

USING DNA TO FIGURE OUT SOFT CORAL TAXONOMY – PHYLOGENETICS
OF RED SEA OCTOCORALS

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LIST OF ABBREVIATIONS

28S:	28S ribosomal DNA
ASGPB:	Advanced Studies of Genomics, Proteomics and Bioinformatics
<i>ATPSα</i> :	The nuclear ATP Synthetase Subunit α
<i>ATPSβ</i> :	ATP Synthetase Subunit β
BI:	Bayesian Inference
BSA:	Bovine Serum Albumin
<i>COI</i> :	The mitochondrial Cytochrome C Oxidase subunit 1
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic acid
E. F. I.:	East Farasan Island
GTR+G:	General time reversible (variable base frequencies, symmetrical substitution matrix) with Gamma distribution (gamma distributed rate variation among sites)
HKY:	Hasegawa-Kishino-Yano (nucleotide substitution model with variable base frequencies, one transition rate and one transversion rate)
HKY+G:	HKY with Gamma distribution (gamma distributed rate variation among sites)
<i>ITS</i> :	Internal Transcribed Spacers
Marmar R.:	Marmar Reef
ML:	Maximum Likelihood
mtDNA:	Mitochondrial DNA
<i>mtMutS</i> :	The mitochondrial protein coding gene MutS homolog
MUSCLE:	MUltiple Sequence Comparison by Log- Expectation
N. B. R.:	North Brown Reef
<i>ND2</i> :	NADH dehydrogenase subunit 2
nDNA:	Nuclear DNA
Palace N.:	Palace North
Palace S.:	Palace South
PCR:	Polymerase Chain Reaction
PHYML:	Phylogenetic estimation using Maximum Likelihood
S. B. R.:	South Brown Reef
SB:	Sodium Borate
SEM:	Scanning Electron Microscope
sp.:	species
spp:	species pluralis
SRP54:	Signal Recognition Particle 54-kDa subunit
TAU:	Tel Aviv University
TrN+I:	Tamura-Nei (variable base frequencies, equal transversion rates, variable transition rates) with proportion of invariable sites (extent of static, unchanging sites in a dataset)
ZMTAU:	Zoological Museum TA

CHAPTER 1. INTRODUCTION

The importance of coral reefs

The health of coral reefs, often called the rainforests of the sea, is of considerable importance for a variety of reasons. Most prominently, these very diverse ecosystems host millions of species that live primarily or exclusively in association with coral reefs, with estimates for the biodiversity ranging from 1–9 million species (Knowlton 2001). Furthermore, coral reefs offer an important source of nutrition for humans. Currently, one-third of the world's six billion people rely on fish and other aquatic products for at least one-fifth of their annual protein intake (Dulvy & Allison 2009). Moreover, healthy coral reefs provide many opportunities for leisure activities, and consequently greatly affect the economy in coastal cities through jobs created by tourism. For example, in Hawai'i, direct economic benefits of the coral reefs are estimated at \$360 million a year (Cesar and Beukering 2004), and U.S. residents recently valued Hawai'i's coral reef ecosystems at \$33.57 billion per year (Bishop et al. 2011). Furthermore, coral reefs form a natural protection against coastline erosion, by breaking land-approaching swells. And lastly, chemical compounds found in corals hold enormous medical potential for the prevention of cancerous cells from dividing and multiplying, for anti-tumor properties, and for use as bone grafts to facilitate the regrowth of skeletal tissue (Sautier et al. 1990, Begley *et al.* 1995). This chapter briefly summarizes how the health of coral reefs affects individuals and communities globally, highlighting the need to conserve these extremely important and diverse ecosystems.

Coral reef health and phylogenetics

Conservation programs seek to maintain biodiversity and resilience among coral reef ecosystems. In order to make management decisions, data is needed regarding the presence and abundance of taxonomic groups (species, genera, families), in specific areas. Before we can evaluate coral reefs in such a manner, we need to know which taxonomic groups exist within the class Anthozoa (corals). This is where the study of phylogenetics and molecular systematics, which is the study of evolutionary relationships between taxonomic groups based on both morphological and molecular data, plays an important role. To gain a better understanding of the evolutionary relationships among corals, in this study we focus on the phylogenetics of 4 out of 30 octocoral families; the Alcyonacea, Nephtheidae, Tubiporidae, and Xeniidae.

Further research

The knowledge of evolutionary relationships between taxonomic groups is fundamental to a range of further studies. For example, it allows for studies on ecological interactions (competition, symbiosis, etc.) between different species of octocorals, or between octocorals and other marine organisms, such as scleractinian (hard) corals. This is of specific interest now, when hard corals are affected by global climate change (Hughes et al. 2003; Hoegh-Guldberg et al. 2007). An ecological shift from scleractinian dominated coral reefs to alcyonacean soft corals may result from a combination of environmental changes impacting hard corals, and opportunistic features of soft corals such as rapid growth rates, high fecundity, and

extensive vegetative reproduction (Fabricius 1995, Tilot et al. 2008, Wood & Dipper 2008). Furthermore, a better understanding of evolutionary relationships allows analyses of ecological differences between taxonomic groups, such as reproduction methods or habitat preference. Moreover, genetic barcoding will allow geographic comparisons which may reveal range-restricted endemics and novel evolutionary lineages. In conclusion, the data produced by this study will provide robust scientific foundations for a variety of further biological studies, as well as conservation management decisions, and we will highlight taxonomic groups that require further attention.

CHAPTER 2. A TAXONOMIC SURVEY OF SOME SAUDI ARABIAN ALCYONACEA (CNIDARIA: OCTOCORALLIA)

Abstract

Here we present the first systematic survey of Saudi Arabian Alcyonacea. We explored 14 locations along the west coast of the Kingdom of Saudi Arabia to assess the taxonomic diversity of alcyonacean corals in the region. We collected a total sample size of 74 individual colonies, distributed among four families: 18 colonies of Alcyoniidae, 14 of Nephtheidae, 9 of Tubiporidae, and 33 of Xenidae. Additionally, we sequenced the corals to distinguish between taxa and to identify the taxa, using multiple nuclear and mitochondrial loci, and with multiple individuals of each species where possible. We obtained sequences of the nuclear ribosomal Internal Transcribed Spacers (*ITS*), the nuclear ATP Synthetase Subunit α (*ATPS α*), the mitochondrial protein coding gene MutS homolog (*mtMutS*), and the mitochondrial Cytochrome C Oxidase subunit 1 (*COI*) from most individuals. This preliminary phylogeny was consistent with monophyletic genera with the exception of *Sarcophyton*, *Ovabunda* and *Xenia*, which merit further attention. Also, we revealed a potential new taxon which, based on morphological characters, appears most similar to the Xenidae genus *Xenia*, however molecular data indicate this taxon is more closely related to the genus *Heteroxenia*.

Introduction

Alcyonacean corals are among the most common of benthic marine species in many parts of the world, including the Mediterranean (Weinberg 1980; Ros *et al.* 1985), the North Atlantic (Sebens 1986; Migne & Davault 1997), the Pacific Northwest coast of North America (McFadden & Hochberg 2003), and even Antarctica (Dayton *et al.* 1970; Slattery & McClintock 1995). They are also conspicuous and ecologically important members of many tropical coral reef habitats, including the Red Sea (Benayahu & Loya 1981), Southern Africa (Williams 1993; Riegl *et al.* 1995) and the Indo-West Pacific (Fabricius 1997). For example, in the Indo-West Pacific, best known for the spectacular diversity of scleractinian corals and reef-associated fishes, soft corals may occupy up to 25% of primary space (Benayahu 1995; Fabricius 1997). An ecological shift from scleractinian dominated coral reefs to ones in which much of the available space is occupied by alcyonacean soft corals results from a combination of environmental changes impacting hard corals, and opportunistic features of soft corals such as rapid growth rates, high fecundity, and extensive vegetative reproduction (Fabricius 1995, Tilot *et al.* 2008, Wood & Dipper 2008). With the myriad range of global climate changes impacting coral reefs (Hughes *et al.* 2003; Hoegh-Guldberg *et al.* 2007), understanding the taxonomy of soft coral taxa, which are increasing in abundance on many reefs, becomes an issue of considerable conservation relevance.

Despite being a common and important taxon, the taxonomy of soft corals remains a subject of considerable uncertainty (McFadden *et al.* 2006a; McFadden *et al.* 2009). There is a substantial literature available for Red Sea coral reefs (e.g., Benayahu & Loya 1981; Benayahu *et al.* 1989; Benayahu 1991; Henning *et al.* 1998; Ben-David-Zaslow & Benayahu 1998; Barneah *et al.* 2006; Zeevi-Ben-Yosef &

Benayahu 2008). However, surveys of the coral taxa along the Saudi Arabian coast remain scarce, and have focused almost exclusively on scleractinian fauna (Sheppard & Sheppard 1985, 1991, Sheppard 1985). Further, there is little correspondence between some alcyonacean groups identified by molecular studies and classical taxonomic groups (McFadden *et al.* 2006a, 2009, Bernston *et al.* 2001 & Daly *et al.* (2007). For example, in a phylogenetic analysis of octocorallia based on the mitochondrial genes *ND2* and *mtMutS*, McFadden *et al.* (2006a) found that topology tests rejected the monophyly of the subordinal groups Alcyoniina, Scleraxonia, and Stolonifera. This molecular and morphological discord has led to questions about the taxonomic value of the currently used morphological characters and the evolutionary relationships among families (Bayer 1981, Fabricius & Alderslade 2001, McFadden *et al.* 2006b). Classification of the majority of octocorals into higher taxonomic levels remains problematic, and in this study we follow the classification of Bayer (1981) for the Alcyonacea. We have made collections of the families Alcyoniidae (Lamouroux 1812), Nephtheidae (Gray 1862), Tubiporidae (Ehrenberg 1828) and Xeniidae (Wright & Studer 1889). We report data from both the nuclear (*ITS & ATPSa*) as well as the mitochondrial (*mtMutS & COI*) genome for all 4 families, including 13 genera: *Anthelia*, *Dendronephthya*, *Heteroxenia*, *Litophyton*, *Ovabunda*, *Paralemnalia*, *Rhytisma*, *Sarcophyton*, *Sinularia*, *Stereonephthya*, *Symphodium*, *Tubipora*, and *Xenia*. Our goal was to clarify evolutionary relationships among Saudi Arabian Alcyonacea species, and to provide a greater scientific foundation for conservation efforts in this region. Furthermore, as more such data sets emerge from different geographical regions, this will allow molecular comparisons to be made among Alcyonacea from around the globe, and help to gain a better understanding of alcyonacean taxonomy globally.

Materials and methods

Sample collection

At each of 14 localities along the west coast of the Kingdom of Saudi Arabia, bordering the Red Sea, we examined and photographed all alcyonacean soft corals we could locate (Table 1). Depending on local abundance, tissue samples of up to 4 individuals of each type (especially for colonies with visible variation or taxonomic uncertainty) were collected. A small piece (1-5 cm) was cut from each coral colony using scissors or dive knife. During collections, multiple photos and live videos were taken of each colony before and during disturbance to record colony response. In addition, observational data on depth, colony pulsation, retraction, contraction, size, stem split, substrate and color were recorded for each colony. This resulted in a total of 74 individual colonies sampled within the order Alcyonacea. Included within this collection were 18 individuals of the family Alcyoniidae, 14 of Nephtheidae, 9 of Tubiporidae, and 33 of Xeniidae. Paired tissue samples from each colony were stored in 95% ethanol and saturated salt DMSO (SSD) buffer for future molecular analyses (Gaither *et al.* 2011), in addition to 70% ethanol for morphological analyses. Field identifications were confirmed by subsequent morphological analysis. Examinations include the shape of the colony, the number of rows of pinnules on polyp tentacles, the number of pinnules on the aboral side of polyp tentacles, and the sclerites' presence, shape, size and

microstructure. Pictures of the sclerites and polyp tentacles were taken with a Nikon light microscope, magnitude 400x. For type material, and any uncertainty, a Scanning Electron Microscope (SEM) was used, with magnifications down to 5 μm . Tentative field identifications of *Sarcophyton* colonies could not be confirmed because we lacked an additional tissue sample from the stem of the colony for those samples (SA053, SA066, SA067, and SA077). Taxonomic samples are deposited at the Bishop Museum, Honolulu, and photo vouchers are included here (Figure 2).

DNA isolation and amplification

DNA for mitochondrial locus amplification was extracted using the DNeasy Blood & Tissue Kit® (Qiagen) following the manufacturer's protocol, as outlined in McFadden *et al.* (2010). The mitochondrial marker MutS (*mtMutS*, previously named *msh1*, ~730bp) was amplified using the previously published primers ND42625F & mut3458R (Lepard 2003, Sánchez *et al.* 2003a), with the following PCR protocol: 94°C for 3 min (1 cycle to activate polymerase); 94°C for 30 s, 50°C for 90 s, 72°C for 60 s (35 cycles); followed by a final extension of 72°C for 5 min (1 cycle). The mitochondrial Cytochrome C Oxidase subunit 1 locus (*CO1*, 770bp) was amplified using the previously published primers COII8068F & CO1 Oct-r (McFadden *et al.* 2004, France & Hoover 2002), and with the following PCR protocol: 94°C for 3 min (1 cycle to activate polymerase); 94°C for 90 s, 57.7°C for 90 s, 72°C for 60 s (35 cycles); followed by a final extension of 72°C for 5 min (1 cycle). In sum, a total of ~1500 bp mitochondrial DNA was analyzed for 57 (*CO1*) and 53 (*mtMutS*) of the 74 colonies sampled. PCR products from the mitochondrial markers were purified and sent to Cogenics (Houston, TX) for sequencing following the methods in McFadden *et al.* (2010).

DNA for nuclear locus amplification was extracted following the coral DNA extraction protocol outlined in Concepcion *et al.* (2006, 2008). The nuclear marker ATP Synthetase Subunit α (*ATPS α* , ~280bp) was amplified using the previously published primers *ATPS α f1* and *ATPS α r1* (Jarman *et al.* 2002; Concepcion *et al.* 2008), and with the following PCR protocol: 94°C for 2 min (1 cycle to activate polymerase); 94°C for 20 s, 57°C for 60 s, 72°C for 60 s (35 cycles); followed by a final extension of 72°C for 6 min (1 cycle). The Ribosomal Internal Transcribed Spacer region (*ITS*, ~1100bp) was amplified using the previously published primers 1S-f & 2SS-r (annealing temperature: 50°C; Wei *et al.* 2006), in addition to the newly designed primers *ITSRHF* TTGGCACCTGTCAGATGRKY and *ITSRHR* CACCCRTTTTRGGCTGCATT (45°C). Primers were designed with Primer3 (Rozen and Skaletsky 2000), and amplifications were conducted with the following PCR protocol: 96°C for 9 min (1 cycle to activate polymerase); 96°C for 10 s, 45 or 50°C for 30 s, 70°C for 4 min (33 cycles); followed by a final extension of 70°C for 5 min (1 cycle). In sum, a total of ~1380 bp of nuclear DNA was analyzed for 47 (*ATPS α*) and 59 (*ITS*) of the 74 colonies sampled. PCR amplification of nuclear loci was performed on a Bio-Rad MyCycler™. Each 25 μl PCR reaction contained 1.0 μl of template DNA (~5 ng/ μL), 12.5 μl BioMix Red 2X PCR reaction mix (Bioline Inc.), 0.325 μl of each primer (10 μM), 0.75 μl of BSA (10mg/ml) and 11.1 μl deionized sterile water. For some PCRs an additional 1 μl of DNA template was added to improve PCR

product (final volume 26µl). PCR products were visualized using 1.0% agarose gels in 1x SB (sodium borate; Brody *et al.* 2004) buffer stained with Gelstar® (Lonza). Amplification reactions for sequencing of nuclear genes were first treated with Exo I FastAP™ (Fermentas) alkaline phosphatase using the following thermocycler profile: 37°C for 60 min, 85°C for 15 min. Treated PCR products were then cycle-sequenced using BigDye Terminators (Applied Biosystems) run on an ABI-3730XL DNA Analyzer at the Advanced Studies of Genomics, Proteomics and Bioinformatics (ASGPB) facility at UH Mānoa.

Sequencing and phasing

Phase (Stephens *et al.* 2001) as implemented in DNAsp 5 (Librado & Rozas 2009) was used to resolve the alleles for each heterozygous individual, when aligning the direct sequenced forward and reverse sequences was possible. The PCR products of heterozygous individuals that could not be phased reliably from direct sequences were ligated into the pGEM®-T Easy cloning vector (Promega Inc.) and transformed into JM109 competent cells following the manufacturer protocol. After blue/white colony selection, up to 12 colonies were screened by PCR to verify an insert of the correct size using the M13 vector primers. Initially, 4 colonies were sequenced with the original primers. Heterozygote alleles were accepted only if two or more copies of the same allele were found and could be matched up with the original mixed template direct sequence read. If the first 4 colonies did not recover 2 copies of each allele, 4 additional colonies were sequenced until 2 copies of each allele were recovered.

Phylogenetic analyses

All mitochondrial sequences were inspected using Lasergene (DNASStar) and aligned using CLUSTALW 2.0.11 (Larkin *et al.* 2007) and MUSCLE 3.6 (Edgar 2004). All nuclear sequences were visually inspected using GENEIOUS Pro 4.7.6 (Drummond *et al.* 2010) and aligned using both CLUSTALW and MUSCLE plugins in GENEIOUS. Subsequently, phylogenetic relations were reconstructed using Bayesian Inference (BI) (Chain Length= 1.1×10^6 , Burn-in Length= 100,000) and Maximum Likelihood (ML) (10,000 bootstrap replicates) methods in GENEIOUS Pro. BI trees were generated with MrBayes 2.0.2 (Huelsenbeck and Ronquist 2001), and ML trees were generated from PHYML 1.0 (Guindon & Gascuel 2003). In all analyses the family Tubiporidae was used as outgroup based on earlier analyses (McFadden *et al.* 2006a)

Results

We located members of 4 out of 28 (Fabricius & Alderslade 2001) families of alcyonaceans; Alcyoniidae, Nephtheidae, Tubiporidae and Xeniidae. Within the Alcyoniidae 4 genera were sampled; *Rhytisma* (*R. fulvum fulvum*), *Sarcophyton* (*S. gemmatum*, *S. glaucum*, and *S. auritum*), *Sinularia* (*S. compressa*, *S. leptoclados*, *S. new densa*, and *S. querciformis*), and *Stereonephthya* (*S. sp.*). Within the Nephtheidae 3 genera were detected; *Dendronephthya* (*D. sp.*), *Litophyton* (*L. sp.*), and *Paralemnalia* (*P. eburnea*, and *P. thyrsoides*). Within the family Tubiporidae 1 genus was observed; *Tubipora musica*. Within the family Xeniidae 5 genera were sampled; *Anthelia* (*A. sp.*), *Heteroxenia* (*H. fuscescens*), *Ovabunda* (*O. ainex*, *O.*

arabica, *O. biseriata*, *O. gohari*, *O. impulsatilla*, *O. macrospiculata*, and *O. verseveldti*), *Sympodium* (*S. caeruleum*), and *Xenia* (*X. actusa* and *X. umbellata*).

The mitochondrial marker *mtMutS* was successfully amplified for 67 individual colonies; 16 from the family Alcyoniidae (*Rhytisma* (N=6), *Sarcophyton* (N=4), *Sinularia* (N=4), *Stereonephthya* (N=2)); 14 from the family Nephtheidae (*Dendronephthya* (N=8), *Litophyton* (N=3), *Paralemnalia* (N=3)); 8 from the family Tubiporidae (*Tubipora* (N=8)); and 31 from the family Xenidiidae (*Anthelia* sp. (N=6), *Heteroxenia* sp. (N=2), *Ovabunda* sp. (N=12) and *Xenia* sp. (N=8), see Table 2). *MtMutS* provided great resolution in comparison to the other loci; most genera formed monophyletic clades with the exception of *Ovabunda* and *Xenia*. *Xenia* had 2 lineages; 4 individuals group within the *Ovabunda* clade, and 4 others group more closely to *Heteroxenia* (Figure 1). The mitochondrial marker *COI* was successfully amplified for a total of 73 individual colonies; 16 from the family Alcyoniidae (*Rhytisma* (N=5), *Sarcophyton* (N=5), *Sinularia* (N=4), *Stereonephthya* (N=2)); 13 from the family Nephtheidae (*Dendronephthya* (N=7) and *Litophyton* (N=3), *Paralemnalia* (N=3)); 8 from the family Tubiporidae (*Tubipora musica* (N=8)); and 36 from the family Xenidiidae (*Anthelia* (N=6), *Heteroxenia* (N=3), *Ovabunda* (N=14), *Sympodium* (N=2), *Xenia* (N=8)), see Table 2. *COI* provided the same resolution as *mtMutS*; all genera form monophyletic clades with exception of *Ovabunda* and *Xenia*.

The nuclear marker *ATPSa* was successfully amplified in 47 colonies; 11 from the family Alcyoniidae (*Rhytisma* (N=3), *Sarcophyton* (N=5), *Sinularia* (N=2), *Stereonephthya* (N=1)); 8 from the family Nephtheidae (*Dendronephthya* (N=6), *Litophyton* (N=2)); 2 from the family Tubiporidae (*Tubipora* (N=2)); and 26 from the family Xenidiidae (*Anthelia* (N=5), *Heteroxenia* (N=3), *Ovabunda* (N=9), *Sympodium* (N=2), *Xenia* (N=7)), see Table 2. *ATPSa* provided the least resolution among all markers tested, and only enough to distinguish monophyletic groups of the genera *Paralemnalia*, and *Rhytisma*. *Xenia* was divided into 2 groups, one nested within *Ovabunda*, and a second being part of a large polyphyletic clade containing all remaining genera (including *Heteroxenia*). This pattern is consistent with the systematic positioning of *Xenia* with both *mtMutS* and *COI*. The nuclear marker *ITS* was successfully amplified for a total of 51 individual colonies; 15 from the family Alcyoniidae (*Rhytisma* (N=6), *Sarcophyton* (N=6), *Sinularia* (N=3), *Stereonephthya* (N=2)); 11 from the family Nephtheidae (*Dendronephthya* (N=7), *Litophyton* (N=1), and *Paralemnalia* (N=3)); 4 from the family Tubiporidae (*Tubipora* (N=4)); and 19 from the family Xenidiidae (*Anthelia* (N=4), *Heteroxenia* (N=3), *Ovabunda* (N=5) and *Xenia* (N=7), see Table 2). *ITS* provided high resolution, distinguishing most genera as monophyletic groups with exception of *Sarcophyton*, *Ovabunda* and *Xenia*. Two *Sarcophyton* individuals form a paraphyletic clade more closely related to *Sinularia*. Furthermore, similar to the pattern in *mtMutS*, *COI*, and *ATPSa*, 3 *Xenia* individuals form a paraphyletic clade more closely related to *Heteroxenia*, and the other *Xenia* individuals are nested within the *Ovabunda* clade (Figure 1).

Phylogenetic analyses

Mitochondrial and nuclear phylogenetic analyses generally agree; most genera are monophyletic except *Sarcophyton*, *Ovabunda*, and *Xenia* (Figure 1). *Sarcophyton* forms a paraphyletic clade only in the nDNA phylogeny, with 97% (Bayesian Inference (BI) posterior probability) and 100% Maximum Likelihood (ML) bootstrap support; 4 individuals group separately from another 2 which group as a sister taxon to *Sinularia* (Figure 1). In the mitochondrial tree *Sarcophyton* forms a monophyletic group with 99% BI and 100% ML support. Furthermore, among all loci and all tree construction methods, a previously unrecognized *Xenia* group of 4 individual colonies consistently forms a monophyletic clade more closely related to the *Heteroxenia* clade than to other *Xenia* species, which nest within the *Ovabunda* clade.

In all analyses the family Tubiporidae consistently formed a monophyletic sister group to the family Xenidiidae and to the family cluster groups of Alcyoniidae and Nephtheidae. Within this family, only *Tubipora musica* was found, and the 3 morphotypes were incongruent with the molecular divergence (different morphotypes are represented in Figure 2). Other genera with multiple morphotypes, but no corresponding molecular divergence, include *Sinularia*, *Dendronephthya*, *Paralemnalia*, and *Rhytisma* (Figure 2). Furthermore, the family Xenidiidae consistently forms a monophyletic sister group to the Tubiporidae. Among the five genera sampled from Xenidiidae, *Anthelia*, *Heteroxenia* and *Sympodium* consistently grouped in a monophyletic clade, whereas *Ovabunda* and *Xenia* did not. The families Alcyoniidae and Nephtheidae consistently group in 3 clades. Two of these clades are polyphyletic (1 consists of monophyletic groups for the genera *Dendronephthya*, *Litophyton* and *Stereonephthya*, the other consists of monophyletic groups for the genera *Paralemnalia* and *Rhytisma*), and 1 paraphyletic group of only Alcyoniidae (consisting of *Sarcophyton* and *Sinularia*).

BI and ML analyses both recovered very similar topologies, and the majority of the nodes were well supported by both the BI posterior probability and ML bootstrap values (Figure 1). One difference between the mitochondrial BI and ML analyses is the topology of *Heteroxenia* and *Xenia*; in the mitochondrial ML phylogeny *Heteroxenia* and *Xenia* individuals form 1 monophyletic clade, whereas in the mitochondrial BI phylogeny they do not (Figure 1).

Discussion

Here we present the first survey of Saudi Arabian Alcyonacea in which we sampled 74 colonies. An earlier survey of Saudi Arabian corals by Sheppard and Sheppard (1985, 1991) and Sheppard (1985) focused almost exclusively on Hexacorallia, with the exception of *Tubipora musica*. In this former survey a decrease in scleractinian cover was observed with a decrease in latitude. In our study, multiple reefs in 3 main areas (From North to South: Thuwal, Al Lith and Farasan Islands) were investigated, and highest coral cover and diversity was observed at the outermost reefs in Al Lith. Concordant with the survey by the Sheppards, the lowest coral cover and diversity was observed at our southernmost site, Farasan Islands. However, we examined only a tiny portion of the coast covered in the Sheppard's survey. By comparison to our study, with 74 colonies from 4 families, a recent survey of the Red Sea at Eilat, Israel (Gulf of Aqaba,

northern Red Sea), sampled a total of 58 individuals from 9 families: Acanthogorgiidae, Alcyoniidae, Briareidae, Melithaeidae, Nephtheidae, Nidaliidae, Plexauridae, Tubiporidae, and Xeniidae (McFadden *et al.* 2010). Although there is a greater diversity observed by McFadden *et al.* (2010), we believe this is likely a result of the amount of time dedicated to taxonomic sampling rather than a paucity of diversity in Saudia Arabian alcyonaceans. Further surveys are clearly needed to address that issue.

In addition to being the first systematic survey of alcyonacean corals of Saudi Arabia, Red Sea, this molecular data set, consisting of 4 loci (2 nDNA: *ITS*, *ATPS α* , and 2 mtDNA: *mtMutS*, *COI*), allows comparisons of Saudi Arabian alcyonaceans to those of other regions. For example, we made a comparison of this Saudi Arabian data set to a previously published dataset from the Israel, Red Sea (McFadden *et al.* 2010). In an assemblage of COI sequences, genera from the families Acanthogorgiidae, Alcyoniidae, Briareidae, Melithaeidae, Nephtheidae, Nidaliidae, Plexauridae, Tubiporidae, and Xeniidae, form monophyletic groups with the exception of a few individuals, which have since been reevaluated and renamed by the authors (McFadden *et al.* 2010). As more such data sets emerge from different geographical regions, this will allow molecular comparisons to be made among Alcyonacea taxa from around the globe.

Phylogenetic analyses were generally consistent among nuclear and mitochondrial loci. Overall, 9 of the 12 genera sampled were confirmed monophyletic, the exceptions being *Sarcophyton*, *Ovabunda*, and *Xenia*. The genus *Sarcophyton* was monophyletic in mitochondrial analyses but paraphyletic in the nuclear analyses. The incongruence between mtDNA and nDNA in *Sarcophyton* can result from many processes, but the most common explanations in corals include incomplete lineage sorting or hybridization. Distinguishing between these alternatives is very difficult in most datasets, however (Marti-Puig *et al.* in prep, Forsman *et al.* 2010). For example, in a study by Forsman *et al.* (2010) on Hawai‘ian Montiporids, the *ATPS β* intron data showed a pattern often interpreted as resulting from hybridization and introgression. However, Forsman *et al.* (2010) conclude that incomplete lineage sorting was more likely, because the multicopy nuclear ITS region was consistent with the mitochondrial data. This exemplifies the importance of using multiple loci of both the mitochondrial and nuclear genome in coral phylogenetics studies. Furthermore, we discovered non-sister monophyletic groups within the nominal genus *Xenia*. Four *Xenia* individuals form a paraphyletic clade more closely related to *Heteroxenia*, hereafter called *Xenia* group ‘A’, and the other *Xenia* individuals are nested within the *Ovabunda* clade, hereafter called *Xenia* group ‘B’ (Figure 1). After discovering this cryptic taxon, reverse taxonomy (sensu Markmann & Tautz 2012) was applied; the search for supporting morphological characters which were previously overlooked, inspired by phylogenetic patterns. Interestingly, we were able to detect differences in colony growth forms between the 2 *Xenia* groups (Figure 2). Individuals of *Xenia* clade ‘A’ are always small, the stem and tentacles not very long, and the color pattern is white tentacles and polyps and brown pinnules. The specimens of *Xenia* clade ‘B’ vary in appearance: some have the same morphological features as *Xenia* group ‘A’, but are much lighter in color (SA037, SA071), whereas others have different morphological features than *Xenia* group

'A'; the stems and polyp arms are much longer, but the color pattern is the same as seen in individuals from *Xenia* group 'A' (SA076, SA085).

This first survey of the Saudi Arabian Alcyonacea fauna yielded a number of questions for future study, and a wealth of specimens that can be used for future molecular and geographically comparative analyses. Overall, 10 of the 13 currently recognized genera match monophyletic groups in our phylogenetic reconstructions, which confirm an accurate understanding of the evolutionary relationships among morphological characters used to distinguish these alcyonacean taxa at the genus level. Additionally, our sampling of 4 loci (2 nDNA: *ITS*, *ATPS α* , and 2 mtDNA: *mtMutS*, *COI*) appears to strike the desirable balance between resolution, cost and labor for future phylogenetic studies of alcyonacean corals. However, further study is needed to determine whether alcyonacean corals mimic scleractinian corals in the diversity gradients across Saudi Arabia as reported by Sheppard and Sheppard (1985, 1991) and Sheppard (1985). Likewise, the discord between nDNA and mtDNA reconstructions with *Sarcophyton* require further study to evaluate whether there is confused taxonomy or potential for hybridization between *Sarcophyton* and *Sinularia*. Finally, our discovery of non-sister monophyletic groups within the genus *Xenia* indicates cryptic diversity that merits additional taxonomic attention. Overall, our study provides both a first taxonomic survey of the region and an initial phylogenetic survey to guide future research efforts in the region and the family.

Table 1. Collection information: Species identification (Genus, Species), collection date in 2011, site, and depth, polyps' pulsation, retraction, and contraction, size of colony, number of splits in stem, color of colony. Palace N. /S. = Palace North/ South, N. B. R./ S. B. R. = North/ South Brown Reef. Color abbreviations: White (Wh), Grey (Gr), Brown (Br), Beige (Be), Yellow (Ye), Green (Gn), Purple (Pu).

	Genus	Species	Date	Site	Depth	Puls.	Retr.	Contr.	Size	Split	Color
SA001	<i>Ovabunda</i>	<i>ainex</i>	04-13	Palace N.	42 ft	No	Constant	No	2x2cm	3x	Wh/ Gr
SA002	<i>Ovabunda</i>	<i>gohari</i>	04-12	Shark Reef	42 ft	No	No	Yes	2cm	no	Wh
SA003	<i>Xenia</i>	sp.	04-12	Shark Reef	28 ft	Yes	No	Yes	2x2cm	1x	Wh & Br
SA004	<i>Ovabunda</i>	<i>biseriata</i>	04-13	Palace N.	42 ft	No	Constant	No	4x4cm	1x	Br
SA005	<i>Ovabunda</i>	<i>arabica</i>	04-13	Palace N.	42 ft	No	No	Yes	2x3cm	2x	Wh & Be
SA006	<i>Xenia</i>	sp.	04-12	Shark Reef	28 ft	Yes	No	Yes	1x2cm	1x	Br
SA007	<i>Ovabunda</i>	<i>macrospiculata</i>	04-13	Palace S.	19 ft	No	No	Yes	2x2cm	1x	Be
SA008	<i>Xenia</i>	sp.	04-12	Shark Reef	27 ft	Yes	No	Yes	4x4cm	4x	Wh/ Gr
SA009	<i>Ovabunda</i>	<i>biseriata</i>	04-12	Shi'b Nazar	37 ft	Yes	No	Yes	1x2cm	No	Wh & Be
SA010	<i>Ovabunda</i>	<i>biseriata</i>	04-13	Palace N.	40 ft	No	Constant	No	3x4cm	4x	Wh/ Be
SA011	<i>Xenia</i>	<i>actuosa</i>	04-15	N. B. R.	22 ft	Yes	No	Yes	2x4cm	2x	Wh & Br
SA013	<i>Heteroxenia</i>	<i>fuscescens</i>	04-15	N. B. R.	27 ft	Yes	No	Yes	5x6cm	No	Wh/ Be
SA014	<i>Litophyton</i>	sp.	04-15	S. B. R.	47 ft	No	Yes	No	15x20cm	N/A	Light Ye/ Be
SA016	<i>Rhytisma</i>	<i>fulvum fulvum</i>	04-15	S. B. R.	42 ft	No	Yes	No	15x15cm	N/A	Gn/ Br
SA017	<i>Rhytisma</i>	<i>fulvum fulvum</i>	04-15	N. B. R.	28 ft	No	Yes	No	5x8cm	N/A	Gr/ Pu
SA019	<i>Tubipora</i>	<i>musica</i>	04-15	S. B. R.	41 ft	No	Yes	No	4x10cm	N/A	Wh/ Gr
SA020	<i>Ovabunda</i>	<i>ainex</i>	04-15	N. B. R.	23 ft	No	No	Yes	4x5cm	No	Be
SA021	<i>Heteroxenia</i>	<i>fuscescens</i>	04-13	Palace S.	12 ft	Yes	Little	Yes	4x4cm	No	Wh & Br
SA022	<i>Ovabunda</i>	<i>ainex</i>	04-12	Shi'b Nazar	25 ft	No	No	Yes	4x3cm	1x	Be
SA023	<i>Anthelia</i>	sp.	04-12	Shi'b Nazar	30 ft	No	No	Yes	1x2cm	No	Wh & Be
SA024	<i>Ovabunda</i>	<i>biseriata</i>	04-12	Shi'b Nazar	24 ft	No	No	Yes	1x2cm	No	Wh
SA026	<i>Heteroxenia</i>	<i>fuscescens</i>	04-13	Palace S.	14 ft	Yes	No	Yes	4x4cm	No	Wh & Br
SA027	<i>Anthelia</i>	sp.	04-12	Shi'b Nazar	34 ft	No	No	Yes	4cm*	No	Be
SA028	<i>Litophyton</i>	sp.	04-15	N. B. R.	27 ft	No	No	No	30x20cm	N/A	Light Ye/ Be
SA029	<i>Tubipora</i>	<i>musica</i>	04-15	N. B. R.	22 ft	No	Yes	No	25x25cm	N/A	Gr/ Be
SA030	<i>Tubipora</i>	<i>musica</i>	04-16	Dora Reef	18 ft	No	No	Yes	20x30cm	N/A	Be
SA031	<i>Tubipora</i>	<i>musica</i>	04-17	Dora Reef	38 ft	No	Yes	Yes	5x10cm	N/A	Wh
SA032	<i>Anthelia</i>	sp.	04-17	Dora Reef	32 ft	No	No	No*	7x20cm	No	Be
SA034	<i>Rhytisma</i>	<i>fulvum fulvum</i>	04-17	Dora Reef	29 ft	No	Yes	No	20x20cm	N/A	Gn/Ye
SA035	<i>Sympodium</i>	<i>caeruleum</i>	04-17	Dora Reef	17 ft	No	Yes	No	4x7cm	No	Wh & Be

Table 1. (Continued) Collection information: Species identification (Genus, Species), collection date in 2011, site, and depth, polyps' pulsation, retraction, and contraction, size of colony, number of splits in stem, color of colony. Marmar R. = Marmar Reef. Color abbreviations: White (Wh), Grey (Gr), Brown (Br), Beige (Be), Yellow (Ye), Green (Gn), Purple (Pu), Orange (Or), Red (Re), Pink (Pi).

	Genus	Species	Date	Site	Depth	Puls.	Retr.	Contr.	Size	Split	Color
SA036	<i>Dendronephthya</i>	sp.	04-17	Dora Reef	27 ft	No	Yes	No	5x10cm	N/A	Ye & Pu
SA037	<i>Xenia</i>	sp.	04-23	Jeddah	3 ft	Yes	No	Yes	3x4cm	1x	Be
SA038	<i>Dendronephthya</i>	sp.	04-17	Dora Reef	31 ft	No	Yes	No	3x5cm	N/A	Pu
SA039	<i>Tubipora</i>	<i>musica</i>	04-17	Dora Reef	37 ft	No	Yes	No	5x20cm	N/A	Wh
SA040	<i>Ovabunda</i>	<i>impulsatilla</i>	04-17	Dora Reef	32 ft	No	No	Yes	2x7cm	3x	Wh & Br
SA041	<i>Tubipora</i>	<i>musica</i>	04-16	Dora Reef	20 ft	No	Yes	No	15x15cm	N/A	Wh
SA042	<i>Anthelia</i>	sp.	04-16	Marmar R.	18 ft	No	No	Yes	2x3cm	No	Wh & Br
SA043	<i>Rhytisma</i>	<i>fulvum fulvum</i>	04-16	Marmar R.	15 ft	No	Yes	No	15x20cm	N/A	Be
SA044	<i>Tubipora</i>	<i>musica</i>	04-16	Dora Reef	31 ft	No	Yes	No	5x7cm	N/A	Wh
SA045	<i>Ovabunda</i>	<i>macrospiculata</i>	04-16	Marmar R.	16 ft	No	No	Yes	3x3cm	3x	Be
SA046	<i>Tubipora</i>	<i>musica</i>	04-16	Dora Reef	30 ft	No	Yes	No	5x7cm	N/A	Wh & Br
SA047	<i>Anthelia</i>	sp.	04-16	Marmar R.	32 ft	No	No	Yes	10x10cm	No	Wh & Br
SA048	<i>Sympodium</i>	<i>caeruleum</i>	04-16	Marmar R.	16 ft	No	Yes	No	4x10cm	No	Be
SA049	<i>Paralemnalia</i>	<i>eburnea</i>	04-16	Marmar R.	16 ft	No	Yes	No	15x20cm	N/A	Be
SA050	<i>Anthelia</i>	sp.	04-16	Marmar R.	17 ft	No	No	Yes	20x30cm	No	Be
SA051	<i>Sinularia</i>	<i>new densa</i>	04-17	Abulatt	24 ft	No	Yes	No	10x25cm	N/A	Be
SA052	<i>Rhytisma</i>	<i>fulvum fulvum</i>	04-17	Abulatt	24 ft	No	Yes	No	5x10cm	N/A	Gr
SA053	<i>Sarcophyton</i>	sp.	04-17	Abulatt	22 ft	No	Yes	No	15x25cm	N/A	Ye
SA054	<i>Sarcophyton</i>	<i>glaucum</i>	04-17	Abulatt	30 ft	No	Yes	Yes	7x10cm	N/A	Be
SA055	<i>Paralemnalia</i>	<i>thyrsoides</i>	04-17	Abulatt	31 ft	No	Yes	No	2x5cm	N/A	Gr
SA056	<i>Sinularia</i>	<i>querciformis</i>	04-17	Abulatt	35 ft	No	No	No	3x4cm	N/A	Gn/Be
SA057	<i>Rhytisma</i>	<i>fulvum fulvum</i>	04-17	Abulatt	29 ft	No	Yes	No	5x6cm	N/A	Gr
SA058	<i>Sinularia</i>	<i>leptoclados</i>	04-17	Abulatt	23 ft	No	Yes	No	5x6cm	N/A	Be
SA059	<i>Litophyton</i>	sp.	04-17	Mulathu	30 ft	No	No	Yes	1x1m	N/A	Light Ye
SA060	<i>Sarcophyton</i>	<i>auritum</i>	04-17	Mulathu	20 ft	No	Yes	Yes	20x20cm	N/A	Be
SA061	<i>Dendronephthya</i>	sp.	04-19	Dahik	27 ft	No	No	No	30x30cm	N/A	Wh/Or & Re/Pi
SA062	<i>Tubipora</i>	<i>musica</i>	04-19	Dahik	22 ft	No	Yes	Yes	15x30cm	N/A	Be
SA063	<i>Dendronephthya</i>	sp.	04-19	Dahik	26 ft	No	No	No	4x6cm	N/A	Wh/Or & Re/Pi
SA064	<i>Dendronephthya</i>	sp.	04-19	Dahik	21 ft	No	No	No	20x30cm	N/A	Wh & Pi

Table 1. (Continued) Collection information: Species identification (Genus, Species), collection date in 2011, site, and depth, polyps' pulsation, retraction, and contraction, size of colony, number of splits in stem, color of colony. E. F. I. = East Farasan Island. Color abbreviations: White (Wh), Brown (Br), Beige (Be), Yellow (Ye), Purple (Pu), Orange (Or), Red (Re), Pink (Pi).

	Genus	Species	Date	Site	Depth	Puls.	Retr.	Contr.	Size	Split	Color
SA065	<i>Dendronephthya</i>	sp.	04-19	Dahik	15 ft	No	No	No	20x20cm	N/A	Wh & Re/Pi & Or/Ye
SA066	<i>Sarcophyton</i>	<i>glaucum</i>	04-19	Dahik	17 ft	No	Yes	No	5x5cm	N/A	Light Ye
SA067	<i>Sarcophyton</i>	<i>auritum</i>	04-19	Dahik	21 ft	No	Yes	No	7x10cm	N/A	Ye/Be
SA068	<i>Dendronephthya</i>	sp.	04-20	Abu Shariah	19 ft	No	No	Yes	5x6cm	N/A	Wh and Re
SA069	<i>Dendronephthya</i>	sp.	04-20	Abu Shariah	18 ft	No	No	Yes	20x20cm	N/A	Wh & Re/Pi & Or/Ye
SA071	<i>Xenia</i>	<i>umbellata</i>	04-23	Jeddah	25 ft	Yes	No	Yes	4x7cm	1x	Be
SA072	<i>Paralemnalia</i>	<i>thyrsoides</i>	04-23	Jeddah	25 ft	No	No	Yes	10x10cm	N/A	Be/ light Ye
SA074	<i>Ovabunda</i>	<i>verseveldti</i>	04-23	Jeddah	42 ft	No	No	Yes	7x7cm	1x	Wh
SA075	<i>Ovabunda</i>	<i>gohari</i>	04-23	Jeddah	43 ft	No	No	Yes	7x7cm	No	Wh & Be
SA076	<i>Xenia</i>	sp.	04-23	Jeddah	34 ft	Yes	No	Yes	7x7cm	2x	Wh & Be
SA077	<i>Sarcophyton</i>	<i>gemmatum</i>	04-23	Jeddah	23 ft	No	Yes	Yes	15x15cm	N/A	Be
SA081	<i>Stereonephthya</i>	sp.	04-20	E.F.I.	30 ft	No	No	Yes	15x15cm	N/A	Wh
SA083	<i>Stereonephthya</i>	sp.	04-20	Abulatt	25 ft	No	No	Yes	5x10cm	N/A	Ye & Pu
SA084	<i>Sinularia</i>	<i>compressa</i>	04-20	Abulatt	36 ft	No	No	Yes	20x40cm	N/A	Wh & Br
SA085	<i>Xenia</i>	<i>umbellata</i>	04-23	Jeddah	23 ft	Yes	No	Yes	4x7cm	1x	Be

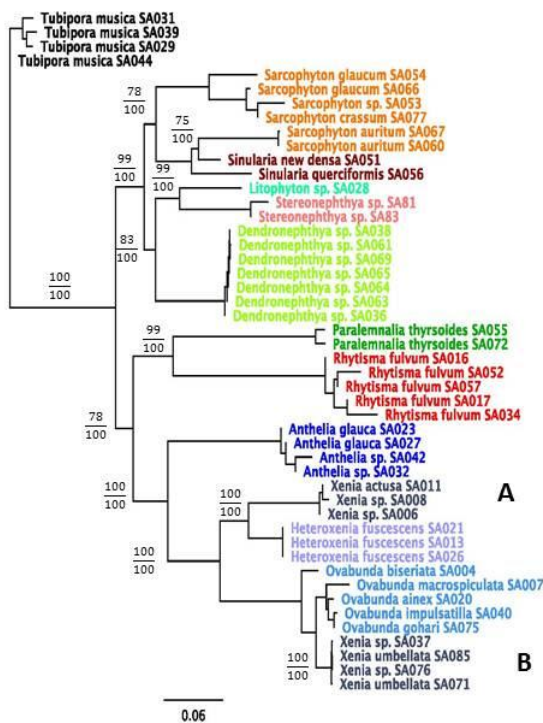
Table 2. Specimens list of acquired molecular markers. * Means heterozygote individuals.

	Family	Genus	Species	ATPSa	ITS	mtMutS	COI
SA001	Xeniidae	<i>Ovabunda</i>	<i>ainex</i>		✓		✓
SA002	Xeniidae	<i>Ovabunda</i>	<i>gohari</i>		✓	✓	✓
SA003	Xeniidae	<i>Xenia</i>	sp.	✓		✓	✓
SA004	Xeniidae	<i>Ovabunda</i>	<i>biseriata</i>		✓	✓	✓
SA005	Xeniidae	<i>Ovabunda</i>	<i>arabica</i>	✓	✓	✓	✓
SA006	Xeniidae	<i>Xenia</i>	sp.	✓*	✓	✓	✓
SA007	Xeniidae	<i>Ovabunda</i>	<i>macrospiculata</i>	✓	✓	✓	✓
SA008	Xeniidae	<i>Xenia</i>	sp.		✓	✓	
SA009	Xeniidae	<i>Ovabunda</i>	<i>biseriata</i>	✓*			✓
SA010	Xeniidae	<i>Ovabunda</i>	<i>biseriata</i>	✓*	✓	✓	✓
SA011	Xeniidae	<i>Xenia</i>	<i>actuosa</i>	✓	✓	✓	✓
SA013	Xeniidae	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓	✓
SA014	Nephtheidae	<i>Litophyton</i>	sp.			✓	✓
SA016	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>		✓		✓
SA017	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>		✓		
SA019	Tubiporidae	<i>Tubipora</i>	<i>musica</i>			✓	✓
SA020	Xeniidae	<i>Ovabunda</i>	<i>ainex</i>	✓	✓	✓	✓
SA021	Xeniidae	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓	✓
SA022	Xeniidae	<i>Ovabunda</i>	<i>ainex</i>	✓	✓	✓	✓
SA023	Xeniidae	<i>Anthelia</i>	sp.	✓	✓	✓	✓
SA024	Xeniidae	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓	✓	✓
SA026	Xeniidae	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓	✓
SA027	Xeniidae	<i>Anthelia</i>	sp.	✓	✓	✓	✓
SA028	Nephtheidae	<i>Litophyton</i>	sp.	✓*	✓*	✓	✓
SA029	Tubiporidae	<i>Tubipora</i>	<i>musica</i>		✓*	✓	✓
SA030	Tubiporidae	<i>Tubipora</i>	<i>musica</i>				
SA031	Tubiporidae	<i>Tubipora</i>	<i>musica</i>		✓*		
SA032	Xeniidae	<i>Anthelia</i>	sp.	✓	✓	✓	✓
SA034	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>	✓	✓	✓	✓
SA035	Xeniidae	<i>Sympodium</i>	<i>caeruleum</i>	✓	✓	✓	✓
SA036	Nephtheidae	<i>Dendronephthya</i>	sp.	✓	✓	✓	✓
SA037	Xeniidae	<i>Xenia</i>	sp.	✓	✓	✓	✓
SA038	Nephtheidae	<i>Dendronephthya</i>	sp.	✓	✓	✓	✓
SA039	Tubiporidae	<i>Tubipora</i>	<i>musica</i>		✓*	✓	✓
SA040	Xeniidae	<i>Ovabunda</i>	<i>impulsatilla</i>		✓	✓	✓
SA041	Tubiporidae	<i>Tubipora</i>	<i>musica</i>	✓		✓	✓
SA042	Xeniidae	<i>Anthelia</i>	sp.		✓	✓	✓
SA043	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>				
SA044	Tubiporidae	<i>Tubipora</i>	<i>musica</i>	✓	✓*		
SA045	Xeniidae	<i>Ovabunda</i>	<i>macrospiculata</i>		✓	✓	✓
SA046	Tubiporidae	<i>Tubipora</i>	<i>musica</i>			✓	✓
SA047	Xeniidae	<i>Anthelia</i>	sp.	✓	✓*	✓	✓
SA048	Xeniidae	<i>Sympodium</i>	<i>caeruleum</i>	✓		✓	✓
SA049	Alcyoniidae	<i>Paralemnalia</i>	<i>eburnea</i>			✓	✓
SA050	Xeniidae	<i>Anthelia</i>	sp.	✓	✓*	✓	✓
SA051	Alcyoniidae	<i>Sinularia</i>	<i>new densa</i>	✓	✓		✓
SA052	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>	✓*	✓*	✓	✓
SA053	Alcyoniidae	<i>Sarcophyton</i>	sp.		✓		
SA054	Alcyoniidae	<i>Sarcophyton</i>	<i>glaucum</i>	✓*	✓*	✓	✓
SA055	Alcyoniidae	<i>Paralemnalia</i>	<i>thyrsoides</i>	✓	✓	✓	
SA056	Alcyoniidae	<i>Sinularia</i>	<i>querciformis</i>	✓	✓	✓	✓
SA057	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>		✓		

Table 2 (Continued). Specimens list of acquired molecular markers. * Means heterozygote individuals.

	Family	Genus	Species	<i>ATPSa</i>	<i>ITS</i>	<i>MtMutS</i>	<i>COI</i>
SA058	Alcyoniidae	<i>Sinularia</i>	<i>leptocladus</i>			✓	✓
SA059	Nephtheidae	<i>Litophyton</i>	sp.				
SA060	Alcyoniidae	<i>Sarcophyton</i>	<i>auritum</i>	✓	✓	✓	✓
SA061	Nephtheidae	<i>Dendronephthya</i>	sp.		✓		
SA062	Tubiporidae	<i>Tubipora</i>	<i>musica</i>				
SA063	Nephtheidae	<i>Dendronephthya</i>	sp.	✓	✓	✓	✓
SA064	Nephtheidae	<i>Dendronephthya</i>	sp.	✓	✓		
SA065	Nephtheidae	<i>Dendronephthya</i>	sp.	✓	✓		
SA066	Alcyoniidae	<i>Sarcophyton</i>	<i>glaucum</i>	✓*	✓*		✓
SA067	Alcyoniidae	<i>Sarcophyton</i>	<i>auritum</i>	✓	✓	✓	✓
SA068	Nephtheidae	<i>Dendronephthya</i>	sp.				
SA069	Nephtheidae	<i>Dendronephthya</i>	sp.	✓	✓		
SA071	Xeniidae	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓	✓
SA072	Nephtheidae	<i>Paralemnalia</i>	<i>thyrsoides</i>	✓	✓		
SA074	Xeniidae	<i>Ovabunda</i>	<i>verseveldti</i>		✓	✓	✓
SA075	Xeniidae	<i>Ovabunda</i>	<i>gohari</i>	✓*	✓	✓	✓
SA076	Xeniidae	<i>Xenia</i>	sp.	✓	✓	✓	✓
SA077	Alcyoniidae	<i>Sarcophyton</i>	<i>gemmatum</i>	✓	✓	✓	✓
SA081	Nephtheidae	<i>Stereonephthya</i>	sp.		✓	✓	✓
SA083	Nephtheidae	<i>Stereonephthya</i>	sp.	✓	✓	✓	✓
SA084	Alcyoniidae	<i>Sinularia</i>	<i>compressa</i>			✓	✓
SA085	Xeniidae	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓	✓
Total				47	59	53	57

ITS & ATP α



Msh1 & CO1

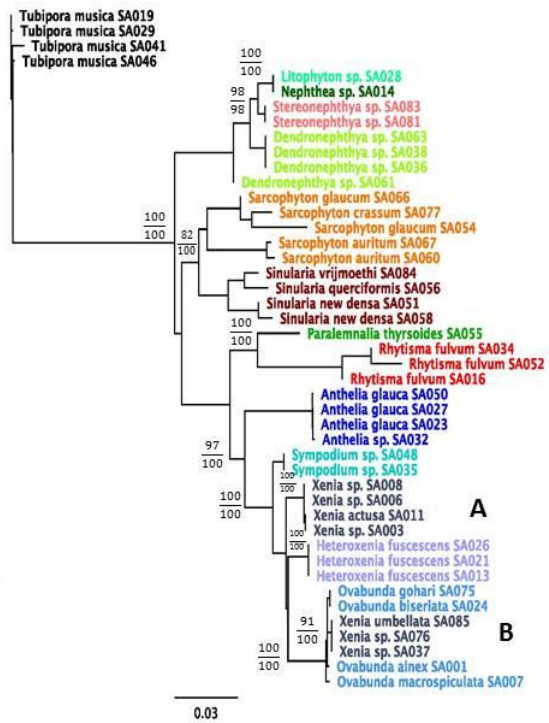


Figure 1. Phylogenetic relationships among 13 genera in the anthozoan order Alcyonacea (subclass Octocorallia). Left: Combined *ATP α* and *ITS* tree. Right: Combined *CO1* and *mtMutS* tree. Bootstrap values at nodes represent (top:) Maximum Likelihood and (bottom:) Bayesian Inference. Color coded by genus: All *Tubipora* specimens are black; All genera of the family Alcyoniidae are shades of red (*Sarcophyton*= light red, *Sinularia*= dark red, *Stereonephthya*= pink, *Rhytisma*= red); All genera of the family Nephtheidae are shades of Gn (*Dendronephthya*= Gn, *Paralemnalia*= dark Gn); All genera of the family Xeniiidae are shades of blue (*Anthelia* = aqua, *Sympodium* = light blue, *Heteroxenia* = bright blue, *Xenia* = dark aqua, *Ovabunda* = blue). *Xenia* group ‘A’ and ‘B’ are marked.

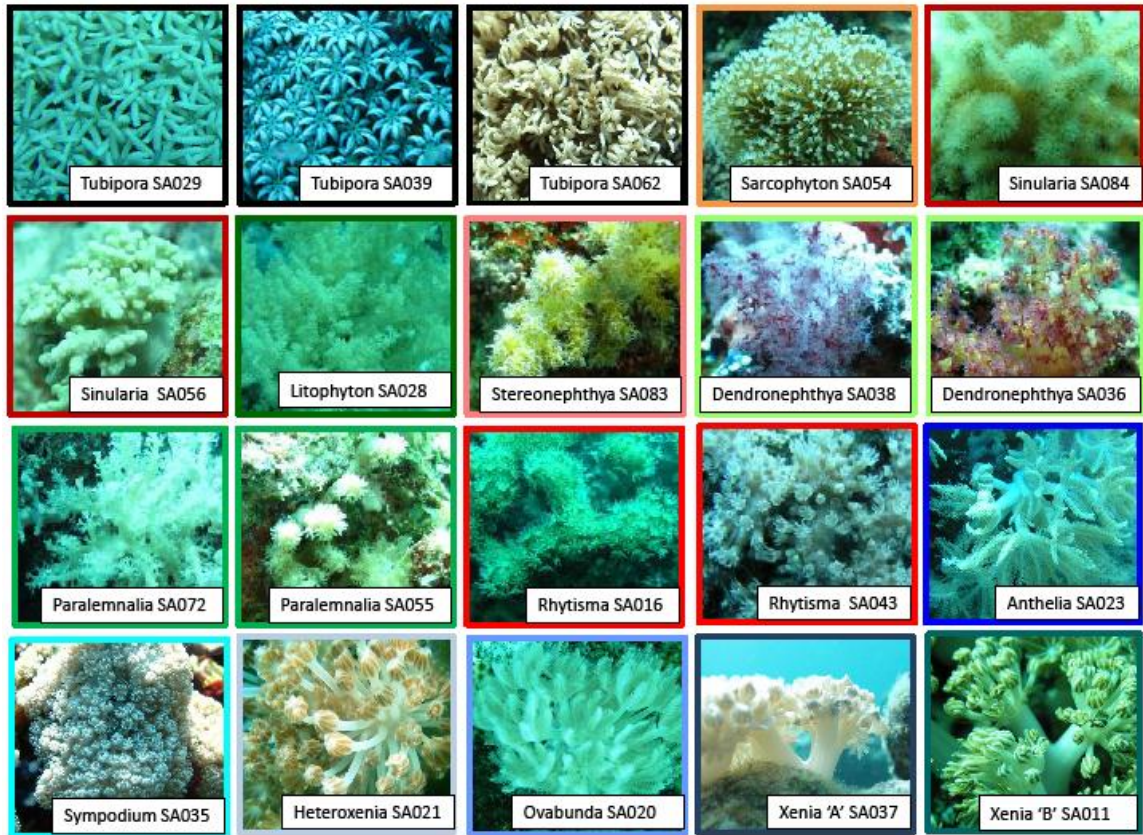


Figure 2. Photographs of all morphotypes that were surveyed. Colors correspond to the taxonomic groups in Figure 1.

CHAPTER 3. DO NUCLEAR MARKERS PROVIDE APPROPRIATE TAXONOMIC RESOLUTION IN PHYLOGENETIC STUDIES OF OCTOCORALS (CNIDARIA: ALCYONACEA)?

Abstract

The development of DNA markers for use in octocoral phylogenetics studies has been lagging for a variety of reasons. It has been hypothesized that better phylogenetic resolution in octocoral studies can be obtained from nuclear DNA markers in comparison to mitochondrial DNA markers based on previous findings with scleractinians. To test this hypothesis we assess the resolution of three mtDNA markers (*COI*, *mtMutS*, *ND2*) and five nDNA markers (*ATP5a*, *ATP5b*, *ITS*, *SRP54*, *28S*) among four octocoral families: Alcyoniidae, Nephtheidae, Tubiporidae, and Xeniidae. Our results indicate that phylogenetic analyses based on mtDNA or nDNA provide similar Maximum Likelihood bootstrap values (respectively 54% to 100% and 55% to 100%). Moreover, we find that combined mtDNA and nDNA analyses result in higher bootstrap values (71% to 100%) than either mtDNA or nDNA analyses. Notably, our analyses revealed six lineages within the genus *Ovabunda* (Alcyonacea: Xeniidae), which do not correspond with accepted taxonomy. This study emphasizes the importance of using multiple markers in octocoral phylogenetics studies. In future studies we recommend the use of the mtDNA markers *COI* and *mtMutS*, and the nDNA markers *ATP5b* and *28S*, because of relative ease of use, availability of newly developed primers presented here, and demonstrated utility in distinguishing taxonomic groups. This combination of markers provides a reasonable compromise between resolution and cost, and allows inferences regarding possible patterns of hybridization, introgression or incomplete lineage sorting.

Introduction

Identifying soft corals to the species level is notoriously difficult due to a variety of issues; uncertainty concerning morphological features to be used for identification, possible polymorphism within species, overlapping morphologies between species, and some ambiguous species descriptions (Fukami et al. 2004; McFadden et al. 2006a; Daly et al. 2007; Forsman et al. 2009; Chapter II). Hence, even though classical taxonomy can provide confident identifications for many taxa, the taxonomy and systematic relationships of octocorals remain the subject of considerable uncertainty (McFadden et al. 2010). The most widely accepted classification (Hickson 1930) divided the soft corals and gorgonians among four orders based on colony growth form (Alcyonacea, Gorgonacea, Stolonifera, Telestacea). Later, recognition that these groups grade into one another without clear morphological distinctions led Bayer (1981) to merge them into a single order, Alcyonacea. This large and morphologically diverse order is not defined by any synapomorphies however. For taxonomic convenience Alcyonacea is often sub-divided into six sub-ordinal groups (Alcyoniina, Calcaxonia, Holaxonia, Protoalcyonaria, Scleraxonia, Stolonifera), representing different grades of colony form and skeletal composition (Fabricius & Alderslade 2001; Daly et al. 2007). Regardless of classification, it is widely acknowledged that these groups do not reflect phylogenetic relationships (Berntson et al. 2001; Fabricius & Alderslade 2001; McFadden et al. 2006b), and current

consensus follows Bayer's classification (Fabricius & Alderslade 2001; McFadden et al. 2006a; Daly et al. 2007). We follow this classification as well. Alcyonacean families are distinguished primarily by overall colony growth form, presence or absence of a supporting skeletal axis, and details of axial composition. The form and distribution of sclerites (microscopic calcite crystals embedded in the coenenchymal tissue and polyps) are the most important characters used to distinguish genera and species of octocorals, but are less important for familial distinctions (Daly et al. 2007).

The utility of traditional morphological classification is limited by the few morphological characters available for identification, the possibility of polymorphism within species, and the overlapping morphologies between species. As a result, traditional morphological classifications often disagree with molecular phylogenetic groups among octocorals (Sánchez et al. 2003b; Fukami et al. 2004; McFadden et al. 2006a; Forsman et al. 2009). To discover additional informative morphological characters, recent studies have applied reverse taxonomy; the search for previously overlooked morphological characters, guided by the clades identified in phylogenies (Markmann & Tautz 2005; McFadden et al. 2006b, 2009; Chapter II). Reverse taxonomy is particularly useful in octocoral phylogenetics. Coral genetics is a relatively new field and lags behind other phylogenetic classifications because the mitochondrial genome of anthozoan cnidarians (e.g., scleractinian corals, sea anemones, and octocorals) evolves roughly 100-fold slower than in other metazoans (France and Hoover 2001, 2002; Shearer et al. 2002; McFadden et al. 2004, 2006a). In contrast, substitution rates in anthozoan nDNA markers are 5x higher compared to anthozoan mtDNA markers, whereas nDNA markers in other metazoans usually evolve more slowly than, or similar to, their mtDNA markers (Shearer et al. 2002; Chen et al. 2009). Therefore, researchers have started to seek nDNA anthozoan markers in addition to the available mtDNA markers (Odorico & Miller 1997; Jarman et al. 2002; Concepcion et al. 2008). To date only one single copy nDNA marker, *SRP54*, has been developed for an octocoral. Concepcion et al. (2008) tested the variability of *SRP54* against 141 colonies of the octocoral *Carijoa riisei*, a few members of the genus *Pocillopora* and members of the order Alcyonacea, and results indicate that this hypervariable nDNA segment is a single-copy marker. *SRP54* was successfully applied to resolve species of *Carijoa*, however other studies had difficulties amplifying the marker reliably across a range of octocorallian taxa (McFadden et al. 2010), or found extreme variability in intron length in keratoisidin bamboo corals (S.C. France, unpublished data). The use of multicopy markers (i.e., intragenomic polymorphisms among repeats within an individual), such as the hypervariable nuclear ribosomal internal transcribed spacer (*ITS*), is of limited value because it requires cloning to isolate and identify intra-individual sequence variants (McFadden et al. 2010). Furthermore, several studies stress the importance of using mtDNA markers in addition to *ITS* because intra-individual variation in the latter can overlap with the within- and between-species variation, and obscure potential phylogenetic signal (Forsman et al. 2005; 2006; Wei et al. 2006; Grajales et al. 2007). Nevertheless, *ITS* has provided statistical phylogenetic support for clades in a number of intrafamilial and intrageneric phylogenetic studies of octocorals (e.g., McFadden et al. 2001; Fujiwara et al. 2003; Ofwegen and Groenenberg 2007), and even in a couple of intraspecific and population level studies (e.g., Sánchez et al. 2007; Gutierrez-Rodriguez et al.

2009). However, *ITS* does not consistently provide sufficient resolution to distinguish evolutionary partitions within or between species (e.g., Lee and Song 2000; Calderon et al. 2006). Thus, the *ITS* region can be useful depending on the desired resolution, but more markers are needed for octocoral phylogenetics studies.

In order to resolve octocoral taxonomy and evaluate the utility of mtDNA versus nDNA markers therein, we examined 212 octocoral colonies from the west coast of Saudi Arabia and the south coast of Israel, within 4 families: Alcyoniidae (Lamouroux, 1812), Nephtheidae (Gray, 1862), Tubiporidae (Ehrenberg, 1828) and Xenidiidae (Wright & Studer, 1889), which were identified according to classical morphology-based taxonomy. From the south coast of Israel we sampled 158 Xenidiidae colonies and obtained the mtDNA markers MutS homolog (*mtMutS*), Cytochrome C Oxidase subunit 1 (*COI*), and NADH dehydrogenase subunit 2 (*ND2*), and the nDNA markers Internal Transcribed Spacers (*ITS*), 28S rDNA (*28S*), ATP Synthetase Subunit α (*ATPS α*), Signal Recognition Particle 54-kDa subunit (*SRP54*), and ATP Synthetase Subunit β (*ATPS β*) for most colonies. In the previous chapter, we obtained from the west coast of Saudi Arabia the mitochondrial markers *COI*, and *mtMutS* and the nuclear markers *ATPS α* , and *ITS* for most colonies, comprising all four families. In this chapter, we additionally obtained the mitochondrial marker *ND2* and the nuclear markers *ATPS β* , and *SRP54* from the Saudi Arabian Xenidiidae colonies, and the nuclear markers *28S* from all four families of Saudi Arabian octocorals. With the combined dataset, we examined the resolution of both mtDNA and nDNA markers in octocoral phylogenetics studies.

Materials and methods

Sample collection

Colonies of the soft coral family Xenidiidae were collected from Eilat, Israel (N=158, Table 3). For the analyses, data from these colonies was combined with the previously reported data from 74 Alcyonacea colonies of the families Alcyoniidae, Nephtheidae, Tubiporidae, and Xenidiidae which were collected from the west coast of Saudi Arabia (Table 4). During collections, a small specimen (1-5 cm) was cut from each coral colony using scissors or dive knife. Depth of collection, type of substrate (hard vs. sand), colony size (measured with ruler side clip board) and color, polyp retraction (process of invagination of the anthocodia within the upper part of the anthostele), tentacle contraction (process of deflation without invagination), stem split (applicable to Xenidiidae), and polyp pulsation (applicable to Xenidiidae), were recorded for each sampled colony. Paired tissue samples from each colony were stored in 95% ethanol and saturated salt DMSO (SSD) buffer for future molecular analyses (Gaither et al. 2011). In addition, a third tissue sample was stored in 70% ethanol for classical taxonomic study. Generic field identifications were verified by subsequent examination of colony morphology, including characterization of sclerites, presence of dimorphic polyps, presence of zooxanthellae, the number of pinnule rows on the polyp tentacles and the number of pinnules on the aboral side of the polyp tentacles (after Reinicke, 1997; Fabricius & Alderslade, 2001). Identifications were facilitated in part by comparisons with permanent sclerite preparations of type material kept at the Zoological Museum, Department of Zoology, Tel Aviv University, Israel (ZMTAU).

For species identification, sclerites were obtained by dissolving small tissue samples in 10% sodium hypochlorite, rinsed in distilled water and examined under a light microscope. Taxonomic samples are deposited at the Museum of Tel Aviv University, Israel.

DNA isolation and amplification

The mitochondrial markers *COI*, *ND2*, and *mtMutS* and the nuclear markers *ATPsa*, *ATPsβ*, *ITS*, *SRP54*, and *28S* were isolated and amplified from most Xeniidae specimens collected from Israel, Red Sea. In the previous chapter, the mitochondrial markers *COI*, and *mtMutS* and the nuclear markers *ATPsa*, and *ITS* were isolated and amplified of most Alcyoniidae, Nephtheidae, Tubiporidae and Xeniidae specimens collected from Saudi Arabia, Red Sea. In this chapter, we additionally isolated and amplified the mitochondrial marker *ND2* and the nuclear markers *ATPsβ*, and *SRP54* from the Saudi Arabian Xeniidae specimens, and the nuclear markers *28S* from all four families of Saudi Arabian octocorals.

DNA extractions for amplification of all mtDNA markers and the nDNA marker *28S* were performed using the DNeasy Blood & Tissue Kit® (Qiagen) following the manufacturer's protocol, as outlined in McFadden et al. (2011), and the primers are listed in Table 5. The mtDNA markers *mtMutS* (previously named *msh1*, ~730 bp) and cytochrome oxidase I (*COI*, ~770 bp) were amplified and sequenced using primers and protocols published by McFadden et al. (2011). The mtDNA marker NADH-dehydrogenase subunit 2 (*ND2*, ~710bp) was amplified and sequenced using primers and protocols published by McFadden et al. (2006a). The ribosomal 28S rDNA (*28S*, ~700bp) was amplified using the newly designed primers 28S-F & 28S-R. The PCR protocol: 94°C for 2 min (1 cycle to activate polymerase); 94°C for 30 s, 50°C for 30 s, 72°C for 60 s (35 cycles); followed by a final extension of 72°C for 5 min (1 cycle). *28S* primers were designed using VISTA (Frazer et al. 2004) and PrimerSelect (DNASTAR Inc.). PCR products from the mtDNA markers and the nDNA marker *28S* were purified and sent to the University of Washington's High-Throughput Genomics Center (Seattle, WA) for sequencing following the methods in McFadden et al. (2011).

DNA for all other nuclear marker amplification was extracted following the coral DNA extraction protocol outlined in Concepcion et al. (2006, 2008), and primers are listed in Table 5. The nDNA markers ATP Synthetase Subunit α (*ATPS α* , ~280bp) and the Ribosomal Internal Transcribed Spacer region (*ITS*, ~1100bp) were amplified and sequenced using primers and protocols outlined in Chapter II. The nDNA marker ATP Synthetase Subunit β (*ATPS β* , ~400bp) was amplified using the previously published primers *ATPS β f1* and *ATPS β r1* (Jarman et al. 2002), in addition to the newly designed primers *XATPS β F* & *XATPS β R* (annealing temperature 53°C), *ATPs β _Het_F1* & *ATPs β _Het_R1* (53°C), *ATPs β _Ova_F1* & *ATPs β _Ova_R1* (57°C), *ATPs β _Xen_F1* & *ATPs β _Xen_R2* (57°C). *ATPS β* primers were designed using Primer3 (Rozen and Skaletsky 2000). The PCR protocol: 95°C for 10 min (1 cycle to activate polymerase); 95°C for 30 s, 53°C or 57°C for 30 s, 70°C for 4 min (35 cycles); followed by a final extension of 72°C for 10 min (1 cycle). The nDNA marker Signal Recognition Particle 54-kDa subunit (*SRP54*, ~440bp) was amplified using the previously published primers *SRP54f1* & *SRP54r1* (49°C, Jarman et al. 2002), in

addition to the newly designed primers XSRP54F & XSRP54R (48°C). *SRP54* primers were designed using Primer3 (Rozen and Skaletsky 2000). The PCR protocol: 95°C for 10 min (1 cycle to activate polymerase); 95°C for 30 s, 48°C or 49°C for 30 s, 70°C for 4 min (35 cycles); followed by a final extension of 72°C for 10 min (1 cycle). PCR amplification of the nDNA markers *ATPS α* , *ITS*, *ATPS β* , and *SRP54* was performed on a Bio-Rad MyCycler™. Each 25 μ l PCR reaction contained 1.0 μ l of template DNA (~5 ng μ L⁻¹), 12.5 μ l BioMix Red 2X PCR reaction mix (Bioline Inc.), 0.325 μ l of each primer (10 μ M), 0.75 μ l of BSA (10mg ml⁻¹) and 11.1 μ l deionized sterile water. For some reactions an additional 1.0 μ l of DNA template was added to improve PCR amplification success (final volume 26 μ l). PCR products were visualized using 1.0% agarose gels in 1x SB (sodium borate; Brody et al. 2004) buffer stained with Gelstar® (Lonza). Amplification reactions were first treated with Exonuclease I and FastAP™ thermo sensitive alkaline phosphatase (Fermentas) using the following thermocycler profile: 37°C for 60 min, 85°C for 15 min. Treated PCR products were then cycle-sequenced using BigDye Terminators (Applied Biosystems) run on an ABI-3730XL DNA Analyzer at the Advanced Studies of Genomics, Proteomics and Bioinformatics (ASGPB) facility at University of Hawai'i Mānoa.

Sequencing and phasing

For nDNA markers, phase (Stephens et al. 2001) as implemented in DNAsp 5 (Librado and Rozas 2009) was used to resolve the alleles for each heterozygous colony, when aligning the direct sequenced forward and reverse sequences was possible. The PCR products of heterozygous colonies that could not be phased reliably from direct sequences were ligated into the pGEM®-T Easy cloning vector (Promega Inc.) and transformed into JM109 competent cells following the manufacturer protocol. After blue/white colony selection, up to 12 colonies were screened by PCR to verify an insert of the correct size using the M13 vector primers. Initially, four colonies were sequenced with the original primers. Heterozygous alleles were accepted only if two or more copies of the same allele were found and could be matched with the direct sequence read from the original mixed template. If the first four colonies did not recover two copies of each allele, four additional colonies were sequenced until two copies of each allele were recovered.

Phylogenetic analyses

All mtDNA sequences were visually inspected using lasergene (DNASStar) and all nDNA sequences were visually inspected using GENEIOUS Pro 4.7.6 (Drummond et al. 2010). All sequences were aligned using the CLUSTALW 2.0.11 (Larkin et al. 2007) plugin in GENEIOUS. Subsequently, phylogenetic relationships for all multi marker analyses were reconstructed using Maximum Likelihood (100 bootstrap replicates) methods in Garli 2.0 (Zwickl 2008). Best substitution models were found with modeltest 3.7 (Nylander 2004) plugin in GENEIOUS Pro. Phylogenetic relationships for all individual markers analyzed were constructed using Bayesian Inference (Chain Length= 1.1 x 10⁶, Burn-in Length= 110,000 (Huelsenbeck and Ronquist 2001)) methods in GENEIOUS Pro (MrBayes 2.0.2).

First, separate multi marker analysis were constructed consisting of all mtDNA, and all nDNA sequences separately to compare the phylogenetic resolution of mtDNA versus nDNA markers. Next,

mtDNA and nDNA sequences were collated to examine the total evidence tree. For all above mentioned analyses the family Tubiporidae was used as outgroup based on earlier analyses (McFadden et al. 2006a). Furthermore, to test the utility of the multi marker analyses to distinguish taxonomic groups within a genus, a tree was constructed consisting of all markers, both mtDNA and nDNA, comprising colonies of the octocoral genus *Ovabunda* (Alcyonacea: Xeniidae). Sister taxa *Heteroxenia* and *Sympodium* were used as outgroups based on the prior analyses described above. Finally, to compare resolution of individual markers, trees were constructed with each marker individually for: 1) species level analyses, comprising of only *Ovabunda* colonies and outgroups *Heteroxenia* and *Sympodium*; 2.) genus level analyses, comprising only Xeniidae colonies and outgroups *Rhytisma* and *Paralemmalia*; and 3) family level analyses, comprising the families Alcyoniidae, Nephtheidae, Tubiporidae, and Xeniidae, with Tubiporidae as an outgroup.

Results

Molecular phylogeny

We obtained a total of ~2210bp of mtDNA comprising three markers (*mtMutS*, *COI*, and *ND2*), and ~2920bp of nDNA comprising five markers (*ITS*, *SRP54*, *ATPs β* , *ATPs α* and *28S*) for most colonies (Table 1, 2). The best substitution model for both *mtMutS* and *COI* was HKY+G (Hasegawa et al. 1985), and therefore the analysis of these markers was combined by concatenating the sequences. The final alignment of *mtMutS* included 738 nucleotide positions and the final alignment of *COI* included 718 nucleotide positions; 404 out of the combined 1456 characters were parsimony-informative, and 42 were variable. The best substitution model for *ND2* was HKY. The final alignment of *ND2* included 711 nucleotide positions; 29 were parsimony-informative, and two were variable. The best substitution model for both *ITS* and *ATPs α* was TrN+I and therefore the analysis of these markers was combined. The final alignment of *ITS* included 973 nucleotide positions and the final alignment of *ATPs α* included 243 nucleotide positions; 663 out of the combined 1216 characters were parsimony-informative, and 40 were variable. The best substitution model for *SRP54* was HKY+G. The final alignment of *SRP54* included 322 nucleotide positions; 183 were parsimony-informative, and 56 were variable. In combined analyses of nDNA and mtDNA, *SRP54* was combined with *mtMutS* and *COI* because these markers share the same substitution model. The best substitution model for *ATPs β* was HKY. The final alignment of *ATPs β* included 361 nucleotide positions; 138 were parsimony-informative, and eight were variable. In combined analyses of nDNA and mtDNA, *ATPs β* was combined with *ND2* because these markers share the same substitution model. The best substitution model for *28S* was GTR+G. The final alignment of *28S* included 699 nucleotide positions; 241 were parsimony-informative, and 19 were variable.

Separate multi marker analysis of all mtDNA and all nDNA markers

The combined phylogeny of mtDNA markers (*mtMutS*, *COI*, and *ND2*) for all four octocoral families (Alcyoniidae, Nephtheidae, Tubiporidae, and Xeniidae) revealed that 11 out of 13 genera were clearly and

consistently distinguishable, the exceptions being *Ovabunda* and *Xenia* (Figure 3). Maximum Likelihood bootstrap support ranged from 54% to 100%. The combined phylogeny of nDNA markers (*ITS*, *SRP54*, *ATPs β* , *ATPs α* and *28S*) for all four octocoral families revealed that 10 out of 13 genera were clearly and consistently distinguishable, the exceptions being *Sarcophyton*, *Sinularia*, and *Xenia* (Figure 3). Bootstrap support ranged from 55% to 100%. In the combined nDNA phylogeny, but not in the combined mtDNA phylogeny, two *Sarcophyton* colonies grouped within the *Sinularia* clade with 100% bootstrap support. In both mtDNA and nDNA analyses, *Xenia* was paraphyletic and had two lineages, one closely related to the genus *Ovabunda* (with 54% bootstrap support in mtDNA analyses and 100% bootstrap support in nDNA analyses), and one as a sister group to *Heteroxenia* (with 71% bootstrap support in mtDNA analyses and 96% bootstrap support in nDNA analyses). In both mtDNA and nDNA analyses Tubiporidae was designated as outgroup, and members of the Alcyoniidae and Nephtheidae consistently grouped in three clades. One of these contained the Nephtheidae genera *Dendronephthya*, *Litophyton* and *Stereonephthya*, the second contained the Alcyoniidae genera *Sarcophyton* and *Sinularia*, and the last was a mixed clade containing *Rhytisma* from Alcyoniidae and *Paralemnalia* from Nephtheidae (Figure 3).

Multi marker analysis consisting of all markers, both mtDNA and nDNA

The combined phylogeny of mtDNA and nDNA markers (*mtMutS*, *COI*, *ND2*, *ITS*, *SRP54*, *ATPs β* , *ATPs α* and *28S*) that includes all four octocoral families revealed that 10 out of 13 genera were clearly and consistently distinguishable, the exceptions being *Sarcophyton*, *Ovabunda* and *Xenia* (Figure 4). Genera grouped as described above, except bootstrap values were higher, ranging from 71% to 100% (Figure 4).

Six distinct clades within the genus Ovabunda

The combined phylogeny of mtDNA and nDNA markers (*mtMutS*, *COI*, *ND2*, *ITS*, *SRP54*, *ATPs β* , *ATPs α* and *28S*) that comprises colonies of the octocoral genus *Ovabunda* (Alcyonacea: Xeniidae), revealed six lineages which are incongruent with classical taxonomy (Figure 5). Additionally, two individual *Ovabunda* colonies did not consistently belong to any of the six clades and were identified with unique haplotypes (SA007 and SA009). Bootstrap support ranged from 58% to 100%.

Individual marker analyses on species, genus, and family level

Analyses of the genus *Ovabunda* with each marker individually (Appendix A) revealed that most clades were recovered by the mtDNA marker *mtMutS* (four out of five) and the nDNA marker *ITS* (four out of six) (Table 6). Colonies affiliated with the sixth clade (Figure 5) were successfully amplified for only the nDNA markers *ITS* and *ATPs β* .

The analyses of the family Xeniidae with each marker individually (Appendix B), revealed that the nDNA markers *ITS* and *28S* provided the greatest resolutions and clearly separated four out of five genera, followed closely by the mtDNA markers *COI* and *ND2* which both clearly separated three out of five genera (Table 7).

Finally, the analyses of the families Alcyoniidae, Nephtheidae, Tubiporidae, and Xeniidae with each marker individually (Appendix C) revealed that the mtDNA markers *mtMutS* and *COI*, and the nDNA markers *ITS* and *28S* provided the greatest resolution.

Heterozygosity

The nDNA marker *SRP54* had the highest proportion of heterozygous sequences (57%), followed by *ATPsβ* (48%), *ITS* (32%), *ATPsα* (30%), and *28S* (3%) (Table 8). Furthermore, proportions of heterozygous sequences derived from direct sequencing (and implementing phase to separate heterozygotes) versus cloning (and sequencing individual heterozygote sequences) ranged from 74% to 100% (Table 8). The marker *28S* revealed the highest proportion of heterozygous sequences derived from direct sequences as opposed to cloning (100%), followed by *ATPsα* (95%), *ITS* (77%), *ATPsβ* (75%), and *SRP54* (74%).

Evaluation of possible cryptic taxa

All morphotypes identified in the field were tested for concordance with the molecular data. Two of the three morphotypes within *Tubipora musica* (differences in tentacle length and form) were distinguished by the mtDNA phylogeny and the nDNA markers *ITS* and *28S*, but not *ATPsα*. The two morphotypes within *Paralemnalia thyrsoides* (multiple polyps connected by stolons or cluster of digitiform branches arising from a common base) were not supported in the mtDNA phylogeny or the nDNA markers *28S* and *ATPsα*. However, the nDNA marker *ITS*, as well as the combined nDNA phylogeny and the combined mtDNA and nDNA phylogeny did support two distinct clades that correspond to colony morphology. The two morphotypes found within *Xenia* spp. (small colonies with short stems, short white tentacles, white polyps, and brown pinnules versus either similar colony morphological features but lighter in color, or different morphological features (longer stems and polyp arms) and a similar color pattern) were supported by all phylogenies. Divergent morphologies within *Ovabunda* spp. colonies (varying stem, polyp, and tentacle lengths and colors, varying presence, form and arrangement of sclerites and varying number and arrangement of pinnules) were not supported by any phylogenies, however, six distinct clades were found in addition to two individual colonies that did not consistently group with any of the six clades and which were identified with unique haplotypes. Furthermore, there were no distinctions among morphotypes within *Rhytisma fulvum fulvum* (Gn/grey color morphs), *Sinularia* species (stalked/ encrusting colony structure), and the genus *Dendronephthya* (dark red and white/ orange and pink color morphs) in any of the phylogenies.

Discussion

The development of DNA markers for octocoral phylogenetics studies has been lagging for a variety of reasons. Since 1996 several studies have revealed and supported the unresolved state of octocoral taxonomy by means of molecular markers such as the partial *16S*, complete *18S*, *ND2*, *mtMutS*, *COI* and complete *28S* (France et al 1996; Bernston et al 2001; Sánchez et al 2003b). However, the poor resolution in most studies to date is a result of the slow substitution rate in anthozoan mtDNA (McFadden et al 2006b;

Concepcion et al 2008). It has been hypothesized that better phylogenetic resolution in octocoral studies can be obtained from nuclear DNA markers in comparison to mitochondrial DNA markers, based on previous findings with scleractinians. Hence, researchers have expressed the need to develop more nDNA markers for soft coral phylogenetics studies. To test this hypothesis we assess the phylogenetic resolution of three mtDNA markers (*ND2*, *mtMutS*, *COI*) and five nDNA markers (*ATPSa*, *ATPSβ*, *ITS*, *SRP54*, *28S*) among four octocoral families: Alcyoniidae, Nephtheidae, Tubiporidae, and Xeniidae.

1) *Do nDNA markers reveal higher resolution than mtDNA markers?*

To date few nDNA markers have been available for cnidarian phylogenetics studies (Concepcion et al 2008). Based on our analyses, 74% to 100% (depending on the marker) of the heterozygous sequences were retrievable by direct sequencing as opposed to cloning, reducing the associated labor and cost. Additionally, we find that combined mtDNA and nDNA phylogenetic analyses result in higher maximum likelihood bootstrap values (71% to 100%) than analyses of either mtDNA (54% to 100%) or nDNA (55% to 100%). This increase in bootstrap values is likely a result of merging mitochondrial and nuclear markers because they have different inheritance patterns and therefore function synergistically. Because of the different inheritance patterns between mitochondrial (maternal) and nuclear (biparental) markers, the use of either mtDNA or nDNA alone may miss valuable information regarding evolutionary history. For example, Forsman et al. (2010) amplified multiple mtDNA (*COI*, *CR*, *Cyt-B*, *16S*, *ATP6*) and nDNA (*ATPSβ*, *ITS*) markers for colonies in the scleractinian genus *Montipora*. The *ATPSβ* intron data showed a pattern often interpreted as resulting from hybridization and introgression; however, Forsman et al. (2010) concluded that incomplete lineage sorting was more likely because the nDNA *ITS* region was consistent with the mtDNA data. This example emphasizes how the use of multiple mtDNA and nDNA markers can lead to stronger inferences about evolutionary history and contemporary demography.

The sequences of the molecular markers *mtMutS*, *COI*, *ITS*, and *ATPSa*, obtained from specimens from the west coast of Saudi Arabia, were previously reported in chapter II. Additionally, we obtained sequences of the mtDNA marker *ND2* and the nDNA markers *28S*, *ATPSβ* and *SRP54* from the Saudi Arabian specimens. These additional markers allow broader testing of phylogenetic resolution, and allow us to re-evaluate patterns previously discussed in chapter II. For example, results represented in chapter II indicate that the three morphotypes in *Tubipora musica*, based on differences in tentacle length and form, were supported by the mtDNA markers *mtMutS* and *COI*, and two out of three morphotypes were supported by the nDNA marker *ITS*, but no morphotypes were supported by the nDNA *ATPSa*. Our additional nDNA marker *28S* support two out of three distinct clades. The third group remains unidentified by the nuclear markers *ITS* and *28S* due to unsuccessful sequencing of these colonies. No *ND2* sequences were recovered from *Tubipora* colonies. Our additional data indicate shared genetic and morphological divergence, which support the hypothesis of potentially new species as discussed in chapter II. Furthermore, based on the data in chapter II, the genus *Xenia* forms 2 monophyletic clades. Our additional markers *ND2* and *28S* support this finding and reinforce the importance of continued research on this group.

2) *Is there an optimal set of markers for future octocoral phylogenetic studies?*
Based on our species level analyses, the mtDNA marker *mtMutS* and the nDNA markers *ATPs β* and *ITS* provided the highest resolution in distinguishing different clades within the genus *Ovabunda*. By amplifying only the mtDNA marker *mtMutS* and the nDNA marker *ITS*, all *Ovabunda* clades can be recovered (Table 6). Previous studies using *ITS* for species level analyses have produced contradicting results; Vollmer and Palumbi (2004) and Calderon et al. (2006) reported that *ITS* does not consistently provide sufficient resolution to distinguish evolutionary lineages within or between species. However, these studies included the scleractinian genus *Acropora* (Vollmer and Palumbi 2004) and gorgonians (Calderon et al. 2006), but no similar findings have so far been reported on non-gorgonian octocorals. A much larger range of studies agree that *ITS* provides ample resolution to distinguish species level taxonomy (McFadden et al. 2001 (soft coral genus *Alcyonium*); Fujiwara et al. 2003 (soft coral genus *Clavularia*); Ofwegen and Groenenberg 2007 (soft coral family Nephtheidae); Sánchez et al. 2007 (gorgonians); and Gutierrez-Rodriguez et al. 2009 (gorgonians)). Our results indicate that in genus level analyses the nDNA markers *ITS* and *28S* provide the highest resolution for distinguishing genera, closely followed by the mtDNA markers *mtMutS* and *COI*. In family level analyses, the mtDNA markers *mtMutS* and *COI*, and the nDNA markers *ITS* and *28S* provide the highest resolution at distinguishing genera. Based on these findings, we recommend the following four markers: *28S*, *ATPs β* , *mtMutS*, and *COI*. Each of these four markers have distinct advantages: *28S*, because of ease of use and low levels of heterozygosity, *ATPs β* , because of its enhanced ability to distinguish clades in species level analyses, and *mtMutS* and *COI*, because of their enhanced ability to distinguish additional clades in species level analyses, as well as genus and family level analyses. Combined, they provide the best compromise between resolution, cost, and ease of use among currently available markers.

3) *Possible cryptic species within the genus Ovabunda (Xeniidae: Alcyonacea)*
The genus *Ovabunda* was only recently described by Alderslade (2001), and since that time several different species have been assigned to the genus based on morphology-based taxonomy. Our phylogenetic analyses of this genus, using the mtDNA markers *mtMutS*, *COI*, and *ND2* and the nDNA markers *ITS*, *28S*, *ATPs α* , *SRP54*, and *ATPs β* , revealed six distinct clades which are incongruent with classical taxonomy. This incongruence indicates that the characters currently used to identify *Ovabunda* species may not reflect evolutionary separations, and highlights the need for further taxonomic attention. Overlapping sclerite morphologies (cemented/ columnar depositions/ integrated) between the clades is not surprising because the formation of the 6 distinct clades is very recent, and not sufficient time may have passed for these clades to develop unique morphological characters. This phylogeny reveals possible cryptic species within *Ovabunda* and will aid the search to possible previously overlooked morphological characters which correspond with evolutionary genetic partitions.

Recommendations for future octocoral phylogenetic studies

In conclusion, mtDNA phylogenetic analyses and nDNA phylogenetic analyses provide low but similar maximum likelihood bootstrap values (54% to 100% in mtDNA analyses and 55% to 100% in nDNA analyses) and provide very similar phylogenetic resolution, and therefore both are effective in phylogenetic studies on the octocoral families Alcyoniidae, Nephtheidae, Tubiporidae, and Xeniidae, and likely others as well. Combined phylogenetic analyses of all mtDNA and nDNA markers result in higher maximum likelihood bootstrap values (71% to 100%) than either class of marker alone. This emphasizes the importance of using both nDNA as well as mtDNA markers in octocoral phylogenetics studies. The newly tested nuclear markers have clear utility and reveal 1) the same cryptic *Sarcophyton* clade reported by McFadden et al (2006b), and 2) cryptic taxa in *Ovabunda*, *Xenia*, and *Tubipora* which were not recognized previously. We find that a combination of the mtDNA markers *mtMutS* and *COI*, and the nDNA markers *28s* and *ATPs β* provide the greatest resolution for the least cost and effort. Moreover, the use of both mtDNA and nDNA markers allows inferences regarding possible patterns of hybridization, introgression, or incomplete lineage sorting.

Table 3. List of Xeniididae collected from Eilat, Israel and acquired molecular markers (✓= homozygote successfully acquired by direct sequencing, ✓*= heterozygote successfully acquired by direct sequencing, ✓**heterozygote successfully acquired by cloning, empty cell = full sequence unavailable).

Sample Name	Genus	Species	ITS	SRP54	ATPsβ	ATPsa	28S	mtMutS	COI	ND2
A01	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓	✓	✓	✓		
A02	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓*	✓	✓	✓	✓	
A03	<i>Ovabunda</i>	<i>macrospiculata</i>	✓*	✓*	✓	✓**	✓	✓	✓	✓
A04	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓*	✓	✓	✓		✓
A05	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓	✓	✓	✓	✓	✓
A06	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓*	✓*	✓*	✓	✓	✓	✓
A07	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓	✓	✓	✓	✓	✓
A08	<i>Ovabunda</i>	<i>verseveldti</i>	✓	✓*	✓		✓	✓	✓	✓
A09	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓*	✓	✓	✓	✓	✓	✓
A10	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓*	✓	✓	✓	✓	✓
A12	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓	✓	✓	✓	✓	✓
A13	<i>Ovabunda</i>	<i>ainex</i>	✓*	✓**	✓*					
A15	<i>Xenia</i>	sp.	✓	✓*	✓	✓	✓		✓	✓
A16	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓	✓*				
A17	<i>Ovabunda</i>	<i>macrospiculata</i>	✓	✓*	✓	✓	✓	✓	✓	✓
A18	<i>Ovabunda</i>	<i>ainex</i>	✓*	✓	✓					
A19	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓	✓*				
A21	<i>Xenia</i>	<i>hicksoni</i>	✓*	✓	✓	✓*				
A22	<i>Ovabunda</i>	<i>verseveldti</i>	✓	✓	✓		✓	✓	✓	✓
A23	<i>Ovabunda</i>	<i>macrospiculata</i>	✓**	✓*	✓*		✓	✓	✓	✓
A24	<i>Ovabunda</i>	<i>ainex</i>	✓	✓	✓	✓*	✓	✓	✓	✓
A25	<i>Ovabunda</i>	<i>ainex</i>	✓	✓*	✓		✓	✓	✓	✓
A26	<i>Ovabunda</i>	<i>farauensis</i>	✓*	✓**	✓	✓	✓	✓	✓	✓
A27	<i>Ovabunda</i>	<i>gohari</i>	✓*	✓*	✓		✓*	✓	✓	✓
A28	<i>Ovabunda</i>	<i>farauensis</i>					✓	✓	✓	✓
A29	<i>Ovabunda</i>	<i>biseriata</i>	✓*	✓*	✓	✓*	✓	✓	✓	✓
A30	<i>Xenia</i>	<i>hicksoni</i>	✓**	✓**	✓	✓*				
A32	<i>Ovabunda</i>	<i>macrospiculata</i>	✓*	✓**	✓*	✓	✓	✓	✓	✓
A33	<i>Xenia</i>	<i>umbellata</i>	✓	✓*	✓**	✓				
A34	<i>Xenia</i>	<i>umbellata</i>	✓*	✓	✓**	✓*		✓		✓

Table 3. (Continued) List of Xeniididae collected from Eilat, Israel and acquired molecular markers (✓= homozygote successfully acquired by direct sequencing, ✓*= heterozygote successfully acquired by direct sequencing, ✓**heterozygote successfully acquired by cloning, empty cell = full sequence unavailable).

Sample Name	Genus	Species	ITS	SRP54	ATPsβ	ATPsa	28S	mtMutS	COI	ND2
A35	<i>Xenia</i>	<i>umbellata</i>	✓*	✓*	✓**	✓				
A36	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓		✓	✓	✓	✓
A37	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓	✓*	✓*	✓	✓	✓	✓
A38	<i>Xenia</i>	<i>puerto-galerae</i>	✓	✓*	✓	✓*				
A39	<i>Ovabunda</i>	<i>farauensis</i>					✓	✓	✓	✓
A40	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓	✓				
A41	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓**					
A42	<i>Xenia</i>	<i>hicksoni</i>	✓	✓*	✓	✓				
A43	<i>Ovabunda</i>	<i>macrospiculata</i>	✓*		✓		✓	✓	✓	✓
A44	<i>Xenia</i>	sp.	✓	✓	✓	✓				
A45	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓	✓*		✓		
A46	<i>Xenia</i>	<i>umbellata</i>	✓*	✓*	✓**	✓				
A47	<i>Xenia</i>	<i>puerto-galerae</i>	✓*	✓*		✓*				
A48	<i>Ovabunda</i>	<i>farauensis</i>	✓	✓	✓	✓	✓	✓	✓	✓
A49	<i>Ovabunda</i>	<i>impulsatilla</i>	✓*	✓	✓*	✓	✓	✓	✓	✓
A50	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓	✓		✓	✓	✓
A51	<i>Ovabunda</i>	<i>biseriata</i>					✓	✓	✓	✓
A52	<i>Ovabunda</i>	<i>farauensis</i>				✓		✓	✓	✓
A53	<i>Ovabunda</i>	<i>biseriata</i>				✓		✓	✓	✓
A54	<i>Xenia</i>	<i>umbellata</i>	✓*	✓**	✓**					
A55	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓	✓				
A56	<i>Xenia</i>	<i>umbellata</i>	✓	✓*	✓**	✓				
A57	<i>Ovabunda</i>	<i>macrospiculata</i>	✓*	✓	✓	✓	✓	✓	✓	✓
A58	<i>Ovabunda</i>	<i>farauensis</i>					✓	✓	✓	✓
A59	<i>Ovabunda</i>	<i>macrospiculata</i>	✓	✓*	✓	✓	✓	✓	✓	✓
A60	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓*	✓	✓	✓		✓	✓
A61	<i>Ovabunda</i>	<i>ainex</i>	✓	✓*	✓*		✓	✓	✓	✓
A62	<i>Ovabunda</i>	<i>biseriata</i>	✓*	✓*	✓	✓	✓	✓		✓
A63	<i>Ovabunda</i>	<i>macrospiculata</i>	✓*	✓*	✓		✓	✓	✓	✓
A64	<i>Anthelia</i>	<i>glauca</i>	✓	✓**						
A65	<i>Xenia</i>	<i>umbellata</i>	✓	✓*	✓	✓*	✓	✓	✓	✓

Table 3. (Continued) List of Xeniidæ collected from Eilat, Israel and acquired molecular markers (✓= homozygote successfully acquired by direct sequencing, ✓*= heterozygote successfully acquired by direct sequencing, ✓**heterozygote successfully acquired by cloning, empty cell = full sequence unavailable).

Sample Name	Genus	Species	ITS	SRP54	ATPsβ	ATPsa	28S	mtMutS	COI	ND2
A66	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓*	✓*		✓	✓	✓	✓
A67	<i>Xenia</i>	<i>hicksoni</i>	✓	✓*	✓*	✓*				
A68	<i>Xenia</i>	<i>umbellata</i>	✓	✓**	✓	✓				
A69	<i>Ovabunda</i>	<i>farauensis</i>	✓**	✓*	✓	✓	✓	✓	✓	✓
A70	<i>Ovabunda</i>	<i>macrospiculata</i>	✓	✓	✓*		✓	✓	✓	✓
A71	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓	✓	✓	✓	✓	✓	✓
A72	<i>Ovabunda</i>	<i>macrospiculata</i>	✓	✓**	✓*	✓	✓	✓	✓	✓
A73	<i>Xenia</i>	<i>membranacea</i>	✓	✓**	✓**	✓*				
A74	<i>Xenia</i>	<i>umbellata</i>	✓	✓	✓	✓	✓	✓	✓	✓
A75	<i>Xenia</i>	<i>umbellata</i>	✓*	✓*	✓	✓				✓
A76	<i>Xenia</i>	<i>umbellata</i>	✓*	✓**	✓**	✓	✓		✓	✓
A77	<i>Ovabunda</i>	<i>impulsatilla</i>	✓*	✓*	✓		✓	✓	✓	✓
A78	<i>Xenia</i>	<i>hicksoni</i>	✓*		✓	✓				
A79	<i>Xenia</i>	<i>hicksoni</i>	✓	✓	✓	✓*	✓	✓		✓
A80	<i>Ovabunda</i>	<i>verseveldti</i>	✓*	✓*	✓		✓*	✓	✓	✓
A81	<i>Ovabunda</i>	<i>impulsatilla</i>	✓*	✓*	✓	✓		✓	✓	✓
A82	<i>Ovabunda</i>	<i>verseveldti</i>	✓*	✓*	✓**	✓*		✓	✓	✓
A83	<i>Ovabunda</i>	<i>macrospiculata</i>	✓	✓*	✓*	✓	✓		✓	✓
A84	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓	✓		✓	✓	✓	✓
A86	<i>Ovabunda</i>	<i>farauensis</i>	✓*	✓*	✓	✓*	✓	✓	✓	
A87	<i>Ovabunda</i>	<i>farauensis</i>	✓*	✓*	✓**		✓	✓	✓	✓
A88	<i>Xenia</i>	<i>umbellata</i>	✓	✓*		✓*	✓	✓	✓	✓
A89	<i>Xenia</i>	<i>hicksoni</i>	✓*		✓**	✓				
A90	<i>Ovabunda</i>	<i>farauensis</i>	✓**	✓	✓*	✓*				
A91	<i>Anthelia</i>	<i>fishelsoni</i>	✓							
A92	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓					
A93	<i>Ovabunda</i>	<i>ainex</i>			✓	✓				
A94	<i>Xenia</i>	<i>gohari</i>	✓	✓*	✓					
A95	<i>Ovabunda</i>	<i>biseriata</i>	✓*	✓*	✓**	✓				
A96	<i>Ovabunda</i>	<i>ainex</i>	✓		✓	✓*				

Table 3. (Continued) List of Xeniidae collected from Eilat, Israel and acquired molecular markers (✓= homozygote successfully acquired by direct sequencing, ✓*= heterozygote successfully acquired by direct sequencing, ✓**heterozygote successfully acquired by cloning, empty cell = full sequence unavailable).

Sample Name	Genus	Species	<i>ITS</i>	<i>SRP54</i>	<i>ATPsβ</i>	<i>ATPsa</i>	<i>28S</i>	<i>mtMutS</i>	<i>COI</i>	<i>ND2</i>
A97	<i>Xenia</i>	<i>umbellata</i>	✓*		✓	✓				
A98	<i>Ovabunda</i>	<i>macrospiculata</i>	✓		✓					
A99	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓		✓*				
A101	<i>Ovabunda</i>	<i>macrospiculata</i>			✓	✓				
A102	<i>Xenia</i>	<i>umbellata</i>	✓*	✓		✓				
A103	<i>Ovabunda</i>	<i>macrospiculata</i>	✓	✓	✓*					
A104	<i>Xenia</i>	<i>umbellata</i>	✓		✓	✓				
A105	<i>Xenia</i>	<i>hicksoni</i>	✓*		✓	✓				
A106	<i>Ovabunda</i>	<i>macrospiculata</i>	✓		✓	✓				
A107	<i>Ovabunda</i>	<i>macrospiculata</i>	✓		✓*	✓				
A108	<i>Ovabunda</i>	<i>farauensis</i>	✓		✓*	✓				
A109	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓*	✓				
A110	<i>Ovabunda</i>	<i>farauensis</i>	✓	✓*	✓*	✓**				
A111	<i>Ovabunda</i>	<i>macrospiculata</i>	✓		✓*	✓				
A112	<i>Ovabunda</i>	<i>biseriata</i>			✓					
A113	<i>Ovabunda</i>	<i>biseriata</i>								
A114	<i>Xenia</i>	<i>umbellata</i>	✓*	✓	✓*	✓				
A115	<i>Ovabunda</i>	<i>farauensis</i>	✓	✓	✓	✓	✓			
A116	<i>Ovabunda</i>	<i>biseriata</i>	✓*	✓*	✓					
A117	<i>Ovabunda</i>	<i>macrospiculata</i>	✓	✓	✓		✓			
A118	<i>Ovabunda</i>	<i>impulsatilla</i>	✓	✓	✓	✓*				
A119	<i>Ovabunda</i>	<i>farauensis</i>	✓	✓*	✓					
A120	<i>Anthelia</i>	<i>glauca</i>								
A121	<i>Ovabunda</i>	<i>impulsatilla</i>	✓*		✓	✓				
A122	<i>Ovabunda</i>	<i>puerto-galerae</i>	✓		✓**	✓	✓			
A123	<i>Xenia</i>	<i>umbellata</i>	✓*		✓*	✓*				
A124	<i>Ovabunda</i>	<i>arabica</i>	✓*	✓**	✓	✓*	✓			
A125	<i>Ovabunda</i>	<i>farauensis</i>	✓*		✓**	✓*				
A126	<i>Ovabunda</i>	<i>biseriata</i>		✓*	✓*					
A127	<i>Xenia</i>	<i>membranacea</i>		✓	✓	✓				

Table 3. (Continued) List of Xeniidae collected from Eilat, Israel and acquired molecular markers (✓= homozygote successfully acquired by direct sequencing, ✓*= heterozygote successfully acquired by direct sequencing, ✓**heterozygote successfully acquired by cloning, empty cell = full sequence unavailable).

Sample Name	Genus	Species	ITS	SRP54	ATPsβ	ATPsa	28S	mtMutS	COI	ND2
A128	<i>Ovabunda</i>	<i>biseriata</i>			✓	✓*				
A129	<i>Xenia</i>	<i>umbellata</i>			✓	✓				
A130	<i>Sympodium</i>	sp.	✓							
A131	<i>Sympodium</i>	sp.	✓							
A132	<i>Ovabunda</i>	<i>farauensis</i>	✓		✓	✓				
A133	<i>Anthelia</i>	<i>glauca</i>								
A134	<i>Anthelia</i>	<i>glauca</i>	✓*							
A135	<i>Ovabunda</i>	<i>ainex</i>	✓*	✓*	✓					
A137	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓					
A139	<i>Ovabunda</i>	<i>ainex</i>			✓*	✓				
CO34076	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓		✓	✓	✓	✓
CO34078	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓		✓	✓	✓	✓
CO34080	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓		✓	✓	✓	✓
CO34119	<i>Heteroxenia</i>	<i>fuscescens</i>	✓		✓		✓	✓	✓	✓
CO34171	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓		✓	✓	✓	✓
CO34172	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓		✓	✓	✓	✓
CO34184	<i>Sympodium</i>	<i>caeruleum</i>	✓				✓	✓	✓	✓
CO34185	<i>Sympodium</i>	<i>caeruleum</i>	✓				✓	✓	✓	✓
CO34188	<i>Sympodium</i>	<i>caeruleum</i>	✓				✓	✓	✓	✓
CO34571	<i>Ovabunda</i>	<i>impulsatilla</i>	✓	✓**	✓	✓	✓	✓	✓	✓
CO34572	<i>Ovabunda</i>	<i>biseriata</i>					✓	✓	✓	✓
CO34573	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓	✓*	✓	✓	✓	✓
CO34574	<i>Ovabunda</i>	<i>farauensis</i>					✓	✓	✓	✓
CO34868	<i>Ovabunda</i>	<i>biseriata</i>					✓	✓	✓	✓
CO34872	<i>Ovabunda</i>	<i>biseriata</i>					✓	✓	✓	✓
CO34874	<i>Ovabunda</i>	<i>biseriata</i>					✓	✓	✓	✓
CO34876	<i>Ovabunda</i>	<i>biseriata</i>	✓*		✓		✓	✓	✓	✓
CO34880	<i>Ovabunda</i>	<i>biseriata</i>		✓*		✓	✓	✓	✓	✓
CO34881	<i>Ovabunda</i>	<i>biseriata</i>	✓		✓*	✓*	✓	✓	✓	✓
CO34882	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓*	✓	✓*	✓	✓	✓	✓

Table 3. (Continued) List of Xeniididae collected from Eilat, Israel and acquired molecular markers (✓= homozygote successfully acquired by direct sequencing, ✓*= heterozygote successfully acquired by direct sequencing, ✓**heterozygote successfully acquired by cloning, empty cell = full sequence unavailable).

Sample Name	Genus	Species	<i>ITS</i>	<i>SRP54</i>	<i>ATPsβ</i>	<i>ATPsa</i>	<i>28S</i>	<i>mtMutS</i>	<i>COI</i>	<i>ND2</i>
CO34884	<i>Ovabunda</i>	<i>farauensis</i>	✓	✓	✓	✓	✓*	✓	✓	✓
CO34886	<i>Ovabunda</i>	<i>farauensis</i>	✓*		✓	✓*	✓*	✓	✓	✓
CO34887	<i>Ovabunda</i>	<i>farauensis</i>		✓*	✓*	✓	✓*	✓	✓	✓
CO34891	<i>Ovabunda</i>	<i>impulsatilla</i>	✓		✓**		✓	✓	✓	✓
CO34893	<i>Ovabunda</i>	<i>impulsatilla</i>					✓	✓	✓	✓
CO34895	<i>Ovabunda</i>	<i>impulsatilla</i>			✓	✓	✓	✓	✓	✓
CO34896	<i>Ovabunda</i>	<i>impulsatilla</i>		✓*	✓	✓	✓	✓	✓	✓
Total: 158			131	100	121	95	77	73	71	74
Heterozygote %			35%	58%	50%	35%	6%	n/a	n/a	n/a
Heterozygote direct sequenced vs cloned			91%	79%	73%	94%	100%	n/a	n/a	n/a

Table 4. List of Alcyonacea collected from Saudi Arabia and acquired molecular markers. Blue = previously published data from Chapter 1, black = new data.

✓ = homozygote successfully acquired by direct sequencing, ✓* = heterozygote successfully acquired by cloning or direct sequencing, empty cell = full sequence unavailable.

	Family	Genus	Species	<i>ITS</i>	<i>SRP54</i>	<i>ATPsβ</i>	<i>ATPsa</i>	<i>28S</i>	<i>mtMutS</i>	<i>COI</i>	<i>ND2</i>
SA001	Xeniidae	<i>Ovabunda</i>	<i>ainex</i>	✓	✓**	✓		✓		✓	✓
SA002	Xeniidae	<i>Ovabunda</i>	<i>gohari</i>	✓	✓*			✓	✓	✓	✓
SA003	Xeniidae	<i>Xenia</i>	sp.		✓	✓	✓	✓	✓	✓	✓
SA004	Xeniidae	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓*	✓*	✓	✓	✓	✓	✓
SA005	Xeniidae	<i>Ovabunda</i>	<i>arabica</i>	✓			✓*	✓	✓	✓	✓
SA006	Xeniidae	<i>Xenia</i>	sp.	✓		✓	✓*	✓	✓	✓	✓
SA007	Xeniidae	<i>Ovabunda</i>	<i>macrospiculata</i>	✓	✓*	✓*	✓	✓	✓	✓	✓
SA008	Xeniidae	<i>Xenia</i>	sp.	✓	✓	✓		✓	✓		✓
SA009	Xeniidae	<i>Ovabunda</i>	<i>biseriata</i>		✓	✓*	✓*	✓		✓	✓
SA010	Xeniidae	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓**	✓*	✓*	✓	✓	✓	✓
SA011	Xeniidae	<i>Xenia</i>	<i>actuosa</i>	✓		✓	✓	✓	✓	✓	✓
SA013	Xeniidae	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓	✓	✓	✓	✓	✓	✓
SA014	Nephtheidae	<i>Litophyton</i>	sp.						✓	✓	
SA016	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>	✓				✓		✓	
SA017	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>	✓				✓			
SA019	Tubiporidae	<i>Tubipora</i>	<i>musica</i>					✓	✓	✓	
SA020	Xeniidae	<i>Ovabunda</i>	<i>ainex</i>	✓			✓	✓	✓	✓	✓
SA021	Xeniidae	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓		✓	✓	✓	✓	✓
SA022	Xeniidae	<i>Ovabunda</i>	<i>ainex</i>	✓	✓**		✓*	✓	✓	✓	✓
SA023	Xeniidae	<i>Anthelia</i>	sp.	✓	✓		✓	✓	✓	✓	✓
SA024	Xeniidae	<i>Ovabunda</i>	<i>biseriata</i>	✓	✓**	✓	✓	✓	✓	✓	✓
SA026	Xeniidae	<i>Heteroxenia</i>	<i>fuscescens</i>	✓	✓		✓	✓	✓	✓	✓
SA027	Xeniidae	<i>Anthelia</i>	sp.	✓	✓**		✓	✓	✓	✓	✓
SA028	Nephtheidae	<i>Litophyton</i>	sp.	✓**			✓	✓	✓	✓	
SA029	Tubiporidae	<i>Tubipora</i>	<i>musica</i>	✓**				✓	✓	✓	
SA030	Tubiporidae	<i>Tubipora</i>	<i>musica</i>					✓			
SA031	Tubiporidae	<i>Tubipora</i>	<i>musica</i>	✓**				✓			
SA032	Xeniidae	<i>Anthelia</i>	sp.	✓	✓**		✓	✓	✓	✓	✓
SA034	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>	✓			✓	✓	✓	✓	

Table 4. (Continued) List of Alcyonacea collected from Saudi Arabia and acquired molecular markers. Blue = previously published data from Chapter 1, black = new data. ✓ = homozygote successfully acquired by direct sequencing, ✓* = heterozygote successfully acquired by cloning or direct sequencing, empty cell = full sequence unavailable.

	Family	Genus	Species	ITS	SRP54	ATPsβ	ATPsa	28S	mtMutS	COI	ND2
SA035	Xeniidae	<i>Sympodium</i>	<i>caeruleum</i>	✓			✓*	✓	✓	✓	✓
SA036	Nephtheidae	<i>Dendronephthya</i>	sp.	✓			✓	✓	✓	✓	
SA037	Xeniidae	<i>Xenia</i>	sp.	✓	✓*	✓	✓	✓	✓	✓	✓
SA038	Nephtheidae	<i>Dendronephthya</i>	sp.	✓			✓	✓	✓	✓	
SA039	Tubiporidae	<i>Tubipora</i>	<i>musica</i>	✓**				✓	✓	✓	
SA040	Xeniidae	<i>Ovabunda</i>	<i>impulsatilla</i>	✓	✓	✓		✓	✓	✓	✓
SA041	Tubiporidae	<i>Tubipora</i>	<i>musica</i>				✓	✓	✓	✓	
SA042	Xeniidae	<i>Anthelia</i>	sp.	✓				✓	✓	✓	✓
SA043	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>	✓**				✓			
SA044	Tubiporidae	<i>Tubipora</i>	<i>musica</i>	✓**			✓	✓			
SA045	Xeniidae	<i>Ovabunda</i>	<i>macrospiculata</i>	✓				✓	✓	✓	✓
SA046	Tubiporidae	<i>Tubipora</i>	<i>musica</i>					✓	✓	✓	
SA047	Xeniidae	<i>Anthelia</i>	sp.	✓*			✓	✓	✓	✓	✓
SA048	Xeniidae	<i>Sympodium</i>	<i>caeruleum</i>		✓		✓	✓	✓	✓	✓
SA049	Alcyoniidae	<i>Paralemnalia</i>	<i>eburnea</i>	✓**				✓	✓	✓	
SA050	Xeniidae	<i>Anthelia</i>	sp.	✓*	✓		✓	✓	✓	✓	✓
SA051	Alcyoniidae	<i>Sinularia</i>	<i>new densa</i>	✓			✓			✓	
SA052	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>	✓**			✓*	✓	✓	✓	
SA053	Alcyoniidae	<i>Sarcophyton</i>	sp.	✓							
SA054	Alcyoniidae	<i>Sarcophyton</i>	<i>glaucum</i>	✓**			✓	✓	✓	✓	
SA055	Alcyoniidae	<i>Paralemnalia</i>	<i>thyrsoides</i>	✓			✓	✓	✓		
SA056	Alcyoniidae	<i>Sinularia</i>	<i>querciformis</i>	✓			✓	✓	✓	✓	
SA057	Alcyoniidae	<i>Rhytisma</i>	<i>fulvum fulvum</i>	✓				✓			
SA058	Alcyoniidae	<i>Sinularia</i>	<i>leptoclados</i>					✓	✓	✓	
SA059	Nephtheidae	<i>Litophyton</i>	sp.								
SA060	Alcyoniidae	<i>Sarcophyton</i>	<i>auritum</i>	✓			✓	✓	✓	✓	
SA061	Nephtheidae	<i>Dendronephthya</i>	sp.	✓*				✓			
SA062	Tubiporidae	<i>Tubipora</i>	<i>musica</i>					✓			
SA063	Nephtheidae	<i>Dendronephthya</i>	sp.	✓			✓	✓	✓	✓	

Table 4. (Continued) List of Alcyonacea collected from Saudi Arabia and acquired molecular markers. Blue = previously published data from Chapter 1, black = new data. ✓ = homozygote successfully acquired by direct sequencing, ✓* = heterozygote successfully acquired by cloning or direct sequencing, empty cell = full sequence unavailable.

	Family	Genus	Species	ITS	SRP54	ATPsβ	ATPsa	28S	mtMutS	COI	ND2
SA064	Nephthidae	<i>Dendronephthya</i>	sp.	✓*			✓	✓			
SA065	Nephthidae	<i>Dendronephthya</i>	sp.	✓			✓	✓			
SA066	Alcyoniidae	<i>Sarcophyton</i>	<i>glaucum</i>	✓**			✓	✓		✓	
SA067	Alcyoniidae	<i>Sarcophyton</i>	<i>auritum</i>	✓			✓	✓	✓	✓	
SA068	Nephthidae	<i>Dendronephthya</i>	sp.								
SA069	Nephthidae	<i>Dendronephthya</i>	sp.	✓			✓	✓			
SA071	Xeniidae	<i>Xenia</i>	<i>umbellata</i>	✓			✓	✓	✓	✓	✓
SA072	Nephthidae	<i>Paralemnalia</i>	<i>thyrsoides</i>	✓			✓*	✓			
SA074	Xeniidae	<i>Ovabunda</i>	<i>verseveldti</i>	✓				✓	✓	✓	✓
SA075	Xeniidae	<i>Ovabunda</i>	<i>gohari</i>	✓	✓*	✓	✓*	✓	✓	✓	✓
SA076	Xeniidae	<i>Xenia</i>	sp.	✓	✓		✓	✓	✓	✓	✓
SA077	Alcyoniidae	<i>Sarcophyton</i>	<i>gemmatum</i>	✓			✓	✓	✓	✓	
SA081	Nephthidae	<i>Stereonephthya</i>	sp.	✓				✓	✓	✓	
SA083	Nephthidae	<i>Stereonephthya</i>	sp.	✓*			✓	✓	✓	✓	
SA084	Alcyoniidae	<i>Sinularia</i>	<i>compressa</i>					✓	✓	✓	
SA085	Xeniidae	<i>Xenia</i>	<i>umbellata</i>	✓			✓	✓	✓	✓	✓
Total				61	22	12	47	68	53	57	33
Heterozygote %				25%	50%	33%	19%	0%	n/a	n/a	n/a
Heterozygote direct sequenced vs. cloned				33%	45%	100%	100%	n/a	n/a	n/a	n/a

Table 4. List of primers.

Locus	Primer	Sequence (5'-3')	Approx. size	Reference	MT	
<i>ATPSα</i>	ATPS α f1	GAGCCMATGCAGACTGGTATTAAGGTCYGT	280 bp	Jarman et al. 2002	57°C	
	ATPS α r1	TTGAANCKCTTCTGGTTGATGATGGTGTC		Jarman et al. 2002		
<i>ATPSβ</i>	ATPS β f1	CGTGAGGGHAAAYGATTTHTACCATGAGATGAT	420 bp	Jarman et al. 2002	53°C	
	ATPS β r1	CGGGCACGGGCRCCDGGNGGTTTCGTTTCAT		Jarman et al. 2002		
	XATPS β F	CATGAGATGATYGARTCAGGTG		This study - GTC	53°C	
	XATPS β R	TGTCCATACACAAGCGCTAC		This study - GTC		
	ATPsB_Het_F1	CTTAAAGATAAAATCCTCAAAGGTACAG		This study - RDH	53°C	
	ATPsB_Het_R1	ACACAAGCGCTACCTACATTG		This study - RDH		
	ATPsB_Ova_F1	CTTAAAGATAAAATCCTCAAAGGTAAAG		This study - RDH	57°C	
	ATPsB_Ova_R1	TGTCCATACACAAGCGCTAC		This study - RDH		
	ATPsB_Xen_F1	AAATCCTCAAAGGTAAAGATATACAAA		This study - RDH	57°C	
	ATPsB_Xen_R2	ACACAAGCGCTACCTACATTG		This study - RDH		
<i>SRP54</i>	SRP54f1	ATGGGTGAYATYGAAGGACTGATWGATAAAGTCAA	410 bp	Jarman et al. 2002	49°C	
	SRP54r1	TTCATGATGTTYTGGAATTGYTCATACATGTC		Jarman et al. 2002		
	XSRP54F	GAGYTGAAAYTGGAAGATAACGA		This study - GTC		48°C
	XSRP54F	TTTGGGAATTGTTTCATACATGTCTCTC		This study - GTC		
<i>ITS</i>	1S-f	GGTACCCTTTGTACACACCCGCCGTCGCT	1100 bp	Wei et al. 2006	50°C	
	2SS-r	CGAAACCCGCCGTCAGGGTTCGTTGGGCTGAG		Wei et al. 2006		
	ITSRHF	TTGGCACCTGTCAGATGRKY		This study - RDH		45°C
	ITSRHR	CACCCRTTTTRGGCTGCATT		This study - RDH		
<i>28S</i>	28S-F	CACGAGACCGATAGCGA ACAAGTA	810bp	This study – CSM	72°C	
	28S-R	TCATTTTCGACCCTAAGACCTC		This study – CSM		
<i>ND2</i>	16S647F	ACACAGCTCGGTTTCTATCTACCA	710bp	McFadden et al. 2004	51°C	
	ND21418R	ACATCGGGAGCCCACATA		McFadden et al. 2004		
<i>mtMutS</i>	ND42475F	TAGTTTTACTGGCCTCTAC	730bp	Brugler & France 2008	51°C	
	ND42599F	GCCATTATGGTTAACTATTAC		France & Hoover 2002		65-72°C
	mut3458R	TSGAGCAAAAAGCCACTCC		Sanchez et al. 2003		
<i>COI</i>	COII8068F	CCATAACAGGACTAGCAGCATC	770bp	McFadden et al. 2004	72°C	
	COII8068xF	CCATAACAGGRCTWGCAGCATC		McFadden et al. 2004		
	COIOCTr	ATCATAGCATAGACCATACC		France & Hoover 2002		

Table 5. Ability of individual markers to distinguish different haplotypes within the genus *Ovabunda* (Xeniidae: Alcyonacea). Numbers represent Bayesian Inference bootstrap support. Column one represents the different haplotypes that were recovered from the phylogenies. Colonies with haplotype six were only successfully amplified from the nDNA markers *ITS* and *ATPs β* .

	<i>ITS</i>	SRP54	<i>ATPsβ</i>	<i>ATPsα</i>	<i>28S</i>	<i>mtMutS</i>	<i>COI</i>	<i>ND2</i>
1	99	No	100	91	63	No	No	No
2	87	No	60	No	90	61	92	70
3	67	No	35	No	100	100	No	No
4	No	No	No	No	No	66	No	No
5	No	No	No	No	No	61	65	No
6	90	n/a	No	n/a	n/a	n/a	n/a	n/a
Total	4/6	0/5	3/6	1/5	3/5	4/5	2/5	1/5

Table 6. Ability of individual markers to distinguish different genera within the family Xeniidae. Column one represents the five genera that part of this analyses. Numbers represent Bayesian Inference bootstrap support. N/a means that colonies of this genus were not successfully amplified this marker.

	<i>ITS</i>	<i>SRP54</i>	<i>ATPsβ</i>	<i>ATPsa</i>	<i>28S</i>	<i>mtMutS</i>	<i>COI</i>	<i>ND2</i>
Anthelia	100	100	n/a	No	100	68	83	100
Heteroxenia	100	100	100	No	100	100	97	68
Ovabunda	96	No	No	No	63	No	No	No
Sympodium	100	n/a	n/a	No	100	n/a	100	100
Xenia	No	No	No	No	No	No	No	No
Total	4/5	2/4	1/3	0/5	4/5	2/4	3/5	3/5

Table 7. Proportions of heterozygote sequences and proportions of heterozygote sequences derived from direct sequencing versus cloning, per marker. E = Eilat, SA = Saudi Arabia, T = Total.

	<i>ITS</i>			<i>28S</i>			<i>SRP54</i>			<i>ATPsβ</i>			<i>ATPsa</i>		
	E	SA	T	E	SA	T	E	SA	T	E	SA	T	E	SA	T
Heterozygote (%)	35	25	32	6	0	3	58	50	57	50	33	48	35	19	30
Direct sequencing (%)	91	33	77	100	n/a	100	79	45	74	73	100	75	94	100	95

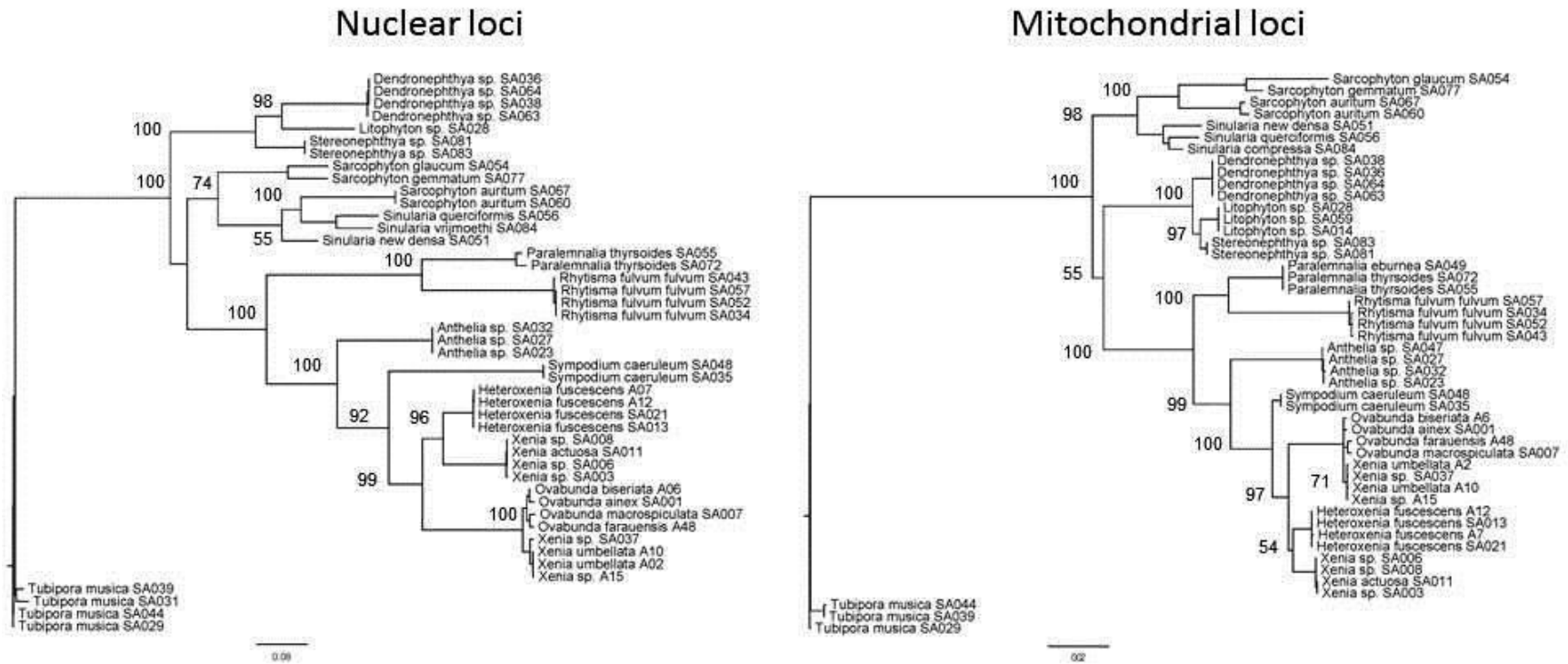


Figure 3. Separate phylogenies of all mtDNA and all nDNA markers including the octocoral families Alcyoniidae, Nephtheidae, Tubiporidae, and Xenidiidae. All mtDNA markers include *COI*, *mtMutS*, and *ND2*. All nDNA markers include *ATPsa*, *ATPsb*, *ITS*, *SRP54* and *28S*. Phylogenetic relations were reconstructed using maximum likelihood (100 bootstrap replicates) methods. The family Tubiporidae was used as outgroup based on earlier analyses (McFadden et al. 2006a).

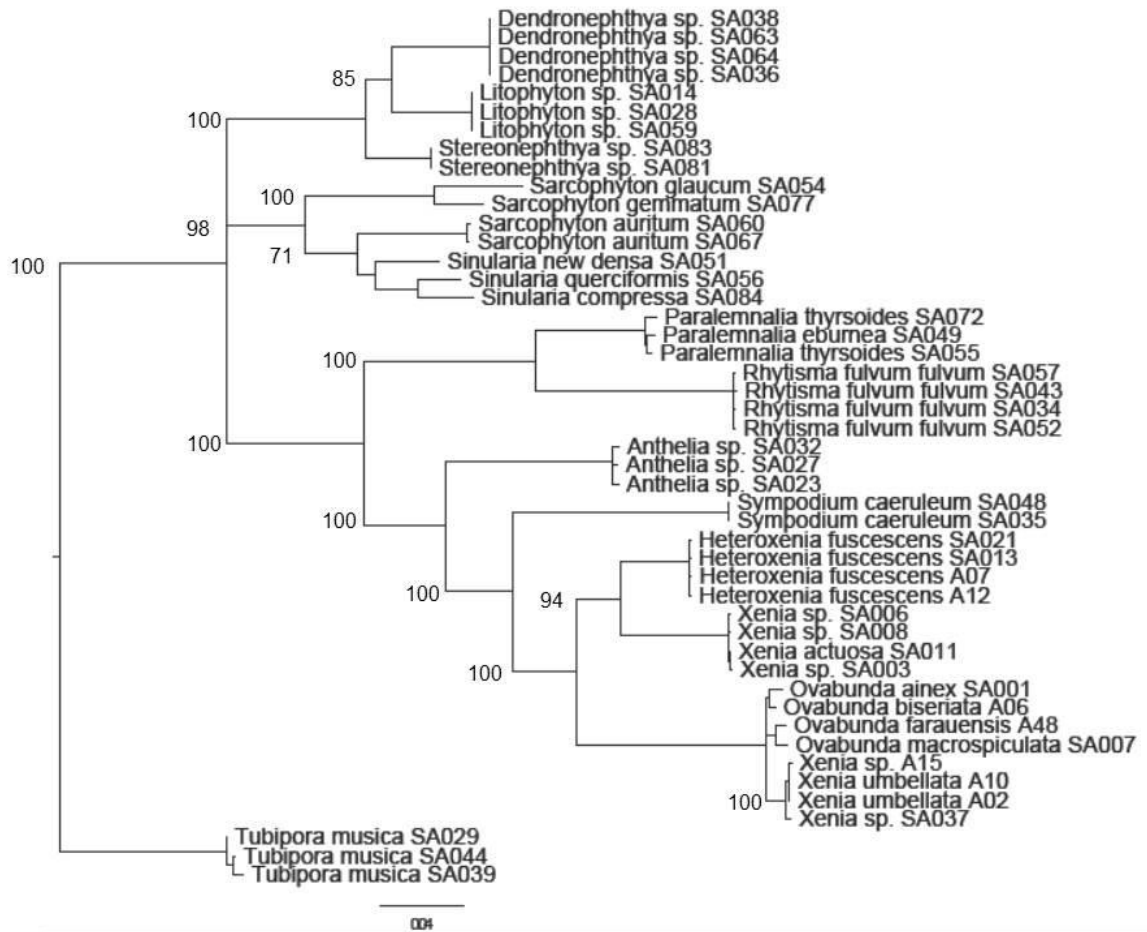


Figure 4. Combined phylogeny of all mtDNA and all nDNA markers (*COI*, *mtMutS*, *ND2*, *ATPsa*, *ATPsβ*, *ITS*, *SRP54* and *28S*) including the octocoral families Alcyoniidae, Nephtheidae, Tubiporidae, and Xeniidae. Phylogenetic relations were reconstructed using maximum likelihood (100 bootstrap replicates) methods. The family Tubiporidae was used as outgroup based on earlier analyses (McFadden et al. 2006a).

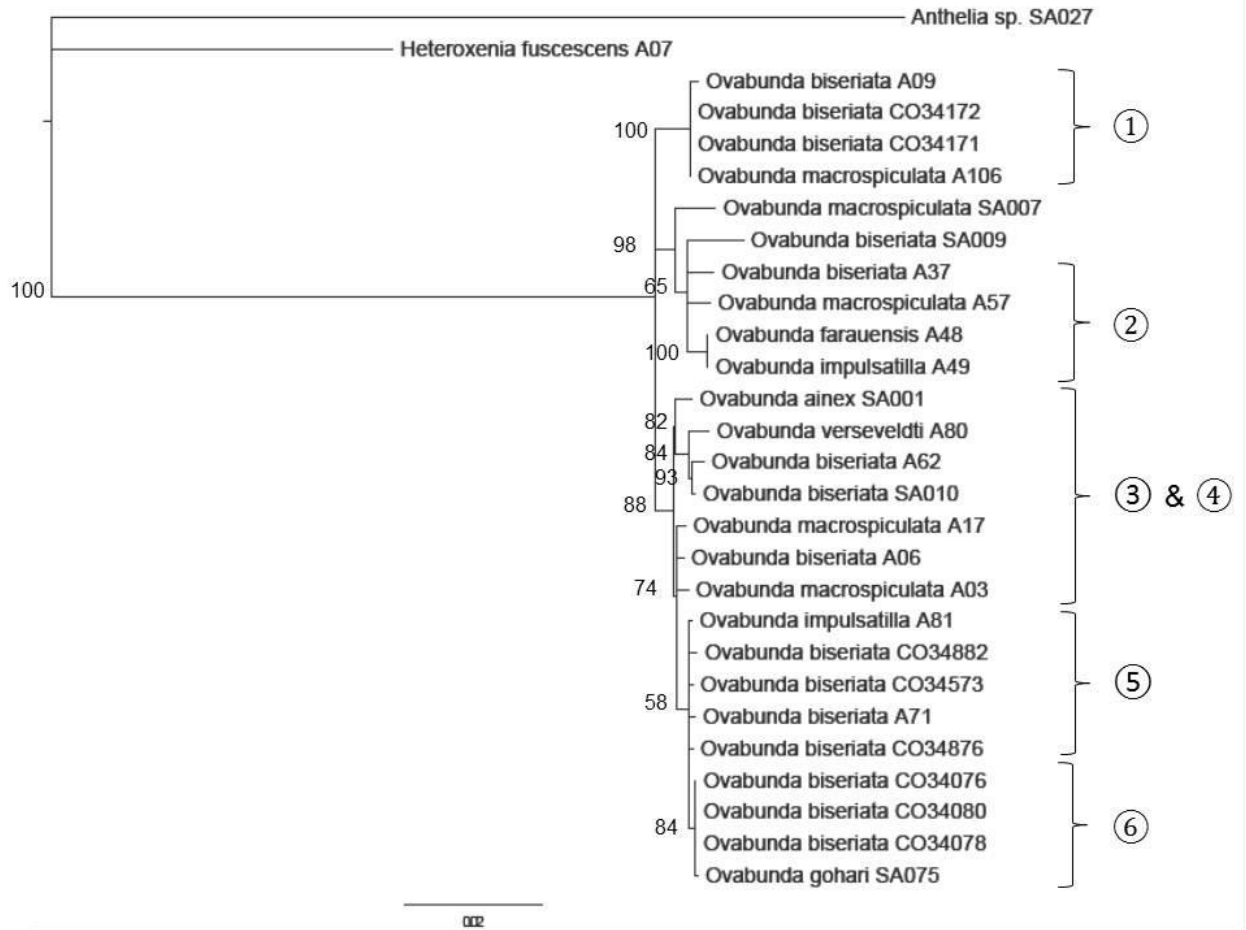


Figure 5. Combined phylogeny of all mtDNA and all nDNA markers (*COI*, *mtMutS*, *ND2*, *ATPsa*, *ATPs β* , *ITS*, *SRP54* and *28S*) including the octocoral genus *Ovabunda* (Alcyonacea: Xeniidae). Phylogenetic relations were reconstructed using maximum likelihood (100 bootstrap replicates) methods. Sister genera *Heteroxenia* and *Anthelia* were used as outgroup based on analyses depicted in Figure 3 and 4.

CHAPTER 4. CONTRIBUTIONS TO ALCYONACEA SYSTEMATICS AND PHYLOGENETICS

Results from this master thesis advance the scientific knowledge of Alcyonacea systematics and phylogenetics on several fronts:

Revisions of the order Alcyonacea

My findings confirm that the order Alcyonacea is in need of major revisions, as argued previously by a range of studies (Daly et al. 2007; Fabricius and Alderslade 2001; McFadden et al. 2006a). Based on our analyses, the families Tubiporidae and Xeniidae are clearly separable. However, members of the families Alcyoniidae and Nephtheidae consistently grouped in three clades. One of these contains genera of only the family Nephtheidae, the second contains genera of only the family Alcyoniidae, and the last was a mixed clade containing genera of both families.

We found that morphological distinctions among genera of the families Alcyoniidae, Nephtheidae, Tubiporidae and Xeniidae were concordant with genetic partitions and current taxonomy in most cases: nine out of 13 genera were clearly separated in our analyses. However there is still need for some revisions on the genus level as well, as outlined below.

Taxa that require further attention

We uncovered evidence of cryptic taxa in the genera *Ovabunda*, *Xenia*, and *Tubipora*. Current taxonomy identifies species of the genus *Ovabunda* based on form and arrangement of sclerites and number and arrangement of pinnules. Our results indicate that these morphological characters are incongruent with the molecular data. We identified six distinct molecular clades within this genus, but further analyses are needed to determine if previously overlooked morphological characters correspond to these genetic lineages. Likewise, the genus *Xenia* was clearly separated into two clades, and we find slight but consistent differences in gross colony morphology between members of these two clades. Members of one clade have typical *Xenia* sclerites, whereas members of the other clade contain no sclerites. Further research is needed to define exact phylogenetic relationships within this group. Lastly, we found possibly cryptic diversity within the species *Tubipora musica*. We found three obvious differences in polyp morphology during collections, and despite our limited sample size, the mitochondrial markers distinguish these three morphospecies. The nuclear markers *ITS* and *28S* are able to distinguish two of the three morphospecies; colonies of the third morphospecies were not successfully sequenced for the nuclear markers due to sample quality. Further research is necessary with an increased sample size, and examination of additional skeletal features to determine whether morphological characters exist to corroborate the genetic distinctions among these three morphs.

Contribution to phylogenetic methods

Previous studies have expressed the need for more nuclear markers for Alcyonacea phylogenetic studies (Jarman et al. 2002; Odorico & Miller 1997; Concepcion et al. 2008), and hypothesized that nuclear

markers might reveal better resolution than mitochondrial markers, similar to results of scleractinian phylogenetics studies (Shearer et al. 2002; Chen et al. 2009). Therefore we tested the resolution of five nuclear markers (*ATPsa*, *ATPsβ*, *ITS*, *SRP54*, *28S*) and three mitochondrial markers (*ND2*, *mtMutS*, *COI*) among four octocoral families: Alcyoniidae, Nephtheidae, Tubiporidae, and Xeniidae. Our results indicate that these mitochondrial and nuclear markers provide similar resolution, and we suggest the use of both in future phylogenetic studies of Alcyonacea. We find that a combination of the mtDNA markers *mtMutS* and *COI*, and the nDNA markers *28s* and *ATPsβ* provide the greatest resolution for moderate cost and effort. Moreover, the use of both mitochondrial and nuclear markers allows inferences regarding possible patterns of hybridization and molecular evolution.

Subsequent research directions

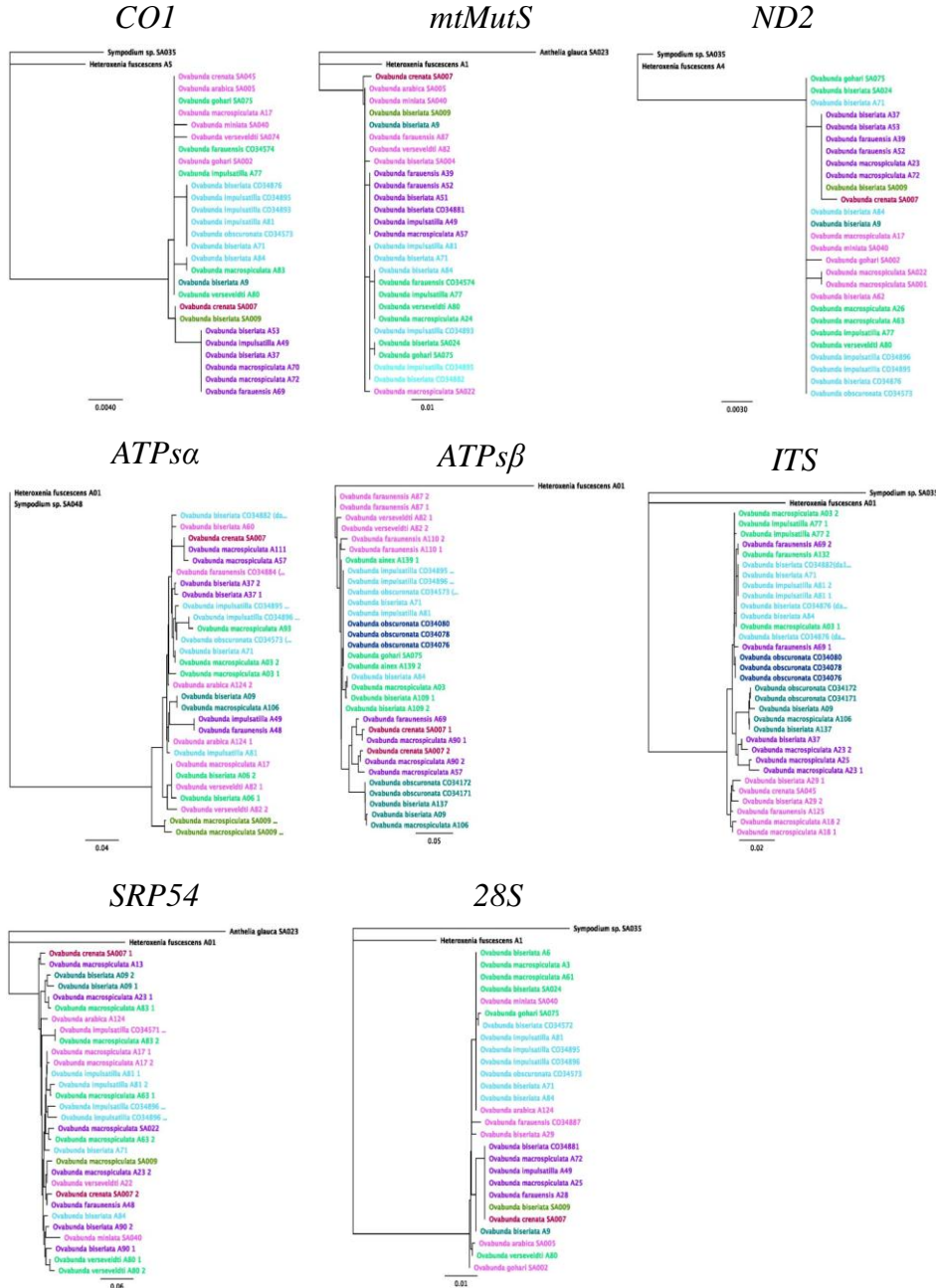
In addition to the contribution to the scientific field of Alcyonacean taxonomy, the understanding of evolutionary relationships between taxonomic groups is fundamental to a range of further studies. For example, our work allows for future studies on ecological interactions between different species of octocorals, or between octocorals and other marine organisms. Also, a better understanding of taxonomy often clarifies ecological differences between taxonomic groups, such as reproductive methods or habitat preference?. Moreover, as more studies arise on Alcyonacean phylogenetics and systematic relationships from other locations, we will be able to combine data and gain a better understanding of the relationships within taxonomic groups between locations, with regards to similarities or endemic species. In sum, the data produced by this study will aid a variety of further biological studies, as well as providing data scientific foundation for conservation and management decisions.

Conservation implications

Barcoding data as provided by this study can strongly influence conservation and management decisions. Conservation management seeks to maintain biodiversity and resilience among coral reef ecosystems. In order to make wildlife management decisions, data is needed regarding the presence and abundance of taxonomic groups (species, genera, families), in specific areas. Because octocoral taxonomy and systematics are so poorly resolved, it is likely that many broadly distributed nominal taxa are in fact cryptic species complexes, some of which may be unknown regional endemics (e.g., *Tubipora musica* cryptic diversity detected in our survey above). Before we can evaluate coral reefs in such a manner, we need to know which taxonomic groups exist within the class Anthozoa. The data provided by our study contributes to the understanding of the taxonomic groups within the Anthozoa families Alcyonacea, Nephtheidae, Tubiporidae, and Xeniidae. Such available sequences with certain identifications enable subsequent less intensive identifications because future studies can simply compare their barcodes (e.g. mtDNA shortcuts) to the available ones to figure out their taxonomy. Consequently, this improves our understanding of species identification, distribution patterns and evolutionary relationships, which subsequently provides a basis for decisions on conservation priorities.

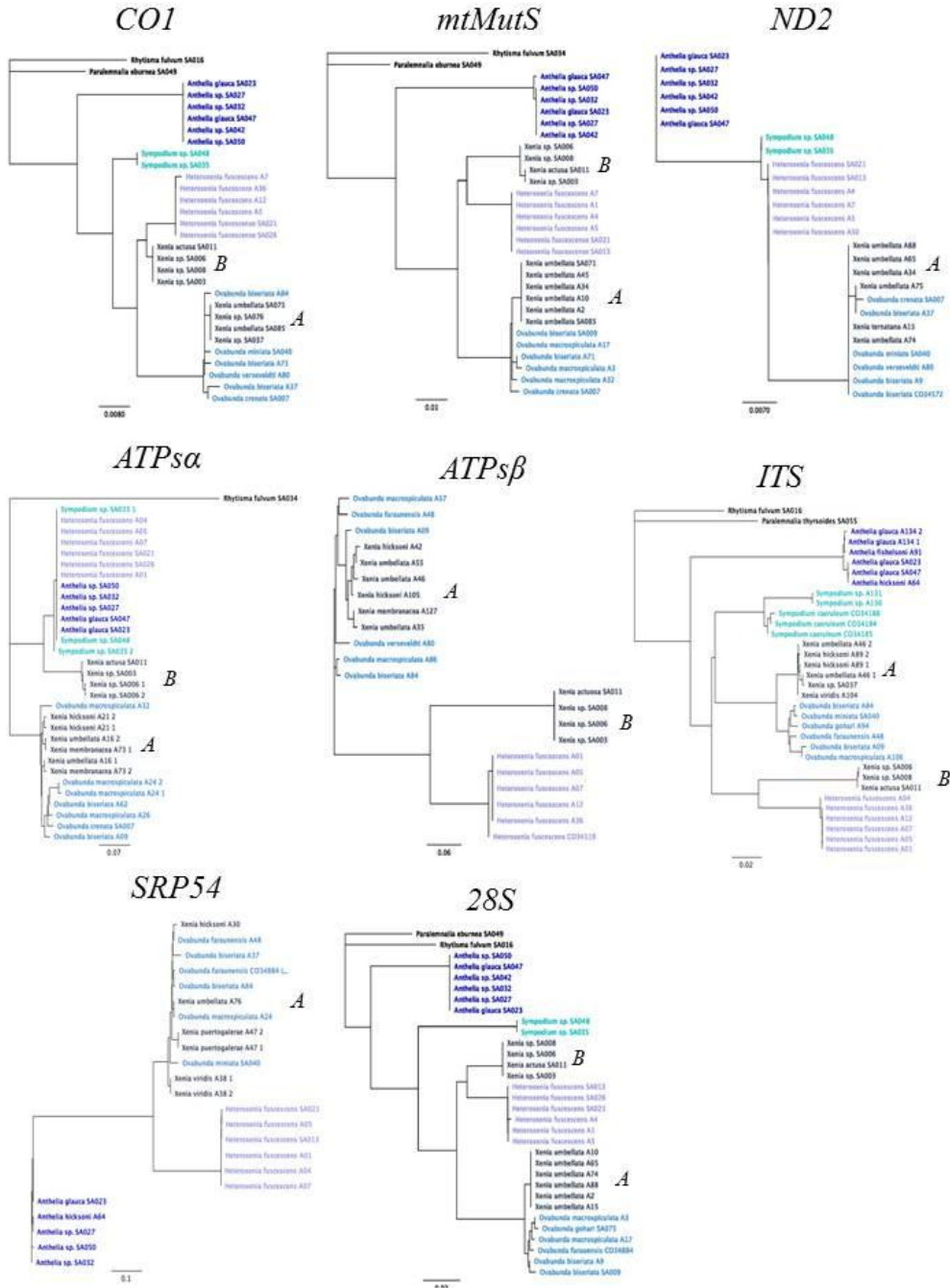
APPENDIX A

Phylogenies of all markers individually (*COI*, *mtMutS*, *ND2*, *ATPsa*, *ATPsb*, *ITS*, *SRP54* and *28S*), including the octocoral genus *Ovabunda* (Alcyonacea: Xeniidae). Phylogenetic relations were reconstructed using bayesian inference (1,100,000 generations and a burnin of 110,000 generations) methods. Sister genera *Heteroxenia* and *Anthelia* were used as outgroup based on analyses depicted in Figure 3 and 4. Clades are color coded as follows: clade 1 is dark aqua, clade 2 is purple, clade 3 is Gn, clade 4 is pink, clade 5 is light blue, and clade 6 is dark blue.



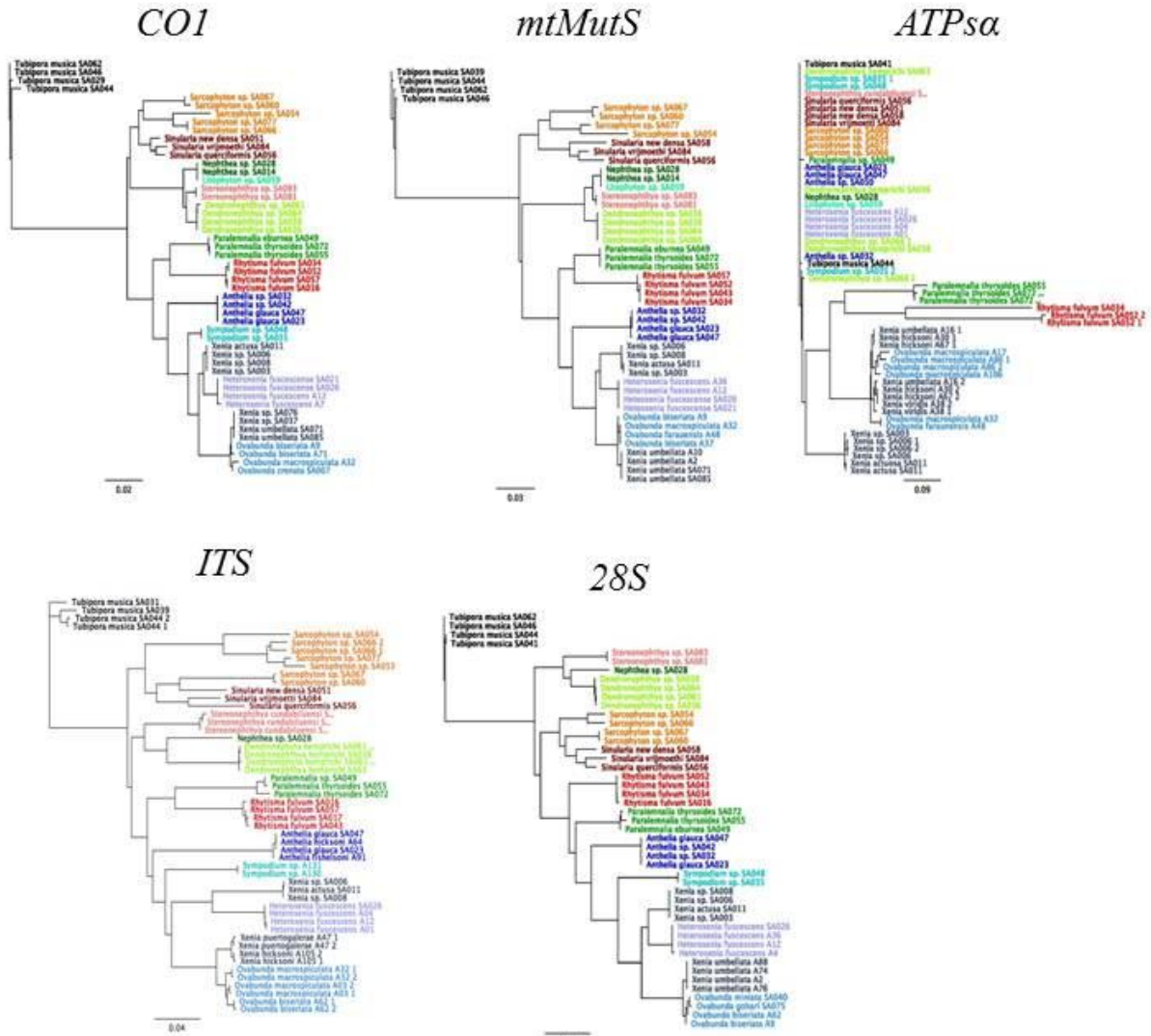
APPENDIX B

Phylogenies of all markers individually (COI, mtMutS, ND2, ATP α , ATP β , ITS, SRP54 and 28S), including the octocoral family Xeniidae (Alcyonacea). Phylogenetic relations were reconstructed using bayesian inference (1,100,000 generations and a burnin of 110,000 generations) methods. Sister genera Rhytisma and Paralemnalia were used as outgroup based on analyses depicted in Figure 3 and 4. Genera are color coded with different shades of blue.



APPENDIX C

Phylogenies of markers individually (*COI*, *mtMutS*, *ATP5α*, *ITS*, and *28S*), including the octocoral families Alcyoniidae, Nephtheidae, Tubiporidae and Xeniidae (Alcyonacea). Phylogenetic relations were reconstructed using bayesian inference (1,100,000 generations and a burnin of 110,000 generations) methods. The family Tubiporidae was used as outgroup based on earlier analyses (McFadden et al. 2006a). Genera are color coded as follows: Members of the family Alcyoniidae are red and genera are distinguished by different shades of red. Members of the family Nephtheidae are Gn and genera are distinguished by different shades of Gn. Members of the family Xeniidae are blue and genera are distinguished by different shades of blue. Members of the family Tubiporidae are black.



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