Utilization of two DNA barcoding techniques, alongside morphological characters, confirms the presence of zoantharian morphotypes (Cnidaria: Hexacorallia: Zoantharia) along the coast of Toco, Trinidad

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ABSTRACT

Many marine invertebrates on coral reefs have cryptic morphological features making them challenging to accurately identify. One such group are the zoantharians, benthic anemone-like organisms found globally and throughout coral reefs in the Caribbean Sea. In past studies, zoantharians surveyed on reefs at Toco, Trinidad, have been visually identified as belonging to genus *Palythoa* and *Zoanthus* spp. This study explored the use of morphology and two DNA barcoding approaches to further classify Toco's zoantharians. Morphological polyp characteristics, including oral disk diameter, tentacle counts, colours, and coenenchyma development were recorded *in situ*. Additionally, mitochondrial 16S ribosomal DNA (mt 16S rDNA) and the mitochondrial cytochrome oxidase subunit I (mt COI) genes of these same zoantharians were amplified using polymerase chain reaction (PCR), then sequenced. Results from mt 16S rDNA and mt COI gene sequences identified four zoantharians: *Z. pulchellus*, *Z. sociatus*, *P. grandiflora*, and for the first time *Z.* aff. *pulchellus*. All new sequences were deposited online at the National Center for Biotechnology Information (NCBI), and GenBank accession numbers were assigned to specimens. Oral disk diameters were smaller (4-10 mm) for *Z. sociatus* than *Z. pulchellus* (4-12 mm), with tentacle counts also following a similar pattern. Oral disk and tentacle colours were variable from blue, green, brown, pink, and orange. Oral disk diameter and tentacle count for *P. grandiflora* were larger and numerous than observed in *P. caribaeorum.* Additionally, coenenchyme growth form easily distinguished these two species. The comparison between morphological features, including oral disk diameter, tentacle count, and coenenchyme development, together with molecular identification using the barcoding technique, characterised zoantharian diversity more accurately than past studies at these sites, and underlines the urgent need for continued work on marine invertebrate species identification in this region.

Key words: barcoding, coral reefs, Palythoa, Zoantharia, Zoantharian, Zoanthus.

INTRODUCTION

Zoantharians (Anthozoa: Hexacorallia: Zoantharia) are found in most marine environments globally, ranging from temperate to tropical areas, and distributed from intertidal zones to the deep sea (Ryland & Lancaster 2003). Zooxanthellate zoantharian species are common colonial cnidarians found in most intertidal tropical and sub-tropical rocky shores, forming dense mats, and sometimes covering rocky substrates throughout the benthic zones of coral reefs (Karlson 1980; Bastidas & Bone 1996; Belford & Phillip 2011; 2012; Belford 2021). These colonial anthozoans are ecologically important as they provide benthic shelter to a variety of invertebrates, such as crustaceans, molluscs, polychaetes, and nudibranchs on reefs (Pérez *et al*. 2005).

Zoantharians frequently cover extensive areas on coral reefs and rocky shorelines (Bastidas and Bone 1996). For instance, López *et al*. (2018) reported a "zoanthid zone" located at Cabo Verde, central eastern Atlantic. In the Caribbean, past research recorded extensive coverage of zoantharians, such as *Palythoa* and *Zoanthus* spp. on coral reefs in Jamaica, Trinidad, Curaçao, and further south in Brazil (Karlson 1981; Belford & Phillip 2011; 2012; Rabelo *et al*. 2015; Reimer *et al*. 2018; Belford 2021). While zoantharians have a wide distribution across the Atlantic and Indo-Pacific regions, they display high intraspecific variation in morphology, thus making them difficult to identify to species level *in situ* (Burnett *et al*. 1997). However, molecular analyses using biomarkers are increasingly being used to clarify taxonomic issues with zoantharians (Reimer *et al.* 2004; Reimer *et al*. 2012; Jaramillo *et al*. 2018

The northeastern coast of Trinidad from the Toco Fishing Depot at Grande L'Anse (10°50.107′N, 60°56.772′W) to the Toco Lighthouse (10°29.37′N, 61°04.14′W) has a rich marine biodiversity. Belford *et al*. (2019) recorded approximately 257 marine species belonging to 134 families, 23 classes, and 11 phyla. Forty-three species of Cnidaria belonging to 9 families were identified in these surveys: Milleporidae, Stomolophidae, Actiniidae, Stichodactylidae, Mussidae, Poritidae, Siderastreidae, Sphenopidae, and Zoanthidae (Belford & Phillip 2011; Belford *et al*. 2019; Belford 2020). They are common, found in variable abundance, and occupy intertidal benthic marine communities along the northeastern coast of Toco, Trinidad. A few of these species

are challenging to identify visually and may benefit from the application of molecular techniques.

From visual observations, Toco's zoantharians were initially identified as belonging to two genera: *Palythoa* and *Zoanthus* spp. However, molecular analyses are required to identify some zoantharians to species level. Belford and Phillip (2011; 2012) further visually recorded two species: *Palythoa caribaeorum* (Duchassaing and Michelotti, 1860) and *Zoanthus sociatus* (Ellis, 1768), during surveys for reefs at Toco, but until recently no information was available for the presence or abundance of other zoantharian species, such as *Palythoa grandiflora* (Verrill, 1900), or *Zoanthus pulchellus* (Duchassaing and Michelotti, 1860), which are challenging to identify yet might be expected to be present.

DNA barcoding has been highly publicized as a method for species identification in biodiversity studies. This technique uses standardized molecular markers that offer a way to use genetic information where morphological analysis is insufficient (Herbert *et al*. 2003; Ngwakum *et al*. 2021). The use of molecular markers has only recently begun to be applied to zoantharians in Trinidad (Belford 2021). Although mitochondrial cytochrome oxidase subunit I (mt COI) gene sequences can be used to identify species, it is impossible to use the COI gene marker alone to identify anthozoans (Shearer & Coffroth 2008). However, when combined with the large mitochondrial ribosomal subunit (16S rDNA) gene marker, different zoantharians may be identifiable (Sinniger *et al*. 2008).

Although initial mt COI data from Belford (2021) successfully identified *Zoanthus pulchellus* and *Zoanthus sociatus*, more specimens with variable morphological characteristics have since been observed *in situ*. This suggests the need to continue using both morphological characteristics and molecular markers for zoantharian identification in understudied regions (Koupaei *et al*. 2018; Reimer *et al*. 2018) including the southern-most region of the Caribbean Sea and NE Trinidad.

The aim of this study is to accurately identify zoantharians in the reefs of NE Trindad using two different molecular approaches, and to match these identifications with morphological data from photographs *in situ*, with the goal of expanding our understanding of local zoantharian diversity.

METHODS

Sample collection and analysis

Polyps were collected from a total of 30 zoantharian colonies at sites along the north-eastern coast of Trinidad: from the Toco Fishing Depot at Grande L'Anse (10°50.107′N, 60°56.772′W) to the Toco Lighthouse (10°29.37′N, 61°04.14′W) from January 2022 to February 2023.

The specific sites sampled were Grande L'Anse (GA) (19 colonies), and Salybia Bay (SB) (2 colonies), Pequelle Bay (ESB) (4 colonies), and the western end of Salybia Bay (WSB) (5 colonies) (see Fig. 1) during extreme low tide (dry tide: < 0.3 metres).

Oral disk diameter (mm), tentacle count, oral disk colour, tentacle colour and coencenchyme development (yes/no) were recorded for each colony. A total of 3-5 polyps per colony were excised using a hand-held scapula for genetic analyses: mitochondrial cytochrome oxidase subunit I (mt COI) and mitochondrial large ribosomal subunit (mt 16S) (3 polyps). Specimens were placed in 1.5 ml vials containing 95% ethanol, then stored in a freezer at -20°C. Hand-held calipers measured oral disk diameter. Polyps were photographed using a digital camera (Nikon Coolpix S5200) with underwater housing, then later re-examined for comparison with the results from molecular analyses.

DNA extraction, PCR amplification and Sequencing

For molecular analyses, DNA was extracted from 1-2 polyps using an E.Z.N.A. tissue extraction kit (Omega-BioTek Model no. D 3396-01 Norcross, GA, USA) following the manufacturer's protocol. The mitochondrial (mt) 16S rDNA was amplified using primers:16Sant 1a 5′-GCC ATG AGT ATA GAC GCA CA 3′, 16Sbm0H 5′-CGA ACA GCC AAC CCT TGG 3′ (see Sinniger *et al*. 2005) and the cytochrome oxidase subunit I (COI) primers: LCOant 5′-TTT TCY ACT AAT CAT AAA GAT AT 3′, COIantr 5′-GCC CAC ACA ATA AAG CCC AAT AYY CCA AT 3′ (Folmer *et al*. 1994). Polymerase chain reaction (PCR) amplifications for 16S rDNA were performed under the following thermal cycler (T-100, Bio-Rad) conditions: 2 min at 94°C; 30 sec at 94°C, 1 min at 52C, 90 sec at 72°C (35 cycles); followed by a final extension for 7 min at 72°C (Koupaei *et al*. 2018). Thermal cycler protocol for COI was 3 min at 94°C; 30 sec at 94°C, 1 min at 52°C, 2 min at 72C (40 cycles); followed by a final extension for 5 min at 72C. PCR were performed in volumes of 10 µl containing 5.0 µl DreamTaq master mix (includes DNA polymerase, dNTPs, KCl, $MgCl_2$, Thermo Scientific), 2.0 µl nuclease-free water (Thermo Scientific), 1.0 µl of each primer (Invitrogen), and 1.0 µl DNA template. A total of 3.0 µl PCR aliquot was mixed with 1.0 µl loading dye (Bio-Rad), and the quality of amplicons were analyzed by gel electrophoresis in 1.0% agarose gel. Run time for gel electrophoresis was 30 mins. at 100 Volts. Visual bands were checked using a gel imaging system (Azure c200, Azure Biosystems), and only bright bands were enzymatically purified with ExoSap-IT (ThermoFisher Scientific, Santa Clara, CA, USA) using 1.0 µl Exosap-IT per 2.5 µl PCR aliquot, then sent for sequencing at Eurofins Genomics (Kentucky, USA). Samples were sequenced in both directions using Sanger sequencing technology on an ABI 3730 analyzer.

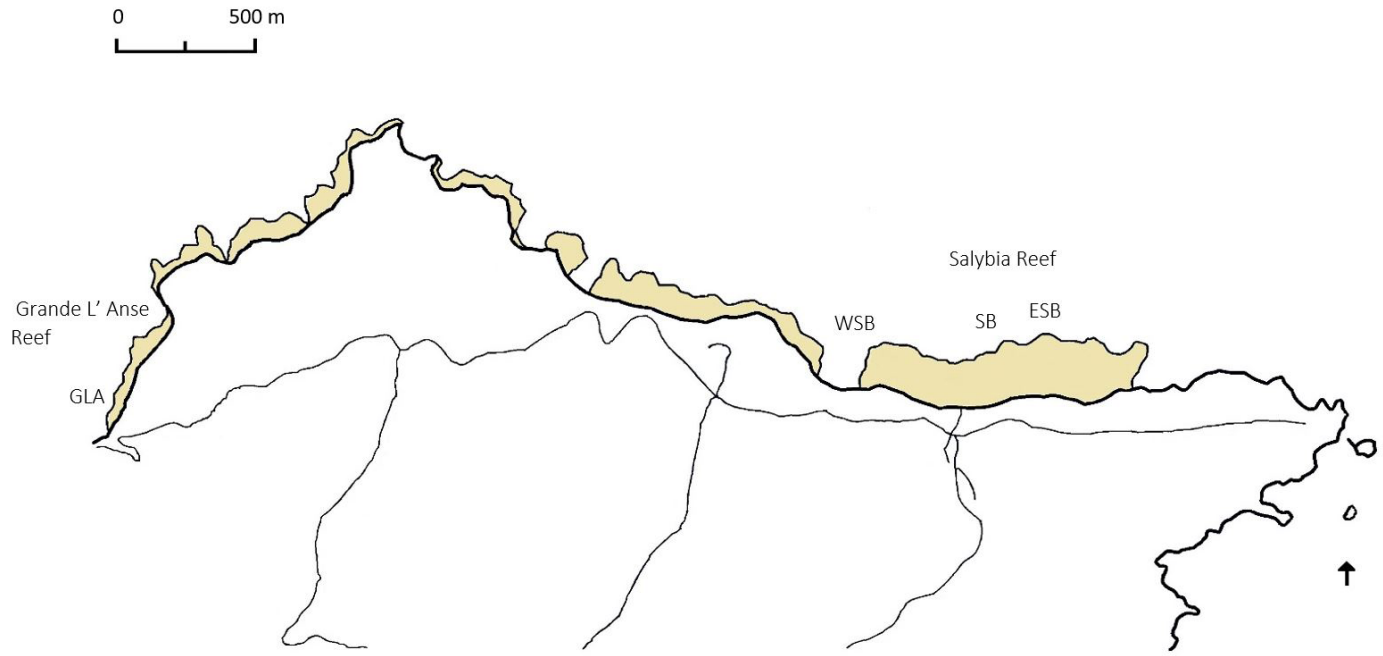

Fig. 1. Location of sample sites at reefs along the north eastern coast of Toco, Trinidad. Key: Salybia Bay (SB), Pequelle Bay (ESB), western Salybia Bay (WSB), and Grande L'Anse (GLA). Map adapted from Belford *et al*. 2019.

Chromatograms were inspected by eye for the presence of multiple "messy" peaks indicative of poor sequence quality using Molecular Evolutionary Genetics Analysis (MEGA X, version 7.0) (Kumar *et al*. 2018). Primer sequences were removed from each end of sample sequences. Basic Local Alignment Search Tool (BLAST) compared sequences with parameters: query cover 99-100%, E value ≤ 0.01, identity ≥ 98%. Both forward and reverse sequences were separately aligned in MEGA X software using ClustalW for phylogenetic analysis. Sequences for mt 16S and COI genes used in this study were blasted against NCBI Genbank collections for comparison and identification, then deposited in GenBank with associated accession numbers allocated to each specimen (Table 1).

RESULTS

PCR and phylogenetic analyses.

DNA was successfully amplified for all 36 samples at both loci. Sequences were obtained for 36 mt COI and 34 mt 16S PCR amplicons. Analyses of carefully inspected high quality amplicons confirmed mt COI (774-800 bp) and mt 16S (722-925 bp) gene sequence lengths, with no chromatograms showing multiple peaks, or unclear sequences. The Basic Local Alignment Search Tool (BLAST) was used to compare sequences from this study to sequences from known species with identities accepted >98%. Results from blasting these sequences identified three *Zoanthus* spp: *Zoanthus pulchellus*, *Zoanthus sociatus* and *Zoanthus* aff. *pulchellus* (Table 1). Maximum likelihood tree for mt COI sequences separated *Z. sociatus* and *Z. pulchellus* (Fig. 2) into two genus level clades for family Zoanthidae. *Z. pulchellus* was moderately supported by phylogenetic analyses (ML: MP >64%), whereas *Z. sociatus* was well supported (ML: MP >98%).

Sequences matched >98% with samples of *Z. sociatus* from Florida (Accession number: JX119154, see Reimer *et al*. 2012), with past studies from specimens at similar sites (SB and WSB), from different sites such as Straight Bay and Toco Lighthouse (see Belford 2021) (Accession numbers: MZ150807, MZ180806, MZ147096, MZ147095), and for *Z. pulchellus* (Accession numbers: MZ150805, MZ147093, MZ147094, OL310195). For mt COI, *Z. pulchellus* (n = 16) and *Z. sociatus* (n = 13) differed by 7 bp using sequences of 793 bp lengths, while *Z.* aff. *pulchellus* (n = 5) differed by 1 bp from *Z. pulchellus*. For mt COI, only *Palythoa grandiflora* (n = 2) was matched with a specimen from Florida (Accession number: JX119165, see Reimer *et al*. 2012). *Palythoa caribaeorum* was only identified using morphological characteristics, and molecular analyses proved to be unsuccessful with the primers used in this study.

For the 34 mt 16S gene marker, 16 sequences identified as *Z. pulchellus*, 13 as *Z. sociatus*, 5 as *Z.* aff. *pulchellus*, and 2 specimens as *P. grandiflora* (Table 1). Blasting results showed four clades well supported by phylogenetic analysis (ML >78%; MP >85%). As shown in Fig. 3, *Z.* aff. *pulchellus* is also well supported (ML:MP 100%).

Table 1. Sample sites (WSB-Western Salybia Bay, ESB- Eastern Salybia Bay, GLA- Grande L'Anse), morphological characteristics (oral disk and tentacle colours), assigned GenBank accession numbers, and molecular identification of each specimen.

Specimen	Oral Disk/Tentacle Colour	COI accession No.	mt 16SrDNA accession No.	Molecular ID
Z44-brgr-WSB	bright green/green	OM982833	OR346866	*Zoanthus pulchellus*
Z50-blu-WSB	blue-green/green	OM982834	OR346867	*Zoanthus sociatus*
Z51-blu-WSB	blue-green/green	OM982835	OR346868	*Zoanthus sociatus*
Z53-gr-WSB	green/green	OM982836	OR346869	*Zoanthus pulchellus*
Z58-blu-WSB	blue-green/green	OM982837	OR351948	*Zoanthus sociatus*
Z61-brgr-ESB	bright green/green	OR346698	OP538560	*Zoanthus pulchellus*
Z65-gr-ESB	green/green	OQ349512	OP538562	*Zoanthus pulchellus*
Z77-gr-SB	bright blue/green	OR346699	OP538557	*Zoanthus* aff. *pulchellus*
Z78-blu-SB	bright blue/green	OR346700	OP538561	*Zoanthus sociatus*
Z84-blgr-GLA	blue-green/green	ON773133	OR351949	*Zoanthus sociatus*
Z85-gr-GLA	green/green	ON773134	ON841655	*Zoanthus* aff. *pulchellus*
Z88-gr-GLA	green/green	OR346702	OP538559	*Zoanthus* aff. *pulchellus*
Z88-br-GLA	brown/brown	OR346701	OP538558	*Zoanthus pulchellus*
Z89-bro-GLA	brown/brown	ON773135	OQ629087	*Zoanthus pulchellus*
Z91-brgr-GLA	bright green/green	ON773136	ON841654	*Zoanthus pulchellus*
Z95-org-GLA	orange/orange	ON773137	OR34670	*Zoanthus pulchellus*
Z96-gr-GLA	green/green	ON773138	ON841656	*Zoanthus sociatus*
Z97-org-GLA	brown/orange	ON773139	OQ629088	*Zoanthus pulchellus*
Z98-gr-GLA	apple-green/green	OR364699	OP538563	*Zoanthus sociatus*
Z100-brgr-GLA	bright green/green	ON773140	OR351950	*Zoanthus pulchellus*
Z102-pink-GLA	pink/green	OR346703	OP484712	*Zoanthus sociatus*
Z105-br-GLA	brown/brown	OP471619	OP484713	*Zoanthus pulchellus*
Z108-brgr-GLA	bright green/green	OP471620	OP484714	*Zoanthus pulchellus*
Z109-brgr-GLA	bright green/green	OP471621	OP484715	*Zoanthus pulchellus*
Z114-blu-GLA	blue-green/green	OP471622	OP484716	*Zoanthus sociatus*
Z115-blu-GLA	blue-green/green	OP471623	OP484717	*Zoanthus sociatus*
Z117-grbr-GLA	green/brown	OQ349511	OP484718	*Zoanthus pulchellus*
Z128-grst-GLA	blue-green/green	OP471624	OP484719	*Zoanthus sociatus*
Z129-grst-GLA	blue-green/green	OP471625	OP484720	*Zoanthus sociatus*
Z130-br-GLA	brown/brown	OR346704	OQ603386	*Zoanthus pulchellus*
Z131-br-GLA	brown/brown	OR346705	OQ603387	*Zoanthus pulchellus*
Z140-grbr-ESB	green/brown	OQ589713	OQ629089	*Zoanthus* aff. *pulchellus*
Z142-grbr-ESB	green/brown	OQ589715	OQ629090	*Zoanthus* aff. *pulchellus*
Z145-apple-SB	apple-green/green	OQ589715	OR351951	*Zoanthus sociatus*
Pa30-gr-GLA	green/brown	OR364697	OR391931	*Palythoa grandiflora*
Pa39-grbr-SB	green/brown	OR364698	OR391934	*Palythoa grandiflora*

Morphological analysis

Preliminary morphological identifications regarding families Zoanthidae (*Zoanthus* spp.) and Sphenopidae (*Palythoa* spp.) were identified using the field guide by Humann *et al*. (2013). These observations showed the presence of *Zoanthus* spp. at all four sites with wide variation in colour (Fig. 4), specifically in oral disk and tentacle colour (Table 1). *Palythoa* spp. were observed at two sites: GLA and SB.

Zoanthus pulchellus and *Z. sociatus* were partly distinguished by their oral disk and tentacle counts. For instance, *Z. pulchellus* (n = 19) had larger oral disk diameter and higher tentacle counts ranging from 4-12 mm and 40-60 respectively, compared to *Z. sociatus* (n = 14) with oral disks ranging from 4-10 mm and tentacle counts between 40-50 (Table 2). Further, *Z. sociatus'* smaller oral disk diameter with fluorescent green middle surrounded by either blue or green colours separated this species from *Z. pulchellus* (Fig. 4). Both *Z. pulchellus* and *Z. sociatus* had polyps clear and free from a developed coenenchyme, however *Z.* aff. *pulchellus* (n = 4) had polyps embedded in a well-developed coenenchyme, hence separating this morphotype from the others. *Z. pulchellus* had the most variable oral disk colours ranging from green, brown, orange, and grey at all sites. *Palythoa caribaeorum* was identified by its characteristic brown oral disks and tentacles. Oral disk diameter ranged between 8-11 mm with tentacle counts between 26-36, embedded in a well-developed coenenchyma. *P. grandiflora* was identified by its larger oral disk diameter, which ranged between 11-13 mm with green oral disk colour and brown tentacles (40-46). Coenenchyma growth form was absent.

DISCUSSION

This study sought to build on the findings of a previous study to confirm the presence of different species of zoantharian in the genera *Zoanthus* and *Palythoa* spp. along Trinidad's north-east coast using a combination of morphological characters and molecular techniques. While the diversity of these taxonomically challenging genus has been previously explored using morphology and mt COI (Reimer *et al*. 2006b; Belford 2021), with two species detected (*Z. sociatus* and *Z. puchellus*), by combining these approaches with 16S markers for the first time, the additional presence of a third morphotype, *Z.* aff. *pulchellus,* is revealed at three of the four locations surveyed. Additionally, *Palythoa grandiflora* and *P. caribaeorum* identification is confirmed for 2 sites: GLA and SB. Both morphological and molecular analyses played crucial roles in confidently identifying zoantharians down to the species level along the coastline of northeast Trinidad, thus laying the groundwork for future studies on zoantharian biodiversity in this area.

Molecular Techniques

This is the first report on zoantharian molecular identification using the mitochondrial 16S rDNA gene marker in conjunction with mitochondrial cytochrome oxidase subunit (mt COI) gene for this region, which now confirms *Z.* aff. *pulchellus* as an additional zoantharian found along the benthic marine habitat of the north eastern coast of Trinidad. This is important in documenting accurate diversity and distribution of these marine colonial anthozoans, which are widespread and provide vital benthic habitat for other reef species. Together, the mt COI and 16S markers appear to be sufficient to identify zoantharians to the species level (Reimer *et al*. 2006b), hence their combined use is recommended for future genetic analyses of samples in this area. Results from these analyses will be used to conduct future surveys to accurately document zoantharian species abundance and distribution along the northeastern coast of Trinidad. While the mt COI is useful on its own, since the mt 16S gene marker gives more variable sequences, it provides additional information for this problematic taxon (Sinniger *et al*. 2008; Reimer *et al*. 2012).

Morphological Characters

Based on morphological characteristics, five zoantharian morphotypes were successfully separated using oral disk diameter and colour, tentacle count, and the presence or absence of a well-developed coenenchyme.

Although some zoantharians are difficult to accurately identify due to large amounts of phenotypic plasticity (Reimer *et al*. 2004; Ong *et al*. 2013), López *et al*. (2018) used morphological characteristics to separate *Palythoa* and *Zoanthus* spp. in the Atlantic Ocean. In fact, Lopez

Table 2. Summary of zoantharian species morphological characteristics.

Species	Oral disk diameter (mm)	Tentacle count	Locations	Well-developed coenenchyme
Zoanthus sociatus	4-10	40-50	GLA ESB SB WSB	No
Z. pulchellus	4-12	40-60	GLA ESB SB WSB	No
Z. aff. *Pulchellus*	4-12	40-60	GLA ESB SB	Yes
Palythoa caribaeorum	8-11	26-36	GLA SB	Yes
P. grandiflora	11-13	40-46	GLA SB	No

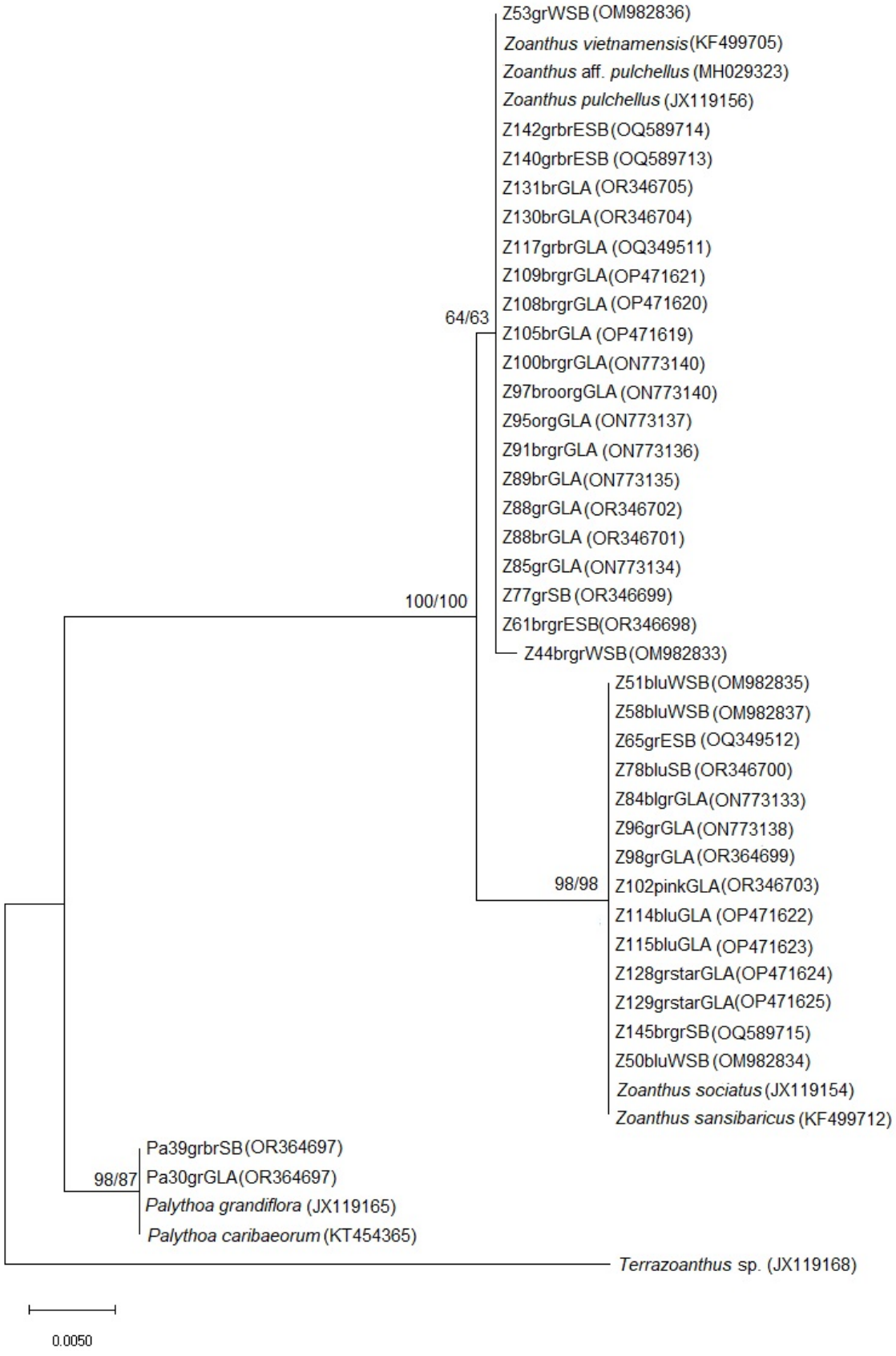

Fig. 2. Maximum-likelihood (ML) tree based on an alignment (793-800 bp long) of mitochondrial cytochrome oxidase subunit I sequences. Numbers above branches represent maximum likelihood and maximum parsimony bootstraps respectively. Specimen identification with GenBank accession numbers (in parentheses) are included. Scale shows substitution per site.

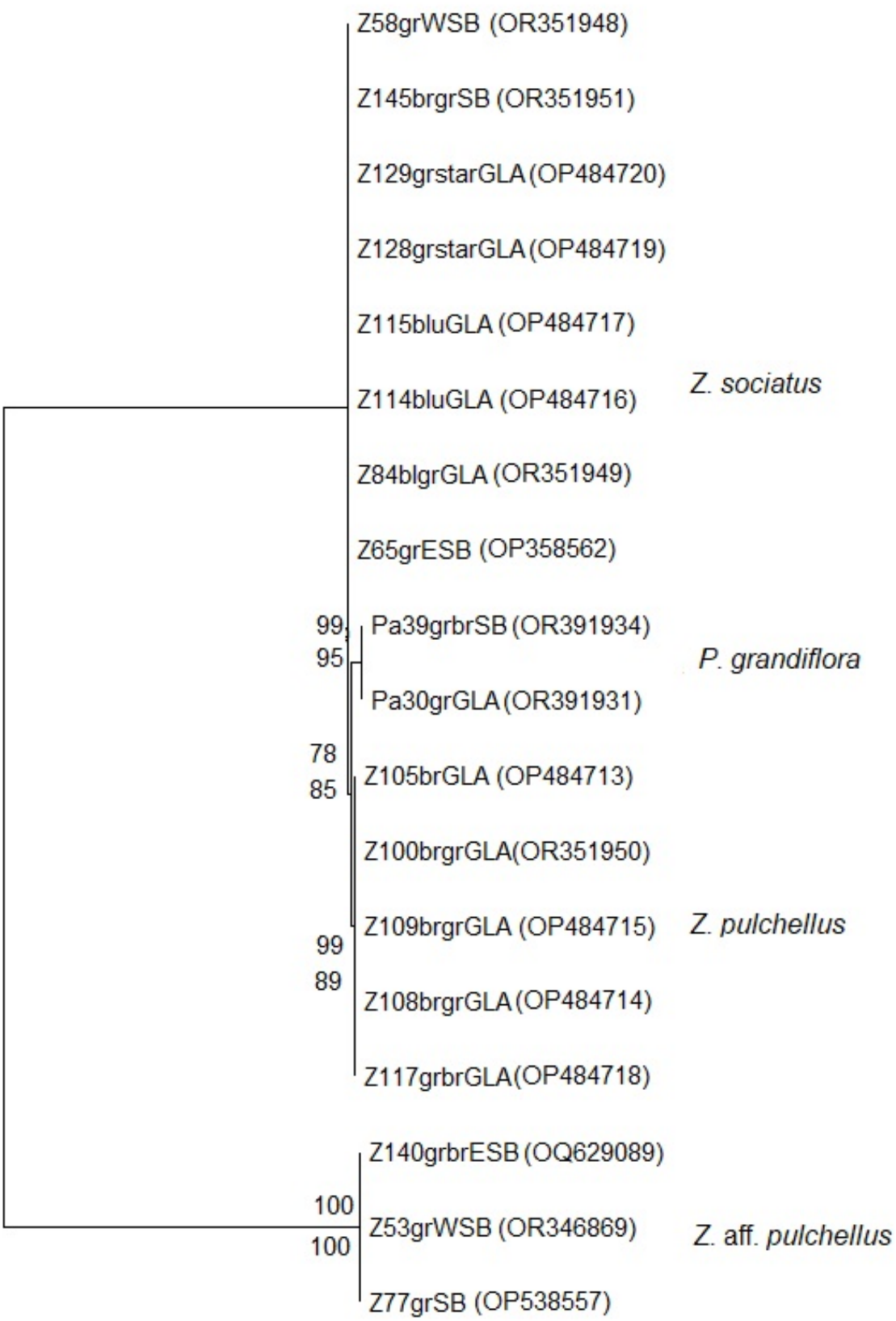

Fig. 3. Maximum-likelihood (ML) tree based on an alignment (884-923 bp long) of mitochondrial 16S rDNA sequences. Values at branches represent maximum likelihood and maximum parsimony bootstrap percentages from 1000 trees respectively. Specimen identification with GenBank accession numbers in parentheses are included.

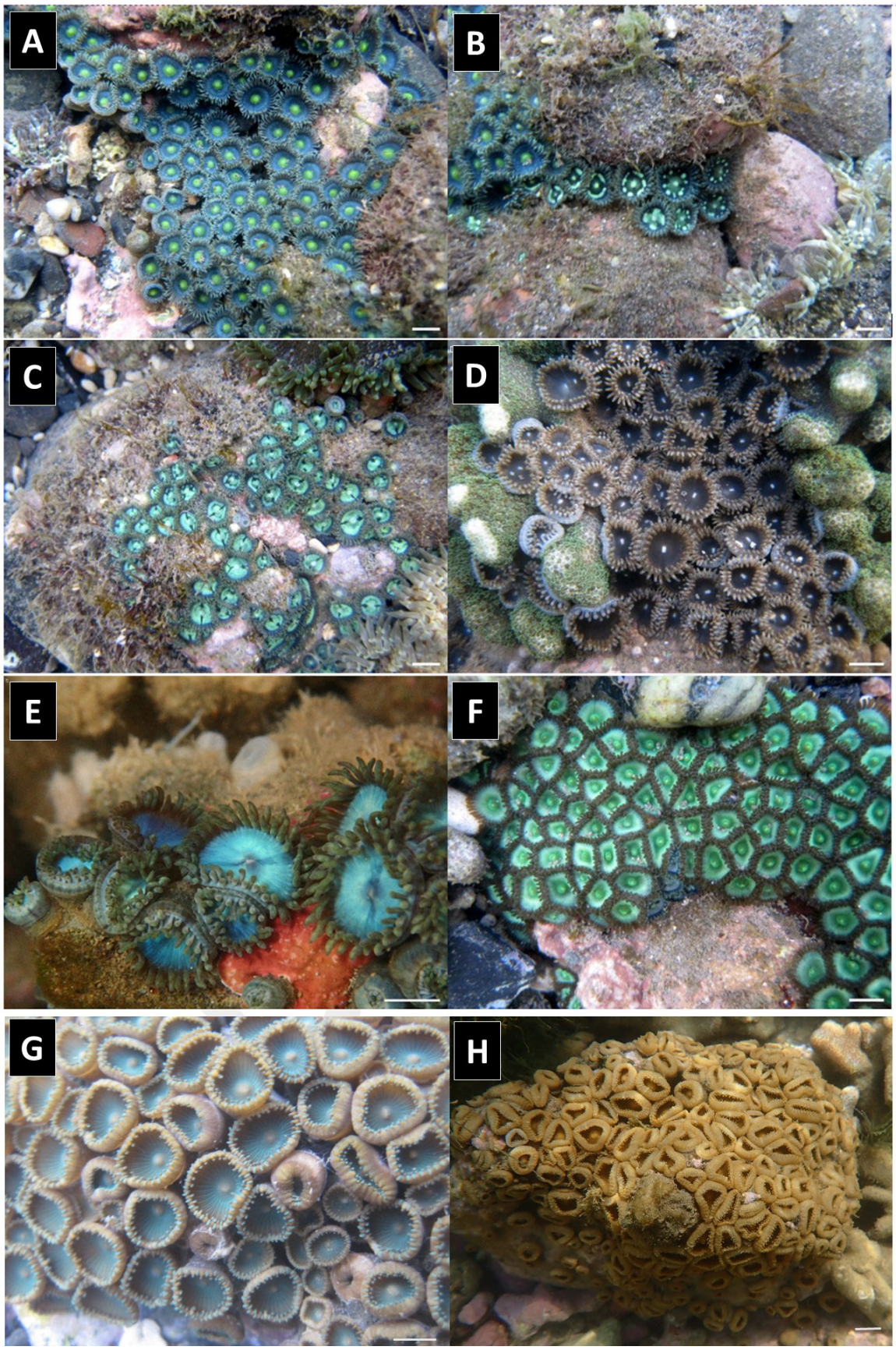

Fig. 4. *In situ* images showing zoantharian biodiversity from marine habitats at Toco, Trinidad showing variation in morphological characteristics. (A) *Zoanthus sociatus* blue colour morph (Z114-blu-GLA); (B) *Zoanthus sociatus* green-star (Z128-gr-star-GLA); (C) *Z.* aff. *pulchellus* bright-green (Z85-gr-GLA); (D) *Z. pulchellus* brown (Z88-br-GLA); (E) *Z. sociatus* blue morph (Z78-blu-SB); (F) *Z.* aff. *pulchellus* green morph (Z140-grbr-GLA); (G) *Palythoa grandiflora*; (H) *P. caribaeorum.* White bar scale = 1 cm.

et al. (2018) identified both *Z. pulchellus*, and *Z. sociatus*, which had polyps free from the coenenchyme, as '*liberae*' (Pax 1910). These were separated morphologically from *Z.* aff. *pulchellus*, which displayed polyps connected to a well-developed coenenchyma (Reimer *et al*. 2010), as observed in this study, and noted as 'immersae' (Pax 1910). In fact, although phylogenetically similar to each other, *Z. pulchellus* and *Z.* aff. *pulchellus* are morphologically well-distinguished, which is also seen with their Indo-Pacific sister species *Z. kuroshio* and *Z. vietnamensis* (Reimer *et al*. 2006a). In this study, two morphotypes were clearly distinguished by polyp size, i.e. oral disk diameter, with *Z. pulchellus* and *Z.* aff. *pulchellus* recording larger oral disk sizes than *Z. sociatus*. Similar polyp sizes for *Z. sociatus* have been reported in other parts of the Caribbean Sea (Reimer *et al*. 2012). Additionally, the larger oral disk of *Z. pulchellus* and *Z.* aff. *pulchellus* displayed a white stripe vertical marking, which can be prominent on polyps. *Z. sociatus* displayed a circular fluorescent green colour surrounded by blue or green outer parts of the disk. Overall, oral disk polyp sizes and tentacle counts for these *Zoanthus* spp. match other studies from the Caribbean and sites in this area (Reimer *et al*. 2012; López *et al*. 2018; Belford 2021).

Two zoantharians from the family Sphenopidae were morphologically identifiable: *Palythoa grandiflora* and *Palythoa caribaeorum*. *P. grandiflora* is easily distinguished from *P. caribaeorum* by a larger oral disk diameter, higher tentacle count, and green morphotype. The main visual differences were a green oral disk and *liberae* growth form in *P. grandiflora*, compared to a brown oral disk and *immersae* growth form in *P. caribaeorum*.

CONCLUSIONS

This study applies new morphological and genetic approaches to invertebrate species identification in Caribbean waters, and in doing so has added to our knowledge of local and regional marine benthic biodiversity. The molecular results matched with the morphological traits means that ecologists can now have better confidence in their species identifications when surveying these valuable reef habitats, and future research can build upon these findings to add to our understanding of the diversity and distributions of the different zoantharian species in shallow Caribbean coral reefs.

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