

# MtDNA diversity of the Indonesian giant barrel sponge *Xestospongia testudinaria* (Porifera: Haplosclerida) – implications from partial cytochrome oxidase 1 sequences

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*The Indonesian archipelago is a 'hotspot' for invertebrate biodiversity ('Coral Triangle'). In this area of 'peak' biodiversity, the origins of this high species diversity have often been debated. Xestospongia testudinaria is one of the sponge species that dominates coral reef sponge communities in this region. The role of the so-called 'giant barrel sponge' for the reef ecosystem has been studied repeatedly, as have its various bioactive compounds. However, the genetic variation of this iconic sponge in the region remains unknown. We investigate over 200 barrel sponge samples from Indonesia, and neighbouring as well as more distant localities (Saudi Arabia, Tanzania, Thailand, Taiwan, Java, Sulawesi and the Great Barrier Reef, Australia) using the mitochondrial cytochrome oxidase subunit 1. We compare our results with those from the studies on the congeneric barrel sponges Xestospongia muta from the Caribbean, and Xestospongia bergquistia from the Indo-Pacific, and observe a high degree of overlapping haplotypes between the three barrel sponge species, likely indicating the presence of ancestral polymorphisms. We discuss the implications of these findings to better interpret the phylogeography of barrel sponge taxa in the Indo-Pacific.*

**Keywords:** Porifera, barrel sponges, *Xestospongia*, Indo-Pacific, taxonomy, mitochondrial DNA

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## INTRODUCTION

The Indonesian archipelago forms a part of the 'Coral Triangle' (Veron, 1995), a region recognized for the high diversity of its marine species (Briggs, 1999; Hoeksema, 2007; Bellwood & Meyer, 2009). However, the genetic diversity of the marine biota in this region remains incompletely studied and poorly understood (Veron, 1995; Hoeksema, 2007). Gaining this knowledge is pivotal to the assessment of genetic diversity within populations and species, and to understand past factors and future triggers of how organisms radiate and disperse in a given region. Such information is important for managing ecosystems and directing conservation efforts towards better resilience in times of global warming and sea-level changes (Sutherland *et al.*, 2004; Jones *et al.*, 2007; Lopez-Legentil & Pawlik, 2009).

Sponges (Phylum Porifera) are an abundant and diverse group of marine organisms in the Indo-Pacific region, for

which the genetic connectivity is also not fully understood (Wörheide *et al.*, 2005; Bentlage & Wörheide, 2007; Blanquer & Uriz, 2007). Sponges play an important role in marine ecosystems due to their impact on substrate (e.g. as bioeroders, sediment aggregators, or for reef restoration), chemical cycling, symbiotic associations (see review in Bell, 2008) and might be a key component for the sustainability of reef ecosystems in oligotrophic seas (de Goeij *et al.*, 2013). Sponge biodiversity studies have been shown to assist in monitoring the influence of environmental factors such as depth, light, tidal amplitude, water flow rate, temperature, velocity, salinity and suspended sediment load (Carballo *et al.*, 1996; Bell, 2007; Cleary & de Voogd, 2007; de Voogd & Cleary, 2007; Becking *et al.*, 2013). In addition, many sponge species contain various secondary metabolites, which have been a focus of drug discovery for decades (e.g. Newman & Cragg, 2012).

An iconic sponge species in the Indonesian archipelago is the petrosiid demosponge *Xestospongia testudinaria* (Lamarck, 1815), also known as the 'Giant Barrel Sponge' (van Soest, 1989; van Soest *et al.*, 2012). *Xestospongia testudinaria* is known to be widespread in the Indo-Pacific and is one of the more common species on Indonesian coral reefs (van

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Soest, 1989; de Voogd & Cleary, 2008). Research on *X. testudinaria* has been focused mainly on new bioactive compounds (Calcul *et al.*, 2003; Cao *et al.*, 2005), ecology (Powell, 2013), aspects of reproduction (Fromont, 1988; Fromont & Bergquist, 1994) and taxonomy (Fromont, 1991). More recently, research was conducted on the genetic connectivity of *X. testudinaria*, but so far only in a narrow geographic scale in northern (Swierts *et al.*, 2013) and south-east Sulawesi (Bell *et al.*, 2014a). Swierts *et al.* (2013) found that in an area of less than 200 km<sup>2</sup>, *X. testudinaria* comprises four distinct morphotypes, which are not obviously affected by environmental factors, and could be differentiated using mitochondrial and nuclear markers. One of the most intriguing findings of that work was the overlap of mitochondrial haplotypes between the Indo-Pacific *X. testudinaria* with the Caribbean barrel sponge *Xestospongia muta* (Schmidt, 1870) (see also Montalvo & Hill, 2011). These intriguing findings highlight the need for a better understanding of barrel sponge species and population structures on a wider, preferably global scale. This study should include a critical re-evaluation of how sponge species have been delineated in the past, and can act as models for the future (see e.g. Solé-Cava & Wörheide, 2007), using barrel sponges as an example.

Throughout the entire history of sponge systematics (see Boury-Esnault, 2006) morphological apomorphies and specific character combinations were the major discriminatory factors in taxonomy (cf. Diagnosable Species Concept, see de Queiroz, 2007). Even now, the most recent supra-specific classification of sponges, *Systema Porifera* (Hooper & van Soest, 2002), is predominantly based on morphological criteria. This concept, however, apparently fails to distinguish between all barrel sponge species. On the one hand few morphological features differentiate *X. testudinaria* from a second Indo-Pacific barrel sponge species, *X. bergquistia* Fromont (Fromont, 1991), as recently supported by molecular (microsatellite) data (Bell *et al.*, 2014a, b). However, so far there have been no unequivocal morphological apomorphies established to differentiate these two Pacific species from the Caribbean *X. muta* barrel sponge. Likewise, biochemical compounds, occasionally used as a source for apomorphies in demosponges (Erpenbeck & van Soest, 2007), are inconclusive for barrel sponges. One *X. muta* specimen shared a similar, but not species-specific sterol composition to *X. bergquistia* specimens, and this was different from those found in specimens of *X. testudinaria* (Fromont *et al.*, 1994).

Ecological parameters can provide apomorphies to differentiate sponge species (see e.g. Wapstra & van Soest, 1987), where taxa are distinguishable by their respective ecological niches (cf. Ecological Species Concept, see de Queiroz, 2007). For example, among the oviparous and gonochoric barrel sponges, different spawning periods act as a potential reproductive barrier between the two Indo-Pacific taxa *X. testudinaria* and *X. bergquistia* (Fromont, 1988; Fromont & Bergquist, 1994). Likewise, the geographic distance and continental barrier that separates the Caribbean *X. muta* from the Indo-Pacific barrel sponges acts as a potential reproductive barrier and is regarded as an important distinguishing feature (Montalvo & Hill, 2011).

The advent of molecular systematics in sponges (Kelly-Borges *et al.*, 1991) facilitated the delimitation of taxa based on the coalescence or common ancestry of lineages (Monophyletic and Genealogical Species Concepts, de Queiroz, 2007) (see examples in Cárdenas *et al.*, 2012).

Many genetic analyses of barrel sponge species use the mitochondrial cytochrome oxidase subunit 1 (*cox1*), particularly its 3' partition, which is shown to better resolve species-level differences than the standard barcoding fragment (Erpenbeck *et al.*, 2006b). The 3' partition enabled the recognition of four *X. muta* haplotypes in the Caribbean (Lopez-Legentil & Pawlik, 2009), and six among Indo-Pacific barrel sponges (Swierts *et al.*, 2013). Swierts *et al.* (2013) however, also corroborated earlier studies that failed to distinguish the geographically separated *X. muta* and *X. testudinaria* based on *cox1* sequences alone (Montalvo & Hill, 2011). So far, barrel sponge *cox1* contradicts earlier studies on the use of this marker for unravelling demosponge species level phylogenies (Erpenbeck *et al.*, 2006a) (see also Lopez-Legentil & Pawlik, 2009; Xavier *et al.*, 2010; Reveillaud *et al.*, 2011).

These findings prompted the present study, which aims to analyse the haplotype diversity of barrel sponges at the largest geographic scale attempted so far, stretching from the western Indian Ocean to the west Pacific, and including the type material of all three barrel sponge species. We explore the suitability of one of the most frequently used mitochondrial markers, *cox1*, to distinguish the three barrel sponge species. Furthermore, this study aims to further our understanding of the genetic population structure of the barrel sponge species at a much wider geographic scope than previous studies that were limited to populations surrounding Sulawesi (Swierts *et al.*, 2013; Bell *et al.*, 2014a).

## MATERIALS AND METHODS

Fragments of 187 specimens of *Xestospongia testudinaria* were freshly collected from three regions: Java (West, Central, and East), Bali and Sulawesi (Lembah, Spermonde, Wakatobi). Immediately after collection, samples were cut, rinsed and soaked in 99% ethanol before being kept in fresh 99% ethanol. An additional 24 samples were studied from Saudi Arabia, Tanzania, Taiwan and Thailand, provided by the Naturalis Biodiversity Center, Leiden, the Netherlands, and samples from the Great Barrier Reef (GBR) and Solomon Islands provided by the Queensland Museum (QM) Brisbane, Australia. Furthermore, the Queensland Museum also provided five individuals of *Xestospongia bergquistia*, including the holotype specimen (see Supplementary Material). In addition, we also obtained the neotype (BMNH 1881.10.21.266) and another, associated specimen of Ridley's (1881) material (BMNH 1881.10.21.267) of *X. testudinaria* from the British Museum of Natural History, London (Hooper & Wiedenmayer, 1994). For *Xestospongia muta*, two syntypes were investigated (MCZ PORa-6449, MCZ PORa-6450, of which we designate the former as lectotype) and additional sequences were taken from GenBank (<http://www.ncbi.nlm.nih.gov>).

Samples were extracted based on previously published methods established for sponge barcoding (Vargas *et al.*, 2012). The I3-M11 partition of *cox1* was amplified with the Polymerase Chain Reaction (PCR) using primers CJ-J2165 and C1-Npor 2760 (Erpenbeck *et al.*, 2002). The 25 µL PCR mix consisted of 5 µL 5 × green GoTaq<sup>®</sup> PCR Buffer (Promega Corp, Madison, WI), 4 µL 25 mM MgCl<sub>2</sub> (Promega Corp, Madison, WI), 2 µL 10 mM dNTPs, 1 µL each primer (5 µM), 9.8 µL H<sub>2</sub>O, 0.2 µL GoTaq<sup>®</sup> DNA

polymerase ( $5 \mu \mu\text{L}^{-1}$ ) and  $2 \mu\text{L}$  DNA template. The PCR regime comprised an initial denaturation at  $94^\circ\text{C}$  for 3 min, 35 cycles of 30 s denaturation at  $94^\circ\text{C}$ , 20 s annealing at  $42^\circ\text{C}$  and 60 s elongation at  $72^\circ\text{C}$  each, followed by a final elongation at  $72^\circ\text{C}$  for 5 min. Re-amplifications with an internal primer set of the partition I3-M11 (CO1porF1 and CO1porR1, Erpenbeck *et al.*, 2003) and combinations (CJ-J2165 – CO1porR1 and CO1porF1 – C1-Npor 2760) were required for obtaining PCR products from the type specimen of *X. bergquistia*. For the 133 year old specimen of *X. testudinaria* (BMNH 1881.10.21.267), the primer set CJ-J2165 and COX1-R1 (5'-TGTTGRGGGAAAAARGTTAAATT-3') (Rot *et al.*, 2006) was amplified under the use of additional  $2 \mu\text{L}$  Bovine Serum Albumin (BSA,  $10 \text{ mg mL}^{-1}$ ) and the PCR regime of 45 s annealing at  $42^\circ\text{C}$ , 45 s elongation at  $72^\circ\text{C}$  and a final elongation at  $72^\circ\text{C}$  for 7.5 min. All of the PCR products were cleaned by ammonium acetate precipitation. Sequencing of forward and reverse strand was performed with the ABI BigDye v3.1 chemistry (Applied Biosystems, CA, USA) and the amplification primers following the manufacturer's protocol on an ABI 3730 Automated Sequencer in the Genomic Sequencing Unit of the LMU Munich. Sequences were assembled, trimmed and analysed by Geneious version 6.1.7 (<http://www.geneious.com>). Sequences were checked with BLAST against GenBank (<http://www.ncbi.nlm.nih.gov>) for contaminations. In total 211 sequences were aligned with MUSCLE version 3.5 (Edgar, 2004) as implemented in Geneious under default settings. Sequences of *X. testudinaria*, which are identical to the haplotype of sequences in the study of Swierts *et al.* (2013), are numbered according to that study (haplotypes C1–C6, GenBank accession numbers KC424439–KC424444). Conversely, new haplotypes (one haplotype C7, two type specimens (*X. testudinaria* and *X. bergquistia*) and one *X. bergquistia* haplotype) were submitted and deposited to NCBI GenBank under accession numbers KM014752–KM014755.

TCS v 1.2.1 (Clement *et al.*, 2000) was used to create a statistical parsimony haplotype network among the analysed barrel sponges (*X. testudinaria*, *X. bergquistia* and *X. muta*) to map barrel sponge haplotype distributions according to their localities. DnaSP v. 5.10.01 (Librado & Rozas, 2009) was recruited to obtain genetic diversity indices ( $\pi$ ) and to test neutral evolution of I3-M11 alleles from *X. testudinaria* by calculating Tajima's D (Tajima, 1989). For the analysis of the genetic structure of *X. testudinaria*, regional samples were pooled as follows: Saudi Arabia (N = 3), Tanzania (N = 13), Taiwan (N = 7), Thailand (N = 12), west Java (N = 14), central Java (N = 9), east Java & Bali & Sumba (N = 10), north Sulawesi (N = 126), south & southeast Sulawesi (N = 10), and the GBR and Solomon Islands (N = 7). A hierarchical analysis of molecular variance (AMOVA) was performed and pairwise  $F_{ST}$  values calculated in Arlequin v 3.5.1.2 (Excoffier *et al.*, 2005) with a permutation test of 10,000 replicates. The significance of  $F_{ST}$  values was amended after a Bonferroni correction (Rice, 1989). Phylogenetic reconstructions among barrel sponges were performed under Maximum-likelihood (ML) and Bayesian inferences criteria. A Maximum-likelihood phylogram was inferred by RAxML v. 7.0.4 in raxmlGUI v. 1.3 (Silvestro & Michalak, 2012) using 1000 rapid bootstrap replications (Stamatakis *et al.*, 2008) and the GTR + I +  $\Gamma$  model as suggested by jModeltest v. 2.1.3 (Darriba *et al.*, 2012) under the Akaike Information Criterion (Akaike, 1974). Likewise, a Bayesian

phylogram was inferred using MrBayes v. 3.2.1 (Ronquist *et al.*, 2012) under the same model of evolution. Each analysis consisted of two independent runs of four Metropolis-coupled Markov-chains under default temperature with trees sampled at every 1000th generation. Analyses were terminated automatically when the chains converged significantly as indicated by an average standard deviation of split frequencies  $<0.01$ .

Spicule re-measurements were made for the three type specimens of barrel sponges *X. testudinaria* (BMNH 1881.10.21.266), *X. bergquistia* (QM G25018) and *X. muta* (MCZ PORa-6449). Spicule preparations were made by dissolving a small piece of the specimen in commercial bleach, after which the residue was rinsed four times with water, and once with 96% ethanol. The spicules were air-dried on microscopic slides and prepared for study with a light microscope by mounting in Ultrabed. Spicule dimensions are given as the mean length times mean width of 25 spicule measurements.

## RESULTS

We obtained partial *cox1* sequences (I3-M11 partition) of 211 specimens of *Xestospongia testudinaria* and six of *X. bergquistia* resulting in an alignment of 544 base pairs with six different haplotypes (Table 1). No amplification product for the neotype of *X. testudinaria* BMNH 1881.10.21.266, and the type material of *X. muta* MCZ PORa-6449 and MCZ PORa-6450 could be obtained. Five haplotypes were identical to haplotypes reported from Swierts *et al.* (2013) (C1, C2, C4–C6), whose labelling system is followed here; one novel haplotype was discovered and was subsequently named C7.

All six haplotypes were present in *X. testudinaria* (Figure 1). Two haplotypes are shared between *X. testudinaria* and *X. bergquistia* (Haplotype C2 and C5). The holotype of *X. bergquistia* (QM G25018) possessed the C5 haplotype and *X. testudinaria* BMNH 1881.10.21.267 possessed the C2 haplotype. These sequences differed by an uncorrected *p*-distance of 0.0092. The inclusion of previously published *Xestospongia cox1* sequences from GenBank revealed that two haplotypes (C2 and C5) are shared between the Indo-Pacific and Caribbean barrel sponge species. The 116 specimens of the Caribbean barrel sponge (*Xestospongia muta*) had a total of four haplotypes. Two of these haplotypes (H2 and H4, as defined by Lopez-Legentil & Pawlik, 2009), are not shared with the Indo-Pacific specimens (see Tables 1 & 2).

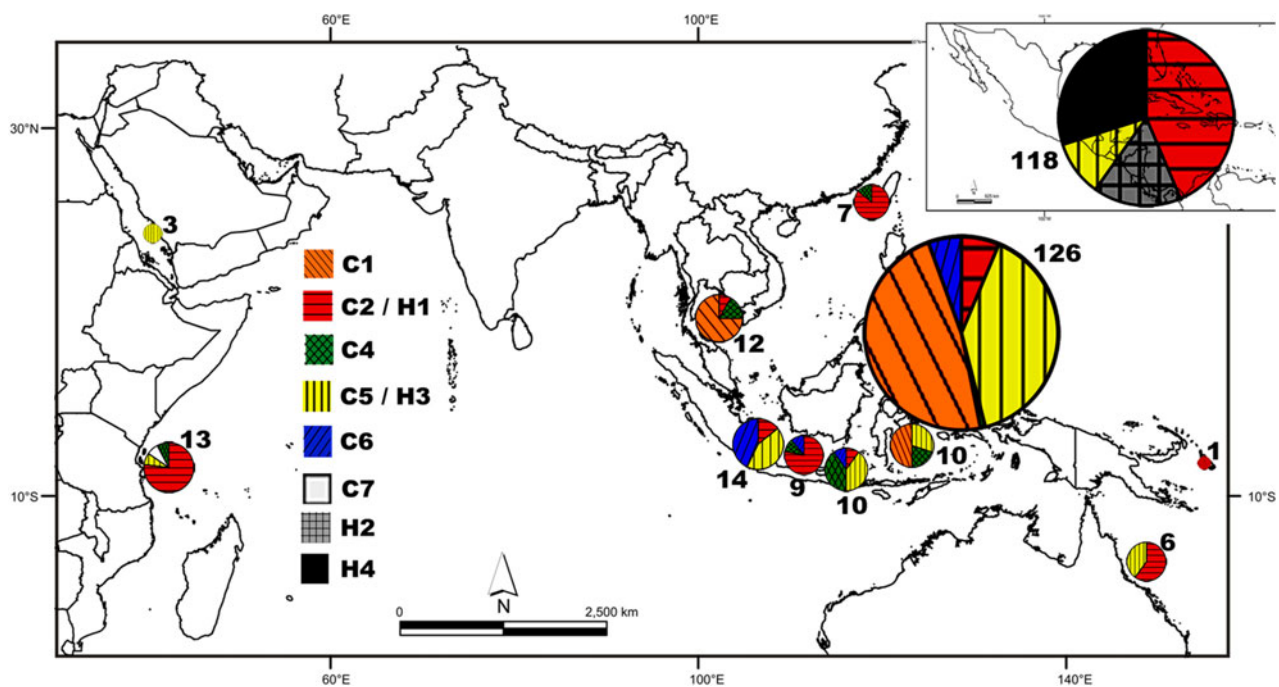
A close genealogical relationship among haplotypes was observed (Figure 2). All haplotypes were connectable with a maximum of one inferred mutational step. Haplotype C1 is the most abundant haplotype among the Indo-Pacific samples, comparable with H1 in the Caribbean (cf. Lopez-Legentil & Pawlik, 2009) – with one mutational step separating those two major haplotypes. Haplotypes C5 and C1 were the dominant haplotypes in the Indonesian archipelago, from which the majority of specimens of *X. testudinaria* used in this study were collected. Haplotype C7 was exclusively found in the 13 samples from Tanzania, which in total displayed four different haplotypes. Among the three samples from the Red Sea, Saudi Arabia, only C5 was found (Figures 1 & 2). In the reconstructed phylogram the barrel sponges are recovered as monophyletic with high support (Figure 3). However, as expected from the overlapping haplotypes, the *cox1* fragment fails to support the monophyly of any of the three barrel sponge species.

**Table 1.** Haplotypes recovered from the *Xestospongia* barrel sponge alignment. Positions refer to the full *cox1* sequence from *Xestospongia muta* EU716653 (Kayal & Lavrov, 2008); C1–C7, haplotype annotation follows Swierts *et al.* (2013); H1–H4, follows the labelling of Lopez-Legentil & Pawlik (2009).

Position (bp)	7	7	7	8	1	1	1	Species; Reference (associated haplotype number as used in reference) and example GenBank accession number
Haplotype	6	7	8	8	1	1	2	
	6	7	3	8	0	8	1	
					2	2	8	
C1 (cf. Swierts <i>et al.</i> 2013)	A	T	C	A	G	G	T	<i>X. testudinaria</i> ; (Montalvo & Hill, 2011) e.g. HQ452959 <i>X. testudinaria</i> ; (Swierts <i>et al.</i> 2013) e.g. KC424439 <i>X. testudinaria</i> ; (this study)
C2 (cf. Swierts <i>et al.</i> 2013)	A	T	C	A	G	G	C	<i>X. muta</i> ; (Lopez-Legentil & Pawlik, 2009) (as H1 e.g. EU716652) <i>X. testudinaria</i> ; (Swierts <i>et al.</i> 2013) e.g. KC424440 <i>X. bergquistia</i> ; KM014752 and <i>X. testudinaria</i> ; (this study)
C4 (cf. Swierts <i>et al.</i> 2013)	A	T	C	C	G	G	C	<i>X. testudinaria</i> ; (Swierts <i>et al.</i> 2013) e.g. KC424442 <i>X. testudinaria</i> ; (this study)
C5 (cf. Swierts <i>et al.</i> 2013)	A	A	C	C	G	G	C	<i>X. muta</i> ; (Lopez-Legentil & Pawlik, 2009) (as H3 e.g. EU716654) <i>X. testudinaria</i> ; (Swierts <i>et al.</i> 2013) e.g. KC424443 <i>X. bergquistia</i> ; KM014753 and <i>X. testudinaria</i> (this study)
C6 (cf. Swierts <i>et al.</i> 2013)	G	A	C	G	G	G	C	<i>X. testudinaria</i> ; (Montalvo & Hill, 2011), e.g. HQ452960 <i>X. testudinaria</i> ; (Swierts <i>et al.</i> 2013) e.g. KC424444 <i>X. testudinaria</i> ; (this study)
C7 (this study)	A	A	C	C	G	T	C	<i>X. testudinaria</i> ; KM014755 (this study)
H2 (cf. Lopez-Legentil & Pawlik, 2009)	A	T	T	A	G	G	C	<i>X. muta</i> ; (Lopez-Legentil & Pawlik, 2009) e.g. EU716653 <i>X. muta</i> ; (Kayal & Lavrov, 2008) e.g. EU237490
H4 (cf. Lopez-Legentil & Pawlik, 2009)	G	A	C	G	A	G	C	<i>X. muta</i> ; (Lopez-Legentil & Pawlik, 2009) e.g. EU716653

The nucleotide diversity ( $\pi$ ) value of *X. testudinaria* was 0.0029 and Tajima's D 1.65911 ( $P > 0.1$ ), which indicated neutral evolution of the I3-M11 fragment. Furthermore, samples from four regions (Thailand, West Java, North Sulawesi and GBR + Solomon Islands) displayed significantly different population structures from the other localities (see

Table 3). Moreover, the hierarchical AMOVA analysis revealed that 32.49% of the genetic variation was found among populations and 67.51% within populations (see Table 4). Likewise, the overall  $F_{ST}$  value (0.32491,  $P < 0.05$ ) indicated the presence of genetic structuring of *X. testudinaria* in the Indo-Pacific area.



**Fig. 1.** Haplotype distribution of *Xestospongia* barrel sponge sequences used in this study. The size in the pie charts is proportional to the sample size. Different haplotypes are colour- and pattern-coded (see Tables 1 & 2 for further details). Inset: Samples from the Caribbean (published by Kayal & Lavrov, 2008; Lopez-Legentil & Pawlik, 2009). C1–C7 haplotype annotations follow Swierts *et al.* (2013), whereas H1–H4 follow Lopez-Legentil & Pawlik (2009).

**Table 2.** Haplotype distribution of *Xestospongia testudinaria* and other barrel sponges according to their geographic localities. Haplotype annotation follows Swierts *et al.* (2013) and Lopez-Legentil & Pawlik (2009)

Haplotype	Saudi Arabia	Tanzania	Taiwan	Thailand & Singapore	Indonesian Archipelago					Great Barrier Reef & Solomons	Florida, Bahamas, Belize <sup>d</sup>	Total
					West Java	Central Java	East Java, Bali, Sumba	North Sulawesi	South and Southeast Sulawesi			
C1	0	0	0	9	0	0	0	60	5	0	0	74
C2	0	10	6	1	2	7	1	8	0	8 <sup>b</sup>	51	94
C4	0	1	1	2	0	1	4	1	2	0	0	12
C5	3	1	0	1 <sup>a</sup>	6	0	4	50	3	4 <sup>c</sup>	12	84
C6	0	0	0	0	6	1	1	7	0	0	0	15
C7	0	1	0	0	0	0	0	0	0	0	0	1
H2	0	0	0	0	0	0	0	0	0	0	19 <sup>e</sup>	19
H4	0	0	0	0	0	0	0	0	0	0	35	35
Total	3	13	7	13	14	9	10	126	10	12	118	334

<sup>a</sup>One *Xestospongia bergquistia* specimen from Singapore.

<sup>b</sup>Including one specimen of *X. bergquistia* from the Great Barrier Reef and one Ridley (1881) specimen of *X. testudinaria* (BMNH 1881.10.21.267).

<sup>c</sup>Four specimens (including one holotype) of *X. bergquistia*.

<sup>d</sup>Taken from Lopez-Legentil & Pawlik (2009).

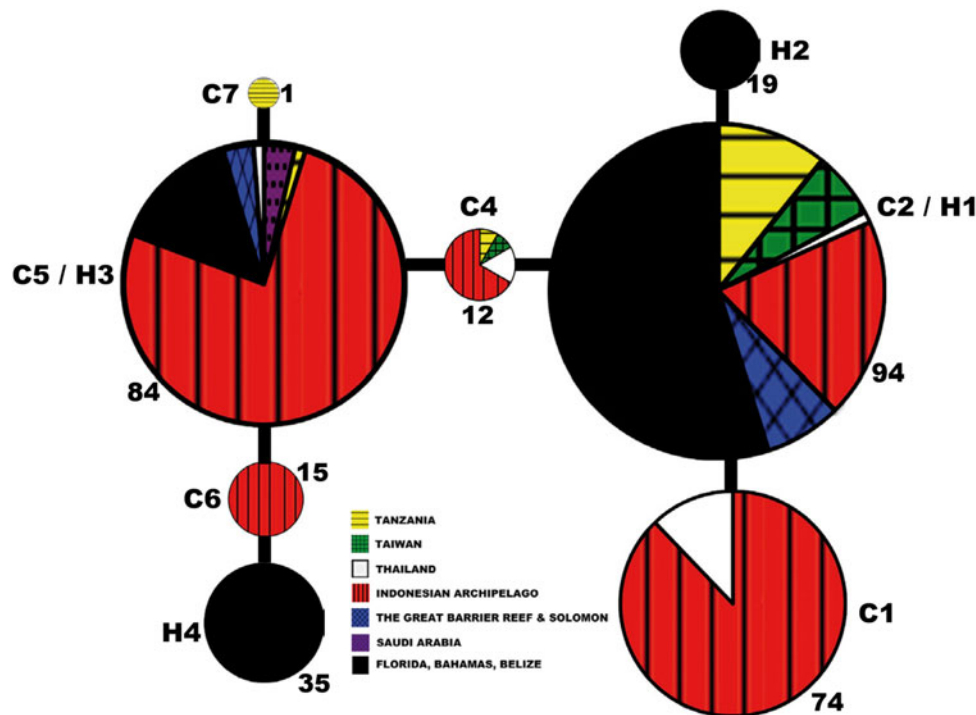
<sup>e</sup>Including full mt genome sequence of *X. muta* (see Kayal & Lavrov, 2008).

DISCUSSION

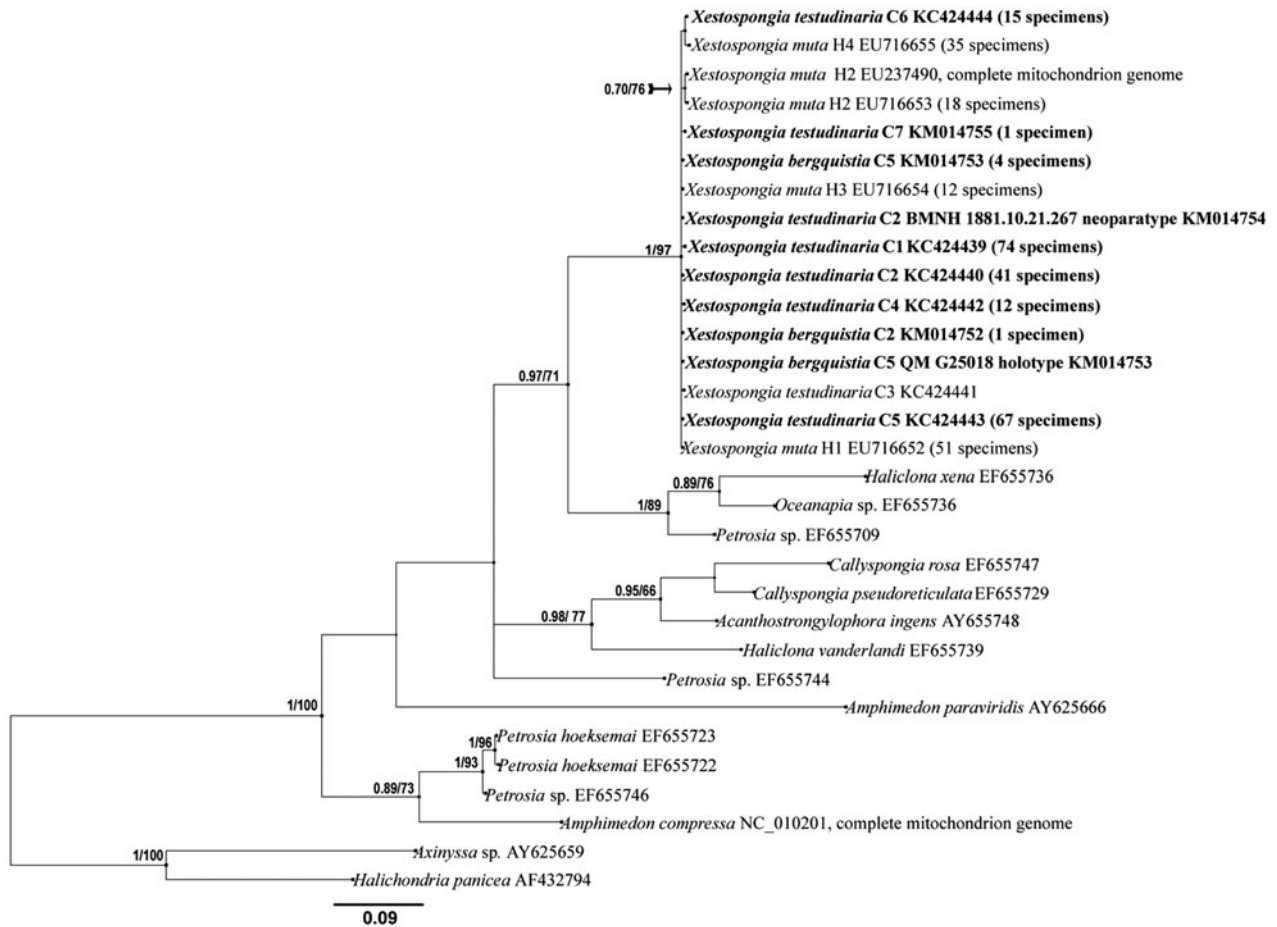
**Cytochrome oxidase 1 and barrel sponge species differentiation**

Currently the three barrel sponge species *Xestospongia testudinaria*, *X. muta* and *X. bergquistia* cannot be differentiated

using the I3-M11 partition of *cox1*, which was suggested as a candidate marker for species level phylogenies other than the standard barcoding marker (Erpenbeck *et al.*, 2006b), or by using any morphological or ecological parameter alone. The three barrel sponge species therefore can be regarded as ‘Gray zone species’ (de Queiroz, 2007) given our current understanding of them. The concept of *Gray zone species*



**Fig. 2.** Haplotype network of the *Xestospongia* barrel sponge specimens with respect to their sampling location (see Tables 1 & 2 for details). *Xestospongia muta* sequences: (Kayal & Lavrov, 2008; Lopez-Legentil & Pawlik, 2009). C1–C7 haplotype annotations follow Swierts *et al.* (2013), whereas H1–H4 follow Lopez-Legentil & Pawlik (2009). Numbers indicate the number of sequences analysed.



**Fig. 3.** Bayesian inference phylogram of *Xestospongia* barrel sponge haplotypes included in the analysis and other marine haplosclerid taxa. New sequences are given in bold and followed by the number of sequences obtained in this study, including GenBank accession numbers. The haplotype annotations follow Swiertz *et al.* (2013) and Lopez-Legentil & Pawlik (2009). Numbers on the branches represent posterior probabilities (PP)/bootstrap proportions (BP) of maximum likelihood analyses. Scale bar indicates the number of substitutions/site.

arises when there are differences in the views of species concepts, which then lead to conflicts in species delimitation (de Queiroz, 2007). Nevertheless, despite the overlapping haplotypes observed here, the three *Xestospongia* taxa cannot constitute a single species as evident from their morphological and biochemical differences. No unique character distinguishing the three species has yet been found. The measured spicules of all three type specimens revealed overlapping spicule dimensions (see Table 5). However, with respect to the two Indo-Pacific species, the magnitude of spongin reinforcement in the skeletons (Fromont, 1991) and microsatellite data (Bell *et al.*, 2014b) supported species separation.

In terms of ecological parameters, the three species of barrel sponges constitute different species based on reproductive behaviour (Fromont, 1988, Ritson-Williams *et al.*, 2005), and by their wide geographic separation (Montalvo & Hill, 2011). Contact zones between the Indo-Pacific and the Caribbean barrel sponges do not exist. While gene flow between barrel sponge populations in the Caribbean and the Indo-Pacific via ballast water from ships or vessels travelling through the Panama Canal is possible (Ruiz *et al.*, 2007), it is unlikely to cause or explain the haplotype patterns observed in this study. No stepping stones bridging the Atlantic or the Pacific populations have been found yet that would facilitate a worldwide distribution of a single species. In addition, sponge

larvae are known to typically have short lifespans (see Maldonado, 2006). There are no data on the present or past existence of barrel sponges in the Eastern Pacific, which might potentially explain the haplotype overlaps as an historical consequence of gene flow between both regions (see in this context Knowlton *et al.*, 1993; Knowlton & Weigt, 1998).

Why does the cytochrome oxidase 1 display overlapping haplotypes? We conclude that *Xestospongia* mitochondrial systematics suffers from factors that mask the distinctiveness of the individual lineages. This can occur if divergence time for the cytochrome marker is longer than the divergence time of the two sibling species (Charlesworth, 2010). In other words, the polymorphisms occurred before descendants split into two separate species. These so-called ancient polymorphisms explain the overlapping haplotype distribution among the widely distributed barrel sponge populations rather than the presence of a single cosmopolitan barrel sponge species, although additional gene loci are required to test this hypothesis (see e.g. Wu, 1991; Takahata & Satta, 1997; Rannala & Yang, 2003; Zhou *et al.*, 2007). The distribution of morphological, ecological and biochemical features of the three barrel sponge species strongly suggest the presence of ancient polymorphisms and explain the presence of identical haplotypes for morphotypes in both *X. muta* and *X. testudinaria* barrel sponges (Lopez-Legentil &

**Table 3.** Pairwise  $F_{ST}$  values between populations of *X. testudinaria*

Population	Saudi Arabia	Tanzania	Taiwan	Thailand	West Java	Central Java	East Java, Bali, Sumba	North Sulawesi	South and South-east Sulawesi	Great Barrier Reef & Solomons
Saudi Arabia	0.00000									
Tanzania	0.64309	0.00000								
Taiwan	0.79412	-0.08484	0.00000							
Thailand	0.65982	0.53306*	0.58250*	0.00000						
West Java	0.20755	0.36607*	0.41935*	0.43917*	0.00000					
Central Java	0.68966	-0.06814	-0.11078	0.53185*	0.34472	0.00000				
East Java, Bali, Sumba	0.18919	0.34050	0.37838	0.37377*	0.09950	0.33154	0.00000			
North Sulawesi	0.24492	0.39836*	0.44124*	0.14689	0.20787*	0.41011*	0.20305	0.00000		
South and South-east Sulawesi	0.32216	0.43221*	0.47371*	0.05110	0.22752	0.43020*	0.11111	-0.01047	0.00000	
Great Barrier Reef & Solomons	1.00000	0.01573	0.00000	0.70612*	0.52703*	0.02778	0.54002*	0.50116*	0.60752*	0.00000

\*Significant values at  $P < 0.005$  after Bonferroni corrections.

**Table 4.** Hierarchical analysis of molecular variance (AMOVA) based on the I3-M11 region of *X. testudinaria*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	9	20.323	0.13530 Va	32.49
Within populations	201	56.506	0.28112 Vb	67.51
Total	210	76.828	0.41643	
Fixation ( $F_{ST}$ ) index	0.32491*			

\*Significant values at  $P < 0.005$  after Bonferroni corrections.

**Table 5.** Spicule dimensions of the barrel sponge type specimens

Species	Spicule length	Spicule width
<i>X. testudinaria</i> (BMNH 1881.10.21.266)	174.95–277.17–331.71 $\mu\text{m}$	8.34–14.67–21.65 $\mu\text{m}$
<i>X. bergquistia</i> (QM G25018)	303.28–352.29–378.09 $\mu\text{m}$	4.96–11.54–16.26 $\mu\text{m}$
<i>X. muta</i> (MCZ PORa-6449)	192.31–367.67–446.44 $\mu\text{m}$	3.39–14.19–22.61 $\mu\text{m}$

Minimum–mean–maximum.

Pawlik, 2009). These ancient polymorphisms, in combination with the slow mitochondrial substitution rates in poriferans, prevent the formation of monophyletic species based on *cox1* data and falsely suggest the presence of cosmopolitan lineages, which is a concept successively rejected in the literature on sponges and other marine taxa (see Palumbi *et al.*, 1997; Solé-Cava & Boury-Esnault, 1999; Wörheide *et al.*, 2008). This is particularly evident since microsatellites, which are faster evolving independent markers, clearly show the distinction between *X. bergquistia* (including the type material) and *X. testudinaria* samples (Bell *et al.*, 2014a, b). These results imply that the I3-M11 cannot be regarded as universally suitable for detecting species boundaries in all demosponges.

### The suitability of cytochrome oxidase 1 to resolve barrel sponge intra-specific relationships

Nucleotide diversity ( $\pi$ ) is a measure of genetic variation, which is applied to calculate the degree of polymorphism in a population. Therefore, the higher the  $\pi$  value, the more suitable is a marker in assessing intraspecific genetic diversity. The low variation in mitochondrial genomes observed in several non-bilaterian taxa (see e.g. Shearer *et al.*, 2002; Huang *et al.*, 2008) generally restricts the use of mitochondrial proteins as markers for population analyses in demosponges. However, a higher  $\pi$  value on the 3' (I3-M11) partition in mitochondrial DNA would suggest that this marker is more suitable to resolve genetic diversity compared with the 5' partition. For *X. muta*, Lopez-Legentil & Pawlik (2009) found a nucleotide diversity of  $\pi = 0.00058$  in the 5' partition, contrasting the much higher nucleotide diversity of  $\pi = 0.0039$  in the I3-M11 partition, which restricts the use of the 5' partition for intraspecific analyses and highlights the use of the I3-M11 partition for these purposes (see additional examples in Uriz & Turon, 2012). Among the Indo-Pacific *X. testudinaria* six haplotypes were sampled over a range of more than 10,000 km extending from the Red Sea to Melanesia with  $\pi = 0.00290$  in the I3-M11 fragment. Five of these haplotypes were detected in specimens from the Indonesian archipelago in an area of 2300 km distance ( $\pi = 0.00302$  west Java to north Sulawesi). Nevertheless, our analysis clearly highlights that the application of I3-M11 in barrel sponges is only suitable when the correct boundaries of the target species are defined. Among Indo-Pacific sponges all haplotypes of *X. bergquistia*, of which the I3-M11 fragment has been sequenced for the first time, overlap with haplotypes of *X. testudinaria*. Likewise 50% of the haplotypes of the Caribbean *X. muta* found by Lopez-Legentil & Pawlik (2009) are shared with Indo-Pacific haplotypes. Consequently, prior to the application of I3-M11 on the intraspecific relationships of barrel sponges, the correct assignment to either barrel sponge species is mandatory.

## Implications for the genetic diversity and population structure of *Xestospongia testudinaria*

In *Xestospongia testudinaria* we recognize a major difference in common haplotypes among regions. For instance, C2 is the most common haplotype in Tanzania, Taiwan and the Great Barrier Reef. On the other hand, C1 is the most common haplotype in Sulawesi and Thailand, and is also only present in those areas. One haplotype is found in Tanzania (Indian Ocean) only. The occurrence of major haplotypes has been suggested to be influenced by environmental factors such as currents or anthropogenic effects (Lopez-Legentil & Pawlik, 2009; Swierts *et al.*, 2013), and shows an eco-geographic heterogeneity in the Indo-Pacific. In addition, the hierarchical AMOVA and overall  $F_{ST}$  results display genetic structuring for *X. testudinaria*, which also corroborated the results of the geographically narrow scale studies of north Sulawesi (Swierts *et al.*, 2013) and south-east Sulawesi (Bell *et al.*, 2014a) populations.

The existence of different external morphotypes in *X. testudinaria* with distinct haplotypes, which likely represent reproductively isolated rather than ecophenotypic varieties, implies the presence of a *X. testudinaria* species complex composed of several sympatric species (Swierts *et al.*, 2013) (see also Bell *et al.*, 2014a). Likewise, in *X. muta*, two morphotypes (smooth and digitate) were found to have different genotypes although the authors assume that hybridization prevents their separation (Lopez-Legentil & Pawlik, 2009). The presence of different *X. muta* lineages is also evident from different sterol compositions in three specimens of this species examined by Fromont *et al.* (1994), although they did not assign the different chemotypes to morphotypes. Nevertheless, our understanding of *Xestospongia* barrel sponge species with respect to their global genetic structuring and diversity is still limited due to unbalanced sample sizes and insufficient numbers of sequences at the regional scale (e.g. in the Solomon Islands, Great Barrier Reef, Thailand, Taiwan, Tanzania, Red Sea), including potentially missed haplotypes in some regions. These should be addressed in future analyses. Because of high sample numbers from one specific area and low numbers from other areas, we might have missed the occurrence of other haplotypes. For this reason, a more balanced sample size is required in order to get more accurate results for the future study of spatial relationships in every barrel sponge species, particularly *X. testudinaria* and *X. bergquistia* in the Indo-Pacific. Likewise, the utilization of nuclear intron markers, such as ATPS-beta (Bentlage & Wörheide, 2007; Wörheide *et al.*, 2008) could be beneficial to support our understanding of barrel sponge relationships.

## SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0025315415001149>.

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