Hindawi Publishing Corporation
Journal of Marine Biology
Volume 2012, Article ID 856079, 14 pages
doi:10.1155/2012/856079

Research Article

Species Diversity of Shallow Water Zoanthids (Cnidaria: Anthozoa: Hexacorallia) in Florida

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Received 22 March 2012; Accepted 25 May 2012

Academic Editor: Pei-Yuan Qian

Shallow water zooxanthellate zoanthids are a common component of the coral reef ecosystems of the Caribbean. Despite this, their species diversity remains poorly understood. In this study, collected *Palythoa*, *Zoanthus*, *Isaurus*, and *Terrazoanthus* specimens from the waters of Florida were phylogenetically examined to obtain a better understanding of zoanthid species diversity in the Caribbean. Surprisingly, the results from analyses utilizing three DNA markers (mitochondrial 16S ribosomal DNA, cytochrome oxidase subunit I, and the internal transcribed spacer of ribosomal DNA) showed the presence of at least eleven species, of which up to four appear undescribed. Additionally, the presence of the genus *Terrazoanthus* in the Caribbean was confirmed for the first time. Attempts to match phylogenetic species or clades with original literature were hampered by vague and short original descriptions, and it is clear that for Atlantic *Palythoa* and *Zoanthus* species an in-depth and multidisciplinary investigation is needed to reconcile recent phylogenetic results such as in this study with traditional taxonomy. Furthermore, most shallow water zoanthid species from Florida were observed to have close, sister-species relationships with previously investigated species in the Pacific Ocean. These results indicate that many brachycnemic zoanthid species likely had a Caribbean-Pacific distribution until the formation of the Isthmus of Panama. However, due to inadvertent redescriptions, overall species diversity in these two common genera is likely much lower than literature indicates.

1. Introduction

The coral reef ecosystems of Florida are the only large area of shallow water coral reefs in the continental United States, and as such have been the subject of studies investigating their ecology [1], biodiversity, and recent history [2]. Despite this, the region has undergone rapid degradation in terms of live coral coverage due to coral bleaching [3] and disease [4], among other stressors, with corresponding large losses of live coral cover [5, 6].

One of the most common groups of organisms on the reefs of Florida and the Greater Caribbean is the zoanthids. In fact, some zoanthids (Anthozoa: Hexacorallia) are so common that a portion of the shallow intertidal zone has been called the "*Zoanthus* zone" [7]. Like many reef-building hard corals, most shallow tropical and subtropical zoanthids are in symbiosis with *Symbiodinium* (=zooxanthellae) species, endosymbiotic, photosynthetic dinoflagellates. Despite being an obvious and ubiquitous part of the Caribbean coral reef ecosystem, the taxonomy and diversity of zoanthids worldwide are poorly understood, and even species identification remains problematic [8–10]. However, recent research utilizing different mitochondrial and nuclear DNA markers has allowed researchers to begin to reassess zoanthid species identification [9, 10]. In this study, we apply these molecular methods to investigate the diversity of shallow water zoanthids in Florida. Phylogenetic species or species groups were then compared with original species descriptions in an attempt to formally identify specimens. Our results (1) demonstrate the utility of molecular methods in zoanthid identification, (2) indicate that previously undescribed zoanthid diversity may be common in the Caribbean Sea despite overall diversity being likely lower than in the taxonomic literature, and (3) highlight the considerable taxonomic problems of shallow water brachycnemic zoanthids in the Caribbean Sea.

2. Materials and Methods

2.1. Specimen Collection. This study primarily focused on zoanthids from the suborder Brachycnemina, which consists of the families Sphenopidae, Zoanthidae, and Neozoanthidae, and we did not collect specimens from Parazoanthidae or Epizoanthidae (suborder Macrocnemina). However, a specimen of Hydrozoanthidae (Macrocnemina), specifically nonepizoic species (resembling *Terrazoanthus* sp.), was collected. All specimens were collected by snorkeling, and collectively we refer to these specimens as "shallow water zoanthids" in this study.

Specimens from Florida ($n = 20$) were collected between December 2009 and January 2010 by SCUBA or snorkeling (see Table 1 for collection details). Two additional specimens were collected from the aquarium trade (Table 1). Specimens were collected, placed in labeled, sealable plastic cups, and subsequently photographed in vitro once acclimated in an aquaculture facility in Miami (Figure 1). Preservation was carried out using 99% analytical grade ethanol.

2.2. DNA Extraction, PCR Reactions, and DNA Sequencing. Genomic DNA was extracted from portions of specimens by following a guanidine extraction protocol as described in [11]. PCR amplification using template genomic DNA was conducted using HotStarTaq DNA polymerase (Qiagen) according to the manufacturer's instructions. An approximately 900 base pair fragment of mitochondrial 16S ribosomal DNA (mt 16S rDNA) was amplified using the primer set 16 SbmoH [10] and 16Sant0a [11]. An approximately 460 base pair portion of cytochrome oxidase subunit 1 (COI, including the common "barcode" region amplified with universal primers) was amplified using the zoanthid-specific primer COIZoanF [11] and the universal reverse primer HCO2198. The nuclear internal transcribed spacer region (ITS-rDNA, including ITS-1, 5.8S rDNA, and ITS-2; approximately 600–650 base pairs) was amplified using the primers Zoan-F and Zoan-R [12, 13]. Amplification conditions followed [10–14], and, for each DNA marker, respectively.

Amplified products were visualized by 1.0% agarose gel electrophoresis, and positive PCR products were treated with Exonuclease I and Shrimp Alkaline Phosphatase (Takara) prior to sequencing reactions. Direct sequencing of each marker in both directions was performed by MacroGen Japan (Tokyo).

2.3. Phylogenetic Analyses. New sequences obtained in this study were deposited in GenBank (accession numbers JX119120-JX119168). Sequences of all three DNA markers were aligned with publically available sequences of *Palythoa* (family Sphenopidae), *Zoanthus, Acrozoanthus,* and *Isaurus* (Zoanthidae), and *Hydrozoanthus* and *Terrazoanthus* (Hydrozoanthidae), with sequences from Parazoanthidae utilized as outgroups for mt 16S rDNA and COI, as the monophylies of these two families and their sister-group relationship has previously been demonstrated [10]. The identity of zoanthid sequences in GenBank is generally believed to be accurate, although asides from some Pacific species' sequences, sequences are not from topotypic specimens. For COI, two alignments were made, a shorter alignment with more taxa ("COI short"), and a longer alignment with less taxa ("COI long"), as many publically available COI sequences are shorter (e.g., <350 b.p.) than the COI sequences acquired in this study. Thus, the "short" alignment has more different taxa, while the "long" alignment has less taxa but longer sequences. For the ITS-rDNA alignment, *Hydrozoanthus* (but not *Terrazoanthus*) sequences were utilized as the outgroup. For the mt 16S rDNA alignment, a base alignment from [11] was used to guide alignment of sequences, while for ITS-rDNA, alignments were guided by alignments from [12] for *Palythoa* and from [13] for *Zoanthus*, with additional secondary structure information [15] utilized.

All alignments were inspected by eye and any ambiguous sites (e.g., only sites with double or ambiguous peaks, $n = 0$ to 1/alignment) in the alignments were removed from the dataset prior to phylogenetic analyses. Four alignment datasets were generated: (1) 603 sites of 46 sequences (mt 16S rDNA), (2) 461 sites of 28 sequences ("COI long"), (3) 280 sites of 35 sequences ("COI short"), and (4) 1013 sites of 32 sequences (ITS-rDNA). Alignment data sets are available from the corresponding author.

For the phylogenetic analyses of the data sets, the same methods were independently applied. Alignments were subjected to analyses with the maximum likelihood (ML) with PhyML [16] and neighbour-joining (NJ) methods. PhyML was performed using an input tree generated by BIONJ with the general time-reversible model [17] of nucleotide substitution incorporating a discrete gamma distribution (eight categories) (GTR+). The discrete gamma distribution and base frequencies of the model were estimated from the dataset. PhyML bootstrap trees (1000 replicates) were constructed using the same parameters as the individual ML tree. The distances were calculated using a Kimura's 2-parameter model [18]. Support for NJ branches was tested by bootstrap analysis [19] of 1000 replicates. CLC Free Workbench 3.0 (Aarhus, Denmark) was used for NJ phylogenetic analyses (1000 replicates).

2.4. Specimen Identification. Specimens were identified using combined molecular, morphological, and ecological data from previous literature. For morphology, colony shape, polyp size and structure, and comparisons with original descriptions were undertaken. For ecological data, specimens' habitats and depths were taken into account. Acquired DNA sequences in this study were compared against previously obtained sequences in GenBank. When morphological date for specimens did not fit perfectly with described

Table 1: Zoanthid specimens from florida examined in this study, their collection details, GenBank accession numbers, and identity.

Specimen number	Collection locality*	Latitude	Longitude	Depth (m)	GenBank accession number mt 16S rDNA	 COI	 ITS-rDNA	Identity
1558	Hawk Channel Patch Reef, Key West	24 29′ 53.21″ N	81 44′ 01.17″ W	6	JX119135	JX119160	JX119122	*P.* aff. *variabilis*
1559	Offshore from Destin, FL	30 20′ 53″ N[2]	86 29′ 57″ W [2]	30	JX119138	JX119164	JX119128	*P. grandis*
1560	Dania Beach	26 05′ 18.49″ N	80 06′ 26.05″ W	5	JX119142	JX119165	JX119124	*P. grandiflora*
1561	Big Pine Key	24 38′ 14.44″ N	81 18′ 50.42″ W	5	JX119139	JX119163	JX119129	*P. grandis*
1562	Pompano Beach	26 15′ 27.30″ N	80 04′ 49.47″ W	2	JX119141	JX119167	JX119126	*P.* aff. *clavata*
1563	Miami Beach	25 45′ 37.48″ N	80 07′ 44.23″ W	1	JX119140	JX119166	JX119125	*P.* aff. *clavata*
1564	Hawk Channel Patch Reef, Key West	24 29′ 53.21″ N	81 44′ 01.17″ W	6	NA	NA	JX119123	*P.* aff. *variabilis*
1565	Miami Beach	25 45′ 37.48″ N	80 07′ 44.23″ W	1	NA	NA	JX119127	*P. caribaeorum*
1566	Aquarium Trade[1]	NA	NA	NA	JX119137	JX119162	JX119121	*P.* aff. *variabilis*
1567	Aquarium Trade[1]	NA	NA	NA	JX119136	JX119161	JX119120	*P.* aff. *variabilis*
1568	Fisher Island	25 45′ 25.08″ N	80 08′ 03.68″ W	1	NA	NA	JX119131	*Z. sociatus*
1569	Lake Worth	26 46′ 24.94″ N	80 02′ 19.70″ W	Intertidal	NA	NA	NA	*Z.* aff. *pulchellus*
1570	Hawk Channel Patch Reef, Key West	24 29′ 53.21″ N	81 44′ 01.17″ W	6	JX119151	JX119157	NA	*Z. solanderi*
1571	Fisher Island	25 45′ 25.08″ N	80 08′ 03.68″ W	1	JX119150	JX119158	NA	*Z. solanderi*
1572	Dania Beach	26 03′ 46.21″ N	80 06′ 26.25″ W	3	NA	NA	NA	*Z. pulchellus*
1573	Dania Beach	26 03′ 46.21″ N	80 06′ 26.25″ W	4	JX119143	JX119156	NA	*Z. pulchellus*
1574	Hawk Channel Patch Reef, Key West	24 29′ 53.21″ N	81 44′ 01.17″ W	6	JX119148	NA	JX119132	*Z. sociatus*
1575	Boynton Beach	26 32′ 42.36″ N	80 02′ 32.85″ W	Intertidal	JX119144	NA	NA	*Z.* aff. *pulchellus*
1576	Jupiter	26 56′ 40.76″ N	80 04′ 17.34″ W	1	JX119147	JX119155	JX119130	*Z. pulchellus*
1577	Jupiter	26 56′ 38.10″ N	80 04′ 25.45″ W	1	JX119146	NA	NA	*Z. pulchellus*
1578	Pompano Beach	26 15′ 27.30″ N	80 04′ 49.47″ W	Intertidal	JX119145	NA	NA	*Z.* aff. *pulchellus*
1579	Key West	24 29′50.05″N	81 45′08.01″W	1	JX119149	JX119154	JX119133	*Z. sociatus*
1580	Big Pine Key	24 38′ 14.44″ N	81 18′ 50.42″ W	5	JX119152	JX119159	NA	*I. tuberculatus*
1581	Pt Everglades Inlet	26 05′ 32.88″ N	80 06′ 26.80″ W	2	JX119153	JX119168	JX119134	*Terrazoanthus* sp.

* All locations Florida unless otherwise noted.

[1] Acquired from the aquarium trade, origin unknown.

[2] Location approximate.

NA: not available, not acquired.

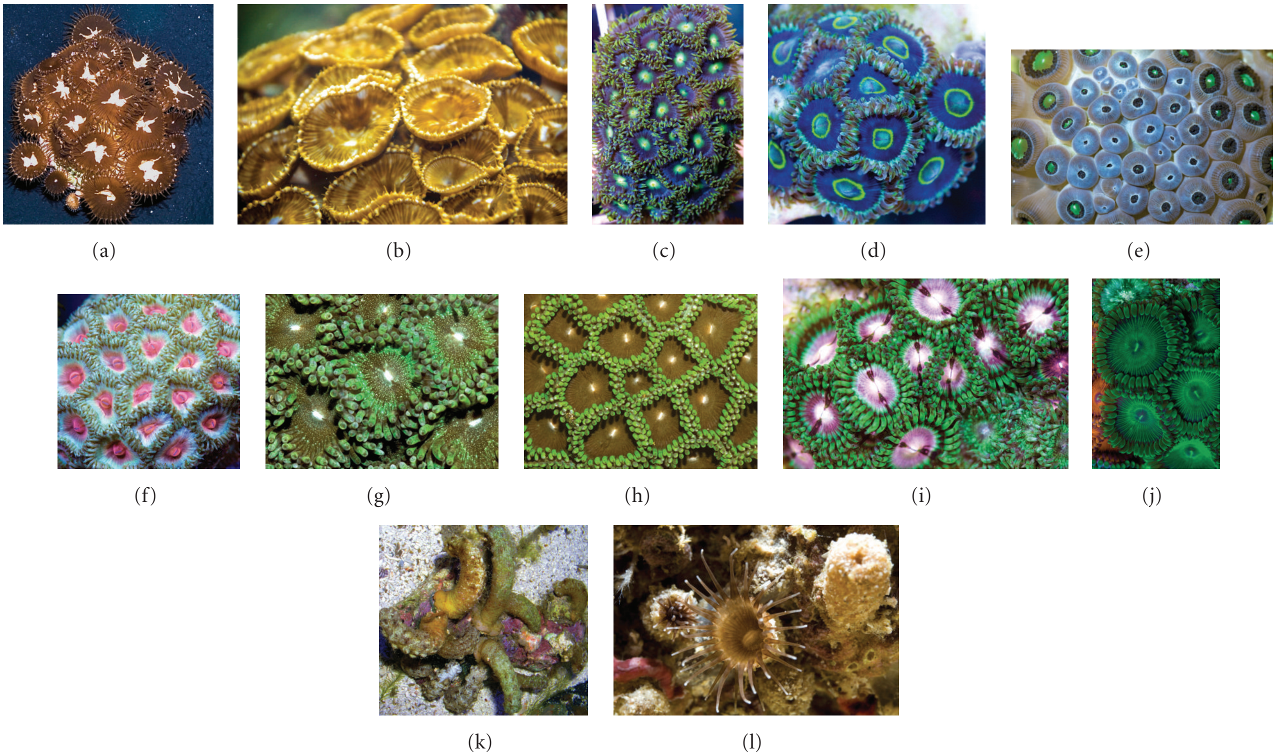


Figure 1: Zoanthid specimens from Florida examined in this study. (a) *Palythoa* aff. *variabilis* 1558; (b) *Palythoa grandis* specimen 1559; (c) *Zoanthus sociatus* specimen 1579 and (d) specimen 1574; (e) *Zoanthus* aff. *pulchellus* specimen 1575 and (f) specimen 1578; (g) *Zoanthus pulchellus* specimen 1572, (h) specimen 1576, and (i) specimen 1577; (j) *Zoanthus solanderi* specimen 1571; (k) *Isaurus tuberculatus* specimen 1580; (l) *Terrazoanthus* sp. specimen 1581. For collection details refer to Table 1.

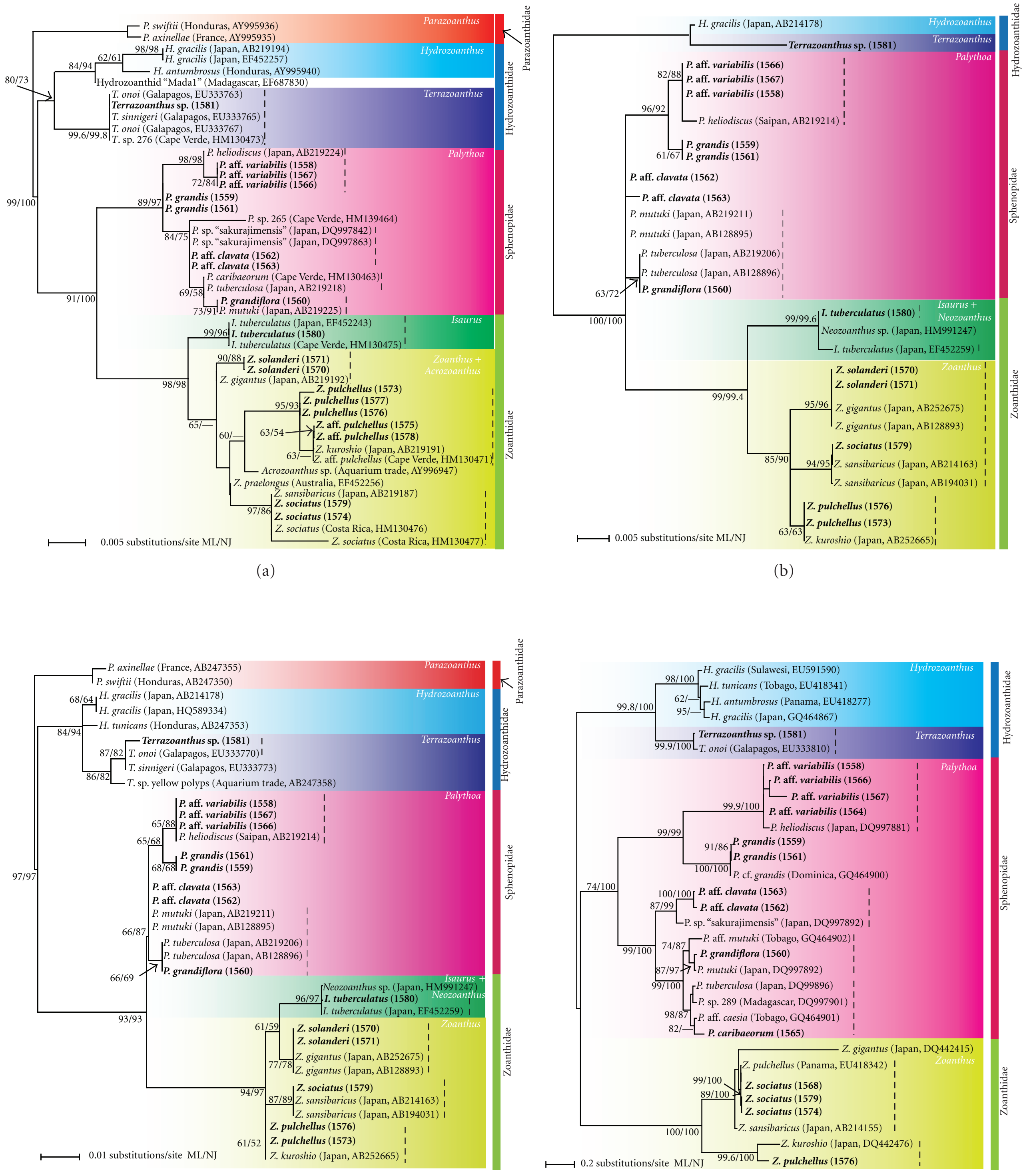
species, we then identified species or species groups utilizing "aff." or "cf." accordingly. All relevant previous zoanthid taxonomic literature was consulted [7, 20–71].

3. Results

3.1. Mitochondrial 16S Ribosomal DNA. mt 16S rDNA sequences from specimens in this study were placed within three large clades, corresponding to the families Sphenopidae, Zoanthidae, and Hydrozoanthidae (Figure 2(a)). Each of these large family-level clades was generally well supported in phylogenetic analyses (e.g., ML = 89%, 98%, 80%, resp.).

Sequences from eight specimens were within the Sphenopidae clade. Sequences from 1558, 1566, and 1567 were identical to each other and were most closely related to *Palythoa heliodiscus* [66] sequence AB219224 from Japan. These four sequences together formed a well-supported subclade (ML = 98%, NJ = 98%). Two other sequences (1559, 1561), although identical to each other, did not match with any previously reported *Palythoa* sequence and were in an unresolved position basal to other *Palythoa*. The remaining large subclade, which was moderately supported (ML = 84%, NJ = 75%), contained the remaining *Palythoa* sequences. Sequences from 1562 and 1563 were identical to each other, and to two sequences from *Palythoa* sp. "sakurajimensis" *sensu* Reimer et al. [12] (DQ997842, DQ997863) in an unresolved position. The sequence from specimen 1560 was identical to one from *Palythoa mutuki* Haddon and Shackleton 1891, from Japan (AB219225) (ML = 73%, NJ = 91%).

In the Zoanthidae clade, the sequence from specimen 1580 was identical to *Isaurus tuberculatus* Gray 1828 sequences from Japan (EF452253) and Cape Verde (HM130475), and these three sequences formed a well-supported *Isaurus* clade (ML = 99%, NJ = 96%). Sequences from specimens 1570 and 1571 were identical and formed a clade (ML = 90%, NJ = 88%) and were close to and derived from a sequence from *Zoanthus gigantus* Reimer and Tsukahara 2006 ([72]; AB219192) from Japan. Sequences from 1574 and 1579 were identical to previously reported sequences from *Z. sansibaricus* Carlgren 1900 [73] (AB219187) in Japan and *Z. sociatus* (Ellis 1768) (HM130476) from Costa Rica, and similar to an additional *Z. sociatus* (HM130477) sequence from Costa Rica, and these sequences formed a well-supported clade (ML = 97%, NJ = 86%). Most remaining Zoanthidae sequences asides from *Z. praelongus* (EF452256) and *Acrozoanthus* (AY996947) formed a well-supported subclade (ML = 95%, NJ = 93%). Within this subclade, two sequences (1575, 1578) were identical to previously sequences from *Z. kuroshio* Reimer and Ono 2006 in Japan (AB219191) and *Z.* aff. *pulchellus* (Duchassaing and Michelotti 1860) from Cape Verde (HM130471). Two sequences (1576, 1577), identical to each other, differed by one base substitution from the former four sequences, and one



(a)

(b)

(c)

(d)

Figure 2: Maximum likelihood trees of (a) mitochondrial 16S ribosomal DNA sequences, (b) "long" alignment of mitochondrial cytochrome oxidase subunit I (COI), (c) "short" alignment of COI, and (d) internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences. Novel sequences from specimens in this study represented in bold. Sequences/species names from previous studies are in regular font with species name, location, and GenBank accession numbers. Values at branches represent maximum likelihood (ML) and neighbor-joining (NJ) bootstrap probabilities, respectively. Atlantic/Pacific sister species groups are represented by dark dashed lines, groups for which resolution in the phylogenetic tree are weak by gray dashed lines. For specimen information see Table 1.

additional sequence (1573) was different from these two sequences by three additional base pairs.

Within the Hydrozoanthidae clade, two subclades were observed, one corresponding to genus *Hydrozoanthus* (ML = 84%, NJ = 94%) and one to genus *Terrazoanthus* (ML = 99%, NJ = 99%). The mt 16S rDNA sequence from specimen 1581 was identical to previously reported sequences from *T. onoi* Reimer and Fujii 2010 [74] (EU333762, EU333767), *T. sinnigeri* Reimer and Fujii 2010 (EU333765) in the Galapagos, and an unidentified *Terrazoanthus* species from Cape Verde (HM130473).

3.2. *Cytochrome Oxidase Subunit I.*

The topologies of the "long" and "short" maximum likelihood trees were generally identical with only a few inconsistencies (Figure 2(b), 2(c), resp.). The largest difference was in the families Hydrozoanthidae and Parazoanthidae, as there were more taxa in the "short" alignment ($n = 9$ as opposed to $n = 2$ in the "long" tree). As well, bootstrap values were generally higher in the "long" tree (compare values at nodes in Figures 2(b) and 2(c)). Furthermore, the placement of *Isaurus* and *Neozoanthus* was inconsistent (Figures 2(b), and 2(c)).

3.2.1. *"Short" COI Alignment.*

As in the mt 16S rDNA tree, there were three generally well-supported family-level clades, corresponding to Sphenopidae (ML = 66%, NJ = 87%), Zoanthidae (ML = 94%, NJ = 97%), and Hydrozoanthidae (ML = 84%, NJ = 94%) (Figure 2(c)).

Eight novel sequences from this study were within the Sphenopidae clade. Sequences from specimens 1558, 1566, and 1567 were identical to a sequence from *P. heliodiscus* from Saipan (AB219214) (ML = 65%, NJ = 88%). This subclade was sister to a subclade consisting of the two identical sequences 1559 and 1561 (ML = 68%, NJ = 68%). Sequences from 1562 and 1563 were identical to two sequences from *P. mutuki* from Japan (AB219211, AB128895), and these four sequences were unresolved in a position basal to both the *P. heliodiscus* and 1559 + 1561 subclades, and a *P. tuberculosa* + specimen 1560 subclade (ML = 66%, NJ = 69%).

Within the Zoanthidae clade, the sequence from 1580 was identical to another *Isaurus* sequence from Japan (EF452259) and also to a sequence from *Neozoanthus* sp. in Japan (HM991247), and these three sequences formed a very well-supported subclade (ML = 96%, NJ = 97%). Sequences for specimens 1570 and 1571 were identical to sequences from *Z. gigantus* in Japan (AB128893, AB252675), and these four sequences formed a moderately well-supported subclade sister to the *Isaurus* subclade (ML = 77%, NJ = 78%). The sequence from specimen 1579 was identical to two sequences from *Z. sansibaricus* in Japan, forming a well-supported subclade (ML = 87%, NJ = 89%). Finally, 1573 and 1576's sequences were identical to a sequence from *Z. kuroshio* in Japan (AB252665).

The novel sequence from specimen 1581 clustered with three other previously reported *Terrazoanthus* spp. sequences and formed a well-supported subclade (ML = 86%, NJ = 82%).

3.2.2. *"Long" COI Alignment.*

Results from the "long" COI phylogenetic analyses were basically the same as reported above for the "short" COI alignment. The only major difference was that the sequence from 1581 was clustered with the only other Hydrozoanthidae sequence in the alignment, *H. gracilis* in Japan (AB214178), as the outgroup (Figure 2(b)), but this is to be expected given the reduction of Hydrozoanthidae sequences in this tree compared with the "short" tree. Furthermore, some small sequence differences not seen in the short COI tree were present (e.g., between *P. heliodiscus* and sequences from specimens 1566, 1567, and 1568).

3.3. *Internal Transcribed Spacer Region of Ribosomal DNA.*

Several *Zoanthus* specimens (1569, 1570, 1571, 1572, 1575, 1577, and 1578) did not amplify, and the single *Isaurus* specimen also did not amplify (1580), despite repeated attempts using all previously reported zoanthid ITS-rDNA primers [12, 13, 69], similar to as in previous reports for both *Zoanthus* [13] and *Isaurus* [75]. However, we could obtain PCR products for the majority of specimens, and their resulting ITS-rDNA sequences had no double peaks.

Topology of the phylogenetic tree for the ITS-rDNA alignment (Figure 2(d)) was generally similar to the topologies of the mt 16S rDNA and COI trees reported above, with more resolution within the species-group level clades. Again, three major family-level clades were seen, corresponding to Sphenopidae (ML = 74%, NJ = 100%), Zoanthidae (ML = 100%, NJ = 100%), and Hydrozoanthidae (ML = 99%, NJ = 100%).

Within Sphenopidae, two large subclades were formed. One subclade contained *P. heliodiscus*, *P.* cf. *grandis* (Verrill 1900), and related sequences, and the other subclade contained *P.* sp. "sakurajimensis", *P.* aff. *mutuki*, *P. mutuki*, *P.* aff. *caesia* Dana 1846, *P. tuberculosa* (Esper 1791) [76], and related sequences. Support for both large subclades was high (ML = 99%, NJ = 99%; ML = 99%, NJ = 100%; resp.).

Within the first large subclade, two smaller subclades were present. Four sequences (1558, 1564, 1566, and 1567) were slightly different but clustered with *P. heliodiscus* from Japan (DQ997881) and formed an almost completely supported subclade (ML = 99.9%, NJ = 100%). This subclade was sister to another subclade that contained a previously reported sequence from *P.* cf. *grandis* from Dominica ([70]; GQ464900) and sequences from 1559 and 1561. This subclade was completely supported (ML = 100%, NJ = 100%).

Within the second large subclade, sequences from 1562 and 1563 formed a grouping (ML = 100%, NJ = 100%) that together with a sequence from *P.* sp. "sakurajimensis" (DQ997887) formed a subclade (ML = 87%, NJ = 99%). The sequence from 1560 was closely related to that of *P. mutuki* from Japan (DQ997892) and *P.* aff. *mutuki* from Tobago (GQ464902) formed a moderately supported subclade (ML = 74%, NJ = 87%). The ITS-rDNA sequence from 1565 was contained within a well-supported *P.* aff. *caesia* (Tobago, GQ464901), *P. tuberculosa* (Japan, DQ997896) and related sequences subclade (ML = 98%, NJ = 87%).

Within Zoanthidae, three specimens' sequences (1568, 1574, and 1579) formed a small clade ($\mathrm{ML} = 99\%, \mathrm{NJ} = 100\%$) with a previously reported sequence from *Z. pulchellus* from Panama (EU418342), while one sequence from specimen (1576) formed a clade with a sequence from *Z. kuroshio* in Japan (DQ442476) ($\mathrm{ML} = 99.6\%, \mathrm{NJ} = 100\%$).

Within Hydrozoanthidae, two subclades were seen. One highly supported subclade corresponded to the genus *Hydrozoanthus* ($\mathrm{ML} = 98\%, \mathrm{NJ} = 100\%$), while the other subclade was made up of sequences from *T. onoi* (EU333810) and specimen 1581 ($\mathrm{ML} = 99.9\%, \mathrm{NJ} = 100\%$).

3.4. Specimen Identification. Overall, 11 species groups were confirmed to exist from the molecular data analyses. The taxonomic identification of each species group is as follows.

(1) *Zoanthus sociatus* (Ellis 1768) [20]. Specimens: 1568, 1574, and 1579.

Although the original description is short, the sum of subsequent literature [7, 21, 23, 30, 39, 40, 42, 44, 45, 54, 55, 58, 60] describes *Z. sociatus* with polyps of approximately 5 mm in diameter with a slightly larger oral disk, polyp height to 25 mm, 48 to 60 short tentacles, with a green, blue, or yellow oral disk sometimes with patterning, with stoloniferous colonies found mostly in shallow water or the intertidal zone. Other former species synonymized with *Z. sociatus* are the Caribbean *Z. flos-marinus* Duchassaing and Michelotti 1860, *Z. poriticola* Pax, 1910, and *Z. proteus* Verrill, 1900 (for a complete taxonomic review, refer to above literature). It is likely *Z. nobilis* Duchassaing and Michelotti 1860 is also within this species, although there is some questions as to whether it is instead a synonym of *Z. solanderi* (see [44, 46]).

In this study, both mtDNA markers grouped these specimens in a clade together with previously acquired *Z. sociatus* sequences from Costa Rica as well as *Z. sansibaricus* sequences from Japan. ITS-rDNA results showed all three specimens forming their own clade together with *Z. pulchellus* (EU418342) from Panama, closely related to *Z. sansibaricus* from Japan. These specimens' shallow distribution (1 to 6 meters; Table 1) and morphology (Figures 1(c), and 1(d)) generally matched with the description of *Z. sociatus* given above, and these specimens were assigned to this species (but see Section 4).

(2) *Zoanthus pulchellus* (Duchassaing and Michelotti 1860) [30]. Specimens: 1572, 1573, 1576, and 1577.

Although the original description is short, it and subsequent literature [37, 40, 42, 44, 45, 55] describe *Z. pulchellus* with polyps of approximately 4–6 mm in diameter with a slightly larger oral disk, polyp height 4 to over 30 mm, 50 to 60 short tentacles, with a green or yellow oral disk sometimes with pink, brown, or yellow patterning, and encrusting colonies with a lamellar coenenchyme found mostly in shallow water, and not intertidal as seen sometimes in *Z. sociatus*. Other former species synonymized with *Z. pulchellus* are the Caribbean *Z. nymphaeus* Duchassaing and Michelotti 1860, *Z. auricula* Duchassaing and Michelotti 1860, *Z. dubius* Lesueur 1817, and *Z. anduzii* Duchassaing and Michelotti 1860 (for a complete taxonomic review, refer to above literature). Additionally, *Zoanthus* sp. 1 (Erdmann, 1886) [77], as well as *Z. flos-marinus* in [39], and some *Z. proteus* in [40] and *Z. dubius sensu* Duerden [37] are likely *Z. pulchellus*.

In this study, analyses of COI and ITS-rDNA sequences placed three specimens (one additional specimen, 1572, did not amplify successfully) in a group together with but not identical to *Z. kuroshio* sequences from Japan. As well, these specimens' mt 16S rDNA sequences were slightly distinct from sequences of specimens 1575 and 1578, as well as previously reported *Z. kuroshio* (AB219191) and *Z.* aff. *pulchellus* (HM130471) sequences. These specimens' distribution (1 to 4 meters; Table 1) and morphology (Figures 1(g)-1(i)) generally matched with the description of *Z. pulchellus* given above, and these specimens were assigned to *Z. pulchellus*.

(3) *Zoanthus* aff. *pulchellus*. Specimens: 1569, 1575, and 1578.

DNA sequences from two specimens (1575, 1578) were only amplifiable for mt 16S rDNA, and despite repeated attempts the other two markers did not amplify, while no amplifications were successful for specimen 1569. A similar situation has been seen previously with *Z. kuroshio* from Japan, in which ITS-rDNA did not amplify using usual zoanthid-specific markers [13]. mt 16S rDNA sequences for these two specimens were identical to both *Z. kuroshio* (AB219191) and *Z.* aff. *pulchellus* (HM130471) from Cape Verde. Morphologically, specimens had a more developed coenenchyme than *Z. sociatus*, similar to as seen in *Z.* aff. *pulchellus* from Cape Verde *sensu* Reimer et al. [71], and had brightly colored oral disks of green or pink (Figures 1(e), and 1(f)).

Furthermore, specimens were found only in the intertidal zone, different from the described habitat of *Z. pulchellus*, and similar to as observed in in *Z.* aff. *pulchellus* [71], and due to the slightly different morphology and ecology as well as small but identical differences in mt 16S rDNA sequences from those of *Z. pulchellus* specimens above, we have designated specimens 1575 and 1578 as *Z.* aff. *pulchellus*. As this designation is based on only mt 16S rDNA and morphology/ecology, it is hoped that future in-depth investigation with more specimens and phylogenetic data will confirm this identification.

(4) *Zoanthus solanderi* Lesueur 1817 [23]. Specimens: 1570, and 1571.

The original description [23] and subsequent literature [7, 37, 40, 54, 55, 58, 60, 63] describe *Z. solanderi* as larger than other Caribbean *Zoanthus* species, with polyps of approximately 8 to 10 mm in diameter with a slightly larger oral disk, polyp height to 50 mm, 50 to 60 short tentacles, with a blue, bright orange, dark brown, dark green, or bright blue oral disks sometimes with "white fleck" patterning, with stoloniferous colonies found mostly on the surf zone, below *Z. sociatus*. Often polyps have white markings around the mouth when semi-contracted [37]. It may be that *Z. nobilis* Duchassaing and Michelotti 1860 is also within *Z. solanderi*, although there is some questions as to whether it is instead a synonym of *Z. sociatus* [44, 45]. As well, *Z. sociatus* in [52] has been thought in fact to have been *Z. solanderi* [7].

In this study, DNA sequences from mtDNA molecular markers placed these specimens in a group together with previously acquired *Z. gigantus* sequences from Japan. These specimens' shallow distribution (1 to 6 meters; Table 1) and morphology (Figure 1(j)) fit with the description of *Z. solanderi* given above, and these specimens were assigned to *Z. solanderi*.

(5) *Palythoa caribaeorum* (Duchassaing and Michelotti, 1860) [30]. Specimen: 1565.

The original description [30] and subsequent literature [7, 31, 32, 42, 45, 47, 48, 53–55, 58, 60, 63, 64, 66] describe *P. caribaeorum* as cream, brown, or yellow in color, with polyps deeply embedded in a well-developed, sand-encrusted coenenchyme (="immersae", [45]). Polyps have 28 to 34 tentacles, and this species is common throughout the Caribbean.

In this study, DNA sequences from ITS-rDNA placed specimen 1565 in a subclade together with previously acquired *P.* aff. *caesia* sequences from Tobago, and this subclade was within a larger clade consisting of *P. tuberculosa* Esper 1791, *P. mutuki* and other specimens from the Pacific and Atlantic. It should be noted that *P. caesia* Dana 1846 is described from Fiji and Australia, and not the Caribbean. Based on morphological and molecular results, this specimen was assigned to *P. caribaeorum*.

(6) *Palythoa grandiflora* (Verrill, 1900) [40]. Specimen: 1560.

Caribbean *Palythoa* species with polyps somewhat or completely clear of their coenenchyme ("intermediate" or "liberae" in [45]) have a particularly long and confused taxonomic history, with at least 39 binomens in the literature. Specimen 1560 morphologically best fit with *P. grandiflora* Verrill 1900, which is described in literature as having polyps 15–20 mm in height, an oral disc diameter of 14–16 mm, and 52–56 tentacles, found in the Caribbean and Brazil [40, 41, 43, 44, 66, 67]. This species is mentioned as one of the only two valid species of genus *Protopalythoa* (=*Palythoa* with liberae polyps) in [66] in the Caribbean.

Furthermore, our obtained DNA sequences from all three molecular markers support lend support to our identification. In [66], Ryland and Lancaster note that Pacific *P. mutuki* is likely closely related to *P. grandiflora*, as they have similar morphologies, while Pacific *P. heliodiscus* may be closely related to *P. variabilis*. Phylogenetic analyses show specimen 1560 sequences to be either very similar to *P. mutuki* (mt 16S rDNA, ITS-rDNA) or very closely related to both *P. mutuki* and *P. tuberculosa* (COI). Previously, *P. mutuki* and *P. tuberculosa* have been shown to be very closely related [12], and our phylogenetic results show specimen 1560 is most closely related to Pacific *P. mutuki*.

Finally, as noted in [62] and [66], *P. grandiflora* may be a junior synonym of *P. fusca* Duerden 1898. We also agree with [66] that this species is not synonymous with *P. mammillosa* [21], as Figure 7 in [40] of this species shows a much more "immersae" *P. mammillosa* colony than as described for *P. grandiflora* or our specimen.

(7) *Palythoa grandis* (Verrill, 1900) [40]. Specimens: 1559, 1561.

This species, originally assigned to *Protopalythoa* by Verrill [40], is easy to identify due to its large size (height = 30–36 mm, oral disc diameter = 10–16 mm), and deeper habitat (e.g., up to >12 m in [40]) than other Caribbean zooxanthellate zoanthids [40, 43–45, 47, 48]. Two specimens (1556, 1561; Table 1) fit well with this species' characteristics (Figure 1(b)). Furthermore, acquired ITS-rDNA sequences from these specimens were identical or very close to sequences from previously acquired *P.* cf. *grandis* sequences [70]. It may be that there is more than one species within the *P. grandis* species group, but until such time as revision occurs, we identify specimens 1559 and 1561 as *P. grandis*.

It should be mentioned that Ryland and Lancaster [66] considered *P. grandis* to be a variant of *P. variabilis* (Duerden 1898), but given *P. grandis*' unique morphology and phylogenetic position, we do not agree with this hypothesis.

(8) *Palythoa* aff. *variabilis* (Duerden 1898) [37]. Specimens: 1558, 1564, 1566, and 1567.

Palythoa variabilis was described from Jamaica [37] as a "liberae" [45] *Gemmaria* (=*Protopalythoa*) species with polyps 10 to 50 mm in height and 7 mm in diameter. This species has 60–80 tentacles, is generally green or brown in color, and has a poorly developed coenenchyme [37, 40, 42, 45]. *Palythoa variabilis* is distributed throughout the Caribbean and the Atlantic coast of South America (e.g., [67]). [66] noted the morphological similarities between *P. variabilis* and Indo-Pacific *P. heliodiscus*, but no sequence data exist for this species and the molecular markers used in this study. However, we support the separation of these two species given their distributions in different oceans.

However, four specimens from this study (1558, 1564 from Hawk Channel, Key West, 1566, 1567 from the aquarium trade) were shown in the phylogenetic results to be similar to previously acquired *P. heliodiscus* sequences. From the ITS-rDNA tree, it appears that the Key West specimens were slightly different from the two aquarium trade specimens. Key West specimens were liberae, with 64–74 tentacles, brown with white patterns on their oral disk, and smaller and less robust than *P. grandis*, and generally fit the description of *P. variabilis* asides from oral disk patterning (Figure 1(a)). On the other hand, the aquarium trade specimens had much brighter oral disk colors, either fluorescent green (1566) or purple (1567), and these are likely a different species from Key West specimens. The original sampling location of these two specimens remains unknown, like many specimens obtained from the aquarium trade [78]. Thus, until *P. variabilis* sequence data as well as localities for specimens 1566 and 1567 are obtained, identification remains open to error, and for this reason we have designated these four specimens as one species group, *P.* aff. *variabilis*.

(9) *Palythoa* aff. *clavata* (Duchassaing 1850) [28]. Specimens: 1562, 1563.

These two specimens were shown through molecular analyses to be closest to *P.* sp. "sakurajimensis" from the Pacific, and did not match with any Atlantic species' sequences. Furthermore, their morphology did not completely match with any hitherto formally described Caribbean zoanthid species, although both specimens did closely resemble images of a species called "brown zoanthid" in a recent marine life guidebook [79], having a brown disc with green or blue overtones of 60–12 mm in diameter and a white center. This "brown zoanthid" has also recently been cited as being similar to *Palythoa* sp. 265 reported from the Cape Verde Islands [71]. The mt 16S rDNA tree in this study showed that although specimens 1562 and 1563 were somewhat closely related to *Palythoa* sp. 265, their sequences were not exact matches.

The closest match from taxonomic literature for these two specimens is *P. clavata* [28] from St Thomas, which is described with polyps approximately 11–14 mm in height, violet or purple oral disk and tentacles, having approximately 60 tentacles, and being smaller than *P. variabilis* [29–32, 37, 40], although [80] stated *P. clavata* and his *P. variabilis* (from Singapore; now likely *P. heliodiscus*) to be very similar. Thus, based on our phylogenetic results and the small amount of past literature dealing with similar specimens, we have designated this group as *P.* aff. *clavata* here, although they may be an undescribed species.

(10) *Isaurus tuberculatus* Gray 1828 [24]. Specimen: 1580.

The single species of *Isaurus* from this study was easily identified as *Isaurus tuberculatus* due to its unique morphology with recumbent (nonerct) polyps with tubercules (Figure 1(k)), and as it is considered the only valid species of this genus in the Atlantic (reviewed in [61]). Currently, this species, described from the Caribbean, has a circumtropical distribution, although Indo-Pacific *I. tuberculatus* is almost certainly a different, sister species, as no other zooxanthellate coral or zoanthid is known to have such a distribution.

Although phylogenetic results showed *Isaurus* specimens as closely related to *Neozoanthus* specimens, these two genera are clearly different in morphology and do have phylogenetic differences as well [81].

(11) *Terrazoanthus* sp. Specimen: 1581.

This specimen was identified as an undescribed member of this new genus, as it was azooxanthellate with sand incrustations, and was not epizoic on other organisms, instead found on boulders (Figure 1(l)), easily distinguishing this genus from other macrocnemic genera. Currently, only two species of *Terrazoanthus* are described, both from the Galapagos Islands. This specimen is very likely to be an unidentified species given its location, different COI and ITS-rDNA sequences from its congeners, and different morphology (dark brown as opposed to bright red for *T. onoi* and clear, white or light brown for *T. sinnigeri*).

4. Discussion

4.1. Palythoa *and* Zoanthus *Species Identification.*

Actual numbers of species in *Palythoa* and *Zoanthus* are likely much lower than the numbers of species for each genus described in the literature (currently 218 and 151 species, resp.; according to [82]) [8, 9], as it has been suggested that many species are actually inadvertent redescriptions due to high levels of intraspecific variation and wide geographic ranges [8, 9]. Despite the comparatively "high" levels of diversity of zoanthid species observed in this study, the numbers of species observed in this and other recent studies (e.g., 5–13 species/region) support the theory of an overall reduction in the actual number of shallow water zoanthid species, as most species appear to be widespread and not endemic to one location.

Although many *Palythoa* and *Zoanthus* species have been described from the Caribbean, the true number of valid *Palythoa* and *Zoanthus* species for the Caribbean is unknown. From our review of the taxonomic literature (listed in Materials and Methods), there are at least 37 *Palythoa* (including *Protopalythoa*) and 11 *Zoanthus* described species in the Caribbean. However, many of these species have been synonymized, or theorized to be synonyms, and the taxonomic situation for these two common genera is very chaotic [8]. Results from this study demonstrate this as one *Zoanthus* species group (*Z.* aff. *pulchellus* [1575, 1578]) and two *Palythoa* species groups (1562 + 1563; 1566 + 1567 + 1558 + 1564) do not perfectly match with any previous description of species from the Caribbean region.

The morphology of *Z. sociatus* specimens in this study matches well with descriptions given in literature listed in the "Identification" section. However, past problems in accurate species identification in *Palythoa* and *Zoanthus* may be inadvertently compounded by molecular investigations of Caribbean species. Our *Z. sociatus* mt 16S rDNA sequences completely matched (=100%) by Nucleotide BLASTn with DNA sequences of specimens identified as *Z. sociatus* from Brazil (e.g., AY049060, EU348615) by Longo et al. (unpublished), and from Cape Verde [71]. However, these specimens' mt 16S rDNA sequences are also identical with sequences designated *Z. pulchellus* (EU828762) in [70]. Furthermore, an examination of GenBank records shows that mt 16S rDNA sequences for specimens identified as *Z. sociatus* by Burnett (AF282933, AF282934; unpublished) in fact are identical to our *Z.* aff. *pulchellus* group's sequences. It is clear from such results that proper identification of *Zoanthus* spp. in the Caribbean remains a difficult undertaking.

This confusion clearly demonstrates that a large-scale, multidisciplinary effort to examine brachycnemic zoanthids in the Caribbean is needed to clarify their diversity and to generate an identification key that can allow future researchers to properly identify these common zoanthids. Until species identification protocols of *Palythoa* and *Zoanthus* in the Caribbean are stabilized, molecular investigations may add to the confusion, as accurate species identifications will remain problematic.

4.2. Zoanthid Species Diversity in Florida.

The results of this study indicate the presence of eleven species or species groups of shallow water zoanthids in Florida; four *Zoanthus* spp. (*Z. sociatus*, *Z.* aff. *pulchellus*, *Z. pulchellus*, and *Z. solanderi*), five *Palythoa* spp. (*P. caribaeorum*, *P. grandiflora*, *P. grandis*, one or more unknown *Palythoa* species related to *P. variabilis*), and *P.* aff. *clavata*, as well as one *Isaurus* (*I. tuberculatus*), and one undescribed *Terrazoanthus* species. This number is unexpectedly high given the relatively small number of specimens and the relatively narrow geographic range of this study. However, specimens were collected after numerous observations and dives in the Florida region, and were conducted to collect as many species as possible.

Results from this and other studies [69–71] suggest that there may not be so much difference in levels of shallow water zoanthid diversity between the Atlantic and the Pacific oceans. This situation is different from that observed in Scleractinia (hard corals) in which Pacific diversity is much higher than Atlantic [83]. Furthermore, in scleractinians, more ancestral groups distinct from Pacific taxa are found in the Atlantic [83]. The similarity in numbers of species of shallow water zoanthids between the Atlantic and Pacific may be due to a combination of factors. First of all, despite being common in many coral reef ecosystems, it appears that most shallow water zoanthid species have wide ranges, and only a few species are known to have narrow ranges (e.g., *Z. praelongus* Carlgren 1954 [84] known only from western Australia, *I. maculatus* Muirhead and Ryland 1985 from Fiji, both discussed in [61]). This situation is likely due to brachycnemic zoanthids' long larval stage (up to 170 d) and subsequent high rates of dispersal [85, 86]. This theory is further supported by the presence of sister species of *Zoanthus*, *Palythoa* and *Isaurus* in the Atlantic and Pacific (shown in Figure 2(d)), which are very likely the result of the formation of the Isthmus of Panama approximately 2.8 to 3.1 million years ago that divided widespread shallow water zoanthid species into two separate populations [87]. Similar transisthmian sister species have been noted in other marine invertebrate taxa, including *Alpheus* snapping shrimp [88, 89]. While the possibility that the slow evolution of mtDNA in zoanthids may also be responsible for the lack of divergence between Atlantic and Pacific zoanthid species, the ITS-rDNA results do show species-level differences between sister species from each ocean. Further examination utilizing additional DNA markers should help researchers more clearly understand zoanthid transisthmian evolution.

Currently, there are no zooxanthellate coral species that inhabit both the Atlantic and Indo-Pacific, and although *I. tuberculatus* is described as being distributed in both oceans [61], due to the isolation of these two ocean regions, we feel this is highly unlikely [90]. Thus, the presence of several pairs of Atlantic-Pacific sister species fits with the ecology, reproduction, and phylogenetic data currently available for zoanthids. In the future, it is likely *Isaurus tuberculatus* will again be split into an Atlantic and Indo-Pacific species.

4.3. DNA Barcoding in Zoanthids. The results of this study also suggest that the utility of mtDNA for the "DNA barcoding" of sister species is not as effective as for most zoanthids in general [91], due to their recent isolation and divergence, and to the fact that benthic cnidarian mtDNA has a very slow rate of evolution [92]. Indeed, most sister species in this study had identical mt 16S rDNA and COI sequences. Still, combined with sampling location data (i.e., Atlantic or Pacific), barcoding should allow correct zoanthid species identification, or at least identification to the nearest species group in the case of closely related species. Furthermore, the utility of ITS-rDNA as a phylogenetic marker appears to be high, as resolution in the phylogeny and bootstrap support values in this study were high. However, this marker has multiple intragenomic copies and also potential signals from past hybridization events and/or ancestral polymorphisms in zoanthids [12, 13]. These potential problems can be overcome with cloning protocols, but these issues make ITS-rDNA unusable as a "DNA barcode", which requires single copy markers. However, based on results of previous research [11–13, 15, 69, 70], ITS-rDNA does appear to have high utility as a phylogenetic marker for zoanthids even if it is not useful as a barcode.

4.4. Terrazoanthus *Distribution.* This paper is the second demonstrating the presence of the genus *Terrazoanthus* in the Atlantic, after [71], and this genus has now been reported from the eastern Atlantic (Cape Verde), Caribbean (this study), and eastern and central Pacific (Indonesia; *T.* sp. "yellow polyps" *sensu* Reimer and Fujii 2010) [74], and this genus has a circumtropical distribution as do *Palythoa*, *Zoanthus*, and *Isaurus*. However, most species in this genus are apparently either not common or inhabit "cryptic" habitats, as asides from *T. onoi* in [74] most species have only been recorded once or a few times. The *Terrazoanthus* specimen from this study was found growing in a darkened crevice in the interstices of an artificial jetty composed of boulders in 2 meters of water. The polyps were heavily encrusted with grains of silica sand and were growing upon crustose coralline algae. The environment in which the *Terrazoanthus* were growing is frequently subjected to heavy wave action and surge. Such environments are not common in the reefs of Florida, excepting man-made structures, and *Terrdzoanthus* may be rare in Florida.

5. Conclusions

This study demonstrates both the difficulties in identifying shallow water zoanthids to the species level, as well as the utility of DNA markers and phylogenetics. Furthermore, despite their regular occurrence in the shallow waters of the Caribbean, it appears that undescribed species potentially exist in this region. In the future, a detailed examination of ITS-rDNA with multiple specimens combined with comparison to the numerous species in literature should help ascertain Caribbean zoanthids' species diversity. The inclusion of specimens from "nonreef" habitats (i.e., docks/pilings, and mangrove swamps) is also needed to obtain a complete picture of zoanthid diversity in this region.

Appendix

A. Scientific Names and Authorities of Species Mentioned in Text, Figures, and Tables

P. caesia Dana 1846 [27]

P. caribaeorum (Duchassaing and Michelotti 1860) [30]

P. clavata (Duchassaing 1850) [28]

P. fusca (Duerden 1898) [37]

P. grandiflora (Verrill 1900) [40]

P. grandis (Verrill 1900) [40]

P. heliodiscus (Ryland and Lancaster 2003) [66]

P. mammillosa (Ellis and Solander 1786) [21]

P. mutuki (Haddon and Shackleton 1891) [35]

P. tuberculosa (Esper 1791) [76]

P. variabilis (Duerden 1898) [37]

H. gracilis (Lwowsky 1913) [46]

I. maculatus Muirhead and Ryland 1985 [61]

I. tuberculatus Gray 1828 [61]

Z. anduzii (Duchassaing and Michelotti 1860) [30]

Z. auricula (Lesueur 1817) [23]

Z. dubius Lesueur 1817 [23]

Z. flos-marinus Duchassaing and Michelotti 1860 [30]

Z. gigantus Reimer and Tsukahara 2006 (in Reimer et al. 2006) [72]

Z. kuroshio Reimer and Ono 2006 (in Reimer et al. 2006) [72]

Z. nobilis Duchassaing and Michelotti 1860 [30]

Z. nymphaeus (Lesueur 1817) [23]

Z. poriticola Pax 1910 [45]

Z. praelongus Carlgren 1954 [84]

Z. proteus Verrill 1900 [40]

Z. pulchellus (Duchassaing and Michelotti 1860) [30]

Z. sansibaricus Carlgren 1900 [73]

Z. sociatus (Ellis 1786) [21]

Z. solanderi Lesueur 1817 [23]

T. onoi Reimer and Fujii 2010 [11]

T. sinnigeri Reimer and Fujii 2010 [11].

Acknowledgments

The first author was supported in part by the Rising Star Program and the International Research Hub Project for Climate Change and Coral Reef/Island Dynamics (both at the University of the Ryukyus). Drs. E. Hirose (University of the Ryukyus), H. Fukami (Miyazaki University), and M. Costello (University of Auckland) are thanked for their help in discussing transisthmithian evolution.

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