Genetic diversity of selected petrosiid sponges

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Contents

Summary

Sponges are simple animals that mostly inhabit the marine ecosystem. The role of sponges in the marine ecosystem and the potential of their bioactive compounds for the pharmaceutical industry have already been reviewed. Because of the extensive investigations of sponges within those two disciplines, marine ecology and chemistry, sponges are among the best-studied Metazoa. Likewise, sponges have been selected as animal models for investigating the origin of the multicellularity because sponges have a simple body structure and physiology (e.g., lack of nervous and circulatory organs). Due to their diversity and abundance in the tropics, particularly in the Indo-Pacific, sponges have also attracted taxonomists, systematists and ecologists to assess their diverseness and their phylogenetic and phylogeographic relationships. Resolving those research questions is difficult, because sponges are categorised as comparatively character poor taxa. By using only conservative taxonomy or systematics, the sponge diversity might therefore be underestimated. Inevitably, sponge biologists have to employ molecular methods as additional tools.

In this research, molecular tools were used in order to analyse the taxonomy, phylogeny and phylogeographic relationships of selected sponge species. *Xestospongia testudinaria* & *Neopetrosia exigua* (Family Petrosiidae, Order Haplosclerida) were selected because of their conspicuousness in the Indo-Pacific coral reef ecosystems, whereby *Xestospongia testudinaria* is prominently known as the Indo-Pacific giant barrel sponge. Additionally, the order Haplosclerida has been described as an example of sponge order that has been examined systematically for a number of years and displays major discrepancy between morphology and molecular phylogenies. Molecular data suggests that the order needs revision at all taxonomic levels, which is the cause for further conflicts between taxonomists and systematists. In my research I focused mostly on sponge samples that originated from South East Asia or the Indo-Australian Archipelago (IAA). This region represents one of the best-explored marine regions in the Indo-Pacific. The aim of my research is to discover to what extent molecular tools are suitable to detect a phylogenetic signal, a phylogeographical break or a genotypic difference in the two selected sponge taxa.

Several markers from the mitochondrial (mtDNA), ribosomal (rRNA) and nuclear (nucDNA) have been utilised. The 3' partition of the cytochrome oxidase subunit 1 (I3-M11 of cox1) from the mtDNA could be used to detect a genetic structure in *Xestospongia testudinaria* in a geographical narrow scale study of < 200 km^2 in Lembeh, North Sulawesi, Indonesia **(Chapter 6)** and throughout the Indo-Pacific despite limitations in the sample

datasets (**Chapter 2**). In addition, the presence of a species complex in *X. testudinaria* was detected with the aid of phylogenetic reconstructions from a concatenation of mtDNA sequences (I3-M11 of cox1 and the Adenosine Triphosphate Synthase F0 subunit 6 / ATP6), and a nucDNA marker, the Adenosine Triphosphate Synthase β subunit intron (ATPS-β intron) **(Chapter 6).** At the same time, the presence of a species complex in *X. testudinaria* was recognised in a broader scale study of the Indo-Australian Archipleago (IAA) (**Chapter 3**). As a result, selected mtDNA and nucDNA markers in this thesis are useful for the investigation of the taxonomical status and phylogeographical relationships of *X. testudinaria*. A phylogeographical break in the IAA region due to the Pleistocene low sea level and Holocene recolonisation events (**Chapter 3)** could not be recovered among *X. testudinaria* in a phylogeographical analysis. Similarly, overlapping I3-M11 cox1 haplotypes between *X. testudinaria, X. muta* and *X. bergquistia* were recovered. This might be due to the presence of ancient polymorphisms on the barrel sponge mtDNA markers.

Molecular tools are also used to help identifying my second selected sponge species (**Chapter 4).** The use of selected cox2 mtDNA and 28S rRNA markers contributed significantly to the identification of. *Neopetrosia exigua* used to be a congeneric of *X. testudinaria*. During my examinations of self-collected and holotype specimens I discovered that the species named *N. exigua* bears a wrong name. For this reason, a taxonomical revision is suggested and, more importantly, according to my findings and the principle of priority in the ICZN (International Code of Zoological Nomenclature) I use the species name '*chaliniformis*' instead of the species name '*exigua*'. Furthermore, the use of selected nucDNA marker, the Lysidyl Aminoacyl Transfer RNA Synthetase (LTRS) intron, also contributes to the detection of phylogeographical breaks in *N. chaliniformis* of the IAA **(Chapter 5)**.

In a nutshell, the success of unravelling sponge taxonomies, phylogenies, and phylogeographic relationships always depends on the suitability of the utilised molecular markers and the significance of environmental influences on the sponges. Haplosclerid sponges possess limited morphological features. These hurdles create several problems, e.g. difficulties with taxa delimitation and unresolved phylogeography relationships. Even though the application of molecular techniques generated some limitations and obstacles in these studies, it has already contributed significantly to a better understanding of the phylogenies, phylogeographic relationships and taxonomical problems of *X. testudinaria* and *N.chaliniformis*, the species I selected for my research.

Author Contributions

Chapter two, Genetic diversity of the Indonesian giant barrel sponge *Xestospongia testudinaria* (Porifera; Haplosclerida)

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Chapter three, Exploring the utility of intron Adenosine Triphosphate Synthase β subunit (ATPS-β intron) for resolving phylogeographic relationships of the Indo-Pacific giant barrel sponge *Xestospongia testudinaria* (Porifera; Haplosclerida)

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Chapter four, Bearing the wrong identity: on the smooth brown Indo-Pacific sponges, *Neopetrosia exigua* (Porifera; Haplosclerida)

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Chapter five, Utilising nuclear intron Lysidyl Aminoacyl Transfer RNA Synthetase (LTRS intron) for resolving phylogeographic relationships of smooth-brown sponges, *Neopetrosia exigua* (Porifera; Haplosclerida)

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Chapter six, Lock, stock and two different barrels: comparing the genetic composition of morphotypes of the Indo-Pacific sponge *Xestospongia testudinaria*

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CHAPTER 1

General Introduction

1.1. General characteristics of sponges

Sponges are multi-cellular animals that constitute the Phylum Porifera Grant 1836. They are considered to be one of the most diverse phyla (e.g., van Soest et al. 2012) and the earliest branching lineage of all metazoans (e.g., Nichols et al. 2012). About 8,000 recent species have been described and the total number of species is estimated at more than 15,000 (see review in Hooper and van Soest 2002a; van Soest et al. 2012). Currently, the number of described taxa has been increasing to more than 10,000, including more than 2,000 species from poriferan fossils (see review in Zhang 2013). Sponges are sessile animals and live predominantly in marine habitats. However, one sponge sub-order (Spongillina) inhabits the freshwater environment (see review in Manconi and Pronzato 2002). Because of their sessile life-style, most sponges depend on organic particles, which are obtained from filtering water and from living symbiotically with other organisms. Only a few groups of sponges are recognised as carnivorous (Vacelet and Boury-Esnault 1995). In order to get nutritious substances, sponge cells capture small food particles and oxygen by filtering large amounts of water. Nutrients in the water enter the outer layer that is composed of pinacocytes. In this outer layer, pores (ostia) and channels are present. The wave-like movements of the flagella drive water with nutrients into the inner part to the flagellated choanocytes (or collar cells). Subsequently, food particles are absorbed into the feeding cells. Besides carrying nutrients into the nuclei of the feeding cells, flagella also drive and propel oligotrophic water out through the osculum (Figure 1.1).

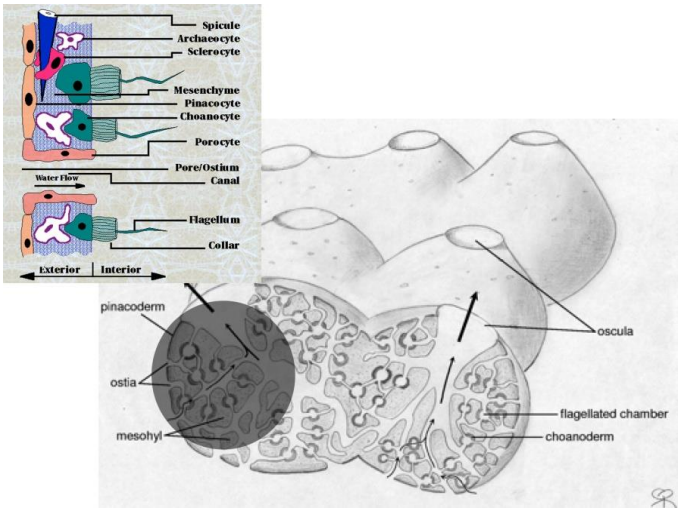


Figure 1.1. Macroscopic and microscopic sponge anatomies. Figure is taken and adapted from Ruppert and Barnes (1994)

The body plan of sponges can be categorised into four aquiferous types: (1) Ascon, the simplest aquiferous system, in which choanocytes line the entire internal cavity of the sponge (2) Sycon, which is an aquiferous system with elongated choanocyte chambers, (3) Sylleibid, a system that is more complex than Sycon because it has radial invaginated choanocyte chambers and (4) Leucon, the most complex aquiferous system, which comprises of discrete and dispersed choanocyte chambers in the mesohyl (Figure 1.2).

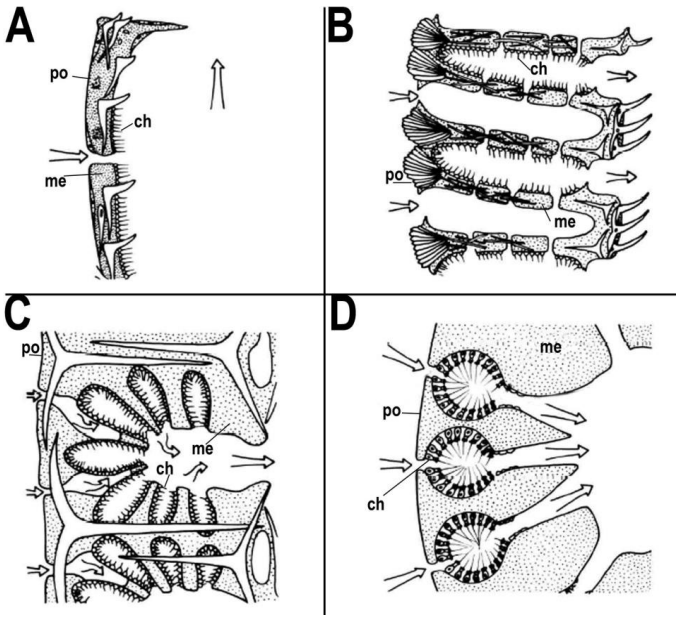


Figure 1.2. Type of aquiferous system in sponges. A. Ascon. B. Sycon C. Sylleibid D. Leucon
po= porocyte ch= choanocyte me= mesenchyme arrows= water flow
Figure is taken from Boury-Esnault and Rützler (1997)

The mesohyl is a layer between pinacocytes and choanocytes, where amoebocytes and spicules are present. Amoebocytes, also named archaeocytes, are unspecialised cell types that can be transformed into specific cells with physiological activities like secretion of spicules (a hard mineralic needle-like structure) and spongin (a protein substance for developing skeletal fibres). Spongin fibres and spicules are the main morphological characters in classical sponge taxonomy (see further 1.2). They stiffen the mesohyl, which is one of the main components that build the endoskeleton. However, in some hard sponge taxa like coralline sponges, exoskeletons are present, which are developed by the calcium carbonates secretion from the pinacocytes.

Sponges have an asexual mode with buds or gemmules, whereas hermaphroditism or gonochorism represent their sexual mode. The reproductive condition of sponges is oviparous

(spawning or depositing their fertilised or unfertilised egg) or viviparous (brooding and nourishing the egg internally) (see review in Pechenik 2000; Müller 2003). In asexual reproduction, a juvenile sponge will grow and attach to the parental sponge, which is called budding. In contrast, some sponge species will bud internally by packing new sponge cells into vesicles that are called gemmules. These gemmules are usually found in freshwater sponges and are released into the water when the environmental conditions are favourable for sponge growth. All sponges that are produced asexually are clones of their parents. In the sexual mode, most sponges are hermaphrodites, in which the function of both sexes is simultaneously present although gonads are absent. Sperms are produced by spermatic cysts that are transformed from choanocytes while eggs are from transformed archaeocytes and sometimes choanocytes. In the spawning period, sperm cells are expelled from the sperm cysts via the osculum and broadcasted into the water to find egg cells of other individuals to cross-fertilise. In contrast to hermaphroditism, gonochorism is a sexual mode in which every individual has only one type of reproductive cell. Nevertheless, in gonochoric individuals, sexual dimorphism is not recognised. Because the sexual mode (hermaphrodism or gonochorism) and reproductive condition (oviparous or viviparous) of sponge species are so specific, the ancestral reproductive strategy of sponges has already been investigated. The outcome of that research is that the reproductive condition of the sponge ancestors was probably viviparity and the sexual mode gonochorism (Riesgo et al. 2013).

According to the current sponge systematics, Phylum Porifera is grouped into four classes (see review in van Soest et al. 2012; Wörheide et al. 2012). The first class is Hexactinellida or commonly known as glass sponges, which mainly inhabit the deep sea. Approximately 500 species of glass sponges are recognised and found mostly in a range of 300 - 600 m depth. Due to limitations on deep-sea habitat exploration and the fact that there are not many experts that can describe taxa of museum samples, the actual number of recognised taxa is still underestimated (Reiswig 2002). Some important morphological characters that make Hexactinellida distinct from other classes are (1) syncytial tissue that is formed through the fusion of early embryonic cells, (2) hexactin shaped spicules or a derivation thereof for both microscleres and megascleres (3) a triaxonal or cubic skeleton, and (4) a square shaped axial filament (Leys et al. 2007). The second sponge class, which is characterised by a siliceous and occasionally calcareous mineralic skeleton in some species, is Demospongiae. Demospongiae is the most diverse group among poriferan classes, which consist of 85 % of the extant sponge species (Hooper and van Soest 2002b). Demospongian

skeletons consist of monaxonic, tetraxonic, or polyaxonic mineral skeletons, but never of triaxonic skeletons like Hexactinellida. In contrast to the syncytial tissue of Hexactinellida, Demospongiae comprise of cellular tissue. Spongin and spicules may also be very reduced. Recent discoveries and state-of-the-art methods revealed that homoscleromorph sponges form a separate class (Gazave et al. 2012), see below. The third fourth sponge class, named Calcarea, is distinguished by calcium carbonate spicules that are secreted extracellularly (Manuel et al. 2002; Sethmann and Wörheide 2008). Furthermore, most of the calcarean skeletons are composed of free spicules and are fused from a rigid basal skeleton or cemented spicules. Diactines, triactines and tetractines are the most common spicule types among calcarean sponges. Unlike Hexactinellida, which mostly inhabit deep marine water, calcarean sponges inhabit mostly marine shallow water with some exceptions of species that inhabit the deep sea (Rapp et al. 2011). The fourth and most recent established sponge class is Homoscleromorpha. Before separated as a valid and monophyletic sponge class, Homoscleromorpha was classified a member of Tetractinellida (Demospongiae) because of their tetractinal siliceous spicules (Lévi 1956). Molecular phylogenetic analyses, indicate that homoscleromorphs are sister to Calcarea (Dohrmann et al. 2008; Philippe et al. 2009; Pick et al. 2010) with potential apomorphic cross–striated rootlets in the ciliated cells of the cinctoblastula larva, (see Cárdenas et al. 2012; Gazave et al. 2012; Wörheide et al. 2012).

As the focus of my studies was the genetic diversity and phylogeographical patterns of selected petrosiid sponges (*Xestospongia testudinaria* and *Neopetrosia exigua*, Class Demospongiae), I will concentrate on a taxonomic and phylogenetic review of the marine haplosclerid family Petrosiidae and some species concepts that are generally used for delimiting sponges, as well as their limitations. I also explore the utilities of molecular biology for resolving the phylogenetic and phylogeographic relationships of sponges in general, particularly haplosclerids. Furthermore, I describe my selected sponge taxa, which are recognised as common and iconic species in the Indonesian archipelago, including their taxonomy and phylogenetic relationships. Finally, I describe two major events in the paleogeographic history of the Indonesian archipelago, which could have influenced the genetic diversity and phylogeographical pattern of the sponge taxa studied in this thesis.

1.2. The taxonomy and phylogeny of marine haplosclerids

Haplosclerida Topsent, 1928 is the second most diverse order of demosponges in terms of species and habitat (van Soest et al. 2012). Haplosclerid sponges belong to an order

that has been systematically researched for a number of years, but whose phylogeny has not been resolved yet. The classification of haplosclerids appears unreliable due to a limited suite of complex characters, high plasticity, large numbers of taxa and discrepancies between morphological and molecular data (Redmond et al. 2007). Prior to the *Systema Porifera* in 2002, several changes in haplosclerid classification have occurred (see details in Fromont 1990). These changes were due to the fact that classification was difficult since the order Haplosclerida has a low diversity of spicule types and simple skeletal structures (Weerdt 1985). Basically, there are two major versions that describe the classification of order Haplosclerida:

First, Bergquist (1980) explained that besides Order Haplosclerida, a second order should be established, which was given the name "Nepheliospongida", and was raised from the family Nepheliospongiidae, (Bergquist 1980). Order Nepheliospongida comprises of the family Oceanapiidae and the family Nepheliospongiidae, to which the selected sponge taxa of this thesis (*Xestospongia*) belong. Bergquist proposed the establishment of Order Nepheliospongida based on four evidences, which distinguish it from Order Haplosclerida. The first was a fossil indication that Nepheliospongida was separated from Haplosclerida since the Devonian. The second was the presence of novel sterols that possess cyclopropene rings in their side chain. This is exclusively the case for Nepheliospongida. The third evidence is oviparity as reproductive condition, and the fourth is the presence of a siliceous skeleton to an extend that made the sponges hard and stony.

Van Soest proposed the second scenario in 1980. He did not support the scenario of Bergquist since oviparity is too primitive to be utilised for taxonomy. Moreover, there was no evidence that petrosiids and oceanapiids would be closer related to each other rather than other haplosclerids (van Soest 1980). In his opinion, a monophyletic group could only be defined by derived character states. Therefore, he kept the order Haplosclerida, which now comprises of five families (Haliclonidae, Petrosiidae, Niphatidae Oceanapiidae and Callyspongiidae). This scenario was later used in the *Systema Porifera* (2002). In the *Systema Porifera*, Order Haplosclerida consists of three suborders, which are Haplosclerina Topsent, 1928, Petrosina Boury-Esnault and Van Beveren, 1982 (consisting of nepheliospongiids described by Bergquist, 1980), and Spongillina Manconi and Pronzato, 2002 (freshwater sponges).

According to the *Systema Porifera*, Order Haplosclerida is mainly characterised by three important characters, namely isodictyal skeletons of diactinal megascleres, short

fusiform oxeas or short compact strongyle megascleres, and several restricted forms of microscleres (strongyles, sigmas, toxas, raphides, and amphidiscs), van Soest and Hooper (2002c) (Figure1.3).

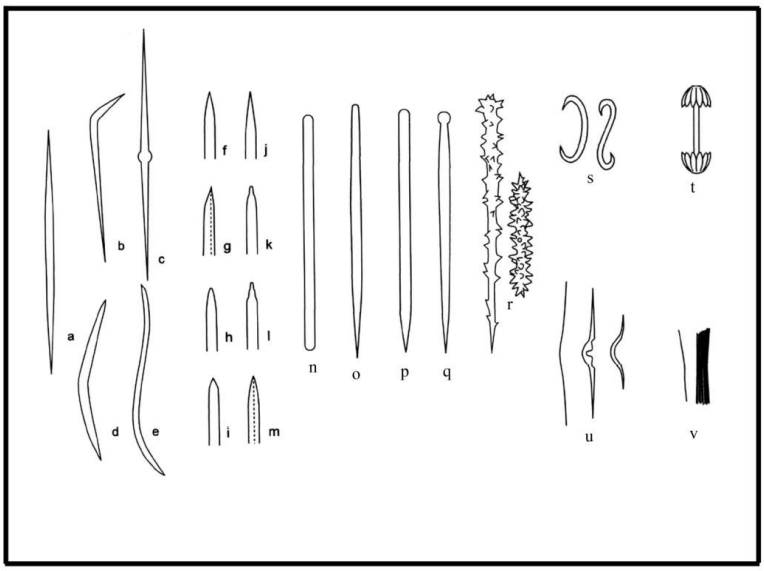


Figure 1.3. Common spicule types of haplosclerid sponges. Monaxonic or diactinal megascleres oxeas (a-m) with different types of shape and tip morphology. Shape (a) fusiform (b) angulate (c) centrotylote (d) curved (e) flexuous. Tips are (f) acerate (g) asymmetrical (h) blunt (i) conical (j) hastate (k) mucronate (l) stepped (m) symmetrical. Other types of megascleres (n-r) are: (n) strongyle (o) strongyloxea (p) style (q) subtylostyle (r) spined acanthostyle. Types s-v are:(s) sigma (t) amphidisc (u) toxa (v) raphide. Figure is taken and adapted from Boury-Esnault and Rützler (1997)

The habitat is the simplest and easiest factor to distinguish suborder Spongillina (freshwater sponges) from other Haplosclerida (Haplosclerina and Petrosina, marine sponges). Furthermore, spined megascleres, amphidisc microscleres, gemmules in the reproduction system, and the absence of tangential ectosomes are the most important morphological characters, which make freshwater sponges very dissimilar to marine haplosclerids. Intensive studies on the molecular phylogenies of the order Haplosclerida (e.g., Itskovich et al. 2006; Redmond et al. 2007) revealed and suggested that the position of suborder Spongillina based on the classical Linnaean taxonomy needed to be revised. Based on the phylogenetic relationship, freshwater sponges are not positioned as a sister group of marine haplosclerids (Clade G3), but placed as a sister group of other sponge orders in Clade G4 like Agelasida, Halichondrida, Poecilosclerida, etc. (see review in Wörheide et al. 2012) (Figure 1.4). Currently, sponge systematists try to identify phylogenetic signal that can be used to delimit the lower rank taxa within the clades. This will help taxonomists to establish a new

taxonomical scheme, which was introduced and advocated as "The bottom up strategy" by Cárdenas et al. (2012). This strategy can also be used to help resolve the morphological and molecular discrepancies (see further 1.5 reported on the marine haplosclerids (McCormack et al. 2002).

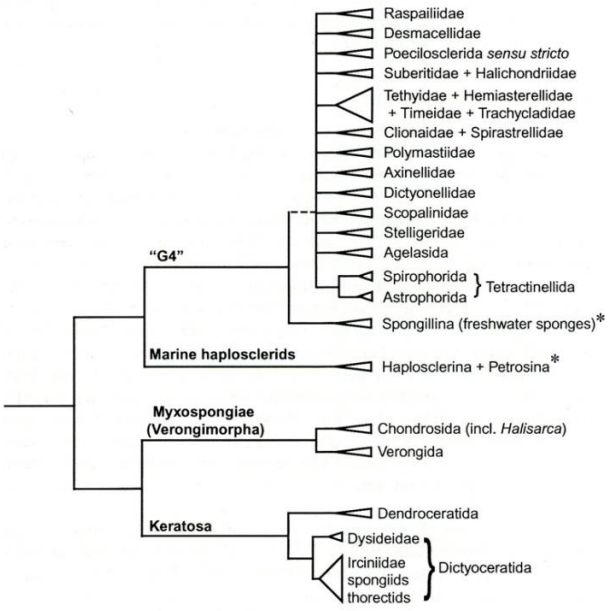


Figure 1.4. Overview of a phylogenetic tree based on nuclear and mitochondrial DNA. Figure is modified from Wörheide et al. (2012). This figure is used to describe the position of marine (G3) and freshwater haplosclerids (G4), which are positioned in different clades (*)

The morphological differences between suborders Haplosclerina and Petrosina are found in the skeleton structure, which is anisotropic for Haplosclerina and isotropic for Petrosina (Figure 1.5).

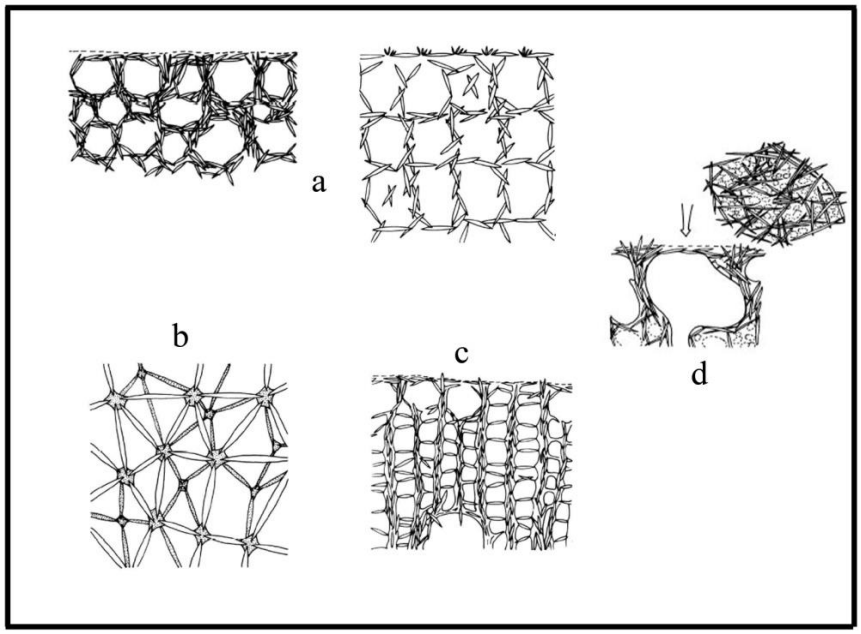


Figure 1.5. Common skeleton arrangements in haplosclerid sponges. (a) isotropic reticulation i.e. no differentiation of reticulation in primary and secondary fibres, tracts or lines. (b) isodictyal reticulation: isotropic reticulation, in which the meshes are spicule-long triangles. (c) anisotropic reticulation: skeleton with secondary tracts, lines or fibres. (d) tangential skeleton, in which ectosomal skeletons are arranged parallel to the surface. Figure is taken and adapted from Boury-Esnault and Rützler (1997)

However, both suborders share spicule sizes and types (van Soest and Hooper 2002c), as well as chemical components (van Soest and Braekman 1999). Therefore, the use of morphological characters is not suitable for distinguishing lower taxa and makes vague and elusive synapomorphies in the suborders Haplosclerina and Petrosina (van Soest and Hooper 2002b; van Soest and Hooper 2002a). Moreover, environmental factors also influence morphological characters, which can create obstacles in resolving taxonomic and phylogenetic relationships of marine haplosclerids.

1.3. Species concepts usually used in delimiting sponges species and their limitations.

Sponges or are often considered as character-poor taxa. Because of this, taxonomic and systematic uncertainties are frequent (Klautau et al. 1999; Knowlton 2000). Several species concepts have been implemented for the delimitation of sponge species. To my knowledge, one of the most conventional and simple species concepts is the Diagnosable Species Concept. This concept delineates a taxon based on qualitative or fixed differences (see de Queiroz 2007). For instance, in sponges morphological characters like spicule types, spicule composition, spicule dimensions, meshes, and skeleton arrangements have already

been used for supra–specific classifications like the *Systema Porifera* (Hooper and van Soest 2002a). These constitutive elements are unreliable as they are shared among sponges from different orders, families, genera etc, particularly within haplosclerids (van Soest and Hooper 2002c). Besides this unreliability, morphological plasticity is also caused by environmental factors such as currents and bathymetric effects. For example, in an area with strong and turbulent current, growth and density of sponges may be small, and delicate forms like pedunculate or arborescent shapes will be absent. Conversely, massive and globular forms will be dominant in that area. At the same time, in areas where the current is weak, the pedunculate or arborescent form is predominantly present to relieve the sedimentation in that area (Bell and Barnes 2000). Another example is the effect of predation on spicule composition (Hill and Hill 2002). Due to such phenotypic plasticity sponge, particularly haplosclerid taxonomy is difficult (Barnes and Bell 2002).

In general, siliceous sponges like Demospongiae take silica from sea water in the form of silicic acid. Obviously, the silicic acid content in any given area is different throughout the geographical location and is influenced by external factors such as upwelling, temperature, salinity, depth, etc. In an in vitro experiment with the halichondrid sponge *Halichondria panicea*, a positive correlation was demonstrated between silica content dissolved in water and the intake of silica by the sponge. In extreme conditions, like during the absence of dissolved silica, *Halichondria panicea* could not even produce spicules (Frøhlich and Barthel 1997). Furthermore, it is reported that environmental factors definitely affect the size and growth of spicules in the halichondrid *Hymeniacidon perleve* (Stone 1970), the poecilosclerid *Crambe crambe* (Maldonado et al. 1999), the hadromerid *Cliona azzaroliae*, and the petrosiid *Petrosia ficiformis* (Bavestrello et al. 1993).

Another approach of the Diagnosable Species Concept is the use of chemical characters (see further 1.4). Another species concept that is currently frequently used, is the Monophyletic Species Concept. This concept examines ancestors and their descendants, including shared derived character properties (see de Queiroz 2007) (see further 1.5). The Monophyletic Species Concept is frequently used because during intensive studies of molecular phylogeny remarkable cryptic species and species boundaries have been discovered in Porifera (Blanquer and Uriz 2007; Cárdenas et al. 2007).

1.4. The use of chemical markers for resolving the phylogenetic relationships of haplosclerid sponges

Biochemical compounds in sponges have been recognised as a novel and valuable source for discoveries and explorations in the medical field (see review in Garson 1994; Munro et al. 1999; Proksch et al. 2002; Belarbi et al. 2003). Biochemical compounds are also utilised for chemotaxonomic purposes. Sterols were investigated as a major component in marine organisms and the sterol structures in sponges have been explored since the 1950s (Bergmann 1949). Furthermore, specific sterol structures in sponges, particularly in Demospongiae, were researched intensively towards the end of the 1970s (e.g., Sica et al. 1978; Kanazawa et al. 1979). In the early 1980s, sterol compositions of Demospongiae were proposed as a robust character for distinguishing four sponge orders (Verongida, Nepheliospongida, Axinellida, and Hadromerida) even though this method was unreliable due to seasonal and geographical differences (Bergquist et al. 1980). Despite the fact that the order Nepheliospongida no longer exists and is now referred to as suborder Petrosina, the use of chemical markers is still suitable for distinguishing it from suborder Haplosclerina (Fromont et al. 1994). The use of chemical markers such as sterols for distinguishing sponges within the order Axinellida should be reconsidered because some families within this order are still assigned to the orders Halichondrida, Hadromerida and Poecilosclerida. Likewise, the nominal taxa of Genus *Axinella* and Family Axinellidae are currently assigned to Order Halichondrida, see World Porifera Database (van Soest et al. 2014).

Besides sterols, other chemical compounds like alkaloids, terpenes, etc. were used for sponge taxonomy. Basically, chemical compounds in sponges, particularly in Demospongiae, are used as additional characters for supporting phylogenetic signals detected by morphology (van Soest and Braekman 1999). However, recent reviews demonstrated that some chemical compounds used for delimiting sponge orders and their lower ranks are unreliable (see review in Erpenbeck and van Soest 2007). For instance, sterol compositions, applied for the delimitation of *Xestospongia testudinaria* and *Neopetrosia exigua* (the two selected taxa for this research), are no longer regarded as reliable to distinguish between the two species of *Xestospongia* (*Xestospongia bergquistia* and *Xestospongia muta*) (Fromont et al. 1994). Fromont et al. (1994) stated that sterols were no longer suitable for delimiting petrosiid sponges. Furthermore, the unsuitability of C27, C28 and C29 Δ^5 sterols for resolving phylogenetic relationships of the taxa genus within *Xestospongia* has also been reported (Gauvin et al. 2004).

In an earlier review, van Soest and Braekman (1999) hypothesised that the straight–chain acetylenes or similar fatty acid compounds and alkylpiperidine derivatives, were exclusively present in haplosclerid sponges. However, after a review in 2004 (Erpenbeck and van Soest 2007), only brominated acetylenes were found suitable as exclusive markers for haplosclerid sponges. On the other hand, two other fatty acid compounds (straight-chain and polyhydroxylated acetylenes) were discovered in *Theonella* (Theonellidae), as well as 3-alkylpiperidine in poecilosclerid and halichondrid sponges. This diminished the suitability of straight-chain and polyhydroxylated acetylenes as a haplosclerid marker.

In general, the unreliability of chemical compounds and therefore the pitfalls of sponge chemotaxonomy are caused by seven factors, namely: (1) the absence or presence of chemical compounds, (2) the compound homology, (3) the compound variability, (4) the origin of compounds, (5) misidentification of the sponges, (6) sponge to sponge contaminations and (7) the insufficient amount of characters for a robust phylogeny (Erpenbeck and van Soest 2007). The first factor, the absence or presence of chemical compounds refers to ideal and suitable chemotaxonomic markers. It means that the specific chemical compounds should be only present in the member of the specific taxon, whereas they should be absent in others. If these criteria are not fulfilled, the markers cannot be defined as ideal and suitable anymore. An example for this is the overlapping fatty acid components (straight-chain and polyhydroxylated acetylenes) in haplosclerid and astrophorid sponges (Erpenbeck and van Soest 2007). The second factor, the compound homology, is probably the biggest problem that hinders chemotaxonomy. Here, the identification of compound production pathways is the largest problem since specific compounds that are usually utilized to distinguish a specific taxon are not necessarily derived from a common precursor. The third factor, the variability of compounds is influenced by biotic factors (precursors), the abundance of symbionts, and other abiotic factors like season and location. If compound compositions are variable and subsequently scored as different characters, the phylogenetic coding based on that variable character would not be reliable. The fourth factor, the origins of the compounds, refers to the fact that compounds produced by symbionts cannot be used as a character since symbionts might switch among different hosts that are genealogically unrelated. This will confuse the phylogenetic analysis. The fifth factor is a classical problem in sponge taxonomy, the misidentification. As we know, the Diagnosable Species Concept using morphological characters is very difficult to implement in sponge identification, especially among lower taxa. The sixth factor, even though it is rare, is the

erroneous identification of the correct compounds if several sponges were overgrowing each other. Finally, the low overall number of characters is an obstacle for robust chemotaxonomy. A popular solution to resolve this problem is the inclusion of compound concentrations as characters in the matrix analysis. However, this also may result in uninformative autapomorphic characters that cannot be used for supporting phylogeny. As all of these obstacles connected with chemotaxonomy remain difficult to resolve, molecular phylogenetic methods appear to be a good alternative for resolving sponge taxonomy and phylogeny.

1.5. The use of molecular markers for resolving the phylogenetic relationships of haplosclerid sponges

In the last few decades, molecular biology approaches have been useful to unravel species level diversity in the sea and recognising cryptic and sibling species (see review in Knowlton 2000). The first application of molecular biology, which utilised allozyme divergence on sponge systematics, was performed in 1980 (see review in Borchiellini et al. 2000). However, this method requires fresh samples, which constitutes a major obstacle. Then, the Polymerase Chain Reaction (PCR) was introduced for use in molecular phylogenetics, in which the availability of fresh samples was not such a pivotal requirement. In addition, several markers that can be utilised for PCR like nuclear and mitochondrial genes have been introduced. Those markers were frequently used for the reconstruction of phylogenetic relationships of sponges (see review in Erpenbeck and Wörheide 2007; Wörheide and Erpenbeck 2007). This technique is not only utilised to resolve host and symbionts phylogenies of halichondrid sponges (Erpenbeck et al. 2002), but also to unravel cryptic speciation (Blanquer and Uriz 2007; Blanquer and Uriz 2008; Xavier et al. 2010b; Reveillaud et al. 2011; see review in Uriz and Turon 2012) as well as to assist in the identification of species in the course of sponge barcoding (see review in Wörheide et al. 2007). Until now, the small (18S) and large (28S) of ribosomal subunits (hereafter referred to as 18S and 28S rRNA) and some of the mitochondrial genes (hereafter referred to as mtDNA) e.g., cytochrome oxidase subunit 1 (cox1), have been among the most popular markers.

In a eukaryotic organism, the energy-producing organelle (mitochondrion) has its own genome, i.e., the mtDNA is unrelated to the nuclear DNA (hereafter referred to as nucDNA), based on its evolutionary history. So far, mtDNA is the most utilised marker in animal phylogeny and phylogeography due to its uniparental inheritance, higher evolutionary rates, a high copy number in the organism, and little or total lack of recombination (see review in

Moritz et al. 1987). Likewise, the use of the cox1 gene from the mtDNA has become more popular for the barcoding of species or as a quick and efficient method for species delimitation (see review in Hebert et al. 2003; Borisenko et al. 2009), except for the lower metazoan taxa (see further). The knowledge of mtDNA in sponges has increased significantly after the first complete mtDNA genomes in sponges were published (Lavrov et al. 2005; see review in Wörheide et al. 2012). Likewise, the mtDNA marker is also utilised on the Sponge Barcoding Project (see http://www.spongebarcoding.org/), in which cox1 markers from the 5' partition (Folmer et al. 1994) and the 3' partition namely I3-M11 (Erpenbeck et al. 2002; Erpenbeck et al. 2006a) are particularly used. The utilisation of mtDNA in sponge phylogeny remains problematic however, because mitochondrial genes in most sponge species evolve slowly (Shearer et al. 2002; Huang et al. 2008). This creates a problem for the delimitation of two different sponge taxa (e.g., Pöppe et al. 2010) and for further utilisation of mtDNA in phylogeographic studies (see 1.6).

In comparison to the mtDNA genes, the genes of nucDNA are evolving even slower. This is referred to as "the three-times rule"(Palumbi and Cipriano 1998); i.e. if the branch length of the mtDNA sequences in the phylogram is three times longer than the average of mtDNA genetic diversity, there is a monophyly of sequences from most of the nuclear genes in the phylogram. Other problem like heterozygosity makes allele distinction difficult. Additionally, unconcerted multicopy genes are problematic. In a multicopy gene family, a homogenisation process is needed to maintain its functionality. This means that transmissions and accumulations of mutations along repetitive regions need to be homogenised, which is known as a concerted evolution. However, with the exception of rRNA, a homogenisation process of repetitive regions is not always completely done. Hence, divergent copies occur and therefore phylogenetic analyses based on those unconcerted multicopy genes are likely to be erroneous. For this reason, the utilisation of classical rRNA markers like 28S rRNA and 18S rRNA in sponge phylogeny has been implemented (see review in Cárdenas et al. 2012). Other markers that are more polymorphic have also been introduced like the Internal Transcribed Spacer between 18S and 28S rRNA (ITS) (e.g., Wörheide et al. 2002; Wörheide et al. 2004) and the non-coding regions of nuclear genes (introns). Some intron markers are assumed to be part of a single copy gene and to be involved in evolutionary processes e.g., introns from Jarman et al. (2002). Therefore, these introns are suitable for phylogenetic analyses in sponges (e.g., Reveillaud et al. 2011) and further sponge intraspecies studies (see further 1.6).

The utilisation of molecular markers is inevitably needed to resolve haplosclerid phylogeny since the diagnosable approaches using morphological and chemical characters are not able to resolve it, particularly below family level. However, molecular phylogenetic analyses based on mtDNA and rRNA markers have not totally resolved haplosclerid phylogeny relationships yet, which is why their phylogeny is still one of the largest mysteries with many unanswered questions (e.g., McCormack et al. 2002; Redmond et al. 2007; Redmond and McCormack 2008,2009; Redmond et al. 2011). Cárdenas et al. (2012) postulated that the outlook of taxonomists, who utilise the presence of a skeleton as a marker for assessing the "internal relationship" within all haplosclerids, is always incongruent to the molecular phylogeny of haplosclerids. For this reason, "re-evaluation of morphological characters in relation to molecular results and starting a bottom up strategy", by studying the type species of each haplosclerid sponge genus first, is suggested and advocated.

1.6. The use of molecular tools in resolving phylogeography relationship of sponges

The slower mtDNA evolution rate in some of the lower metazoan taxa has prompted researchers to consider other markers for sponge intraspecies studies. Basically, the mtDNA is still the preferred marker in intraspecies studies because of its matrilineal inheritance, its faster coalescence time, and its high copy numbers in the organism (Avise 2000,2009). In order to assess the degree of polymorphism within a selected gene in intraspecies studies, usually the nucleotide diversity (π) value is measured. The nucleotide diversity is the proportion of nucleotide differences per site of two DNA sequences that are chosen randomly from one population (Nei and Li 1979). The higher the π value, the more polymorphic sites are present and the more suitable the marker is for intraspecies studies. Thus, the fixation index (F_{ST}), which provides insights into genetic structures and evolutionary history among selected populations, is inferred from the π value (see review in Holsinger and Weir 2009). In sponges, cox1 is utilised for assessing phylogeographic patterns. The 5' (Folmer) partition has lower π values (e.g., Duran et al. 2004; Duran and Rützler 2006; Wörheide 2006) compared to the 3' (I3-M11) partition (e.g., Lopez-Legentil and Pawlik 2009; Xavier et al. 2010a), which suggests the higher suitability of the I3-M11 fragment for intraspecies studies. At the same time, other polymorphic mitochondrial markers have also been introduced and tested in several sponge taxa (Rua et al. 2011). These consist of two genes (ATP Synthase 6 (ATP6) and cytochrome oxidase 2 (cox2)), and two intergenic regions (spacer between ATP6 and cox2 and the spacer between the NADH dehydrogenase subunit 5 (ND5) and the small

subunit of ribosomal RNA (rns)). However, the use of mtDNA should be reconsidered, because of its "patchiness". Especially when used for phylogeographic studies of organisms, mtDNA has its limitations (see review in Zhang and Hewitt 2003). The first potential problem is the interference of mtDNA pseudogenes. The second limitation is due to the matrilineal nature. Due to the occurrence of distinct alleles or haplotypes the interpretation of the population history may be biased. Finally, faster lineage sorting and high allele extinction rates also occur due the one-fourth of effective population size of mtDNA compared to nucDNA (e.g., Moore 1995). All of those limitations lead to oversimplification of evolutionary relationships, underestimation of the genetic diversity values, uncertainties in genealogical analysis and erroneous detections during analysis of coalescence between two distant populations.

At the same time, ITS has also been used in intraspecies studies of sponges (e.g., Wörheide et al. 2002; Wörheide et al. 2004). However, ITS turned out not be suitable for haplosclerids due to the lack of polymorphic sites (Redmond and McCormack 2009). Microsatellite primers from the nucDNA, which are able to amplify more variable sites, have been considered to replace mtDNA and rRNA markers. Several sponge microsatellite primers have been developed specifically, for instance in *Crambe crambe, Halichondria panicea, Scopalina lophyropoda, Hymeniacidon sinapium, Spongia lamella, Spongia officinalis, Ephydatia fluviatilis,* and *Paraleucilla magna* (see review in Uriz and Turon 2012), as well as recent microsatellite primers for *Xestospongia testudinaria,* a selected sponge taxon studied in this thesis, (Bell et al. 2014a; Bell et al. 2014b). However, the utilisation of microsatellites in intraspecies studies is not perfect either because of some issues. The first problem is that the different allele size, which is usually common in microsatellite sequences, may not directly relate to their divergence, but refer to homoplasy. The second difficulty is that mutation rates are different between organisms and may even vary. These inconsistent mutation rates might not refer to the divergence but to the dynamic process among alleles. Finally, the neutrality of some microsatellites is speculated as an abundant source of mutations, which only contributes to quantitative trait variations. Hence, the neutrality has recently been increasingly questioned (see review in Zhang and Hewitt 2003).

In contrast to the ITS markers, intron markers have been explored and developed from non-coding genomic regions of several nucDNA genes. These introns are spliced prior to translation in order to facilitate protein synthesis by mRNA. Exon-Primed, Intron-Crossing (EPIC) is a common method to amplify intron sequences using exon flanking regions

(Palumbi and Baker 1994). Polymerase Chain Reaction (PCR) primers are easily annealed on this region because of conserved exons in the intron flanking regions. EPIC loci also have several advantages compared to microsatellites as they are applicable across widespread species, less prone to null alleles and less susceptible to homoplasy (see review in Chenuil et al. 2010).

The intron of the Adenosine Triphosphate Synthase β subunit gene (ATPS-β intron) is an intron marker already applied to resolve phylogeographic relationships in sponges (Bentlage and Wörheide 2007; Reveillaud et al. 2010; see review in Uriz and Turon 2012). However, since introns are derived from nucDNA, several obstacles still hamper their application in the molecular phylogenetic and phylogeographic analyses. When nucDNA markers are utilised, the following obstacles are possible: i) distortion of valid information of evolutionary history caused by allele recombination, ii) ambiguous alignments caused by the high variability and different alleles length, and iii) misidentification of the allele type due to heterozygosity (Zhang and Hewitt 2003). Therefore, when using a nucDNA marker in a molecular analysis, its thorough understanding is required.

1.7. Selected haplosclerid taxa from the Indonesian archipelago

Sponges from the order Haplosclerida are considered to be important elements of tropical benthic communities, such as coral reefs and mangrove communities in the Indonesian marine ecosystem (van Soest 1989; Amir 1992; de Voogd and van Soest 2002; de Voogd 2004; de Voogd et al. 2004; de Voogd and Cleary 2008). Also, the order is recognised as the most abundant and diverse sponge group in this region. Likewise, *Xestospongia testudinaria* (Figure 1.6. A) and *Neopetrosia exigua* (Figure 1.7. A) are examples of conspicuous sponge taxa from the family Petrosiidae in the Indonesian marine ecosystem.

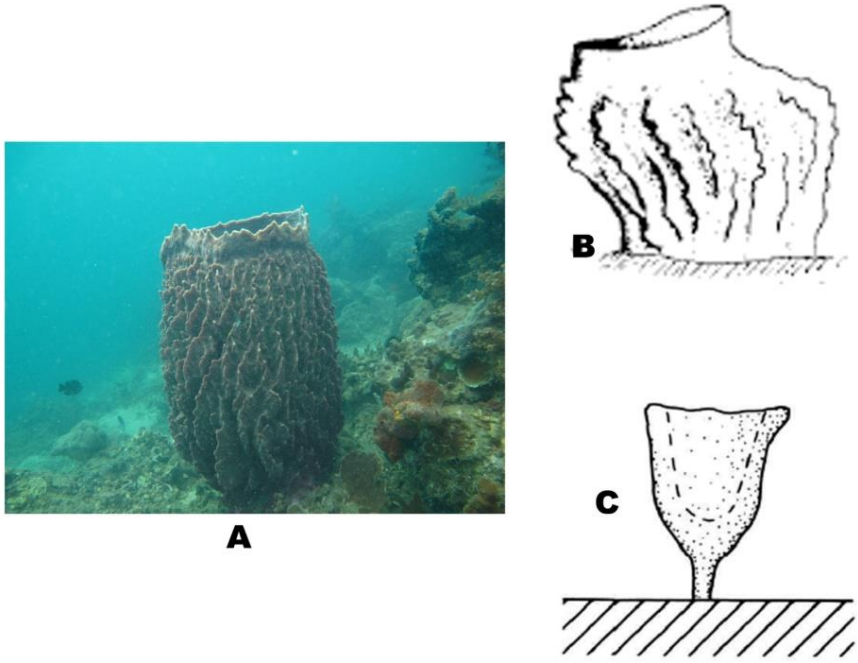


Figure 1.6. *X. testudinaria*. A is a picture of a *X. testudinaria* specimen that was sampled by the author in Pecaron Bay, Situbondo, East Java, Indonesia on 11 May 2011. B is a *X. testudinaria* sketch from the figure of van Soest (1989) and C is a simplified sketch of the cup-shaped growth form Boury-Esnault and Rützler (1997).

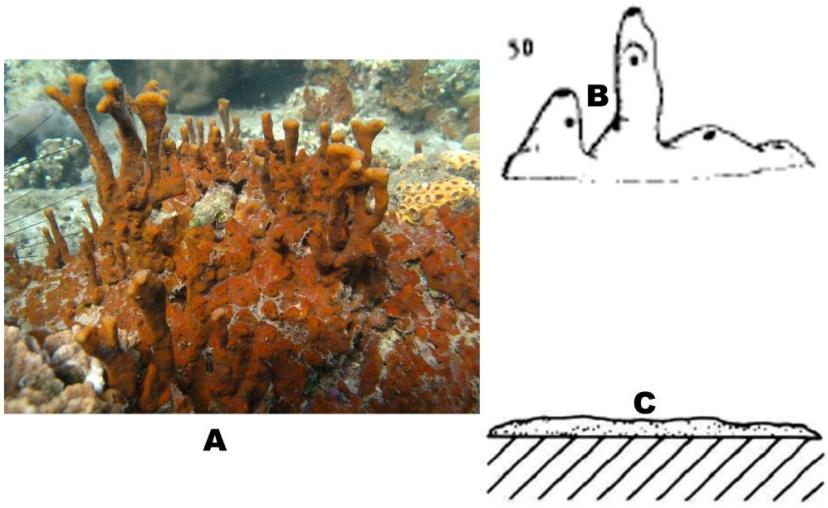


Figure 1.7. Main macroscopical features of *N. exigua*. A is a picture of a *N. exigua* specimen that was sampled by the author on the Probolinggo coast, East Java, Indonesia on 18 May 2011. B is a *N. exigua* sketch from the figure of van Soest (1989) and C is a sketch of an encrusting growth form Boury-Esnault and Rützler (1997).

Xestospongia testudinaria (Lamarck, 1815) is described as the Indo-Pacific or Indonesian giant barrel sponge. This barrel sponge has been known to contain bioactive compounds, which could be useful for application in the pharmaceutical industry. Aaptamines, for example, have a cytotoxic effect and could act as an anticancer component. Furthermore, halenaquinones and xestoquinones can be used in antimalarial drugs (Calcul et al. 2003; Cao et al. 2005). Besides potential application in the pharmaceutical industry, *Xestospongia testudinaria* has an important role in the reef structure since it is one of the major species and the dominant constituent of the Indonesian coral reefs (van Soest 1989; de Voogd and Cleary 2008). Based on the morphological description, *X. testudinaria* possesses an "erect-cup" or "tube" shape (van Soest 1980), often giantly sized, heavily fitted with vertical flukes or a ridged outer wall and having a stony hard consistency (Figure 1.6. B, C). When the sponge is alive, its colour is red-brown, but when it is preserved in alcohol it changes to beige. The sponge is "firm, springy and slightly compressible" when alive and it can be torn with some force (Fromont 1991). The tissues are interspersed with canals of 0.5 cm in diameter and consist of very compact tissue. The skeleton surface is constructed by an extension of the choanosome, which consists of an isotropic isodictyal spicule tract reticulation with a small mesh size of 90-227 μm width (Figure 8b). The tracts are 2-6 spicules wide, which corresponds to 34-84 μm across. A multispicular reticulation with irregular oval meshes of 360-640 μm, constructs the internal skeleton. The interior of the skeleton consists of reticulated tracts of 100-150 μm with 10-20 strongyloxea type spicules (Figure 1.8. c). Thin spicules that are more oxeote in shape occur interstitially. Spongin fibres are formed around the spicule tracts, especially at the nodes of the reticulation and lighten up under the microscope when stained with haematoxylin eosin. The spicule type is basically monaxonic and extremely variable in size (Figure 1.8. d, e). Many spicules have strongylote or tapering oxeote ends. Other spicules occasionally have knob-like protuberances near the end of the spicules. Spicule dimensions vary from short and squatty to long and thin in any combination of length and width. Strongyloxeas spicules range from 168-361 μm in length x 5.3-19.0-μm in width, whereas thin forms measure 151-336 μm in length x 2.0-5.3-μm in width.

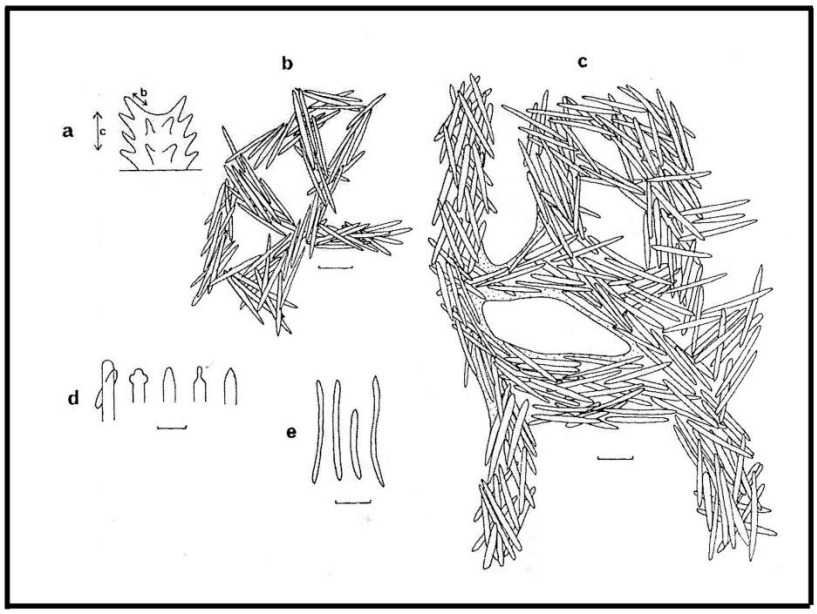


Figure 1.8. Skeleton and spicules of *Xestospongia testudinaria*. (a) The orientation of skeletal sectioning as depicted further in figure b and c. (b) Isodictyal reticulation of the skeleton, scale bar = 100 µm. (c) choanosomal skeleton, The dotted area represents spongin development, scale bar = 100 µm. (d) variability of tips, scale bar= 20 µm. (e). Megascleres of strongyloxea type, scale bar= 100 µm. Figure is taken from Fromont (1991)

The distribution of the species is widespread throughout the Indo-Pacific. At the same time, another sibling species of *X. testudinaria*, namely *Xestospongia bergquistia* Fromont, 1991, which inhabits only inshore reefs, is reported to live sympatrically in the Indo-Australian Archipelago (Fromont 1991). The distribution of *X. testudinaria* is broader than *X. bergquistia* since the latter inhabits only inshore reefs and mid-shelf reefs (description by Fromont 1991). *Xestospongia bergquistia* is characterised by a more open skeleton and a lack of sponge fibres. Therefore, *X. bergquistia* is less robust, easier to tear and more compressible than *X. testudinaria*. Also, it has a different colour than *X. testudinaria* (maroon to reddish brown in life and a uniform fawn colour in alcohol).

Neopetrosia exigua (Kirkpatrick, 1900) is another sponge species, which used to be classified under the same genus as *Xestospongia testudinaria* although its morphological structure is very different (giant sized cup form in *Xestospongia testudinaria* and massively encrusting form in *Neopetrosia exigua*) (Figure 1.7. B, C) (van Soest 1989; Fromont 1991; Desqueyroux-Faundez and Valentine 2002). Based on the morphological description (cf. description in van Soest 1989; Fromont 1991), *N. exigua,* has a variable morphology from a massively encrusting form of 1.3 cm thickness to an erect form with lobes or turrets or additional branches or laminations up to 15 cm. In life specimens, it has a light ochre to dark

brown colouration with a yellowish to beige interior, whereas it turns to medium to dark chocolate brown in alcohol. The texture is compressible, crumbly and sticky when it is touched, leaving some of the ectosomes sticking to the fingers. The exterior surface is smooth but under the microscope it looks hispid. A thin layer of tissue and pigmented cells cover the superficial spicule. Unlike *Xestospongia*, *Neopetrosia* can be identified by its ectosome, choanosome, and the separative layer in between. The ectosomal skeleton is a continuation of the dense and irregular uni- or multispicular isodictyal reticulation, which forms the internal skeleton of a sponge. The internal meshes are triangular or circular and compact, 58-168 μm wide (Figure 1.9. b). Vertical parallel tracts are occasionally present in the centre of the sponge. The principle spicules are consistently oxeote but may have hastate of fusiform ends. The small oxeas are 88-170 μm in length x 2.2-7.4-μm in width, whereas the thin forms are 80-168 μm in length x 0.6-3.8-μm in width (Figure 1.9. c). This sponge species is distributed widely in the Indonesian archipelago (van Soest 1989; de Voogd and Cleary 2008) and mentioned extensively in scientific literature for its bioactive metabolites. Biochemical studies of *Neopetrosia exigua* have demonstrated that this sponge also contains some bioactive compounds like araguspongines, which has a cytotoxic and antifungal activity and xestosin which possesses a vasodilatory or ichtyotoxic effect (Iwagawa et al. 2000; Orabi et al. 2002; Limna Mol et al. 2009).

Some sponges, like *Neopetrosia chaliniformis* (Thiele, 1899), are morphologically similar to *N. exigua*, which makes them difficult to distinguish (Fromont 1991). Further examinations using molecular techniques as suggested by Fromont (1991) might reveal their differences, resolve their phylogenetic relationships and detect other cryptic species within *N. exigua*.

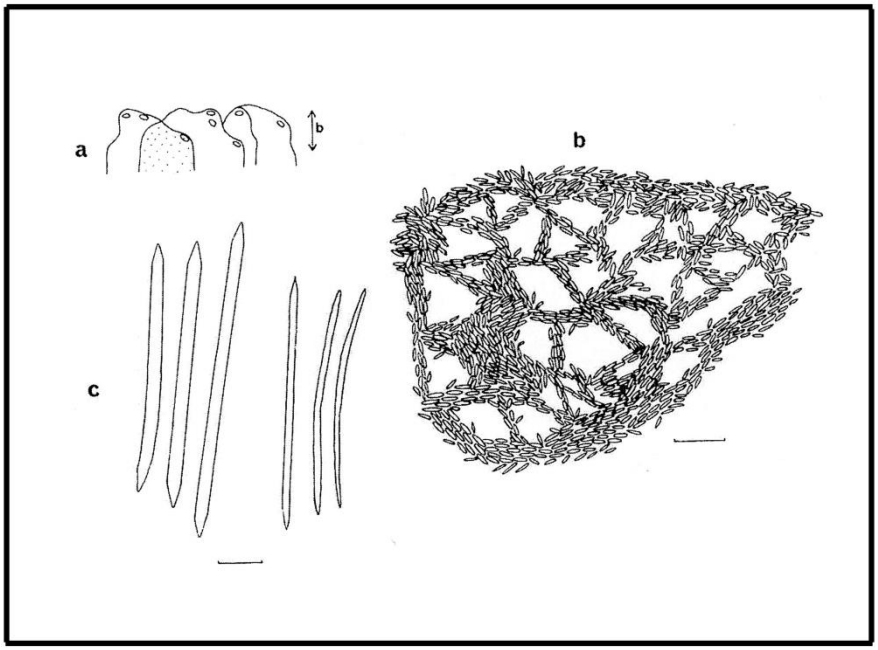


Figure 1.9. Skeleton and spicules of *Neopetrosia exigua*. (a) An orientation of the skeletal sectioning as depicted further in figure b (b) isodictyal reticulations are visible beneath the less dense choanosomal skeleton, scale bar = 100 µm. (c) thick oxeas with thinner ends, scale bar= 20 µm. Figure is taken from Fromont (1991)

1.8. The Indo-West Pacific (IWP) biodiversity and the paleogeographic history of South East Asia

The tropical underwater world reveals a wealth of biodiversity resources. Mora et al. (2003) state that the Indo-Malayan tropical waters are recognised as one of the regions with the highest diversity of marine species. It is suggested that the majority of tropical marine species are concentrated within the relatively small East Indies Triangle. This triangle refers to the Indo-West Pacific (IWP) region (Briggs 1999). The Indo-Australian Archipelago (IAA) belongs to this IWP region, which consists of the Malay Peninsula in the west, the Philippines in the north, Papua New Guinea in the east and the Indo-Australian Archipelago in the south. The Indo-Australian Archipelago (IAA) is also considered as a prominent hotspot for studying biodiversity and radiation of marine organisms (Bellwood and Meyer 2009).

The high biodiversity and high number of endemic species in the IAA are the result of the geological and geographical history of that region. The region is also recognised as an "immense, intriguing and understudied region" (Hall 2009). Geologically, this region can be divided into four plates; i.e. the continental core in the west (Sundaland), the Australian continent in the south, which collided with the easternmost point of Eurasia, the oceanic sea plate in the east (known as the Philippine Sea Plate) that is attached to the Pacific, and the

Wallacea deep sea bed that is located between Sundaland, Australia and the Pacific. Wallacea is recognised as a region that changed progressively since the Cenozoic and possesses a great biodiversity.

The geological formation of the IAA ended 5 Ma in the early Pliocene. In the Pleistocene, seas around Sundaland (including Borneo, Java and the South China Seas) and straits on the Australian continent (called Sahul), between New Guinea and Australia were exposed during the last glacial maximum. At that time the sea levels in those regions were approximately 110-150 m lower than today. During the Pleistocene the sea basins had dried up and in the Holocene that followed, the sea basins were filled with water again. These two extreme conditions that succeeded one another during the Pleistocene and Holocene, as well as the sea currents had a great influence on the genetic diversity and patterns of marine organisms in the IAA (Hoeksema 2007).

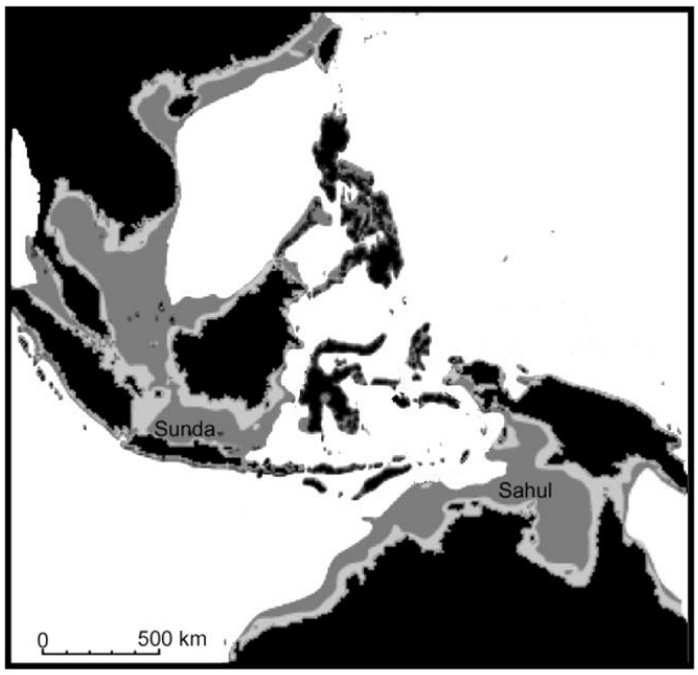


Figure 1.10. Map of the Indo-Australian (IAA) archipelago during the Pleistocene low sea level. Light and dark grey indicates the Pleistocene shorelines, which is 30 until 120 m below the current sea level. Picture is taken and adapted from Voris (2000) and Rowe et al. (2008)

The genetic diversity of marine taxa among Sundaland, Wallacea, (including the Philippine seabed) and the Australian continent is diverse (Hoeksema 2007). Diversity patterns related to those four regions have been observed in crustaceans (Barber et al. 2000), molluscs (Kochzius and Nuryanto 2008; Nuryanto and Kochzius 2009), echinoderms (Kochzius et al. 2009) and fishes (Timm et al. 2012). However, further information on the

genetic diversity and diversity patterns of sponges in this region have not been fully unravelled yet.

1.9. Aim, objective and outline of the thesis

The aim of this thesis is to answer fundamental questions regarding the biodiversity and radiation of selected Indonesian sponges, especially the family Petrosiidae, in order to have a better understanding of taxonomy, ecology and evolutionary patterns. The studies on the giant barrel sponge (*Xestospongia testudinaria*) and its con-family, *Neopetrosia exigua*, will give further information on marine biodiversity and conservation management since these species are conspicuous taxa. More importantly, an assessment of genetic diversity of both species will demonstrate whether divergences in various different geographical areas (within the IAA and the Indo-Pacific) have an effect on its genetic composition.

The objective of this research is to investigate the genetic diversity of samples from two selected species of the family Petrosiidae (*Xestospongia testudinaria* & *Neopetrosia exigua*) from several different geographic locations. I use samples collected by myself, and also museum specimens from various locations (mostly from the IAA region and other locations of the Indo-Pacific). I extracted DNA and characterised the molecular differences among samples of different localities. I utilised several molecular primers from mitochondrial and nuclear DNA in order to understand their genetic diversity. I compared and analysed all of those molecular data with statistical methods like Bayesian inference and maximum likelihood to establish whether *Xestospongia testudinaria* and *Neopetrosia exigua* are a species complex. In addition, I assessed further markers, especially intron markers, to determine whether they are suitable for further phylogeographic studies on both taxa.

Chapter 6 describes the genetic diversity, including the morphotype of *Xestospongia testudinaria* in a small spatial scale (Lembeh islands, North Sulawesi, Indonesia) based on two mitochondrial markers (I3-M11 partition of cox1 and ATP6) and the ATPS-β intron. In this chapter, recognition of environmental factors and genetic diversity on morphotypes, as well as the probability of the existence of a *X. testudinaria* species complex is disputed and questioned. Therefore, in **Chapter 2** I study the genetic diversity of *Xestospongia testudinaria* based on a broader geographical scale and a small phylogenetic study, as well as a close relative species (*X. muta, X. bergquistia*), based on the I3-M11 partition of cox1. This study emphasises the recent taxonomical status of barrel sponges since not all of the barrel sponge taxa (*X. testudinaria*, *X. muta and X. bergquistia*) could be resolved as monophyletic based on

the Monophyletic Species Concept utilising cox1. **Chapter 3** describes the utilisation of the ATPS-β intron in resolving the genetic diversity of *Xestospongia testudinaria*. I describe why the ATPS-β intron is more suitable for further phylogeographic studies of *X. testudinaria* than other mitochondrial markers (I3-M11 partition of cox1 and ATP6). In this chapter, the presence of at least three complex species is detected and reconfirms the result of chapter 2. The taxonomical problem of *Neopetrosia exigua* is described in **chapter 4**. I find that this species bears a wrong name and might be a cryptic species. The current taxonomic status reveals that *N. exigua* consists of two junior synonyms, which are *N. pandora* and *X. pacifica*. In addition, there is another sponge species named *N. chaliniformis*, which resembles *N. exigua*. Based on the type specimen assessments, we found that the sponge species named *N. chaliniformis* is identical to *N. exigua* and *X. pacifica*. In contrast, *N. pandora* is a different taxon. For this reason, taxonomy revisions according to the findings, are suggested and more importantly, the sponge species named *N. exigua* should be renamed as *N. chaliniformis*, according to our findings in combination with the priority rule in the ICZN (International Code of Zoological Nomenclature). In **chapter 5,** I describe an intron marker from the Lysidyl Aminoacyl Transfer RNA Synthetase gene (LTRS intron), which can be utilised for further phylogeography studies of *N. exigua*. I find that the LTRS intron is capable of resolving the phylogeographical pattern of *N. chaliniformis* samples that originate from Queensland, Australia and Papua New Guinea - Solomon Islands. **Chapter 7** is a general discussion on the implications of the discovered genetic diversity and phylogeographical patterns in order to answer the fundamental research questions about taxonomy, phylogenetic and phylogeographic relationships of *X. testudinaria* and *N. chaliniformis.*

CHAPTER 2

Genetic diversity of the Indonesian giant barrel sponge *Xestospongia testudinaria* (Porifera; Haplosclerida).

2.1. Introduction

The archipelago of Indonesia forms a part of the coral triangle, a region recognized for its high diversification of marine organisms (Briggs 1999; Hoeksema 2007; Bellwood and Meyer 2009). However, the genetic diversity of marine organisms in Indonesia remains incompletely studied and understood (Veron 1995; Hoeksema 2007). Conversely, the assessment of genetic diversity within populations and species is pivotal to understand past factors and future triggers of how organisms radiate and disperse in a given region. Such information is important for conservation efforts in times of global warming and sea level changes (Sutherland et al. 2004; Jones et al. 2007; Lopez-Legentil and Pawlik 2009).

Sponges (Phylum Porifera) are an abundant group of marine organisms in the Indo-Pacific region, for which the genetic connectivity is not fully understood (Wörheide et al. 2005; Bentlage and Wörheide 2007; Blanquer and Uriz 2007). Similarly, sponges play an important role in marine ecosystems due to their impact on substrate (e.g., as bioeroders 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 aid 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 and de Voogd 2007; de Voogd and Cleary 2007; Becking et al. 2013a). In addition, many sponge species contain various secondary metabolites, which have been a focus of drug discovery for decades (e.g., Munro et al. 1999).

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 as widespread in the Indo-Pacific and therefore one of the more common species on Indonesian coral reefs (van Soest 1989; de Voogd and Cleary 2008). Research on *X. testudinaria* has mainly been focussed on new bioactive compounds (Calcul et al. 2003; Cao et al. 2005), ecology (Powell 2013), reproduction aspects (Fromont 1988; Fromont and Bergquist 1994). Recently research concentrated on the genetic connectivity, but the so far only in a narrow geographical scale in

in Northern (Swierts et al. 2013) and Southeast Sulawesi (Bell et al. 2014b). The study of Swierts et al. 2013 uncovered that in an area of less than 200 km^2, *X. testudinaria* comprises distinct morphotypes, which are not obviously affected by environmental factors and could genetically be differentiated with mitochondrial and nuclear markers. One of the most intriguing findings of this work was the overlap of mitochondrial haplotypes between *X. testudinaria* with the Caribbean barrel sponge *Xestospongia muta* (Schmidt, 1870) (see also Montalvo and Hill 2011). These intriguing findings invoke the need for understanding the sponge species structures of this common sponge on a wider, preferably global, scale including a critical look on past and present modes of drawing species delimitations in sponges (see e.g., Solé-Cava and Wörheide 2007), particularly barrel sponges.

Throughout the entire history of sponge systematics (see Boury-Esnault 2006) morphological apomorphies and specific character combinations were the major discriminatory factors (cf. "Diagnosable Species Concept", see de Queiroz 2007) and the most recent supra-specific classification of sponges, *Systema Porifera* (Hooper and van Soest 2002a) is predominantly based on such morphological criteria. For barrel sponges, this approach appears to have only limited capabilities. On the one hand morphological features distinguish *X. testudinaria* from a second Indo-Pacific barrel sponge species, *Xestospongia bergquistia* Fromont (Fromont 1991), recently supported by microsatellite data (Bell et al. 2014a; Bell et al. 2014b), however, no clear-cut morphological apomorphies distinct from to *X. muta*, the Caribbean barrel sponge, have yet been elaborated. Likewise biochemical compounds, occasionally used as source for apomorphies in demosponges (Erpenbeck and van Soest 2007) are non-conclusive as *X. muta* and *X. bergquistia* share a similar, but not species-specific sterol composition different from *X. testudinaria* (Fromont et al. 1994).

Ecological parameters, however, provide for some sponge species delimitating features (see e.g., Wapstra and van Soest 1987). In this "Ecological Species Concept" (see de Queiroz 2007) taxa are distinguished by their respective niches. Among the oviparous and gonochoristic barrel sponges, different spawning periods as reproductive barriers are observed between the two Indo-Pacific taxa *X. testudinaria* and *X. bergquistia* (Fromont 1988; Fromont and Bergquist 1994). Certainly the geographical distance of the Caribbean *X. muta* to the Indo-Pacific barrel sponges acts as potential reproductive barrier and most important distinguishing feature (Montalvo and Hill 2011).

The advent of molecular systematics in sponges (Kelly-Borges et al. 1991) facilitated the taxon delimitation based on coalescence or the 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 recruit the mitochondrial cytochrome oxidase subunit 1 (cox1), particularly its 3' partition, which is shown to better resolve species on a lower taxonomic level than the standard barcoding fragment (Erpenbeck et al. 2006b). This fragment identified four haplotypes of *X. muta* in the Caribbean (Lopez-Legentil and Pawlik 2009) and six in the Indo-Pacific (Swierts et al. 2013). These findings corroborate earlier results from Montalvo and Hill (2011) who failed to distinguish the two allegedly geographical strictly separated species based on cox1 sequences alone and contradict a general suitability of cox1 for demosponge species level phylogenies (Erpenbeck et al. 2006a; Xavier et al. 2010b; Reveillaud et al. 2011).

These findings prompt me to analyse the haplotype diversity of barrel sponges in the so far largest geographical scale stretching from the Western Indian Ocean to the West Pacific including the type material of all three barrel sponge species. I explore the suitability of one of the most frequently used mitochondrial marker to distinguish the three barrel sponge species. Furthermore this study aims to further our understanding of the common and iconic barrel sponge species structure further only the region around Sulawesi (Swierts et al. 2013; Bell et al. 2014b).

2.2. Materials and Methods

2.2.1. Sampling

Some 187 specimens of *Xestospongia testudinaria* were freshly collected from three regions: Java (West, Central and East), Bali, and Sulawesi (Lembeh, Spermonde, Wakatobi). Immediately after collection, samples were cut, rinsed and soaked in 99% ethanol before kept in 99% ethanol. Further 24 samples from Saudi Arabia, Tanzania, Taiwan and Thailand were provided by the Naturalis Biodiversity Centre, Leiden, the Netherlands, including samples from the Great Barrier Reef (GBR) and Solomon Island that were 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 2.1). In addition, we also obtained the neotype (BMNH 1881.10.21.266) and a neoparatype (BMNH 1881.10.21.267) of *X. testudinaria* from the British Museum of Natural History, London (Hooper and Wiedenmayer 1994). For *Xestospongia muta*, two syntype materials were investigated (MCZ PORa-6449, MCZ PORa-6450) and additional sequences were taken from GenBank (www.ncbi.nlm.nih.gov).

2.2.2. DNA extraction, amplification and sequencing

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 5x green Go*Taq*® PCR Buffer (Promega Corp, Madison, WI), 4µL 25mM $MgCl_2$ (Promega Corp, Madison, WI), 2 µL 10mM dNTPs, 1 µL each primer (5µM), 9.8 µL H_2O, 0.2 µL Go*Taq*® DNA polymerase (5u/µl) and 2 µL DNA template. The PCR regime comprised an initial denaturation at 94° C for 3 min, 35 cycles of 30 s denaturation at 94° C, 20 s annealing at 42° C and 60 s elongation at 72° C each, followed by a final elongation at 72° 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 *X. bergquistia* type specimen. For the more than one century 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 utilised with 2 µL Bovine Serum Albumin (BSA, 10 mg/ ml) and the PCR regime of 45 s annealing at 42° C, 45 s elongation at 72° C each and a final elongation at 72° 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, California USA) and the amplification primers following the manufacturers 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 (available from http://www.geneious.com/). Sequences were checked with BLAST against GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for contaminations. In total 211 sequences were aligned with MUSCLE version 3.5 (Edgar 2004) as implemented in Geneious under default settings. Those 211 *X. testudinaria* sequences, 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.

2.2.3. Data analysis

TCS v 1.21 (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*). This network is used to map and describe barrel sponges haplotypes distribution according to the localities. Conversely, Dna SP v. 5.10.01 (Librado and Rozas 2009) was utilised for obtaining genetic diversity indices (π) and testing neutral evolution of I3-M11 alleles from *X. testudinaria* by calculating Tajima's D (Tajima 1989). For the analysis of *X. testudinaria* genetic structure, regional samples were pooled as follows (1) Saudi Arabia [n=3], (2) Tanzania [n=13], (3) Taiwan [n=7], (4) Thailand [n=12], (5) West Java [n=14], (6) Central Java [n=9], (7) East Java, Bali, Sumba [n=10], (8) North Sulawesi [n=126], (9) South and Southeast Sulawesi [n=10], and (10) the GBR and Solomon Islands [n=7]. Then, 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 were 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 and Michalak 2012) using 1000 rapid bootstrap replications (Stamatakis et al. 2008) and the GTR + I + Γ as optimized as the "best-fit" model via hierarchical likelihood ratio test in jModeltest v. 2.1.3 (Darriba et al. 2012) under the Akaike Information Criterion (Akaike 1974). Likewise, Bayesian phylogram was inferred using MrBayes v. 3.2.1 (Ronquist et al. 2012) under the ML 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.

2.2.4. Spicules analysis on type specimens

Spicules re-measurements were conducted 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, once with 96% ethanol. The spicules were air-dried on microscopic slides and prepared for study with the light microscope by mounting them in Ultrabed. Spicule dimensions are given

as the mean length x mean width of 25 spicule measurements.

2.3. Results

2.3.1. Sequence variation and genealogical relationship

I obtained partial cox1 sequences (I3-M11 partition) of 211 *Xestospongia testudinaria* and six *X. bergquistia* specimens resulting in an alignment of 544 base pairs with six different haplotypes (Table 2.1**)**. Unfortunately no amplification product for BMNH 1881.10.21.266, MCZ PORa-6449 and MCZ PORa-6450 could be obtained (but see also chapter 7). Five haplotypes were identical to haplotypes reported from Swierts et al. (2013) (C1, C2, C4-C6) whose labelling system will be followed; one novel haplotype is subsequently named C7.

All six haplotypes were present in *X. testudinaria*. Two haplotypes are shared between *X. testudinaria* and *X. bergquistia* (Haplotype C2 and C5). The holotype specimen of *X. bergquistia* (QM G25018) possessed the C5 haplotype and the *X. testudinaria* neoparatype 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 (Haplotype C2 and C5) are shared between the Indo-Pacific and Caribbean barrel sponge species. The 116 Caribbean barrel sponge samples (*Xestospongia muta*) comprised in total four haplotypes. Two of their haplotypes (Haplotypes H2 and H4, as defined by Lopez-Legentil & Pawlik 2009) are not shared with the Indo-Pacific specimens (see Tables 2.1 and 2.2).

A close genealogical relationship among haplotypes was observed (Figure 2.2). All haplotypes were connectable with a maximum of one assumed mutational step. Haplotype C1 is the most abundant haplotype among the Indo-Pacific samples, comparable with H1 in the Caribbean (cf. Lopez-Legentil and Pawlik 2009) - one mutational step separates those two major haplotypes. Haplotypes C5 and C1 were the dominant haplotypes in the Indonesian archipelago, which has been the primarily sample region for *X. testudinaria* in this study. Haplotype C7 was exclusively found in the 13 samples of Tanzania, which in total displayed four different haplotypes. Among the three Red Sea samples from Saudi Arabia only C5 was found (Figure 2.1 and 2.2).

In the reconstructed phylogram the barrel sponges are recovered monophyletic with high support (Figure 2.3). However, as expected from the overlapping haplotypes the cox1 fragment fails to support the monophyly of any of the three barrel sponge species.

Table 2.1. Haplotypes recovered from the *Xestospongia* alignment. Positions refer to the full cox1 sequence from *Xestospongia muta* EU716653 (Kayal and Lavrov 2008). C1-C7 haplotype numeration follows Swierts et al. 2013, whereas H1-H4 follows the labelling of Lopez-Legentil and Pawlik

Position (bp) / **Haplotype**	**766**	**777**	**783**	**888**	**1102**	**1182**	**1218**	**Species; Reference (associated haplotype number as used in reference) and example GenBank accession number**
C1 (cf. Swierts et al. 2013)	A	T	C	A	G	G	T	*X. testudinaria*; (Montalvo and Hill 2011) e.g., HQ452959 *X. testudinaria*; (Swierts et al. 2013) e.g., KC424439 **(this study)**
C2 (cf. Swierts et al. 2013)	A	T	C	A	G	G	C	*X. muta*; (Lopez-Legentil and Pawlik 2009) (as H1 e.g., EU716652) *X. testudinaria*; (Swierts et al. 2013) e.g., KC424440 **KM014752; (this study)**
C4 (cf. Swierts et al. 2013)	A	T	C	C	G	G	C	*X. testudinaria*; (Swierts et al. 2013) e.g., KC424442 **(this study)**
C5 (cf. Swierts et al. 2013)	A	A	C	C	G	G	C	*X. muta*; (Lopez-Legentil and Pawlik 2009) (as H3 e.g., EU716654) *X. testudinaria*; (Swierts et al. 2013) e.g., KC424443 **KM014753 and (this study)**
C6 (cf. Swierts et al. 2013)	G	A	C	G	G	G	C	*X. testudinaria*; (Montalvo and Hill 2011), e.g., HQ452960 *X. testudinaria*; (Swierts et al. 2013) e.g., KC424444 **(this study)**
C7 (this study)	A	A	C	C	G	T	C	**KM014755 (this study)**
H2 (cf. Lopez-Legentil & Pawlik 2009)	A	T	T	A	G	G	C	*X. muta*; (Lopez-Legentil and Pawlik 2009) e.g., EU716653 *X. muta*; (Kayal and Lavrov 2008) e.g., EU237490
H4 (cf. Lopez-Legentil & Pawlik 2009)	G	A	C	G	A	G	C	*X. muta*; (Lopez-Legentil and Pawlik 2009) e.g., EU716653

Table 2.2. Haplotype distribution of *Xestospongia testudinaria* and other barrel sponges according to their geographical localities.

Haplotype numeration follows Swierts et al. 2013 and Lopez-Legentil and Pawlik 2009.

[1] One *Xestospongia bergquistia* specimen from Singapore

[2] Including one specimen of *X. bergquistia* from the Great Barrier Reef and one neoparatype specimen of *X. testudinaria* (BMNH 1881.10.21.267)

[3] Four specimens (including one holotype) of *X. bergquistia*

[4] Taken from Lopez-Legentil and Pawlik (2009).

[5] Including full mt genome sequence of *X. muta* (see Kayal and Lavrov 2008)

Haplotype	Saudi Arabia	Tanzania	Taiwan	Thailand & Singapore	Indonesian Archipelago					Great Barrier reef & Solomon	Florida, Bahamas, Belize [4]	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[2]	51	94
C4	0	1	1	2	0	1	4	1	2	0	0	12
C5	3	1	0	1[1]	6	0	4	50	3	4[3]	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[5]	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**

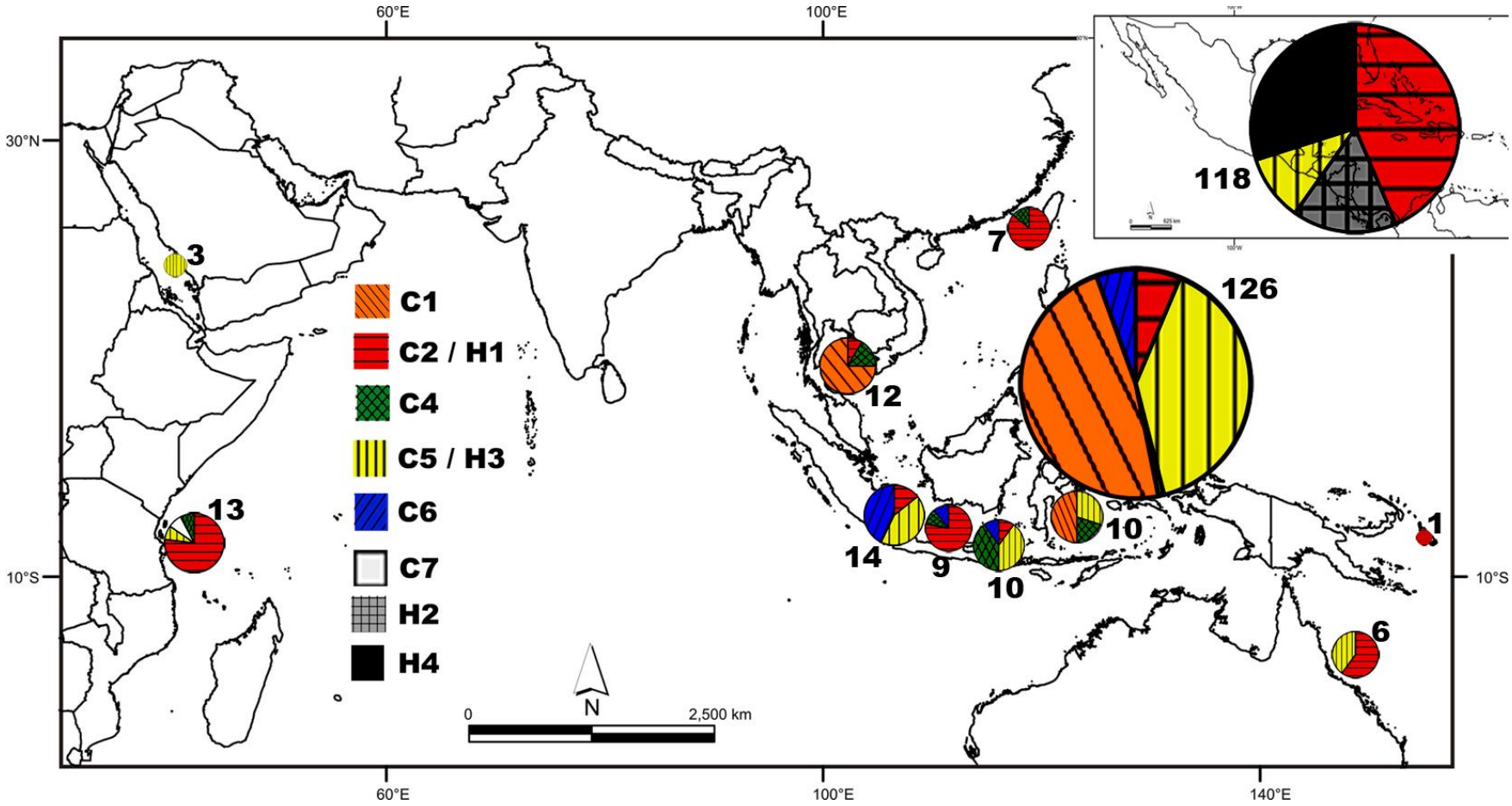


Figure 2.1. Haplotype distribution of *Xestospongia* sequences used in this study. The size in the pie charts is proportional to the sample size. Different haplotypes are colour-coded (see Tables 1 and 2 for further details).

Inset: Samples from the Caribbean (published by Kayal and Lavrov 2008; Lopez-Legentil and Pawlik 2009).

C1-C7 haplotype numerations follow Swierts et al. (2013), whereas H1-H4 follow Lopez-Legentil and Pawlik (2009)

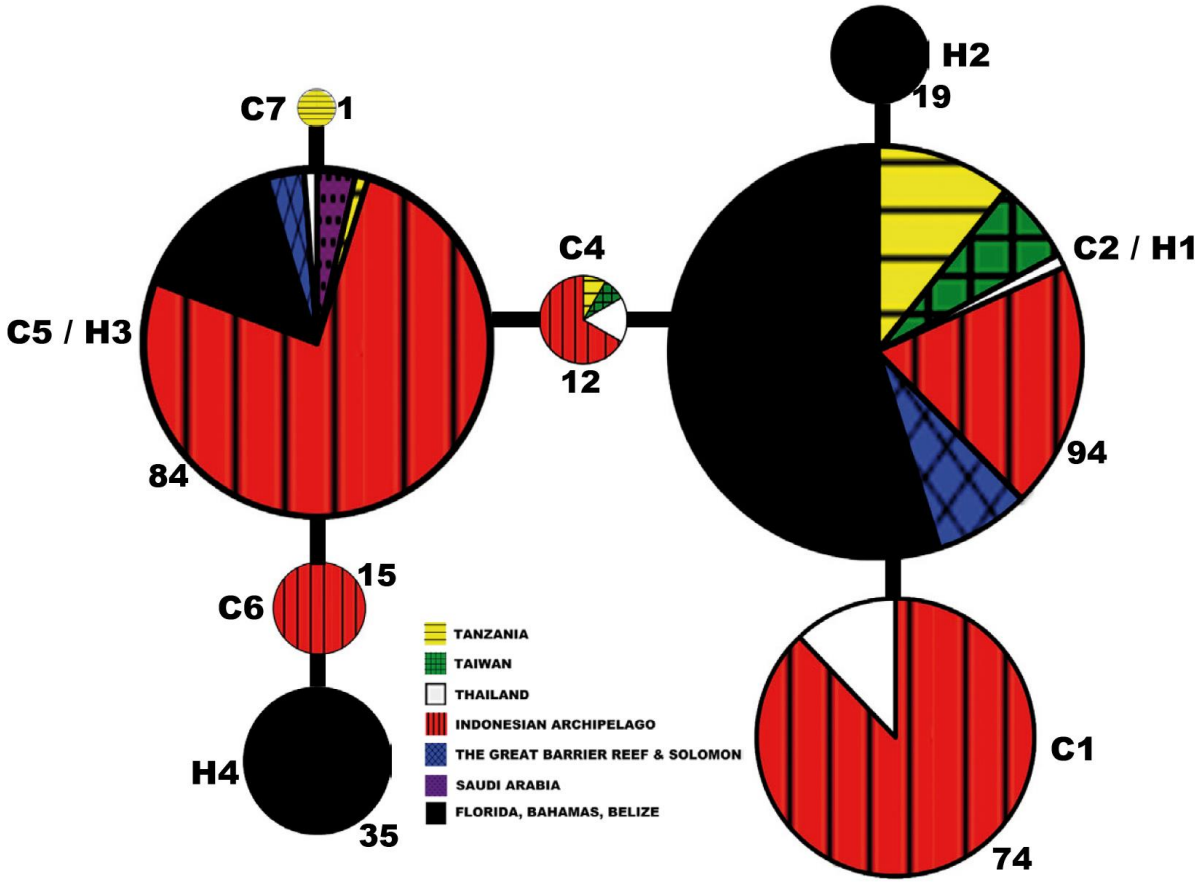


Figure 2.2. Haplotype network of the *Xestospongia* specimens of this analysis in respect to their sampling location (see Tables 2.1 and 2.2 for details). *Xestospongia muta* sequences were obtained from Kayal and Lavrov (2008) and Lopez-Legentil and Pawlik (2009). C1-C7 haplotype numerations follow Swierts et al. (2013), whereas H1-H4 follow Lopez-Legentil and Pawlik (2009). Numbers are described the amount of the analysed sequences.

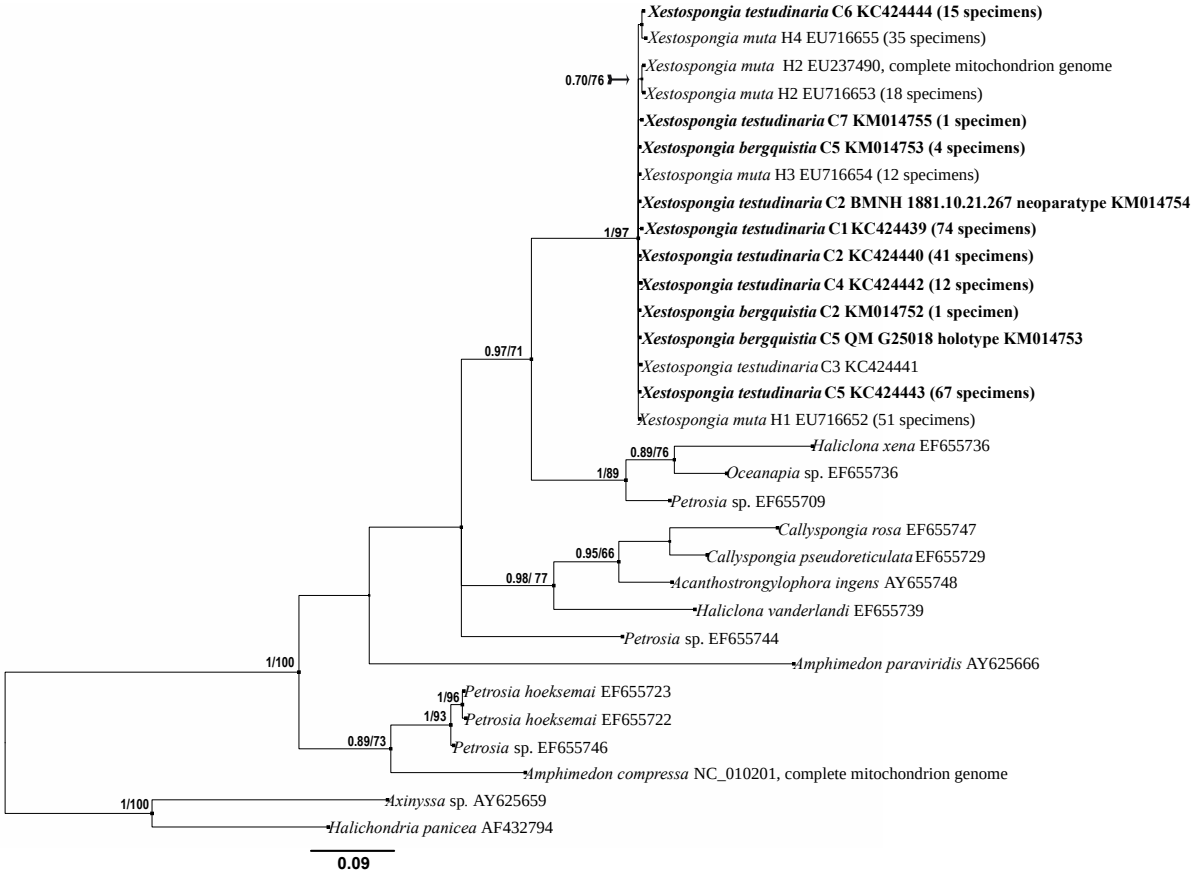


Figure 2.3. Bayesian inference phylogram of haplotypes of *Xestospongia* species included in the analysis with other marine haplosclerid taxa. New sequences are given in bold and followed by number of sequences obtained in this study, including GenBank accession numbers. The haplotype numerations follow Swierts et al. (2013) and Lopez-Legentil and Pawlik (2009). Numbers on the branches represent posterior probabilities (PP) / bootstrap proportions (BP) of maximum likelihood analyses. Scale bar indicates the number of substitutions by site.

2.3.2. Genetic diversity and spatial relationship

The nucleotide diversity (π) 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, four regional samples (Thailand, West Java, North Sulawesi and GBR + Solomon Islands) displayed significantly different structures from other localities (see Table 2.3). Moreover, the hierarchical AMOVA analysis revealed that 32.49 % of the genetic variation was found among populations and 67.51 % within populations (see Table 2.4). Likewise, the overall F_{ST} value (0.32491, $P < 0.05$) indicated the presence of *X. testudinaria* genetic structuring in the Indo-Pacific area researched.

Table 2.3. Pairwise F_{ST} values between populations of *X. testudinaria*. Significant values at $P < 0.005$ after Bonferroni corrections are indicated by an asterisk.

Population	Saudi Arabia	Tanzania	Taiwan	Thailand	West Java	Central Java	East Java, Bali, Sumba	North Sulawesi	South and Southeast Sulawesi	Great Barrier reef & Solomon
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 Southeast Sulawesi	0.32216	0.43221*	0.47371*	0.05110	0.22752	0.43020*	0.11111	-0.01047	0.00000	
Great Barrier reef & Solomon	1.00000	0.01573	0.00000	0.70612*	0.52703*	0.02778	0.54002*	0.50116*	0.60752*	0.00000

Table 2.4. Hierarchical analysis of molecular variance (AMOVA) based on the I3-M11 sequences of *X. testudinaria*. Significant value at $P < 0.005$ after Bonferroni correction is indicated by an asterisk

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*			

2.4. Discussion

2.4.1. Cytochrome oxidase 1 and barrel sponge species distinction

Currently the three barrel sponge species *Xestospongia testudinaria*, *X. muta* and *X. bergquistia* can neither be distinguished with the I3-M11 partition of the cytochrome oxidase subunit 1, suggested as more suitable marker for species level phylogenies than the standard barcoding marker (Erpenbeck, 2006), nor by any diagnosable or ecological parameter alone. The three barrel sponges therefore can be regarded as what de Quieroz (2007) referred to as *Gray zone species* under our current knowledge. *Gray zone species* occur when disagreements among different views of species concepts are observed, which initiate conflicts in species delimitation (de Queiroz 2007). Nevertheless, despite the overlapping haplotypes our *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 spicules of all three type specimens were also re-measured, which revealed overlapping spicule dimensions (see Table 2.5), while the magnitude of spongin enforcements distinguished the two Indo-pacific species (Fromont et al. 1991).

Table 2.5. Spicule dimensions of the barrel sponge type specimens (minimum-mean-maximum).

Species:	Spicule length	Spicule width:
X. testudinaria (BMNH 1881.10.21.266)	174.95 – **277.17** – 331.71 μm	8.34 – **14.67** – 21.65 μm
X. bergquistia (QM G25018)	303.28 – **352.29** – 378.09 μm	4.96 – **11.54** –16.26 μm
X. muta (MCZ PORa-6449)	192.31– **367.67** – 446.44 μm	3.39 – **14.19** – 22.61 μm

In terms of ecological parameters, however, the barrel sponges constitute different species based on reproductive behaviour (Fromont 1988; Ritson-Williams et al. 2005) and based on their respective geographical habitat (Montalvo and Hill 2011). Contact zones between the Indo-Pacific and the Caribbean barrel sponges do not exist. Hence, gene flow between barrel sponges of the Caribbean and the Indo-Pacific via water-ballast of ship or vessel through Panama Canal is possible (Ruiz et al. 2007), but might not be sufficient to result in the haplotype patterns as observed in this study. No stepping stones bridging the Atlantic or the Pacific populations are found, that would facilitate a world-wide distribution of a single species despite the typical short life-span of sponge larvae (see Maldonado 2006). No data exists on present or past existence of barrel sponges in the East Pacific, which would

explain the haplotype overlaps as a consequence of gene flow between both regions (see also Knowlton et al. 1993; Knowlton and Weigt 1998; Duran and Rützler 2006).

Why does the cytochrome oxidase 1 display overlapping haplotypes? I 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). 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 and Satta 1997; Rannala and 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 in the mitochondrial lineage of their last common ancestor and explain the presence of identical haplotypes for digitate morphotypes in both *X. muta* and *X. testudinaria* barrel sponges. 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 cosmopolitic lineages, which constitute a concept successively rejected in sponge and other marine taxa (see Palumbi et al. 1997; Solé-Cava and Boury-Esnault 1999; Wörheide et al. 2008). This is particularly evident as microsatellites, which are faster and independent markers, clearly show the distinction between *X. bergquistia* (including the type material) and different *X. testudinaria* samples (Bell et al. 2014a; Bell et al. 2014b). These results imply that the I3-M11 cannot be regarded as universally suitable for detecting species boundaries in all demosponges.

2.4.2. The suitability of cytochrome oxidase 1 to resolve intra-specific relationships

Nucleotide diversity (π) is a measure of the genetic variation, which is applied to calculate the degree of polymorphism in a population. Therefore, the higher the π 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 marker for population analyses in demosponges. However, a higher π value on the 3' (I3-M11) partition in mitochondrial DNA would suggest that this marker is more suitable to resolve genetic

diversity compared to the 5' partition. For *X. muta* Lopez-Legentil et al. (2009) found a nucleotide diversity of π=0.00058 in the 5' partition, contrasting the much higher nucleotide diversity of π=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 and Turon 2012). Among the Indo-Pacific *X. testudinaria* six haplotypes were sampled over a range of more than 10,000 km from the Red Sea to Melanesia with π=0.00290 in the I3-M11 fragment. Five of these haplotypes were detected in specimens from the Indonesian archipelago in an area of 2,300 km distance (π=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 in Lopez-Legentil et al. (2009) are shared with Indo-Pacific haplotypes. Consequently, prior to the application of I3-M11 on barrel sponges intraspecific relationships the correct assignment to either barrel sponge species is mandatory.

2.4.3. Implications for *Xestospongia testudinaria* genetic diversity and structure

For *Xestospongia testudinaria* I recognize a major difference in common haplotypes among regions. One haplotype is found in Tanzania (Indian Ocean) only. The occurrence of major haplotypes is discussed as influenced by environmental factors such as currents or anthropogenic effects (Lopez-Legentil and Pawlik 2009; Swierts et al. 2013) and shows an eco-geographical 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 in a geographical narrow scale of North Sulawesi (Swierts et al. 2013) and Southeast Sulawesi (Bell et al. 2014b).

The finding of different external morphotypes in *X. testudinaria* with distinct haplotypes, which likely represent reproductively isolated rather than ecophenotypic varieties, displays the presence of a *Xestospongia testudinaria* species complex with several sympatric species (Swierts et al. 2013), see also (Bell et al. 2014b). Likewise, in *X. muta*, two morphotypes (smooth and digitate) were found to have different genotypes although the authors assume that hybridization prevent their distinct separation (Lopez-Legentil and

Pawlik 2009). The presence of different *X. muta* lineages is also evident from different sterol compositions as found by Fromont et al. (1994), although they did not assign the different chemotypes to morphotypes. Nevertheless, our understanding of *Xestospongia* concerning its global genetic structuring and diversity is still limited due to imbalanced sampling size and regionally insufficient number of sequences (e.g., in Solomon, Great Barrier Reef, Thailand, Taiwan, Tanzania, Red Sea), including potentially missed haplotypes in some regions for subsequent analyses.

2.5. Conclusion

Despite of several limitations such as imbalanced sample size in the selected area studied, a genetic structuring of *X. testudinaria* can be detected. However, the species boundaries among barrel sponges could not be resolved due to the possession of slow substitution rates in the marker, in addition to ancient polymorphism. In the future, more improvements are needed such as correction on the sample size and geographical boundaries for measuring the genetic structure more precisely. Likewise, concatenation with other molecular markers can be used for delimiting the species boundaries of barrel sponges.

CHAPTER 3

The utility of the Adenosine Triphosphate Synthase β subunit intron for resolving phylogeographic relationships of the Indo-Pacific giant barrel sponge *Xestospongia testudinaria* (Porifera; Haplosclerida).

3.1. Introduction

The use of nuclear genes in addition to mitochondrial genes for resolving phylogenetic relationships and the assessment of phylogeographic patterns among taxa is beneficial because it allows the differentiation between maternal and biparental components of dispersal (see e.g., Karl et al. 1992). Crosschecking mitochondrial DNA (mtDNA) and nuclear DNA (nucDNA) helps to clarify hidden patterns in natural populations like a discrepancy between mtDNA and nucDNA due to the influence of the male allele (Palumbi and Baker 1994). NucDNA markers can be classified by their variability into a several main classes of can be distinguished. Among these are: (1) Nuclear Protein Coding Loci (NPCL) (2) introns, and (3) Anonymous Nuclear Markers (ANM) like AFLPs (Amplified Fragment Length Polymorphisms), RFLPs (Restriction Fragment Length Polymorphisms), RAPD (Randomly Amplified Polymorphic DNA), SNIPs (Single Nucleotide Polymorphisms) and microsatellites (see review in Thomson et al. 2010). Nuclear Protein Coding Loci are usually applicable for deep phylogenetic analyses because they constitute more conserved genes, whereas introns and Anonymous Nuclear Marker are mostly utilised for phylogeographic studies due to their faster evolutionary rates. Anonymous Nuclear Markers are the among the most suitable markers in phylogeographic studies due to their higher substitution rate than e.g., introns, however, several factors such assuccess rate and paralogy, remain a major concern (Thomson et al. 2010). Likewise, the development of Anonymous Nuclear Marker frequently requires comparatively more intensive laboratory work in addition and higher costs, particularly when working with non-model organisms (e.g., Bertozzi et al. 2012)..

Typically, nuclear protein coding genes in Metazoa possess a lower evolutionary rate in comparison to their mtDNA (Brown et al. 1979; 1982). However, in several non bilaterian invertebrate groups like demosponges and anthozoans, a particularly slow mitochondrial evolutionary rate has been recognized (Shearer et al. 2002; Huang et al. 2008). This constitutes a limiting factor for the use of mtDNA use in intra-specific studies. In addition, mtDNA has limitations for intraspecies studies due to its uniparental inheritance and fourfold smaller effective population size, which may result in oversimplified evolutionary

relationships and underestimated genetic diversity (see review in Zhang and Hewitt 2003).

Nuclear markers suitable for intraspecies studies should fulfil several specific requirements. They should be (i) evolutionary well conserved across different taxon groups, (ii) resulting in amplicons of sufficient size (iii) sufficiently variable and (iv) derive from single copy genes (see review in Zhang and Hewitt 2003). Several nuclear introns potentially fulfilling these requirements for invertebrates (Jarman et al 2002). A popular method to amplify such introns known as EPIC (Exon-Primed, Intron-Crossing) where the primers anneal to the more conserved flanking exon regions and the intron is subsequently bridged during amplification (Palumbi and Baker 1994).

In sponges, the intron of the Adenosine Triphosphate Synthase β subunit (ATPS-β intron) has been shown to be suitable for unravelling phylogenetic and phylogeographic relationships including the recognition of species complexes (Bentlage and Wörheide 2007; Wörheide et al. 2008; Reveillaud et al. 2010; see review and example detials in Uriz and Turon 2012). Swierts et al. 2013, applied this marker marker in a species structure analysis of the Indo-pacific barrel sponge *Xestospongia testudinaria*.

Xestospongia testudinaria (Lamarck, 1815) (Porifera; Haplosclerida) is a conspicuous sponge species in the Indo-Pacific. *Xestospongia testudinaria* inhabits and dominates Indonesian coral reef structures (van Soest 1989; de Voogd and Cleary 2008) but only recently molecular phylogenetic work on this iconic species has been attempted (Swierts et al. 2013; see also Bell et al. 2014b). Swierts et al. 2013, recognised the presence of a *Xestospongia testudinaria* species complex, the impact of environmental factor on morphotypes and the genetic structure in a geographical restricted area around Lembeh (Sulawesi, Indonesia). As assessments of genetic connectivity and phylogeographic relationships of marine organisms help us to understand their evolution and to facilitate their conservation (Jones et al. 2007; Lopez-Legentil et al. 2008), a larger scale genetic study of *Xestospongia testudinaria* throughout the Indo-Australian Archipelago is needed.

The Indo-Australian Archipelago (IAA) is a unique region with an abundance of marine diversity (Mora et al. 2003). The region is spread across an area of 5000 km between 95^0 E and 140^0 E. The IAA consists of four main zones of marine organism diversity (see also review in Hoeksema 2007) and originated in two simultaneous events, the Pleistocene low sea level and the Holocene recolonisation. During the Pleistocene, the narrow sea surrounding Sundaland and Australia fell dry. Water bodys only remained in the deep regions around Wallacea and the Philippines, forming barriers for organisms in Sulawesi and the Philippines.

During the Holocene recolonisation, the ocean currents refilled the shallow areas around Sundaland and Australia, forming (i) the Sundaland continental core that covers Thai-Malay Peninsula, Sumatra, Borneo and Java (ii) the Australian plate that covers Australia and Papua (iii) the Oceanic plate of the Philippines that is directly connected to the Pacific and (iv) Wallacea that covers Sulawesi and is surrounded by deep seas (Hall 2009), see Figure 3.1.

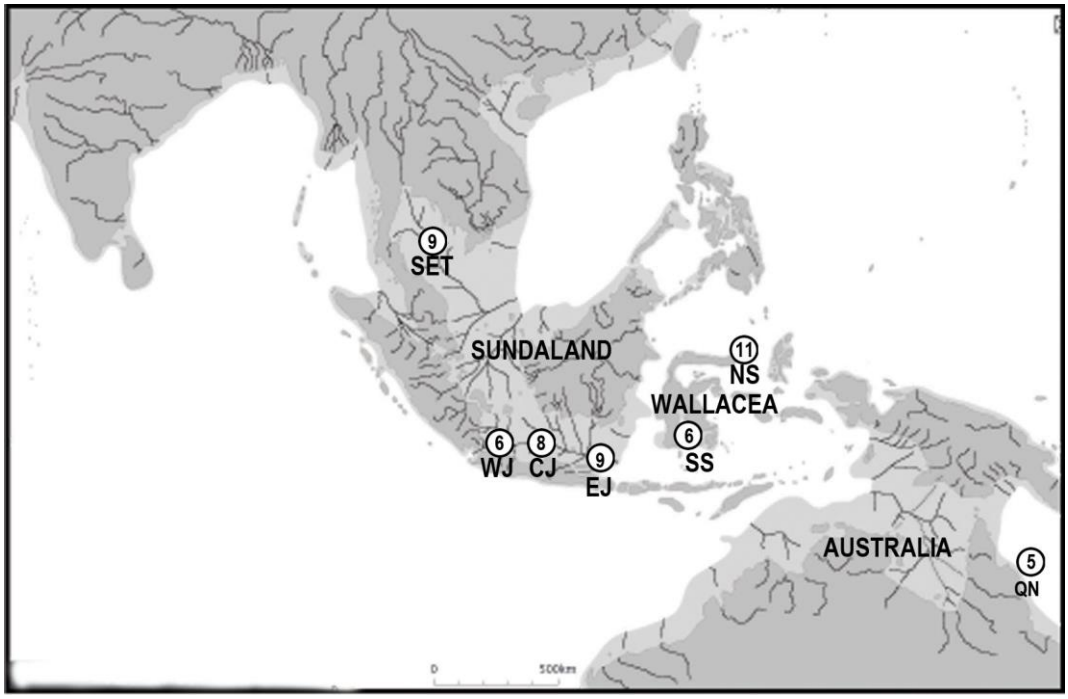


Figure 3.1. Map of the IAA archipelago during the Pleistocene low sea level. The depth of the shallow sea around Sundaland and Australia (bright grey area) is less than 120m. The circles represent localities and numbers of *Xestospongia testudinaria* samples. SET= Southeast Thailand, WJ= West Java, CJ= Central Java, EJ= East Java, NS= North Sulawesi, SS= Southeast Sulawesi, QN= Queensland. Figure is adapted and taken from the original map of Field Museum of Natural History (© 2000 Field Museum of Natural History, Chicago, Illinois, USA) and Voris, 2001

Several studies investigated the effect of Pleistocene low sea level and Holocene recolonisation for the genetic diversity of marine organisms in the IAA e.g., for crustaceans (Barber et al. 2000), molluscs (Kochzius and Nuryanto 2008; Nuryanto and Kochzius 2009), echinoderms (Kochzius et al. 2009) and fish (Timm et al. 2012). However, similar data on sponges is scarce. For this reason I analyse whether the genetic diversity as revealed from the ATPS-β intron in *X. testudinaria* also reveals a similar phylogeographic zonation for sponges following Pleistocene low sea level and Holocene recolonisation similar. I aim to analyze the suitability of the ATPS-β intron in comparison to two mtDNA markers to detect the genetic

diversity of *X. testudinaria* and its phylogeographical relationship in a wider scale and in the palaeogeographical context in the Indo-Australian (IAA) archipelago.

3.2. Materials and Methods

3.2.1. Specimen collections

We collected 41 fresh specimens of *Xestospongia testudinaria* from three regions of Java (West, Central and East), Bali and Sulawesi. Immediately after collecting, the specimens were cut, rinsed and soaked in 99% ethanol before they were preserved in 99% ethanol. Other 19 samples, which covered localities in Southeast Thailand, were provided from the Naturalis Biodiversity Centre, Leiden, The Netherlands. In addition, the Queensland Museum Brisbane, Australia provided samples from Queensland localities (see Supplementary Material 3.1).

3.2.2. DNA extraction, amplification and sequencing

The DNA extraction method published for sponge barcoding (Vargas et al. 2012) was applied The 3' partition of the cytochrome oxidase subunit 1 (I3-M11 of cox1) was amplified with the polymerase chain reaction (PCR) using primers CJ-J2165 and C1-Npor 2760 (Erpenbeck et al. 2002). The Adenosine Triphosphate Synthase F0 subunit 6 (ATP6) was amplified using primers ATP6 PorF and ATP6 PorR (Rua et al. 2011). Specifically designed intron primers (Xt_ATPSβ_F & Xt_ATPSβ_R) were required because the ATPS-β intron from several *X. testudinaria* samples could not be amplified with the primers published from from Jarman et al. (2002, ATPSβF1 and ATPSβR1). The primer sequences including annealing temperatures are listed in Table 3.1**.** The 25 µL PCR mix consisted of 5 µL 5x green Go*Taq* ® PCR Buffer (Promega Corp, Madison, WI), 4µL 25mM $MgCl_2$ (Promega Corp, Madison, WI), 2 µL 10mM dNTPs, 1 µL each primer (5µM), 9.8 µL water, 0.2µL Go*Taq*® DNA polymerase (5u/µl) (Promega Corp, Madison, WI) and 2 µL DNA template. The PCR regime comprised an initial denaturation phase of 94° C for 3 min followed by 35 cycles of 30 s denaturation at 94° C, 20 s annealing and 60 s elongation at 72° C each and a final elongation at 72° C for 5 min. All of the PCR products were cleaned using ammonium acetate precipitation (Sambrook et al. 1989). Sequencing of forward and reverse strands was performed with the ABI BigDye v 3.1 (Applied Biosystems, California USA) chemistry 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 are labelled according to the haplotypes of Swiert et al. (2013(, when identical (C1-C6 for I3-M11 cox1,

A1 and A2 for ATP6 haplotypes; GenBank accession number KC424439 - KC424446). In addition, haplotype sequences that are not recovered in that study are submitted and deposited at NCBI GenBank under accession numbers KM014757, KM014758, KM014763 for A4-A6 ATP6 and KM030066-KM030094 for β1- β29 ATPS-β intron haplotypes.

Table 3.1. Primers used in this study

Primer Name	Primer Sets	Marker	References	Annealing Temperature
C1-J2165 C1-Npor2760	5'-GAAGTTTATATTTTAATTTTACCNGG-3' 5'-TCTAGGTAATCCAGCTAAACC-3'	I3-M11 (cox1)	Erpenbeck et al. 2002	42° C
ATP6porF ATP6porR	5'-GTAGTCCAGGATAATTTAGG-3' 5'-GTTAATAGACAAAATACATAAGCCTG-3'	ATP6	Rua et al. 2011	44° C
ATPSβF1 ATPSβR1	5'-CGTGAGGGHAAYGATTTHTACCATGAGATGAT-3' 5'-CGGGCACGGGCRCCDGGNGGTTCGTTCAT-3'	ATPS-β intron	Jarman et al. 2002	54° C
Xt_ATPS-β_F Xt_ATPS-β_R	5'-ATGAGATGATCACATCAGGTG-3' 5'-GGTTCGTTCATCTGTCC-3'	ATPS-β intron	This study	50° C

3.2.3. Intron annotation and specific primer design

The ATPS-β intron fragment of four only samples (GW2317, GW2321, GW1484, and G317830) could be amplified using the primers of Jarman et al. (2002). These sequences were aligned using MUSCLE version 3.5 (Edgar 2004) as implemented in Geneious version 6.1.7 (available from http://www.geneious.com/) under default settings and subsequently trimmed. This yielded a fragment of 275 bp for all samples. The consensus sequence was checked with BLAST against GenBank (http://www.ncbi.nlm.nih.gov/) to check its poriferan origin. The highest BLAST match, *Ircinia strobilana* ATPS-β (Accession Number GQ330993), indicated that the targeted gene was indeed derived from sponge origin and not from an associate DNA template. ORFs and intron splice sites were identified and annotated with Geneious to distinguish both exon and intron regions. Conforming to the general splicing site motifs (Clancy 2008), the intron region of the ATPS-β gene started with GT in the 5'splice site (the donor site), a branch site with pyrimidine nucleotides, and AG at the 3'splice site (acceptor site). The partial ATPS-β gene consisted of 40 bp of exon 1, a 214 bp intron, and 21 bp of exon 2. These annotations were used to design *X. testudinaria* specific primers for the ATPS-β intron, a few basepairs further downstream (5' primer) respectively upstream (3' primer) on the exons (see Table 3.1 for the sequence).

3.2.4. Data analyses

SeqPHASE (Flot 2010) was used to resolve ATPS-β alleles that could not be directly distinguished due to heterozygosity as detected in form of double peaks in the electropherograms. *P*-distances were calculated manually. The genetic diversity indices (π) and Tajima's D (Tajima 1989) were obtained with Dna SP v. 5.10.01 (Librado and Rozas 2009). For genetic structure analysis, regional samples were pooled as follows (1) Southeast Thailand [SET, N=9], (2) West Java [WJ, N=6], (3) Central Java [CJ, N=8], (4) East Java [EJ, N=9], (5) North Sulawesi [NS, N=11], (6) Southeast Sulawesi [SS, N=6], and (7) Queensland [QN, N=5]. In addition, an analysis of molecular variance (AMOVA) and the calculation of pairwise F_{ST} values were performed in Arlequin v 3.5.1.2 (Excoffier et al. 2005) with a permutation test under 10,000 replicates. The significance of F_{ST} values was amended following a Bonferroni correction (Rice 1989).

Phylogenetic reconstructions for all markers were performed under Bayesian inference (BI) and Maximum-likelihood (ML) criteria. Inferences under the Bayesian framework were performed with MrBayes v. 3.2.1 (Ronquist et al. 2012). Each analysis consisted of two independent runs of four Metropolis- coupled Markov-chains under default temperature with trees sampled at every 1000^{th} generations. Analyses were terminated automatically when the chains converged significantly as indicated by an average standard deviation of split frequencies < 0.01. ML analysis was performed using RAxML v. 7.0.4 in raxmlGUI v. 1.3 (Silvestro and Michalak 2012) with 1,000 rapid bootstrap replications (Stamatakis et al. 2008). Hierarchical Likelihood Ratio Tests (hLRTs) for identifying the relatively best fitting model during tree searches in both inferences were performed with jModeltest 2.1.3 (Darriba et al. 2012) under the Akaike Information Criterion (AIC) (Akaike 1974). The HKY + I, HKY + Γ, and HKY substitution models were suggested for ATP6, ATPS-β intron, and I3-M11 alignments respectively but were changed into their GTR equivalents as the HKY model is not implemented in RAxML (see Stamatakis 2008). In the Bayesian analyses, the more complex GTR + Γ model was used as overparameterization does not negatively affect bayesian analyses (Huelsenbeck and Rannala 2004).

3.3. Results

3.3.1. The ATPS- β intron, heterozygosity and amplification success

The newly designed primers amplified 214-235 bp ATPS-β intron that is flanked by 29 bp exon 1 and 15 bp exon 2 of ATPS-β (see Figure 3.2). The final alignment comprised

289 characters. The sixty samples resulted into 82 sequences as 21 individuals possessed two different sequence types due to alledged heterozygosity. After allele determination, four samples (GW4858, GW1249, GW2316, and GW2317) were excluded from the analysis due to a PHASE value lower than 0.900, which indicates haplotype ambiguity (Flot 2010). Consequently these samples were excluded from the analyses. ATP6 sequences from all 60 samples were successfully obtained. In contrast, I3-M11 sequences of two samples from Southeast Thailand (GW4844) and Queensland (G306508) could not be amplified. For subsequent analyses only samples with a complete sequence set for all three fragments were included (see supplementary Material 4.1). Therefore, in total 162 sequences from 54 individuals were available, which comprised of nine samples from Southeast Thailand (SET), six samples from West Java (WJ), eight samples from Central Java (CJ), nine samples from East Java (EJ), eleven samples from North Sulawesi (NS), six samples from Southeast Sulawesi (SS) and five samples from Queensland (QN) (see Figure 3.1).

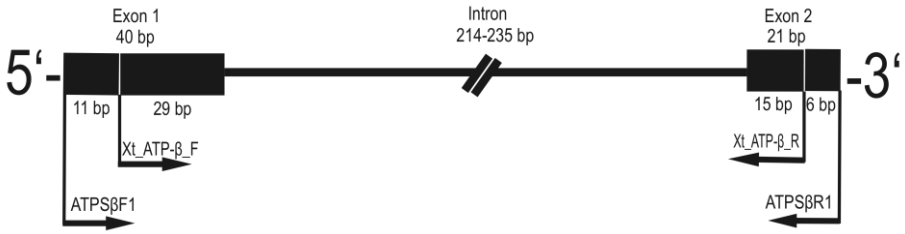


Figure 3.2. Intron splice sites from the ATP-β gene. ATPSβF1 & ATPSβR1 are universal EPIC primers for a ATPS-β fragment. Xt_ATPS-β_F & Xt_ATPS-β_R are specific ATPS-β intron primers for *X. testudinaria*

3.3.2. DNA polymorphisms, genetic structure and spatial relationships

Tajima's D ($P > 0.1$) indicated neutral evolution among the three markers utilised in this study. Likewise, overall F_{ST} values from the hierarchical AMOVA test revealed that the signal of genetic structuring was present among the markers analysed ($P < 0.05$, see table 3.2). Furthermore, ATPS-β intron sequences yielded the highest *p*-distances and nucleotide diversity values (π) in comparison to mtDNA markers (see Table 4.2). Due to the highest number of polymorphic sites (18%), the ATPS-β intron possessed the highest number of haplotypes in comparison to the mtDNA markers (0.67% for ATP6 and 0.76% for I3M11) (see Table 3.2 and Supplementary Material 3.1). Since gaps were utilised to align sequences of different lengths, gaps were also included in the calculation of *p*-distances and π values.

Due to the unambiguous nature of the alignment, gaps were treated as a fifth character. The ATPS-β intron displayed the most significant genetic structuring of sequences in almost all localities, in comparison to the mtDNA markers (see Table 3.3).

Table 3.2. DNA polymorphism among markers tested, including their neutral evolution (Tajima's D, $P > 0.1$), and genetic structuring (F_{ST}, $P < 0.05$ after Bonferroni correction)

Markers	Numbers of sequences	Length of sequences	Number of polymorphic sites	Numbers of haplotypes	*P*-distance values	Nucleotide diversity (π) values	Tajima 's D	F_{ST}
ATPS-β intron	70	258- 279 bp	53	29	0.18	0.045	-0.23286	0.18812
I3-M11	54	544 bp	4	5	0.0076	0.0026	1.34108	0.37471
ATP6	54	445 bp	3	5	0.0067	0.0026	1.40512	0.25809

Table 3.3. Pairwise F_{ST} values between populations of *X. testudinaria* in the ATPS-β intron / I3-M11/ ATP6. Significant values at $P < 0.005$ after Bonferroni corrections are indicated by asterisks

Population	Southeast Thailand	West Java	Central Java	East Java	North Sulawesi	Southeast Sulawesi	Queensland
Southeast Thailand (N=9)	0.00000						
West Java (N=6)	0.08338/ 0.42466/ 0.36842	0.00000					
Central Java (N=8)	0.19180*/ 0.62059*/ -0.09363	0.13889/ 0.33518/ 0.46745	0.00000				
East Java (N=9)	0.09008/ 0.37829*/ 0.37321	0.05032/ 0.12855/ 0.09244	0.11355/ 0.39935/ 0.44964*	0.00000			
North Sulawesi (N=11)	0.09631/ 0.22401/ 0.16636	0.06651/ 0.14304/ 0.07799	0.14344*/ 0.37356*/ 0.22775	0.02314/ 0.05104/ 0.07918	0.00000		
Southeast Sulawesi (N=6)	0.45050*/ -0.12971/ -0.07143	0.46221*/ 0.46667/ 0.40000	0.55120*/ 0.70771*/ -0.07131	0.37701*/ 0.38667/ 0.42466*	0.24660/ 0.24892/ 0.18821	0.00000	
Queensland (N=5)	0.14554/ 0.70395/ -0.02273	0.21979*/ 0.41281/ 0.56835	0.28516*/ -0.06870/ -0.06870	0.20516*/ 0.48864/ 0.54082*	0.21556*/ 0.43012/ 0.29487	0.65843*/ 0.81707/ -0.03448	0.00000

3.3.3. Phylogenetic analyses

The phylograms from the three different fragments resulted in trees with a high degree of similarity as three major taxon groups (A-C) could be identified throughout all markers (see Figures 3.3. A-C). These taxon groups represent supported clades in some of the trees, while in others their monophyly cannot be shown, due to a strong degree of conservation. However, separation according to geography could not be identified. Sequences from Sundaland (East Java [EJ], West Java [WJ]) and Wallacea (North Sulawesi [NS], South Sulawesi [SS]) are dispersed over all three groups. Only samples from Queensland fell homogeneously into group C but they share this position with samples from other localities. Two samples (GW2331 and GW4861) display discordant positions in their mtDNA and ATPS- β phylograms.

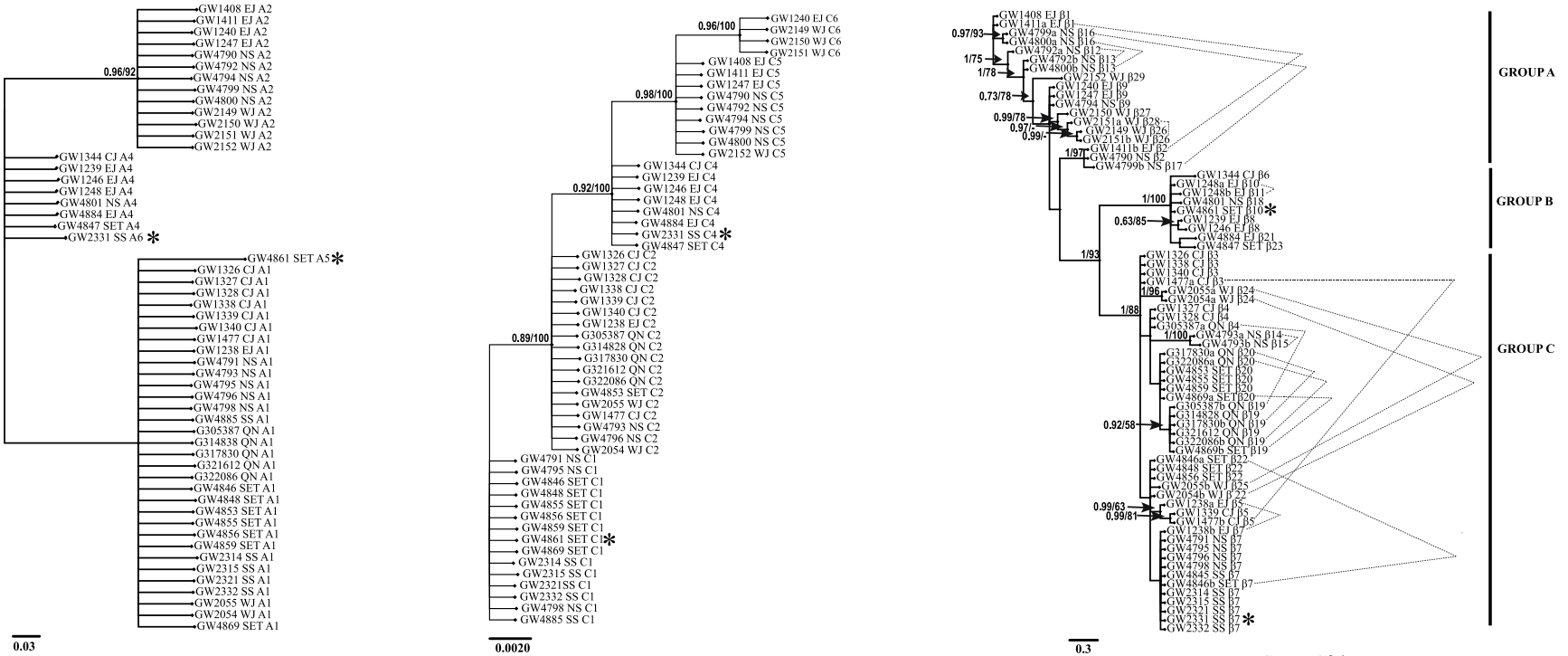


Figure 3.3 Unrooted bayesian inference phylograms of *Xestospongia testudinaria* sequences, which is generated from: **A**. partial ATP6, **B** the I3-M11 partition of cox1 gene, and **C** the ATPSβ intron. Numbers on the branches represent posterior probabilities (PP) / bootstrap proportions (BP) of maximum likelihood analyses. Scale bar indicates the number of substitutions per sites and dashed lines indicate a connectivity of two haplotypes from one individual. SET= Southeast Thailand, WJ= West Java, CJ= Central Java, EJ= East Java, NS= North Sulawesi, SS= Southeast Sulawesi, QN= Queensland. A1, A2, A4, A5, and A6: ATP6 haplotypes recovered in this study following Swierts et al. 2013 numeration. C1-C6: I3-M11 haplotypes recovered in this study following Swierts et al. 2013 numeration. Asterisks indicate samples with discordant mtDNA (ATP6 andI3-M11) and ATPSβ positions

3.4. Discussion

3.4.1. ATPS-β intron suitability for studying *X. testudinaria* phylogeography

The capability of the ATPS-β intron marker for detecting phylogeographical breaks in *X. testudinaria* according to the palaeogeographical history in the Indo-Australian (IAA) archipelago is evaluated. The ATPS-β intron is a potentially suitable marker for *X. testudinaria* intraspecies studies because it possesses a faster evolutionary rate than two mtDNA markers tested. The *p*-distance and the nucleotide diversity (π) are basic parameters for assessing the polymorphism of molecular markers (Nei 1987; Nei and Kumar 2000). In this study, the ATPS-β intron generated the highest *p*-distance and π value in comparison to the other (mtDNA) markers. In addition, the strongest genetic structuring signal in *X. testudinaria* can be generated out of the ATPS-β intron according to the pairwise F_{ST} values.

Despite possessing the highest *p*-distance, π value, and pairwise F_{ST}, the ATPS-β intron did not separate *X. testudinaria* sequences according to the common pattern of marine organisms during the Pleistocene low sea level and Holocene recolonisation in the IAA. The pattern of genetic diversity in this region is usually fragmented into Sundaland, Wallacea, Philippine and Australian populations (e.g., Barber et al. 2000; Kochzius and Nuryanto 2008; Kochzius et al. 2009; Nuryanto and Kochzius 2009; Timm et al. 2012). This common pattern could not yet be observed for sponges. In *Leucetta chagosensis*, ATPS-β intron sequences from Bali (part of Sundaland) could not be separated from sequences of the central Coral Sea (central GBR, part of Australia). However, deep lineage separations are still observed among sequences of Sulawesi (part of Wallacea), Bali (part of Sundaland) and the other three Australian parts (QLD Plateau, Northern GBR, and Southern GBR) (Wörheide et al. 2008). In a different study *Suberites diversicolor* cox1 and ITS sequences from Sundaland (Singapore and Derawan, Kalimantan) are likewise mixing with Wallacea (Raja Empat, Papua) sequences despite separations between to Darwin populations (Australia) are recognised (Becking et al. 2013b). Becking et al. (2013) explained that phylogeographic studies on sponges based on less conserved markers (e.g., Wörheide et al. 2005; Wörheide et al. 2008; Lopez-Legentil and Pawlik 2009; DeBiasse et al. 2010; Xavier et al. 2010a) occasionally uncover sponge taxa that posses strong genetic structures and long distance dispersal events. This movement pattern includes the ability of sponges to disperse viable fragments in currents or to raft on various floating material (Wulff 1995; Maldonado and Uriz 1999) and results in the absence of genetic separation between two isolated localities as proposed by Wörheide et al. (2008) for *Leucetta chagosensis*.

3.4.2. *Xestospongia testudinaria* species complex

The inclusion of cryptic species in phylogenetic analyses negatively influences the accuracy of π values (Goodall-Copestake et al. 2012). The high degree of similarity among phylograms in this study indicates that *X. testudinaria* consists of a species complex according to the Monophyletic (a.k.a. Genealogical) species concept (de Queiroz 2007). This concept is based on the coalescence or common ancestry of lineages, in which species are required to be monophyletic. Therefore only those species, which are clearly distinguishable by apomorphies, are accepted as valid. Further taxa, which are not clearly distinguishable by apomorphies are denoted as metataxa (see explanation in de Pinna 1999; de Queiroz 2007).

I did not utilise the Diagnosable species concept in distinguishing the boundaries among analysed *X. testudinaria* species because my scope of this chapter is the investigation of the ATPS-β intron suitability marker to detect phylogeographical breaks with the monophyletiv species concept. However, as several studies indicate that *X. testudinaria* constitutes a species complex, additional robust diagnosable characters need to be determined to delimit its members. For example the *X. testudinaria* species complex unravelled by Swierts et al. (2013) genetically on a narrow spatial scale is supported by morphotypes. Likewise, independent (microsatellite) markers recently emphasised the presence of a different *X. testudinaria* species complex in Southeast Sulawesi (Bell et al. 2014b). These findings are in congruence, on the general view that widespread or cosmopolitic sponge species alludes a high degree of cryptic speciation and endemism. Cosmopolitism has been rejected for almost every sponge species investigated (see e.g., Wörheide et al. 2008; Pöppe et al. 2010; Reveillaud et al. 2010; Xavier et al. 2010b; Reveillaud et al. 2011) and suggests the presence of cryptic species due to the overestimation of widely distributed taxa (Palumbi et al. 1997; Klautau et al. 1999; Solé-Cava and Boury-Esnault 1999; Bierne et al. 2003).

3.4.3. The mtDNA and nucDNA discordance

Two samples fall in divergent positions in the ATPS-β intron and mtDNA phylograms. A similar mito-nuclear discordance was discovered in a study on *Callyspongia vaginalis* and explained based on two factors (DeBiasse et al. 2013). First, the discordance is caused by the effective population size difference between mtDNA and nucDNA. Male alleles influence the effective population size in nucDNA (see e.g., Palumbi and Baker 1994; Palumbi et al. 2000). Thus, effective population size of mtDNA is only one fourth compared nucDNA and therefore mtDNA responses faster to population contractions, which is indicated

by a higher Tajima's D of mtDNA compared to nucDNA. In our study, the ATPS-β intron possessed a lower Tajima's D (-0.23286) than mtDNA (1.34108 for cox1 and 1.40512 for ATP6). Therefore, mito-nuclear discordance could have occurred due to differences of effective population size (see also Palumbi and Baker 1994; DeBiasse et al. 2013).

Second, the discordant tree might be the result of incomplete lineage sorting of the ATPS-β intron and cox1. The two affected samples possessed unique ATP6 haplotypes (A5 and A6), but divergent haplotypes in cox1 and the ATPS-β intron (C1 and C4 for cox1, β7 and β10 for the ATPS-β intron). The presence of only homozygote ATPS-β intron alleles would reduce the possibility of such discordances. However, this discordance would not be restricted to two samples only, but affect a larger subset as observed in the study of DeBiasse et al. (2013) and Palumbi and Baker (1994) if the discordance is caused by the different of effective population size. The presence of unique haplotypes only in one marker (ATP6), homozygote alleles and effect of discordance for only those two samples suggest that the discordance is due to incomplete lineage sorting in the ATPS-β intron and I3-M11 markers.

3.5. Conclusion

Despite of several limitations, the study revealed that the ATPS-β intron could be utilised in the intraspecies studies of *X. testudinaria*. The ATPS-β intron possessed a faster substitution rate than the mtDNA markers tested. Likewise, the ATPS-β intron can also be utilised for detecting the presence of a species complex. For resolving phylogeographic relationships of *X. testudinaria* improvements, e.g., using uniform samples sizes for each location, genotyping of all heterozygotic alleles, additional tests on narrower geographical scales and avoidance of species complexes would enhance the capability of the ATPS-β intron. The genetic diversity of *X. testudinaria* in the IAA is not identical to the pattern of common marine taxa during Pleistocene low sea level and Holocene recolonisation, presumably since sponges occasionally posses long time dispersal measurements and the capability to disperse asexually with the water current.

CHAPTER 4

Bearing the wrong identity: on the smooth brown Indo-Pacific sponges, *Neopetrosia exigua* (Porifera; Haplosclerida)

4.1. Introduction

Sponges are common and important elements of reef and mangrove communities in the Indonesian marine ecosystem, where haplosclerid sponges are categorised as one of the most conspicuous orders due to their abundance in terms of diversity and quantity (van Soest 1989; Amir 1992; de Voogd and van Soest 2002; de Voogd 2004; de Voogd et al. 2004; de Voogd and Cleary 2008). However, the published knowledge of haplosclerids is far from complete (Weerdt and van Soest 2001; de Voogd and van Soest 2002; de Voogd 2004). Taxonomically, haplosclerids are recognised as one of the most complicated and unreliable groups in demosponges (Borchiellini et al. 2004), and "a sound classification of the order is a long way from being established" (Redmond et al. 2011). For this reason, the use of classical taxonomy that employs spicule dimensions, type, and arrangement of skeletal meshes often fails in delimiting lower taxa and creates polyphyletic groups because of "vague and elusive morphological synapomorphies" within the suborders Haplosclerina and Petrosina (van Soest and Hooper 2002c; van Soest and Hooper 2002b; van Soest and Hooper 2002a).

The genus *Neopetrosia* (suborder Petrosina; family Petrosiidae) is an example of a haplosclerid genus that is very difficult to recognise and identify. De Laubenfels (1949) erected this genus for the previously described species *Haliclona longleyi*, although he did not define this new genus. The genus was further discussed by de Laubenfels (1954), Bergquist (1965), Wiedenmayer (1977), and van Soest (1980), but was only properly defined in 2002 (see Systema Porifera Hooper and van Soest 2002a). The genus differs from other genera within the family Petrosiidae by its relatively small size of spicules and a recognisable anisotropic pattern in its skeleton (Desqueyroux-Faundez and Valentine 2002). So far, 28 nominal species have been recognised within the genus *Neopetrosia* (World Porifera Database, van Soest et al. 2014).

Neopetrosia exigua (Kirkpatrick, 1900) is a very common species in many Indo-Pacific shallow water reefs and has extensively been mentioned in the literature, primarily for its interesting bioactive properties (Orabi et al. 2002; Liu et al. 2004; de Almeida Leone et al. 2008; Abdillah et al. 2013a; Abdillah et al. 2013b). *Neopetrosia exigua* was originally described as *Petrosia exigua* from Christmas Island (south of Java, Indonesia) by Kirkpatrick

(Kirkpatrick 1900). He noted that the species possessed very small oxeas as opposed to its most allied species, *Petrosia similis*. Currently, two sponge taxa are accepted as junior synonyms of *N. exigua*, namely *Neopetrosia pandora* de Laubenfels, 1954 from Ponape (de Laubenfels 1954), Micronesia, and *Xestospongia pacifica* (Kelly-Borges and Bergquist 1988) from Motupore Island, Papua New Guinea (World Porifera Database, van Soest et al. 2014).

During my phylogeographic study, I observed a striking similarity between freshly collected specimens and *Neopetrosia chaliniformis* (Thiele 1899). This species was described one year before *N. exigua* from Sulawesi, Indonesia, however, this name was never used after its original description. Here, I aim to unravel the true identity of these common shallow water Indo-Pacific sponges. I investigate type material and examine freshly collected specimen materials, including additional specimens that are deposited at several institutes. Main morphological characters such as spicule measurements and other skeleton features are studied. In addition, molecular phylogenetic methods employing fragments of the mitochondrial cytochrome oxidase subunit 2 (cox2) and 28S ribosomal DNA (28S rRNA) sequences are compared for assisting the morphological examinations, since haplosclerids possess a reduced suite of phylogenetic informative characters (Weerdt 1985).

4.2. Materials and Methods

4.2.1. Samples (Porifera; Haplosclerida)

Fourteen samples were freshly collected from several localities in Indonesia. Directly after being collected, the samples were cut, rinsed and soaked in 99% ethanol before being preserved in 99% ethanol. Additional 18 samples were received from the Naturalis Biodiversity Center, Leiden, The Netherlands (samples coded with ZMAPOR, are at present the Zoological Museum Amsterdam collection housed in the Naturalis Biodiversity Center in Leiden), and further specimens were provided by the Queensland Museum (QM) Brisbane, Australia (G). Holotype specimens were retrieved from the British Museum of Natural History, London, UK (BMNH, *Neopetrosia exigua*), the Zoological Museum Berlin, Germany (ZMB, *Neopetrosia chaliniformis*), the Smithsonian Museum, Washington DC, USA (USNM, *Neopetrosia pandora*), and the Australian National Museum, Sydney, Australia (Z, *Xestospongia pacifica*), see Table 4.1.

The descriptions presented below are based on external morphology, skeletal architecture and shape and size of the spicules. Spicule dimensions are given as the minimum-**mean**-maximum of length measurements x minimum-**mean**-maximum of width

measurements from 25 spicule measurements. For study of the skeletal architecture, hand-cut perpendicular sections were made. These sections were air-dried, mounted in Ultrabed on a microscope slide, and studied under a Leica high power light microscope. 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 the light microscope by mounting them in Ultrabed.

4.2.2. Statistical Analyses on the spicules

The statistical analyses were carried out using PASW 20.00 (SPSS Inc, 2012). The statistic significance of phenotypic differences among selected localities is tested by the Univariate analysis of variance (ANOVA, with Duncan's post hoc test, $p < 0.05$). These analyses were only conducted for some localities where at least three specimens were sampled (Thailand, West Java, North Sulawesi, South East Sulawesi, Solomon Island and the Great Barrier Reef Australia, see Table 4.1.

4.2.3. DNA extraction and sequencing

DNA extraction based on the previously published and established methods in sponge barcoding (Vargas et al. 2012) was performed for all specimens except the holotypes. *Neopetrosia pandora and X. pacifica* were extracted separately using the DNeasy Blood & Tissue kit (Qiagen) following the instructions of the manufacturer. Because first attempts to amplify two >100-year-old holotypes (*N. chaliniformis and N. exigua*) failed, DNA extractions for those two holotypes were repeated using a modification from the CTAB phenol-chloroform method of Porebski et al. (1997). In this modified method, phenol-octanol and RNase solutions steps were skipped. With this modified method I also gained amplifiable DNA for the two 100-year-old holotypes as opposed to the spin column method. The Polymerase Chain Reaction (PCR) using primers CO2F Por, 5'-TTTTTCACGATCAGATTATGTTTA-3' and CO2R Por, 5'-ATACTCGCACTGAGTTTGAATAGG-3' (Rua et al. 2011), was performed for amplifying a fragment of the cox2 gene, and with primer 28S-C2-fwd, 5'-GAAAAGAACTTTGRARAGAGAGT-3' and 28S-D2-rev and 5'-TCCGTGTTTCAAGACGGG-3' (Chombard et al. 1998) for a fragment of 28S rRNA. The 25 µL PCR mix consisted of 5 µL 5x green Go*Taq*® PCR Buffer (Promega Corp, Madison, WI),

4µL 25mM $MgCl_2$ (Promega Corp, Madison, WI), 2 µL 10mM dNTPs, 1 µL each primer (5µM), 9.8 µL water, 2 µL DNA template, and 0.2 µL Go*Taq*® DNA polymerase (5u/µl) (Promega Corp, Madison, WI). The PCR regime comprised an initial denaturation at 94° C for 3 min, 35 cycles of 30 s denaturation at 94° C, 20 s annealing at 40° C and 60 s elongation at 72° C each, followed by a final elongation at 72° C for 5 min. For the holotypes of *N. chaliniformis and N. exigua* 2 µL Bovine Serum Albumin (BSA 10 mg/ml) were added in the PCR mix and PCR regime was modified with 45 s annealing at 40° C, 45 s elongation at 72° C each and a final elongation at 72° C for 7.5 min. All of the PCR products were cleaned with the ammonium acetate precipitation method. Sequencing of the forward and reverse strand was performed with the ABI BigDye v3.1 (Applied Biosystems, California USA) chemistry and the amplification primers following the manufacturers protocol on an ABI 3730 Automated Sequencer in the Genomic Sequencing Unit of the LMU Munich (http://www.gi.bio.lmu.de/sequencing/). All sequences are deposited at NCBI GenBank under accession numbers KM030095-KC030119 (cox2 mtDNA) and KM030120-KM030145 (28S rRNA fragment C2-D2).

4.2.3. Molecular Phylogenetic Analysis

Geneious version 6.1.7, (http://www.geneious.com) was used for assembling, trimming and analysing the sequences. Additionally, sequences were checked with BLAST against GenBank (http://www.ncbi.nlm.nih.gov/) for contaminations. Sequences were aligned with MUSCLE version 3.5 program (Edgar 2004) as implemented in Geneious under default settings. Phylogenetic reconstructions with cladistic analyses under probabilistic criteria were inferred for cox2 sequences using Bayesian Inferences (BI) and Maximum Likelihood (ML). The HKY+ Γ model of evolution as suggested by jModeltest v. 2.1.3 (Darriba et al. 2012) under the Akaike Information Criterion (Akaike 1974) was selected. BI was performed in MrBayes v. 3.2.1 (Ronquist et al. 2012). Each analysis consisted of two independent runs of four Metropolis-coupled Markov-chains under default temperature with trees sampled at every 1000^{th} generation. Analyses were terminated automatically when the chains converged significantly as indicated by an average standard deviation of split frequencies <0.01. Similarly ML analyses were inferred using RAxML v. 7.0.4 in the raxmlGUI v. 1.3 program (Silvestro and Michalak 2012) with a rapid bootstrap of 1000 replications (Stamatakis et al. 2008). As the HKY+ Γ model is not implemented in RAxML, the model utilised in RAxML was changed to GTR+ Γ (Stamatakis 2008).

Cladistic analyses were not performed for the 28S rRNA sequences because an unambiguous final alignment was not possible due to the abundance of highly variable sites. Therefore a simple phenetic analysis was carried out instead to display the grade of similarities between the sequences. For this purpose a data set of the 28S rRNA sequences was approached with MUSCLE (Edgar 2004) under default settings and analyzed with Neighbour-Joining (NJ) analysis with observed distances in SeaView version 4.4.2 (Gouy et al. 2010).

A sample of *X. testudinaria* (Lamarck, 1815) (GW1341: Indonesia, Central Java, Kep Karimun Jawa, Pulau Sintok, South side, 5° 47' 06" S, 110° 30' 18" E, 27 May 2011, coll. E. Setiawan) was sequenced and utilised only for the molecular analysis as additional non-*Neopetrosia* sequence.

4.3. Results

4.3.1. Spicule analyses and skeleton observations and description of type material

Systematics

Phylum Porifera Grant, 1835

Class Demospongiae Sollas, 1885

Order Haplosclerida Topsent, 1928

Suborder Petrosina Boury-Esnault & Van Beveren, 1982

Family Petrosiidae van Soest, 1980

Genus *Neopetrosia* de Laubenfels, 1949

Neopetrosia chaliniformis (Thiele, 1899) Figure 4.1A, 4.2A, and 4.3A, Table 4.1

Petrosia chaliniformis Thiele, 1899 (Page 21, Plate 2. Figure 9; Plate 5. Figure 15 in its original description)

Holotype: ZMB2889, Indonesia, North Sulawesi, Kema, Minahasa.

Description amended from Thiele, 1899: Sponge consists of several fragments. Frequently found in the form like plates with branches. Branches have a diameter of 5- 6 mm. Size of osculum is measured 1-2 mm. Height is measured in average of 12 cm.

Consistency. – Grainy.

Colour. – Brown in dry condition and light brown in alcohol.

Skeleton. – Skeleton has structure of isodictyal tangential spicule network with one size of spicule.

Spicules. – Oxeas in a range of 100-**140.8**-165 µm x 7.5-**9.9** -12.5 µm.

Remarks: Thiele (1899) described that the specimen is more properly related to and placed in the genus *Petrosia* rather than in *Pellina* because it has an easy removable epidermis layer. However, Thiele was also not sure about placing the specimen in the genus *Petrosia* because the consistency of the specimen was more elastic than in common *Petrosia*. Likewise, Thiele observed that the skeleton structure of the specimen is similar to the skeleton structure from *Reniera* or *Rhizochalina*. Nevertheless, all genera similarities that are mentioned by Thiele no longer exist, because *Reniera* and *Pellina* have already been transferred to several genera within the family Petrosiidae e.g., *Petrosia*, *Haliclona*, *Rhizochalina*, the latter of which was renamed as *Oceanapia* (World Porifera Database, van Soest et al. 2014).

Neopetrosia exigua (Kirkpatrick, 1900) (Figure 4.1B, 4.2B, and 4.3B, Table 4.1).

Petrosia exigua Kirkpatrick, 1900 (Page 139, Plate XII. Figure 7; Plate XIII. Figure 4 in its original description)

Holotype: BMNH1898.12.20.49, Australia, Christmas Island.

Description amended from Kirkpatrick, 1900: Sponge forms a hard, thick, nodulated crust, smooth surface, showing an irregular reticulate pattern formed by pore-areas, oscules are measured in a range of 1-5 mm in diameter.

Colour. – Pale gray brown in the dry condition.

Skeleton. – Forms of slender main lines of fibres passing vertically to the surface and connected at right angles to this plane by closely packed single spicules, so as to form circular or obscurely polygonal tubes about 70 mm in diameter. The skeleton network is a dense and irregularly oriented.

Spicules. – Oxeas in a size range of 70-**113.8**-130 x 5-**7.3**-10 µm.

Remarks: Kirkpatrick (1900) described that spicules are considerably smaller than their allied species *Petrosia similis* (less than half size). We also discovered that only a small fragment of the type material is left.

Xestospongia pacifica Kelly-Borges & Bergquist, 1988 (Figure 4.1C, 4.2C, and 4.3C, Table 4.1; in its original description Page 155, Plate 6c, 6d).

Holotype: Z4999, Papua New Guinea, Buna Motu Reef, Bootless Bay, South Papua New Guinea.

Description amended from Kelly-Borges & Bergquist, 1988: A sponge, which grows as an

encrustation of 5-10 mm thickness, frequently with papillae 20-60 mm high and 5-10 wide. It has small oscules of 1-3 mm width.

Colour. – In life, the colour is brownish orange to olive. In alcohol, the colour changes into olive black and slightly browner.

Consistency. – Tough, compressible and extremely brittle.

Spicules. – Oxeas in a size range of 100-**149.6**-175 x 5-**9.2**-10 µm

Skeleton. – Dense and an isodictyal tangential spicule network with one size of spicule.

Remarks:

This species was synonymised with *Neopetrosia exigua* by van Soest, see World Porifera Database (van Soest et al. 2014) since it has a morphological characteristic similarity to *N. exigua* e.g., the spicule is only consist of oxeas smaller than 200 µm and the meshes posses a more compact structure than the meshes of *Xestospongia* (Desqueyroux-Faundez and Valentine 2002).

Neopetrosia pandora de Laubenfels, 1954.

(Figure 4.1D, 4.2D, and 4.3D, Table 4.1; in its original description Page 81, Figure 49).

Holotype: USNM 23046, Palau, Matalim, East Ponape, 1 August 1949, 5m depths, coll. de Laubenfels.

Description amended from de Laubenfels, 1954: A repent or sprawling ramose sponge, reaches a maximum length of at least 13 cm. The diameter of the branches is 9 mm; oscules are about 2 mm in diameter and 2 cm apart.

Colour. – Colour in life is dull olive drab.

Consistency. – Slightly spongy but also somewhat stiff, tearing very easily.

Spicules. – Oxeas in a range of 100-**119.8**-150 x 2.5-**2.9**-5 µm.

Skeleton. – Less dense, more irregular isodictyal spicule network with one size of spicule.

Remarks:

De Laubenfels described that the consistency of the type specimen was slightly spongy, somewhat stiff and tearing very easily. The surface of the type specimen does not possess an ectosomal specialisation, which is a typical characteristic of sponges from Family Haliclonidae. Little or lack of spongin in skeleton was observed. The spicules are also much thinner than the other spicules of type specimens and samples analysed in this study.

Additional material

I examined additional material identified as *Neopetrosia exigua* (coded with GW voucher samples and museum numbers) and freshly collected specimens identified as *Neopetrosia chaliniformis* (only coded with GW voucher samples) from various location in Mauritius, Thailand, Japan, the Philippines, Singapore, Indonesia, Palau, Papua New Guinea, Australia, Solomon Islands, Vanuatu, Palau, and Tonga (see detail bellow, Table 4.1 and Figure 4.4 A-C).

GW18429, G303302: Australia, Northern Territory, Dudley Point Reef, East Point, Darwin, intertidal reef, 12° 25' 03" S, 130° 49' 00" E, 20 September 1993; GW18478, G313113: Singapore, Pulau Tembakul (Kusu I), Freyberg Channel, very silty patchy coral reef, 1° 13' 05" N, 103° 51' 07" E, 2 May 1995, 18.7m depth; GW18491, G313297: Tonga, Vaipuua, at end of channel, highly silted fringing reef, Porites spires, 18° 37' 55" S, 173° 58' 47" W, 14 November 1997, 15 m depth; GW18598, G306321; Palau, Ongingiang, W of, W. Palau, fringing coral reef surrounding channel in outer barrier reef, strong current, spur and grooves, 7° 16' 05" N, 134° 14' 05" E, 10 December 1995, 31m depth; GW18777, G311804: Papua New Guinea, North of Lion Island, S/w Of Motupore Island; Near Port Moresby, PNG, top ridge or reef, dead coral, 19 September 1990, 6-18m depths; GW18793, G315226: Australia, Queensland, Hook Reef lagoon, coral reef lagoon, 19° 45' 14" S, 149° 10' 45" E, 5 June 1999, 9.4m depth; GW 18804, QM G312397: Australia, Western Australia, North Head, Beagle Bay, NW WA, rock salt , 16° 30' 00" S, 122° 19' 12" E, 12 August 1991; GW18806, G315374: Australia, Queensland, Stevens Reef, GBR, back reef, 20° 32' 34" S, 150° 6' 26" E, 7 June 1999, 30m depth; GW19027, G322668: Solomon Islands, Rendova Island Tetepare, Fringing reef, inshore slope northwest side, 8° 42' 04" S, 157° 28' 14" E, 6 July 2009, 20-50m depth; GW19041, G322710: Solomon Islands, Rendova Island Tetepare, Fringing reef, inshore slope northwest side, 8° 42' 04" S, 157° 28' 14" E, 6 July 2009, 40-50m depth; GW19052, G322696: Solomon Islands, Vangunu Island, Barrier reef, external slope of slope facing northwest vertical wall, richly covered with various organisms, 8° 40' 19" S, 157° 50' 15" E, 5 July 2009; GW2037: Indonesia, West Java, Thousand Island, JAK01- Pulau Air, 5° 45' 35" S, 106° 34' 44" E, 26 July 2011, coll. N.J de Voogd; GW2112: Indonesia, West Java, Thousand Island, JAK03 - Semak Daun NW, 5° 43' 40" S, 106° 33' 57" E, 26 July 2011, 16m depth, coll. N.J de Voogd; GW2113: Indonesia, West Java, Thousand Island, JAK03 - Semak Daun NW, 5° 43' 40" S, 106° 33' 57" E, 26 July 2011, 16m depth, coll. N.J de Voogd; GW18758, QM G315299: Australia, Queensland, Edgell Reef, back reef, 20° 8' 53" S, 149° 55' 09" E, 6 June 1999, 18m depth; GW4870, ZMA POR16482: Japan, Ryukyu Islands, Saki-shima Islands, Hatoma Island, coral reef, 24° 26' 60" N, 123° 49' 60" E, coll. K. Watanabe; GW4782: Indonesia, N Sulawesi, Lembeh, W Sarena Kecil, 1° 27' 25.5234"N, 125° 13' 31.1874"S, 17 Feb 2012, coll. N.J de Voogd; GW4783: Indonesia, N Sulawesi, Lembeh, Tanjung Nanas I, 1° 27' 40.212"N, 125° 13' 36.408"S, 3 Feb 2012, coll. N.J de Voogd; GW4784: Indonesia, N Sulawesi, Lembeh, Tanjung Kelapasatu, 1° 25' 38.568"N, 125° 11' 0.7794"S, 15 Feb 2012, coll. N.J de Voogd; GW4785: Indonesia, N Sulawesi, Lembeh, N Tanjung Pandean, 1° 24' 21.7074"N, 125° 10' 4.5114"S, 14 Feb 2012, coll. N.J de Voogd; GW4788: Indonesia, N Sulawesi, Lembeh, S Pulau Dua, 1° 23' 17.016"N, 125° 12' 43.1274"S, 13 Feb 2012, coll. N.J de Voogd; GW4811, ZMA POR16473: Palau, Koror Island, Abe's Traverse, smooth; GW4828, ZMA POR21753: Philippines, Calamian Group: Busuanga Island; GW4829, ZMA POR21752: Philippines, Calamian Group: Busuanga Island; GW4843, ZMA POR18793: Thailand, Laem Tum-Pung, South of Ko Kram, Sattahip, Chonburi, rock, 25 February 2007, coll. Sumaitt Putchakarn; GW4845, ZMA POR18754: Thailand, South of Ko Mark, Chang Islands, Trad, rock, 11° 47' 10" N, 102° 29' 14" E, 8 August 2012, coll. Sumaitt Putchakarn; GW4849, ZMA POR18737: Thailand, West side of Ko Klum, Chang Islands, Trad, rock, 11° 55' 02" N, 102° 21' 43" E, 8 August 2012, coll. Sumaitt Putchakarn; GW4866, ZMA POR17251: Mauritius, 25 June 2006, identified by D. Marie; GW4867, ZMA POR17229: Indonesia, South Sulawesi, Makassar, 8 May 2012, identified by R.A. Edrada; GW7173: Indonesia, South Sulawesi, Barangbaringan, 5° 2' 59.60"S, 119° 25' 12"E, 5 Aug 2012; GW7174: Indonesia, South Sulawesi, Barangbaringan, 5° 2' 59.60"S, 119° 25' 12"E, 5 Aug 2012; GW7175: Indonesia, South Sulawesi, Lankai, 5° 1' 44.7"S, 119° 5' 8.8" E, 8 Aug 2012.

The length of oxeas are overlapping among the type specimens of *N. chaliniformis, N. exigua, X. pacifica* and most of the additional analysed material. Conversely, the oxeas from the type of *N. pandora* and specimens identified as *N. exigua* from the Great Barrier Reef,

Australia are much thinner (Table 4.1). Further statistical tests based on the 20 collected samples from six localities (Thailand, West Java, North Sulawesi, Southeast Sulawesi, Great Barrier Reef and Solomon Islands) revealed that there was a significant difference of spicules length (0.005, $p < 0.05$) and width (0.001, $p < 0.05$) from every locality.

The spicule arrangement of *Neopetrosia chaliniformis*, *N. exigua*, and *Xestospongia pacifica* types is similar (Figure 4.3A, B, C). They share a high degree of dense similarity and possess an isodictyal tangential skeleton. On the other hand, the type specimen of *N. pandora* exhibited a less dense spicular network and more irregular spicule arrangements despite of its similarity to an isodictyal tangential skeleton (Figure 4.3D).

Table 4.1. *Neopetrosia* spp. examined in this study (X=could not be amplified its DNA, *= two sequences type).

Voucher Numbers	Museum code	Length (µm)	Width (µm)	Localities	Cox2 Genbank accession numbers	28S rRNA Genbank accession numbers
GW7107	ZMB2889 (Holotype *N. chaliniformis*)	100–**140.8**–165	7.5–**9.9**–12.5	North Sulawesi, Indonesia	KM030103	X
GW7185	BMNH1898.12.20.49 (Holotype *N. exigua*)	70–**113.8**–130	5–**7.3**–10	Christmas Island, Australia	KM030104	X
GW4805	Z4999 (Holotype *N. pacifica*)	100–**149.6**–175	5–**9.2**–10	Bootless Bay Papua New Guinea	KM030105	KM030128
GW4806	USNM23046 (Holotype *N. pandora*)	100–**119.8**–150	2.5–**2.9**–5	East Ponape, Palau	KM030106	KM030129
GW4843		110–**136.8**–155	5–**6.1**–7.5	Southeast Thailand	KM030113	KM030137
GW4845		100–**134.4**–155	5–**6.3**–7.5		KM030114	KM030138
GW4849		100–**137.2**–160	5–**7.6**–10		KM030115	KM030139
GW2037		100–**138.8**–170	5–**7.4**–7.5	Thousand Island, West Java Indonesia	KM030116	KM030140 KM030141*
GW2112		110–**151.8**–170	5–**7.3**–10		KM030117	KM030142
GW2113		100–**140.2**–175	5–**7.3**–7.5		KM030118	KM030143 KM030144*
GW4782		110–**141**–160	5 –**8**– 10	Lembeh, North Sulawesi	KM030095	KM030120
GW4783		120–**146.4**–175	7.5–**7.8**–10		KM030096	KM030121
GW4784		105–**148**–175	5–7.**6**–10		KM030097	KM030122
GW4785		100–**144.2**–175	7.5–**8.2**–10		KM030098	KM030123
GW4788		110–**141.6**–155	5–**5.4**–7.5		KM030099	KM030124

Voucher Numbers	Museum code	Length (µm)	Width (µm)	Localities	Cox2 Genbank accession numbers	28S rRNA Genbank accession numbers
GW7173		125–**151.8**–175	5–**7.3**–10	Makassar, South Sulawesi	KM030100	KM030125
GW7174		100–**134.8**–175	5–**7.5**–10		KM030101	KM030126
GW7175		80–**141.6**–155	5–**5.4**–7.5		KM030102	KM030127
GW18793	G315226	95–**112.8**–130	2.5–**4**.9–5	GBR, Australia	KM030107	KM030130
GW18758	G315299	80–**119.8**–150	2.5–**4.9**–5		KM030108	KM030131 KM030132 *
GW18806	G315374	100–**128.6**–150	2.5–**4**.5–5		KM030109	KM030133
GW19027	G322668	100–**137.4**–150	5–**5.6**–7.5	Solomon Islands	KM030110	KM030134
GW19052	G322696	100–**129.8**–150	2.5–**5**–7.5		KM030111	KM030135
GW19041	G322710	100–**133.8**–150	2.5–**3.8**–5		KM030112	KM030136
GW18429	G303302	100–**135.4**–155	5–**5.8**–7.5	Northern Territory, Australia		
GW18478	G313113	105–**143**–185	5–**6.5**–7.5	Pulau Tembakul Singapore		
GW18491	G313297	105–**126.4**–150	5–**6.4**–7.5	Vaipuaa, Tonga		
GW18598	G306321	74–**104**–130	5–**6**–7.5	Ongiangiang, Palau		
GW18777	G311804	105–**128.6**–155	5–**7.4**–7.5	Motupure Island Papua New Guinea		
GW18804	G312397	105–**123.4**–150	5–**5.5**–7.5	Western Australia		
GW4870	ZMA POR16482	100–**113.8**–170	5–**5.2**–7.5	Ryukyu Island, Japan		
GW4811	ZMA POR16473	100–**121.8**–145	2.5–**4.9**–5	Koror Islands, Palau		
GW4828	ZMA POR21753	100–**143**–175	5–**5.38**–7.5	Busuanga Island, the Philippines		
GW4829	ZMA POR21752	105–**145.4**–175	5–**6.1**–7.5	Busuanga Island, the Philippines		
GW4866	ZMA POR17251	115–**158.8**–190	7.5–**8.1**–10	Mauritius		
GW4867	ZMA POR17229	100–**132.2**–165	5–**5.7**–7.5	Makassar, South Sulawesi		

Figure 4.1. A *Neopetrosia chaliniformis* (Thiele, 1899) ZMB2889, **B** *Neopetrosia exigua* (Kirkpatrick, 1901), BMNH1898.12.20.49, **C** *Xestospongia pacifica* Kelly-Borges & Bergquist, Z4999, and **D** *Neopetrosia pandora* de Laubenfels, 1954, USNM4806 type specimens. Scale bar= 1 cm

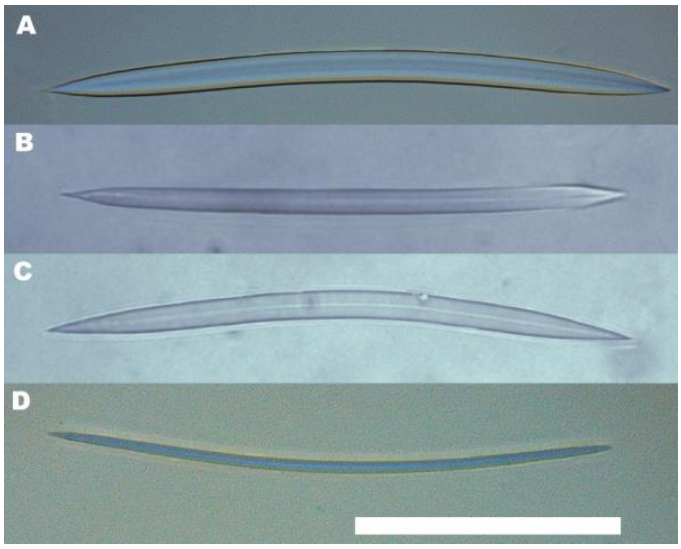


Figure 4.2. Oxea of **A** *Neopetrosia chaliniformis* (Thiele, 1899) ZMB2889, **B** *Neopetrosia exigua* (Kirkpatrick, 1901), BMNH1898.12.20.49, **C** *Xestospongia pacifica* Kelly-Borges & Bergquist, Z4999, and **D** *Neopetrosia pandora* de Laubenfels, 1954, USNM4806 type specimens. Scale bar= 50 µm

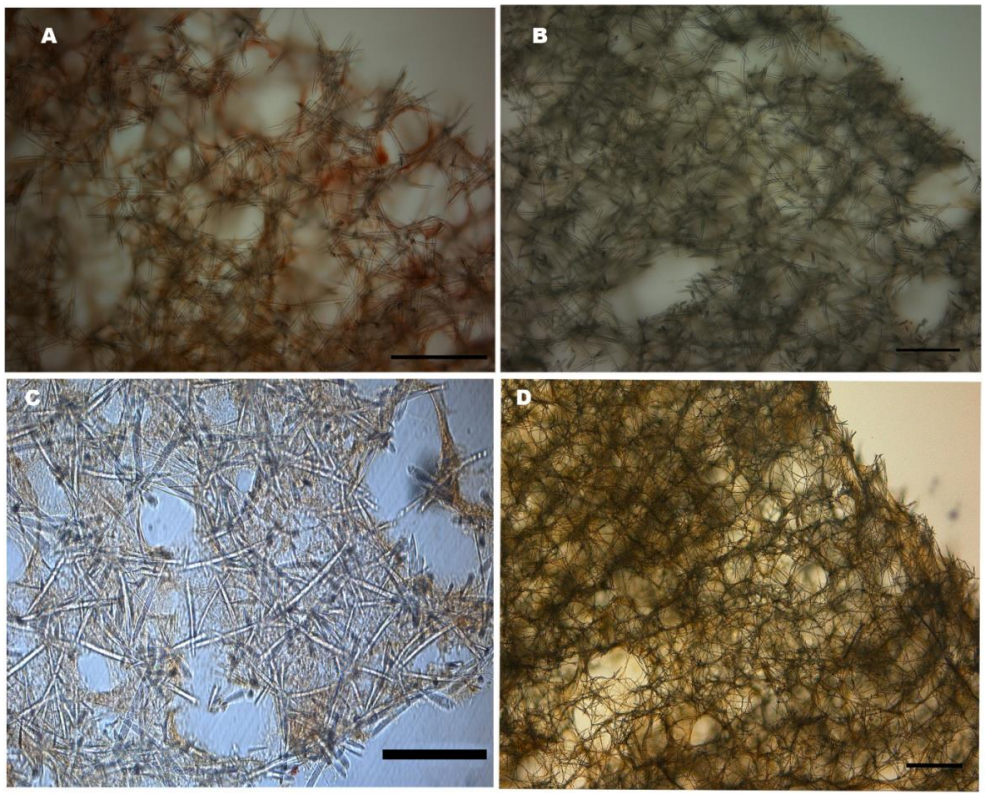


Figure 4.3. Spicule arrangement of **A** *Neopetrosia chaliniformis* (Thiele, 1899) ZMB2889, **B** *Neopetrosia exigua* (Kirkpatrick, 1901), BMNH1898.12.20.49, **C** *Xestospongia pacifica* Kelly-Borges & Bergquist, Z4999, and **D** *Neopetrosia pandora* de Laubenfels, 1954, USNM4806 type specimens. Scale bar= 150 µm

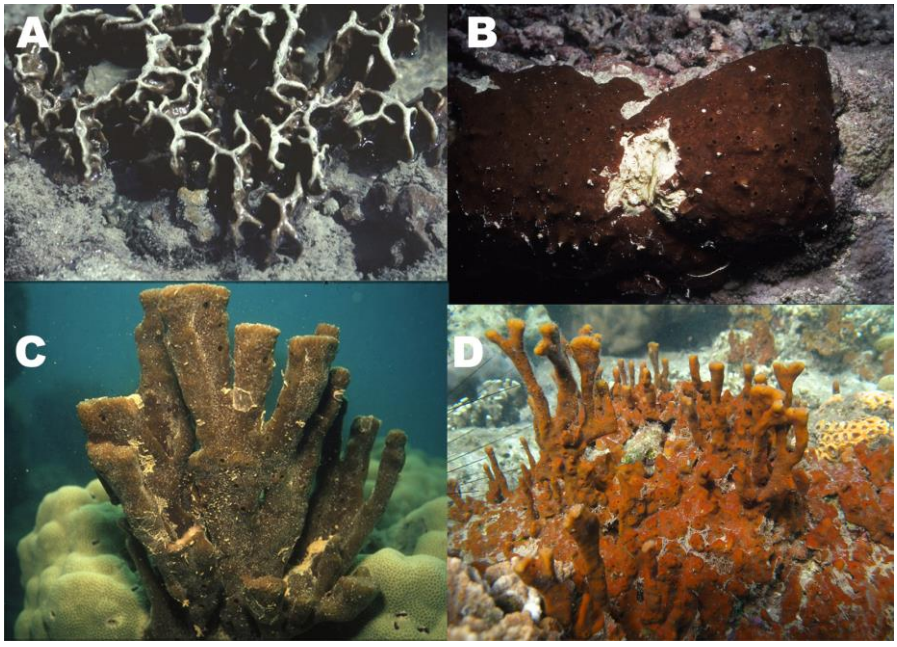


Figure 4.4. In situ photo of sponges identified as *N. exigua*, that were collected in (A) Northern Territory Australia, G303302, (B) Palau, G306321, (C) Tonga, G313297 and (D) a sponge identified as *N. chaliniformis* that was photographed in Probolinggo, East Java, Indonesia.

4.3.2. Molecular phylogenetic analyses

Only the 28S rRNA sequences from two type specimens (*N. pandora* and *X. pacifica*) could be generated. However, the cox2 mtDNA fragments were successfully amplified in all of the type specimens (see Table 4.1). The cox2 alignment length with all taxa included was 350 base pairs with 129 variable sites.

The sequences number in the 28S rRNA phylogenetic tree increased because three individuals (G315229, GW2037, and GW2113) consist of two sequence types. In order to resolve the sequence type ambiguities, SeqPHASE (Flot 2010) were implemented. The alignment length for the 28S sequences was 606 characters. It comprises only 69 % alignable characters from the two holotypes, collected specimens, and outgroups.

The resulting trees are shown in Figures 4.8 and 4.9. The cox2 holotype sequences from *N chaliniformis*, *N. exigua*, and *X. pacifica* were identical in terms of cox2 sequences. This sequence type is shared with several other samples collected in the field, which is corroborated in the 28S rRNA analyses. The *N. pandora* holotype is different and clusters distant with both markers.

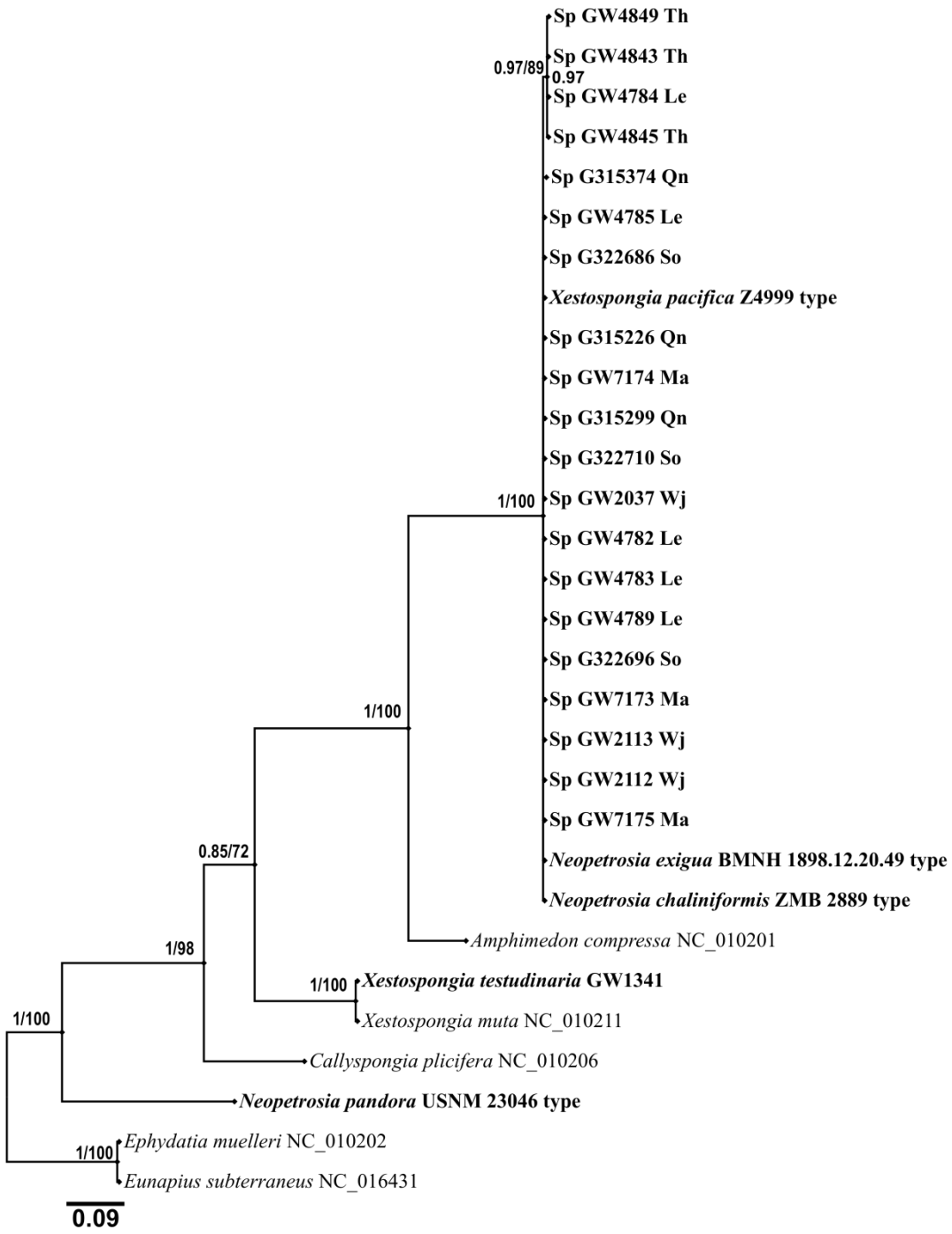


Figure 4.8. Bayesian cox2 phylogram of *Neopetrosia* spp.. Numbers on the branches represent posterior probabilities (PP) / bootstrap proportions (BP) of maximum likelihood analyses. Scale bar indicates the number of substitutions per site. Bold is sequences, which are obtained from this study. Wj= Pulau Seribu, West Java, Indonesia, Indonesia Le= Lembeh, North Sulawesi, Indonesia, Ma = Makassar, South Sulawesi, Indonesia Th= Southeast Thailand, Qn= The Great Barrier Reef, Queensland, Australia, So= Solomon Islands

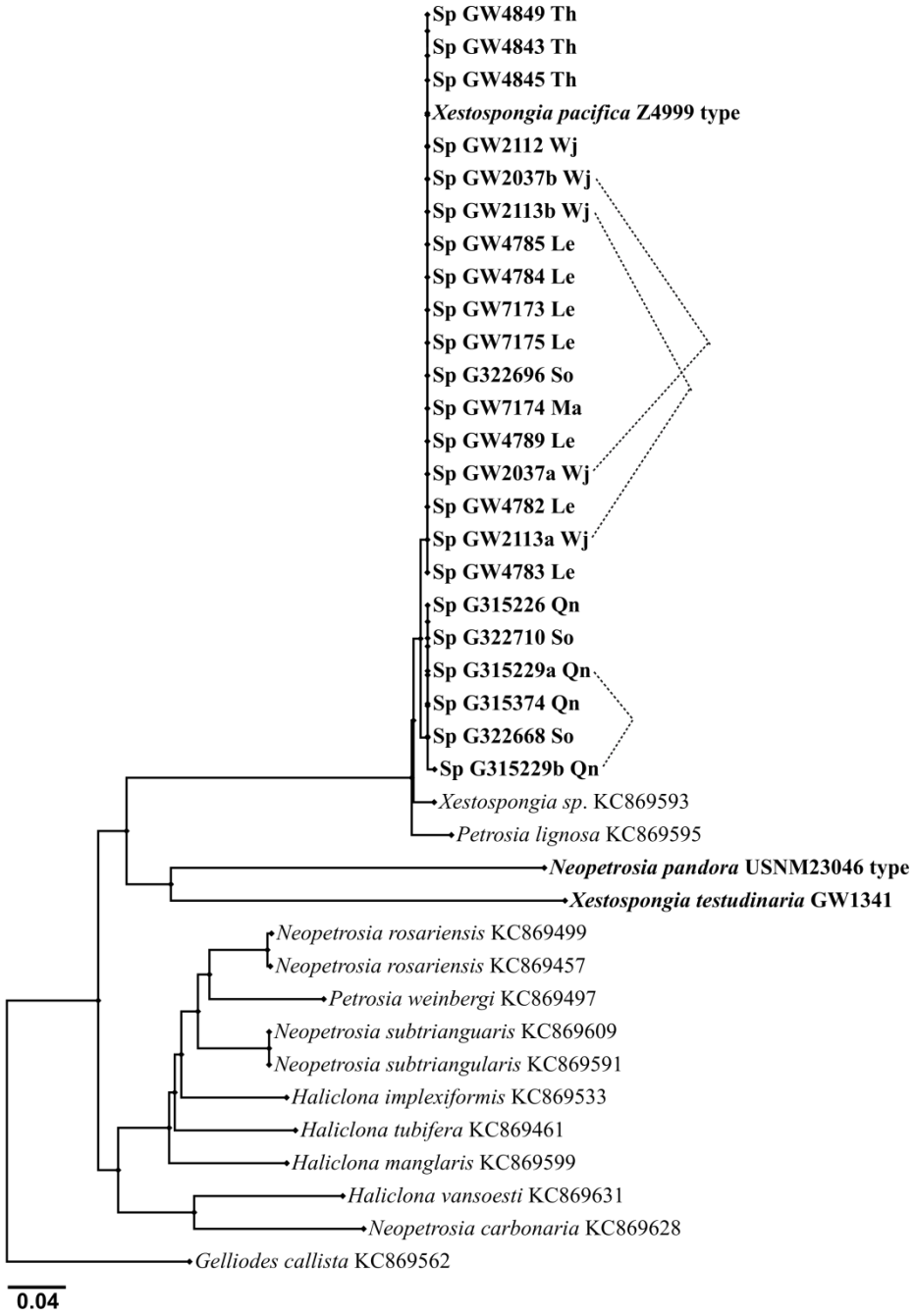


Figure 4.9. Neighbour Joining phylogram of 28S rRNA sequences from *Neopetrosia* spp. The scale bar indicates the number of substitutions per site and dashed lines indicate connectivity between two sequence types from one individual. Bold are sequences, which are obtained from this study. Wj= Pulau Seribu, West Java, Indonesia, Le= Lembeh, North Sulawesi, Indonesia, Ma = Makassar, South Sulawesi, Indonesia Th= Southeast Thailand, Qn= The Great Barrier Reef, Queensland, Australia, So= Solomon Islands

4.4. Discussion

The morphology is important for distinguishing sponges species within the family Petrosiidae. In general, all holotypes and collected samples have an overall encrusting appearance and occasionally possessing branches. Likewise, all of those specimens possess a dark until light brown colouration, a sticky structure, and a brittle and compressible consistency that refer to the field characteristics of *Neopetrosia* spp. We conclude that *N. chaliniformis* and *N. exigua* are the same species based on both morphological and genetic analyses. The former species name was described only one year before *N. exigua* (Thiele 1899; Kirkpatrick 1900; see World Porifera Database, van Soest et al. 2014). Thus, based on the principle of priority of the code in ICZN, *N. chaliniformis* is the preferred valid name and *N. exigua* is considered as a junior synonym of *N. chaliniformis.* In addition, the type material is well recognizable and conforms to recent freshly collected material of this species, whereas the material of *N. exigua* is only a small fragment of a crust.

The difference between spicule widths of the *N. pandora* type specimen and collected samples from the GBR localities might be affected by silica influxes as recognized for other demosponges (e.g., Stone 1970; Bavestrello et al. 1993; Maldonado et al. 1999; Uriz et al. 2003). According to the morphological and molecular analyses, only *N. pandora* should be raised as a valid species since it has a distinctive skeleton and sequence types in comparison to the other type specimens and collected samples. Conversely, the GBR samples, which possess a similar skeleton structure and sequence types (partial cox2 mtDNA and 28S rRNA genes) to the other type specimens of *N. chaliniformis*, *N. exigua*, and *N. pacifica* could not be defined and raised as a new species. This finding also contradicts previous studies, which placed *N. pandora* and *X. pacifica* as the junior synonyms of *N. exigua* due to similarity of morphological characters, particularly spicules, see World Porifera Database (van Soest et al. 2014).

It is difficult to identify haplosclerid sponges, especially to distinguish *Neopetrosia chaliniformis* and *N. exigua* in this study, only based on spicule dimensions and outer morphology. Misidentifications are frequent. Many descriptions merely with drawings do frequently not suffice for the identification of species, and this may lead to unintentional descriptions of new species. In addition, English descriptions (for *N. exigua*) are more accessible than descriptions in German (for *N. chaliniformis*) and therefore, Thiele's material was apparently overlooked.

Growth forms are frequently regarded to be static (only encrusting or only branching

species). In situ observations helped to discover that encrusting species (assumed as *N. exigua*) forms branches under certain circumstances (cf. *N. chaliniformis*). This species is most of the times encrusting, but in favorable conditions (see figure 4.4 A-D) it can grow branches.

Identifying and recognizing the different haplosclerids in the field is less problematic than the identification of small, preserved museum samples and the link to existing species based on incomplete and old descriptions like *N. chaliniformis* and *N. exigua*. Molecular markers are indispensible for this purpose and also in this case facilitated the identification of a common species. The use of correct taxonomic names should be sought consistently in all aspects of organismal research.

Further attempts are required for obtaining DNA sequences of the *N. chaliniformis* and *N. exigua* holotypes. This will contribute to "the bottom up strategy" (see review in Cárdenas et al. 2012) in the phylogenetic study of haplosclerids since the genus *Neopetrosia* is still recovered as a polyphyletic group (Redmond et al. 2011; Redmond et al. 2013; Thacker et al. 2013). The bottom up strategy helps taxonomist on the revision or "re-evaluation (as the best alternative) of morphological characters under the light of molecular results" (studying type species of each haplosclerid sponges genus first) to resolve the discrepancies between current morphology and phylogeny in haplosclerids.

4.5. Conclusion

Here I conclude that the true identity of the common shallow water Indo-Pacific sponge should be *Neopetrosia chaliniformis*. *Neopetrosia exigua* and *Xestospongia pacifia* should be considered as junior synonyms of *N. chaliniformis*. Conversely, *Neopetrosia pandora* should not be assigned as a junior synonym of *N. exigua* and should be erected as a valid species based on the morphological and molecular differences.

CHAPTER 5

The suitability of the Lysidyl Aminoacyl Transfer RNA Synthetase (LTRS intron) for resolving phylogeographic relationships of the smooth-brown sponge, *Neopetrosia chaliniformis* (Porifera; Haplosclerida)

5.1. Introduction

The name "smooth-brown sponge" refers to the Indo-Pacific sponge species *Neopetrosia exigua* (Haplosclerida: Petrosida) (Lim et al. 2008). *Neopetrosia exigua* is distributed abundantly in the Indonesian archipelago (van Soest 1989; de Voogd and Cleary 2008). Similarly, bioactive compounds of *N. exigua* intensively been explored (e.g., Orabi et al. 2002; de Almeida Leone et al. 2008; Abdillah et al. 2013a; Abdillah et al. 2013b). Nevertheless, Fromont (1991) reminds that other sponge species are morphologically similar to *N. exigua* and difficult to distinguish. One of such sponge species that morphologically resemble *N. exigua* is *Neopetrosia chaliniformis* (Thiele, 1899). After examination of type specimens, both species have been shown to be identical and *N. chaliniformis* is proposed as the only valid species with *N. exigua* as its junior synonym (see chapter 4 of this thesis). Studies on the genetic diversity of *N. chaliniformis* need to be conducted in order to understand its genetic connectivity and phylogeographical relationships.

Assessments of the genetic diversity are also important for “closing real gaps in knowledge concerning organismal behaviours, natural histories and current and past population demographic factors that in turn can be highly relevant to conservation efforts” (Avise 1998). At the same time, phylogeographic studies on sponges, which usually deal with assessments of genetic diversity from different localities, have become problematic due to their strong morphological plasticity (Bell and Barnes 2000; Hill and Hill 2002) in combination with conservation of mitochondrial DNA (mtDNA) genes in most sponge classes (Shearer et al. 2002; Huang et al. 2008). Thus, selecting a suitable molecular marker for resolving sponge intraspecies relationship have become an essential and a crucial matter.

Introns are examples of molecular markers that consist of non-coding regions of genes from the nuclear DNA. Currently, introns are the most widely used marker for the purpose of intraspecies studies due to the possession of a higher substitution rate in comparison to exons. The central challenges in utilising introns are how to find regions that are variable enough and highly informative, with flanking exon regions conserved enough to facilitate primer binding

and amplification of the intron target regions (see review in Zhang and Hewitt 2003; Thomson et al. 2010). Therefore, PCR with the combination of exon flanking regions as binding sites for the primers and a sufficiently variable intron, represent a method of choice and is described as EPIC (Exon-Primed, Intron-Crossing) strategy (Palumbi and Baker 1994).

In sponges, the intron of the Adenosine Triphosphate Synthase β subunit (ATPS-β intron, see Jarman et al. 2002) has successfully been utilised for the detection of geographical breaks in two species of calcarean sponges (Bentlage and Wörheide 2007; Wörheide et al. 2008). Likeweise it has been used for species complex detections in *Hexadella* spp. (Reveillaud et al. 2010) and *Xestospongia testudinaria* (Swierts et al. 2013).

This study aims to test the intron markers suggested by Jarman et al. (2002) for their suitability in the intraspecies study in sponges, particularly *N. chaliniformis* as hitherto only the ATPS-β intron is utilised among poriferans (see review in Uriz and Turon 2012). Additionally, I would like to compare the intron suitability with some mtDNA markers that have already been tested and suggested for intraspecies studies of sponges in the past, e.g., ATP synthase 6 [ATP6], cytochrome oxidase 2 [cox2], the spacer between ATP6 and cox2 [ATP6-cox2] and sthe pacer between the NADH dehydrogenase subunit 5 and the small ribosomal RNA subunit [ND5-rns] (Rua et al. 2011). For this reason, I assessed and compared substitution rates, genetic structure among selected *N. chaliniformis* populations, and phylogeographical patterns. I hypothesised that the intron markers will display a higher substitution rates in comparison to the polymorphic mtDNA markers and therefore, a better displays the genetic structure of selected *N. chaliniformis* populations.

5.2. Materials and Methods

5.2.1. Sample collections

Twenty-eight specimens of *N. chaliniformis* were collected by SCUBA diving in depths of ranging from < 10 to 30m in the localities of West Java, North Sulawesi and South East Sulawesi. Directly after being collected, samples were cut, rinsed and soaked in 99% ethanol before preserved within 99% ethanol. Other 27 samples from localities of Thailand, The Philippines, Japan, Mauritius and Singapore were provided by the Naturalis Biodiversity Centre, Leiden, The Netherlands. In addition, the Queensland Museum Brisbane, Australia provided samples from other localities in the Australasia region (e.g., Queensland, Solomon Islands, Papua New Guinea, Palau, and Vanuatu), see Supplementary Material 5.1.

5.2.2. DNA extraction, amplification, and sequencing

The previously published method for DNA extraction, established for sponge barcoding has been used for extracting the samples (Vargas et al. 2012). Polymerase Chain Reaction (PCR) was performed with an annealing temperature gradient ranging from 40-60^{0}C to find the optimal annealing temperatures for the selected mitochondrial (ATP6, cox2, ATP6-cox2, and ND5-rns, Rua et al. 2011) and intron (ATPα, ATP β, ANT, SRP54, LTRS, TBP, and ZMP, Jarman et al. 2002) markers. The 25 µL PCR mix consisted of 5 µL 5x green Go*Taq*® PCR Buffer (Promega Corp, Madison, WI), 4µL 25mM $MgCl_2$ (Promega Corp, Madison, WI), 2 µL 10mM dNTPs, 1 µL each primer (5µM), 9.8 µL water, 2 µL DNA template and 0.2 µL Go*Taq*® DNA polymerase (5u/µl) (Promega Corp, Madison, WI). The PCR regime comprised an initial denaturation at 94° C for 3 min, 35 cycles of 30 s denaturation at 94° C, 20 s annealing with the gradient temperatures in a range of 40°- 60° C with 2° C different temperature per lane and 60 s elongation at 72° C each, followed by a final elongation at 72° C for 5 min. Detailed PCR conditions including primers, annealing temperatures and amplified genes, are shown in Table 6.1. Only the cox2 from the mitochondrial markers and LTRS from the intron markers could be amplified from *N. chaliniformis* despite tests with varying $MgCl_2$ concentration and additives such as Bovine Serum Albumin (2 µL of10 mg/ml).

Specific primer design was required for the amplification of the LTRS intron since only DNA templates from five specimens (GW4866, GW2112, GW4843, GW4845, and GW4849) could be amplified with the LTRS intron primers published in Jarman et al 2002. All of the PCR products were cleaned by ammonium acetate precipitation. Sequencing of forward and reverse strands was performed with the ABI BigDye v3.1 (Applied Biosystems, California USA) chemistry and the PCR primers following the manufacturers protocol on an ABI 3730 Automated Sequencer in the Genomic Sequencing Unit of the LMU Munich (http://www.gi.bio.lmu.de/sequencing/). Sequences were assembled, analysed with Geneious version 6.1.7 (available from http://www.geneious.com/) and subsequently trimmed. MUSCLE version 3.5 (Edgar 2004) as implemented in Geneious under default settings was used to align sequences. A BLAST test against GenBank (http://www.ncbi.nlm.nih.gov/) was performed in order to check for contaminations in sequences.

Table 5.1. The primers that are utilised in the experiment

Primer Name	Primer Sets	References	Intron of	Remarks
ATPSαf	5'-GAGCCMATGCAGACTGGTATTAAGGCYGT-3'	Jarman et al. 2002	ATPSα	X
ATPSαr1	5'-TTGAANCKCTTCTGGTTGATGATGGTGTC-3'			
ATPSβf1	5'-CGTGAGGGHAAYGATTTHTACCATGAGATGAT-3'		ATPSβ	X
ATPSβr1	5'-CGGGCACGGGCRCCDGGNGGTTCGTTCAT-3'			
ANTf1	5'-TGCTTCGTNTACCCVCTKGACTTTGC-3'		ADP-ATP	X
ANTr1	5'-CCAGACTGCATCATCATKCGRCGDC-3'		Translocase	
SRP54f1	5'-ATGGTGAYATYGAAGGACTGATWGATAAAGTCAA-3'		SRP54	X
SRP54r1	5'-TTCATGATGTTYTGGAATTGYTCATC TATGTC-3'			
ZMPf1	5'-CATGARRTTGGMCATAAYTTTGGATC-3'		TBP	X
ZMPr1	5'-CCDCTYCTTACRCTRACACCKA-3'			
TBPf1	5'-GCNCGAAATGCHGAGTATAATCC-3'		ZMP	X
TBPr1	5'-TCYTTTATRCGNTCTCAACATGTCTT-3'			
LTRSf1	5'-CAYTTTGGSYTBAARGACAAGGA-3'		LTRS	1
LTRSr1	5'-GCCATGTAGAACTCRCAVGTGGTG-3'			
Ne_LTRS_f	5'- CACTTCCTGGACAACCTCGG-3'	This study	LTRS	2
Ne_LTRS_r	5'- CCTACCTTCATTCCTGAAC-3'			
Primer Name	Primer Sets	References	Gene	Remarks
ATP6porF	5'-GTAGTCCAGGATAATTTAGG-3'	Rua et al. 2011	ATP6	X
ATP6porR	5'-GTTAATAGACAAAATACATAAGCCTG-3'			
CO2F	5'-TTTTTCACGATCAGATTATGTTTA-3'		cox2	3
CO2R	5'-ATACTCGCACTGAGTTTGAATAGG-3'			
CO2Fc	5'-TGTKGCGCAAATCATTCWTTTATGC-3'		4	X
ATP6R	5'-TGATCAAAATAWGCTGCTAACAT-3'			
ND5F	5'-GTGTTCAACTATGCTTTAATWATGAT-3'		5	X
rnsR	5'-CGTACTTTCATACATTGYAC-3'			

X. No PCR product

1. Difficult amplification on the most of samples, especially old museum specimens. Annealing temperature: 60° C
2. Easy amplifications and these primer set can be utilised in all of our specimens. Annealing temperature: 53°C.
3. Annealing temperature: 40°C
4. Intergenic region between ATP6 and cox2 (Spacer1)
5. Intergenic region between ND5 and rns (Spacer2)

All sequences are deposited at NCBI GenBank under accession numbers KM030095, KMC030097, KM030109 (haplotypes C1, C2, C3 cox2 mtDNA) and KM030146-KM030171 (haplotypes L1-L26 LTRS intron).

5.2.3. Specific primer design for the LTRS intron and heterozygotic alleles

An annealing temperature of 60° C has been found to be optimal for the amplification of the LTRS intron marker (Table 5.1). The five successfully amplified LTRS sequences were utilised to design specific primers for all other *N. chaliniformis* samples as following: First, the consensus sequence from the five samples was queried in BLAST against GenBank (http://www.ncbi.nlm.nih.gov/). It matched a predicted protein sequence of Lysyl-tRNA Synthetase (Accession Number XP_003383808) from *Amphimedon queenslandica*. This confirmed that the targeted LTRS gene was indeed from sponge origin and not from an associated organism. Second, the intron splicing site was annotated with Geneious to distinguish both exon and intron regions. Exons were recognised by their amino acid translation according to their open reading frame (ORF). According to the general splicing site motifs (Clancy 2008), the intron region of the LTRS gene started with GT in the 5'splice site (the donor site), and possessed a branch site with pyrimidine nucleotides, and AG at the 3'splice site (acceptor site). The partial LTRS gene constituted of 99 bp exon 1, 85 bp intron 1, 192 bp exon 2, 72 bp intron 2, and 28 bp exon 3. Out of this information a pair of *N. chaliniformis* specific primer (Ne_LTRS_f & Ne_LTRS_r) that has more capability to amplify all *N. chaliniformis* specimens was designed. I also tried to amplify the LTRS fragment combinations from my designed and Jarman's primers (LTRSf1- Ne_LTRS_r and Ne_LTRS_f – LTRSr1) under PCR conditions as listed in 5.2.2. Here, a PCR product was difficult to be obtained, especially when I used DNA of museum samples. Finally, SeqPHASE (Flot 2010) was used to determine the haplotype of ambiguous alleles.

5.2.4. Data analyses

The differnt haplotypes, the genetic diversity indices (π), and Tajima's D (Tajima 1989) values were calculated by Dna SP v. 5.10.01 (Librado and Rozas 2009). For genetic structure analysis of *N. chaliniformis*, only four geographic regions possessed a number of more than 5 samples. Therefore, regional samples were pooled and categorised as follows: (1) West Java [n=11], (2) North Sulawesi [n=7], (3) South Sulawesi [n=8], and (4) Queensland

[n=8]. An analysis of molecular variance (AMOVA) and the calculation of pairwise F_{ST} values were performed in Arlequin v 3.5.1.2 (Excoffier et al. 2005) with a permutation test under 10,000 replicates. The significance of F_{ST} values was amended following a Bonferroni correction (Rice 1989).

The phylogeographic patterns were analysed by reconstruction of phylograms. These were performed under Maximum-likelihood (ML) and Bayesian inferences. The ML phylogram was generated by RAxML v. 7.0.4 in raxmlGUI v. 1.3 (Silvestro and Michalak 2012) with a rapid bootstrap of 1,000 replications (Stamatakis et al. 2008). Conversely, the Bayesian phylogram was generated by MrBayes v. 3.2.1 (Ronquist et al. 2012) under the ML model of evolution. Each analysis consisted of two independent runs of four Metropolis-coupled Markov-chains under default temperatures with trees sampled at every 1000th generations. Analyses were terminated automatically when the chains converged significantly as indicated by an average standard deviation of split frequencies <0.01. The F81 model for the cox2 and SYM + I for the LTRS intron were suggested by the hierarchical likelihood ratio test as implemented in jModeltest v. 2.1.3 (Darriba et al. 2012) under the Akaike Information Criterion (Akaike 1974). As SYM + I and F81 models are not implemented in the RAxML, ML analyses under the GTR model equivalents were applied respectively (see Stamatakis 2008).

5.3. Results and Discussions

5.3.1. The LTRS intron and its polymorphism

In comparison to the fragment amplified by the universal LTRS intron primers from Jarman et al. (2002) my specific primers only prime on the first and second exons and therefore amplify a fragment 210 bp shorter in length. This resulted in a 266 bp fragment that constituted of a 3 bp exon 1, 85 bp intron 1, and a 178 bp exon 2 (see details in Figure 5.1). After analyses with SeqPHASE, six samples (GW4782, GW4783, GW7181, GW7183, G313297 and G312397) were excluded due to their PHASE values (<0.900). Alleles with values of less than 0.900 should be excluded from the analyses because their haplotypes cannot not be unambiguously distinguished (Flot 2010). Therefore, in total 71 sequences out of 49 samples were yielded (see Supplementary Material 5.1 and Figure 5.2 for the origin of localities).

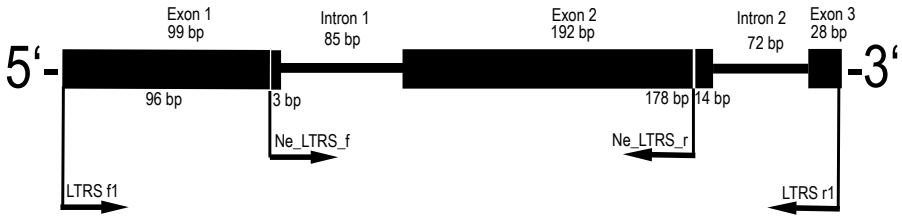


Figure 5.1. Primer positions and intron splicing site of the LTRS gene amplified by the universal LTRS intron primers from Jarman et al. 2002 (LTRS f1 and LTRS r1) and the newly designed specific LTRS intron primers for *Neopetrosia chaliniformis* (Ne_LTRS_f and Ne_LTRS_r)

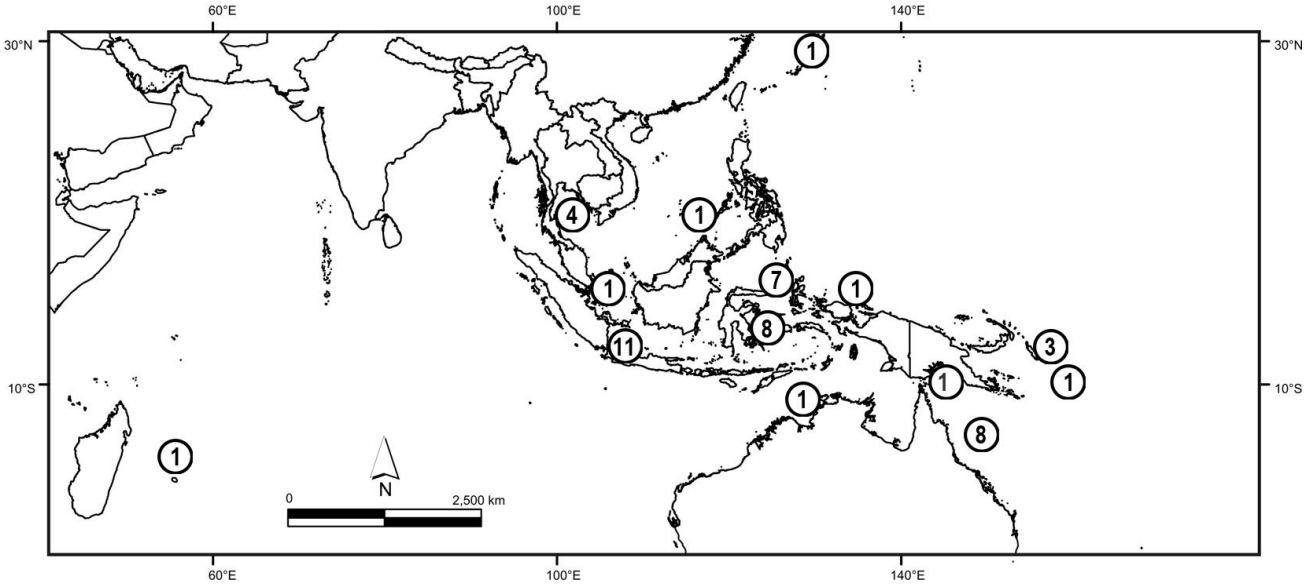


Figure 5.2. Localities and number of *Neopetrosia chaliniformis* samples used in this study.

Twenty-three variable sites were observed within the fragment of the LTRS gene. Both exon and intron parts possessed an uncorrected *p*-distance of 8.65% (π=0.01922). The neutral evolution of the LTRS intron was indicated by Tajima's D value 0.22873 ($P > 0.10$). Seventy-one sequences were collapsed into 26 unique sequences types by removing identical sequences (see list of recovered haplotypes in Supplementary Material 6.1). Because this is the first application of the LTRS intron in sponges, we could only compare the intron polymorphism to the ATPS-β intron, which is the only intron marker that has already been tested in sponges (see review in Uriz and Turon 2012). The uncorrected *p*-distance of the LTRS intron was higher in comparison to the ATPS-β intron of two *Hexadella* taxa (in a range of 1.3-6.3% Reveillaud et al. 2010). Furthermore, the uncorrected *p*-distance of the LTRS intron was in the same range as the ATPS-β intron in calcarean sponges, i.e. *Pericharax heteroraphis* (8.3%; Bentlage and Wörheide 2007) and *Leucetta chagosensis*

(9.57%, π=0.03524; Wörheide et al. 2008).

5.3.2. Phylogeographical relationships among *N. chaliniformis* as inferred by LTRS intron sequences.

The F_{ST} value from the AMOVA test indicated the presence of genetic structuring among the selected populations (West Java, North Sulawesi, South Sulawesi and Queensland: 0.18102, $P < 0.05$ after Bonferroni correction). A spatial analysis showed that the Queensland population was strongly (F_{ST} between 0.20718-0.26142, $P < 0.05$ after Bonferroni correction) and significantly different from West Java, North Sulawesi and South Sulawesi. Genetic structuring was absent between populations of North and South Sulawesi (see Table 6.2).

Table 5.2. Pairwise F_{ST} values between populations of *N. chaliniformis* (LTRS intron/ cox2 mtDNA). Significant values at $P < 0.005$ after Bonferroni corrections are indicated by asterisks.

Population	West Java	North Sulawesi	South Sulawesi	Queensland
West Java N=11	0.00000			
North Sulawesi N=7	0.16284*/ 0.06855	0.00000		
South Sulawesi N=8	0.12528*/ 0.04199	0.05938/ -0.15305	0.00000	
Queensland N=8	0.26142*/ 0.19757	0.26364*/ -0.00380	0.20718*/ 0.01299	0.00000

From the LTRS intron phylogenetic analyses, only two monophyletic groups could be identified in accordance to their geographic locations. These are defined as Groups A, and B and allow for the resolution of a phylogeographic break of *N. chaliniformis*. The group of sequences from Queensland, which are in the phylogenetic trees (Figure 5.3) is Group A, Solomon Islands & Papua New Guinea comprise Group B. The LTRS intron could not fully resolve phylogeographic breaks of the remaining specimens (Group C), which here comprises sequences of various localities in the Indonesian Archipelago (West Java, North Sulawesi, South Sulawesi) and Thailand, including single samples from Mauritius, Japan, The Philippines, Singapore, Northern Territory, Palau and Vanuatu. Furthermore, this group contained one sample from Queensland as the only sample of that region outside of Group A.

The lack of geographic separation in Group C might be based on two factors. First, the

reduction of sequence length from the LTRS intron fragment amplified by our specific primers reduced the number of informative sites. Second, according to Becking et al. (2013b), long distance dispersal events are occasionally observed in some sponges taxa (e.g., Wörheide et al. 2005; Wörheide et al. 2008; Lopez-Legentil and Pawlik 2009; DeBiasse et al. 2010; Xavier et al. 2010a). This ability of sponges to disperse asexual fragments in currents or to raft on various floating material (Wulff 1995; Maldonado and Uriz 1999) might result in the absence of genetic separation between two isolated localities, as proposed by Wörheide et al. (2008) for *Leucetta chagosensis*. *Neopetrosia chaliniformis* possesses a variable shape of mostly encrusted form and sometimes has branches including a structure like turrets. The consistency of *N. chaliniformis* is compressible and extremely brittle. Such morphological characteristics facilitate the dispersal of asexual parts through water current or some floating materials. The asexual mode of this sponge species facilitates the ability to perform a rapid local colonisation (Fromont and Bergquist 1994). Therefore, the role of asexual reproduction in combination to the sponge dispersal ability by various floating material is the most likely explanation for the absence of phylogeographical signals in Group C.

Despite of the low signal for geographical separation in Group C, the pattern for the other groups resemble findings for calcarean sponges in the Indo Australian Archipelago (IAA). Using the ATPS-β intron on *Pericharax heteroraphis*, Bentlage and Wörheide (2007), recognised a similar phylogeographical break among sequences from the Great Barrier Reef (Queensland, Australia), Vanuatu, and Sulawesi populations (Bentlage and Wörheide 2007), and a similar pattern in *Leucetta chagosensis*, among the Great Barrier Reef, Vanuatu, and Sulawesi, (Wörheide et al. 2008). However, in the LTRS intron phylogram, the sequence from Vanuatu is mixing with sequences from Singapore, Thailand and Sulawesi. In my opinion, further phylogeography investigations are still needed with the use of additional sequences from another intron marker such as ATPS-β, and an enlarged sample set. This will be used to assess either whether different taxa of sponges posses a similar phylogeographical pattern to *N. chaliniformis* or whether the LTRS intron marker is unsuitable to resolve the phylogeographical break among sequences from Vanuatu, Thailand, Sulawesi and Singapore.

5.3.3. DNA polymorphism among cox2 mtDNA sequences in *N. chaliniformis*

Partial cox2 from 55 specimens could be sequenced. For a better comparison, those samples that could not be utilised in the LTRS intron analyses were excluded from the

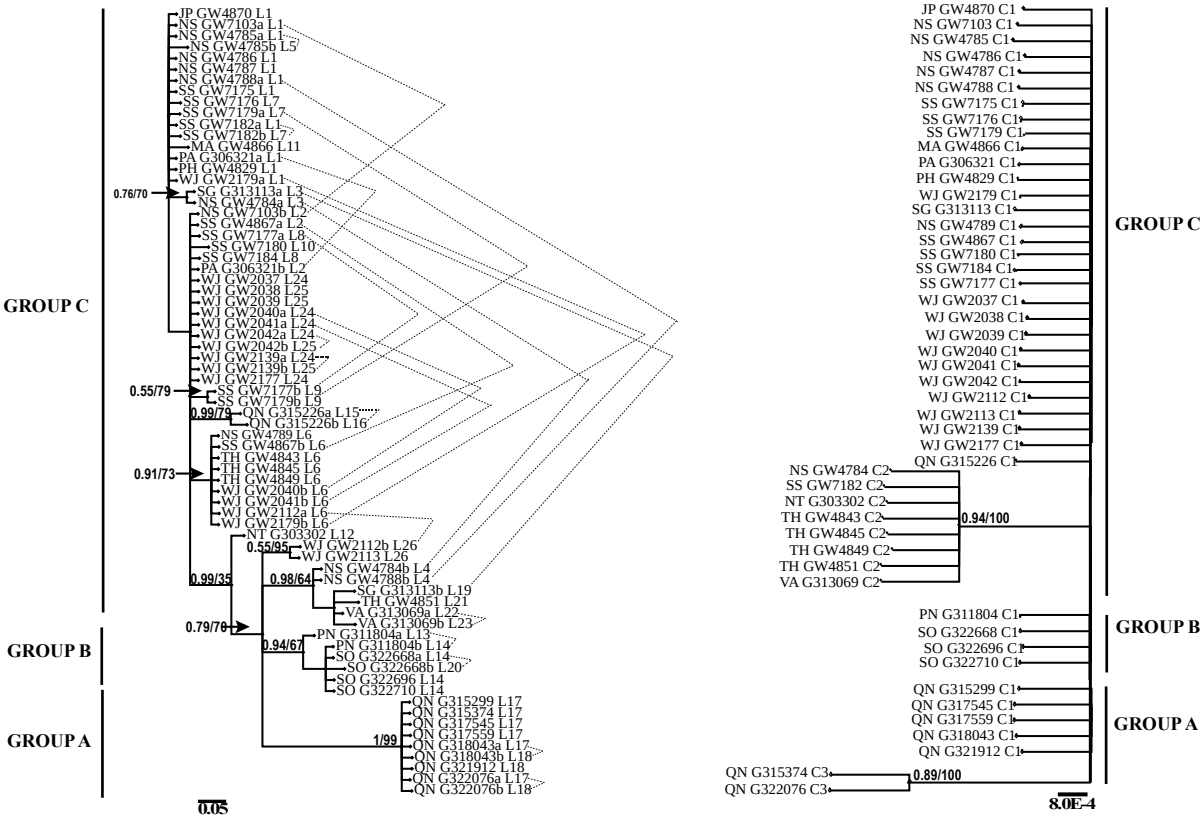
phylogenetic analysis to avoid taxon discrepancy (see Supplementary Material 5.1). The fragments yielded 350 base pairs (bp) with 2 variable sites. Therefore 49 sequences could be collapsed into only three unique haplotypes (see list of recovered haplotypes in Supplementary Material 5.1). The sequences possessed an uncorrected *p*-distance of 0.58% (π=0.00103). The neutral evolution of the cox2 marker was indicated by Tajima's D value -0.35857 ($P > 0.10$). This π value has a higher magnitude in comparison to other haplosclerids, *Amphimedon erina* (π=0.00000) (π=0.00000, Rua et al. 2011). So far, the highest π value of mtDNA cox2 in demosponge sequences is only recovered from the spirophorid *Cinachyrella* sp. (π=0.03, Rua et al. 2011). Due to the possession of very low substitution rates of the cox2 marker in *N. exigua*, only weak phylogeographic signal was detected in this study.

5.3.4. Phylogeographical relationships in *N. chaliniformis* that are detected by the cox2 mtDNA sequences

Cox2 does not contradict the LTRS marker in the resulting phylogenetic tree topologies, which supports the suitability of the LTRS intron as a phylogeographic marker. However, the resolution of the former is very weak. The F_{ST} from AMOVA test (0.03263, $P < 0.05$ after Bonferroni correction) and Pairwise F_{ST} values indicated the absence of genetic structuring among selected populations (West Java, North Sulawesi, South Sulawesi and Queensland, see detail Table 5.2). A very weak signal for a phylogeographical break was only observed in some sequences in Group A and C. Due to the possession of slow evolutionary rates (only two among 350 base pairs polymorphic sites), incomplete lineage sorting is present, which is also evident by the topology of the cox2 mtDNA phylogram different to the LTRS intron phylogram (Figure 5.3).

5.4. Conclusion

Despite the LTRS intron could not fully resolve a geographical break between sequences that originated from various localities in this study, the intron can be used as a potential marker for analysing the phylogeographical relationships of *N. chaliniformis*. Higher substitution rates and a higher genetic structuring on the selected population are observed in comparison to the mtDNA marker tested (cox2). However, some improvements should be made. For example, redesigning intron primers amplifying a longer fragment.



LTRS intron cox2 mtDNA

Figure 5.3. Unrooted phylograms of Bayesian inference from *N. chaliniformis* LTRS intron and cox2 mtDNA sequences. Numbers on the branches represent posterior probabilities (PP) / bootstrap proportions (BP) values. Scale bars indicate the number of substitutions per site, whereas dashed lines indicate connectivity from two sequence types of one individualas detected by PHASE. JP= Japan, MA= Mauritius, NS= North Sulawesi, NT=Northern Territory, PA= Palau, PH= Philippine, PN= Papua New Guinea, QN= Queensland, SG= Singapore, SO= Solomon Islands, SS= South Sulawesi, TH= Thailand, WJ= West Java, VA= Vanuatu. C1- C3: recovered mtDNA cox2 haplotypes; L1- L26: recovered LTRS intron haplotypes

CHAPTER 6

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PLOS ONE

Lock, Stock and Two Different Barrels: Comparing the Genetic Composition of Morphotypes of the Indo-Pacific Sponge *Xestospongia testudinaria*

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Abstract

The giant barrel sponge *Xestospongia testudinaria* is an ecologically important species that is widely distributed across the Indo-Pacific. Little is known, however, about the precise biogeographic distribution and the amount of morphological and genetic variation in this species. Here we provide the first detailed, fine-scaled (<200 km²) study of the morphological and genetic composition of *X. testudinaria* around Lembeh Island, Indonesia. Two mitochondrial (CO1 and ATP6 genes) and one nuclear (ATP synthase β intron) DNA markers were used to assess genetic variation. We identified four distinct morphotypes of *X. testudinaria* around Lembeh Island. These morphotypes were genetically differentiated with both mitochondrial and nuclear markers. Our results indicate that giant barrel sponges around Lembeh Island, which were all morphologically identified as *X. testudinaria*, consist of at least two different lineages that appear to be reproductively isolated. The first lineage is represented by individuals with a digitate surface area, CO1 haplotype C5, and is most abundant around the harbor area of Bitung city. The second lineage is represented by individuals with a predominantly smooth surface area, CO1 haplotype C1 and can be found all around Lembeh Island, though to a lesser extent around the harbor of Bitung city. Our findings of two additional unique genetic lineages suggests the presence of an even broader species complex possibly containing more than two reproductively isolated species. The existence of *X. testudinaria* as a species complex is a surprising result given the size, abundance and conspicuousness of the sponge.

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Introduction

Marine sponges are diverse and structurally important components of coral reefs [1,2,3]. They provide a substrate for numerous organisms, are involved in marine nutrient dynamics, and are a key source of pharmaceutical compounds [4,5,6,7]. Many aspects of their biology, biogeography and genetic structuring across space and time, however, remain unknown [8,9,10]. This is in large part due to a paucity of variable single-copy markers with sufficient resolution to differentiate between sponge taxa and populations compared to other marine organismal groups [8,10].

The broad distribution of many marine organisms, including sponges, has been attributed to the lumping of morphologically similar but often evolutionarily distinct lineages into single species [11,12]. Molecular studies have indicated the presence of multiple genetically differentiated lineages within morphologically identical samples, i.e. cryptic species [13,14,15,16]. Molecular studies of sponges from the Indo-Pacific are scarce [17,18,19], particularly in the Coral ,Triangle. The Coral ,Triangle is known to be region of highest marine biodiversity and consequently represents an important region for conservation and economic management [20,21].

Giant barrel sponges *Xestospongia testudinaria* (Lamarck, 1813) and *Xestospongia bergquistia* (Fromont, 1991) in the

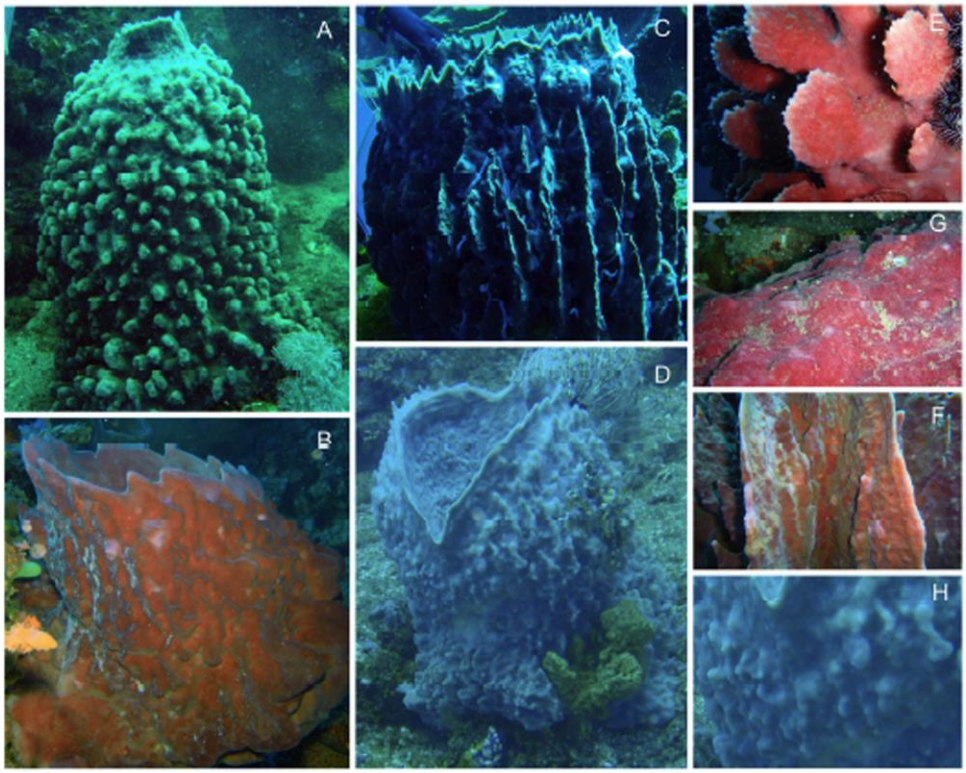

Figure 1. Pictures of identified morphotypes of *Xestospongia testudinaria* around Lembeh Island. a: Digitate; b: Smooth; c: Lamellate; d: Intermediate; e: Digitate close-up; f: Smooth close-up; g: Lamellate close-up; h: Intermediate close-up.
doi: 10.1371/journal.pone.0074396.g001

Indo-Pacific and *Xestospongia muta* (Schmidt, 1870) in the Caribbean, are among the largest known sponges (Demospongiae; Haplosclerida), measuring up to 2.4 meters in height and width. These iconic animals have a large, erect, barrel-shaped appearance and individuals vary in size, shape (Figure 1) and biochemical composition. The external morphology of giant barrel sponges can vary from smooth to highly digitate or lamellate surfaces [22]. They have very long life spans (possibly in excess of 2000 years); *X. muta* has been called the 'redwood of the reef' due to its size, longevity and ecological importance [23]. So far, *X. bergquistia* is only known from the northern Great Barrier Reef where it is sympatric with *X. testudinaria*. In contrast, the known biogeographical distribution of *X. testudinaria* extends from the Red Sea and East Africa to the Great Barrier Reef in Australia and Tonga [2,24]. Its almost ubiquitous presence in coral reefs makes it a good model species to study detailed spatial patterns of morphological and genetic variation in sponges.

The sponges *X. testudinaria* and *X. muta* share many characteristics. Similar morphologies (smooth, digitate, lamellate) have been described for both species [22,25,26] and their mitochondrial Cytochrome Oxidase 1 (CO1) sequences revealed little divergence between both taxa (99-100% identical). Furthermore, both species displayed similar bacterial community compositions [27]. The above mentioned resemblances prevail despite the fact that these taxa must have been separated for at least three million years, since the closing of the Isthmus of Panama [28]. Hence, it has been proposed that the most significant difference between *X. testudinaria* and *X. muta* is the geographic region in which they occur [27].

Strong spatial genetic structuring based on CO1 sequence data was found for samples of *X. muta* in Caribbean reefs. This

structuring was suggested to be related to patterns of ocean currents [26]. While the Caribbean *X. muta* is currently among the most intensively studied sponge species in the world, comparative studies of the Indo-Pacific *X. testudinaria* are lacking. This hampers our understanding of the evolutionary history of giant barrel sponges and specifically, the relationship between *X. testudinaria* and *X. muta*. Moreover, the link between external morphotype and genealogy remains unclear, as suggested elsewhere [26,29].

Here, we provide the first detailed, fine-scaled (<200 km²) study of the morphological and genetic diversity of *X. testudinaria*. Our study took place in the reefs surrounding Lembeh Island (northeast Sulawesi, Indonesia). Our main goal was to test for differences in genetic composition among the morphotypes of *X. testudinaria*. We were particularly interested to assess whether this conspicuous sponge taxon represents a species complex, as opposed to a widespread single species. To achieve our goal, individuals of *X. testudinaria* were sampled around Lembeh Island, identified to morphotype and sequenced at the I3-M11 partition of the mitochondrial CO1 gene [26,30]. In addition to this, we sequenced the mitochondrial adenosine triphosphate synthase subunit 6 (ATP6) gene [31] and the nuclear adenosine triphosphate synthase β (ATPSβ) intron for a subset of individuals [18,19]. Nuclear genetic markers evolve independently from mitochondrial markers. Hence, congruent patterns across marker types, including morphology, mitochondrial, and nuclear genetic markers, would corroborate the existence of reproductively isolated units, i.e. distinct biological species, e.g. [32,33,34].

Materials and Methods

Sampling

Samples of *X. testudinaria* were collected by SCUBA diving from 33 different sites around Lembeh Island, off the northeast coast of Sulawesi, Indonesia from January 30th to February 18th 2012 (Table 1). All collections were done during the International Seminar on Conservation of Marine Biodiversity, UNSRAT (University Sam Ratulangi, Manado) as part of the Marine Biodiversity workshop based at the field station of the Research Centre for Oceanography of the Indonesian Institute of Sciences (in Indonesian, Lembaga Ilmu Pengetahuan Indonesia, or LIPI) in Bitung. The workshop was hosted by the Sam Ratulangi University in cooperation with LIPI. We were allowed to collect samples for our research based on a Memorandum of Understanding between LIPI and the Netherlands Centre for Biodiversity Naturalis in Leiden, The Netherlands. LIPI is the governmental authority for science and research in Indonesia. It consists of 47 research centers in the fields ranging from social to natural sciences. LIPI is the authority to collect and export samples. We operated from out of their Research Station in Bitung in cooperation with the Sam Ratulangi University. This university is also authorized to collect by LIPI.

A long, narrow and sheltered channel with a maximum depth of around 30m in the southern part and 70m in the northern part runs between Lembeh Island and the main island of

Table 1. Specifications of sampled dive sites around Lembeh Island.

Dive Site Number	Dive Site Name	Date	Coordinates	CO1	ATP6	ATPSβ
1	Tanjung Nanas I	30-Jan-12	1°27'40.43"N; 125°13'36.41"E	3	3	1
2	SE Sarena Kecil	30-Jan-12	1°27'15.80"N; 125°13'29.53"E	2	2	1
3	E Sarena Besar	31-Jan-12	1°27'34.16"N; 125°14'01.90"E	1	1	1
4	Tanjung Mawali	31-Jan-12	1°26'36.42"N; 125°13'45.98"E	4	4	2
5	Tanjung Nanas II	01-Feb-12	1°27'43.67"N; 125°13'41.63"E	3	3	0
6	Tanjung Kubur	01-Feb-12	1°28'44.69"N; 125°14'59.14"E	1	1	1
7	Pantai Perigi	02-Feb-12	1°28'10.02"N; 125°14'38.80"E	4	1	1
8	Tanjung Nanas I	03-Feb-12	1°27'40.21"N; 125°13'36.41"E	4	1	1
9	Pulau Abadi	03-Feb-12	1°26'00.74"N; 125°12'22.61"E	4	1	0
10	Tanjung Labuhankompeni	04-Feb-12	1°25'55.85"N; 125°11'10.64"E	6	1	1
11	Kelapadua	04-Feb-12	1°26'08.38"N; 125°12'34.09"E	3	1	0
12	Baturiri	06-Feb-12	1°27'34.70"N; 125°14'23.10"E	3	0	0
13	Lobangbatu	06-Feb-12	1°26'02.65"N; 125°12'09.72"E	2	1	0
14	SW Sarena Kecil	07-Feb-12	1°27'19.84"N; 125°13'25.03"E	6	3	2
15	Lobangbatu Besar	07-Feb-12	1°25'49.40"N; 125°11'26.81"E	3	0	0
16	Teluk Rarandam	08-Feb-12	1°27'03.20"N; 125°14'17.52"E	5	1	1
17	Teluk Makawide	09-Feb-12	1°29'05.06"N; 125°14'26.12"E	4	0	0
18	Kelapadua	09-Feb-12	1°26'19.07"N; 125°12'49.00"E	3	0	0
19	Tanjung Kungkungan	10-Feb-12	1°27'58.39"N; 125°14'02.26"E	2	0	0
20	Pulau Abadi	10-Feb-12	1°26'01.03"N; 125°12'22.28"E	2	0	0
21	Tanjung Kuning	11-Feb-12	1°23'10.79"N; 125°10'23.23"E	3	0	0
22	Tanjung Pandea	11-Feb-12	1°23'52.69"N; 125°09'58.93"E	3	3	1
23	N Pulau Dua	13-Feb-12	1°23'28.64"N; 125°12'58.72"E	4	3	1
24	S Pulau Dua	13-Feb-12	1°23'17.02"N; 125°12'43.13"E	6	1	1
25	N Tanjung Pandean	14-Feb-12	1°24'21.71"N; 125°10'04.51"E	3	0	0
26	Desa Pandean	14-Feb-12	1°25'16.07"N; 125°10'52.68"E	4	2	0

Table 1 (continued).

Dive Site Number	Dive Site Name	Date	Coordinates	CO1	ATP6	ATPSβ
27	Teluk Walemetodo	15-Feb-12	1°24'11.34"N; 125°10'20.32"E	1	0	0
28	Tanjung Kelapasatu	15-Feb-12	1°25'38.57"N; 125°11'00.78"E	7	3	2
29	Tanjung Kusukusu	16-Feb-12	1°27'13.75"N; 125°14'12.95"E	5	3	2
30	N Sarena Kecil	16-Feb-12	1°27'26.86"N; 125°13'37.69"E	6	6	2
31	W Sarena Kecil	17-Feb-12	1°27'25.52"N; 125°13'31.19"E	7	5	4
32	Batu Kapal	18-Feb-12	1°32'56.83"N; 125°17'31.85"E	7	2	0
33	Pulau Putus	18-Feb-12	1°31'20.75"N; 125°16'37.27"E	5	2	0
Total				128	64	26

Dive sites, sampling dates and coordinates followed by the number of sequences collected of *Xestospongia testudinaria* per genetic marker. Mitochondrial DNA markers: Cytochrome Oxidase 1 (CO1) and adenosine triphosphate synthase subunit 6 (ATP6), and nuclear DNA marker: nuclear adenosine triphosphate synthase β intron (ATPSβ).

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Sulawesi (see Figure 1). This channel, Lembeh Strait, stretches for more than twelve kilometers and has a width of between one and four kilometers. The port of Bitung is located on the main island of Sulawesi, facing the southern part of Lembeh Island. This results in busy shipping traffic in this part of the strait. Lembeh Island lies within the center of maximum marine biodiversity, located in the Indo-Malayan region [20,35].

All sponges were photographed with a digital camera from above and in profile with a unique tag number. A fragment of about 20 grams of each sponge was collected using an apple corer. For each living sponge, we recorded habitat and depth in addition to details of outer morphology and dimensions. After each sampling dive, sponge tissue for DNA extraction was immediately stored in absolute ethanol (98%) in a cool box. After 6-12 hours, the ethanol was changed and samples were stored at -20°C.

Top-view and profile pictures of all sampled giant barrel sponges were independently analyzed by two of us (TS and NJV) and sorted into four different morphotypes (Figure 1a-f) according to the morphotypes described for *X. muta* [22,34]. Individuals that were too small to be assigned to a morphotype or for which there was no consensus were classified as 'Unknown' (X). The main morphotypes were 'digitate' (D), 'lamellate' (L), 'smooth' (S) and 'intermediate' (I). Digitate sponges had digitate or spiky projections covering their outer body surface (Figure 1a, 1e). Lamellate sponges had closely spaced, pronounced and smooth flanges extending from the base to the apex of their exterior (Figure 1c, 1f). Smooth sponges had a smooth surface area with no surface projections present (Figure 1b, 1g). Intermediate sponges had a rough or bulged surface area in which no distinct pattern was detectable (Figure 1d, 1h).

Molecular analysis

DNA was extracted from sponge tissue using the DNeasy Blood & Tissue kit (Qiagen) following the instructions of the manufacturer. We sequenced 126 samples from reefs surrounding Lembeh Island for the CO1 mitochondrial gene following [36]. The primers used were C1-J2165 (GAAGTTTATATTTTAATTTTACCDGG) and C1-Npor2760 (TCTAGGTAATCCAGCTAAACC), which amplified a fragment of 544 base pairs (bps). Amplification was performed in a 25µl total reaction volume with: 13.95µl sterile water, 5µl dNTPs (1 mM), 2.5µl 10x buffer, 1.5µl $MgCl_2$, 0.4µl forward primer, 0.4µl reverse primer, 0.25µl Taq polymerase and 1µl DNA. PCR profiles consisted of an initial denaturing step (95°C for 5 min), followed by 35 cycles of denaturing (95°C for 30 s), annealing (42°C for 45 s) and extension (68°C for 1.30 min), and a final extension step (72°C for 10 min) executed in a Biorad DNAengine model ptc-200 PCR machine. Following PCR, amplification success was checked on a 1.5% agarose gel. A subset of 54 samples was sequenced for the ATP6 mitochondrial gene following [35], using primers ATP6porF (GTAGTCCAGGATAATTTAGG) and ATP6porR (GTTAATAGACAAAATACATAAGCCTG), which amplified a product of 445 bps. Variation in the mitochondrial genome is typically low for sponges [8,10,17] and the more variable ATP6 gene has been shown to provide valuable additional information [31]. In this subset all CO1 haplotypes were represented by at least one ATP6 sequence. Amplifications were performed in a 20µl total reaction volume containing 9.6µl sterile water, 4µl dNTPs (1 mM), 4µl 5x Phire® Reaction Buffer, 0.3µl forward primer, 0.3µl reverse primer, 0.3µl Phire® Hotstart Taq polymerase DNA (Thermo Scientific, Finnzymes), and 1.5µl DNA. PCR profiles consisted of an initial denaturing step (98°C for 3 min), 35 cycles of denaturing (98°C for 5 s), annealing (38°C for 5 s) and extension (72°C for 20 s), and a final extension step (72°C for 1 min). Sequencing was performed by Macrogen Europe using the PCR primers.

Sequences were checked using CodonCode Aligner version 3.7.1.2 (CodonCode Corporation). primer sequences were trimmed and a final alignment was obtained using CLUSTALW in MEGA 5.05 [37]. Total sequence length was 544 bps for the CO1 alignment and 445 bps for the ATP6 alignment. The best matches from BLAST searches of GenBank for CO1 and ATP6 sequences were with sequences from *X. muta*, 99-100% identity (EU716652-EU716655 [30], and 99% identity EU237490 [38], respectively).

To test for congruent patterns at an independent genetic locus, the ATPSβ nuclear intron was amplified for a subset of 25 samples using primers modified from [39]. The ATPSβ intron was chosen because it has proved to be useful and informative in previous studies [18,19]. Detailed methods for this new genetic marker for sponges are described in [18,19]. All sequences are deposited at NCBI Genbank under accession numbers KC424439-KC424444 (CO1), KC424445-KC424447 (ATP6) and KF577733-KF577766 (ATPSβ).

Data analysis

Mitochondrial CO1 haplotypes were identified and genetic summary statistics were calculated in Arlequin version 3.11 [40]. Statistical parsimony networks displaying evolutionary relationships between haplotypes were obtained with TCS v 1.21 [41]. We combined CO1 haplotypes of *X. testudinaria* with data from *X. muta* reported in [26] (Genbank accession numbers EU716652-EU716655).

We tested for significant differences in CO1 haplotype composition among morphotypes (n=126) using the adonis function from the vegan library [42] in R (http://www.r-project.org/). The adonis function is an analysis of variance with distance matrices using permutations (also known as a PERMANOVA [43]) that partitions distance matrices among sources of variation; in this case morphotype. Permutational ANOVA's and adonis analyses are frequently used to test hypotheses related to species composition [44,45,46,47], but they are a general implementation of an ANOVA framework using distances. In the adonis analysis, a distance matrix of pairwise genetic distances from DNA sequences was the response variable with morphotype as independent variable. The number of permutations was set at 1999; all other arguments used the default values set in the function. The distance matrix of pairwise CO1 distances was generated by first importing a fasta file containing sequences of all individuals into R using the read. dna function from the ape library [48]. The sequences were aligned using the muscle function in ape. Additional arguments for the muscle alignment included -gapopen -400.0, -gapextend -0.1, -seqtype dna, -cluster1 neighborjoining, -cluster2 neighborjoining. Finally, a distance matrix of pairwise distances from CO1 sequences was constructed using the dist.dna function in ape with the model argument set to the TN93 model [49].

In order to compare phylogenetic relationships between samples based on mitochondrial and nuclear loci, we constructed maximum likelihood phylogenetic trees in MEGA v 5.05 [37]. We used two final alignments, the first consisted of a combined mitochondrial dataset of CO1 and ATP6 sequences (54 samples, 989 bps) and the second represented 34 sequences of the nuclear intron ATPSβ (258-270 bps in a total alignment of 278 bps). Gaps in the nuclear data were treated as complete deletion. The best-fit DNA substitution model was selected using the Akaike Information Criterion [50], which were the GTR+I model [51] and the HKY+I model [52] for the mtDNA and nuclear DNA (nrDNA) dataset, respectively. Maximum likelihood trees were rooted using the midpoint rooting method as a suitable outgroup was not available [53,54]. Maximum likelihood bootstrap analyses (1000 replicates) were carried out for both datasets.

Results

Morphotypes

The most abundant morphotype of 126 *X. testudinaria* sponges sampled around Lembeh Island displayed the digitate growth form (D, n=47), followed by smooth (S, n=34), intermediate (I, n=21) and lamellate (L, n=19). Five individuals could not be assigned to a morphotype with confidence. The four different morphotypes occurred in close proximity to each other and were present at most of the sampled sites (Figure 2). The digitate morphotype was most abundant in waters adjacent to the harbor buildings and other areas of human settlement of Bitung City (Figure 2A). Morphotypes I, L and S were also present in this area, but in much lower abundances (Figure 2B-D). Morphotype S was most abundant in the center of Lembeh Strait to the north of the harbor area, while morphotype L had a relatively high abundance in the northernmost part of the channel. No relation was found between morphotypes and depth.

Mitochondrial variation

Genetic composition based on CO1 sequences differed significantly among morphotypes (adonis: $F_{4, 121} = 25.42$, $P < 0.001$, $R^2 = 0.457$). As can be seen in Figure 2, the major difference in composition was between the digitate morphotype (Figure 2A) and the other morphotypes (Figure 2B-D). In total, 126 CO1 sequences yielded six different haplotypes (named C1-C6) based on a total of four variable sites (Table 2). Two variable sites resulted in non-synonymous substitutions. Haplotypes C1 and C5 were the most abundant haplotypes (n=61 and n=49, respectively) and were found along the entire Lembeh Strait at almost every sampled site (Figure 2). Haplotypes C2, C3, C4 and C6 were far less common (n<10) and were only found in the sheltered center of Lembeh strait to the north of the harbor and city of Bitung (Figure 2). Three mutational steps separate the two most abundant haplotypes; C1 and C5 (see Appendix S1). Haplotypes C2 and C6 represent two smaller groups of sponges (n=6 and n=8, respectively), while only one individual was found representing haplotypes C3 and C4.

Digitate sponges consisted mainly of CO1 haplotype C5 (91.5%), whereas smooth sponges mainly consisted of C1 (88.2%) (Table 3). Mitochondrial diversity estimates for the total dataset (based on CO1 sequences, n=126) were 0.6128 (S.D. =0.0250) (haplotype diversity) and 0.002987 (S.D. =0.001968) (nucleotide diversity). Mitochondrial diversity was much higher for intermediate and lamellate morphotypes (nucleotide diversities of 0.21% and 0.26%, respectively) compared to digitate and smooth morphotypes (nucleotide diversities of 0.046% and 0.071%, respectively).

The 54 ATP6 mitochondrial sequences yielded three haplotypes (named A1-A3) identified by a total of three variable sites (Table 2). None of these variable sites resulted in a non-synonymous substitution. The most common haplotypes for ATP6 were A1 (n=26) and A2 (n=27). Haplotype A3 was only found in a single specimen (the same individual in which C4 was found). The concatenated mitochondrial data set resulted in 989 base pairs. All CO1 haplotypes were represented by at least one ATP6 sequence and networks based on both markers were congruent (not shown, but see Table 2). Table 2 also shows that ATP6 contributed additional information, useful for inferring phylogenetic patterns (see combined mitochondrial haplotypes (C1A1-C6A2) for *X. testudinaria*).

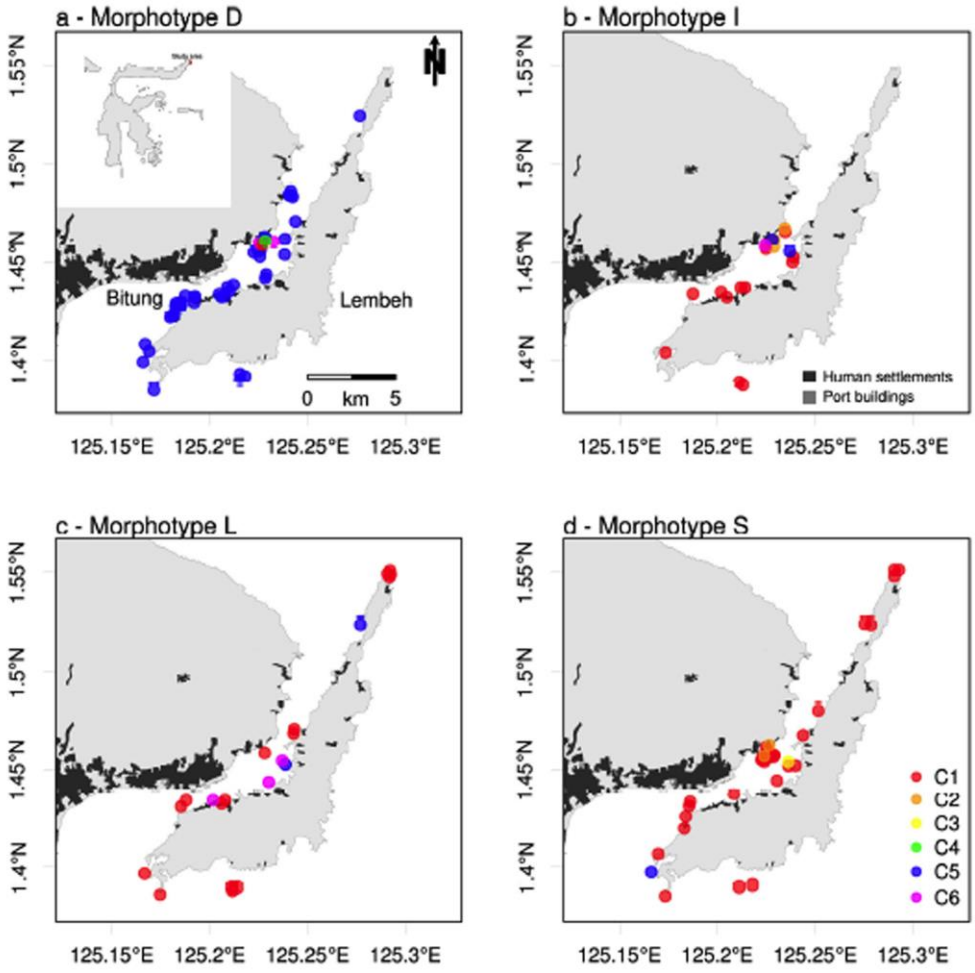

Figure 2. Distribution of mitochondrial haplotypes per morphotype of *Xestospongia testudinaria*. Spatial distribution of mitochondrial Cytochrome Oxidase 1 (CO1) haplotypes (C1-C6, see also Tables 2 & 3) per morphotype of *Xestospongia testudinaria* (see Figure 1). C1 (red) and C5 (blue) are present at all sampled sites of Lembeh Island. C2 (orange), C3 (yellow), C4 (green) and C6 (purple) are only present in the sheltered center of Lembeh strait north east of the port of Bitung.

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Nuclear variation

A subset of 25 specimens representing all morphotypes was sequenced for the nuclear ATPSβ intron yielding 16 different alleles with a total of 67 variable sites in a total alignment of 278 bps. Nine individuals were heterozygotes; their allelic sequences are labeled with "a" and "b" (Figure 3). This nuclear DNA (nrDNA) dataset revealed a pattern that is largely consistent with that of the combined mitochondrial DNA (mtDNA) data and morphotype assignment given that individuals sharing mtDNA haplotypes are grouped together in the nrDNA phylogenetic tree as well (Figure 3).

Generally, two major clades are visible in the more resolved nrDNA phylogeny with bootstrap support of 82% and 96%, respectively (Figure 3). The first clade consists of 17 nuclear

Table 3. Genetic composition and mitochondrial diversity (based on CO1 sequence data, n=126) of *Xestospongia testudinaria* per morphotype.

Morphotype	N	H	π	C1	C2	C3	C4	C5	C6
Digitate	47	0.1637 (0.0720)	0.000463 (0.000588)	0.021	-	-	0.021	0.915	0.043
Intermediate	19	0.5731 (0.1101)	0.002128 (0.001605)	0.632	0.211	-	-	0.105	0.053
Lamellate	21	0.4095 (0.1205)	0.002574 (0.001834)	0.762	-	-	-	0.095	0.143
Smooth	34	0.2228 (0.0929)	0.000711 (0.000763)	0.882	0.059	0.029	-	0.029	-
Undetermined	5	0.8000 (0.1640)	0.004412 (0.003345)	0.400	-	-	-	0.200	0.400
Total	*126*	*0.6126 (0.0260)*	*0.002987 (0.001968)*	*0.464*	*0.048*	*0.008*	*0.008*	*0.389*	*0.064*

Genetic composition and mitochondrial diversity (based on CO1 sequence data, n=126) of *Xestospongia testudinaria* per morphotype. N = number of samples, H = haplotype diversity, π = nucleotide diversity (standard deviation between brackets). C1-6 refer to six CO1 haplotypes as in Figure 2 and Table 2.

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Table 2. Nucleotide differences for mitochondrial markers CO1 and ATP6.

mtDNA		CO1			ATP6				mtDNA combined
CO1 (n=126)	ATP6 (n=54)	11	22	133	483	678	726	786	CO1+ATP6 (n=54)
C1	A1	A	T	A	T	T	T	T	C1A1 (n=18)
C1	A2	.	.	.	.	C	.	C	C1A2 (n=1)
C2	A1	.	.	.	C	.	.	.	C2A1 (n=5)
C3	A1	.	A	.	C	.	.	.	C3A1 (n=1)
C4	A3	.	.	G	C	C	C	.	C4A3 (n=1)
C5	A1	.	A	G	C	.	.	.	C5A1 (n=3)
C5	A2	.	A	G	C	C	.	C	C5A2 (n=19)
C6	A2	G	A	G	C	C	.	C	C6A2 (n=6)

Nucleotide differences in mitochondrial markers Cytochrome Oxidase I (CO1) and adenosine triphosphate synthase subunit 6 (ATP6). Six haplotypes (C1-C6) are found for the CO1 fragment (basepairs 1-544; n=126) with a total of four variable sites. Three haplotypes (A1-A3) are found for the ATP6 fragment (basepairs 545-989; n=54) with a total of three variable sites. Eight different haplotypes are found when the CO1 and ATP6 markers combined (e.g. C1A1, basepairs 1-989; n=54).

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alleles with mitochondrial haplotypes C5A2 (filled blue box), C5A1 (outlined blue box) and C6A2 (pink box). All samples in this clade are digitate and all but one individual (XT176) are heterozygous. The second major clade consists primarily of mitochondrial haplotype C1A1 (filled red box), however, C1A2, C5A1 and C6A2 are also represented by a single individual each in this clade. All samples in this clade are homozygotes for a shared nuclear allele and the clade consists predominantly of samples with a smooth outer morphology, although other morphotypes (D, L, I) are represented in this clade as well. Two single individuals of unique mitochondrial haplotypes C3A1 (yellow box) and C4A3 (green box) represent unique nuclear genetic lineages as well. All in all, samples with similar mitochondrial haplotypes group together in the nuclear phylogenetic tree as well, with the exception of individuals XT029 and XT123. In this respect, sample XT123 is notable because it is the only sample with mitochondrial haplotype C5A1 that has a smooth morphotype. Sample XT123 grouped together in the nuclear phylogeny with the other smooth samples, whereas the two digitate morphotypes with haplotype C5A1 grouped with the other digitate samples (Figure 3).

Discussion

Four distinct morphotypes of *Xestospongia testudinaria* were identified around Lembeh Island, Indonesia. There was, however, a noticeable higher occurrence of sponges with a digitate exterior in waters surrounding the port of Bitung and other human settlements compared to sponges with intermediate, lamellate and smooth morphotypes. Shipping traffic and human activities have been linked to more turbid waters and higher nutrient values [55]. This may favor digitate sponges given that they have higher surface/volume ratios compared to the other morphotypes; the digitate surface projections may also aid in the removal of heavy sediment loads as has been demonstrated for lamellate structures [56]. To better understand the distribution of morphotypes, abiotic measurements such as wave-action, light attenuation and sediment composition need to be taken into account in future studies. The degree and type of morphological variation observed were similar to those described for *X. muta* [30,26]. Different functional roles have been proposed to explain the morphological variation, e.g., lamellate structures have been linked to the removal of heavy sediment loads [56]. Nevertheless, no correlation was found between morphology and depth, micro-habitat or geographical locality for *X. muta* [22].

We found significant differences in genetic composition among the morphotypes of *X. testudinaria* (Figures 2-3), which suggests that these are not mere ecophenotypic varieties, but rather reproductively isolated units. Our total dataset of 126 CO1 sequences showed that haplotype C5 was far more abundant in the digitate morphotype, whereas haplotype C1 was more abundant in intermediate, lamellate and smooth morphotypes (Table 3). ATP6 proved to be a good addition to the CO1 gene to study mitochondrial variation in giant barrel sponges. This yielded more mtDNA variation, but the number of variable sites was still relatively low, hampering phylogenetic support of the clades. This is illustrated by the fact that most clades in the mtDNA tree received relatively low bootstrap support (<70%) (Figure 3). It is well known that sponges (and other non-bilaterian animals) share a comparatively lower

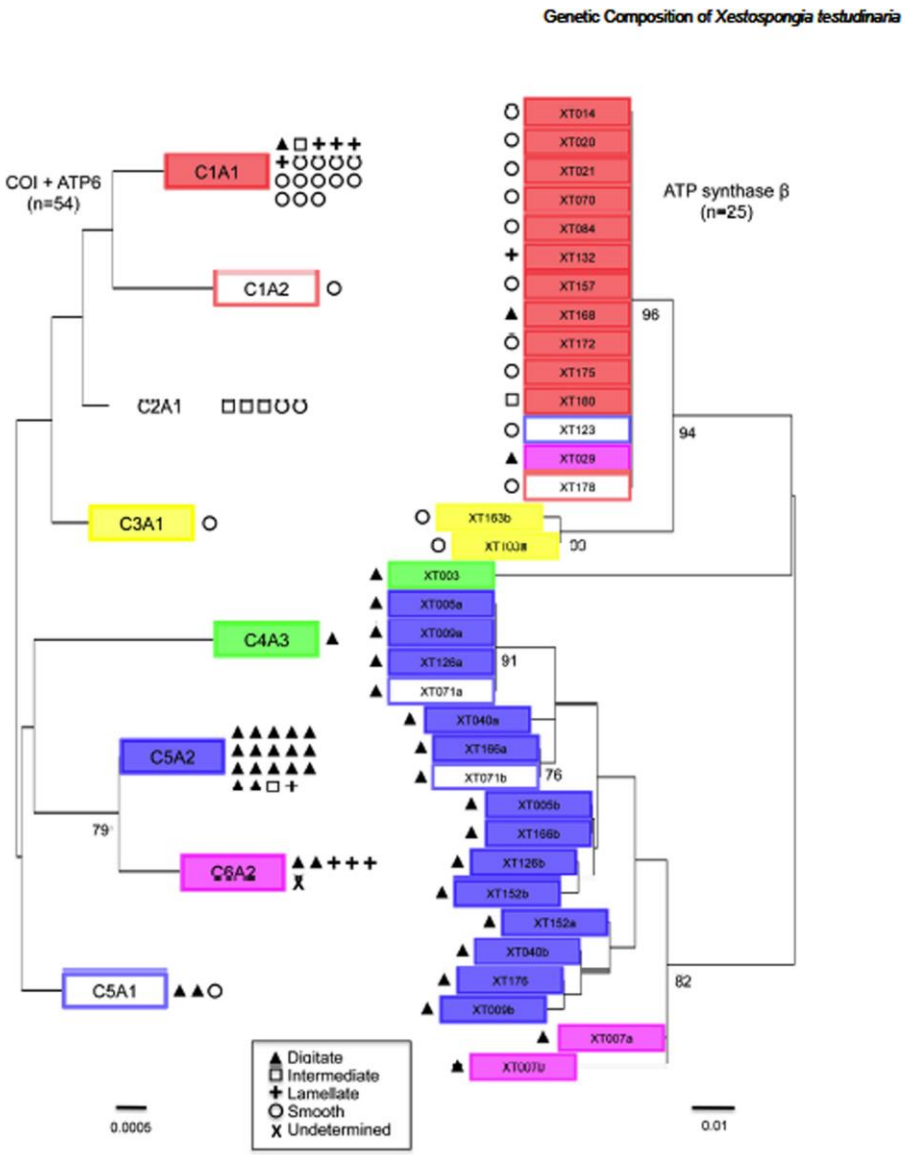

Figure 3. Phylogenetic trees based on mitochondrial and nuclear DNA of *Xestospongia testudinaria*. Unrooted maximum likelihood phylogenetic trees of the combined mitochondrial DNA (CO1+ATP6, n=54, 989 base pairs, left) and nuclear intron ATPSβ (n=34, 258-270 base pairs, right) sequences of *Xestospongia testudinaria*. Symbols denote the assigned morphotype of different individuals, with the number of symbols indicating the number of sampled sponges of a given morphotype with that specific DNA sequence (see legend). The colored boxes of the individual sample numbers in the nuclear DNA phylogeny correspond to the same colored boxes of haplotypes in the mitochondrial tree. These colors correspond with the CO1 haplotypes in Figure 2. Haplotypes with an outlined box in a particular color share the CO1 sequence but vary in ATP6 sequence, resulting in a unique combined haplotype (CO1+ATP6). The letters "a" and "b" in the nuclear gene tree represent heterozygote alleles. Bootstrap values are only shown when > 70%. Scale bars depict substitutions per site.

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degree of mtDNA variation compared to Bilateria [8,10,57]. The nuclear intron sequenced here from *X. testudinaria* contained much more genetic variability than the mtDNA fragments, and yielded congruent patterns with the mtDNA phylogeny. Individuals that shared a haplotype for the combined mtDNA (CO1+ATP6) dataset grouped together in the nuclear phylogenetic tree. If extensive genetic exchange is present between the different morphotypes in *X. testudinaria*, we would expect a random distribution of both mitochondrial and nuclear genetic types across the phylogenies. This, however, is not the case in *X. testudinaria* around Lembeh Island. In the future, we hope to sequence individuals from other areas and to sequence more individuals for the nuclear gene.

The mitochondrial and nuclear intron data presented here provide strong support for the existence of a species complex of *X. testudinaria* around Lembeh Island. A species complex was previously proposed for the closely related *X. muta* by Kerr and Baker [29] in the Caribbean based on sterol chemistry. Our data show that there are much larger morphological and genetic differences within *X. testudinaria* from Lembeh Island than between individuals of *X. testudinaria* and *X. muta* sharing the same haplotypes and morphotypes (Figure 4). In total, six haplotypes were found for the CO1 mitochondrial fragment in giant barrel sponges around Lembeh Island. López-Legentil & Pawlik [26] only found four haplotypes at this same locus for *X. muta* sampled throughout the Caribbean, and thus encompassing a much larger area. Our haplotypes C2 and C5 are identical to haplotypes H1 and H3 respectively, found in *X. muta* [26]. The most abundant haplotypes in our dataset, C1 and C5, occurred at all sites around the island. The four other haplotypes, all of which were found in less than ten individuals, only occurred at the sheltered center of Lembeh Strait away from the port and city of Bitung (Figure 2). This undisturbed sheltered area, which has a less oceanic profile compared to sites outside of Lembeh Strait, appears to be a local hotspot of genetic diversity. The exact reason for this particular distribution is unknown [58]. The fact that four of the six haplotypes are only found in a very small portion of the reefs surrounding Lembeh Island illustrates the importance of fine-scaled sampling.

A third giant barrel sponge species, *Xestospongia bergquistia* was described from the Northern Great Barrier Reef. This sponge is sympatric with *X. testudinaria* and is very similar in outer morphology and spicule dimensions. The differences between the two species are very subtle with the main difference being that *X. testudinaria* has some sponge fiber development around the spicules and therefore has a more compact consistency than *X. bergquistia* and it was noted that the live texture is the best field guide to species identification [25]. In the present study, we noticed a large variation in consistency and thus in sponge fiber development, but all of our specimens contained spongin, and were thus assigned to *X. testudinaria*. Therefore the possible presence of *X. bergquistia* cannot explain the morphological and genetic variation in the giant barrel sponge populations around Lembeh Island and we conclude that at least two distinct, and possibly more, species coexist in the Lembeh Strait. In addition to the dissimilarity in sponge fiber development, *X. testudinaria* and *X. bergquistia* spawn during different time periods [59]. For Great Barrier Reef sponges, spawning only occurs during a short period between October-November [59,60]. This has been linked to annual changes in seawater temperature [61]. During fieldwork early in 2012, we observed several individuals of *X. testudinaria* spawning during the full moon lunar cycle and it has also been observed in August in Ambon [62]. Given that waters around Indonesia show little seasonal variation in temperature [63], it is possible that barrel sponges in these waters spawn during the whole year. The as yet unidentified differences in the timing of spawning events, however, may explain the persistence of a species complex in *X. testudinaria*.

The fact that Caribbean and Indo-Pacific giant barrel sponges share CO1 haplotypes is perhaps more surprising than the existence of a species complex around Lembeh Island. After more than three million years of separation [28] the CO1 mitochondrial genes of giant barrel sponges have still not accumulated mutations to differentiate these taxa or alternatively, this may be the result of secondary contact between the two species. Figure 4 shows a combined CO1 haplotype network from this study with results from López-Legentil and Pawlik [26]. This network shows that two individuals from different regions (i.e., Caribbean and Indo-Pacific) can be more closely related than two individuals occurring in sympatry. This strongly suggests that there are in fact different species in the Indo-Pacific and possibly in the Caribbean as well. It is unlikely that barrel sponges from the Indo-Pacific and Caribbean exchange genes nowadays, but this needs to be tested with more variable genetic markers than CO1, such as the nuclear ATPSβ intron that we used.

In summary, giant barrel sponges found around Lembeh Island that are morphologically identified as *X. testudinaria* consist of at least two different lineages that probably represent reproductively isolated species. The first lineage has a digitate surface area and mitochondrial CO1 haplotype C5 or C6, and is most abundant around the harbor area of Bitung city. The second lineage is represented by CO1 haplotypes C1 and C2 and usually has a smooth surface area, though other morphotypes have also been found to belong to this lineage. This lineage can be found all around Lembeh Island though to a lesser extent around the harbor of Bitung city. More species may be present in this species complex as some unique genetic lineages for both mitochondrial and nuclear markers were found in the sheltered and relatively undisturbed center of Lembeh Strait. In addition to detailed spatial sampling in the Indo-Pacific, as was done in this study, a wide global-scale sampling of genetic and morphological diversity in giant barrel sponges is necessary to elucidate further biogeographic and evolutionary relationships in this taxon.

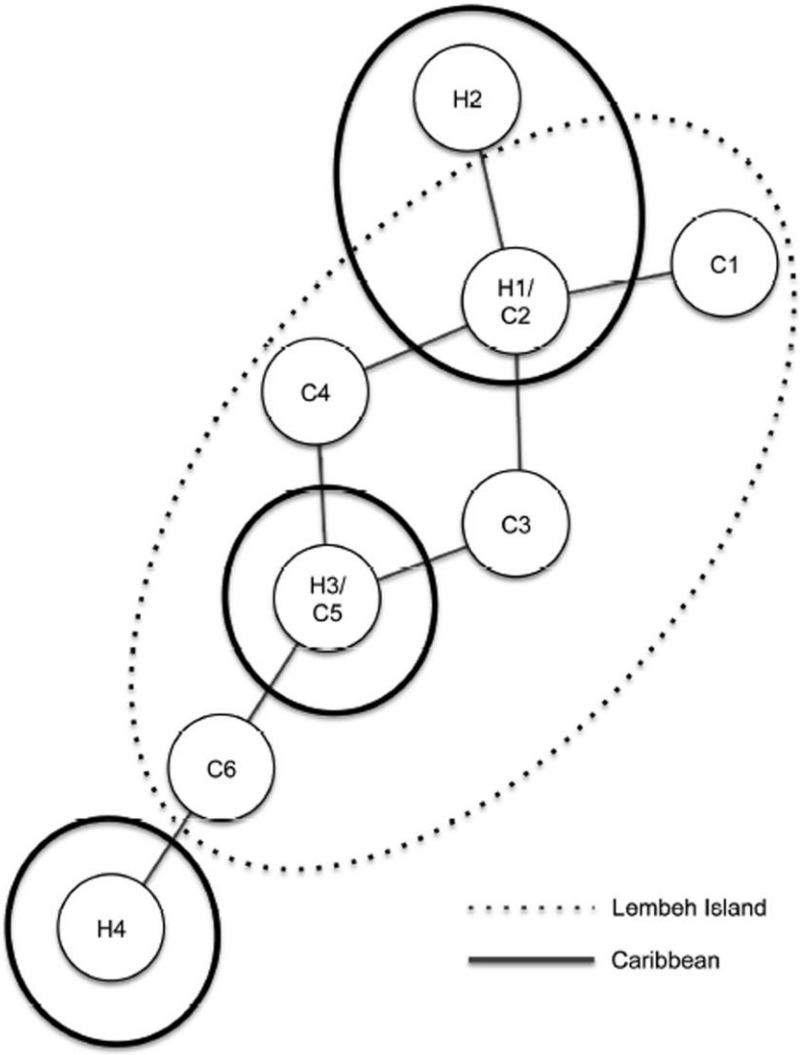

Figure 4. Combined haplotype network of *Xestospongia sp.* from the Caribbean and Lembeh Island. Haplotype network of eight Cytochrome Oxidase 1 (CO1) mitochondrial haplotypes of giant barrel sponges *Xestospongia testudinaria* and *X. muta*. Dotted line encircles haplotypes found in our study of 126 *X.* testudinaria around Lembeh Island (C1-6). Solid lines encircle haplotypes found in 116 samples of *X. muta* from the Caribbean (H1-4) [26]. Each line connecting the haplotypes represents a single nucleotide substitution.

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Supporting Information

Appendix S1. Haplotype network based on mitochondrial DNA for *Xestospongia testudinaria* around Lembeh Island. (DOC)

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Author Contributions

Conceived and designed the experiments: NdV DC KP. Performed the experiments: TS CL CH ES NdV. Analyzed the data: TS CL DC KP ES DE. Contributed reagents/materials/analysis tools: TS KP CL DC CH ES GW DE NdV. Wrote the manuscript: TS KP CL DC CH ES GW DE NdV.

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CHAPTER 7

General Results and Discussion

7.1. Taxonomy and phylogeny of the selected haplosclerids

In this study, molecular tools were applied for the detection of cryptic species and species complexes in the petrosiid sponges *Xestospongia testudinaria* and *Neopetrosia exigua.* The aim of the study was to analyse the suitability of these tools for this purpose, as well as for resolving the taxonomical status and phylogenetic relationships of these two taxa. In the last two decades a significant increase in the discovery of cryptic marine species (Knowlton 2000), particularly among sponges, with the aid of molecular tools was observed (see e.g., Wörheide et al. 2008; Pöppe et al. 2010; Reveillaud et al. 2010; Xavier et al. 2010b; Reveillaud et al. 2011). With molecular tools, species complexes were discovered in sponges, which indicated that the number of sponge species had been not properly estimated thus far (Palumbi et al. 1997; Klautau et al. 1999; Solé-Cava and Boury-Esnault 1999; Bierne et al. 2003). In my opinion it is very likely that, with the application of molecular tools, more species complexes will be discovered in the future. Current discoveries were the presence of a *X. testudinaria* species complex in the Indo-Australian Archipelago (**chapter 3)**, in North Sulawesi and in Southeast Sulawesi (Swierts et al. 2013; Bell et al. 2014b), see **chapter 6**. In spite of these achievements, future investigations are needed, particularly to define robust characters to delineate the members of the *X. testudinaria* species complex. The composition of the species complex in *X. testudinaria* is still to be determined because one of the mtDNA markers utilised in the analyses (I3-M11 cox1), displays overlapping haplotypes with its Caribbean con-generic *X. muta* (**chapter 2 and chapter 6)**. These overlapping sequence types were detected previously by Montalvo and Hill (2011) and Swierts et al. (2013), between *X. testudinaria* and its sibling species in the Indo-Pacific (*X. bergquistia* see **chapter 2**)**.** For this reason, I investigated the type specimens of the barrel sponge species using molecular tools in addition to the spicule measurements.

In **chapter 2**, I took spicule measurements of all type specimens, but I could not find any striking spicule characters to define species boundaries. All of the barrel sponge taxa overlapped in spicule length and width. I was only able to retrieve two I3-M11 cox1 sequences from the type specimens of *X. bergquistia* (holotype G25018) and *X. testudinaria* (neoparatype BMNH 1881.10.21.267), whose haplotypes were different. However, a species

boundary between them could not be distinguished since the haplotype C5 of *X. bergquistia* holotype and samples is identical to the C5 *X. testudinaria* samples. Similarly the haploytpe C2 of *X. testudinaria* neoparatype is identical to the haplotype C2 *X. bergquistia* samples. I assume that ancient polymorphisms affect the barrel sponge cox1 phylogeny, or that the cox1 marker has insufficient capability to resolve lineage sorting among those three taxa. Bell et al. (2013) and (2014) have introduced microsatellites that can distinguish *X. bergquistia* (including the holotype) from *X. testudinaria*. Nevertheless, further tests should confirm whether the use of microsatellites would facilitate delimiting *X. muta* as well. These approaches should also include holotype sequences.

I decided to apply a partial ATP6 mtDNA fragment in a phylogenetic approach to delimit the three barrel sponge taxa since I also successfully utilised that fragment for *X. testudinaria* in previous chapters. In addition, I yielded a large number of cox1 sequences from *X. testudinaria* from **chapter 2**. However, I could unfortunately not amplify the specifically designed ATPS-β intron primer on the type specimens although the ATP6 amplifications were successful.

I also discovered that the ATP6 marker was more suitable for delimiting barrel sponge species than the I3-M11 fragment. I measured the *p*-distances and π values for a range of more than 13,000 km (from the Red Sea until the Coral Sea in the Great Barrier Reef). Despite the fact that the π values between both mtDNA markers were almost equal (π= 0.0027 for ATP6 and π= 0.0029 for I3-M11), the *p*-distances of ATP6 are slightly higher than the I3-M11 cox1 (0.018 for ATP6 and 0.013 for I3-M11). (Material and methods for the amplifications of the partial ATP6 in barrel sponges, the phylogenetic analysis, a list of the haplotypes and the utilised samples are provided in the Supplementary materials 7.1, 7.2, and 7.3.)

I included neither the *X. testudinaria* neotype sequence (BMNH 1881.10.21.266) nor the sequence of the *X. muta* syntype (MCZ Pora-6449) in the phylogenetic analyses, because they comprised only very short fragments (144 bp). However, the (short) sequence from the *X. testudinaria* neotype BMNH 1881.10.21.266 is identical to the neoparatype specimen of *X. testudinaria*, BMNH 1881.10.21.267. Similarly, the (short) syntype sequence of *X. muta* MCZ Pora-6449 is identical to the syntype sequence of *X. muta* MCZ Pora-6450 (see Table 7.1).

Table 7.1. Haplotypes recovered from the *Xestospongia* alignment. Positions refer to the full ATP6 sequence from *Xestospongia muta* EU237490 (Kayal and Lavrov 2008). Haplotype numeration follows Swierts et al. 2013. Asterisks represent the position of unique polymorphic sites, which are discovered only in the *X. muta* taxon. Undetermined haplotypes are almost identical to A1 or A6. However, they may also represent other, unique haplotypes.

Position (bp) **Haplo-Type**	**32**	**181**	**205**	**214**	**241**	**347**	**358**	**389**	**Species; Reference (associated haplotype number as used in reference) and example GenBank accession number**
A1 (cf. Swierts et al. 2013)	T	T	G	C	T	G	G	T	*X. testudinaria* A1; (Swierts et al. 2013); e.g., KC42445 **(this study)**
A2 (cf. Swierts et al. 2013)	C	T	G	C	C	G	G	T	*X. testudinaria* A2; (Swierts et al. 2013); e.g., KC42446 **(this study)**
A4 (this study)	C	C	G	C	T	G	G	T	**(this study); KM014757**
A5 (this study)	T	C	G	C	T	G	G	T	**(this study); KM014758**
A6 (this study)	C	T	G	C	T	G	G	T	**(this study); KM014763**
A7 (this study)	C	T	A	C	T	G	G	T	**(this study); KM014760**
A8 (this study)	C	T	G	T	T	G	G	T	**(this study); KM014761**
A9 (this study)	C	T	G	C	T	G	G	C	**(this study); KM014759**
Undetermined haplotypes (this study)	?	T	G	C	T	G	G	T	**BMNH 1881.10.21.267 (this study); KM014764** **Type QM G25018; (this study); KM014762**
Undetermined haplotypes (this study)	?	T	G	C	T	?	?	?	**Type BMNH 1881.10.21.266; (this study)**
A10 (this study)	?	C	G	C	T	A*	A*	T	**Type MCZ Pora-6450; (this study); KM014756** *X. muta*; (Kayal and Lavrov 2008); e.g., EU237490
Undetermined haplotypes (this study)	?	C	G	C	T	?	?	?	**Type MCZ Pora-6449; (this study)**

Not all of the barrel sponge taxa could be determined with the partial ATP6 marker. In the phylogram, only *X. muta* can be distinguished from *X. testudinaria* and *X. bergquistia* with high Posterior Probability (PP) and Bootstrap Support (BS), whereas *X. testudinaria* and *X. bergquistia* sequences remain unresolved (see Figure 7. 1). However, additional data is still needed as the *X. muta* sequence is short and therefore states of (at least) two polymorphic sites cannot be assessed. Nonetheless, the taxon can be delimited from other barrel sponge taxa by two out of 445 bp distinctive sites from the partial ATP6 gene in this chapter (see Table 7.1). Therefore a more thorough genetic diversity study of *X. muta* utilising ATP6 should be conducted in the future to assess whether other *X. muta* haplotypes overlap with *X. testudinaria*, parallel to cox1 as in **chapter 2** and Swierts et al. 2013.

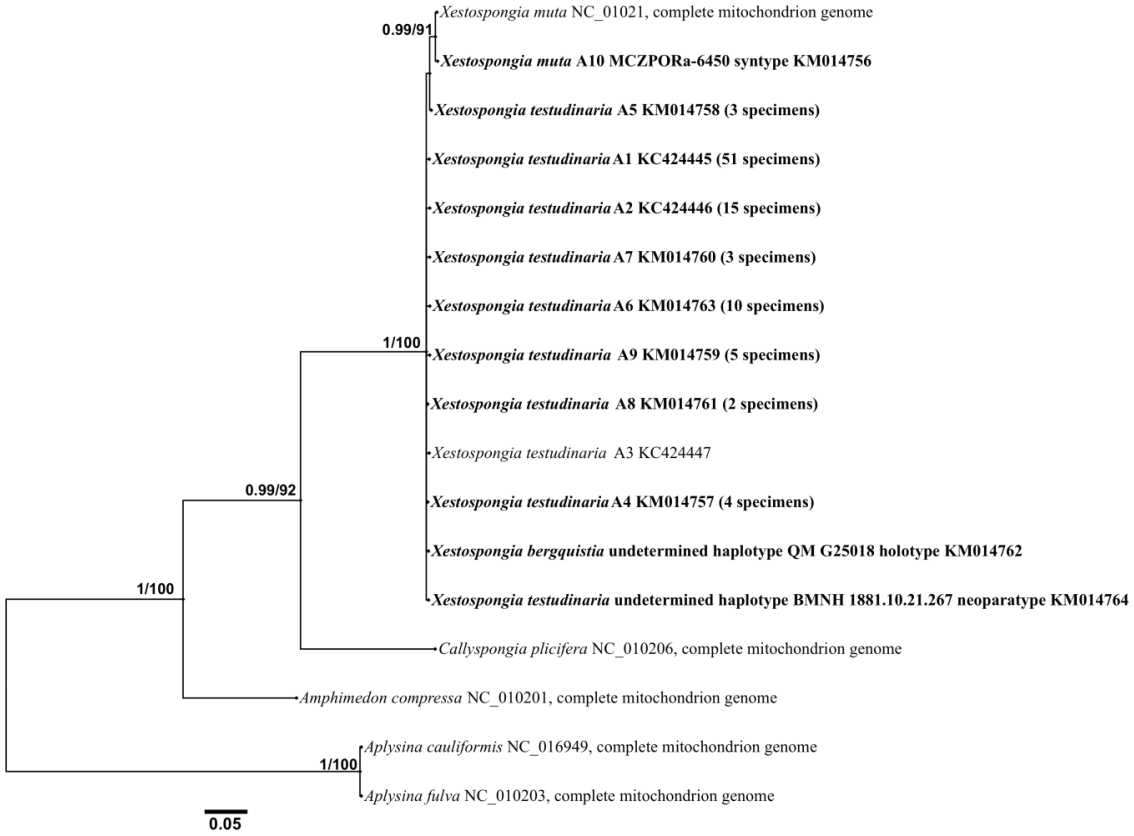

Figure 7.1. Bayesian inference phylogram of ATP6 sequences from barrel sponges and other marine haplosclerid taxa. Sequences obtained in this study are written in bold, followed by the ATP6 haplotype numeration according to Swierts et al. (2013) and the number of specimens. Two haplotypes could not be fully determined (see Table 7.1). The numbers on the branches represent posterior probabilities (PP) / bootstrap proportions (BP) of maximum likelihood analyses. The scale bar indicates the number of substitutions per site.

In my opinion, all of the three barrel sponges studied represent different species according to the results of ATP6. I would predict that either ancient polymorphisms affect the mtDNA barrel sponge systematics or that the mtDNA markers tested (cox1 and ATP6) could not fully resolve the lineage sorting among barrel sponges and mask the species boundaries. Perhaps, due to the slow evolutionary rates in the mtDNA of demosponges (Shearer et al. 2002; Huang et al. 2008), mtDNA markers cannot fully delimit demosponge taxa until genus or species level.

In chapter 4, molecular markers help delimit two different species that appear morphologically similar. I was able to distinguish *Neopetrosia pandora*, which is currently a junior synonym of *Neopetrosia exigua*, as a different species. Likewise, I discovered that the sponge species named *Neopetrosia exigua*, which was described one year earlier (see **chapter 4)** should be considered as a junior synonym of *Neopetrosia chaliniformis* because both have identical cox2 mtDNA and 28S rRNA sequences. Therefore, a taxonomical revision is suggested to avoid misidentifications on specimens resembling *Neopetrosia chaliniformis*.

For the reconstruction of the phylogenetic relationships of my two selected species *Neopetrosia* and *Xestospongia,* I only used cox2 and 28S rRNA and demonstrated their different phylogenetic positions in the phylograms of **chapter 4**. Here I conduct a similar analysis with the cox1 fragment because many barrel sponge cox1 sequences were yielded in the course of **chapters 2** and **6** and a large number of haplosclerid cox1 sequences is published in GenBank. The following phylogram was reconstructed with Bayesian inference and Maximum likelihood methods similar to **chapter 2** (Figure 7.2).

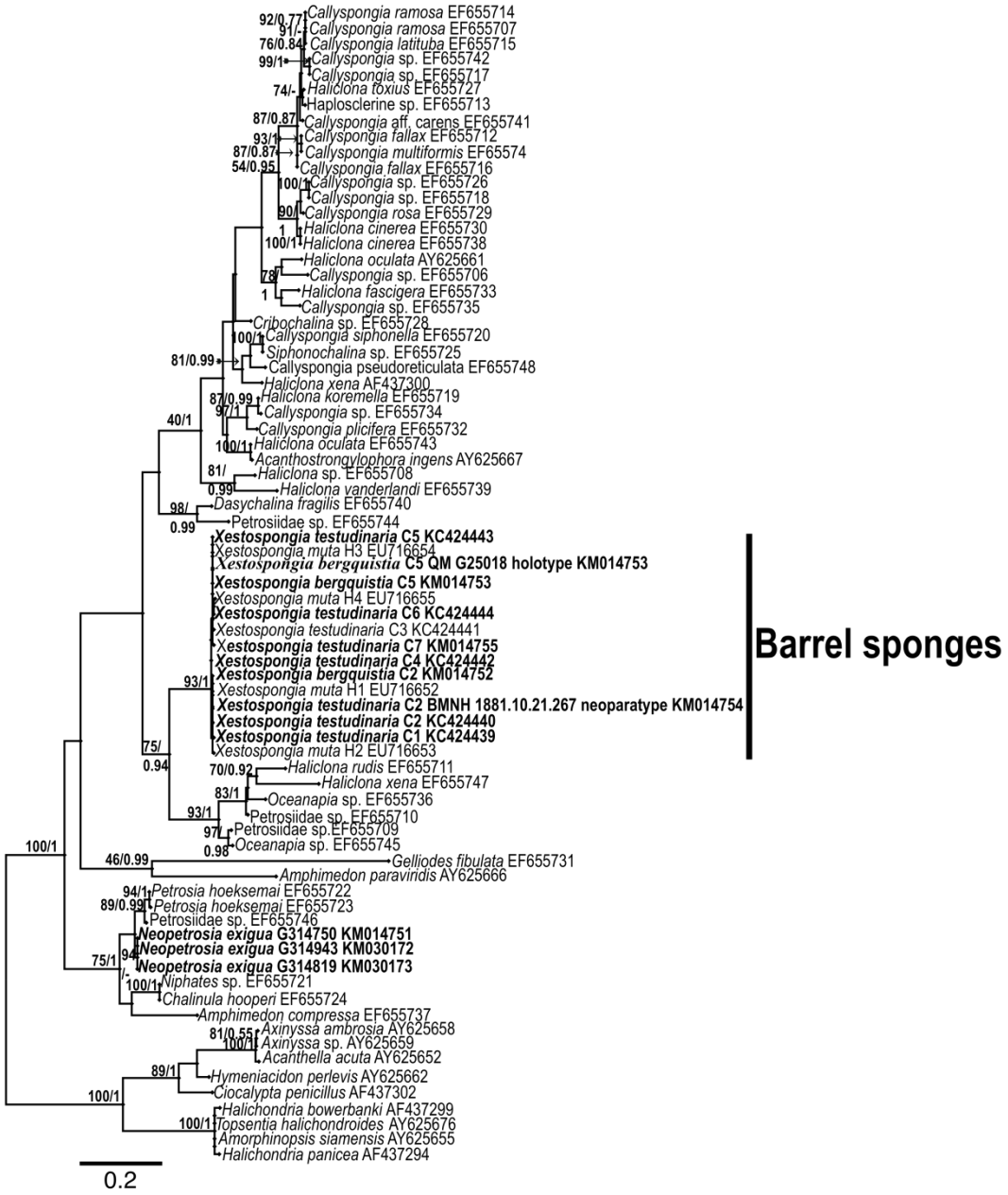

Figure 7.2. ML phylogram of selected marine haplosclerid taxa using the I3-M11 partition of cox1, rooted with halichondrid sponges. Sequences generated from this experiment are written in bold, whereas the others are retrieved from GenBank. The numbers on the branches represent bootstrap proportions (BP) / posterior probabilities (PP) of maximum likelihood analyses. The scale bar indicates the number of substitutions per site.

The I3-M11 phylogram (Figure 7.2) confirms the monophyly of barrel sponges, however we could not confirm the status of the genus *Neopetrosia,* because no I3-M11 cox1 sequences other than *N. exigua* were available. However, this I3-M11 phylogram can still be used to demonstrate the different phylogenetic positions of the genera *Xestospongia* and *Neopetrosia*,

which corroborates the current classification. Before the publication of *Systema Porifera*, the validity of the genus *Neopetrosia* had become a subject of disputations before separated from the genus *Xestospongia* (e.g., Bergquist 1965; van Soest 1980; Desqueyroux-Faundez and Valentine 2002). Both sponge genera were placed in the genus *Petrosia* (see Thiele 1899; Kirkpatrick 1900; Topsent 1933) and later in the genus *Xestospongia* (see Bergquist 1965,1980). The difference between both genera *Xestospongia* and *Neopetrosia* is also confirmed by the molecular phylogenies of cox1 (5' partition), 18S and 28S rRNA from Redmond et al. (2011), Redmond et al. (2013) and Thacker et al. (2013).

In brief, the utilisation of molecular markers contributed to the detection of cryptic species and species complexes in sponges, particularly in the selected haplosclerid taxa of this study. However, molecular phylogenies could not corroborate the monophyly of suborders within the marine haplosclerids on family and genus levels, which remain to be unravelled. Recent supertree analyses based on seven nuclear housekeeping genes revealed paraphyletic relationships within Suborder Petrosina and Family Petrosiidae, as well as within Suborder Haplosclerina and the two families, Chalinidae and Niphatidae (Hill et al. 2013). Paraphyly within suborders, families and genera among marine haplosclerids has been confirmed elsewhere with mtDNA markers in Erpenbeck et al. (2007) and Redmond et al. (2011). I agree with Cárdenas et al. (2012) that the findings of early taxonomists, who presumed that skeleton characters are sufficient for the delimitation of internal relationships, contradict the molecular findings. It is difficult to unravel the internal relationship of marine haplosclerids using morphological analyses alone due to their lack of complex and phylogenetic relevant morphological characters. To resolve this problem, Cárdenas et al. (2012) advocated the re-evaluation of haplosclerid taxonomy based on molecular results.

7.2. The phylogeographical relationships of the selected haplosclerids

Several mtDNA and nuclear markers are discussed as suitable for sponge intraspecies studies, e.g., mtDNA I3-M11 cox1 (Erpenbeck et al. 2006a), several mtDNA markers introduced by Rua et al. (2011), and several nuclear intron markers introduced by Jarman et al. (2002), see also review in Uriz and Turon (2012). I utilised three mitochondrial (I3-M11 cox1, ATP6, and cox2 fragments) and two nuclear markers (the ATPS-β and LTRS intron) to investigate the phylogeographical relationships of the haplosclerids selected for my research.

In a narrow geographical scale (**chapter 6),** the I3-M11 marker can be used to detect

the genetic structure of *X. testudinaria*. This confirmed previous findings with its congeneric *X. muta* in the Caribbean (Lopez-Legentil and Pawlik 2009). The I3-M11 can also be used to detect genetic structures of the Atlanto-Mediterranean poecilosclerid *Phorbas fictitius* (Xavier et al. 2010a). The suitability of I3-M11 for intraspecies studies of *X. testudinaria* in a wider geographical range of the Indo-Pacific is described in **chapter 2**. However, further research is still required, because the sample sizes per location vary too much. **Chapter 3** describes the suitability of the intron marker (ATPS-β intron) for *X. testudinaria* intraspecies studies in comparison to other mtDNA markers. I discovered that the phylogeographical patterns of *X. testudinaria* do not follow the phylogeographical patterns of other marine organisms studied for this area, which follow the Pleistocene low sea level and the Holocene recolonisation pattern. The ATPS-β intron is unable to unravel the phylogeographic relationships of *X. testudinaria,* although it is capable to display the vicariance of calcarean sponges e.g., *Pericharax heteroraphis* (Bentlage and Wörheide 2007) and *Leucetta chagosensis* (Wörheide et al. 2008). **Chapter 6** describes why an intron is more suitable for resolving the phylogeographic relationships of *N. chaliniformis* rather than mtDNA. The LTRS intron in *N. chaliniformis* can be utilised for the detection of signals for a phylogeographical break between the Great Barrier Reef in Australia and Papua New Guinea & Solomon Island.

Most intraspecies studies on sponges display a highly structured population with limited exchange of genetic material (Duran et al. 2004; Lopez-Legentil et al. 2008; Xavier et al. 2010a; Swierts et al. 2013; Bell et al. 2014b). These phenomena are caused by the typical lecithotrophic and meroplankontic sponge larvae, which have limited swimming and dispersal abilities (Mariani et al. 2006; Uriz and Turon 2012). However, several factors can influence genetic patterns and result in a weak genetic structuring and an overlapping haplotypes among selected populations. First, the slow substitution rate of the marker may affect lineage sorting and therefore reduces its phylogeographic resolution. However, we observe taxon-specific differences for this phenomenon among sponges. For instance, the 5’ partition of cox1 appears suitable for resolving the phylogeographical patterns of *Callyspongia vaginalis* (DeBiasse et al. 2010), but is not suitable for resolving the phylogeographical patterns of *X. muta* (Lopez-Legentil and Pawlik 2009). In my study, cox2 could not resolve the phylogeographical patterns of *N. chaliniformis* although it was published as suitable for sponge phylogeography (Rua et al. 2011).

Second, external factors like ocean currents affect the gene flow and genetic structures of sponges in the different populations (Lopez-Legentil and Pawlik 2009; Swierts et al. 2013).

Also, the paleogeographical history affected the current genetic pattern among sponge populations (Bentlage and Wörheide 2007). For this reason, I hypothesised that the genetic pattern of *X. testudinaria* and *N. chaliniformis* will follow the currents and the Pleistocene low sea level and the Holocene recolonisation event in the IAA. However, none of the intron markers could fully resolve the phylogeographical pattern of my selected sponge taxa. Becking et al. (2013b) discussed the effects of long time dispersal as occasionally observed among sponge taxa. This long time dispersal may result in the haplotype overlap between two different sponge populations.

The third factor is the influence of asexual reproduction in combination with abiotic factors, e.g., currents. The ability of sponge fragments to disperse and raft on various floating materials has been observed (Wulff 1995; Maldonado and Uriz 1999). Such scenario might be likely for *N. chaliniformis*, given its morphological characteristics (see discussion of chapter 5), which suggests a dispersal of the asexual part through water currents or floating materials.

Finally, unintentional inclusion of cryptic species in the phylogenetic analysis of *X. testudinaria* in this study and in Bell et al. (2014b) may lead to less accurate phylogeographic data and interpretations.

In summary, introns are potentially suitable for future applications in intraspecies studies of sponges, particularly haplosclerids. In spite of several limitations, e.g., imbalances among sample numbers per locality, intron fragments have shown to possess high numbers of polymorphic sites, which are inevitably needed for intraspecies studies of sponges. However, technical hurdles, extra costs and more laborious works for genotyping heterozygotic alleles, which can only be genotyped by cloning in the worst case, provide pitfalls in the utilisation of nuclear DNA for interspecies studies. For instance, the unresolved heterozygote haplotypes in the intraspecies analyses of my haplosclerids weaken the phylogeographic signal due to undetected unique haplotypes. I could have cloned all those heterozygotic samples to achieve correct genotyping, but this would have involved unpredictable efforts in money and time. These obstacles also refrained me from applying other tools like microsatellites, SNIPS and RAD-SEQ in spite of their higher resolution, as they would have demanded e.g., library development and fragment selection.

In my opinion, the use of mtDNA markers on intraspecies studies is still unrivalled, since they are easy to amplify and simpler to genotype, i.e., almost no extra costs, time and laborious work. Likewise, mtDNA markers can also be used for pilot studies in phylogeographic relationships despite of their slower evolution rates. Actually, it would be possible use other intergenic mtDNA markers like those introduced byRua et al. (2011), which have a higher substitution rate in comparison to conventional mtDNA gene fragments. However, the variety of gene arrangements in sponge mtDNA (Wang and Lavrov 2008) creates difficulties for primer design, particularly among haplosclerids.

To conclude, my future direction for intraspecies studies in sponges—haplosclerids in particular— is 1) finding more polymorphic introns markers with the simplest and most economically methods for genotyping heterozygote alleles and 2) the discovery of intergenic mtDNA markers, which can be easily amplified and utilised.

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Supplementary material

2.1. List of samples that are utilised in chapter 2 and sorted by the haplotype

Legend

- GW and XT are internal voucher numbers of the Molecular Geo- & Palaeobiology Lab, Department of Earth and Environmental Sciences, Palaeontology & Geobiology, Ludwig-Maximilians - Universität München, Germany and Naturalis Biodiversity Centre Leiden, The Netherlands.
- ZMA POR numbers refer to samples of the Zoological Museum Amsterdam, which is currently merged into Naturalis Biodiversity Centre Leiden, The Netherlands.
- G numbers refer to samples of the Queensland Museum, Brisbane, Australia.
- BMNH numbers refer to samples of the British Museum Natural History, London, UK.
- BSPG numbers refer to samples of the Bavarian State Collection for Palaeontology and Geology, Munich, Germany.

Haplotype numeration (C1-C6) follows Swierts et al. 2013

Xestospongia testudinaria samples

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
GW2314	BSPG 2014 XIV 19 (GW2314)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08 Aug 2011	C1
GW2315	BSPG 2014 XIV 20 (GW2315)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08 Aug 2011	C1
GW2321	BSPG 2014 XIV 23 (GW2321)	Indonesia	SE Sulawesi, Wakatobi, Hoga, Pak Kasim's	GPS not available		07 Aug 2011	C1
GW2332	BSPG 2014 XIV 27(GW2332)	Indonesia	SE Sulawesi, Wakatobi, Tomia, NW P. Sawa	5° 45' 00" S, 123° 53' 33" E		14 Aug 2011	C1

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
GW4791		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 25"N, 125° 13' 31" E		12 Feb 2012	C1
GW4795		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 28' 10"N, 125° 14' 38"E		02 Feb 2012	C1
GW4798		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 26' 8"N, 125° 12' 34"E		04 Feb 2012	C1
GW4846	ZMA POR18747	Thailand	Ko Rad, Kood Islands, Trad	11° 57' 21" N, 102° 21' 46" E		08 May 2012	C1
GW4848	ZMA POR18738	Thailand	West side of Ko Klum, Chang Islands, Trad	11° 55' 02" N, 102° 21' 43" E		08 May 2012	C1
GW4854	ZMA POR18692	Thailand	Ao Pagarung, South of Ko Samet, Samet Islands, Rayong	12° 33' 14" N, 101° 28' 11" E		28 Oct 2005	C1
GW4855	ZMA POR18688	Thailand	Hin Khan Na, West side of Ko Samet, Samet Islands, Rayong	12° 33' 14" N, 101° 28' 11" E		27 Oct 2005	C1
GW4856	ZMA POR18687	Thailand	Hin Khan Na, West side of Ko Samet, Samet Islands, Rayong	12° 33' 14" N, 101° 28' 11" E		08 May 2012	C1
GW4859	ZMA POR18627	Thailand	West side of Ko Khrok, Ko Lan IslandFs, Pattaya, Chonburi	12° 55' 13" N, 100° 48' 27" E		26 Sept 2005	C1
GW4860	ZMA POR18618	Thailand	Ko Thai-ta-Mun, South of Ko Si-chang, Chonburi	13° 6' 09" N, 100° 48' 28" E		08 May 2012	C1
GW4861	ZMA POR18616	Thailand	Ko Thai-ta-Mun, South of Ko Si-chang, Chonburi	13° 6' 09" N, 100° 48' 28" E		08 May 2012	C1
GW4869	ZMA POR16708	Thailand	Pattaya, Klungbadan Island, Gulf of Thailand	12° 45' 00" N, 100° 52' 00" E		24 Jun 2009	C1
GW4885	ZMA POR8250	Indonesia	S Sulawesi, Tukang Besi Islands, southern reef of Karang Kaledupa, east of entrance	5° 55' 60" S, 123° 47' 60" E		07 Sept 1988	C1
XT025		Indonesia	N Sulawesi, Lembeh, Pantai Perigi	1° 28' 10.0194"N, 125° 14' 38.796"E		02 Feb 2012	C1
XT026		Indonesia	N Sulawesi, Lembeh, Pantai Perigi	1° 28' 10.0194"N, 125° 14' 38.796"E		02 Feb 2012	C1
XT028		Indonesia	N Sulawesi, Lembeh, Tanjung Nanas I	1° 27' 40.212"N, 125° 13' 36.408"E		03 Feb 2012	C1
XT035		Indonesia	N Sulawesi, Lembeh, Pulau Abadi	1° 26' 0.744"N, 125° 12' 22.608"E		03 Feb 2012	C1
XT044		Indonesia	N Sulawesi, Lembeh, Tanjung Labuhankompeni	1° 25' 55.848"N, 125° 11' 10.6434"E		04 Feb 2012	C1
XT045		Indonesia	N Sulawesi, Lembeh, Tanjung Labuhankompeni	1° 25' 55.848"N, 125° 11' 10.6434"E		04 Feb 2012	C1
XT046		Indonesia	N Sulawesi, Lembeh, Tanjung Labuhankompeni	1° 25' 55.848"N, 125° 11' 10.6434"E		04 Feb 2012	C1
XT047		Indonesia	N Sulawesi, Lembeh, Tanjung Labuhankompeni	1° 25' 55.848"N, 125° 11' 10.6434"E		04 Feb 2012	C1
XT048		Indonesia	N Sulawesi, Lembeh, Tanjung Labuhankompeni	1° 25' 55.848"N, 125° 11' 10.6434"E		04 Feb 2012	C1

Supplementary material 2.1: List of *Xestospongia testudinaria* and *X. bergquistia* I3-M11 cox1 haplotypes

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
XT054		Indonesia	N Sulawesi, Lembeh, Kelapadua	1° 26' 8.3754"N, 125° 12' 34.0914"E		04 Feb 2012	C1
XT066		Indonesia	N Sulawesi, Lembeh, Lobangbatu	1° 26' 2.6514"N, 125° 12' 9.7194"E		06 Feb 2012	C1
XT070		Indonesia	N Sulawesi, Lembeh, SW Sarena Kecil	1° 27' 19.836"N, 125° 13' 25.0314"E		07 Feb 2012	C1
XT072		Indonesia	N Sulawesi, Lembeh, SW Sarena Kecil	1° 27' 19.836"N, 125° 13' 25.0314"E		07 Feb 2012	C1
XT073		Indonesia	N Sulawesi, Lembeh, SW Sarena Kecil	1° 27' 19.836"N, 125° 13' 25.0314"E		07 Feb 2012	C1
XT074		Indonesia	N Sulawesi, Lembeh, SW Sarena Kecil	1° 27' 19.836"N, 125° 13' 25.0314"E		07 Feb 2012	C1
XT083		Indonesia	N Sulawesi, Lembeh, Teluk Rarandam	1° 27' 3.2034"N, 125° 14' 17.52"E		08 Feb 2012	C1
XT084		Indonesia	N Sulawesi, Lembeh, Teluk Rarandam	1° 27' 3.2034"N, 125° 14' 17.52"E		08 Feb 2012	C1
XT087		Indonesia	N Sulawesi, Lembeh, Teluk Rarandam	1° 27' 3.2034"N, 125° 14' 17.52"E		08 Feb 2012	C1
XT088		Indonesia	N Sulawesi, Lembeh, Teluk Rarandam	1° 27' 3.2034"N, 125° 14' 17.52"E		08 Feb 2012	C1
XT095		Indonesia	N Sulawesi, Lembeh, Kelapadua	1° 26' 19.068"N, 125° 12' 48.996"E		04 Feb 2012	C1
XT097		Indonesia	N Sulawesi, Lembeh, Kelapadua	1° 26' 19.068"N, 125° 12' 48.996"E		04 Feb 2012	C1
XT106		Indonesia	N Sulawesi, Lembeh, Tanjung Kungkungan	1° 27' 58.392"N, 125° 14' 2.2554"E		10 Feb 2012	C1
XT110		Indonesia	N Sulawesi, Lembeh, Pulau Abadi	1° 26' 1.0314"N, 125° 12' 22.284"E		03 Feb 2012	C1
XT111		Indonesia	N Sulawesi, Lembeh, Pulau Abadi	1° 26' 1.0314"N, 125° 12' 22.284"E		03 Feb 2012	C1
XT113		Indonesia	N Sulawesi, Lembeh, Tanjung Kuning	1° 23' 10.788"N, 125° 10' 23.2314"E		11 Feb 2012	C1
XT117		Indonesia	N Sulawesi, Lembeh, Tanjung Kuning	1° 23' 10.788"N, 125° 10' 23.2314"E		11 Feb 2012	C1
XT120		Indonesia	N Sulawesi, Lembeh, Tanjung Pandea	1° 23' 52.6914"N, 125° 09' 58.932"E		11 Feb 2012	C1
XT130		Indonesia	N Sulawesi, Lembeh, N Pulau Dua	1° 23' 28.6434"N, 125° 12' 58.7154"E		13 Feb 2012	C1
XT131		Indonesia	N Sulawesi, Lembeh, S Pulau Dua	1° 23' 17.016"N, 125° 12' 43.1274"E		13 Feb 2012	C1
XT132		Indonesia	N Sulawesi, Lembeh, S Pulau Dua	1° 23' 17.016"N, 125° 12' 43.1274"E		13 Feb 2012	C1
XT133		Indonesia	N Sulawesi, Lembeh, S Pulau Dua	1° 23' 17.016"N, 125° 12' 43.1274"E		13 Feb 2012	C1

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
XT134		Indonesia	N Sulawesi, Lembeh, S Pulau Dua	1° 23' 17.016"N, 125° 12' 43.1274"E		13 Feb 2012	C1
XT135		Indonesia	N Sulawesi, Lembeh, S Pulau Dua	1° 23' 17.016"N, 125° 12' 43.1274"E		13 Feb 2012	C1
XT136		Indonesia	N Sulawesi, Lembeh, S Pulau Dua	1° 23' 17.016"N, 125° 12' 43.1274"E		13 Feb 2012	C1
XT140		Indonesia	N Sulawesi, Lembeh, N Tanjung Pandean	1° 24' 21.7074"N, 125° 10' 4.5114"E		14 Feb 2012	C1
XT149		Indonesia	N Sulawesi, Lembeh, Desa Pandean	1° 25' 16.068"N, 125° 10' 52.6794"E		14 Feb 2012	C1
XT151		Indonesia	N Sulawesi, Lembeh, Teluk Walemetodo	1° 24' 11.3394"N, 125° 10' 20.3154"E		15 Feb 2012	C1
XT157		Indonesia	N Sulawesi, Lembeh, Tanjung Kelapasatu	1° 25' 38.568"N, 125° 11' 0.7794"E		15 Feb 2012	C1
XT160		Indonesia	N Sulawesi, Lembeh, Tanjung Kusukusu	1° 27' 13.7514"N 125° 14' 12.948"E		16 Feb 2012	C1
XT167		Indonesia	N Sulawesi, Lembeh, N Sarena Kecil	1° 27' 26.8554"N, 125° 13' 37.5954"E		16 Feb 2012	C1
XT168		Indonesia	N Sulawesi, Lembeh, N Sarena Kecil	1° 27' 26.8554"N, 125° 13' 37.5954"E		16 Feb 2012	C1
XT169		Indonesia	N Sulawesi, Lembeh, N Sarena Kecil	1° 27' 26.8554"N, 125° 13' 37.5954"E		16 Feb 2012	C1
XT172		Indonesia	N Sulawesi, Lembeh, N Sarena Kecil	1° 27' 26.8554"N, 125° 13' 37.5954"E		16 Feb 2012	C1
XT173		Indonesia	N Sulawesi, Lembeh, N Sarena Kecil	1° 27' 26.8554"N, 125° 13' 37.5954"E		16 Feb 2012	C1
XT177		Indonesia	N Sulawesi, Lembeh, W Sarena Kecil	1° 27' 25.5234"N, 125° 13' 31.1874"E		16 Feb 2012	C1
XT178		Indonesia	N Sulawesi, Lembeh, W Sarena Kecil	1° 27' 25.5234"N, 125° 13' 31.1874"E		17 Feb 2012	C1
XT180		Indonesia	N Sulawesi, Lembeh, W Sarena Kecil	1° 27' 25.5234"N, 125° 13' 31.1874"E		17 Feb 2012	C1
XT182		Indonesia	N Sulawesi, Lembeh, Batu Kapal	1° 32' 56.832"N, 125° 17' 31.848"E		18 Feb 2012	C1
XT183		Indonesia	N Sulawesi, Lembeh, Batu Kapal	1° 32' 56.832"N, 125° 17' 31.848"E		18 Feb 2012	C1
XT185		Indonesia	N Sulawesi, Lembeh, Batu Kapal	1° 32' 56.832"N, 125° 17' 31.848"E		18 Feb 2012	C1
XT186		Indonesia	N Sulawesi, Lembeh, Batu Kapal	1° 32' 56.832"N, 125° 17' 31.848"E		18 Feb 2012	C1
XT187		Indonesia	N Sulawesi, Lembeh, Batu Kapal	1° 32' 56.832"N, 125° 17' 31.848"E		18 Feb 2012	C1
XT188		Indonesia	N Sulawesi, Lembeh, Batu Kapal	1° 32' 56.832"N, 125° 17' 31.848"E		18 Feb 2012	C1

Supplementary material 2.1: List of *Xestospongia testudinaria* and *X. bergquistia* I3-M11 cox1 haplotypes

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
XT190		Indonesia	N Sulawesi, Lembeh, Batu Kapal	1° 32' 56.832"N, 125° 17' 31.848"E		18 Feb 2012	C1
XT192		Indonesia	N Sulawesi, Lembeh, Pulau Putus	1° 31' 20.7474"N, 125° 16' 37.2714"E		18 Feb 2012	C1
XT198		Indonesia	N Sulawesi, Lembeh, Pulau Putus	1° 31' 20.7474"N, 125° 16' 37.2714"E		18 Feb 2012	C1
XT199		Indonesia	N Sulawesi, Lembeh, Pulau Putus	1° 31' 20.7474"N, 125° 16' 37.2714"E		18 Feb 2012	C1
GW1238	BSPG 2014 XIV 1 (GW1238)	Indonesia	E Java, Situbondo, Pantai Pecaron, Takat Palapa, E atol	7° 40' 52" S, 113° 51' 08" E		09 May 2011	C2
GW1326	BSPG 2014 XIV 8 (GW1326)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Menjagan Kecil, W side	5° 53' 22" S, 110° 24' 18" E		26 May 2011	C2
GW1327	BSPG 2014 XIV 9 (GW1327)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Menjagan Kecil, N side	5° 53' 22" S, 110° 24' 18" E		26 May 2011	C2
GW1328	BSPG 2014 XIV 10 (GW1328)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Menjagan Kecil, E side	5° 53' 22" S, 110° 24' 18" E		26 May 2011	C2
GW1338	BSPG 2014 XIV 11 (GW1338)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Sintok, W side	5° 47' 06" S, 110° 30' 18" E		27 May 2011	C2
GW1339	BSPG 2014 XIV 12 (GW1339)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Sintok, N side	5° 47' 06" S, 110° 30' 18" E		27 May 2011	C2
GW1340	BSPG 2014 XIV 13 (GW1340)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Sintok, E side	5° 47' 06" S, 110° 30' 18" E		27 May 2011	C2
GW1477	BSPG 2014 XIV 18 (GW1477)	Indonesia	C. Java, Kep Karimun Jawa, Takat Menjangan Kecil, E side	5° 53' 56" S, 110° 24' 54" E		27 May 2011	C2
GW18547	G305387	Australia	Queensland, Gannet Cay,S side reef, fore-reef slope, Swain Rfs	21° 59' 05" S, 152° 28' 06" E	24m	23 Jul 1995	C2
GW18642	G314838	Australia	Queensland, Alcyonarian Point, Hook Island, Whitsunday Grp	20° 3' 56" S, 149° 55' 24" E	15m	03 June 1999	C2

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
GW18740	G317830	Australia	Queensland, Mackerel Reef, Swain Reefs	21° 50' 10" S, 151° 58' 19" E	30m	12 Feb 2002	C2
GW18743	G321612	Australia	Queensland, East Heron Is	23° 26' 04" S, 151° 56' 20" E	5-17m	09 Nov 2004	C2
GW19046	G323994	Solomon Islands	Three sisters, the island most north	10° 9' 04" S, 161° 55' 04" E	15-30m	20 June 2009	C2
GW2994	BMNH 1881.10.21.267	Australia	Queensland, Port Denison				C2
GW19049	G322086	Australia	Queensland, Cayley Reef	17° 29' 11" S, 146° 26' 28" E	11.9-12m	29 Nov 2008	C2
GW2054		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26 Jul 2011	C2
GW2055		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26 Jul 2011	C2
GW4793		Indonesia	North Sulawesi, Pulau Lembeh, Bitung	1° 27' 19"N, 125° 13' 25"E		07 Feb 2012	C2
GW4796		Indonesia	North Sulawesi, Pulau Lembeh, Bitung	1° 28' 25"N, 125° 14' 2"E		01 Feb 2012	C2
GW4797		Indonesia	North Sulawesi, Pulau Lembeh, Bitung	1° 27' 40"N, 125° 13' 36"E		30 Jan 2012	C2
GW4853	ZMA POR18701	Thailand	Khao Gek, West of Ko Samet, Samet Islands, Rayong	12° 31' 01" N, 101° 26' 46" E		28 Oct 2005	C2
GW7149		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2
GW7150		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2
GW7151		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2
GW7152		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2
GW7153		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2

Supplementary material 2.1: List of *Xestospongia testudinaria* and *X. bergquistia* I3-M11 cox1 haplotypes

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
GW7154		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2
GW7155		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2
GW7157		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2
GW7159		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2
GW7160		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C2
GW7164		Taiwan	Cingwan, Penghu island	23°31'45. 23"N, 119°33'14. 07"E			C2
GW7165		Taiwan	Cingwan, Penghu island	23°31'45. 23"N, 119°33'14. 07"E			C2
GW7167		Taiwan	Fongguei, Penghu island	23°32'15. 42"N, 119°32'39. 26"E			C2
GW7168		Taiwan	Fongguei, Penghu island	23°32'15. 42"N, 119°32'39. 26"E			C2
GW7169		Taiwan	Fongguei, Penghu island	23°32'15. 42"N, 119°32'39. 26"E			C2
XT001		Indonesia	N Sulawesi, Lembeh, Tanjung Nanas I	1° 27' 40.428"N, 125° 13' 36.408"E		30 Jan 2012	C2
XT002		Indonesia	N Sulawesi, Lembeh, Tanjung Nanas I	1° 27' 40.428"N, 125° 13' 36.408"E		30 Jan 2012	C2
XT103		Indonesia	N Sulawesi, Lembeh, Tanjung Kungkungan	1° 27' 58.392"N, 125° 14' 2.2554"E		10 Feb 2012	C2
XT170		Indonesia	N Sulawesi, Lembeh, N Sarena Kecil	1° 27' 26.8554"N, 125° 13' 37.5954"E		16 Feb 2012	C2
XT179		Indonesia	N Sulawesi, Lembeh, N Sarena Kecil	1° 27' 25.5234"N, 125° 13' 31.1874"E		17 Feb 2012	C2
GW1239	BSPG 2014 XIV 2 (GW1239)	Indonesia	E Java, Situbondo, Pantai Pecaron, Takat palapa, S atol	7° 40' 52" S, 113° 51' 08" E		09 May 2011	C4
GW1246	BSPG 2014 XIV 4 (GW1246)	Indonesia	E Java, Situbondo, Pantai Pecaron, Karang mayit, E fringing reef	7° 40' 52" S, 113° 51' 08" E		09 May 2011	C4

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
GW1248	BSPG 2014 XIV 6 (GW1248)	Indonesia	E Java, Situbondo, Pantai Pecaron, Karang mayit, S fringing reef	7° 41' 13" S, 113° 49' 38" E		09 May 2011	C4
GW1344	BSPG 2014 XIV 15 (GW1344)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Sintok, E Side	5° 47' 06" S, 110° 30' 18" E		27 May 2011	C4
GW2323	BSPG 2014 XIV 25 (GW2323)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		07 Aug 2011	C4
GW2331	BSPG 2014 XIV 26 (GW2331)	Indonesia	SE Sulawesi, Wakatobi, Tomia, NW P. Sawa	5° 45' 00" S, 123° 53' 33" E		14 Aug 2011	C4
GW4801		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 40"N, 125° 13' 36"E		30 Jan 2012	C4
GW4847	ZMA POR18745	Thailand	Ko Rad, Kood Islands, Trad	11° 57' 21" N, 102° 21' 46" E		08 May 2012	C4
GW4858	ZMA POR18632	Thailand	West side of Ko Khrok, Ko Lan Islands, Pattaya, Chonburi	12° 55' 13" N, 100° 48' 27" E		26 Sept 2005	C4
GW4884	ZMA POR8275	Indonesia	Sumba, NE coast of Sumba, E of Melolo	9° 54' 00" S, 120° 42' 30" E	6m	14 Sept 1988	C4
GW7161		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C4
GW7166		Taiwan	Cingwan, Penghu island	23°31'45. 23"N, 119°33'14. 07"E			C4
GW1247	BSPG 2014 XIV 5 (GW1247)	Indonesia	E Java, Situbondo, Pantai Pecaron, Karang Mayit, S fringing reef	7° 41' 13" S, 113° 49' 38" E		09 May 2011	C5
GW1249	BSPG 2014 XIV 7 (GW1249)	Indonesia	E Java, Situbondo, Pantai Pecaron, Karang Mayit, S fringing reef	7° 41' 13" S, 113° 49' 38" E		09 May 2011	C5
GW1408	BSPG 2014 XIV 16 (GW1408)	Indonesia	W Bali, Menjangan Island.	8° 5' 55" S, 114° 30' 43" E		01 May 2011	C5
GW1411	BSPG 2014 XIV 17 (GW1411)	Indonesia	W Bali, Menjangan Island.	8° 5' 55" S, 114° 30' 43" E		01 May 2011	C5

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Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
GW2152		Indonesia	W Java, Thousand Island, Lancang Besar	5° 55' 27" S, 106° 34' 51" E		01 Aug 2011	C5
GW2186		Indonesia	W Java, Thousand Island, Tidung Besar	5° 47' 28" S, 106° 28' 42" E		31 Jul 2011	C5
GW2238		Indonesia	W Java, Thousand Island, Pulau Kelapa	5° 39' 20" S, 106° 33' 27" E		02 Aug 2011	C5
GW2300		Indonesia	W Java, Thousand Island, Dapur	5° 55' 23" S, 106° 43' 23" E		05 Aug 2011	C5
GW2303		Indonesia	W Java, Thousand Island, Dapur	5° 55' 23" S, 106° 43' 23" E		05 Aug 2011	C5
GW2316	BSPG 2014 XIV 21 (GW2316)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08 Aug 2011	C5
GW2317	BSPG 2014 XIV 22 (GW2317)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08 Aug 2011	C5
GW2322	BSPG 2014 XIV 24 (GW2322)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08 Aug 2011	C5
GW4790		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 25"N, 125° 13' 31" E		17 Feb 2012	C5
GW4792		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 19"N, 125° 13' 25"E		07 Feb 2012	C5
GW4794		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 40"N, 125° 13' 36"E		30 Jan 2012	C5
GW4799		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 26' 8"N, 125° 12' 34"E		04 Feb 2012	C5
GW4800		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 19"N, 125° 13' 25"E		07 Feb 2012	C5
GW4823	ZMA POR22222.10	Saudi Arabia	FSAR reef	22° 13' 50" N, 39° 1' 44" E	12-14m	08 May 2012	C5
GW4824	ZMA POR22222.2	Saudi Arabia	FSAR reef	22° 13' 50" N, 39° 1' 44" E	12-14m	08 May 2012	C5
GW4825	ZMA POR22222	Saudi Arabia	FSAR reef	22° 13' 50" N, 39° 1' 44" E	12-14m	08 May 2012	C5
GW7158		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C5

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
GW7172		Taiwan	Fongguei, Penghu island	23°32'15. 42"N, 119°32'39. 26"E			C5
XT006		Indonesia	N Sulawesi, Lembeh, SE Sarena Kecil	1° 27' 15.804"N, 125° 13' 29.5314"E		30 Jan 2012	C5
XT012		Indonesia	N Sulawesi, Lembeh, Tanjung Mawali	1° 26' 36.4194"N, 125° 13' 45.9834"E		31 Jan 2012	C5
XT017		Indonesia	N Sulawesi, Lembeh, Tanjung Nanas II	1° 27' 43.6674"N, 125° 13' 41.6274"E		01 Feb 2012	C5
XT018		Indonesia	N Sulawesi, Lembeh, Tanjung Nanas II	1° 27' 43.6674"N, 125° 13' 41.6274"E		01 Feb 2012	C5
XT023		Indonesia	N Sulawesi, Lembeh, Pantai Perigi	1° 28' 10.0194"N, 125° 14' 38.796"E		02 Feb 2012	C5
XT030		Indonesia	N Sulawesi, Lembeh, Tanjung Nanas I	1° 27' 40.212"N, 125° 13' 36.408"E		30 Jan 2012	C5
XT031		Indonesia	N Sulawesi, Lembeh, Tanjung Nanas I	1° 27' 40.212"N, 125° 13' 36.408"E		30 Jan 2012	C5
XT032		Indonesia	N Sulawesi, Lembeh, Pulau Abadi	1° 26' 0.744"N, 125° 12' 22.608"E		03 Feb 2012	C5
XT038		Indonesia	N Sulawesi, Lembeh, Pulau Abadi	1° 26' 0.744"N, 125° 12' 22.608"E		03 Feb 2012	C5
XT039		Indonesia	N Sulawesi, Lembeh, Pulau Abadi	1° 26' 0.744"N, 125° 12' 22.608"E		03 Feb 2012	C5
XT040		Indonesia	N Sulawesi, Lembeh, Tanjung Labuhankompeni	1° 25' 55.848"N, 125° 11' 10.6434"E		04 Feb 2012	C5
XT049		Indonesia	N Sulawesi, Lembeh, Kelapadua	1° 26' 8.3754"N, 125° 12' 34.0914"E		04 Feb 2012	C5
XT051		Indonesia	N Sulawesi, Lembeh, Kelapadua	1° 26' 8.3754"N, 125° 12' 34.0914"E		04 Feb 2012	C5
XT057		Indonesia	N Sulawesi, Lembeh, Baturiri	1° 27' 34.704"N, 125° 14' 23.1"E		06 Feb 2012	C5
XT058		Indonesia	N Sulawesi, Lembeh, Baturiri	1° 27' 34.704"N, 125° 14' 23.1"E		06 Feb 2012	C5
XT071		Indonesia	N Sulawesi, Lembeh, SW Sarena Kecil	1° 27' 19.836"N, 125° 13' 25.0314"E		07 Feb 2012	C5
XT075		Indonesia	N Sulawesi, Lembeh, Lobangbatu Besar	1° 25' 49.404"N, 125° 11' 26.808"E		07 Feb 2012	C5
XT076		Indonesia	N Sulawesi, Lembeh, Lobangbatu Besar	1° 25' 49.404"N, 125° 11' 26.808"E		07 Feb 2012	C5
XT078		Indonesia	N Sulawesi, Lembeh, Lobangbatu Besar	1° 25' 49.404"N, 125° 11' 26.808"E		07 Feb 2012	C5
XT089		Indonesia	N Sulawesi, Lembeh, Teluk Rarandam	1° 27' 3.2034"N, 125° 14' 17.52"E		08 Feb 2012	C5
XT090		Indonesia	N Sulawesi, Lembeh, Teluk Makawide	1° 29' 5.0634"N, 125° 14' 26.1234"E		09 Feb 2012	C5
XT091		Indonesia	N Sulawesi, Lembeh, Teluk Makawide	1° 29' 5.0634"N, 125° 14' 26.1234"E		09 Feb 2012	C5

Supplementary material 2.1: List of *Xestospongia testudinaria* and *X. bergquistia* I3-M11 cox1 haplotypes

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
XT092		Indonesia	N Sulawesi, Lembeh, Teluk Makawide	1° 29' 5.0634"N, 125° 14' 26.1234"E		09 Feb 2012	C5
XT093		Indonesia	N Sulawesi, Lembeh, Teluk Makawide	1° 29' 5.0634"N, 125° 14' 26.1234"E		09 Feb 2012	C5
XT098		Indonesia	N Sulawesi, Lembeh, Kelapadua	1° 26' 19.068"N, 125° 12' 48.996"E		04 Feb 2012	C5
XT115		Indonesia	N Sulawesi, Lembeh, Tanjung Kuning	1° 23' 10.788"N, 125° 10' 23.2314"E		11 Feb 2012	C5
XT123		Indonesia	N Sulawesi, Lembeh, Tanjung Pandea	1° 23' 52.6914"N, 125° 09' 58.932"E		11 Feb 2012	C5
XT125		Indonesia	N Sulawesi, Lembeh, Tanjung Pandea	1° 23' 52.6914"N, 125° 09' 58.932"E		11 Feb 2012	C5
XT126		Indonesia	N Sulawesi, Lembeh, N Pulau Dua	1° 23' 28.6434"N, 125° 12' 58.7154"E		13 Feb 2012	C5
XT127		Indonesia	N Sulawesi, Lembeh, N Pulau Dua	1° 23' 28.6434"N, 125° 12' 58.7154"E		13 Feb 2012	C5
XT128		Indonesia	N Sulawesi, Lembeh, N Pulau Dua	1° 23' 28.6434"N, 125° 12' 58.7154"E		13 Feb 2012	C5
XT139		Indonesia	N Sulawesi, Lembeh, N Tanjung Pandean	1° 24' 21.7074"N, 125° 10' 4.5114"E		14 Feb 2012	C5
XT143		Indonesia	N Sulawesi, Lembeh, N Tanjung Pandean	1° 24' 21.7074"N, 125° 10' 4.5114"E		14 Feb 2012	C5
XT144		Indonesia	N Sulawesi, Lembeh, Desa Pandean	1° 25' 16.068"N, 125° 10' 52.6794"E		14 Feb 2012	C5
XT145		Indonesia	N Sulawesi, Lembeh, Desa Pandean	1° 25' 16.068"N, 125° 10' 52.6794"E		14 Feb 2012	C5
XT150		Indonesia	N Sulawesi, Lembeh, Desa Pandean	1° 25' 16.068"N, 125° 10' 52.6794"E		14 Feb 2012	C5
XT152		Indonesia	N Sulawesi, Lembeh, Tanjung Kelapasatu	1° 25' 38.568"N, 125° 11' 0.7794"E		15 Feb 2012	C5
XT153		Indonesia	N Sulawesi, Lembeh, Tanjung Kelapasatu	1° 25' 38.568"N, 125° 11' 0.7794"E		15 Feb 2012	C5
XT154		Indonesia	N Sulawesi, Lembeh, Tanjung Kelapasatu	1° 25' 38.568"N, 125° 11' 0.7794"E		15 Feb 2012	C5
XT155		Indonesia	N Sulawesi, Lembeh, Tanjung Kelapasatu	1° 25' 38.568"N, 125° 11' 0.7794"E		15 Feb 2012	C5
XT156		Indonesia	N Sulawesi, Lembeh, Tanjung Kelapasatu	1° 25' 38.568"N, 125° 11' 0.7794"E		15 Feb 2012	C5
XT158		Indonesia	N Sulawesi, Lembeh, Tanjung Kelapasatu	1° 25' 38.568"N, 125° 11' 0.7794"E		15 Feb 2012	C5
XT162		Indonesia	N Sulawesi, Lembeh, Tanjung Kusukusu	1° 27' 13.7514"N 125° 14' 12.948"E		16 Feb 2012	C5
XT193		Indonesia	N Sulawesi, Lembeh, Pulau Putus	1° 31' 20.7474"N, 125° 16' 37.2714"E		18 Feb 2012	C5
XT196		Indonesia	N Sulawesi, Lembeh, Pulau Putus	1° 31' 20.7474"N, 125° 16' 37.2714"E		18 Feb 2012	C5

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplo-type
GW2306		Indonesia	W Java, Thousand Island, Untung Jawa	5° 58' 26" S, 106° 42' 12" E		08 Sep 2011	C5
GW1240	BSPG 2014 XIV 3 (GW1240)	Indonesia	E Java, Situbondo, Pantai Pecaron, Takat Palapa, S side Atol	7° 40' 52" S, 113° 51' 08" E		09 May 2011	C6
GW1341	BSPG 2014 XIV 14 (GW1341)	Indonesia	C Java, Kep Karimun Jawa, Pulau Sintok, S side	5° 47' 06" S, 110° 30' 18" E		27 May 2011	C6
GW2149		Indonesia	W Java, Thousand Island, Bokor	5° 56' 32" S, 106° 37' 39" E		01 Aug 2011	C6
GW2150		Indonesia	W Java, Thousand Island, Lancang Besar	5° 55' 27" S, 106° 34' 51" E		01 Aug 2011	C6
GW2151		Indonesia	W Java, Thousand Island, Bokor	5° 56' 32" S, 106° 37' 39" E		01 Aug 2011	C6
GW2211		Indonesia	W Java, Thousand Island, Hantu Besar NW	5° 31' 50" S, 106° 32' 19" E		09 Aug 2011	C6
GW2212		Indonesia	W Java, Thousand Island, Hantu Besar NW	5° 31' 50" S, 106° 32' 19" E		09 Aug 2011	C6
GW2299		Indonesia	W Java, Thousand Island, Untung Jawa	5° 58' 26" S, 106° 42' 12" E		05 Aug 2011	C6
XT007		Indonesia	N Sulawesi, Lembeh, E Sarena Besar	1° 27' 34.1634"N, 125° 14' 1.896"E		31 Jan 2012	C6
XT011		Indonesia	N Sulawesi, Lembeh, Tanjung Mawali	1° 26' 36.4194"N, 125° 13' 45.9834"E		31 Jan 2012	C6
XT056		Indonesia	N Sulawesi, Lembeh, Baturiri	1° 27' 34.704"N, 125° 14' 23.1"E		06 Feb 2012	C6
XT064		Indonesia	N Sulawesi, Lembeh, Lobangbatu	1° 26' 2.6514"N, 125° 12' 9.7194"E		06 Feb 2012	C6
XT069		Indonesia	N Sulawesi, Lembeh, SW Sarena Kecil	1° 27' 19.836"N, 125° 13' 25.0314"E		07 Feb 2012	C6
XT164		Indonesia	N Sulawesi, Lembeh, Tanjung Kusukusu	1° 27' 13.7514"N 125° 14' 12.948"E		16 Feb 2012	C6
XT181		Indonesia	N Sulawesi, Lembeh, W Sarena Kecil	1° 27' 25.5234"N, 125° 13' 31.1874"E		17 Feb 2012	C6
GW7148		Tanzania	Octopus Wall & Laura's Garden, just off the Dar es Salaam Yacht Club	6° 47' 0"S, 39° 17' 300"E		24 Oct 2012	C7

Supplementary material 2.1: List of *Xestospongia testudinaria* and *X. bergquistia* I3-M11 cox1 haplotypes

Xestospongia bergquistia samples

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplotype
GW18652	G314887	Australia	Queensland, Cateran Bay, Border Island, Whitsunday Grp	20° 9' 09" S, 149° 2' 32" E	30m	04 Jun 1999	C2
GW18394	G303438	Australia	Northern Territory, Fish Reef, W side near Brisbane, Bynoe Harbour	12° 26' 01" S, 130° 26' 05" E	11m	26 Sep 1993	C5
GW18746	G321736	Australia	Queensland, Eastern side, Polmaise Reef	23° 34' 08" S, 151° 42' 47" E	5-14m	11 Nov 2011	C5
GW18494	G314515	Australia	Queensland, Stanley Reef on SW corner	19° 18' 50" S, 148° 2' 34" E	30m	25 Jan 1999	C5
	G25018 (Holotype)	Australia	Queensland, Pioneer Bay, Orpheus Island, GBR	18° 36' S, 146° 29' 34 E	15m	18 Feb 1987	C5
GW18502	G313133	Singapore	Pulau Tekukor, Buran Channel	1° 13' 08" N, 103° 50' 05" E	13m	02 May 1997	C5

3.1. List of samples that are utilised in chapter 3 and sorted by the country and localilities

Legend

- A1- A6: ATP6 haplotypes recovered in this study following Swierts et al. (2013).
- C1-C6: I3-M11 haplotypes recovered in this study following Swierts et al. (2013).
- β1- β29: ATPS- β intron haplotypes recovered in this study. The presence of two haplotypes in one sample indicates allele heterozygosity.
- X: samples that could neither be amplified with the selected primers nor their haplotypes could be determined due to a PHASE value <0.900 (Flot 2010). Therefore, these samples were excluded from the analysis.
- GW and XT are internal voucher numbers of the Molecular Geo- & Palaeobiology Lab, Department of Earth and Environmental Sciences, Palaeontology & Geobiology, Ludwig-Maximilians -Universität München, Germany and Naturalis Biodiversity Centre Leiden, The Netherlands.
- ZMA POR numbers belong to samples of the Zoological Museum Amsterdam, which is currently merged into Naturalis Biodiversity Centre Leiden, The Netherlands.
- G numbers belong to samples of the Queensland Museum, Brisbane, Australia.
- BSPG numbers belong to samples of the Bavarian State Collection for Palaeontology and Geology, Munich, Germany.
- Haplotype numeration (C1-C6) follows Swierts et al. 2013

Voucher numbers	Museum code	Country	Localities	Coordinates	Date collection	ATP6 haplotypes	I3-M11 haplotypes	ATPS-β intron haplotypes
GW19049	G322086	Australia	Queensland, Cayley Reef	17° 29' 11" S, 146° 26' 28" E	20 June 2009	A1	C2	β19, β20
GW18642	G314838	Australia	Queensland, Alcyonarian Point, Hook Island, Whitsunday Grp	20° 3' 56" S, 149° 55' 24" E	03 June 1999	A1	C2	β19
GW18743	G321612	Australia	Queensland, East Heron Is	23° 26' 04" S, 151° 56' 20" E	09 Nov 2004	A1	C2	β19
GW18740	G317830	Australia	Queensland, Mackerel Reef, Swain Reefs	21° 50' 10" S, 151° 58' 19" E	12 Feb 2002	A1	C2	β19, β20
GW18575	G306508	Australia	Queensland, South side Porpoise Cay	22° 11' 09" S, 155° 21' 04" E	11 Jan 1996	A6	X	β20
GW18547	G305387	Australia	Queensland, Gannet Cay, S side reef, fore-reef slope, Swain Rfs	21° 59' 05" S, 152° 28' 06" E	23 Jul 1995	A1	C2	β4, β19

Supplementary material 5.1: List of *Xestospongia testudinaria* ATP6, I3-M11 cox1 and ATPS- β intron haplotypes

Voucher numbers	Museum code	Country	Localities	Coordinates	Date collection	ATP6 haplotypes	I3-M11 haplotypes	ATPS-β intron haplotypes
GW1328	BSPG 2014 XIV 10 (GW1328)	Indonesia	C Java, Kep Karimun Jawa, Pulau Menjagan Kecil, East side	5° 53' 22" S, 110° 24' 18" E	26 May 2011	A1	C2	β4
GW1327	BSPG 2014 XIV 9 (GW1327)	Indonesia	C Java, Kep Karimun Jawa, Pulau Menjagan Kecil, North side	5° 53' 22" S, 110° 24' 18" E	26 May 2011	A1	C2	β4
GW1326	BSPG 2014 XIV 8 (GW1326)	Indonesia	C Java, Kep Karimun Jawa, Pulau Menjagan Kecil, West side	5° 53' 22" S, 110° 24' 18" E	26 May 2011	A1	C2	β3
GW1340	BSPG 2014 XIV 13 (GW1340)	Indonesia	C Java, Kep Karimun Jawa, Pulau Sintok, East side	5° 47' 06" S, 110° 30' 18" E	27 May 2011	A1	C2	β3
GW1344	BSPG 2014 XIV 15 (GW1344)	Indonesia	C Java, Kep Karimun Jawa, Pulau Sintok, East side	5° 47' 06" S, 110° 30' 18" E	27 May 2011	A4	C4	β6
GW1339	BSPG 2014 XIV 12 (GW1339)	Indonesia	C Java, Kep Karimun Jawa, Pulau Sintok, North side	5° 47' 06" S, 110° 30' 18" E	27 May 2011	A1	C2	β5
GW1338	BSPG 2014 XIV 11 (GW1338)	Indonesia	C Java, Kep Karimun Jawa, Pulau Sintok, West side	5° 47' 06" S, 110° 30' 18" E	27 May 2011	A1	C2	β3
GW1477	BSPG 2014 XIV 18 (GW1477)	Indonesia	C Java, Kep Karimun Jawa, Takat Menjangan Kecil, East side	5° 53' 56" S, 110° 24' 54" E	27 May 2011	A1	C2	β3, β5
GW1246	BSPG 2014 XIV 4 (GW1246)	Indonesia	E Java, Pantai Pecaron Situbondo Jatim, Karang mayit, East side of fringing reef	7° 40' 52" S, 113° 51' 08" E	09 May 2011	A4	C4	β8
GW1247	BSPG 2014 XIV 5 (GW1247)	Indonesia	E Java, Pantai Pecaron Situbondo Jatim, Karang mayit, East side of fringing reef	7° 41' 13" S, 113° 49' 38" E	09 May 2011	A2	C5	β9
GW1248	BSPG 2014 XIV 6 (GW1248)	Indonesia	E Java, Pantai Pecaron Situbondo Jatim, Karang mayit, South side of fringing reef	7° 41' 13" S, 113° 49' 38" E	09 May 2011	A4	C4	β10, β11

Voucher numbers	Museum code	Country	Localities	Coordinates	Date collection	ATP6 haplotypes	I3-M11 haplotypes	ATPS-β intron haplotypes
GW1249	BSPG 2014 XIV 7 (GW1249)	Indonesia	E Java, Pantai Pecaron Situbondo Jatim, Karang mayit, South side of fringing reef	7° 41' 13" S, 113° 49' 38" E	09 May 2011	A2	C5	X
GW1238	BSPG 2014 XIV 1 (GW1238)	Indonesia	E Java, Pantai Pecaron Situbondo Jatim, Takat palapa, East side atoll	7° 40' 52" S, 113° 51' 08" E	09 May 2011	A1	C2	β5, β7
GW1239	BSPG 2014 XIV 2 (GW1239)	Indonesia	E Java, Pantai Pecaron Situbondo Jatim, Takat palapa, South side atoll	7° 40' 52" S, 113° 51' 08" E	09 May 2011	A4	C4	β8
GW1240	BSPG 2014 XIV 3 (GW1240)	Indonesia	E Java, Pantai Pecaron Situbondo Jatim, Takat palapa, South side atoll	7° 40' 52" S, 113° 51' 08" E	09 May 2011	A2	C6	β9
GW4791		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 25"N, 125° 13' 31" E	12 Feb 2012	A1	C1	β6
GW4792		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 19"N, 125° 13' 25"E	07 Feb 2012	A2	C5	β12, β13
GW4793		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 19"N, 125° 13' 25"E	07 Feb 2012	A1	C2	β14, β15
GW4794		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 40"N, 125° 13' 36"E	30 Jan 2012	A2	C5	β9
GW4795		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 28' 10"N, 125° 14' 38"E	02 Feb 2012	A1	C1	β7
GW4796		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 28' 25"N, 125° 14' 2"E	01 Feb 2012	A1	C2	β7
GW4798		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 26' 8"N, 125° 12' 34"E	04 Feb 2012	A1	C1	β7
GW4799		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 26' 8"N, 125° 12' 34"E	04 Feb 2012	A2	C5	β16, β17
GW4800		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 19"N, 125° 13' 25"E	07 Feb 2012	A2	C5	β13, β16
GW4801		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 40"N, 125° 13' 36"E	30 Jan 2012	A4	C4	β18
GW4885	ZMA POR8250	Indonesia	S Sulawesi, Tukang Besi islands, Southern reef of Karang Kaledupa, East of entrance	5° 55' 60" S, 123° 47' 60" E	07 Sep 1988	A1	C1	β7

Supplementary material 5.1: List of *Xestospongia testudinaria* ATP6, I3-M11 cox1 and ATPS- β intron haplotypes

Voucher numbers	Museum code	Country	Localities	Coordinates	Date collection	ATP6 haplotypes	I3-M11 haplotypes	ATPS-β intron haplotypes
GW2321	BSPG 2014 XIV 23 (GW2321)	Indonesia	SE Sulawesi, Wakatobi, Hoga, Pak Kasim's	GPS not available	07 Aug 2011	A1	C1	β7
GW2315	BSPG 2014 XIV 20 (GW2315)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E	08 Aug 2011	A1	C1	β7
GW2316	BSPG 2014 XIV 21 (GW2316)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E	08 Aug 2011	A2	C5	X
GW2317	BSPG 2014 XIV 22 (GW2317)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E	08 Aug 2011	A2	C5	X
GW2331	BSPG 2014 XIV 26 (GW2331)	Indonesia	SE Sulawesi, Wakatobi, Tomia, NW P. Sawa	5° 45' 00" S, 123° 53' 33" E	14 Aug 2011	A6	C4	β7
GW2332	BSPG 2014 XIV 27(GW2332)	Indonesia	SE Sulawesi, Wakatobi, Tomia, NW P. Sawa	5° 45' 00" S, 123° 53' 33" E	14 Aug 2011	A1	C1	β7
GW4884	ZMA POR8275	Indonesia	Sumba, NE coast of Sumba, E of Melolo	9° 54' 00" S, 120° 42' 30" E	14 Sep 1988	A3	C1	β21
GW1408	BSPG 2014 XIV 16 (GW1408)	Indonesia	W Bali, Menjangan Island.	8° 5' 55" S, 114° 30' 43" E	01 May 2011	A2	C5	β1
GW1411	BSPG 2014 XIV 17 (GW1411)	Indonesia	W Bali, Menjangan Island.	8° 5' 55" S, 114° 30' 43" E	01 May 2011	A2	C5	β1, β2
GW2054		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E	26 Jul 2011	A1	C2	β22, β24
GW2055		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E	26 Jul 2011	A1	C2	β24, β25
GW2149		Indonesia	W Java, Thousand Island, Bokor	5° 56' 32" S, 106° 37' 39" E	01 Aug 2011	A2	C6	β26
GW2151		Indonesia	W Java, Thousand Island, Bokor	5° 56' 32" S, 106° 37' 39" E	01 Aug 2011	A2	C6	β26, β28

Voucher numbers	Museum code	Country	Localities	Coordinates	Date collection	ATP6 haplotypes	I3-M11 haplotypes	ATPS-β intron haplotypes
GW2150		Indonesia	W Java, Thousand Island, Lancang Besar	5° 55' 27" S, 106° 34' 51" E	01 Aug 2011	A2	C6	β27
GW2152		Indonesia	W Java, Thousand Island, Lancang Besar	5° 55' 27" S, 106° 34' 51" E	01 Aug 2011	A2	C5	β29
GW4790		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 25"N, 125° 13' 31" E	17 Feb 2012	A2	C5	β2
GW2314	BSPG 2014 XIV 19 (GW2314)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E	08 Aug 2011	A1	C1	β7
GW4844	ZMA POR18773	Thailand	Hin Kum-Pan, Ko Rung, Chang Islands, Trad	11° 46' 36" N, 102° 23' 48" E	08 May 2012	A7	X	β20
GW4858	ZMA POR18632	Thailand	West side of Ko Khrok, Ko Lan Islands, Pattaya, Chonburi	12° 55' 13" N, 100° 48' 27" E	26 Sep 2005	A4	C4	X
GW4855	ZMA POR18688	Thailand	Hin Khan Na, West side of Ko Samet, Samet Islands, Rayong	12° 33' 14" N, 101° 28' 11" E	27 Oct 2005	A1	C1	β20
GW4856	ZMA POR18687	Thailand	Hin Khan Na, West side of Ko Samet, Samet Islands, Rayong	12° 33' 14" N, 101° 28' 11" E	08 May 2012	A1	C1	β22
GW4853	ZMA POR18701	Thailand	Khao Gek, West of Ko Samet, Samet Islands, Rayong	12° 31' 01" N, 101° 26' 46" E	28 Oct 2005	A1	C2	β20
GW4846	ZMA POR18747	Thailand	Ko Rad, Kood Islands, Trad	11° 57' 21" N, 102° 21' 46" E	08 May 2012	A1	C1	β7, β22
GW4847	ZMA POR18745	Thailand	Ko Rad, Kood Islands, Trad	11° 57' 21" N, 102° 21' 46" E	08 May 2012	A4	C4	β23
GW4861	ZMA POR18616	Thailand	Ko Thai-ta-Mun, South of Ko Si-chang, Chonburi	13° 6' 09" N, 100° 48' 28" E	08 May 2012	A5	C1	β10
GW4869	ZMA POR16708	Thailand	Pattaya, Klungbadan Island, Gulf of Thailand	12° 45' 00" N, 100° 52' 00" E	24 June 2009	A1	C1	β19, β20

Supplementary material 5.1: List of *Xestospongia testudinaria* ATP6, I3-M11 cox1 and ATPS- β intron haplotypes

Voucher numbers	Museum code	Country	Localities	Coordinates	Date collection	ATP6 haplotypes	I3-M11 haplotypes	ATPS-β intron haplotypes
GW4859	ZMA POR18627	Thailand	West side of Ko Khrok, Ko Lan Islands, Pattaya, Chonburi	12° 55' 13" N, 100° 48' 27" E	26 Sep 2005	A1	C1	β20
GW4848	ZMA POR18738	Thailand	West side of Ko Klum, Chang Islands, Trad	11° 55' 02" N, 102° 21' 43" E	8 May 2012	A1	C1	β22
Total number of samples that can be genotyped						60	58	**54**
Discrepancy						6		

3.2. Haplotype list and GenBank accession numbers

ATP6	Accession numbers		ATPS- β intron	Accession numbers	ATPS- β intron	Accession numbers
A1	KC424445		β1	KM030079	β16	KM030076
A2	KC424446		β2	KM030078	β17	KM030081
A4	KM014757		β3	KM030091	β18	KM030070
A5	KM014758		β4	KM030094	β19	KM030087
A6	KM014763		β5	KM030090	β20	KM030078
			β6	KM030083	β21	KM030088
I3-M11	Accession numbers		β7	KM030092	β22	KM030080
C1	KC424439		β8	KM030066	β23	KM030093
C2	KC424440		β9	KM030082	β24	KM030067
C4	KC424442		β10	KM030069	β25	KM030089
C5	KC424443		β11	KM030068	β26	KM030086
C6	KC424444		β12	KM030075	β27	KM030073
			β13	KM030077	β28	KM030074
			β14	KM030084	β29	KM030072
			β15	KM030085		

5.1. List of samples that are utilised in chapter 5 and sorted by the country and localities (chapter 5)

- C1- C3: recovered mtDNA cox2 haplotypes in this study.
- L1- L26: recovered LTRS intron haplotypes in this study. The presence of two haplotypes in one sample indicates allele heterozygosity.
- X: Unresolved heterozygote haplotypes due to low PHASE value (< 0.900, see Flot 2010). Therefore, these samples are excluded from the analysis to avoid discrepancy.
- GW refers to internal voucher numbers in the internal voucher numbers in The Molecular Geo- & Palaeobiology Lab, Department of Earth and Environmental Sciences, Palaeontology & Geobiology, Ludwig-Maximilians Universität München, Germany
- ZMA POR and RMNH numbers are accession numbers for samples that belong to the Zoological Museum Amsterdam and Rijksmuseum van Natuurlijke Historie Leiden, which are currently merged into the Naturalis Biodiversity Center, Leiden, The Netherlands
- G numbers are accession numbers for samples that belong to the Queensland Museum, Brisbane, Australia

Sample	Museum code	Country	Localities	Coordinates	Depth	Collection Date	Cox2 haplotypes	LTRS intron haplotypes
GW18429	G303302	Australia	Northern Territory, Dudley Point Reef, East Point, Darwin	12° 25' 03" S, 130° 49' 00" E		20 Sept 1999	C2	L12
GW18793	G315226	Australia	Queensland, Hook Reef lagoon	19° 45' 14" S, 149° 10' 45" E	9.4m	05 June 1999	C1	L15, L16
GW18806	G315374	Australia	Queensland, Stevens Reef, GBR	20° 32' 34" S, 150° 6' 26" E	30m	07 June 1999	C3	L17
GW18836	G321912	Australia	Queensland, Gibson Reef	17° 18' 21" S, 146° 20' 39" E	22.4-24m	27 Nov 2008	C1	L18
GW18840	G322076	Australia	Queensland, Shoal, NW of Farquharson Reef	17° 46' 48" S, 146° 28' 39" E	22.9-25m	08 Feb 2005	C3	L17, L18
GW18862	G317559	Australia	Queensland, Lagoon at Reef 21-505, Swain Reefs	21° 41' 57" S, 152° 21' 26" E	11m	29 Nov 2008	C1	L17
GW18874	G317545	Australia	Queensland, Reef 21-505, Swain Reefs	21° 42' 19" S, 152° 20' 25" E	21m	08 Feb 2002	C1	L17
GW18900	G318043	Australia	Queensland, Prong No.2 Reef, northern Swain Reefs	21° 42' 21" S, 151° 44' 32" E	10.4m	12 Mar 2004	C1	L17, L18

Sample	Museum code	Country	Localities	Coordinates	Depth	Collection Date	Cox2 haplotypes	LTRS intron haplotypes
GW18758	G315299	Australia	Queensland, Hook Reef lagoon	19° 45' 14" S, 149° 10' 45" E	9.4m	06 Jun 1999	C1	L17
GW18804	G312397	Australia	Western Australia, North Head, Beagle Bay, NW WA	16° 30' 00" S, 122° 19' 12" E		12 Aug 1991	C2	X
GW4867	ZMA POR17229	Indonesia	S Sulawesi, Makassar			08 May 2012	C1	L2, L6
GW7103	RMNH2632	Indonesia	N Sulawesi, Bunaken	1° 36' 07" N, 124° 45' 09" E	74m	17 Jul 2006	C1	L1, L2
GW7179		Indonesia	S Sulawesi, Makasar, Karanrang	4° 51' 21.12"S, 119° 22' 37.33"E		09 Aug 2012	C1	L7, L9
GW7180		Indonesia	S Sulawesi, Makasar, Barangbaringan	5° 3' 2.739"S, 119° 25' 21.93"E		15 Aug 2012	C1	L10
GW7181		Indonesia	S Sulawesi, Makasar, Padjenekang	4° 58' 1.99"S, 119° 19' 21.36"E		12 Aug 2012	C2	X
GW7182		Indonesia	S Sulawesi, Makasar, Padjenekang	4° 58' 1.99"S, 119° 19' 21.36"E		12 Aug 2012	C2	L1, L7
GW7183		Indonesia	S Sulawesi, Makasar, Lae Lae	5° 8' 12.7"S, 119° 23' 14.23"E		13 Aug 2012	C2	X
GW7184		Indonesia	S Sulawesi, Makasar, Lae Lae	5° 8' 12.7"S, 119° 23' 14.23"E		13 Aug 2012	C1	L8
GW4782		Indonesia	N Sulawesi, Lembeh, W Sarena Kecil	1° 27' 25.5234"N, 125° 13' 31.1874"E		17 Feb 2012	C1	X
GW4783		Indonesia	N Sulawesi, Lembeh, Tanjung Nanas I	1° 27' 40.212"N, 125° 13' 36.408"E		03 Feb 2012	C1	X
GW4784		Indonesia	N Sulawesi, Lembeh, Tanjung Kelapasatu	1° 25' 38.568"N, 125° 11' 0.7794"E		15 Feb 2012	C2	L3, L4
GW4785		Indonesia	N Sulawesi, Lembeh, N Tanjung Pandean	1° 24' 21.7074"N, 125° 10' 4.5114"S		14 Feb 2012	C1	L1, L5
GW4786		Indonesia	N Sulawesi, Lembeh, N Tanjung Pandean	1° 24' 21.7074"N, 125° 10' 4.5114"E		14 Feb 2012	C1	L1
GW4787		Indonesia	N Sulawesi, Lembeh, N Tanjung Pandean	1° 24' 21.7074"N, 125° 10' 4.5114"E		14 Feb 2012	C1	L1

Supplementary material 5.1: List of *Neopetrosia chaliniformis* cox2 and LTRS intron haplotypes

Sample	Museum code	Country	Localities	Coordinates	Depth	Collection Date	Cox2 haplotypes	LTRS intron haplotypes
GW4788		Indonesia	N Sulawesi, Lembeh, S Pulau Dua	1° 23' 17.016"N, 125° 12' 43.1274"S		13 Feb 2012	C1	L1, L4
GW4789		Indonesia	N Sulawesi, Lembeh, Tanjung Kuning	1° 23' 10.788"N, 125° 10' 23.2314"E		11 Feb 2012	C1	L6
GW7175		Indonesia	S Sulawesi, Makasar, Lankai	5° 1' 44.7"S, 119° 5' 8.8" E		08 Aug 2012	C1	L1
GW7176		Indonesia	S Sulawesi, Makasar, Lankai	5° 1' 44.7"S, 119° 5' 8.8" E		08 Aug 2012	C1	L7
GW7177		Indonesia	S Sulawesi, Makasar, Polewali	0' 44.36S"S, 119° 24' 4.28"E		09 Aug 2012	C1	L8, L9
GW2037		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26 Jul 2011	C1	L24
GW2038		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26 Jul 2011	C1	L25
GW2039		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26 Jul 2011	C1	L25
GW2040		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26 Jul 2011	C1	L24, L26
GW2041		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26 Jul 2011	C1	L6, L24
GW2042		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26 Jul 2011	C1	L24, L25
GW2112		Indonesia	W Java, Thousand Island, Semak Daun NW	5° 43' 40" S, 106° 33' 57" E	16m	28 Jul 2011	C1	L6, L26
GW2113		Indonesia	W Java, Thousand Island, Semak Daun NW	5° 43' 40" S, 106° 33' 57" E	16m	28 Jul 2011	C1	L26
GW2139		Indonesia	W Java, Thousand Island, Belanda NW	5° 36' 15" S, 106° 36' 09" E		30 Jul 2011	C1	L24, L25
GW2177		Indonesia	W Java, Thousand Island, Tidung Besar	5° 47' 28" S, 106° 28' 42" E		31 Jul 2011	C1	L24
GW2179		Indonesia	W Java, Thousand Island, Tidung Kecil NW	5° 47' 58" S, 106° 31' 04" E		31 Jul 2011	C1	L1, L6
GW4870	ZMA POR16482	Japan	Ryukyu Islands, Saki-shima Islands, Hatoma Island	24° 26' 60" N, 123° 49' 60" E			C1	L1

Sample	Museum code	Country	Localities	Coordinates	Depth	Collection Date	Cox2 haplotypes	LTRS intron haplotypes
GW4866	ZMA POR17251	Mauritius				25 Jun 2009	C1	L11
GW18598	G306321	Palau	Ongingiang, W of, W. Palau	7° 16' 05" N, 134° 14' 05" E	31m	10 Des 1995	C1	L1, L2
GW18777	G311804	Papua New Guinea	North Of Lion Island, S/w Of Motupore Island; Near Port Moresby, PNG	9° 19' 19" S, 147° 9' 40" E	6-18m	19 Sept 1990	C1	L13, L14
GW4829	ZMA POR21752	Philippines	Calamian Group: Busuanga Island				C1	L1
GW18478	G313113	Singapore	Pulau Tembakul (Kusu I), Freyberg Channel	1° 13' 05" N, 103° 51' 07" E	18.7m	02 May 1997	C1	L3, L19
GW19027	G322668	Solomon Islands	Rendova Island Tetepare	8° 42' 04" S, 157° 28' 14" E	20-50m	06 Jul 2009	C1	L14, L20
GW19041	G322710	Solomon Islands	Rendova Island Tetepare	8° 42' 04" S, 157° 28' 14" E	40-50m	06 Jul 2009	C1	L14
GW19052	G322696	Solomon Islands	Vangunu Island	8° 40' 19" S, 157° 50' 15" E		05 Jul 2009	C1	L14
GW4843	ZMA POR18793	Thailand	Laem Tum-Pung, South of Ko Kram, Sattahip, Chonburi			25 Feb 2007	C2	L6
GW4845	ZMA POR18754	Thailand	South of Ko Mark, Chang Islands, Trad	11° 47' 10" N, 102° 29' 14" E		08 May 2012	C2	L6
GW4849	ZMA POR18737	Thailand	West side of Ko Klum, Chang Islands, Trad	11° 55' 02" N, 102° 21' 43" E		08 May 2012	C2	L6

Supplementary material 5.1: List of *Neopetrosia chaliniformis* cox2 and LTRS intron haplotypes

Sample	Museum code	Country	Localities	Coordinates	Depth	Collection Date	Cox2 haplotypes	LTRS intron haplotypes
GW4851	ZMA POR18725	Thailand	North of Ko Mon Nai, Mon Islands, Klang, Rayong	12° 36' 26" N, 101° 41' 26" E		30 Oct 2005	C2	L21
GW18491	G313297	Tonga	Vaipuua, at end of channel	18° 37' 55" S, 173° 58' 47" W	15m	14 Nov 1997	C1	X
GW18525	G313069	Vanuatu	Canal, Luganville, Espiritu Santo	15° 31' 56" S, 167° 11' 46" E	3-43m	24 May 1997	C2	L22, L23
Total number of samples that are able to be genotyped							55	**49**
Discrepancy							**6**	

5.2. Haplotype list and GenBank accession numbers

Cox2 mtDNA	Accession numbers		LTRS intron	Accession numbers	LTRS intron	Accession numbers
C1	KM030095		L1	KM030146	L16	KM030161
C2	KM030097		L2	KM030147	L17	KM030162
C3	KM030109		L3	KM030148	L18	KM030163
			L4	KM030149	L19	KM030164
			L5	KM030150	L20	KM030165
			L6	KM030151	L21	KM030166
			L7	KM030152	L22	KM030167
			L8	KM030153	L23	KM030168
			L9	KM030154	L24	KM030169
			L10	KM030155	L25	KM030170
			L11	KM030156	L26	KM030171
			L12	KM030157	L16	
			L13	KM030158	L17	
			L14	KM030159	L18	
			L15	KM030160	L19	

7.1. Supplementary material and methods of DNA extractions, PCR, sequencing, and data analysis for the ATP6 mtDNA phylogenetic analysis in chapter 7

The previously published extraction method for sponge barcoding (Vargas et al. 2012) was applied on 54 freshly collected and 51 museums samples preserved in ethanol 99 % (see Supplementary 7.2). An ATP6 fragment was amplified by the primers ATP6 PorF and ATP6 PorR (Rua et al. 2011). The 25 µL PCR mix consisted of 5 µL 5x green Go*Taq* ® PCR Buffer (Promega Corp, Madison, WI), 4µL 25mM $MgCl_2$ (Promega Corp, Madison, WI), 2 µL 10mM dNTPs, 1 µL each primer (5µM), 9.8 µL water, 0.2µL Go*Taq*® DNA polymerase (5u/µl) (Promega Corp, Madison, WI) and 2 µL DNA template. The PCR regime comprised an initial denaturation at 94° C for 3 min, 35 cycles of 30 s denaturation at 94° C, 20 s annealing and 60 s elongation at 72° C each, followed by a final elongation at 72° C for 5 min. However, an additional ATP6 internal primer set modified from Rua et al. 2011 (ATP6_Xt_f1: 5'- TAGGGGTAACTTTGTTAGGG-3'; ATP6_Xt_r1: 5'- CCAATGAAATAGCACGAGCC-3'), additional 2 µL Bovine Serum Albumin (BSA, 10mg/ ml) in a final volume of 25 µL PCR mix and modification on PCR regime with 45 s annealing at 42° C, 45 s elongation at 72° C each and a final elongation at 72° C for 7.5 min) were required to obtain PCR products from the type specimens of barrel sponges, which are *X. bergquistia* (holotype QM G25018) *X. testudinaria* (neotype BMNH 1881.10.21.266), *X. testudinaria* (neoparatype BMNH 1881.10.21.267) and *X. muta* (syntypes MCZ Pora-6449 and MCZ Pora-6450). All of the PCR products were cleaned using the ammonium acetate precipitation (Sambrook et al. 1989). Sequencing of forward and reverse strands was performed with the ABI BigDye v 3.1 (Applied Biosystems, California USA) chemistry and the amplification primers following the manufacturers' protocol on an ABI 3730 Automated Sequencer in the Genomic Sequencing Unit of the LMU Munich (http://www.gi.bio.lmu.de/sequencing). Sequences obtained that were identical to the haplotypes of Swierts et al. (2013) are numbered accordingly (A1 and A2, GenBank accession number KC424439- KC424446). In addition, haplotype sequences that are not recovered in that study are submitted and deposited at NCBI GenBank under accession numbers KM014756 – KM014764 for A4-A10, including type specimens of barrel sponges (see detail list in Supplementary 7.2 and 7.3).

Phylogenetic reconstructions among all sequences from the tested markers were

performed under Bayesian inferences (BI) and Maximum-likelihood (ML) criteria. Inferences under the Bayesian framework were performed with MrBayes v. 3.2.1 (Ronquist et al. 2012). Each analysis consisted of two independent runs of four Metropolis- coupled Markov-chains under default temperature with trees sampled at every 1000^{th} generations. Analyses were terminated automatically when the chains converged significantly as indicated by an average standard deviation of split frequencies < 0.01. ML analysis was performed using RAxML v. 7.0.4 in the raxmlGUI v. 1.3 program (Silvestro and Michalak 2012) with 1,000 rapid bootstrap replication (Stamatakis et al. 2008). Hierarchical Likelihood Ratio Tests (hLRTs) for identifying the best fitting model during tree searches in both inferences were acquired by jModeltest 2.1.3 (Darriba et al. 2012) under the Akaike Information Criterion (AIC) (Akaike 1974). The HKY + I substitution model was suggested for but were changed into their GTR equivalents as the HKY model is not implemented in RAxML (see Stamatakis 2008).

7.2. List of samples that are utilsed in chapter 7 and sorted by the haplotype

Legend

- GW refers to internal voucher numbers in the internal voucher numbers in the Molecular Geo- & Palaeobiology Lab, Department of Earth and Environmental Sciences, Palaeontology & Geobiology, Ludwig-Maximilians Universität München, Germany
- ZMA POR number is an accession number for samples belong to Zoological Museum Amsterdam, which is currently merged into Naturalis Biodiversity Centre Leiden, The Netherlands.
- G number is an accession number for samples belong to Queensland Museum, Brisbane, Australia
- BMNH number is an accession number for samples belong to British Museum Natural History, London, UK
- MCZ Pora- is the accession number for samples belong to museum of comparative zoology, Harvard, USA
- A1- A10: ATP6 haplotypes recovered in this study, which are identical (A1, A2) and follow Swierts et al. 2013 numeration.
- BSPG number is an accession number for samples belong Bavarian State Collection for Palaeontology and Geology, Munich, Germany.

Xestospongia testudinaria samples

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplotype
GW18441	G303333	Australia	Northern Territory, East Point Bommies, Darwin Harbour	12° 24' 05" S, 130° 48' 08" E	10m	23.09.1993	A1
GW18642	G314838	Australia	Queensland, Alcyonarian Point, Hook Island, Whitsunday Grp	20° 3' 56" S, 149° 55' 24" E	15m	03.06.1999	A1
GW19049	G322086	Australia	Queensland, Cayley Reef	17° 29' 11" S, 146° 26' 28" E	11.9-12m	29.11.2008	A1
GW18743	G321612	Australia	Queensland, East Heron Is	23° 26' 04" S, 151° 56' 20" E	5-17m	09.11.2004	A1
GW18547	G305387	Australia	Queensland, Gannet Cay, S side reef, fore-reef slope, Swain Rfs	21° 59' 05" S, 152° 28' 06" E	24m	23.07.1995	A1
GW18740	G317830	Australia	Queensland, Mackerel Reef, Swain Reefs	21° 50' 10" S, 151° 58' 19" E	30m	12.02.2002	A1

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplotype
GW4830	ZMA POR21696	China	Weizhou Island		10m	30.04.2009	A1
GW1328	BSPG 2014 XIV 10 (GW1328)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Menjagan Kecil, E side	5° 53' 22" S, 110° 24' 18" E		26.05.2011	A1
GW1327	BSPG 2014 XIV 9 (GW1327)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Menjagan Kecil, N side	5° 53' 22" S, 110° 24' 18" E		26.05.2011	A1
GW1340	BSPG 2014 XIV 13 (GW1340)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Sintok, E side	5° 47' 06" S, 110° 30' 18" E		27.05.2011	A1
GW1339	BSPG 2014 XIV 12 (GW1339)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Sintok, N side	5° 47' 06" S, 110° 30' 18" E		27.05.2011	A1
GW1338	BSPG 2014 XIV 11 (GW1338)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Sintok, W side	5° 47' 06" S, 110° 30' 18" E		27.05.2011	A1
GW1477	BSPG 2014 XIV 18 (GW1477)	Indonesia	C. Java, Kep Karimun Jawa, Takat Menjangan Kecil, E side	5° 53' 56" S, 110° 24' 54" E		27.05.2011	A1
GW1470	BSPG 2014 XIV 28 (GW1470)	Indonesia	Gulf of Bone, Kolaka, Pomala, Pelabuhan	4° 15' 06" S, 121° 15' 06" E		20.08.2011	A1
GW4791		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 25"N, 125° 13' 31" E		12.02.2012	A1
GW4795		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 28' 10"N, 125° 14' 38"E		02.02.2012	A1
GW4798		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 26' 8"N, 125° 12' 34"E		04.02.2012	A1
GW4793		Indonesia	North Sulawesi, Pulau Lembeh, Bitung	1° 27' 19"N, 125° 13' 25"E		07.02.2012	A1
GW4796		Indonesia	North Sulawesi, Pulau Lembeh, Bitung	1° 28' 25"N, 125° 14' 2"E		01.02.2012	A1
GW4797		Indonesia	North Sulawesi, Pulau Lembeh, Bitung	1° 27' 40"N, 125° 13' 36"E		30.01.2012	A1
GW4885	ZMA POR8250	Indonesia	S Sulawesi, Tukang Besi Islands, southern reef of Karang Kaledupa, east of entrance	5° 55' 60" S, 123° 47' 60" E		07.09.1988	A1
GW2319		Indonesia	SE Sulawesi, Wakatobi, Hoga, Pak Kasim's			07.08.2011	A1

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplotype
GW2321		Indonesia	SE Sulawesi, Wakatobi, Hoga, Pak Kasim's	GPS not available		07.08.2011	A1
GW2314	BSPG 2014 XIV 19 (GW2314)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08.08.2011	A1
GW2315	BSPG 2014 XIV 20 (GW2315)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08.08.2011	A1
GW2316	BSPG 2014 XIV 29 (GW2316)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08.08.2011	A1
GW2317	BSPG 2014 XIV 30(GW2317)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08.08.2011	A1
GW2329	BSPG 2014 XIV 31 (GW2329)	Indonesia	SE Sulawesi, Wakatobi, Tomia, NW P. Sawa	5° 45' 00" S, 123° 53' 33" E		14.08.2011	A1
GW2332	BSPG 2014 XIV 27(GW2332)	Indonesia	SE Sulawesi, Wakatobi, Tomia, NW P. Sawa	5° 45' 00" S, 123° 53' 33" E		14.08.2011	A1
GW2055		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26.07.2011	A1
GW1326	BSPG 2014 XIV 8 (GW1326)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Menjagan Kecil, W side	5° 53' 22" S, 110° 24' 18" E		26.05.2011	A1
GW1238	BSPG 2014 XIV 1 (GW1238)	Indonesia	E Java, Situbondo, Pantai Pecaron, Takat Palapa, E atoll	7° 40' 52" S, 113° 51' 08" E		09.05.2011	A1
GW4884	ZMA POR8275	Indonesia	NE coast of Sumba, E of Melolo	9° 54' 00" S, 120° 42' 30" E	6m	14.09.1988	A1
GW2054		Indonesia	W Java, Thousand Island, Pulau Air	5° 45' 35" S, 106° 34' 44" E		26.07.2011	A1
GW4878	ZMA POR11065	Japan	Hachijo Island, 300 km S of Tokyo			30.11.1995	A1
GW18949	G321075	Malaysia	SW peninsular, rocks just offshore of Indonesian border	2° 4' 18" N, 109° 38' 39" E		29.05.2007	A1
GW18465	G312947	Papua New Guinea	Keppel Point, Hood Bay, SE. Papuan Lagoon	10° 8' 08" S, 147° 54' 09" E	35m	16.12.1996	A1

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplotype
GW18756	G312340	Philiphine	Alas Diyot Rf; NE Face Of Silino Island Bohol Sea	8° 30' 50" N, 123° 15' 11" E	20-30m	20.04.1991	A1
GW4879	ZMA POR9537	Singapore	Raffles Lighthouse	12° 53' 02" N, 103° 45' 23" E		07.01.1982	A1
GW19034	G323993	Solomon Islands	Three sisters, the island most north	10° 7' 59" S, 161° 55' 09" E	30m	20.07.2009	A1
GW19046	G323994	Solomon Islands	Three sisters, the island most north	10° 9' 04" S, 161° 55' 04" E	15-30m	20.06.2009	A1
GW19106	G324373	Solomon Islands	Trios soeurs, ile la plus nord	10° 9' 04" S, 161° 55' 04" E	15-30m	21.07.2008	A1
GW4854	ZMA POR18692	Thailand	Ao Pagarung, South of Ko Samet, Samet Islands, Rayong	12° 33' 14" N, 101° 28' 11" E		28.10.2005	A1
GW4855	ZMA POR18688	Thailand	Hin Khan Na, West side of Ko Samet, Samet Islands, Rayong	12° 33' 14" N, 101° 28' 11" E		27.10.2005	A1
GW4856	ZMA POR18687	Thailand	Hin Khan Na, West side of Ko Samet, Samet Islands, Rayong	12° 33' 14" N, 101° 28' 11" E		08.05.2012	A1
GW4853	ZMA POR18701	Thailand	Khao Gek, West of Ko Samet, Samet Islands, Rayong	12° 31' 01'' N, 101° 26' 46'' E		28.10.2005	A1
GW4846	ZMA POR18747	Thailand	Ko Rad, Kood Islands, Trad	11° 57' 21" N, 102° 21' 46" E		08.05.2012	A1
GW4860	ZMA POR18618	Thailand	Ko Thai-ta-Mun, South of Ko Si-chang, Chonburi	13° 6' 09" N, 100° 48' 28" E		08.05.2012	A1
GW4869	ZMA POR16708	Thailand	Pattaya, Klungbadan Island, Gulf of Thailand	12° 45' 00" N, 100° 52' 00" E		24.06.2009	A1
GW4859	ZMA POR18627	Thailand	West side of Ko Khrok, Ko Lan Islands, Pattaya, Chonburi	12° 55' 13" N, 100° 48' 27" E		26.09.2005	A1
GW4848	ZMA POR18738	Thailand	West side of Ko Klum, Chang Islands, Trad	11° 55' 02" N, 102° 21' 43" E		08.05.2012	A1
GW1341		Indonesia	C. Java, Kep Karimun Jawa, Pulau Sintok, S side	5° 47' 06" S, 110° 30' 18" E		27.05.2011	A2
GW1247	BSPG 2014 XIV 5 (GW1247)	Indonesia	E Java, Situbondo, Pantai Pecaron, Karang Mayit, S fringing reef	7° 41' 13" S, 113° 49' 38" E		09.05.2011	A2
GW1240	BSPG 2014 XIV 3 (GW1240)	Indonesia	E Java, Situbondo, Pantai Pecaron, Takat Palapa, S side Atol	7° 40' 52" S, 113° 51' 08" E		09.05.2011	A2

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplotype
GW4790		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 25"N, 125° 13' 31" E		17.02.2012	A2
GW4792		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 19"N, 125° 13' 25"E		07.02.2012	A2
GW4794		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 40"N, 125° 13' 36"E		30.01.2012	A2
GW4799		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 26' 8"N, 125° 12' 34"E		04.02.2012	A2
GW4800		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 19"N, 125° 13' 25"E		07.02.2012	A2
GW1408	BSPG 2014 XIV 16 (GW1408)	Indonesia	W Bali, Menjangan Island.	8° 5' 55" S, 114° 30' 43" E		01.05.2011	A2
GW1409	BSPG 2014 XIV 32 (GW1409)	Indonesia	W Bali, Menjangan Island.	8° 5' 55" S, 114° 30' 43" E		01.05.2011	A2
GW1411	BSPG 2014 XIV 17 (GW1411)	Indonesia	W Bali, Menjangan Island.	8° 5' 55" S, 114° 30' 43" E		01.05.2011	A2
GW2149		Indonesia	W Java, Thousand Island, Bokor	5° 56' 32" S, 106° 37' 39" E		01.08.2011	A2
GW2151		Indonesia	W Java, Thousand Island, Bokor	5° 56' 32" S, 106° 37' 39" E		01.08.2011	A2
GW2150		Indonesia	W Java, Thousand Island, Lancang Besar	5° 55' 27" S, 106° 34' 51" E		01.08.2011	A2
GW2152		Indonesia	W Java, Thousand Island, Lancang Besar	5° 55' 27" S, 106° 34' 51" E		01.08.2011	A2
GW1344	BSPG 2014 XIV 15 (GW1344)	Indonesia	C. Java, Kep Karimun Jawa, Pulau Sintok, E Side	5° 47' 06" S, 110° 30' 18" E		27.05.2011	A4
GW1246	BSPG 2014 XIV 4 (GW1246)	Indonesia	E Java, Situbondo, Pantai Pecaron, Karang Mayit, E fringing reef	7° 40' 52" S, 113° 51' 08" E		09.05.2011	A4
GW1248	BSPG 2014 XIV 6 (GW1248)	Indonesia	E Java, Situbondo, Pantai Pecaron, Karang Mayit, S fringing reef	7° 41' 13" S, 113° 49' 38" E		09.05.2011	A4
GW1249	BSPG 2014 XIV 7 (GW1249)	Indonesia	E Java, Situbondo, Pantai Pecaron, Karang Mayit, S fringing reef	7° 41' 13" S, 113° 49' 38" E		09.05.2011	A4

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplotype
GW1239	BSPG 2014 XIV 2 (GW1239)	Indonesia	E Java, Situbondo, Pantai Pecaron, Takat Palapa, S atoll	7° 40' 52" S, 113° 51' 08" E		09.05.2011	A4
GW4865	ZMA POR17284	Indonesia	Lae-lae Island, off Ujung Pandang			08.05.2012	A4
GW4801		Indonesia	N Sulawesi, Pulau Lembeh, Bitung	1° 27' 40"N, 125° 13' 36"E		30.01.2012	A4
GW19078	G323745	Malaysia	Mabul, Collin's Patch just west of mabul island	4° 15' 12" N, 118° 37' 17" E	5-30m	14.01.2008	A4
GW18440	G303101	Papua New Guinea	Bootless Inlet, I. between Motupore and mainland	9° 31' 01" S, 147° 17' 02" E	10m	24.10.1992	A4
GW4847	ZMA POR18745	Thailand	Ko Rad, Kood Islands, Trad	11° 57' 21" N, 102° 21' 46" E		08.05.2012	A4
GW4858	ZMA POR18632	Thailand	West side of Ko Khrok, Ko Lan Islands, Pattaya, Chonburi	12° 55' 13" N, 100° 48' 27" E		26.09.2005	A4
GW4836	ZMA POR19017	China	Xidao Island, Sanya			28.06.2009	A5
GW18764	G311686	P New Guinea	Ne Wongat Is; Ne Of C.r.i.; Madang	5° 4' 48" S, 145° 29' 51" E	12m	02.09.1990	A5
GW4861	ZMA POR18616	Thailand	Ko Thai-ta-Mun, South of Ko Si-chang, Chonburi	13° 6' 09" N, 100° 48' 28" E		08.05.2012	A5
GW18361	G303250	Australia	Northern Territory, East Arm, Darwin, reef N of boatramp, S of S Shell I	12° 29' 08" S, 130° 53' 05" E	1m	19.09.1993	A6
GW18515	G313570	Australia	Northern Territory, SW of Groote Eylandt	14° 24' 54" S, 136° 19' 59" E	21.2m	12.10.1997	A6
GW18575	G306508	Australia	Queensland, south side Porpoise Cay	22° 11' 09" S, 155° 21' 04" E	30m	11.01.1996	A6
GW18553	G306055	Australia	Western Australia, NE of Dampier	19° 45' 08" S, 117° 57' 07" E	47m	02.09.1995	A6
GW4882	ZMA POR8675	Indonesia	Pulau-pulau Maisel, reef edge N of Mai	5° 28' 00" S, 127° 31' 00" E	6-13m	08.09.1988	A6
GW2322	BSPG 2014 XIV 24 (GW2322)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		08.08.2011	A6
GW2323	BSPG 2014 XIV 25 (GW2323)	Indonesia	SE Sulawesi, Wakatobi, Kaledupa, Sampela I	5° 28' 55" S, 123° 44' 21" E		07.08.2011	A6

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplotype
GW2331	BSPG 2014 XIV 26 (GW2331)	Indonesia	SE Sulawesi, Wakatobi, Tomia, NW P. Sawa	5° 45' 00" S, 123° 53' 33" E		14.08.2011	A6
GW4832	ZMA POR216990	Japan	Matsugaura, Kagoshima		10-15m	08.05.2012	A6
GW18973	G321083	Malaysia	SW peninsular, Rock in centre of bay w. of T. Melano	2° 1' 42" N, 109° 39' 12" E	8m	31.05.2007	A6
GW4837	ZMA POR19012	China	Xidao Island, Sanya			28.06.2009	A7
GW18537	G313104	Singapore	Pulau Tembakul (Kusu I), Freyberg Channel	1° 13' 05" N, 103° 51' 07" E	18.7m	02.05.1997	A7
GW4844	ZMA POR18773	Thailand	Hin Kum-Pan, Ko Rung, Chang Islands, Trad	11° 46' 36" N, 102° 23' 48" E		08.05.2012	A7
GW18816	G312436	Papua New Guinea	Manko Maru, Simpson Harbour, Rabaul, E. New Britain	4° 7' 26" S, 152° 6' 15" E	30m	09.10.1991	A8
GW18479	G313256	Tonga	Kolotaki Bay SE side of W end of Vava'u I.	18° 38' 24" S, 174° 1' 34" W	14m	10.11.1997	A8
GW4823	ZMA POR22222.10	Saudi Arabia	FSAR reef	22° 13' 50" N, 39° 1' 44" E	12-14m	08.05.2012	A9
GW4824	ZMA POR22222.2	Saudi Arabia	FSAR reef	22° 13' 50" N, 39° 1' 44" E	12-14m	08.05.2012	A9
GW4825	ZMA POR22222	Saudi Arabia	FSAR reef	22° 13' 50" N, 39° 1' 44" E	12-14m	08.05.2012	A9
GW4833	ZMA POR21449	Saudi Arabia	Obhor Sharm, Jeddah		8m	08.05.2012	A9
GW4835	ZMA POR21461	Saudi Arabia	Obhor Sharm, Jeddah		15m	08.05.2012	A9

Type specimens

Voucher numbers	Museum code	Country	Localities	Coordinates	Depth	Date	Haplotype
GW2993	BMNH 1881.81.10.21.266 (neotype of *X. testudinaria*)	Australia	Queensland, Port Denison				Unidentified
GW2994	BMNH 1881.81.10.21.267 (neoparatype of *X. testudinaria*)	Australia	Queensland, Port Denison				Unidentified
	G25018 *(X. bergquistia* holotype)	Australia	Queensland, Pioneer Bay, Orpheus Island, GBR	18° 36' S, 146° 29' 34 E	15m	18.02.1987	Unidentified
GW2997	MCZ Pora-6450 (*X. muta* syntype)						A10
GW2996	MCZ Pora-6449 (*X. muta* syntype)						Unidentified

7.3. Haplotype list and GenBank accession numbers

ATP6	Accession numbers	ATP6	Accession numbers
A1	KC424445	A8	KM014761
A2	KC424446	A9	KM014759
A4	KM014757	A10	KM014756
A5	KM014758	*X. muta* type specimens	KM014756
A6	KM014763	*X. testudinaria* type specimens	KM014764
A7	KM014760	*X. bergquistia* type specimen	KM014762

Curriculum Vitae

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Since 01.01.2008 Sepuluh November Institute of Technology Surabaya, Faculty of Mathematic and Natural Science, Biology Department; Zoology Laboratory; Lecturer; Responsible for teaching, researching, and community service

Education

2010-2014 Ludwig-Maximilians-Universität München, PhD student in Molecular Geo-and Palaeontology Lab

2001-2003 Utrecht University the Netherlands, MSc student in behavioural biology

1996-2000 Airlangga University Surabaya, Indonesia, BSc student in biology

1993-1996 St Albertus Catholic High School Malang, Indonesia

Working experience

2008-present Sepuluh November Institute of Technology Surabaya, Faculty of Mathematic and Natural Science, Biology Department; Lecturer

2004-2007 Cita Hati Christian High School Surabaya, Biology teacher

Publication: peer-reviewed article

2013 Swierts T, Peijnenburg KTCA, de Leeuw C, Cleary DFR, Hörnlein C, Setiawan E, Wörheide G, Erpenbeck D, de Voogd NJ Lock, Stock and Two Different Barrels: Comparing the Genetic Composition of Morphotypes of the Indo-Pacific Sponge *Xestospongia testudinaria*. PLoS One 8:e74396

Scientific Communications: oral presentations

2013 Setiawan. E., Swierts. T., Wörheide. G., de Voogd. N.J., Erpenbeck. D., Genetic diversity of giant barrel sponges *Xestospongia testudinaria* -9th World Sponge Conference, Fremantle, WA, Australia.

2013 Setiawan. E., Swierts. T., Wörheide. G., Erpenbeck. D., de Voogd. N.J., The pandora box of *Neopetrosia exigua*- Biosyst EU 2013, Vienna, Austria.

2012 Setiawan. E., Swierts. T., Wörheide. G., de Voogd. N.J., Erpenbeck. D., Genetic diversity of giant barrel sponges *Xestospongia testudinaria* -2nd German Young Corral Reef Scientist Meeting, Berlin, Germany.

Training

2011 Ecology, Evolutionary and Systematic (EES) summer school, LMU München, Facultyof Biology, Prien am Chiemsee, Germany.

Languages

Indonesian	native
English	business fluent, IELTS test in 2010, overall score 6.0
German	fluent conversation, certificate B2 Goethe Institute in 2011
Dutch	simple or basic conversation and understanding, certificate A2 Erasmus Taalcentrum in 2003