

**Molecular revision of Zoantharia (Anthozoa  
Hexacorallia) on the east Coast of South Africa**

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As the candidate's supervisor, I have approved this thesis for submission.

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## Abstract

The order Zoantharia (Cnidaria: Anthozoa) is ubiquitous on the east coast of South Africa, and despite their widespread distribution they are poorly represented in literature. No molecular identification has been carried out on these organisms on the South African shoreline. *Zoanthus sansibaricus* Carlgren, 1900 has a global distribution and has been reported having numerous morphotypes in terms of polyp shape, size, colour and oral disk colour. The initial aim in this study was to examine the molecular characteristics of three *Zoanthus* species; *Z. sansibaricus*, *Z. durbanensis* Carlgren, 1938 and *Z. natalensis* Carlgren, 1938, to determine whether they are three separate species or merely morphotypes of one another. Following on from this research, the aim was to conduct a molecular revision of all zoanths found in the intertidal zone along the east coast of South Africa, and to identify the *Symbiodinium* spp. within zoanths for comparisons with conspecifics elsewhere. Samples were collected at sites along the coast from Umgazana (31.7024° S, 29.4175° E) to Sodwana (27.6594° S, 32.6477° E) and at one site in Libanona, Madagascar (25.0421° S, 46.9952° E). Sequences of cytochrome oxidase subunit one (COI), mitochondrial 16S ribosomal DNA (mt 16S rDNA), the nuclear internal transcribed spacer region of ribosomal DNA (ITS rDNA) for zoanths and ITS-rDNA region for *Symbiodinium* spp. were used in this study to run phylogenetic analyses and examine the molecular characteristics for comparisons with zoanths elsewhere using GenBank. Seven species were identified; *Isaurus tuberculatus*, *Palythoa nelliae*, *Palythoa tuberculosa*, *Z. durbanensis*, *Z. gigantus*, *Z. natalensis* and *Z. sansibaricus*. The COI sequences (for *Z. sansibaricus*, *Z. natalensis* and *Z. durbanensis*) had little variation between species groups, while the mt 16S rDNA tree showed that *Z. sansibaricus* matched with sequences of previously reported *Z. sansibaricus* from the Pacific. *Zoanthus natalensis* was identical to *Z. kuroshio* Reimer & Ono, 2006 and *Z. durbanensis* was identical to *Z. vietnamensis* Pax & Müller, 1957. The ITS rDNA sequences were very

similar for these four species; *Z. natalensis*, *Z. kuroshio*, *Z. durbanensis* and *Z. vietnamensis*. *Palythoa nelliae* Pax, 1935 appears to match with Pacific species *Palythoa mutuki* Haddon & Shackleton, 1891, and this is supported by the mt 16S and ITS rDNA markers. *Symbiodinium* subclade A1 was most often found with *Z. natalensis* and subclade C15/C91 was most often found with *Z. durbanensis*. Subclade C1 *sensu* LaJeunesse (2002) was found with all *Isaurus* and *Palythoa* samples, and most of *Z. sansibaricus* samples. The results of this study indicate that *Z. natalensis* is likely conspecific to *Z. kuroshio*, *Z. durbanensis* is likely conspecific to *Z. vietnamensis*, and *P. nelliae* is likely conspecific to *P. mutuki*, however, this is only a tentative hypothesis as no formal morphological analyses were done on proposed conspecifics. This work highlights the importance for similar studies in the clarification of zoanthid taxonomy.

Key words: COI, ITS rDNA, mt 16S rDNA, *Symbiodinium*, Zoantharia, South Africa, Madagascar

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## Preface

The following MSc thesis was undertaken at the School of Life Science, University of KwaZulu-Natal, Westville. The work described was carried out from June 2012 to July 2014 under the supervision of Dr A.H.H. Macdonald

The work presented in this thesis has not been submitted previously for any degree or diploma to any other tertiary institution. The studies are the original work of the author and where the work of others has been used, the sources have been acknowledged in the text.

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## Plagiarism declaration

I, Michelle Risi declare that:

The research reported in this thesis, except where otherwise stated, is my own original work.

This thesis has not been submitted for any other degree, diploma or examination at any other tertiary institution.

This thesis does not contain data, pictures, graphs or other information which are not my own, unless specifically acknowledged as being sourced from another's work.

This thesis has not been written by anyone but myself, unless specifically acknowledged as being the work of published sources. Where the work of other researchers has been quoted:

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b) Where their exact words have been used, these statements have been placed inside quotation marks and after which they have been appropriately referenced.

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## Publication declaration

The following details the contribution to the publication which forms part of the research presented in this thesis.

### Publication 1:

Risi, M., Macdonald, A. (2015) Possible synonymies of *Zoanthus* (Anthozoa: Hexacorallia) species on the east coast of South Africa with Pacific congeners. Systematics and Biodiversity, 13:1, 93-103, DOI: 10.1080/14772000.2014.968233

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Collection of data: Michelle Risi. Analysis of data: Michelle Risi and Angus Macdonald.

Wrote the draft paper: Michelle Risi. Angus Macdonald and anonymous reviewers contributed comments on the manuscript.

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Wrote the draft paper: Michelle Risi. Angus Macdonald contributed comments on the manuscript.

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## Other research outputs

### Oral presentation:

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### Poster presentations:

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Risi, M., Macdonald, A. July 2014. Zoanthid phylogenetics: molecular revision of zoanthids in South Africa. 15th Southern African Marine Science Symposium (SAMSS), Stellenbosch, South Africa.

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## Chapter 1: General introduction

Zoanthids belong to the class Anthozoa, subclass Hexacorallia in the order Zoantharia. There are two suborders of Zoantharia; Brachycnemina (imperfect 5th dorsal mesentery) and Macrocnemina (perfect 5th dorsal mesentery) (Haddon and Shackleton, 1891).

Brachycnemina zoanthids are found in tropical and subtropical shallow marine environments, attached to rocks, show high intraspecific colour variation, produce planktonic larvae (Semper's larvae) (Ryland *et al.*, 2000), and are generally zooxanthellate (contain symbiotic algal cells of genus *Symbiodinium*). Macrocnemina zoanthids are generally found on other marine organisms, show little intraspecific colour variation, have no record of having produced planktonic larvae (Ryland *et al.*, 2000), and are rarely zooxanthellate. There are nine families in Zoantharia, Brachycnemina contains three (Neozoanthidae, Zoanthidae and Sphenopidae), Abysoanthidae does not belong to a grouping, and the remaining five belong to Macrocnemina (Parazoanthidae, Epizoanthidae, Hydrozoanthidae, Nanozoanthidae and Microzoanthidae). Although suborders have been carefully defined, genera and family classifications are less clear, especially within Macrocnemina (Sinniger *et al.*, 2005).

Species in Zoantharia are characterized by having features such as two rows of tentacles, polyps joined by a coenenchyme, and a single siphonoglyph. Zoanthids generally integrate sand or other bits of hard material into their tissue, however those in the family Zoanthidae (genera *Acrozoanthus*, *Isaurus* and *Zoanthus*) are non-sand encrusting. Zoanthids are found in marine waters worldwide and range from a diversity of carpet forming species on rocky shores to deep oceanic species. They are found in polar, temperate and tropical areas and their occurrence on coral reefs lists them as crucial organisms to study to determine reef health when facing pressures such as global warming due to their association with zooxanthellae (Irei *et al.*, 2011). Zoanthids are most well known for their trade in aquaria and

their production of unique biochemical compounds (Fukuzawa *et al.*, 1995; Labas *et al.*, 2002).

In the last decade zoanthids have attracted attention due to the gap in information on this Cnidarian order and the chaotic state of their taxonomy. A number of factors contribute to this problem, these include: the high level of morphological plasticity in terms of oral disk and tentacle colour within species groups, a non-uniform set of characters used when defining species, as well as lack of comparisons with previous descriptions made elsewhere before describing new species.

Despite their cosmopolitan range and dominance in certain habitats, these organism have largely been left out of ecological surveys due to the inability to identify different species with confidence (Burnett *et al.*, 1995). The difficulty in classifying species has resulted in re-descriptions of the same species and therefore a probable overestimation of species numbers, particularly within the genera *Zoanthus* and *Palythoa* (Burnett *et al.*, 1995; Burnett *et al.*, 1997). There are 358 zoanthid species reported in Fautin (2014) to date, and only 84 are valid descriptions. Molecular techniques have allowed for the revision of the zoanthid group with the description of new families, genera and species as well as the identification of synonymies (Reimer *et al.*, 2004; Reimer *et al.*, 2007c; Sinniger and Haussermann, 2009; Reimer and Fujii, 2010; Fujii and Reimer, 2013).

Zoanthids are prevalent along rocky shores in South Africa. Reimer *et al.* (2011b) reported a zoanthid diversity of Brachycnemic zoanthids between seven to ten species on coral reef locations in Taiwan. In this, the first molecular revision of zoanthids in South Africa, there was a strong possibility that species ranges would grow to include the South African coastline, and we also expected to find endemic species. The aim of this study was to utilize both molecular and morphological techniques to determine the species diversity of zoanthids

found along the east coast of South Africa in rocky-shore/shallow habitats. Comparisons of DNA sequences generated using molecular markers cytochrome oxidase subunit (COI), mitochondrial 16S ribosomal DNA (mt 16S rDNA), internal transcribed spacer region of ribosomal DNA (ITS rDNA) for zoanthids and ITS rDNA of *Symbiodinium* spp. were made with species found elsewhere using GenBank, It was predicted that synonymies would be found with global species, and also that endemics will be identified with molecular characterization.

## **Chapter 2: Possible synonymies of *Zoanthus* (Anthozoa: Hexacorallia) species on the east coast of South Africa with Pacific congeners**

### **2.1 Introduction**

The taxonomy of Hexacorallia in general has come under scrutiny because the diagnostic features used to identify groups have failed to resolve the phylogenetic histories of taxa (Daly *et al.*, 2003). Although zoanthids (class Anthozoa, subclass Hexacorallia, order Zoantharia) have a cosmopolitan distribution, until recently there was little research done on these organisms in comparison to close relatives, the hard corals (Scleractinia) and anemones (Actinaria) (Burnett *et al.*, 1995). Zoanthids have been described as sand encrusting anemones and are ecologically important to the systems they reside in (Herberts, 1972b). Zoanthids are typically colonial with polyps linked by common tissue called coenenchyme and most species integrate sand grains into their tissue for support.

A number of factors contribute to the complicated taxonomy of zoanthids. They are morphologically plastic and a single species can be found having various polyp shapes and sizes, and differing polyp, oral disk and tentacle colours (Burnett *et al.*, 1997; Reimer, 2007; Aguilar and Reimer, 2010). Other reasons include encrusting of sand which hinders histology, lack of comparison with previously described zoanthids when describing new species and inadequate descriptions based on poorly preserved voucher specimens (Burnett *et al.*, 1994; Aguilar and Reimer, 2010). The poor conservation of voucher specimens on which descriptions were based has left these samples either lost or unidentifiable (Sinniger *et al.*, 2008). These reasons have led to an overestimation of species diversity for the *Zoanthus* and *Palythoa* genera in particular (Burnett *et al.*, 1997).

The genus *Zoanthus* Lamarck, 1801, has been reported as having amongst the highest number of species (Reimer *et al.*, 2011b). Another area of research interest for this genus is their

production of Green Fluorescent Protein (GFP, which controls host tissue colour) (Labas *et al.*, 2002; Kelmanson, 2003). *Zoanthus* species, when found, can be the dominant invertebrates in their habitat (Karlson, 1983; Reimer *et al.*, 2011a). They mainly occur in shallow tropical and subtropical waters and have an association with endosymbiotic dinoflagellates from the genus *Symbiodinium*, from which they obtain a large portion of their diet (Trench, 1974). *Zoanthus* species are generally found in regions with high wave energy or currents and these environmental factors along with shading can influence polyp morphology (Koehl, 1977; Ong *et al.*, 2013). With over 150 *Zoanthus* species described (Fautin, 2014), molecular work has shown that several descriptions may belong to conspecifics (Burnett *et al.*, 1995; Burnett *et al.*, 1997).

In the last decade there has been an increase in zoanthid research due to the development and ease of molecular techniques that allow for standardization in creating taxonomic boundaries (Reimer and Fujii, 2010). This work has led to a complete revision of the taxonomy of the group where new families, genera and species have been described (Reimer *et al.*, 2006e; Reimer *et al.*, 2007c; Sinniger and Haussermann, 2009; Reimer and Fujii, 2010; Fujii and Reimer, 2013). The use of molecular technology and analysis techniques such as mitochondrial DNA (Reimer *et al.*, 2004; Sinniger *et al.*, 2005) and nuclear DNA (Reimer *et al.*, 2007a; Swain, 2009) phylogenies may result in a number of species names becoming junior synonyms due to the misidentification of groups. Some morphological characters previously used in identification have been shown to be incorrect in delineating species boundaries (Boscolo and Silveira, 2005; Sinniger and Haussermann, 2009). Phylogenetic analyses allow for the identification of individuals which cannot be done using only morphological characters (Reimer and Fujii, 2010).

Genetic markers that have been previously utilized for zoanthids included cytochrome oxidase subunit (COI) and mitochondrial 16S ribosomal DNA (mt 16S rDNA).

Mitochondrial markers (COI and mt 16S rDNA), which are usually fast evolving in metazoans, have been found to be slow evolving in anthozoan species (Shearer *et al.*, 2002), whereas nuclear genes are slow-evolving in metazoans, but the internal transcribed spacer region of ribosomal DNA (ITS rDNA) in particular, is comparatively fast evolving in anthozoans and has much greater variation at the species level (Reimer *et al.*, 2007b; Aguilar and Reimer, 2010). Therefore a combination of the COI, mt 16S rDNA and ITS rDNA genes has been expected to reveal species level variation in zoanthids.

*Zoanthus sansibaricus* Carlgren, 1900 is common throughout the Pacific and Indian Oceans and there are a large number of colour morphotypes within this species (Reimer, 2007; Reimer and Hickman, 2009; Reimer, 2014). *Zoanthus sansibaricus* is commonly found in shallow water, is more tolerant to desiccation, and harbours a variety of different zooxanthellae clades (Reimer *et al.*, 2006b; Reimer *et al.*, 2011b). *Zoanthus sansibaricus* is found along the east coast of South Africa with other *Zoanthus* species; *Zoanthus durbanensis* Carlgren, 1938 and *Zoanthus natalensis* Carlgren, 1938. Of the several *Zoanthus* species that were described in Carlgren (1938), these species were the only *Zoanthus* species found at sampling locations, on the rocky shore in this study.

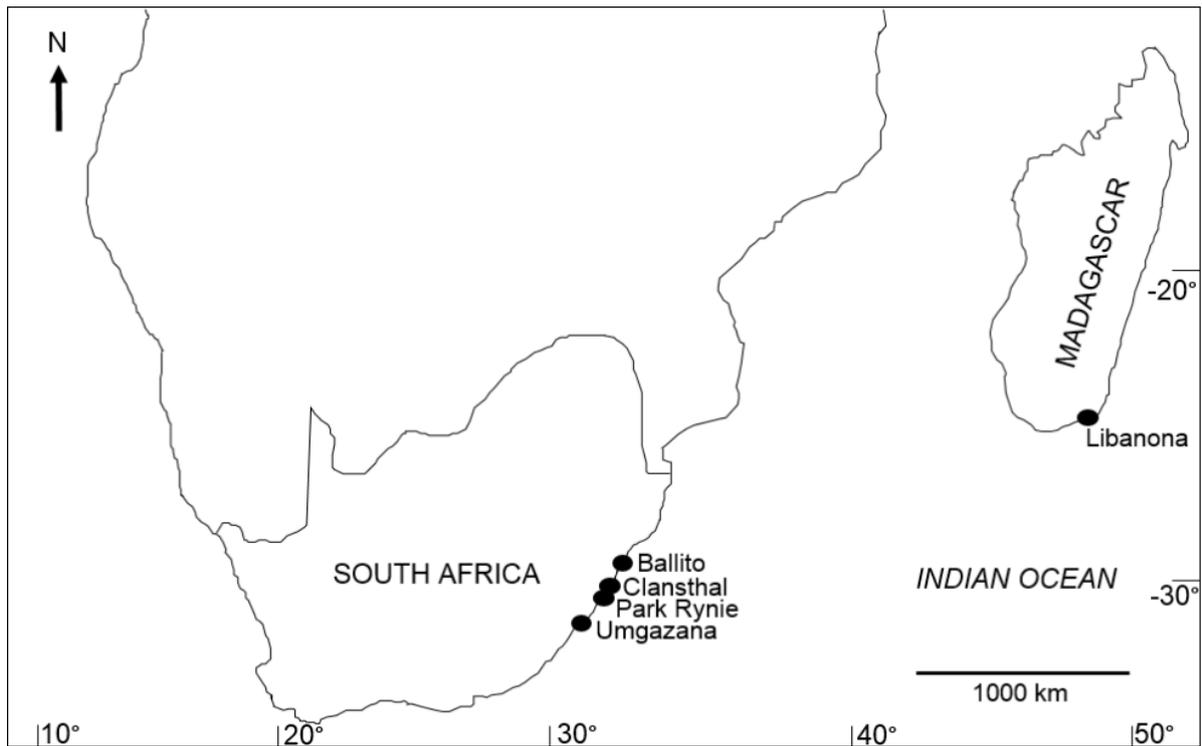
Due to the high morphological plasticity within *Z. sansibaricus* and the work published by Reimer *et al.* (2004) which found that four previously described *Zoanthus* species were likely all *Z. sansibaricus*, it was hypothesised that *Z. natalensis* and *Z. durbanensis* were conspecifics of *Z. sansibaricus* and would therefore have the same or highly similar DNA sequence for chosen genes. The objective was to sample polyps from these three morphological species along the east coast of South Africa, using the original species description in Carlgren (1938) and Branch *et al.* (2008) as species identification guides, and then to use COI, mt 16S rDNA and ITS rDNA markers to determine the relatedness of these specimens.

## 2.2 Materials and methods

### 2.2.1 Specimen collection and preservation

Specimens were collected from the rocky shores at Park Rynie, Ballito, Umgazana, Clansthal and Madagascar-Libanona (Figure 1). *Zoanthus* species *Z. sansibaricus*, *Z. natalensis* and *Z. durbanensis* were actively searched for on the rocky shore. The species were identified according to the original description in Carlgren (1938). Individual samples were taken from different patches using a flat scraper and placed into vials containing 70% ethanol for further analysis. Twenty-two specimens were used in this study (Table 1). Photographs were taken of the three different species *in situ* (Figure 2).

*Zoanthus durbanensis* (Figure 2a) is termed the “durban zoanthid”, polyps have a height close to their width (10 mm tall, 8 mm wide), and a grey coloured column with *liberae* polyps and green-brown oral disk, and green/grey tentacles (Carlgren, 1938; Branch *et al.*, 2008). *Zoanthus natalensis* (Figure 2b) is termed the “green zoanthid” due to the light green colour of the column and it is found higher up on the shore than both *Z. sansibaricus* and *Z. durbanensis*. Lower parts of the column are light grey and are generally light green near the top of the polyp, with brown tentacles and green oral disk colour (Carlgren, 1938). *Zoanthus natalensis* is short and squat with *immersae* polyps, with a polyp length of 4 mm and width of 4 mm. *Zoanthus sansibaricus* (Figure 2c) is termed the “violet zoanthid” as the column is generally violet in colour with *liberae* polyps (10 mm), has a vivid green oral disk and green-brown tentacles (Branch *et al.*, 2008). “*Liberae*” refers to the polyps standing free from the thin coenenchyme whereas “*immersae*” polyps are embedded within the thick coenenchyme (Pax, 1910).



**Figure 1:** Map showing the sample sites in this research, on the east coast of South Africa and Libanona in Madagascar.



**Figure 2:** *In situ* photographs of the *Zoanthus* species collected in this study. Scale bars all represent 5mm. (a) *Zoanthus durbanensis*. (b) *Zoanthus natalensis*. (c) *Zoanthus sansibaricus*.

### 2.2.2 DNA extraction, PCR amplification and sequencing

DNA was extracted from the zoanthid tissue (25 mg) following the protocol for solid tissue samples using a Zymogen Quick-gDNA MiniPrep extraction kit (Zymo Research Corporation, California, United States of America). PCRs were run with 12.5 µl of EconoTaq (Lucigen Corporation, Wisconsin, United States of America), 0.84 µl of both forward and reverse primer each at a concentration of 10 µM, 1 µl of bovine serum albumin and 10 µl of PCR water and 2 µl of extracted DNA. The COI region was amplified using the universal primers HCO2198 and LCO1490 (Folmer *et al.*, 1994), following the thermal cycler conditions used in Reimer *et al.* (2004). The mt 16S rDNA region was amplified using the zoanthid specific primers 16Sant1a and 16S bmoH (Sinniger *et al.*, 2005), following the thermal cycler conditions used in Sinniger *et al.* (2005). For *Z. sansibaricus* and *Z. durbanensis*, ITS rDNA was amplified using zoanthid specific primers ZoanF and ZoanR (Reimer *et al.*, 2007b), following the thermal cycler conditions used in Reimer and Sinniger (2010).

The ITS rDNA primers (ZoanF and ZoanR) did not generate amplicons for *Z. natalensis*. ITS rDNA primers had to therefore be generated using ITS rDNA sequences that had been uploaded to GenBank for *Z. kuroshio* (proposed conspecific to *Z. natalensis*). *Zoanthus kuroshio* sequences were aligned and a consensus sequences was generated using BioEdit v7.0.9 (Hall, 1999). The accession numbers for *Z. kuroshio* ITS rDNA sequences were: DQ442468-DQ442493 (Reimer *et al.*, 2007b). This consensus sequence was uploaded to Primer 3 (Rozen and Skaletsky, 2000) and the following primer set was generated; ZkITS-F (5'-TAACGCGTCTCGTCTTGTTG-3') and ZkITS-R (5'-TTCCGACACTCAGACAGACG-3'). The thermal cycler conditions used were an initial denaturation step of 3 min at 94°C, 40 cycles of: 1 min at 94°C, 1 min at 56°C and 2 min at 72°C, followed by a 10 min extension at 72°C. The PCR-amplified products were checked

using 1 % agarose gel electrophoresis and a 100 bp molecular weight marker. The expected PCR product size was  $\pm 600$ -900 bp for ITS rDNA and mt 16S rDNA,  $\pm 650$  bp for COI, and lastly  $\pm 450$  bp for *Z. natalensis* ITS rDNA. Negative and positive controls were run in all PCRs to check for signs of contamination. The PCR-amplified DNA fragments were sequenced in the forward direction with an ABI 3730 capillary sequencer (Inqaba Biotechnical Industries, Pretoria, South Africa) using Big End Dye technology (Life Technologies, Johannesburg, South Africa).

### **2.2.3. Phylogenetic analyses**

The new sequences obtained in this study were deposited into GenBank (KJ416418-KJ416465) (Benson *et al.*, 2005). Sequences were verified by using the BLAST tool on GenBank (Altschul *et al.*, 1997). Sequences were aligned and edited using a Clustal W multiple-alignment algorithm on BioEdit v7.0.9 (Hall, 1999). Three alignments were generated; COI containing 14 sequences with 244 sites, mt 16S rDNA containing 17 sequences with 426 sites and ITS rDNA containing 17 sequences with 380 sites. All alignments were inspected by eye and manually edited, and all ambiguous sites were removed from the dataset for phylogenetic analyses. Multiple copies of ITS-rDNA were found in some of the samples and the sequences were sorted manually by analysing the chromatogram and choosing the strongest signal (highest peak). Intrapopulation and interpopulation diversity indexes, and neutrality tests, were calculated using DNAsp v5 software (Librado and Rozas, 2009). These analyses were run for all markers' datasets.

The outgroups for each alignment, that were used to root the trees, were downloaded from GenBank for *Palythoa tuberculosa* (KF499761, AB219219 and KF499775) and *Parazoanthus gracilis* (EF672668, AB219194, and AB214161). Sequences from *Zoanthus gigantus* (AB252675, AB235411, and DQ442467) were included in all of the alignments, *Acrozoanthus australiae* was included in the COI (HM171914) and mt 16S rDNA

(HM171921) alignments, and *Zoanthus praelongus* was included in only the COI (EF452276) alignment. Sequences of COI, mt 16S rDNA and ITS rDNA were downloaded from GenBank for *Z. sansibaricus* (AB214173, AB214162, HM749065, AB219188, AB235412, JX845320, HM749064, AB214146, AB214143, AB214133 and AB214142), *Z. kuroshio* (AB252668, AB219183, AB252665, AB219182, JF419760, AB235410, AB219191, DQ442492, DQ442491, DQ442481 and DQ442468) and *Z. vietnamensis* (EU333687, EU333678, EU333677, EU333685, AB235409, AB235408 and AB235397).

Sequences with identical sequence codes were grouped for each marker separately using DNAsp v5 (Librado and Rozas, 2009). The following phylogenetic outputs were produced for COI, mt 16S rDNA and ITS rDNA alignments (fig 5-7). Evolutionary models were calculated using MrModeltest v2.3 (Nylander, 2004) from which the best fit evolutionary model under Akaike information criterion (Posada and Buckley, 2004) was utilized in the following analyses; bayesian analysis using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) and neighbor-joining (NJ) analysis using PAUP\* v4 (Swofford, 2002). Maximum parsimony (MP) analysis was also run using PAUP\* v4.

The MrBayes analysis was run for 1 000 000 Monte-Carlo Markovian chain (MCMC) generations with a 10% burnin, using the Kimura 2 frequency class model of evolution (Kimura, 1981), equal nucleotide frequencies and a gamma distribution of rates. Four MCMC chains were run simultaneously; convergence was measured by likelihood values approaching one another for in excess of 90% of the run (i.e. excluding the burnin). The NJ tree (1000 bootstrap replicates) was constructed using the same model of evolution, and the model was the same for each marker analyzed. Phylogenetic trees were edited using TreeViewX (Page, 1996).

**Table 1:** *Zoanthus* specimens examined in this study and their relevant GenBank accession numbers

Specimen no.	Specimen identification	Date collected	Location	GPS coordinates	COI (Acc. No.)	mt 16S rDNA (Acc. No.)	ITS rDNA (Acc. No.)
71z	<i>Z. durbanensis</i>	06/05/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416448	KJ416434	KJ416457
78z	<i>Z. sansibaricus</i>	06/05/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416443	KJ416423	KJ416463
79z	<i>Z. sansibaricus</i>	06/05/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416440	KJ416422	KJ416462
111z	<i>Z. durbanensis</i>	06/05/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416447	NA	KJ416456
112z	<i>Z. durbanensis</i>	06/05/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416446	KJ416431	KJ416455
114z	<i>Z. sansibaricus</i>	06/05/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416444	KJ416420	KJ416464
115z	<i>Z. durbanensis</i>	06/05/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416445	KJ416430	NA
116z	<i>Z. sansibaricus</i>	06/05/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416441	KJ416419	KJ416460
118z	<i>Z. sansibaricus</i>	06/05/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416442	KJ416418	KJ416465
143z	<i>Z. natalensis</i>	27/06/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416437	KJ416429	KJ416450
144z	<i>Z. natalensis</i>	27/06/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416438	NA	NA
145z	<i>Z. natalensis</i>	27/06/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416435	NA	NA
146z	<i>Z. natalensis</i>	27/06/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416439	NA	NA
147z	<i>Z. natalensis</i>	27/06/12	RSA: Park Rynie	30.3183° S, 30.7426° E	KJ416436	NA	NA
Zn4	<i>Z. natalensis</i>	27/02/13	RSA: Ballito	29.5451° S, 31.2160° E	NA	KJ416424	KJ416451
Zd13	<i>Z. sansibaricus</i>	27/02/13	RSA: Ballito	29.5451° S, 31.2160° E	NA	KJ416421	KJ416461
Um2_15	<i>Z. natalensis</i>	10/07/13	RSA: Umgazana	31.7024° S, 29.4175° E	NA	KJ416428	KJ416453
Um3_17	<i>Z. natalensis</i>	11/07/13	RSA: Umgazana	31.6849° S, 29.4416° E	NA	KJ416426	KJ416452
Cl_24	<i>Z. natalensis</i>	22/06/13	RSA: Clansthal	30.2391° S, 30.7865° E	NA	KJ416427	KJ416449
Mad_Z1	<i>Z. durbanensis</i>	01/07/13	MDG: Libanona	25.0421° S, 46.9952° E	NA	KJ416432	KJ416458
Mad_Z2	<i>Z. durbanensis</i>	01/07/13	MDG: Libanona	25.0421° S, 46.9952° E	NA	KJ416433	KJ416459

## 2.3 Results

### 2.3.1. Molecular diversity

For both COI and mt 16S rDNA (Table 2) standard molecular diversity statistics were low when calculated for all sequences, with two haplotypes in COI and three in mt 16S rDNA. The interpopulation pairwise nucleotide difference ( $\pi$ ) for COI and mt 16S rDNA showed no variation within any of the species ( $h=0.000$ ), but markedly different for the ITS region. The ITS rDNA (Table 2) showed greater variation both within and between populations. In total 10 haplotypes were formed with 154 variable sites. *Zoanthus durbanensis* had eight polymorphic sites, *Z. natalensis* had six, whilst *Z. sansibaricus* had none. The GC content was highest for *Z. natalensis* (0.578) and lowest for *Z. sansibaricus* (0.440). The results of the neutrality tests for Tajima's D and Fu and Li's D were negative for the intraspecific test.

**Table 2:** Molecular diversity statistics for the COI, mt 16S rDNA and ITS rDNA sequences for *Z. sansibaricus*, *Z. durbanensis* and *Z. natalensis*, S, number of variable sites; H, number of haplotypes;  $h$ , haplotype (genetic) diversity;  $\pi$ , nucleotide diversity;  $k$ , mean number of pairwise differences

COI	n	S	GC content	H	$h$	$\pi$	$k$	Tajima's D	$P$	Fu and Li's D	$P$
<i>Z. sansibaricus</i>	5	0	0.467	1	0.000	0.00000	-	-	-	-	-
<i>Z. durbanensis</i>	4	0	0.475	1	0.000	0.00000	-	-	-	-	-
<i>Z. natalensis</i>	5	0	0.475	1	0.000	0.00000	-	-	-	-	-
All sequences	14	2	0.472	2	0.495± 0.088	0.00405± 0.00072	0.98901	-	-	-	-

mt 16S rDNA	n	S	GC content	H	<i>h</i>	$\pi$	<i>k</i>	Tajima's D	<i>P</i>	Fu and Li's D	<i>P</i>
<i>Z. sansibaricus</i>	6	0	0.474	1	0.000	0.00000	-	-	-	-	-
<i>Z. durbanensis</i>	5	0	0.474	1	0.000	0.00000	-	-	-	-	-
<i>Z. natalensis</i>	6	0	0.472	1	0.000	0.00000	-	-	-	-	-
All sequences	17	3	0.473	3	0.706± 0.042	0.00342± 0.00035	1.45588	-	-	-	-
ITS rDNA	n	S	GC content	H	<i>h</i>	$\pi$	<i>k</i>	Tajima's D	<i>P</i>	Fu and Li's D	<i>P</i>
<i>Z. sansibaricus</i>	6	0	0.440	1	0.000	0.00000	-	-	-	-	-
<i>Z. durbanensis</i>	5	8	0.571	5	1.000± 0.126	0.01088± 0.00251	4.10000	-0.36190	<i>P</i> > 0.10	-0.19740	<i>P</i> > 0.10
<i>Z. natalensis</i>	6	6	0.578	6	1.000± 0.096	0.00676± 0.00137	2.53333	-0.20563	<i>P</i> > 0.10	-0.41639	<i>P</i> > 0.10
All sequences	17	154	0.531	1 0	0.868± 0.070	0.19735± 0.03036	73.80882	-	-	-	-

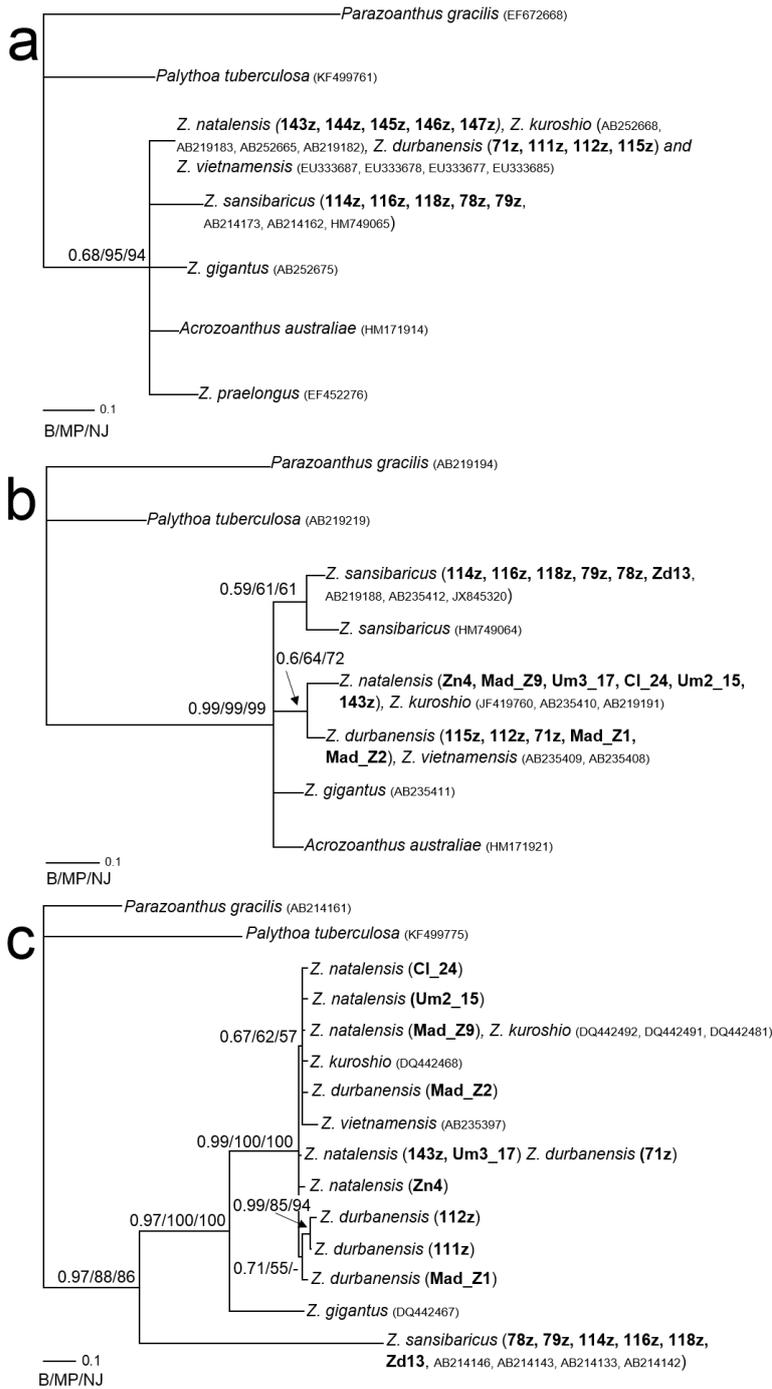
### 2.3.2. DNA sequence and phylogenetic identification

**COI.** New COI sequences were obtained for 14 specimens and the phylogenetic tree is shown in Figure 3a. The COI region was highly conserved with *Z. natalensis* and *Z. durbanensis* belonging to the same haplotype. This haplotype also contained *Z. kuroshio* and *Z. vietnamensis* sequences from the Pacific. *Zoanthus sansibaricus* from this study share a haplotype with *Z. sansibaricus* from the Pacific. All *Zoanthus* specimens were grouped in a single clade (B=0.68, MP=95% and NJ=94%). There was not enough genetic difference for *Acrozoanthus australiae* to separate out from the *Zoanthus* clade due to the conservative nature of the marker.

**mt 16S rDNA.** New mt 16S rDNA sequences were obtained for 17 specimens. The mt 16S rDNA tree is shown in Figure 3b. All *Zoanthus* specimens formed a well supported clade (B=0.99, MP=99% and NJ=99%). Within this clade a subclade of *Z. sansibaricus* sequences formed, which was not well supported (B=0.59, MP=61% and NJ=61%). This contained a

haplotype with all samples from this study and several from the Pacific, whereas the other haplotype was a single sequence from the Pacific. Further resolution was seen in the separation of *Z. durbanensis* and *Z. natalensis*. *Zoanthus natalensis* remained in the same haplotype as *Z. kuroshio* and *Z. durbanensis* remained with *Z. vietnamensis*. This subclade was not well supported (B=0.6, MP=64% and NJ=72%). There was not enough genetic difference for *Acrozoanthus australiae* to separate out from the *Zoanthus* clade.

**ITS rDNA.** New ITS rDNA sequences were obtained for 17 specimens. The ITS rDNA tree is shown in Figure 3c. There was greater resolution in this tree with *Z. sansibaricus* emerging as the most basal *Zoanthus* specimen. The separation of the *Z. sansibaricus* clade from the rest of the *Zoanthus* group was well supported (B=0.97, MP=88% and NJ=86%). *Z. sansibaricus* specimens from this study shared a haplotype with previously reported specimens from the Pacific. The next most basal clade was the separation of *Z. gigantus* from the remaining *Zoanthus* specimens. This node was very well supported (B=0.97, MP=100% and NJ=100%). The ITS rDNA sequences for *Z. natalensis*, *Z. kuroshio*, *Z. durbanensis* and *Z. vietnamensis* were all on a well supported branch of (B=0.99, MP=100% and NJ=100%). Within this clade the sequences are highly similar with small subclades appearing (Table 2). A *Z. natalensis* sequence (Mad\_Z9) matched with three of the GenBank *Z. kuroshio* sequences. One haplotype was formed with a *Z. natalensis* and a *Z. durbanensis* sequence. Most specimens from this study differed in their haplotype with approximately one base pair differentiating them from one another.



**Figure 3:** Bayesian consensus tree (3a) cytochrome oxidase subunit one (COI), (3b) mitochondrial 16S ribosomal DNA (mt 16S rDNA) and (3c) internal transcribed spacer region of ribosomal DNA (ITS rDNA) for the genus *Zoanthus* from South Africa. Values on the branches represent posterior probability values, maximum parsimony and neighbor-

joining bootstrap values. Taxa labeled without accession numbers were sequenced in the present study.

## 2.4. Discussion

It was hypothesized that *Z. durbanensis* and *Z. natalensis* were conspecific with *Z. sansibaricus*. These two species are different from *Z. sansibaricus*, however they do form possible synonymies with other species described in the western Pacific. The neutrality tests for the intraspecific analyses indicated no signal of selection and that the species groups had little diversity within groups. The greater diversity found for ITS rDNA is due to the faster evolution of these sequences compared to mitochondrial COI and mt 16S rDNA, and this result has been found previously (Reimer *et al.*, 2007b; Aguilar and Reimer, 2010).

Mitochondrial 16S rDNA has been extremely useful in classifying taxa to species level (Reimer *et al.*, 2013a) and in this study the mt 16S rDNA sequences for *Z. natalensis* were identical to *Z. kuroshio*, and *Z. durbanensis* was identical to *Z. vietnamensis*. *Zoanthus sansibaricus* formed isolated clades in mt 16S rDNA and ITS rDNA trees, and in the ITS rDNA tree it came out as the most basal *Zoanthus* specimen. Both *Z. natalensis* and *Z. durbanensis* were described in Carlgren (1938) on the KwaZulu-Natal coast of South Africa. *Zoanthus vietnamensis* was initially described in Pax and Müller (1957), whereas *Z. kuroshio* was described by Reimer and Ono in Reimer *et al.* (2006e). Although there has been some confusion about whether *Z. kuroshio* and *Z. vietnamensis* are conspecific due to their high molecular similarity but morphological diversity (Reimer *et al.*, 2006c), Reimer *et al.* (2013a) found that there is a 1-2bp difference with the concatenated COI and mt 16S rDNA sequences, and this has been enough to show species level differences for other species.

*Zoanthus kuroshio* was described as having a light purple coenenchyme and a pink oral disk (Reimer *et al.*, 2006e). *Zoanthus natalensis* specimens with pink oral disks were found along

the Eastern Cape coastline in Umgazana. *Zoanthus natalensis* is generally found to have a vivid green polyp column, which is much lighter around the ring of the top and the oral disk is usually a very pale shade of green or purple. *Zoanthus vietnamensis* was described as having small colonies, large polyps, and a pale pink oral disk with white mouth (Pax and Müller, 1957). Another indication to the synonymy between these two species is that the zoanthid ITS primers ZoanF and ZoanR did not generate amplicons for *Z. kuroshio* (Reimer *et al.*, 2007b), this phenomenon was also seen in *Z. natalensis*. *Zoanthus durbanensis* was found in smaller colonies, however the oral disk colour varied from orange to green with the mouth a grey-white in colour. Therefore if these are conspecifics then *Z. kuroshio* would become a junior synonym to *Z. natalensis* and *Z. vietnamensis* would become a junior synonym to *Z. durbanensis*.

Reimer *et al.* (2006e) stated that previous research on the phylogeny of zoanthids utilizing the COI marker showed that there were at most two species despite many morphotypes.

Therefore, the result obtained for the COI sequences in this study support the notion that COI is conservative in zoanthids. The rate of divergence of COI has been reported in corals as < 0.1% per million years in comparison to 2% per million years on average for vertebrates (Knowlton, 2000). The mt 16S marker includes insertions and deletions which add to variability between genera (Sinniger *et al.*, 2008). ITS rDNA was much more variable and the use of ITS and mitochondrial markers combined has been shown to be a powerful tool in investigating hybridization, which has been reported in the *Zoanthus* genus (Reimer *et al.*, 2007b). Microsatellite markers have been developed for *Z. sansibaricus* (Wham *et al.*, 2013) and have shown great genotypic diversity that suggests a large effective population size.

Brachycnemina zoanthids are hermaphroditic broadcast spawners which usually spawn in phase with the moon (Ono *et al.*, 2005; Reimer *et al.*, 2007b). Zoanthid larvae (Semper's larvae) are long lived, with a minimum duration of three weeks allowing them to cover vast

geographic distances if transported in oceanic currents (Babcock and Ryland, 1990; Burnett *et al.*, 1995; Swain, 2009). The long larval duration has been suggested as a reason why the same species are found over a wide range (Reimer *et al.*, 2004). Given the proposed synonymies with species in the Pacific, the same species are found with massive geographic distances and gene flow between regions could be due to long larval duration. A study by Ryland *et al.* (2000) showed that zoanthinae larvae (family Zoanthidae), which are adapted to warm temperatures, were found off the coast of KwaZulu-Natal in the warm Agulhas current. Therefore larvae would travel from north to south with the Agulhas but would not be able to move northward in a cold current, this would indicate the direction of movement of zoanthid larvae along the coastline (Ryland *et al.*, 2000).

The different strains of symbiotic *Symbiodinium* spp. may also allow for the adaptation to many habitats (Reimer *et al.*, 2004). Species in the *Zoanthus* genus have been successful due to rapid recolonization of substrates after disturbances such as storm events (Burnett *et al.*, 1995). This is enabled by successful recruitment of fragments following disturbances, and rapid growth rates (Karlson, 1986; Burnett *et al.*, 1995).

Studies on zoanthids have been focused in the western Pacific (Reimer and Fujii, 2010). Other authors have investigated the species diversity of zoanthids in understudied regions such as the Galápagos Islands (Reimer *et al.*, 2008a), Cape Verde Islands (Reimer *et al.*, 2010a), British Columbia (Reimer and Sinniger, 2010), New Caledonia (Sinniger, 2006) and Palau (Reimer *et al.*, 2013b). The east coast of South Africa consists of three regions; tropical, subtropical and warm temperate (Teske *et al.*, 2011). Brachycnemina zoanthid diversity has been found to be higher in warmer tropical waters (Ryland *et al.*, 2000; Reimer *et al.*, 2011b) and hence on the KwaZulu-Natal coastline there is the potential for a higher diversity of species than previously thought. SCUBA surveys should be conducted to assist in potentially sampling greater species diversity.

Although the results from this study indicate synonymies, it is only a tentative hypothesis as no formal morphological analyses were used and these species need to be investigated further using the various morphological identification characters. It should be noted that the *Z. vietnamensis* specimens were obtained in southern Japan, far from the location of the type specimen (Vietnam) and therefore molecular analyses would need to be carried out on specimens from the type location for comparison (Reimer *et al.*, 2006c). Molecular markers may not be able to distinguish between very closely related species (Reimer *et al.*, 2012), and presently a combination of morphological and molecular identification is needed (Sinniger *et al.*, 2008; Swain and Swain, 2014). Care should be taken to record the following data; depth, location, and high-resolution photographs of open and closed polyps. Specimens should be preserved in 4% sea water formalin for future morphological analyses (Reimer and Sinniger, 2010).

## **Chapter 3: Molecular examination of rocky shore brachynermic zoanthids (Anthozoa: Hexacorallia) and their *Symbiodinium* symbionts (Dinophyceae) in the southwest Indian Ocean**

### **3.1. Introduction**

The east Coast of South Africa is dominated by high wave action and is broken up into three regions: tropical, subtropical and warm temperate (Teske *et al.*, 2011). Reef coral communities only occur in the most northern (tropical) reaches of the KwaZulu-Natal province within the iSimangaliso Wetland Park. The east coast has intervals of rocky shores and sandy beaches. The rocky shores are home to several well studied groups including the brown mussel *Perna perna* (Lasiak and Dye, 1988) and the east coast rock lobster *Panulirus homarus* (Steyn *et al.*, 2008). Due to high extractive effort of these species, which are utilized by artisanal fisheries, they are well monitored. However when paying a visit to these rocky shores at low tide it is apparent that large stretches of intertidal rocky shelf are covered in patches of zoanthids.

Zoanthids belong to the order Zoantharia within class Anthozoa, and are sometimes the dominant species on coral reefs and rocky shore ecosystems (Karlson, 1983). Zoanthids have two suborders; Brachynermina (imperfect 5th dorsal mesentery) and Macrocnemina (perfect 5th dorsal mesentery) (Haddon and Shackleton, 1891). Brachynermina zoanthids are found in tropical shallow marine environments, show high intraspecific colour variation, produce planktonic larvae (Semper's larvae) and are generally zooxanthellate (Ryland, 1997; Ryland *et al.*, 2004). Brachynermina contains the families Neozoanthidae (genus *Neozoanthus*), Zoanthidae (genera *Acrozoanthus*, *Isaurus* and *Zoanthus*) and Sphenopidae (genera *Palythoa* and *Sphenopus*).

Zooxanthellae are dinoflagellates (genus *Symbiodinium*) and form mutualistic relationships with some zoanthids. *Symbiodinium* spp. have a complicated taxonomy in which there are

nine/ten clades (Pochon *et al.*, 2014), and there are several subclades with different host species in each clade (LaJeunesse, 2002). At the subclade level there are differences in their physiologies, for examples, cell growth, thermal sensitivity and photosystem II (Tchernov *et al.*, 2004; Robinson and Warner, 2006). Many zoanthids host only one type/subclade of zooxanthellae and this has been a suggested tool for identification of cryptic species as there are genetic markers for the internal transcribed spacer of ribosomal DNA (ITS rDNA) of *Symbiodinium* spp. (Reimer *et al.*, 2006a; Silverstein *et al.*, 2011). Therefore in recent publications it is common to find the ITS rDNA of the *Symbiodinium* spp. included in the study to help understand species delineations (Reimer *et al.*, 2013a).

Cryptic morphology and discrepancies of characters used to describe zoanthids has led to confused taxonomy and has resulted in zoanthids being neglected in ecological surveys and research (Ryland *et al.*, 2004; Aguilar and Reimer, 2010). Examples of characters that have been used to describe zoanthids are; oral disk colour, tentacle number, mesentery number, shape and position of the sphincter, type and distribution of nematocysts, polyp diameter, ecological characteristics (substrate and habitat), and the geographic distribution of the organism. However, both tentacle count and mesentery number are said to be inappropriate as these numbers increase with age, and identification by nematocysts has proven to be flawed as morphology can be influenced by a number of factors ranging from polyp age to colony size (Burnett *et al.*, 1997). The analysis of the sphincter muscle and the nematocyst also requires near perfect histological samples of zoanthids which is hindered by sand encrustation by some zoanthid groups (Sinniger *et al.*, 2005; Reimer *et al.*, 2010b).

The use of modern molecular techniques has fuelled research on zoanthids and has led to a revision of the taxonomy in this group with the description of several new species, genera and families (Reimer and Fujii, 2010). With reliable identification to the generic level possible when allozyme electrophoretic analysis was introduced (Burnett *et al.*, 1997), now a number

of genetic markers are now available to identify to species level with a degree of confidence (Reimer *et al.*, 2008a). New species are now being described and others are being listed as synonymies, and our knowledge of the diversity and distribution of zoanthids is broadening (Reimer *et al.*, 2004; Reimer *et al.*, 2007c; Sinniger and Haussermann, 2009; Reimer and Fujii, 2010).

The mitochondrial 16S rRNA region has been utilized on zoanthids because of their successful use in octocorals (Sanchez *et al.*, 2003) and scleractinians (Le Goff-Vitry *et al.*, 2004). The 16S marker is more variable than cytochrome oxidase subunit 1 (COI) because it has insertions and deletions which can be used to indicate genetic difference and species delineations (Sinniger *et al.*, 2008). The internal transcribed spacer region of ribosomal DNA (ITS-rDNA) has much greater variation at the species level (Knowlton, 2000; Aguilar and Reimer, 2010). However, ITS-rDNA marker is highly variable and should not be used on its own to indicate species level differences (Reimer *et al.*, 2006e). Additionally, many zoanthids host only one type/subclade of zooxanthellae and this has been a suggested tool for identification of cryptic species as there are genetic markers for the internal transcribed spacer of ribosomal DNA (ITS-rDNA) of *Symbiodinium* (Reimer *et al.*, 2006a). It is important to note that neither morphological nor molecular characterizations can stand on their own with complete reliability, therefore one needs to use both sets of information when identifying zoanthid species (Sinniger *et al.*, 2008).

Although an over estimation of species numbers is likely for *Zoanthus* and *Palythoa* spp. (Burnett *et al.*, 1995; Burnett *et al.*, 1997; Reimer *et al.*, 2004), research suggests that there are new species that are being discovered in locations where zoanthids have not been previously catalogued (Reimer and Fujii, 2010). Species in the genus *Isaurus* were previously thought rare, however they are cryptic in appearance and are found in hard to reach places such as ocean facing rocks (Reimer *et al.*, 2008b). Therefore the probability of an

unidentified species being found in an area where no genetic analyses have been done on zoanthids is possible.

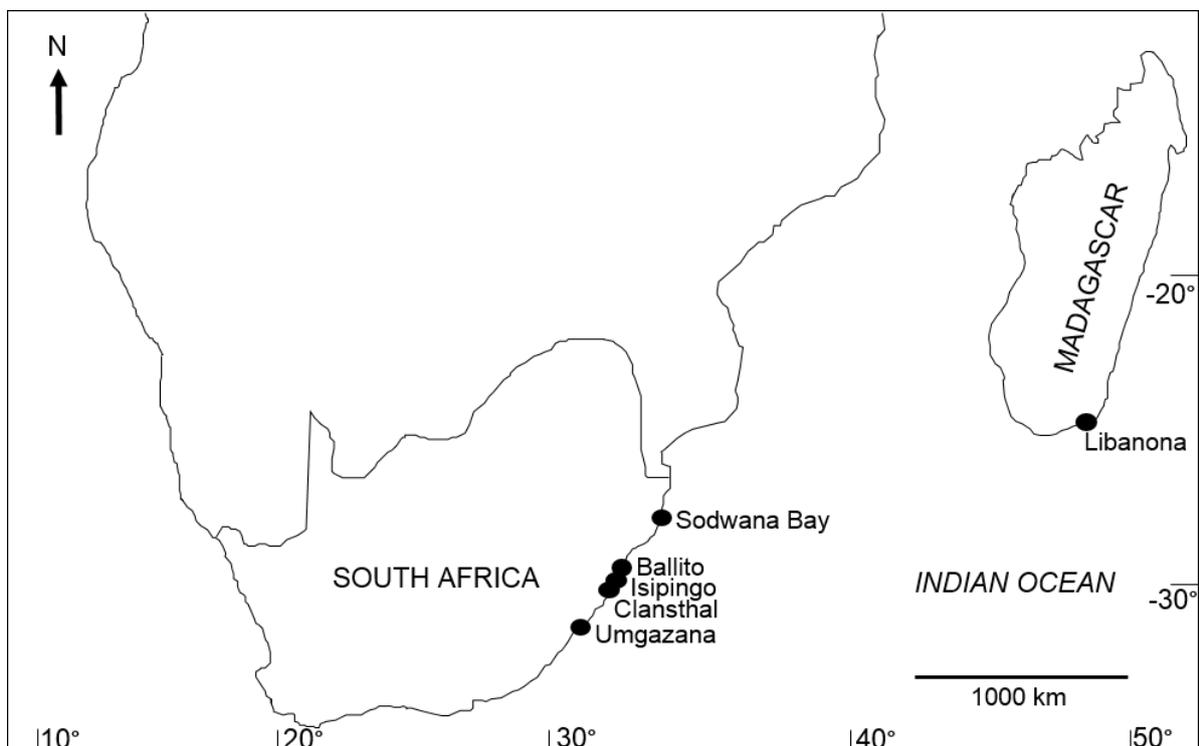
Carlgren (1938) described 27 zoanthid species on the South African coast. Aside from Carlgren's work, literature on zoanthids in South Africa has been scarce, and zoanthids are only mentioned in species identification books of South Africa's marine species (Branch *et al.*, 1981; Sink *et al.*, 2005; Branch *et al.*, 2008) with only a small number of species mentioned in such publications. As well, an ecological study (Herberts, 1972b) and a description of species was carried out in Madagascar, in which 15 species were described (Herberts, 1972a). Tropical zoanthids generally show greater colour variation and hence have higher morphological plasticity (Reimer *et al.*, 2004; Sinniger and Haussermann, 2009), and it has been noted previously that higher species diversity is expected in warmer waters (Irei *et al.*, 2011), and therefore there is a possibility that there are unknown zoanthid species on the east coast of South Africa.

Risi and Macdonald (2015) found that two South African intertidal rocky shore *Zoanthus* species *Z. natalensis* Carlgren, 1938 and *Z. durbanensis* Carlgren, 1938 are likely conspecific with species found in the west Pacific. Therefore it is possible that once more South African species are examined using molecular methods, it is likely to find more synonymies. The aim of this study was to examine zoanthid species found along sites on the east coast of South Africa using molecular techniques, to record certain morphological characters in the field, and to conduct analyses utilizing the molecular markers mt 16S rDNA and ITS rDNA, and for the first time on South African zoanthids, the ITS rDNA region of *Symbiodinium* spp. These results will then be utilized to suggest how to revise species and the taxonomy of these zoanthids.

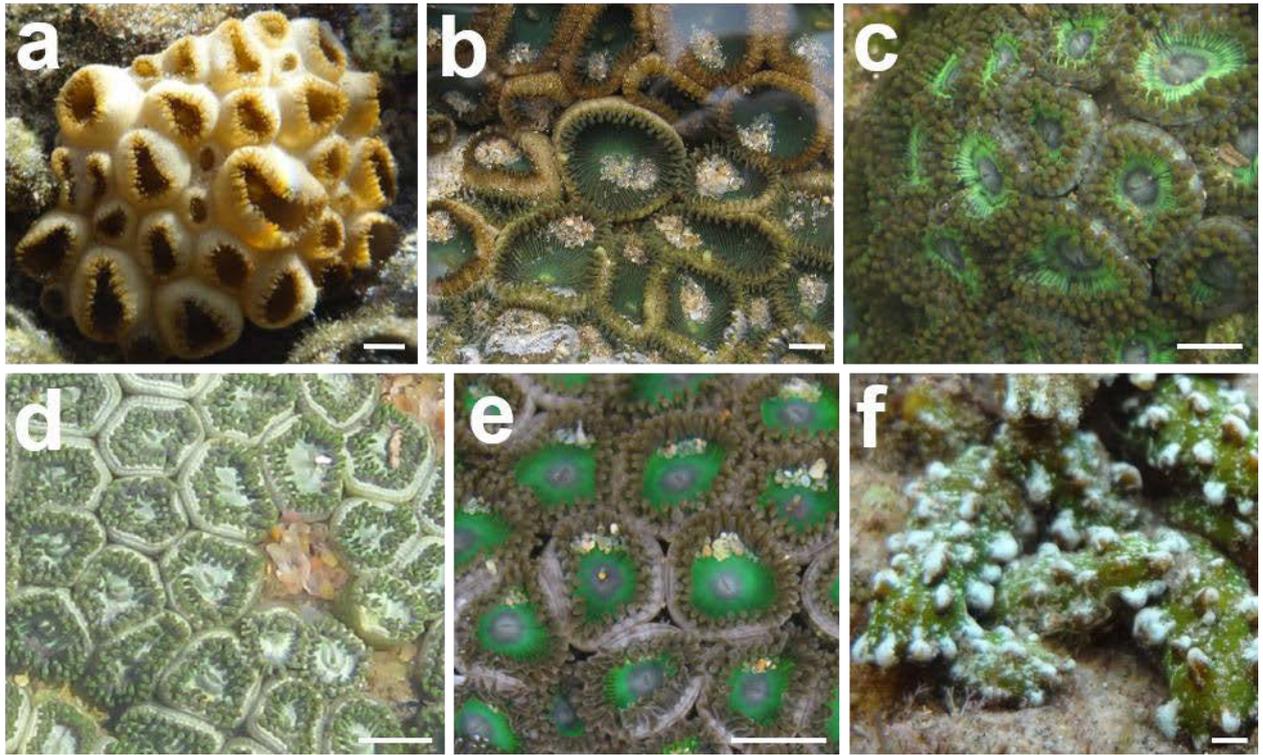
## 3.2. Methods and materials

### 3.2.1. Specimen collection and preservation

Specimens were collected from the rocky shores at Umgazana, Clansthal, Isipingo, Ballito, and Sodwana (Figure 4, Table 3). Zoanthids were actively searched for on the rocky shore during spring low tide. The species were identified following Carlgren (1938) and Branch *et al.* (2008). Zoanthids were also collected at one site in Libanona, Madagascar (Figure 4), and were identified to genus level. Individual specimens were taken from different patches using a flat scraper and placed into vials containing 70% ethanol for further analyses. Specimens collected in Ballito for morphological analysis had the following information recorded: oral disk (OD) diameter, polyp height, and column and OD colour (Table 4). Photographs of zoanthids were taken *in situ* (Figure 5 a-f).



**Figure 4:** Map showing the sample sites in this project, on the east coast of South Africa and Libanona in Madagascar.



**Figure 5:** *In situ* photographs of the zoanthid species collected in this study. Scale bars all represent 5 mm. (5a) *Palythoa tuberculosa*, (5b) *Palythoa nelliae*, (5c) *Zoanthus durbanensis*, (5d) *Zoanthus natalensis*, (5e) *Zoanthus sansibaricus* and (5f) *Isaurus tuberculatus*.

### 3.2.2. DNA extraction, PCR amplification and sequencing

DNA was extracted from the zoanthid polyp (25 mg) using two different techniques. *Zoanthus* species, which do not encrust sand, were extracted by following the protocol for solid tissue specimens using a Zymogen Quick-gDNA MiniPrep extraction kit (Zymo Research Corporation, California, United States of America). *Palythoa* species (sand encrusting) and *Isaurus* species were extracted using a Phenol Chloroform Isoamyl extraction technique (Sambrook *et al.*, 1989). The reason for the two different extraction methods was because the Zymogen kit was the preferred method, however, did not yield enough quality DNA for *Palythoa* and *Isaurus* specimens and hence the PCI method was used. PCRs were run using the following protocol; 12.5  $\mu$ l of EconoTaq (Lucigen Corporation, Wisconsin,

United States of America), 0.84 µl of both forward and reverse primer each at a concentration of 10 µM, 1 µl of bovine serum albumin and 10 µl of PCR water and 2 µl of extracted DNA. The mt 16S rDNA region was amplified using the zoanthid specific primers 16Sant1a and 16S bmoH (Sinniger *et al.*, 2005), following the thermal cycler conditions described in Sinniger *et al.* (2005). ITS rDNA was amplified using zoanthid specific primers ZoanF and ZoanR (Reimer *et al.*, 2007b), following the thermal cycler conditions used in Reimer and Sinniger (2010). ZoanF and ZoanR did not generate product for *Z. natalensis* or *Isaurus* specimens, and therefore ZkITS-F and ZkITS-R (Risi and Macdonald, 2015) were used for *Z. natalensis* specimens. An initial denaturation step of 3 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 72°C, followed by a 10 min extension at 72°C. The ITS-rDNA region for *Symbiodinium* spp. present in zoanthids was amplified with ITS-4 and zITSf and following the procedures in Reimer *et al.* (2006b). The PCR-amplified products were checked using 1 % agarose gel electrophoresis and a 100 bp molecular weight marker. The expected PCR product size was ±600-900 bp for ITS rDNA and mt 16S rDNA, ±500-700bp for *Symbiodinium* spp. ITS rDNA, and lastly ± 450 bp for *Z. natalensis* ITS rDNA. Negative and positive controls were run in all PCRs to check for signs of contamination. The PCR-amplified DNA fragments were sequenced with an ABI 3730 capillary sequencer (Inqaba Biotechnical Industries, Pretoria, South Africa) using Big End Dye technology (Life Technologies, Johannesburg, South Africa).

### **3.2.3. Phylogenetic analyses**

The new sequences obtained in this study were deposited into GenBank (KM032370-KM032604) (Benson *et al.*, 2005). Sequences were aligned and edited using a Clustal W multiple-alignment algorithm on BioEdit v7.0.9 (Hall, 1999). Four alignments were generated; mt 16S rDNA having 428 sites of 142 sequences, ITS rDNA having 399 sites of 52 sequences, *Symbiodinium* ITS rDNA clade C having 493 sites of 74 sequences, and

Symbiodinium ITS rDNA Clade A having 501 sites of 19 sequences. All alignments were inspected by eye and manually edited, and all ambiguous sites (observed only at the 5' and 3' ends of alignments), or double peaks (< two per alignment) were removed from the dataset for phylogenetic analyses. Multiple copies of zoanthid ITS-rDNA were found in some of the specimens and the sequences were sorted manually by analysing the chromatogram and choosing the strongest signal (highest peak). Intraspecies and interspecies diversity indexes were calculated using Dnasp5 software (Librado and Rozas, 2009). These analyses were run for mt 16s rDNA, ITS rDNA of zoanthids and ITS rDNA of *Symbiodinium* spp. Substitution saturation was tested using Data Analysis in Molecular Biology and Evolution (DAMBE) for the zoanthid ITS rDNA sequences and the results were that there was little saturation (Iss= 0.1994, Iss.c= 0.6653, Iss < Iss.c) (Xia *et al.*, 2003; Xia and Lemey, 2009).

The outgroup for mt 16S rDNA and ITS rDNA alignment were downloaded from GenBank for *Parazoanthus gracilis* (family Parazoanthidae). Sequences of mt 16S rDNA and ITS rDNA were downloaded from GenBank for *Palythoa mutuki*, *P. tuberculosa*, *Z. sansibaricus*, *Z. kuroshio*, *Z. gigantus*, *Z. natalensis*, *Z. durbanensis* and *Z. vietnamensis*. Sequences for mt 16S rDNA were downloaded for *Isaurus tuberculatus*, *Neozoanthus* sp., *Palythoa cf. heliodiscus* and *Palythoa* aff. *sakurajimensis*. *Symbiodinium* ITS-rDNA sequences were highly variable for different clades, and therefore two separate trees were made for clade C and clade A, respectively. GenBank accession numbers of all downloaded sequences used are shown in the phylogenetic trees.

Haplotype groups were created using DNAsp5 (Librado and Rozas, 2009). The following phylogenetic outputs were produced for zoanthid mt 16S rDNA, ITS rDNA, and *Symbiodinium* ITS-rDNA alignments. Evolutionary models were calculated using jModelTest version 2.1.3 (Darriba *et al.*, 2012) from which the Akaike Information Criterion was utilized (Posada and Buckley, 2004). The Hasegawa, Kishino and Yano model (HKY) was suggested

for the mt 16S rDNA, and *Symbiodinium* ITS rDNA clade C datasets, and the K80 model was suggested for the zoantharian ITS-rDNA. Lastly the Three Parameter Model (TPM3) was suggested for the *Symbiodinium* ITS rDNA clade A dataset. Bayesian analysis was run using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003), and maximum parsimony (MP) analysis was run using PAUP\* v4 (Swofford, 2002). MrBayes was run for 1 000 000 generations with a 10% burnin, using the Kimura model of evolution (Kimura, 1981), equal nucleotide frequencies and a gamma distribution of rates. The maximum-likelihood (ML) analyses were run using GARLI 2.0 (Zwickl, 2006; Bazinet *et al.*, 2014) with the models selected using the Aikake Information Criterion in jModelTest (Posada and Buckley, 2004). Phylogenetic trees were edited using TreeViewX (Page, 1996). The information for the *Symbiodinium* subclade was indicated by a symbol on the mt 16S rDNA tree.

**Table 3:** Identity of zoanthid specimens from South Africa and Madagascar examined in this study, their collection information, and associated GenBank accession numbers.

*Symbiodinium* subclades are indicated in bold within the *Symbiodinium* ITS-rDNA column.

Specimen number	Location	GPS co-ordinates	Date	Collector (s)	mt 16S rDNA	ITS-rDNA	<i>Symbiodinium</i> ITS-rDNA	Identity
Um1_7	Umgazana	31.7052° S, 29.4157° E	09/07/13	MM Risi	KM032454	KM032401	<b>C1</b> KM032590	<i>Palythoa tuberculosa</i>
Um1_8	Umgazana	31.7052° S, 29.4157° E	09/07/13	MM Risi	NA	NA	<b>C1</b> KM032591	<i>Palythoa tuberculosa</i>
Um1_10	Umgazana	31.7052° S, 29.4157° E	09/07/13	MM Risi	KM032452	KM032399	NA	<i>Palythoa tuberculosa</i>
Um1_12	Umgazana	31.7052° S, 29.4157° E	09/07/13	MM Risi	KM032453	KM032402	<b>C1</b> KM032587	<i>Palythoa tuberculosa</i>
Um1_13	Umgazana	31.7052° S, 29.4157° E	09/07/13	MM Risi	KM032519	KM032374	<b>C1</b> KM032588	<i>Zoanthus sansibaricus</i>
Um1_19	Umgazana	31.7052° S, 29.4157° E	09/07/13	MM Risi	KM032482	KM032378 <i>Z. sansibaricus</i>	<b>C15</b> KM032589	<i>Zoanthus durbanensis</i>
Um2_4	Umgazana	31.7024° S, 29.4175° E	10/07/13	MM Risi	KM032439	NA	<b>C1</b> KM032595	<i>Palythoa nelliae</i>
Um2_6	Umgazana	31.7024° S, 29.4175° E	10/07/13	MM Risi	KM032440	NA	<b>C1</b> KM032596	<i>Palythoa nelliae</i>
Um2_8	Umgazana	31.7024° S, 29.4175° E	10/07/13	MM Risi	KM032524	KM032371	<b>A1</b> KM032597	<i>Zoanthus sansibaricus</i>
Um2_10	Umgazana	31.7024° S, 29.4175° E	10/07/13	MM Risi	KM032522	KM032373	<b>A1</b> KM032592	<i>Zoanthus sansibaricus</i>

Um2_12	Umgazana	31.7024° S, 29.4175° E	10/07/13	MM Risi	KM032420	NA	<b>C1</b> KM032593	<i>Isaurus tuberculatus</i>
Um2_15	Umgazana	31.7024° S, 29.4175° E	10/07/13	MM Risi	KJ416428	KJ416453	<b>C15</b> KM032594	<i>Zoanthus natalensis</i>
Um3_6	Umgazana	31.6849° S, 29.4416° E	11/07/13	MM Risi	KM032441	NA	<b>C1</b> KM032604	<i>Palythoa nelliae</i>
Um3_16	Umgazana	31.6849° S, 29.4416° E	11/07/13	MM Risi	KM032456	KM032404	<b>C1</b> KM032598	<i>Palythoa tuberculosa</i>
Um3_20	Umgazana	31.6849° S, 29.4416° E	11/07/13	MM Risi	KM032509	NA	<b>A1</b> KM032599	<i>Zoanthus natalensis</i>
Um3_21	Umgazana	31.6849° S, 29.4416° E	11/07/13	MM Risi	KM032525	KM032375	<b>C1</b> KM032600	<i>Zoanthus sansibaricus</i>
Um3_23	Umgazana	31.6849° S, 29.4416° E	11/07/13	MM Risi	KM032487	NA	<b>C15</b> KM032601	<i>Zoanthus durbanensis</i>
Um3_24	Umgazana	31.6849° S, 29.4416° E	11/07/13	MM Risi	KM032457	KM032390	<b>C1</b> KM032602	<i>Palythoa tuberculosa</i>
Um3_25	Umgazana	31.6849° S, 29.4416° E	11/07/13	MM Risi	KM032488	NA	<b>A1</b> KM032603	<i>Zoanthus durbanensis</i>
Cl_5	Clansthal	30.2391° S, 30.7865° E	22/06/13	L Cele, RA Van Rooyen	KM032415	NA	<b>C1</b> KM032553	<i>Isaurus tuberculatus</i>
Cl_10	Clansthal	30.2391° S, 30.7865° E	22/06/13	L Cele, RA Van Rooyen	KM032512	KM032376	<b>C1</b> KM032550	<i>Zoanthus sansibaricus</i>
Cl_21	Clansthal	30.2391° S, 30.7865° E	22/06/13	L Cele, RA Van Rooyen	KM032426	KM032396	<b>C1</b> KM032551	<i>Palythoa nelliae</i>
Cl_24	Clansthal	30.2391° S, 30.7865° E	22/06/13	L Cele, RA Van Rooyen	KJ416427	KJ416449	<b>A1</b> KM032548	<i>Zoanthus natalensis</i>
Cl_25	Clansthal	30.2391° S, 30.7865° E	22/06/13	L Cele, RA Van Rooyen	KM032445	NA	<b>C1</b> KM032552	<i>Palythoa tuberculosa</i>
Cl_28	Clansthal	30.2391° S, 30.7865° E	22/06/13	L Cele, RA Van Rooyen	KM032427	KM032393	NA	<i>Palythoa nelliae</i>
Cl_30	Clansthal	30.2391° S, 30.7865° E	22/06/13	L Cele, RA Van Rooyen	KM032470	NA	<b>C15</b> KM032549	<i>Zoanthus durbanensis</i>
Mad_z1	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KJ416432	KJ416458	<b>C15</b> KM032582	<i>Zoanthus durbanensis</i>
Mad_z4	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032517	KM032379	<b>C1</b> KM032583	<i>Zoanthus sansibaricus</i>
Mad_z6	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032489	KM032389	<b>C1</b> KM032584	<i>Zoanthus gigantus</i>
Mad_z7	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032481	NA	<b>C15</b> KM032585	<i>Zoanthus durbanensis</i>
Mad_z8	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032518	NA	<b>C1</b> KM032586	<i>Zoanthus sansibaricus</i>
Mad_p1	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032432	KM032397	<b>C1</b> KM032577	<i>Palythoa nelliae</i>
Mad_p2	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032433	KM032394	<b>C1</b> KM032578	<i>Palythoa nelliae</i>
Mad_p3	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032434	KM032405	<b>C1</b> KM032579	<i>Palythoa nelliae</i>
Mad_p4	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032435	KM032395	NA	<i>Palythoa nelliae</i>
Mad_p5	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032436	KM032410	NA	<i>Palythoa nelliae</i>
Mad_p6	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032437	KM032408	NA	<i>Palythoa nelliae</i>
Mad_p7	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032450	KM032403	<b>C1</b> KM032580	<i>Palythoa tuberculosa</i>
Mad_p8	Madagascar -Libanona	25.0421° S, 46.9952° E	01/07/13	M Schleyer	KM032451	KM032400	<b>C1</b> KM032581	<i>Palythoa tuberculosa</i>
Is_1	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032428	NA	<b>C1</b> KM032554	<i>Palythoa nelliae</i>

Is_2	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032515	KM032380	C1	KM032561	<i>Zoanthus sansibaricus</i>
Is_3	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032471	Short Z. <i>durbanensis</i>	C15	KM032562	<i>Zoanthus durbanensis</i>
Is_4	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032472	NA	C15	KM032563	<i>Zoanthus durbanensis</i>
Is_5	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032473	NA	C15	KM032564	<i>Zoanthus durbanensis</i>
Is_7	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032448	KM032398	C1	KM032565	<i>Palythoa tuberculosa</i>
Is_8	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032500	NA	A1	KM032566	<i>Zoanthus natalensis</i>
Is_10	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032416	NA	C1	KM032555	<i>Isaurus tuberculatus</i>
Is_11	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032417	NA	C1	KM032556	<i>Isaurus tuberculatus</i>
Is_12	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032495	KM032385	C1	KM032557	<i>Zoanthus natalensis</i>
Is_13	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032496	NA	A1	KM032558	<i>Zoanthus natalensis</i>
Is_14	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032497	KM032387	C1	KM032559	<i>Zoanthus natalensis</i>
Is_16	Isipingo	29.9967° S, 30.9501° E	17/11/13	MM Risi	KM032447	NA	C1	KM032560	<i>Palythoa tuberculosa</i>
Bal_1	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032511	KM032381	C1	KM032538	<i>Zoanthus sansibaricus</i>
Bal_2	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032424	KM032407	C1	KM032544	<i>Palythoa nelliae</i>
Bal_3	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032467	NA	C15	KM032545	<i>Zoanthus durbanensis</i>
Bal_5	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032425	NA	C1	KM032546	<i>Palythoa nelliae</i>
Bal_6	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032469	KM032383	NA		<i>Zoanthus durbanensis</i>
Bal_7	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032492	KM032386	A1	KM032547	<i>Zoanthus natalensis</i>
Bal_10	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032462	Short Z. <i>durbanensis</i>	C15	KM032539	<i>Zoanthus durbanensis</i>
Bal_11	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032463	NA	C15	KM032540	<i>Zoanthus durbanensis</i>
Bal_12	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032491	NA	A1	KM032541	<i>Zoanthus natalensis</i>
Bal_15	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032466	Short Z. <i>durbanensis</i>	C15	KM032542	<i>Zoanthus durbanensis</i>
Bal_17	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	KM032412	NA	NA		<i>Isaurus tuberculatus</i>
Bal_18	Ballito	29.5451° S, 31.2160° E	18/01/14	MM Risi	NA	NA	C15	KM032543	<i>Zoanthus durbanensis</i>
A_2	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032490	NA	A1	KM032531	<i>Zoanthus natalensis</i>
A_4	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032442	NA	C1	KM032533	<i>Palythoa tuberculosa</i>
A_5	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032443	KM032391	C1	KM032534	<i>Palythoa tuberculosa</i>
A_6	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032444	NA	C1	KM032535	<i>Palythoa tuberculosa</i>
A_7	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032510	NA	C1	KM032536	<i>Zoanthus sansibaricus</i>
A_9	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032461	NA	C15	KM032537	<i>Zoanthus durbanensis</i>

A_12	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032421	NA	C1 KM032526	<i>Isaurus tuberculatus</i>
A_13	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032411	NA	C1 KM032527	<i>Isaurus tuberculatus</i>
A_17	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032458	NA	C15 KM032528	<i>Zoanthus durbanensis</i>
A_18	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032459	NA	C15 KM032528	<i>Zoanthus durbanensis</i>
A_19	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032460	NA	C15 KM032530	<i>Zoanthus durbanensis</i>
A_20	Sodwana	27.6594° S, 32.6477° E	30/04/14	MM Risi	KM032422	KM032406	C1 KM032532	<i>Palythoa nelliae</i>
M_1	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032429	KM032409	C1 KM032567	<i>Palythoa nelliae</i>
M_2	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032475	KM032384	C15 KM032571	<i>Zoanthus durbanensis</i>
M_3	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032430	NA	C1 KM032572	<i>Palythoa nelliae</i>
M_4	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032501	KM032388	A1 KM032573	<i>Zoanthus natalensis</i>
M_5	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032502	NA	A1 KM032574	<i>Zoanthus natalensis</i>
M_6	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032503	NA	A1 KM032575	<i>Zoanthus natalensis</i>
M_8	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032476	NA	C15 KM032576	<i>Zoanthus durbanensis</i>
M_10	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032418	NA	C1 KM032568	<i>Isaurus tuberculatus</i>
M_11	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032516	KM032382	C1 KM032569	<i>Zoanthus sansibaricus</i>
M_14	Sodwana	27.3314° S, 32.7520° E	29/04/14	MM Risi	KM032449	NA	C1 KM032570	<i>Palythoa tuberculosa</i>

**Table 4:** Morphological analyses of zoanths collected in terms of polyp height, polyp colour, oral disk colour, oral disk width, tentacle colour.

Species	Polyp height (mm)	Polyp colour	Oral disk diameter (mm)	Oral disk colour	Tentacle colour
<i>Palythoa tuberculosa</i>	4-10	Light brown- yellow polyp colour	10-15	Range from light brown to dark brown	Ranging between light yellow to dark brown
<i>Palythoa nelliae</i>	15-45	Sandy brown column sometimes with green tinge near the top of the polyp	10-20	Variations from light green to dark green, with brown ODs observed on occasion	Dark brown, dark green, light green
<i>Zoanthus durbanensis</i>	5-20	Powdery grey with white ring around top of polyp, grey/ brown and dark grey	3-8	Grey OD, orange OD, green OD, shading from light green outside to grey inner OD, shading from orange outside to grey inside OD	Range from light to dark brown, range from light to dark green, grey with green/brown tips,

<i>Zoanthus natalensis</i>	2-5	Grey column with light green around top of polyp, dark green to light green at top, light green to white ring around top of polyp	3-4	Light purple OD, bright pink OD, white OD, light green OD,	Green-brown, light brown, light brown with green tips
<i>Zoanthus sansibaricus</i>	10-25	Light/dark grey, purple, dark purple to light purple/ light grey, and brown column	3-5	Ranges from light green to vivid green OD	Dark brown-light brown, dark green,
<i>Isaurus tuberculatus</i>	15-50	Light brown, light grey, and green columns observed with lighter coloured knobs on polyp	NA	NA	NA

### 3.3. Results

#### 3.3.1 Molecular diversity

The standard molecular diversity statistics (Table 5) were much lower for mt 16S rDNA than for ITS rDNA when calculated for all sequences, with seven haplotypes in 16S rDNA and 21 for ITS rDNA. The inter-population pairwise nucleotide difference ( $\pi$ ) for mt 16S rDNA showed no variation within any of the species ( $\pi = 0.000$ ), but were markedly different for the ITS region, where all species had a degree of variation. The most variation was seen in *P. tuberculosa* ( $h = 1.000 \pm 0.076$ ) and *Z. durbanensis* ( $h = 1.00 \pm 0.272$ ) sequences sets. Mean number of pairwise differences was very high for *Z. durbanensis* ( $k = 102.66667$ ). For the *Symbiodinium* ITS rDNA, subclade C1 had the highest number of haplotypes ( $H = 9$ ), however subclade C15/C91 had the greatest haplotype (genetic) diversity ( $h = 0.758 \pm 0.081$ ) and largest mean number of pairwise differences ( $k = 1.31818$ ). Subclade A1 had the lowest number of haplotypes ( $H = 2$ ), as well as the lowest genetic diversity ( $h = 0.513 \pm 0.082$ ).

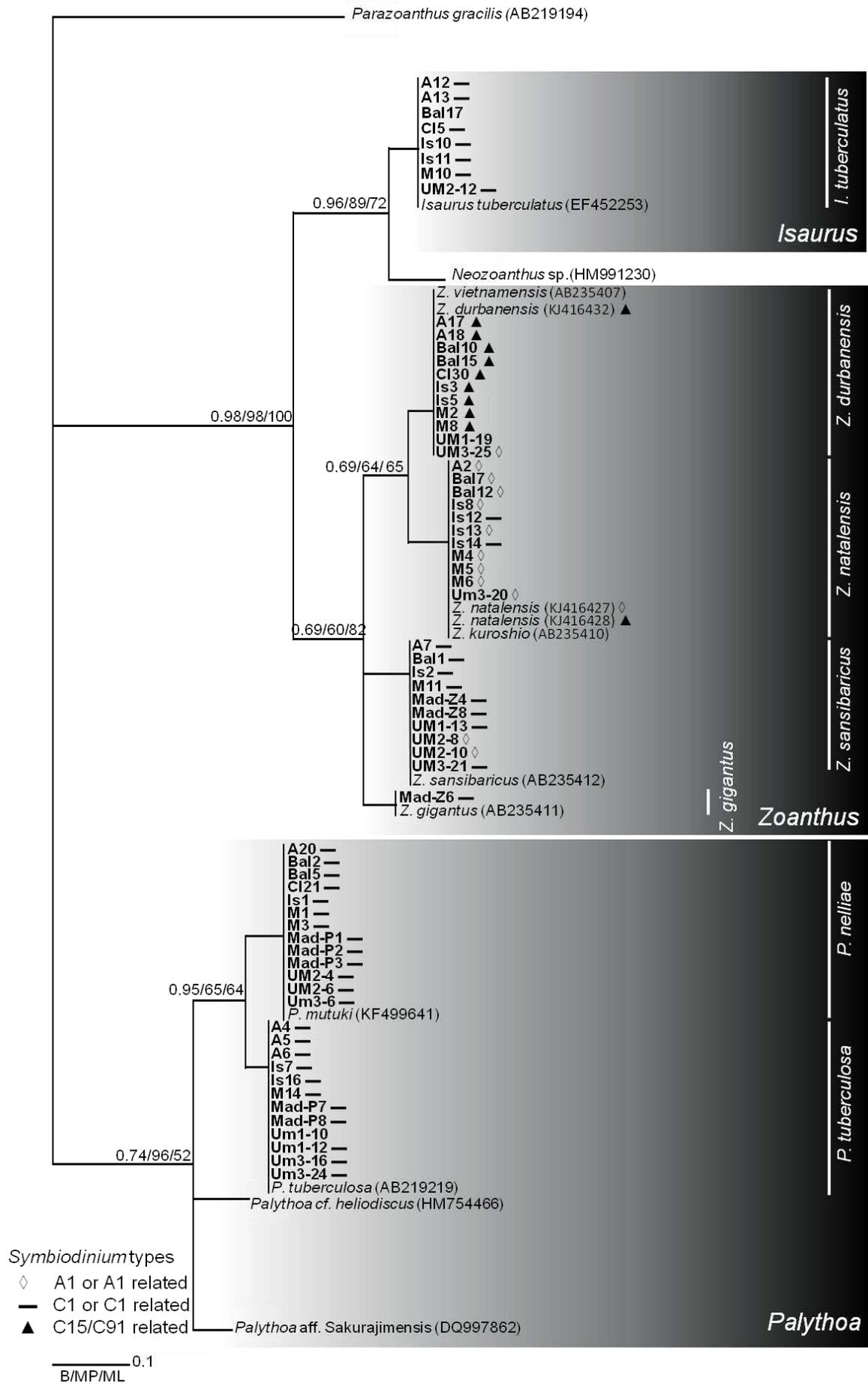
**Table 5:** Molecular diversity statistics for the mt 16S rDNA, ITS rDNA and *Symbiodinium* ITS rDNA sequences for species/clades collected in this study, S, number of variable sites; H, number of haplotypes;  $h$ , haplotype (genetic) diversity;  $\pi$ , nucleotide diversity;  $k$ , mean number of pairwise differences

mt 16S rDNA	n	S	GC content	H	<i>h</i>	$\pi$	<i>k</i>
<i>I. tuberculatus</i>	8	0	0.506	1	0.000	0.00000	-
<i>P. nelliae</i>	13	0	0.521	1	0.000	0.00000	-
<i>P. tuberculosa</i>	12	0	0.525	1	0.000	0.00000	-
<i>Z. durbanensis</i>	11	0	0.509	1	0.000	0.00000	-
<i>Z. gigantus</i>	1	-	-	1	-	-	-
<i>Z. natalensis</i>	11	0	0.506	1	0.000	0.00000	-
<i>Z. sansibaricus</i>	9	0	0.510	1	0.000	0.00000	-
All sequences	65	23	0.515	7	0.847 ± 0.011	0.02322 ± 0.00087	8.94087
ITS rDNA	n	S	GC content	H	<i>h</i>	$\pi$	<i>k</i>
<i>P. nelliae</i>	11	16	0.506	6	0.800 ± 0.114	0.00944 ± 0.00330	3.16364
<i>P. tuberculosa</i>	7	26	0.497	7	1.000 ± 0.076	0.03090 ± 0.00613	9.23810
<i>Z. durbanensis</i>	3	152	0.524	3	1.000 ± 0.272	0.27599 ± 0.12018	102.66667
<i>Z. gigantus</i>	1	-	-	1	-	-	-
<i>Z. natalensis</i>	4	2	0.564	3	0.833 ± 0.222	0.00266 ± 0.00090	1.00000
<i>Z. sansibaricus</i>	8	1	0.438	2	0.250 ± 0.180	0.00064 ± 0.00046	0.25000
All sequences	34	145	0.492	21	0.929 ± 0.032	0.21112 ± 0.01471	60.16934
<i>Symbiodinium</i> ITS-rDNA	n	S	GC content	H	<i>h</i>	$\pi$	<i>k</i>
subclade C1	43	11	0.508	9	0.533 ± 0.087	0.00156 ± 0.00040	0.85714
subclade C15	12	5	0.501	4	0.758 ± 0.081	0.00239 ± 0.00071	1.31818
subclade A1	13	1	0.477	2	0.513 ± 0.082	0.00097 ± 0.00016	0.51282
All sequences	68	163	0.503	15	0.791 ± 0.044	0.09691 ± 0.01644	50.58472

### 3.3.2. DNA sequence and phylogenetic identification

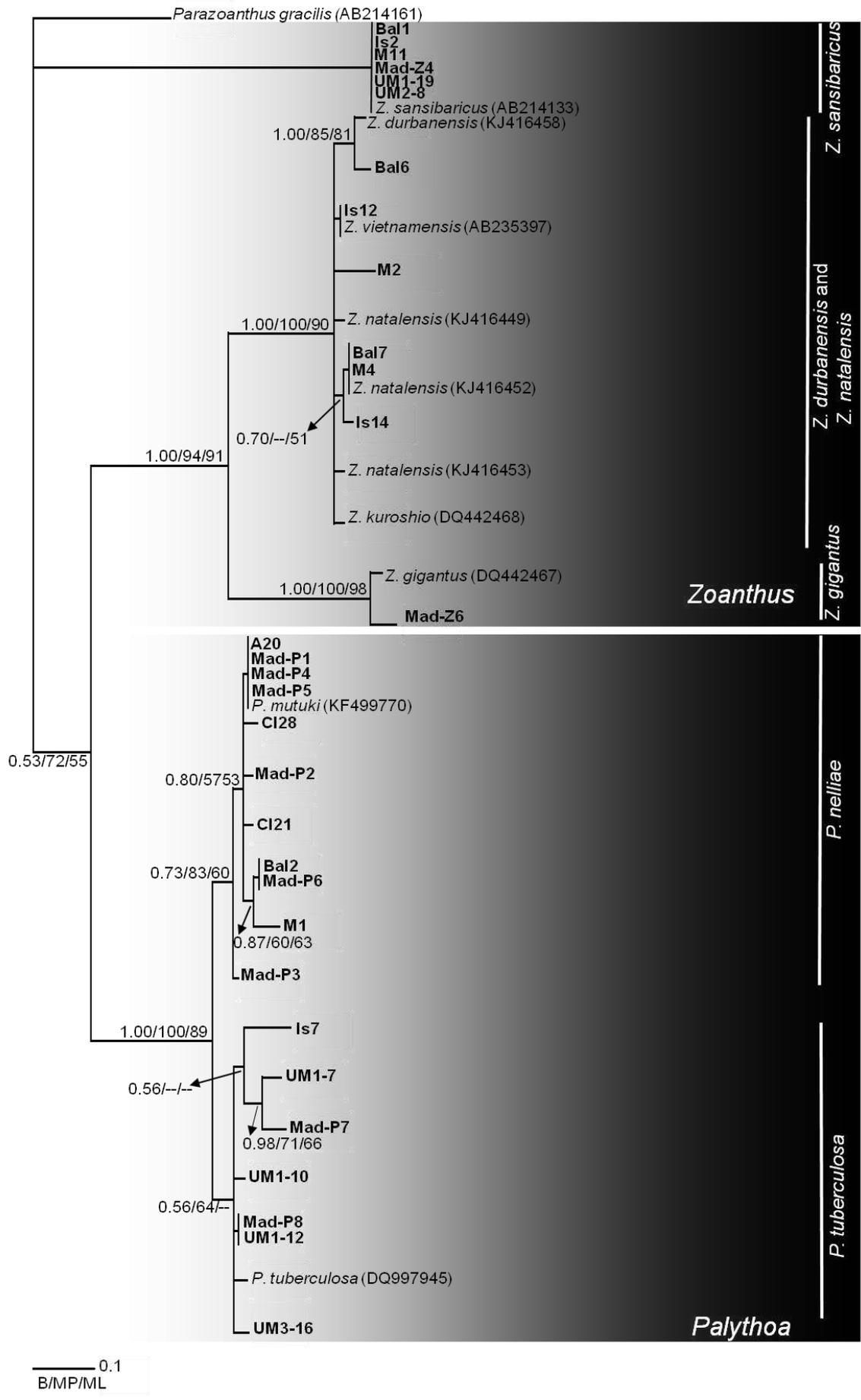
**mt 16S rDNA.** mt 16S rDNA sequences were obtained for 115 specimens. The mt 16S rDNA tree is shown in Figure 6. The 16S tree represented three zoanthid genera; *Isaurus*, *Zoanthus* and *Palythoa*. All *Isaurus* samples clustered with *I. tuberculatus* and the clade with *Isaurus* and *Neozoanthus* was well supported for Bayesian and MP (B=0.96, MP=89% and ML=72%). *Zoanthus* sequences from this study matched with previously reported sequences from four species; *Z. durbanensis*, *Z. natalensis*, *Z. sansibaricus* and *Z. gigantus*. The *Zoanthus* clade, however, was not well supported (B=0.69, MP=60% and ML=82%). *Zoanthus natalensis* formed a single group with *Z. kuroshio* and *Z. durbanensis* with *Z. vietnamensis*. These two groups were very closely related but their common ancestor was not

well supported (B=0.69, MP=64% and ML=65%). All *Palythoa* sequences formed a monophyletic clade which was highly supported in the MP analysis (B=0.74, MP=96% and ML=52%). All *Palythoa* sequences obtained in this study belonged to one of two species; *P. tuberculosa* and *P. nelliae*. The clade with these two species has strong support in the Bayesian analysis (B=0.95, MP=65% and ML=64%). All *P. tuberculosa* sequences in this study formed a single group with a *P. tuberculosa* sequence from Japan and all *P. nelliae* sequences from this study formed a single group with *P. mutuki*.



**Figure 6:** Bayesian consensus tree of mitochondrial 16S ribosomal DNA (mt 16S rDNA) for sequences obtained in this study. Values on the branches represent posterior probability values, maximum parsimony and maximum-likelihood bootstrap values. Taxa labeled without accession numbers were sequenced in the present study, and their corresponding *Symbiodinium* types are represented by symbols as indicated in the legend.

**ITS-rDNA.** New ITS rDNA sequences were obtained for 41 specimens. The ITS rDNA tree is shown in Figure 7. The ITS-rDNA for *Z. sansibaricus* was dissimilar from other *Zoanthus* sequences and therefore this species does not cluster together with other *Zoanthus* sequences. The *Z. sansibaricus* sequences in this study were identical to the “distant”/”sansi” ITS rDNA type mentioned in previous studies (Reimer *et al.*, 2007b; Aguilar and Reimer, 2010). *Zoanthus natalensis* and *Z. durbanensis* formed a well-supported clade with *Z. kuroshio* and *Z. vietnamensis* with the ITS rDNA region (B=1.00, MP=100% and ML=90%). One of the *Z. durbanensis* specimens- UM1-19 (confirmed by 16S) had ITS rDNA that matched up with *Z. sansibaricus*. *Zoanthus gigantus* formed a well-supported clade with *Z. gigantus* from Japan (B=1.00, MP=100% and ML=98%). The *Palythoa* clade was as in the 16S analysis well supported (B=1.00, MP=100% and ML=89%). The ITS rDNA *Palythoa* sequences grouped into two separate clades for *P. tuberculosa* and *P. nelliae*. *Palythoa nelliae* sequences grouped with *P. mutuki* ITS rDNA, and *P. tuberculosa* sequences from this study belonged to the same clade as *P. tuberculosa* from Japan.



**Figure 7:** Bayesian consensus tree of internal transcribed spacer region of ribosomal DNA (ITS rDNA) for sequences collected in this study. Values on the branches represent posterior probability values, maximum parsimony and maximum-likelihood bootstrap values. Taxa labeled without accession numbers were sequenced in the present study.

***Symbiodinium* ITS-rDNA.** New ITS rDNA sequences were obtained for 79 specimens of *Symbiodinium* (Figure 6). The tree for the clade C sequences is shown in the supplementary material in Figure S1, and the tree for clade A is shown in Figure S2. The ITS rDNA sequences obtained for the *Symbiodinium* spp. did not exhibit multiple peaks and hence the sequences obtained were assumed to be the dominant *Symbiodinium* clade within the zoanthid. The *Symbiodinium* spp. found in this study belonged to three different subclades; C1 *sensu* LaJeunesse (2002) and C1 related, C15/C91 related, and lastly, A1 and A1 related. Sequences often differed by 1-2 bp and these were referred to as C1/A1 and related types. C1 was the most common subclade and was found in all of the *I. tuberculatus*, *P. nelliae* and *P. tuberculosa* specimens and most of the *Z. sansibaricus* specimens. The single *Z. gigantus* specimen that was found also had subclade C1. *Zoanthus natalensis* specimens mostly had subclade A1/A1 related (10 out of the 13 represented in Figure 6) with only two specimens collected in Isipingo having C1/C1 related and one specimen collected in Umgazana having C15/C91 related. *Zoanthus durbanensis* specimens were mostly found associated with subclade C15/C91 related (10 out of 11 represented in Figure 6) where one specimen collected in Umgazana had subclade A1/A1 related.

### 3.4 Discussion

Zoanthid species found at five sites along the east coast of South Africa and at one site on the east coast of Madagascar belonged to six different species; *I. tuberculatus*, *P. nelliae*, *P. tuberculosa*, *Z. durbanensis*, *Z. natalensis* and *Z. sansibaricus*. *Zoanthus gigantus* was only

found at the site in Madagascar. Zoanthids in the genera *Palythoa* and *Zoanthus* have been reported elsewhere as common benthic organisms on coral reefs (Irei *et al.*, 2011), the same can be said for these groups on the east coast of South Africa as species from these groups can be found covering large portions of the rocky shores on the KwaZulu-Natal and Eastern Cape coasts.

#### **3.4.1 Molecular examination of zoanthids**

The mt 16S tree showed one haplotype per monophyly (species group) for the species identified in this study. Another possible synonymy was found in this study with *P. nelliae* and *P. mutuki*, this was found in both the mt 16S rDNA and ITS rDNA trees. Images of *P. mutuki* (Reimer *et al.*, 2006d) are very similar to the morphotypes of *P. nelliae* that have been observed on the South African coastline. *Palythoa mutuki* Haddon and Shackleton, 1891 was described prior to *P. nelliae* Pax, 1935, therefore if this synonymy is confirmed *P. nelliae* would become a junior synonym to *P. mutuki*.

Of interest was that the ITS rDNA for one of the *Z. durbanensis* specimens (UM1-19) had 16S rDNA that matched with *Z. durbanensis*, however the ITS rDNA region was identical to that of *Z. sansibaricus*. Hybridization has been investigated in *Zoanthus* spp. previously (Reimer *et al.*, 2007b), and this result could add further evidence to support this hypothesis. *Zoanthus sansibaricus* ITS rDNA sequences were the same for all *Z. sansibaricus* specimens, and, as seen in Risi and Macdonald (2015), *Z. natalensis* and *Z. durbanensis* specimens formed a single clade of sequences that were very closely related. The *Palythoa* species had more divergent ITS rDNA with both *P. nelliae* and *P. tuberculosa* having several haplotypes of ITS rDNA, however despite the diversity, ITS rDNA sequences still grouped on the same clade as indicated by their species identification for 16S rDNA.

Risi and Macdonald (2015) has shown that *Z. natalensis* Carlgren, 1938 is likely conspecific to *Z. kuroshio* Reimer & Ono, 2006 and *Z. durbanensis* Carlgren, 1938 likely conspecific to

*Z. vietnamensis* Pax & Müller, 1957, this result was found again in this study for support in both mt 16S rDNA sequences and ITS rDNA sequences. It should be noted that the *Z. vietnamensis* from which the sequences were derived was found far (southern Japan) from the actual location in which the species was described (Vietnam), and a type specimen from Vietnam needs to be evaluated.

Of all the species found on the South African coastline, *I. tuberculatus* was the rarest and often only one or two patches were found per sampling site, if at all. *Isaurus tuberculatus* was also found to be rare in the Ryukyus (Reimer *et al.*, 2011a). *Zoanthus gigantus* was not found at any sites in South Africa, but the distribution of this species must be confirmed after sampling at depth.

#### **3.4.2. *Symbiodinium* ITS rDNA**

This study was the first to identify the *Symbiodinium* subclades found in South African zoanthids and the results were very interesting. In Reimer and Todd (2009) it is shown that *Z. vietnamensis* (proposed conspecific to *Z. durbanensis*) was also found to be predominately associated with *Symbiodinium* from subclade C15/C91. In Reimer *et al.* (2011a) only one of the four *Z. kuroshio* (proposed conspecific to *Z. natalensis*) specimens had a *Symbiodinium* from subclade A1, and this was the first time that *Z. kuroshio* had ever been recorded with *Symbiodinium* from this subclade. Whereas, in this study the majority of *Z. natalensis* had *Symbiodinium* from subclade A1. *Isaurus* spp. from Japan (Reimer *et al.*, 2008b) and Cape Verde Islands (Reimer *et al.*, 2010a) found that *Isaurus* species were associated with subclade C1, the same was found for *I. tuberculatus* in this study. The results from studies in the western Pacific Ocean (Reimer *et al.*, 2011a; Reimer *et al.*, 2013a) show that *Palythoa* species; *P. mutuki* and *P. tuberculosa* specimens were all found with a *Symbiodinium* subclade C1, C1 related or C3. However Burnett (2002) and Reimer and Todd (2009) found that *Palythoa* sp. were found with clade D in the Indian Ocean. In this study however, both *P.*

*tuberculosa* and *P. nelliae* (proposed synonym to *P. mutuki*) had all specimens containing *Symbiodinium* belonging to clade C, and no clade D was found in any of the specimens.

*Zoanthus sansibaricus*, was also found with the generalist subclade C1, as in Reimer *et al.* (2011a). The trend that most zoanthid species tend to be found with the same *Symbiodinium* subclade is also found in the South African/ Madagascan zoanthids and therefore *Symbiodinium* typing could be a potentially useful tool in identifying zoanthids. Species that have proposed conspecifics are found with the same *Symbiodinium* subclade even though they are separated by huge geographical distances and exchange of genetic material is likely minimal. The different physiologies of the *Symbiodinium* clades has been suspected to have an effect on the distribution as environmental factors such as light intensity and temperature could have an effect on distribution (Rodriguez-Lanetty *et al.*, 2001; Reimer *et al.*, 2006b; Kamezaki *et al.*, 2013).

This is however conflicting with results as *Z. vietnamensis* which is generally found in shallow waters and influenced by sea surface temperature (Reimer *et al.*, 2006c), has been found at a number of locations ranging from Singapore (Reimer and Todd, 2009) to Japan (Reimer *et al.*, 2007d) and *Z. durbanensis* in South Africa and Madagascar, and are all associated with subclade C15/91 despite differences in light intensity, and temperature. It has been proposed that clade A is better adapted to high levels of UV light (LaJeunesse, 2002), and *Z. natalensis* is often found higher up on the shore than other *Zoanthus* species (Branch *et al.*, 2008). However even though the majority of *Z. natalensis* specimens were found with subclade A1, this has not been reported for *Z. kuroshio*, and other zoanthid species (*P. nelliae*) found at the same height up the shore as *Z. natalensis*. Therefore the underlying factors, including host related factors, contributing to symbiont specificity appear to be complicated.

### 3.4.3. Conclusion

Of all seven species examined, three were found to have synonyms and none were unique to this region. Despite the anticipation of endemic species or zoanthid species that had never been identified before using molecular tools, all species identified in this study belonged to a haplotype group that contained a previously reported GenBank sequence in the mt 16S tree. Four of the species found in this study are widespread in the Indo-Pacific and were even found in isolated locations such as the Galapagos (Reimer and Hickman, 2009), namely *P. tuberculosa*, *P. mutuki* (*P. nelliae*), *Z. kuroshio* (*Z. natalensis*) and *Z. sansibaricus* (Reimer *et al.*, 2011a). This study highlights the importance of molecular examinations in previously understudied regions, and the likelihood of finding species that have been already been described elsewhere has again been proven.

## Chapter 4: General conclusions including recommendations for future work

The taxonomy of zoanthids has been in a chaotic state, however, studies such as this bring to light the importance of molecular work for the revision of zoanthid taxonomy and the apparent number of synonymies in previously unstudied regions. Although often excluded from studies in South Africa, the presence of zoanthids on our coastline should not be ignored and this study was sorely needed and long overdue. Carlgren (1938) described 27 zoanthid species along the South African coastline although less than 10 had been included in South African field guides (Branch *et al.*, 2008). Therefore a molecular examination of all South African zoanthids was necessary, and it was conducted on three *Zoanthus* species, followed by a study on all rocky shore zoanthids collected.

The results from the study on *Zoanthus* spp. (Chapter 2) demonstrated that there are differences between *Z. sansibaricus*, *Z. natalensis* and *Z. durbanensis*, although *Z. natalensis* and *Z. durbanensis* are very closely related. In all three of the markers utilized (COI, mt 16S rDNA and ITS rDNA), the *Z. sansibaricus* specimens in this study matched with individuals of this species sampled elsewhere. In the case of the mt 16S rDNA; *Z. natalensis* formed a monophyly with *Z. kuroshio* and *Z. durbanensis* formed a monophyly with *Z. vietnamensis*. For ITS rDNA sequences, these four species had extremely similar sequences and grouped together in a well-supported clade. More importantly, the primers that were used for the ITS rDNA region did not generate amplicons for *Z. natalensis*, as previously seen for *Z. kuroshio* (Reimer *et al.*, 2007b). Therefore primers were designed for this study by using ITS rDNA sequences that had been uploaded to GenBank for *Z. kuroshio*. This study indicates that *Z. natalensis* is likely conspecific to *Z. kuroshio* and *Z. durbanensis* is likely a conspecific to *Z. vietnamensis*. In Burnett *et al.* (1997), it was alluded to the fact that there is likely an

overestimation in *Zoanthus* species numbers. In this study only three species were analysed and two of the three were found likely to be synonymies of species from other regions.

In the examinations of all zoanthids found along the east coast of South Africa and at one site in Madagascar (Chapter 3), COI was not included as this marker was found to be conservative, and for the first time the ITS rDNA region of *Symbiodinium* was amplified for South African zoanthids. Six zoanthid species were found along the east coast of South Africa and an additional species, *Z. gigantus*, was found only in Madagascar. No endemic species were identified. This study revealed a potential synonymy with *P. nelliae* and *P. mutuki*. Three of the seven species found (*Z. natalensis*, *Z. durbanensis* and *P. nelliae*) have proposed synonymies. With regards to the large number of zoanthid species reported in Carlgren (1938), it would therefore appear that most of them are likely to be conspecifics as the zoanthid diversity we found was lower than projected in his descriptions.

The ITS rDNA region for *Symbiodinium* spp. could potentially be used as a tool in zoanthid species identification as one species generally hosted only one particular subclade. *Zoanthus natalensis* was generally found with subclade A1 (10 out of 13 specimens), and *Z. durbanensis* was generally found with subclade C15/C91 (10 out of 11 specimens). The generalist subclade C1 *sensu* LaJeunesse (2002) was found with *P. nelliae*, *P. tuberculosa*, *I. tuberculatus* and most of the *Z. sansibaricus* specimens. The zoanthid species were generally found with the same subclade as their conspecifics elsewhere, except that *Z. kuroshio* had only been previously recorded with subclade A1 once before (Reimer *et al.*, 2011a).

The results of this study are important for highlighting the possibility that many zoanthid species that are currently described are likely to have synonymies with other species found in other locations. Therefore it is important that molecular examinations are carried out initially when describing new species so not as to create another synonym, and that the original species descriptions are referred to. The results of this study have further served to clarify the

taxonomy of zoanths and will hopefully encourage future endeavours in zoanthid work such as population genetic studies to understand the connectivity of populations. Further work needs to include histological analysis of *Z. natalensis*, *Z. durbanensis* and *P. nelliae* to compare diagnostic features with their conspecifics, and sampling with SCUBA, which may lead to more species being found in southern Africa. Zoanths from the *Zoanthus* and *Palythoa* genera have both been seen diving off the South African coastline. Potential changes in *Symbiodinium* subclades would be interesting to study over a depth gradient as light intensity may have an effect on *Symbiodinium* association (Kamezaki *et al.*, 2013).

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<sup>1</sup> The referencing style of this thesis follows that of Systematics and Biodiversity.

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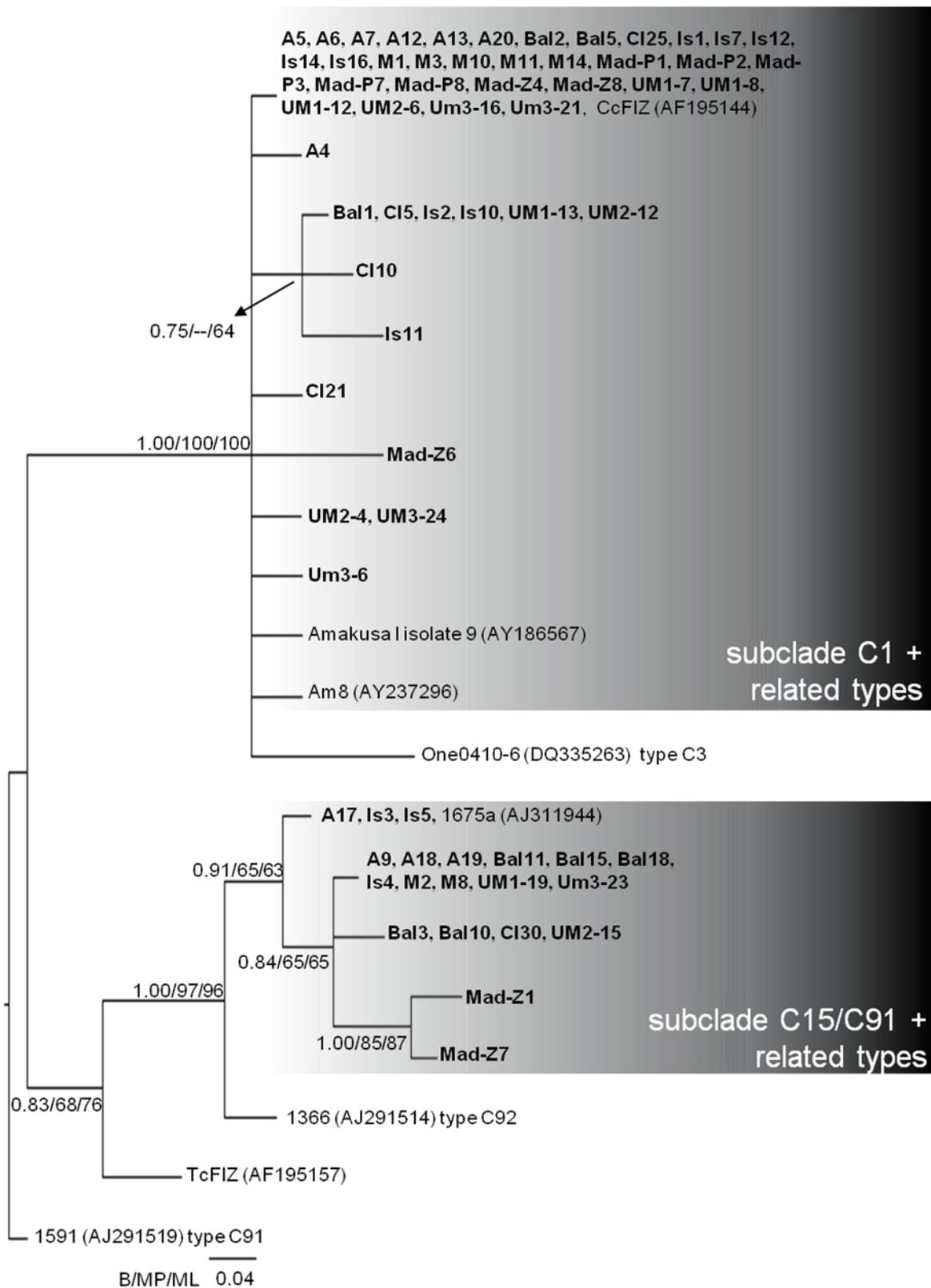
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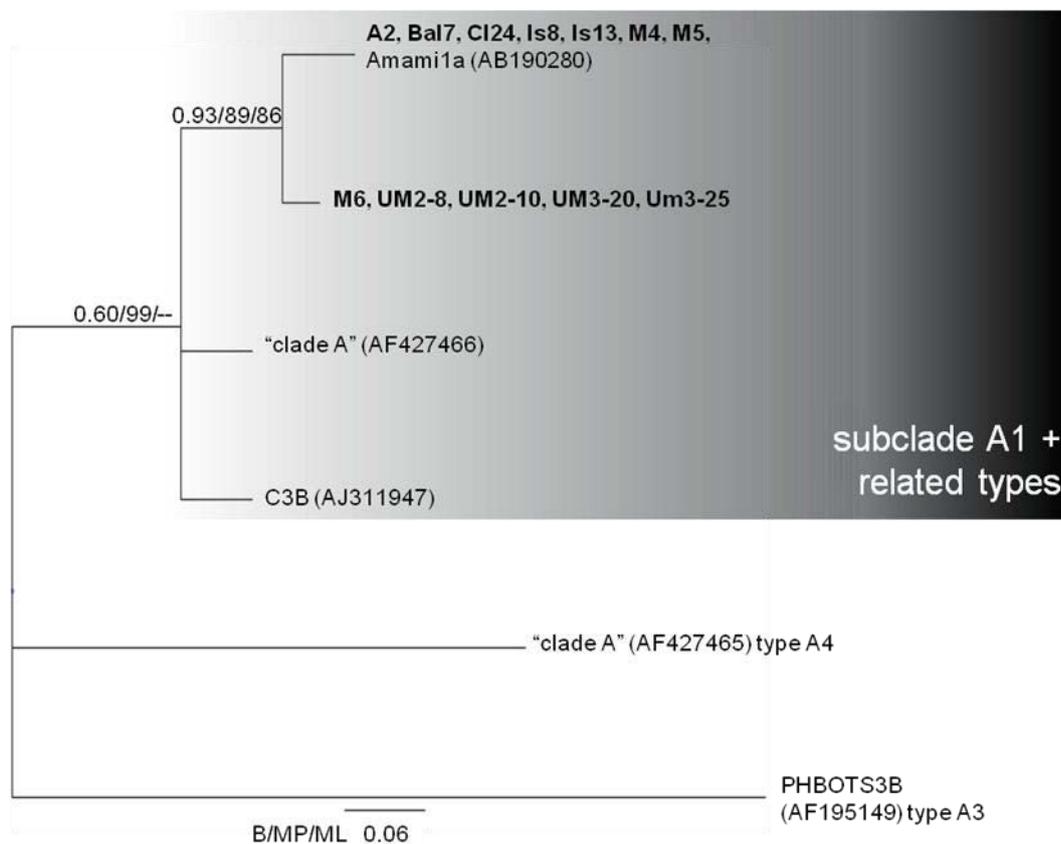
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## Supplementary material



**Figure S1:** Bayesian consensus tree of the internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences for *Symbiodinium* including sequences from this study for clade C. Values on the branches represent posterior probability values, maximum parsimony and maximum-likelihood bootstrap values. Taxa labeled without accession numbers were sequenced in the present study. For specimen information see Table 3.



**Figure S2:** Bayesian consensus tree of the internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences for *Symbiodinium* including sequences from this study for clade A. Values at branches represent posterior probability values, maximum parsimony and maximum-likelihood bootstrap values. Taxa labeled without accession numbers were sequenced in the present study. For specimen information see Table 3.