARLEQUIN ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47-50.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome

- c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294-299.
- France S, Hoover L. 2002. DNA sequences of the mitochondrial COI gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). *Hydrobiologia* 471: 149-155.
- Hall T, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41, 95-98.
- Hayes A, Kucera M, Kallel N, Sbaifi L, Rohling EJ. 2005. Glacial Mediterranean Sea surface temperatures based on planktonic foraminiferal assemblages. *Quaternary Science Reviews* 24: 999-1016.
- Hentschel U, Fieseler L, Wehr M, Gernert C, Steinert M, Hacker J, Horn M. 2003. Microbial diversity of marine sponges. *Progress in Molecular and Subcellular Biology* 37: 59-88.
- Hewitt GM. 2000. The genetic legacy of the Quaternary Ice Ages. *Nature* 405: 907-913.
- Johnson MS, Watts R, Black R. 1994. High levels of genetic subdivision in peripherally isolated populations of the antherinid fish *Craterocephalus capreoli* in the Houtman Abrolhos Islands, Western Australia. *Marine Biology* 119: 179-184.
- Johnson MS, Black R. 2006. Islands increase genetic subdivision and disrupt patterns of connectivity of intertidal snails in a complex archipelago. *Evolution* 60: 2498-2506.
- Knowles LL. 2008. Why does a method that fails continue to be used? *Evolution* 62: 2713-2717.
- Lavrov DV, Forget L, Kelly M, Lang BF. 2005. Mitochondrial genomes of two demosponges provide insights into and early stage of animal evolution. *Molecular Biology and Evolution* 22: 1231-1239.
- López-Legentil S, Pawlik JR. 2009. Genetic structure of the Caribbean giant barrel sponge *Xestospongia muta* using the I3-M11 partition of COI. *Coral Reefs* 28: 157-165.
- Maggs CA, Castilho R, Foltz D, Henzler C, Jolly MT, Kelly J, Olsen J, Perez KE, Stam W, Väinölä, Viard F, Wares J. 2008. Evaluating signatures of glacial refugia for North Atlantic benthic marine taxa. *Ecology* 89: 108-122.
- Maldonado M. 2006. The ecology of sponge larvae. *Canadian Journal of Zoology* 84: 175-194.
- Maldonado M, Uriz MJ. 1999. Sexual propagation by sponge fragments. *Nature* 398: 476.
- Mariani S, Uriz M, Turon X. 2005. The dynamics of sponge larvae assemblages from northwestern Mediterranean nearshore bottoms. *Journal of Plankton Research* 27: 249-262.
- Mariani S, Uriz M, Turon X, Alcoverro T. 2006. Dispersal strategies in sponge larvae: integrating the life history of larvae and the hydrologic component. *Oecologia* 149: 174-184.
- Panchal M. 2007. The automation of Nested Clade Phylogeographic Analysis. *Bioinformatics* 23: 509-510.
- Patarnello T, Volckaert FAMJ, Castilho R. 2007. Pillars of Hercules: is the Atlantic-Mediterranean transition a phylogeographical break? *Molecular Ecology* 16: 4426-4444.
- Petit RJ. 2008. The coup de grâce for the nested clade phylogeographic analysis? *Molecular Ecology* 17: 516-518.
- Pflaumann U, Sarnthein M, Chapman M, d'Abreu L, Funnell B, Huels M, Kiefer T, Maslin M, Schulz H, Swallow J, Kreveld S, Vautravers M, Vogelsang E, Weinelt M. 2003. Glacial North Atlantic: sea-surface conditions reconstructed by GLAMAP 2000. *Paleoceanography* 18: 1065-1093.
- Posada D, Crandall KA, Templeton AR. 2000. GEODIS: a program for the cladistic nested clade analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology* 9: 487-488.
- Posada D, Crandall KA, Templeton AR. 2006. Nested clade analysis statistics. *Molecular Ecology Notes* 6: 590-593.
- Reverdin G, Niiler PP, Valdimarsson H. 2003. North Atlantic Ocean surface currents. *Journal of Geophysical Research* 108: 3002.
- Rozas J, Sanchez-Delbarrio J, Messeguer X, Rozas R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19: 2496-2497.
- Sá-Pinto A, Branco M, Sayanda D, Alexandrino P. 2008. Patterns of colonization, evolution and gene flow in species of the genus *Patella* in the Macaronesian Islands. *Molecular Ecology* 17: 519-532.
- Sarà M, Vacelet J. 1973. Écologie des Démosponges. Pp 462-575 in: Grassé P, *Traité de Zoologie, Spongiaires*. Paris: Masson et Cie.
- Shearer T, Van Oppen MJH, Romano S, Wörheide G. 2002. Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). *Molecular Ecology* 11: 2475-2487.
- Swofford DL. 1998. PAUP: Phylogenetic Analysis Using Parsimony and Other Methods. Sunderland (MA): Sinauer Associates.
- Templeton AR. 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* 7: 381-397.
- Templeton AR. 2004. Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology* 13: 789-809.
- Templeton AR. 2008. Nested clade analysis: an extensively validated method for strong phylogeographic inference. *Molecular Ecology* 17: 1877-1880.
- Templeton AR. 2009. Statistical hypothesis testing in intraspecific phylogeography: nested clade phylogeographical analysis vs. approximate Bayesian computation. *Molecular Ecology* 18: 319-331.
- Templeton AR, Routman E, Phillips CA. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics* 140: 767-782.
- Wörheide G, Hooper JNA, Degnan B. 2002. Phylogeography of western Pacific *Leucetta* 'chagosensis' (Porifera: Calcarea) from ribosomal DNA sequences: implications for population history and conservation of the Great Barrier Reef World Heritage Area (Australia). *Molecular Ecology* 11: 1753-1768.
- Wörheide G. 2006. Low variation in partial cytochrome oxidase subunit I (COI) mitochondrial sequences in the coralline demosponge *Astrosclera willejana* across the Indo-Pacific. *Marine Biology* 148: 907-912.
- Wörheide G, Epp L, Macis L. 2008. Deep genetic divergence among Indo-Pacific populations of the coral reef sponge *Leucetta chagosensis* (Leucettidae): founder effects, vicariance or both? *BMC Evolutionary Biology* 8: 24.

Received: 17 June 2009

Revised and accepted: 31 August 2010

Published online: 1 October 2010

Editor: J.W. Arntzen