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Applying Integrative Systematics to the Poorly Explored Symbiotic Relationships Between Echinoderms and Zoanthidea

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NOVA SOUTHEASTERN UNIVERSITY

HALMOS COLLEGE OF ARTS AND SCIENCES

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Abstract

Epizoic Zoanthidea are highly diverse and live symbiotically with at least six invertebrate phyla, but little is known about zoanthideans living on urchin spines. To better understand symbiotic interactions between Echinodermata and Zoanthidea, Atlantic deep-sea Cidaridae urchin specimens from five museums were surveyed to characterize Zoanthidea colonies on their spines. Specimens were obtained from the Gilbert and Nancy Voss Marine Invertebrate Collection at University of Miami, the Florida Museum Invertebrate Zoology Collection, and the collections of James Reimer and Hiroki Kise at the University of the Ryukyus. This research uses an integrative approach to species discovery and systematics. Integrative systematics aims to understand the evolution of morphology, phylogenetics, cytology, and ecology of many available specimens, described taxa, and species not yet identified. To assess species boundaries of museum specimens, known species and binned specimens were screened using species-level genetic markers, host preferences, and collection location and bathymetry, followed by construction of gross and microanatomical profiles of specimens within those bins to compare with previous descriptions or identify novel species. DNA extraction, PCR amplification, and DNA sequencing targeted the nuclear ribosomal internal transcribed spacer gene region for identification of molecular operational taxonomic units followed by molecular phylogenetic tree reconstruction. Although a single Cidaridae-symbiotic Zoanthidea species had been previously described from the Indo-Pacific deep-sea and cnidarian colonies associated with Atlantic deep-sea Cidaridae spines had been previously observed, this is the first research to attempt to verify their identity in the Atlantic deep-sea. Multiple Zoanthidea species were detected associated with at least four Cidaridae host species among the museum specimens examined. These Zoanthidean species here are assigned to genus *Epizoanthus* and have close affinity with two described Indo-Pacific *Epizoanthus* species (only one of which is known to associate with urchins). Given the novelty in hosts, ocean basin, morphology, and genetics, these species are likely new to science. The challenging nature of collections-based research resulted in incomplete data collection from all specimens examined which necessitates expansion of this effort to additional collections or fresh sampling.

Keywords: deep-sea, Zoanthidea, Echinodermata, integrative systematics, symbiosis, phylogenetics, morphology

Acknowledgments

The completion of this study would not have been possible without specimens provided by the Florida Museum (UF), the Gilbert and Nancy Voss Marine Invertebrate Collection (VMIC) of the University of Miami, and our collaborators at the University of the Ryukyus in Japan, Dr. James Reimer, and Dr. Hiroki Kise. I would like to thank my advisor, Dr. Timothy Swain, for his diligent guidance and mentoring. I appreciated his conscientious and attentiveness throughout the duration of this research. My gratitude extends to my committee members Dr. Jose Lopez and Dr. Gustav Pauly for their time and expertise. Thank you to Dr. Abigail Renegar for generously providing access to laboratory equipment for this project. Additionally, I would like to thank my friends and family for all the advice and immense support given to me over the last two years. This research was supported by a NSU President's Faculty Research and Development Grant to T. Swain.

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Introduction

Symbiosis is defined as the living together of different species in intimate associations (De Bary, 1879). Intimacy refers to their physical contact and physiological integration for most or all their respective life cycles. Symbiosis includes mutualism, parasitism, and commensalism. Changes in abiotic and biotic conditions including host species, host developmental stage, and accessibility to light or nutrition might influence how symbionts affect their host and can move symbiotic outcomes along the mutualism to parasitism continuum (De Bary, 1879).

Because species connections and interactions define the field of symbiosis, it calls for innovative study methods across multiple disciplines including evolutionary biology, ecology, physiology, and cellular and molecular biology (Saffo, 1992). The diversity of species involved in symbiotic associations and their specificity to symbiotic partners are two related features of symbiotic interactions that contribute to the knowledge of the ecology and evolution of symbiotic species. As a first step in describing a symbiosis, specificity in relationships including genotypes, ecotypes, species or genera, families, and orders is necessary (Swain & Wulff, 2007). The assessment of how biological systems are structured, function, and evolve is directly impacted by the ability to identify species as individual units of evolution. Interspecific interactions correlate with the fitness of associated species, especially in symbiotic systems. To provide sufficient rigor for species discovery and description, current taxonomic procedures require numerous lines of evidence (Swain & Wulff, 2007).

Molecular parataxonomy attempts to replace the existing morphology-based taxonomic system that has been used for hundreds of years with an entirely novel system based almost exclusively on DNA barcodes. These competing systems do not share characters and, therefore, taxa described in one system are unrecognizable to the second (Collins & Cruickshank, 2013, Desalle et al., 2005; Swain, 2018; Swain & Swain, 2014; Vogler & Monaghan, 2007). DNA barcodes are not available for most described species and cannot be obtained from most museum specimens. A DNA barcode is short nucleotide sequence from a single gene that is informationally limited, which make it inappropriate and uninformative for further analyses such as molecular phylogenetics because it results in poorly supported gene tree rather than robust species tree (Swain, 2018; Swain & Swain, 2014).

To bridge the molecular parataxonomic divide, an integrative approach that includes multiple data streams such as DNA, morphology, physiology, ecology, and other characters from the existing taxonomic system is necessary. Integrative systematics strives to better understand the evolution of characters of many available specimens, described taxa, and species that have not yet been discovered (Swain, 2017). The field known as "integrative taxonomy" attempts to identify diversity from a variety of complementing data (Dayrat, 2005). Compared to species supported by just one form of data, the degree of confidence in these species will be substantially higher. Because of the need for accuracy in species delineation, a variety of approaches should be used (Dayrat, 2005). An integrative systematics approach generates more robust hypotheses by integrating comprehensive studies from complementary data streams (Swain, 2017).

Zoanthidea are an understudied order of anthozoan cnidarians that are one of the most diverse of hexacorallians, behind actinarians (anemones) and sclaractinians (corals). The most common and best documented Zoanthidea are found on shallow coral reefs, but they occur globally from the intertidal to the deep sea (Swain, 2010). Zoanthidea of the suborder Macrocnemina are epizoic, living as symbionts on various host organisms such as sponges, hermit crabs, octocorals, and echinoderms at photic, mesophotic, and deeper depths (Swain, 2010). Zoanthidea appear diverse but largely unexplored at depths greater than 200 m. There are approximately 280 described Zoanthidea species, but an estimated 1,200 undescribed species, making them a taxonomic research priority (Appeltans et al., 2012).

The specific arrangement of tentacles and mesenteries, marginal musculature, a single siphonoglyph, mesoglea permeated by canals, encrustations of exterior tissue and/or mesoglea, and colonial growth forms are all features of zoanthidean morphology (Figure 1) (Swain et al., 2017). On the outside of the oral disc, the tentacles arrange in two cycles. The number of tentacles is equal to the number of mesenteries. The inner cycle of tentacles erects and arises from the spaces within mesentery pairs (endocoels), whereas the outer cycle of tentacles is reflexed and arises from gaps between mesentery pairs (exocoels). Tentacles are hidden by the capitulum in the retracted position and are only visible when sectioned. The columnar mesoglea projects centripetally into the gastrovascular cavity to form the mesenteries. Macrocneme and microcneme make up the pairs of mesenteries. “Perfect” mesenteries called macrocnemes link to the actinopharynx or terminate in a mesenterial filament below it. The actinopharynx is not

connected to micronemes, which are "imperfect" mesenteries that are almost vestigial and do not wield a mesenterial filament. The siphonoglyph of the actinopharynx serves as the directing axis, and polyps are biradially symmetrical along this axis (Swain et al., 2017). The actinopharynx joins the mouth to the coelenteron. At the ventral tip of the actinopharynx, there is a single siphonoglyph, which is a ciliated groove that runs its entire length. In addition to having longitudinal ridges above the endocoels, the capitulum is frequently heavily furrowed and may have protuberances. The marginal teeth alternate with the outer cycle and often make up half of the tentacles and mesenteries as the endocoels give rise to the inner cycle of tentacles. Repetitive structures within the polyp are organized as components within modules. A single marginal tooth, capitular ridge, and esophageal furrow, along with a pair of tentacles, oral disc furrows, mesenteries, and coelic chambers, make up each module (Swain et al., 2017).

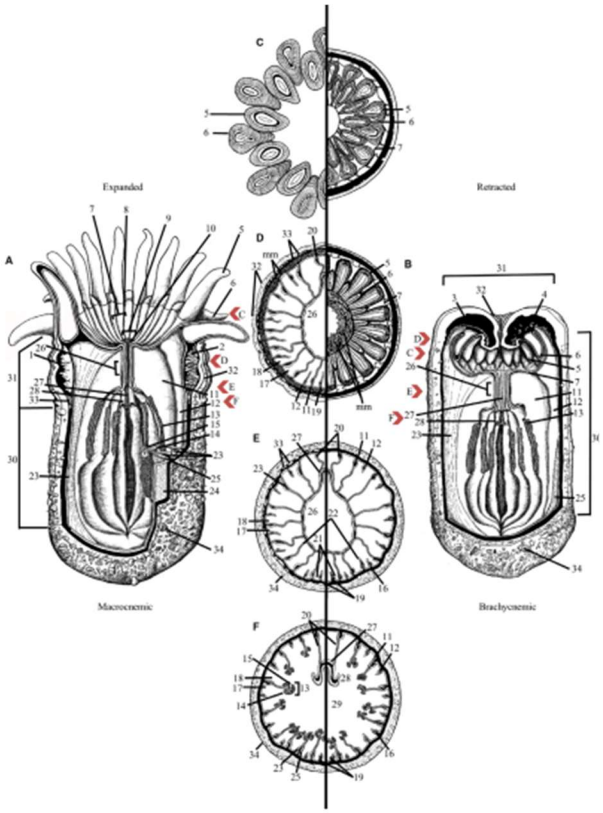

Fig. 1 Zoanthidea anatomy. Polyps are shown with an external longitudinal cutaway in the expanded (A) and retracted (B) state to demonstrate the transformation due to retraction. The marginal musculature (mm) is shown (unnaturally chimeric and enlarged in each polyp) in cteniform endodermal (1), reticulate mesogleal (2), linear mesogleal (3) and discontiguous mesogleal (4) arrangements. Transverse sections shown at the height of the tentacles (C), capitulum (D), actinopharynx (E) and mesenterial filaments (F). Visible anatomical features include inner endocoelic tentacle (5), outer exocoelic tentacle (6), oral disc (7), oral furrows (8), peristome (9), mouth (10), macrocneme (11), microcneme (12), mesenterial filament (13), lateral ciliated tract (14), central cnidoglandular tract (15), mesenterial canal (16), endocoel (17), exocoel (18), dorsal directives (19), ventral directives (20), proliferation chambers (21), fifth couple (22), longitudinal mesenteric retractor (23), columnar circular muscle (24), oblique mesenteric parietobasilar muscles (25), actinopharynx (26), siphonoglyph (27), hyposulcus (28), coelenteron (29), scapus (30), capitulum (31), capitular ridges (32), ring sinus (33) and crust shell (34). Drawings by W.B.

Figure 1: Features of Zoanthidean morphology (Fig. 1 from Swain 2017 (Swain et al., 2017))

Zoanthideans challenge the ability to detect species boundaries because of their simple morphologies and varied coloration (Swain, 2009). Over the past two decades, molecular parataxonomy has been intensively implemented in Zoanthidea taxonomy and systematics research to delineate species (Swain, 2018; Swain & Swain, 2014).

Many recently described zoanthidean taxa are defined solely by nucleotide sequence, with little or no description of the characteristics that identify higher taxa and relate nucleotide-based data to the existing taxonomic system. Identifying conspecifics, congeners, and confamiliars of emerging taxa without knowledge of form may be challenging among the hundreds of specimens and reported species for which nucleotide sequencing is not accessible. Furthermore, some nucleotide sequences are invariant or inconsistently distinguished amongst congeners, reducing the applicability of nucleotide-based taxon classifications (Swain & Swain, 2014). Systematic knowledge has greatly benefited from molecular approaches. But to optimize knowledge, explanation, and stability, many systematists are recognizing the importance of multidisciplinary studies and fusing as many information sources as they can. It is impossible for a single systematic data set to concurrently provide information at all phylogenetic levels. Phylogeny is the evolutionary history of organisms inferred from characters. Phylogenetic systematics uses evolutionary history to understand the relationships between species and organize them into higher taxa (Wiley & Lieberman, 2011). Some methods are helpful for answering phylogeny-related problems among closely related species, whereas other methods are helpful for spanning ancient time periods. For phylogenetic resolution within a group of interest to be maximized, many alternative approaches are frequently needed (Hillis, 1987).

There is almost nothing known about zoanthidean symbiosis with Echinodermata except for two Indo-Pacific species living on sea urchins and crinoids. There is an undescribed *Abyssoanthus* species associated with the crinoid *Metacrinus rotundus* (Carpenter & von Graff, 1885) from mesophotic depths (108–146 m) off the Japanese coast (Zapalski et al., 2021) and *Epizoanthus planus* (Carlgren, 1923) associated with the spines of Cidaridae sea urchins from aphotic depths (741–1019 m) in the Indian Ocean and East China Sea (Kise et al., 2018). There are no described echinoderm-symbiotic zoanthidean species from the Atlantic, but presumed cnidarians have been observed on the urchin *Stylocidaris lineata* (Mortensen, 1910) and there are certain to be other potential examples buried in the literature and natural history collections of

the world (Ryland & Ward, 2016). This thesis aims to make use of those observations and collections to discover zoanthidean species symbiotic with Atlantic echinoderms.

Although zoanthideans are an infamously understudied order of cnidarians, the suborder containing invertebrate-symbiotic species (suborder Macrocnemina) (Haddon & Shackleton, 1891) has been sufficiently examined to suggest multiple testable hypotheses about hypothetical Atlantic Echinodea symbionts. Most macrocnemic zoanthidean species are linked to a variety of marine invertebrates through symbiosis including glass sponges, demosponges, hermit crabs, gastropods, gorgonians, antipatharians, and eunicid worms (Swain, 2010). The only described zoanthidean species associated with echinoderms is *Epizoanthus planus* from the Indian Ocean between 741–1019 m and further observed in the Indian Ocean and the East China Sea at depths ranging from 420–1019 m. In the Atlantic, specimens presumed to be *Epizoanthus* have been observed on the spines of sea urchins in the Caribbean and the Azores at 500m (Kise et al., 2018). Because of striking phylogenetic conservatism among macrocnemic Zoanthidea in both symbiosis and anatomical characters (Swain, 2010; Swain, 2014; Swain, 2017), any hypothetical Atlantic Zoanthidea species should share similarities with *Epizoanthus planus* and its sister species. Synapomorphy is a shared derived character from a group of organisms whereas homoplasy is a trait without a common ancestorial origin. Synapomorphic characters determine a clade, which is a grouping of organisms comprised of two or more species that includes the ancestral species and its decedents. The retention index is the inverse of homoplasy where the higher the retention index, the lower the homoplasy (Wiley & Lieberman, 2011). The hypotheses below stemmed from phylogenetically conserved characteristics (Table 1) that have been previously identified (Swain, 2017).

Table 1: Retention Values extracted from Fig. 5 from Swain, 2017

Synapomorphy Characters:	**Retention Index Values (0-1):**
Coloniality	≅ 0.7
Arrangement of Marginal Musculature	≅ 0.8
Symbiotic Invertebrate host	≅ 0.9
Max # of Modules	≅ 0.5

Hypothesis 1: Atlantic urchin-symbiotic Zoanthidea species should have colony and gross polyp morphology similar to *Epizoanthus planus* and its sister species (Swain, 2017).

Epizoanthus planus has approximately ten polyps that make up the colony, joined by well-developed coenenchyme on Cidaridae sea urchin spines. The oral disk has the same number of oral furrows as tentacles. Each polyp has between 20–22 tentacles and 10–12 capitular ridges. The tentacles are organized in two rows extended from the oral disk. When the polyps are expanded, the oral disk has a diameter of 5–10 mm, and the height of living polyps is 2–4 mm. Contracted polyps measure 3–5 mm in diameter and 1–3 mm in height from the coenenchyme when preserved (Kise et al., 2018). Sand particles of varied sizes are deeply embedded in the ectoderm of polyps and coenenchyme. The outer edge of the oral disk similarly develops encrustation; however, it lessens as it gets closer to the mouth. (Kise et al., 2018).

Hypothesis 2: Atlantic urchin-symbiotic Zoanthidea species should have microanatomical characters, particularly features of the marginal musculature, similar to *Epizoanthus planus* and its sister species (Swain, 2015).

Epizoanthus planus has reticulate mesogleal marginal musculature characterized by haphazardly formed irregularly shaped lacunae along its length, giving the supporting mesoglea the appearance of a reticulate mesh. The muscle fills the entire distal diameter of the mesoglea and narrows near the proximal terminus (Swain et al., 2015). *Epizoanthus planus* is closely related to *Epizoanthus arenaceus* and share a similar marginal musculature structure (Figure 2 & 3). While the specific dimensions of the marginal musculature of *E. planus* are unknown, *E. arenaceus* has been measured. The marginal musculature dimensions within reticulate mesogleal muscle of *E. arenaceus* are 884–2080µm in length, 132–445µm in width, with 51–298 lacunae that are 56–339µm in size, and a muscle cross sectional-area of 3.55–17.4 x 10^4 μm^2 (Swain et al., 2015).

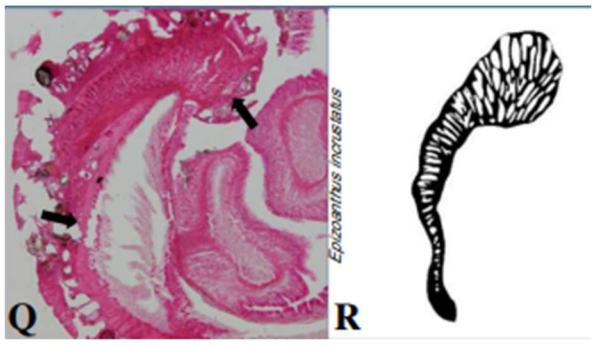

Figure 2: Reticulate mesoglea arrangements (Fig.1 from (Swain et al., 2015)

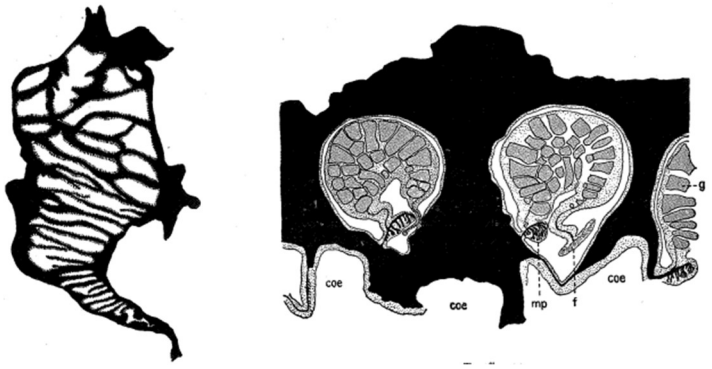

Figure 3: Marginal musculature of *Epizoanthus planus* (Fig.19 & 20 from (Carlgren, 1923)

Hypothesis 3: Zoanthideans associated with echinoids will share a recent common ancestor. Atlantic urchin-symbiotic Zoanthidea species should be a member of genus *Epizoanthus* (Swain, 2015, Swain ,2020).

Epizoanthus planus is closely related to free-living *Epizoanthus arenaceus* according to phylogenetic trees (Figures 4 and 5) from Kise 2018, Fig. 3 (Kise et al., 2018) and Swain 2018, Fig. 2(Swain, 2018). Zoanthideans associated with echinoids should fall into this clade.

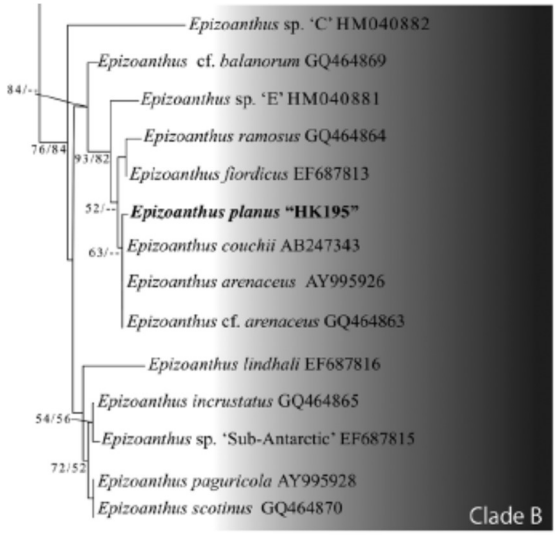

Figure 4: *Epizoanthus planus* phylogenetic tree (Fig. 3 from (Kise et al., 2018)

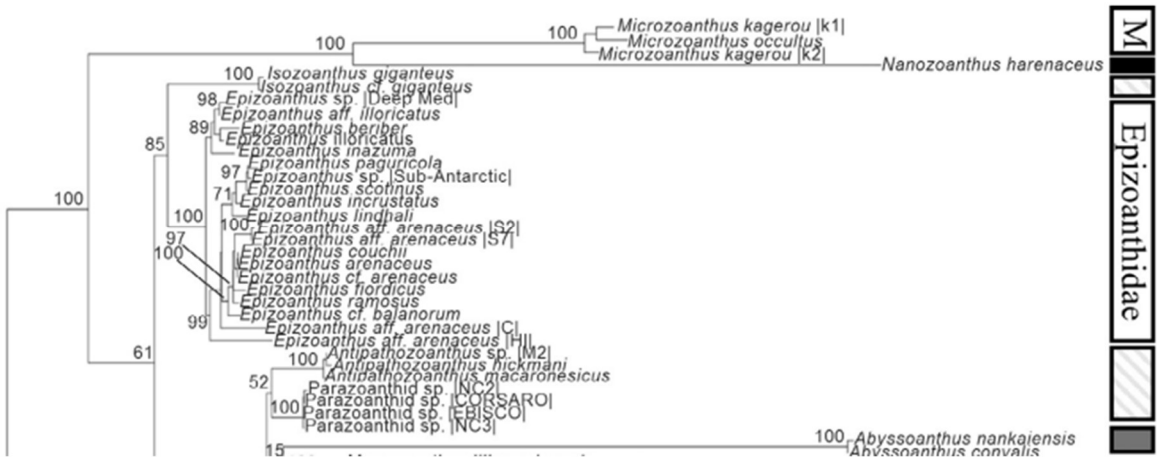

Figure 5: Portion of a phylogenetic tree with *Epizoanthus arenaceaus* (Fig. 2 from Swain 2018 (Swain, 2018)

Hypothesis 4: Atlantic urchin-symbiotic Zoanthidea species should associate with hosts within the Cidaridae family of sea urchins (Swain, 2017).

Zoanthidea symbioses have host species associations reflective of their phylogenetic position. *Epizoanthus planus* has been described living on spines of Pacific sea urchins of the family Cidaridae (Kise et al., 2018). There are no described echinoderm-symbiotic zoanthidean species from the Atlantic, but potential zoanthideans have been observed on the urchin *Stylocidaris lineata* (Mortensen, 1910), also from the family Cidaridae. Variation from the known host species ranges can be an indication of cryptic symbiont species among zoanthideans (Swain, 2018; Swain & Swain, 2014).

This thesis aims to: (1) detect and identify Atlantic zoanthidean species associated with echinoderms; (2) compare morphology among potential new Atlantic species and with known Pacific species; (3) determine phylogenetic position of potential new Atlantic species within the echinoderm-symbiotic and sister taxon clade; (4) identify host species for Atlantic echinoderm symbiotic zoanthideans; and (5) identify next steps for further development of this system.

Materials and Methods

Collection:

Echinoderm-symbiotic zoanthideans are deep-sea organisms and as such are difficult and costly to collect. To maximize sample sizes while minimizing collection costs, existing collections in five natural history museums were used to investigate the unexplored symbiotic

relationships between Atlantic Echinodermata and Zoanthidea. At this point, the only described zoanthidean-urchin symbioses are from the Indo-Pacific and the only indication that these symbioses might occur in the Atlantic are from a photograph (Fig #2 in Ryland & Ward, 2016). The invertebrate zoology echinoderm collections of deep-sea urchins in order Cidaroida contained in the Florida Museum, Yale Peabody Museum, Harbor Branch Oceanographic Institute, the University of Miami (Gilbert and Nancy Voss Marine Invertebrate Collection), and University of the Ryukyus were visually inspected to identify specimens with potential zoanthidean colonies encrusting their spines. Deep-sea sea urchin species with unidentified zoanthideans on the spines were found in the Florida Museum (UF) and the Gilbert and Nancy Voss Marine Invertebrate Collection (VMIC). Our collaborators at the University of the Ryukyus in Japan, Dr. James Reimer, and Dr. Hiroki Kise, also provided preserved specimens of *Epizoanthus planus* and other unidentified urchin symbionts (Table 2).

Table 2: Examined Museum Specimens

Source	**Specimen ID**	**Species**	**Host**	**Collection date**	**Site**	**Depth (m)**	**Lat**	**Long**	**Fixative**
VMIC G-179 July 1-63	371	*Epizoanthus sp.*	Echinoid	7/1/1963	N of Grand Bahama	558	27°46' N	79°13' W	Unknown
VMIC 42.115	372	unknown	*Cidaris abyssicola*	1/30/1964	W of Bimini	463	25°42' N	79°23' W	Unknown
VMIC 42.447	373	unknown	*Cidaris blakei*	7/22/1965	Freeport, Grand Bahama	650	26°27' N	78°47' W	Unknown
VMIC 42.237	374	*Epizoanthus sp.*	*Cidaris blakei*	3/3/1965	Freeport, Grand Bahama	614	26°19' N	78°43' W	Unknown
VMIC 42.306	375	unknown	*Cidaris blakei*	2/5/1964	N of Grand Bahama	490	27°30' N	78°38' W	Unknown
VMIC 42.289	376	*Epizoanthus sp.*	*Cidaris ragosa*	1/30/1964	E of Bimini	415	25°41' N	79°12' W	Unknown
H. Kise	383	*Epizoanthus sp.*	Cidaridae	03/12/2008	Hirajisone, Nagasaki, Japan	280	NA	NA	70% EtOH
H. Kise	384	*Epizoanthus sp.*	Cidaridae	NA	Suruga Bay, Shizuoka, Japan	140-340	34°50' 03 N	138°30 '37 E	99% EtOH
H. Kise	385	*Epizoanthus*	Cidaridae	10/28/2018	Sagami Bay,	300-400	35°4′50.0″N	139°35′53.0″	99% EtOH

		planus			Kanagawa, Japan			E	
H. Kise	386	*Epizoanthus planus*	Cidaridae	10/29/2015	Sagami Bay, Kanagawa, Japan	300-400	35°4′50.0″N	139°35′53.0″E	10% formalin SW -> 70% EtOH
H. Kise	387	*Epizoanthus sp.*	Cidaridae	02/05/2020	Sagami Bay, Kanagawa, Japan	200-400	35°09'34 N	139°35'45 E	99% EtOH
UF 17185	455	unknown	*Cidaris blakei*	04/02/2018	Bahamas	675	24.78503	-76.33962	75% ethanol
UF 17186	456	*Epizoanthus* aff. *ramosus*	Cidaridae	08/03/2014	French Guiana	195-200	6.6576667	-52.509	75% ethanol
UF 17187	457	*Epizoanthus* aff. *planus*	Cidaridae	08/07/2014	French Guiana	200	5.49	-50.9715	75% ethanol
UF 17188	458	*Epizoanthus* aff. *planus*	Cidaridae	08/07/2014	French Guiana	225	5.5108333	-50.9326667	75% ethanol

All cidaroid specimens were pulled from the shelves and inspected for zoanthideans on the spines. The spines with zoanthideans were taken from the urchin host and placed into a test tube with 100% EtOH. Spines were taken back to the Evolution of Marine Symbiosis Laboratory at Nova Southeastern University for analysis. All loaned specimens were given back to the museums with identifications, histological slides, and DNA sequences.

Morphology:

Polyps were decalcified in formic acid and desilified in hydrofluoric acid to remove foreign encrustations and mesogleal inclusions before being embedded. Acid digested polyps were incubated in an ethanol series of increasing concentration before being switched to xylene, xylene paraffin mixture, and finally immersed in pure paraffin wax and mounted to a tissue cassette. Using a Leica RM2125 RTS microtome, four of the embedded polyps were cut into serial 10µm longitudinal (2 polyps) and cross sections (2 polyps). The 10µm serial sections were attached to poly-L-lysine coated glass slides and stained with Harris' hematoxylin and eosin Y and covered with Permount and a glass slip. Slides were examined with a Leica DM 2500 LEP microscope and photographed using a Leica DMC 4500 digital camera. Anatomical features

were measured using Leica LAS-X micrograph analysis software and images were submitted to MorphoBank as digital vouchers documenting the serial sections.

DNA Extraction, PCR Amplification, and Sequencing:

Total genomic DNA was extracted using the cetyl-trimethyl-ammonium bromide (CTAB) method (Doyle & Doyle, 1987). Successful extraction was assessed through agarose gel (3%) electrophoresis and visualization of ethidium bromide-stained DNA under ultraviolet illumination. Specimens that failed to result in detectable DNA through gel electrophoreses were reextracted with a modified DNA extraction protocol used for formalin-fixed paraffin embedded medical specimens outlined in Nguyen et al. (2022). This extraction protocol is intended to partially reverse formaldehyde modification of nucleic acids from formalin-fixed specimens through extended proteinase digestion and heat shock. Heating at high temperature (94°C) at an alkaline pH (pH = 8.0) has been shown to be crucial for improving the quantity and quality of the extracted DNA by reversing the crosslinking between nucleic acids and proteins (Nguyen et al., 2022).

A polymerase chain reaction (PCR) selectively amplified ITS markers. Targeted genes included the complete ITS region, 18S, ITS1, 5.8S, and ITS2, ribosomal RNA (rRNA) genes using the primers in Table 3 from (Swain, 2010). PCR parameters were 94 ℃ for 3 minutes, followed by 32 cycles of 94 °C. for 30 seconds, 50 °C for 60 seconds, and 72 °C for 90 seconds, with a final extension step of 72 °C. for 10 minutes. Illustra ExoProStar enzymatic digestion was used to purify the PCR product, which was sequenced in both forward and reverse directions using the amplification primers and BigDye chemistry at the Florida State University Sequencing Facility. The complete ITS region is a species-level marker and was compared to all other known zoanthidean sequences through BLAST searches of GenBank to bin specimens of the same species and provide a first estimate of the number of species and their clade membership.

Table 3: Description and corresponding amplification information for PCR primers used to generate the sequence data

Gene	Primer	Sequence	Annealing temp	Fragment size
ITS	f r	CTAGTAAGCGCGAGTCATCAGC GGTAGCCTTGCCTGATCTGA	50C	770–943

Morphological analysis:

All specimens were examined for external and internal anatomical features. The number of polyps per colony were counted and averaged based on the number of colonies observed. The number of capitular ridges per polyp were counted and averaged based on the number of polyps observed. Individual polyps were selected from each sample, dissected from their colonies, and sectioned to identify and measure the important morphological characteristics that lead to assessment of species identification. The information gathered from the photographed portions were examined according to Swain 2017 (Swain et al., 2017). The diameter of the muscle, the longest attachment site, the muscle surface area, the length of the marginal muscle, and the number of attachment sites were measured in longitudinal histological sections.

Phylogenetic analysis:

Forward and reverse sequence reads and chromatographs were visualized, verified, and assembled into contigs using the Contig Assembly Program CAP in BioEdit version 7.2.5 (Hall, 1999). Museum specimen sequences were coupled with *Epizoanthus* ITS sequences downloaded from GenBank. BioEdit was used to remove amplification primers from GenBank sequences. Through multi-sequence alignment, homologous sites are deduced from sequence similarity. DNA sequences were assembled into an alignment using the default penalty settings of Clustal W (Thompson, Higgins, & Gibson, 1994) within BioEdit .

The molecular data was analyzed using a model inferential type (Stamatakis, 2014). The multi-sequence alignment serves as the homology statement and character data for phylogenetic tree inference. The alignment was partitioned at the boundary of each gene (18S, ITS1, 5.8S, & ITS2) to be fitted to a molecular evolution mode. Phylogenetic tree inference was performed on the partitioned sequence alignment using maximum-likelihood in the Randomized Axelerated Maximum Likelihood (RAxML) v8.2.8 (Stamatakis, 2014) program within the CIPRES Science Gateway v3.3. The Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway is the NSF & NIH-sponsored project that allows remote access to clustered supercomputing assets for rapid phylogenetic inference. General Time Reversable (GRT) + gamma shape + invariable sites model parameters were empirically determined from the partitioned data by RAxML simultaneously with tree inference. Four taxa were preassigned to the outgroup based on previous molecular phylogenies of Zoanthidea (Swain, 2018, Kise et al., 2022). An outgroup is organisms that are more distantly related and are used as a reference point when determining

evolutionary relationships (Wiley & Lieberman, 2011). This reference point allows the determination of directionality in evolutionary relationships in phylogenetic analyses (Wiley & Lieberman, 2011). The taxa used in the outgroup were *Epizoanthus inazuma, Epizoanthus beriber, Epizoanthus illoricatus,* and *Epizoanthus aff illoricatus.* Node support was determined from 1000 pseudoreplicates generated by RAxML.

Results:

Polyp and colony morphology

The mean number of polyps per colony ranged from 8-18 and the number of mesenteries per polyp ranged from 14-24 (Table 4). All polyps had retracted to a point that completely hid their tentacles, except for specimen 386, so capitulary ridges were counted and doubled to approximate tentacle and mesentery counts (Figure 6, 7, & 8).

Table 4: Colony and Gross Polyp Morphology

Source	**Specimen ID**	**Species**	**# of mesenteries**	**avg. # polyps per colony**	**n_{polyps}, $n_{colonies}$**
VMIC G-179 July 1-63	371	*Epizoanthus sp.*	22-24	13	39, 3
VMIC 42.115	372	unknown	18-20	18	18, 1
VMIC 42.447	373	unknown	20-22	7	14, 2
VMIC 42.237	374	*Epizoanthus sp.*	14-20	7-8	29, 4
VMIC 42.306	375	unknown	indistinct	13	13, 1
VMIC 42.289	376	*Epizoanthus sp.*	14-16	6	6, 1
H. Kise	383	*Epizoanthus sp.*	indistinct	16	32, 2
H. Kise	384	*Epizoanthus sp.*	18-24	8	8, 1
H. Kise	385	*Epizoanthus planus*	20-22	8	8, 1
H. Kise	386	*Epizoanthus planus*	18-20	7	7, 1
H. Kise	387	*Epizoanthus sp.*	indistinct	7	7, 1
UF 17185	455	unknown	24	11-12	67, 6
UF 17186	456	*Epizoanthus* aff. *ramosus*	indistinct	6	6, 1
UF 17187	457	*Epizoanthus* aff. *planus*	16-18	12	24, 2

UF 17188	458	*Epizoanthus* aff. *planus*	indistinct	6-7	31, 5

Figure 6: Capitulary ridges of specimen 455

Figure 7: Tentacles of specimen 386

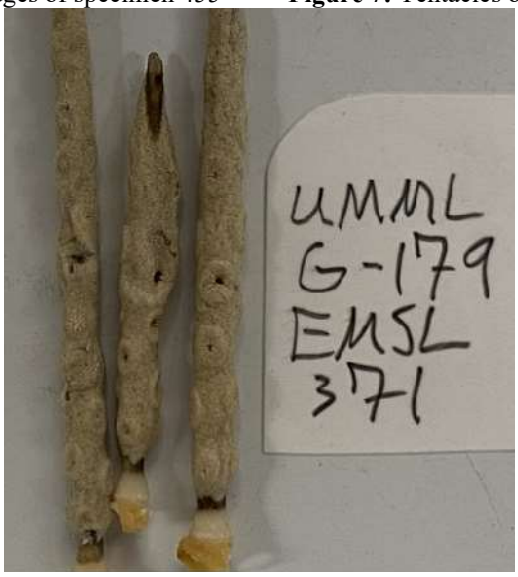

Figure 8: Specimen 371

Microanatomy

Four out of 14 specimens produced longitudinal sections where diameter of the muscle, the longest attachment site, the muscle surface area, the length of the marginal muscle, and the number of attachment sites could be measured (Table 5, Figure 9). Specimens 371, 374, and 376 were unknown species of Zoanthidea from the University of Miami. Specimen 386 was identified by H. Kise as *Epizoanthus planus*. The histological sections of these specimens revealed similar reticulate mesogleal marginal musculature (Figure 9). The marginal musculature dimensions were 285–380µm in length, 90–125µm in width, with 51–298 lacunae that are 47–90

μm in size, and a muscle cross sectional-area of 9,630–20,000 μm^2. The number of attachment sites varied from 15–35 (Table 5).

Table 5: Marginal Musculature

			Log of CS-area of marginal muscle (um^2)	Length of marginal muscle (μm)	Diameter of marginal muscle (μm)	Height of attachment sites of marginal muscle (μm)	# of attachment sites of the marginal muscle
Source	**Species**	**Specimen ID**					
VMIC G-179 July 1-63	*Epizoanthus sp.*	371	17672.28	328.5	122.16	56.17	30
VMIC 42.237	*Epizoanthus sp.*	374	13361.3	355.74538	124.65687	89.32244404	23
VMIC 42.289	*Epizoanthus sp.*	376	19659.52	380.3231	98.45318	75.824632	35
H. Kise	*Epizoanthus planus*	386	9631.111	285.3847	90.61673	47.71083428	15

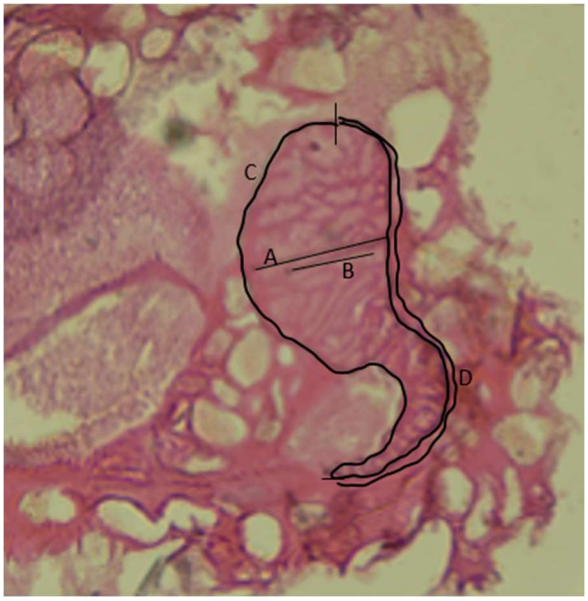

Figure 9: *Epizoanthus* (associated with *Cidaris ragosa*) polyp specimen 376 shown in longitudinal histological section. The labeled features are the diameter of the muscle (A), longest attachment site (B), muscle surface area (C), and length of the marginal muscle (D).

Sequence alignment and Phylogeny

DNA sequencing of ITS rRNA for Atlantic Cidaridae-associated zoanthideans assembled into contigs of 695-743 nucleotides per specimen and were matched by BLAST search of GenBank at >96% to sequences of Pacific *Epizoanthus planus* and *Epizoanthus ramosus*, but not identical to either nor to each other (Table 6, 7, 8). Compilation of newly collected sequences with 12 *Epizoanthus* sequences from GenBank resulted in an alignment of 731 positions which was partitioned along gene boundaries (152-230 positions per partition; Table 9) for molecular evolution model fitting and maximum likelihood phylogenetic tree inference and nodal support bootstrapping. The resulting tree is completely resolved with a final likelihood score of -2651.55 and well-supported with bootstrap values ranging between 41-100 and significant support (>70) at 87% (13/15) of internal nodes (Figure 11, Table 9). All of the Atlantic specimens are positioned within a well-supported *Epizoanthus planus* / *Epizoanthus ramosus* clade and support the interpretation that there are at least two new Atlantic species of urchin-symbiotic Zoanthidea.

Gene Sequence Data

Gel electrophoresis was run through a sodium borate (SB) medium. SB is a conductive medium for DNA detection. PCR product, ladder, and controls were loaded into the gel. The ladder used was Fisher GENERULER 100BP (Table 6, Figure 10).

Table 6: Gel Electrophoresis Interpretation

Lane #	Specimen #	Genus	Primers	PCR Detection Gel Band size	Extraction Gel Band presence	PCR Detection Gel Band Presence
1	371	*Epizoanthus*	ITS-ZoanF/R		O	O
2	372	*Epizoanthus*	ITS-ZoanF/R		O	O
3	373	*Epizoanthus*	ITS-ZoanF/R		O	O
4	374	*Epizoanthus*	ITS-ZoanF/R		O	O
5	375	*Epizoanthus*	ITS-ZoanF/R		O	O
6	376	*Epizoanthus*	ITS-ZoanF/R		X	O
7	383	*Epizoanthus*	ITS-ZoanF/R		X	O
8	384	*Epizoanthus*	ITS-ZoanF/R	Band @ 1.2K nt	X	X
9	ladder					

10	385	*Epizoanthus*	ITS-ZoanF/R	Band @ 1K nt	X	X
11	387	*Epizoanthus*	ITS-ZoanF/R		X	O
12	455	*Epizoanthus*	ITS-ZoanF/R		O	O
13	456	*Epizoanthus*	ITS-ZoanF/R	Faint band @ 900 nt	O	X
14	457	*Epizoanthus*	ITS-ZoanF/R	Faint band @ 900 nt	X	X
15	458	*Epizoanthus*	ITS-ZoanF/R	Faint band @ 1K nt	X	X
Not shown	411	Zoanthidea	ITS-ZoanF/R	Positive Control	X	
Not shown	-	Neg Control	ITS-ZoanF/R	Blank	O	

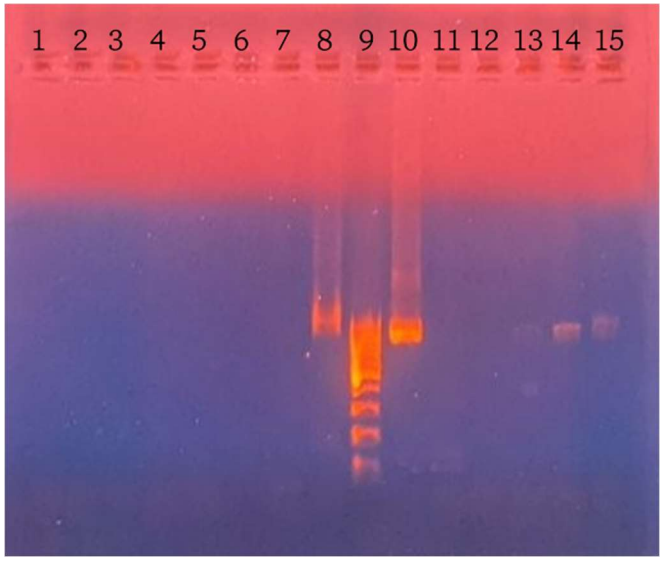

Figure 10: PCR Detection Gel of ITS1

Table 7: Genbank BLAST search results

Specimen ID	Taxa	Assembled contig length	% ID match	Closest taxon match
384	*Epizoanthus*	702	98.13	*Epizoanthus planus* MISE-HK195
385	*Epizoanthus*	743	98.11	*Epizoanthus cf. ramosus* TDS-2010 NIP154
456	*Epizoanthus*	721	98.6	*Epizoanthus cf. ramosus* TDS-2010 isolate NIP154
457	*Epizoanthus*	695	97.38	*Epizoanthus planus* MISE-HK195
458	*Epizoanthus*	700	96.7	*Epizoanthus planus* MISE-HK219

Table 8: DNA sequences

Specimen ID	Complete ITS region sequence (18S, ITS1, 5.8S, ITS2)
384	CTTGTTCTACTACTAACTGTTGGCTGATCGAGACCGTCTGATGGCCGCCACCCCCCCGCATTCTG ATCACATTATGTCAAATAATTGGTCGCTACCGATCATTTAGAGGAAGTAAAAGTCGTAACAAGG TTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGATCATACAAGACCCTACGCGGTTTTGCACT GTGAACCTTACCAAACTTTAGGGGTAGGCTTCGGTCTTTAAATGGTGTGGGTTTAATTTGCCAGC CCCGCTGGTACTTTTTTTCCCACACCGGCCCCGCTCGTGTTTTACCATTTTTAATACACCTTCTCG ACTGAAACTGCGAGATTTGCTTCTCGCGACAAACTGAAAAGAAGACAACTTTTAACGGTGGATC TCTTGGCTCGTGCATCGATGAAGAACGCAGCCAGGTGCGATAAGTAGTGTGAATTGCAGAATTC AGTGAATCATCGAATCTTTGAACGCAAATGGCGCTCTTGGGTTCTCCCAGGAGCATGTCTGTCTG AGTGTCGGTTTACAAACTAACGCGCGACGCTTTTTCTAAGCGTGTCGGTGCGGCGTTGAGGCGTC ATGGCTTACCTACCGGCAGTCTAGGTCGTTCGAATCGCAGCCAGATTCGAGACGGTCGCGCGAA ACCCACCGTCATCAGCCAAAAACGATGTGTCCTTTGGGTGGTCCAAGATGTTCCA
385	CCTGACCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGGCCTCCTGATT GGCGCCGCCGCCCCGGAAACGGAGCAGCGGATGTCGAAAAGTTGGTCAAACTTGATCATTTAGA GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGATCATAC AAGACCCAATGCGGTTTTGCACTGTGAACCTTACCAAACTTTAGGGGTAGGCTTCGGTCTTTAAA TGGTGTGGGTTTAATTTGCCAGCCCCGCTGGTACTTTTTTTCCCACACCGGCCCCGCTCGTGTTTT ACCATTTTTAACACACCTTCTCGACTGAAACTGCGAGATTCACTTCTCGCGACAAACTGAAAAGA AGACAACTTTTAACGGTGGATCTCTTGGCTCGTGCATCGATGAAGAACGCAGCCAGGTGCGATA AGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAAATGGCGCTCTTGGGTT CTCCCAGGAGCATGTCTGTCTGAGTGTCGGTTTACAAACTAACGCGCGACGCTTTTCCTAAGCGT GTCGGCGCGGCGTTGAGGCGTCATGGCCTACCTACCACGGCAGGCTGTGTCCTTCGAAATGCAG CGAGACTCGGTTGCGTAGATTCCGCATCGCAACTCAAGAGGCCAAAATTATTTCGTCCTCTCGGT GCGTTGCGGCTCTCCTACCTGGGTCGAATGG
456	TGTACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGGCCTCCTGATTGGCGCCGCC GCCCCGGAAACGGAGCAGCGGATGTCGAAAAGTTGGTCAAACTTGATCATTTAGAGGAAGTAAA AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGATCATACAAGACCCAA TGCGGTTTTGCACTGTGAACCTTACCAAACTTTAGGGGTAGGCTTCGGTCTTTAAATGGTGTGGG TTTAATTTGCCAGCCCCGCTGGTACTTTTTTTCCCACACCGGCCCCGCTCGTGTTTTACCATTTTTA ACACACCTTCTCGACTGAAACTGCGAGATTCGCTTCTCGCGACAAACTGAAAAGAAGACAACTT TTAACGGTGGATCTCTTGGCTCGTGCATCGATGAAGAACGCAGCCAGGTGCGATAAGTAGTGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAAATGGCGCTCTTGGGTTCTCCCAGGA GCATGTCTGTCTGAGTGTCGGTTTACAAACTAACGCGCGACGCTTTTCCTAAGCGTGTCGGCGCG GCGTTGAGGCGTCATGGCCTACCTACCACGGCAGGCTGTGTCCTTCGAAATGCAGCGAGACTCG GTTGCGTAGATTCCGCATCGCAACTCAAGAGGCCAAAATCATTTCGTCCTCTCGGTGCGTTGCGG CGATCCTAC
457	GCTCACCTCCTACCGATTGAATGGTTTAGTGAGGCCTCCTGATTGGCGCCGCCGCCCCGGAAACG GAGCAGCGGATGTCGAAAAGTTGGTCAAACTTGATCATTTAGAGGAAGTAAAAGTCGTAACAAG GTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGATCATACAAGACCCTATGCGGTTTTGCAC TGCGAACCTTACCAAACTTTAGGGGAAGGCTCCGGCCTTTAATGGGGGGGGGTTAAATTTCCA GCCCCGCTGGTACTTTTTTTCCCACACCGGCCCCGCTCGTGTTTTACCATTTTTAATACACCTTCT CGACTGAAACTGCGAGATTCGCTTCTCGCGACAAACTGAAAAGAAGACAACTTTTAACGGTGGA TCTCTTGGCTCGTGCATCGATGAAGAACGCAGCCAGGTGCGATAAGTAGTGTGAATTGCAGAAT TCAGTGAATCATCGAATCTTTGAACGCAAATGGCGCTCTTGGGTTCTCCCAGGAGCATGTCTGTC TGAGTGTCGGTTTACAAACTAACGCGCGACGCTTTTCCTAAGCGTGTCGGCGCGGCGTTGAGGCG TCATGGCCTACCTACCACGGCAGGCCGTGTCCTTCGAAATGCAGCGAGACTCGGTTGCGTAGATT CCGCATCGCAACTCAAGAGGCCAAAATCTATTTCGTCCTCTCGGTGCG
458	GCTCAGCTCCTACCGATTGAATGGTTTAGTGAGGCCTCCTGATTGGCGCCGCCGCCCCGGAAACG GAGCAGCGGATGTCGAAAAGTTGGTCAAACTTGATCATTTAGAGGAAGTAAAAGTCGTAACAAG GTTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGATCATACAAGACCCTATGCGGTTTTGCAC TGCGAACCTTACCAAACTTTAAGGGGAAGGTTCCGGCCTTTAATGGGGGGGGTTTAATTTTCCCA CCCCCCTGGGACTTTTTTTCCCACACCGGCCCCGCTCGTGTTTTCCCATTTTTAACACACCTTCTC GACTGAAACTGCGAGATTCGCTTCTCGCGACAAACTGAAAAGAAGACAACTTTTAACGGTGGAT CTCTTGGCTCGTGCATCGATGAAGAACGCAGCCAGGTGCGATAAGTAGTGTGAATTGCAGAATT CAGTGAATCATCGAATCTTTGAACGCAAATGGCGCTCTTGGGTTCTCCCAGGAGCATGTCTGTCT GAGTGTCGGTTTACAAACTAACGCGCGACGCTTTTCCTAAGCGTGTCGGCGCGGCGTTGAGGCGT CATGGCCTACCTACCACGGCAGGCCGTGTCCTTCGAAATGCAGCGAGACTCGGTTGCGTAGATTC CGCATCGCAACTCAAGAGGCCAAAATCATTTCGTCCTCTCGGTGCGTTGCGG

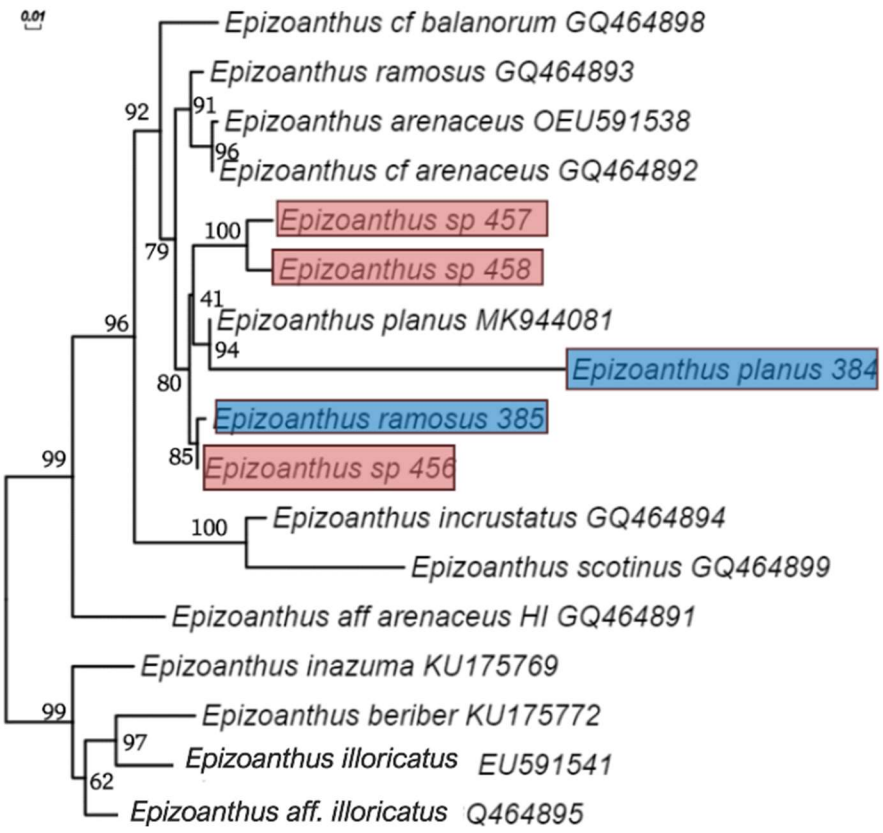

Figure 11: Maximum likelihood phylogeny of collected Zoanthidea specimens and their close relatives based on an alignment of concatenated nuclear (18S, ITS1, 5.8S, & ITS2) nucleotide sequences. Bootstrap values based on 1000 pseudoreplicates. Specimens from the Atlantic in red. Specimens from Indo-Pacific in blue.

Table 9: Partition parameter estimates used to model sequence evolution for phylogenetic inference. The base frequencies and substitution rates provided from RAxML

Partition	Alignment positions	Base Frequencies				Substitution rates (G-T = 1)					Gamma Shape
		A	C	G	T	A-C	A-G	A-T	C-G	C-T	
18S	1-153	0.275573	0.204145	0.291446	0.228836	1.333206	0.467537	0.475535	0.481662	5.389279	0.501896
ITS1	154-384	0.232084	0.256752	0.20803	0.303134	1.315757	2.159901	1.406812	0.812918	2.484954	0.731903
5.8S	385-541	0.235838	0.197452	0.281753	0.284956	52.214812	0.0001	0.0001	0.0001	0.800737	0.572151
ITS2	542-731	0.206134	0.278892	0.278737	0.236238	1.120235	0.909078	1.261261	0.629361	2.188769	0.642559

Host urchins

Collected zoanthidean specimens were found on several Cidaridae host taxa. These urchin species include *Cidaris abyssicola* (Agassiz, 1871), *Cidaris blakei* (Agassiz, 1888), and

Cidaris rugosa (Clark, 1907) (Table 10). While in literature, undescribed specimens have been documented living on the urchin *Stylocidaris lineata* (Mortensen, 1910). These genera make up a small proportion of the Cidaroida generic diversity.

Table 10: Collected specimens and their host

Source	Sample ID	Host	Site
VMIC G-179 July 1-63	371	Echinoid	N of Grand Bahama
VMIC 42.115	372	*Cidaris abyssicola*	W of Bimini
VMIC 42.447	373	*Cidaris blakei*	Freeport, Grand Bahama
VMIC 42.237	374	*Cidaris blakei*	Freeport, Grand Bahama
VMIC 42.306	375	*Cidaris blakei*	N of Grand Bahama
VMIC 42.289	376	*Cidaris ragosa*	E of Bimini
H. Kise	383	Cidaridae	Hirajisone, Nagasaki, Japan
H. Kise	384	Cidaridae	Suruga Bay, Shizuoka, Japan
H. Kise	385	Cidaridae	Sagami Bay, Kanagawa, Japan
H. Kise	386	Cidaridae	Sagami Bay, Kanagawa, Japan
H. Kise	387	Cidaridae	Sagami Bay, Kanagawa, Japan
UF 17185	455	*Cidaris blakei*	Bahamas
UF 17186	456	Cidaridae	French Guiana
UF 17187	457	Cidaridae	French Guiana
UF 17188	458	Cidaridae	French Guiana

Taxonomy of Cidaroida urchins:

Order Cidaroida
- Family Ctenocidaridae
  - Genus *Aporocidaris*
  - Genus *Ctentocidaris*
  - Genus *Homalocidaris*
  - Genus *Notocidaris*
- Family Paurocidaridae
  - Genus *Paurocidaris*
- Family Cidaridae
  - Subfamily Cidarinae
    - Genus *Almucidaris*
    - Genus *Calocidaris*
    - Genus *Centrocidaris*
    - Genus *Chonodrocidaris*
    - Genus *Chorocidais*

Genus *Cidaris*
Genus *Compsocidaris*
Genus *Cynthocidaris*
Genus *Eucidaris*
Genus *Hesperocidaris*
Genus *Kionocidaris*
Genus *Lissocidaris*
Genus *Tretocidaris*
Genus *Triassicaris*
Genus *Prionocidaris*
Subfamily Goinocidarinae
Genus *Austrocidaris*
Genus *Goniocidaris*
Genus *Ogmocidaris*
Genus *Psilocidaris*
Genus *Rhopalocidaris*
Genus *Schizocidaris*
Genus *Phyllacanthus*
Genus *Plegiocidaris*
Subfamily Stereocidarinae
Genus *Hirudocidaris*
Genus *Phalacrocidaris*
Genus *Sinaecidaris*
Genus *Sterocidaris*
Genus *Temnocidaris*
Subfamily Stylocidarinae
Genus *Acanthocidaris*
Genus *Plococidaris*
Genus *Stylocidaris*
Subfamily Typocidarinae
Genus *Typocidaris*
Family Histocidaridae
Genus *Histocidaris*
Family Psychocidaridae
Genus *Psychocidaris*

Genera found to be hosts of Atlantic *Epizoanthus* symbionts bolded. Taxonomy from WoRMS Editorial Board, accessed on 2023-07-17.

Discussion:

The number of polyps per colony varied from 6-8 and the number of mesenteries varied between 14-24 per polyp. (Table 4). The polyps of *Epizoanthus planus* have 7-8 polyps per colony and 18-22 mesenteries per polyp. Colony and gross polyp morphology of the museum specimens were similar but not identical to *Epizoanthus planus*. The first hypothesis, that any hypothetical Atlantic urchin-symbiotic Zoanthidea species should have colony and gross polyp morphology similar to *Epizoanthus planus* and its sister species, is supported.

The polyps used in this study were museum specimens collected between 1963-2020. They were around 3mm in size and fragile, making it difficult to produce interpretable histological slides. The 10um sections consume the entire actinopharynx of the polyp within 5 sections and the tissues tore or shattered easily. Only 4 of 14 specimens yielded interpretable longitudinal sections and none of the specimens yielded interpretable cross sections. The longitudinal sections revealed similar reticulate mesogleal marginal musculature structure. Each specimen had an irregularly shaped lacunae along its length, giving the appearance of a reticulate mesh. The muscles filled the entire distal diameter of the mesoglea and narrowed near the proximal terminus. Longitudinal sections of specimens 371, 374, 376, and 386 provided measurements for the length of the longest pleat, muscle thickness, surface area of the muscle, length of the muscle, and number of attachment sites. The features of the marginal musculature are similar but not identical to *Epizoanthus planus*. Having a reticulate mesogleal muscle is definitive for the genus *Epizoanthus* as there is no other zoanthidean genus that has reticulate mesogleal muscles. The second hypothesis, that any hypothetical Atlantic urchin-symbiotic Zoanthidea species should have microanatomical characters, particularly features of the marginal musculature, similar to *Epizoanthus planus* and its sister species, is supported.

The analysis of DNA showed similar sequences to *Epizoanthus planus* and *Epizoanthus ramosus.* The examination of 18S, ITS1, 5.8S, & ITS2 genes confirmed that the collected specimens were from the genus *Epizoanthus.* DNA sequences were entered into the nucleotide BLAST search in GenBank and specimens 384, 457, and 458 were highly matched to *Epizoanthus planus* whereas specimens 385 and 456 were highly matched to *Epizoanthus ramosus* (Table 7). None of the samples matched 100% and that could be due to the different fragment sizes of the DNA entered into the database or intra-specific variation. It is now demonstrated that the Atlantic urchin-symbiotic zoanthideans collected in this study belong to the genus *Epizoanthus*. The third hypothesis, that zoanthideans associated with echinoids will share a recent common ancestor and, therefore form a monophyly on a well-supported molecular phylogeny and any hypothetical Atlantic urchin-symbiotic Zoanthidea species should be a member of genus *Epizoanthus*, is supported.

Stylocidaris was the only genus of sea urchins that was previously documented to host zoanthideans. After examining the museum collections, Zoanthidea specimens were found living

on *Cidaris abyssicola*, *Cidaris blakei*, and *Cidaris rugosa.* These species all belong to the subfamily Cidarinae in the family Cidaridae. This study expanded the knowledge of urchin host species and potential urchin host genera. It is now demonstrated that Zoanthidea can be found living on *Cidaris,* but have the potential to live on other genera of the family Cidaridae. There are 5 families of the order Cidaroida which include Ctenocidaridae, Paurocidaridae, Cidaridae, Histocidaridae, and Psychocidaridae. The genus *Stylocidaris* belongs to the subfamily Stylocidarinae in the family Cidaridae. The family Cidaridae seems to be the favored host among zoanthideans associated with sea urchin spines.

While surveying specimens at the Florida Museum, symbionts such as clams, barnacles, and tube worms were also found on sea urchin spines in the families Ctenocidaridae and Histocidaridae. While zoanthideans were not found on these specimens, they are families which I observed to host a diversity of other marine invertebrates and should be investigated further in the future as well. The fourth hypothesis, that any hypothetical Atlantic urchin-symbiotic zoanthidean species should associate with hosts within the Cidaridae family of sea urchins, is supported.

Conclusion:

Because the targeted taxa are mobile deep-sea organisms this research was completely reliant on museum collections for specimens. I surveyed five museum collections and compiled 10 accessioned specimens. With the aid of collaborators Dr. James Reimer and Dr. Hiroki Kise, an additional four preserved specimens were added, totaling 14 specimens to work with. Integrative systematics was used to assess the morphology and phylogeny of the available specimens. Unfortunately, no single specimen yielded all targeted characters and our concept of Atlantic urchin-symbiotic zoanthidean species remains partially obscured. However, Atlantic zoanthidean species were detected and identified associated with echinoderms. Morphology was compared among potential new Atlantic species and with known Pacific species. This research determined the phylogenetic position of potential new Atlantic species within the echinoderm-symbiotic clade, specifically the *Epizoanthus planus*/*Epizoanthus ramosus* clade. There are at least two new Atlantic species of urchin-symbiotic Zoanthidea. The Atlantic urchin-symbiotic Zoanthidea species in this study have polyp morphology and marginal musculature similar to *Epizoanthus planus* and its sister species. The urchin host families Cidaridae, Ctenocidaridae,

and Histocidaridae should be surveyed in the future to find more potential specimens to further develop and explore the symbiotic relationships between Echinoderms and Zoanthidea.

References

Agassiz, A. (1871). *Preliminary Report on the Echini and Star-fishes, Dredged in Deep Water Between Cuba and the Florida Reef by LF de Pourtalès: App*. Museum of Comparative Zoology at Harvard College.

Agassiz, A. (1888). *Three cruises of the United States Coast and Geodetic Survey steamer" Blake", in the Gulf of Mexico, in the Caribbean Sea, and along the Atlantic coast of the United States, from 1877 to 1880* (Vol. 14). Sampson Low, Marston, Searle & Rivington.

Appeltans, W., Ahyong, S. T., Anderson, G., Angel, M. V., Artois, T., Bailly, N., Bamber, R., Barber, A., Bartsch, I., & Berta, A. (2012). The magnitude of global marine species diversity. *Current Biology*, *22*(23), 2189-2202. https://doi.org/10.1016/j.cub.2012.09.036

Carlgren, O. (1923). *Ceriantharia und Zoantharia der Deutschen Tiefsee-Expedition*. G. Fischer.

Carpenter, P. H., & von Graff, L. (1885). XVII. On three new Species of Metacrinus. *Transactions of the Linnean Society of London. 2nd Series: Zoology*, *2*(14), 435-446. https://doi.org/10.1111/j.1096-3642.1885.tb00296.x

Clark, H. L. (1907). The cidaridae. Bulletin of the Museum of Comparative Zoology at Harvard College, 51(7), 165-231

Collins, R.A. & Cruickshank, R.H. (2013). The seven deadly sins of DNA barcoding. *Molecular Ecology Resources*, 13, 969–975. https://doi.org/10.1111/1755-0998.12046

Dayrat, B. (2005). Towards integrative taxonomy. *Biological Journal of the Linnean society*, *85*(3), 407-417. https://doi.org/10.1111/j.1095-8312.2005.00503.x

De Bary, A. (1879). *Die erscheinung der symbiose: Vortrag gehalten auf der versammlung deutscher naturforscher und aerzte zu cassel*. Trübner.

DeSalle, R., Egan, M.G. & Siddall, M. (2005) The unholy trinity: Taxonomy, species delimitation and DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 360, 1905–1916. http://dx.doi.org/10.1098/rstb.2005.1722

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*. https://doi.org/10.1038/nprot.2006.384

Haddon, A.C. & Shackleton, A.M. (1891) A Revision of the British Actini. Part II: The Zoanthe. *Scientific Transactions of the Royal Dublin Society, 4*, 609–672

Hall, T. A. (1999, January). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41, 95-98.

Hillis, D. M. (1987). Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics*, 18(1), 23-42. https://doi.org/10.1146/annurev.es.18.110187.000323

Huang X. (1992). A contig assembly program based on sensitive detection of fragment overlaps. *Genomics*, *14*(1), 18–25. https://doi.org/10.1016/s0888-7543(05)80277-0

Kise, H., Dewa, N., & Reimer, J. D. (2018). First record of sea urchin-associated Epizoanthus planus from Japanese waters and its morphology and molecular phylogeny. *Plankton and Benthos Research*, *13*(3), 136-141. DOI:10.3800/pbr.13.136

Kise, H., Montenegro, J., Santos, M. E., Hoeksema, B. W., Ekins, M., Ise, Y., ... & Reimer, J. D. (2022). Evolution and phylogeny of glass-sponge-associated zoantharians, with a description of two new genera and three new species. *Zoological Journal of the Linnean Society*, *194*(1), 323-347. https://doi.org/10.1093/zoolinnean/zlab068

Mortensen, T. (1910). On some west Indian echinoids. Bulletin of the United States National Museum, 74.

Nguyen, H. T., Tatipamula, V. B., Do, D. N., Huynh, T. C., & Dang, M. K. (2022). Retrieving high-quality genomic DNA from formalin-fixed paraffin-embedded tissues for multiple molecular analyses. *Preparative Biochemistry & Biotechnology*, *52*(1), 48-55. https://doi.org/10.3389/fmolb.2023.1087982

Reimer, J. D., Kise, H., Santos, M. E., Lindsay, D. J., Pyle, R. L., Copus, J. M., Bowen, B. W., Nonaka, M., Higashiji, T., & Benayahu, Y. (2019). Exploring the biodiversity of understudied benthic taxa at mesophotic and deeper depths: examples from the order Zoantharia (Anthozoa: Hexacorallia). *Frontiers in Marine Science*, *6*, 305. https://doi.org/10.3389/fmars.2019.00305

Ryland, J. S., & Lancaster, J. E. (2004). A review of zoanthid nematocyst types and their population structure. *Hydrobiologia*, *530*(1), 179-187. https://doi.org/DOI 10.1007/s10750-004-2685-1

Ryland, J. S., & Ward, H. (2016). Carcinoecium-forming Epizoanthus (Hexacorallia: Zoantharia) and the biology of E. papillosus in the eastern Atlantic, with special reference to the cnidom. *Zootaxa*, *4088*(4), 489-514. https://doi.org/10.11646/zootaxa.4088.4.2

Saffo, Mary. (1992). Coming to Terms with a Field: Words and Concepts in Symbiosis. *Symbiosis, 14*, 17-31.

Sinniger, F., & Häussermann, V. (2009). Zoanthids (Cnidaria: Hexacorallia: Zoantharia) from shallow waters of the southern Chilean fjord region, with descriptions of a new genus and two new species. *Organisms Diversity & Evolution*, *9*(1), 23-36. https://doi.org/10.1016/j.ode.2008.10.003

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*(9), 1312-1313. https://doi.org/10.1093/bioinformatics/btu033

Swain, T. D. (2009). Phylogeny-based species delimitations and the evolution of host associations in symbiotic Zoanthusids (Anthozoa, Zoanthusidea) of the wider Caribbean region. *Zoological Journal of the Linnean Society*, *156*(2), 223-238. https://doi.org/10.1111/j.1096-3642.2008.00513.x

Swain, T. D. (2010). Evolutionary transitions in symbioses: dramatic reductions in bathymetric and geographic ranges of Zoanthidea coincide with loss of symbioses with invertebrates. *Molecular Ecology*, *19*(12), 2587-2598. https://doi.org/10.1111/j.1365-294X.2010.04672.x

Swain, T. D. (2018). Revisiting the phylogeny of Zoanthidea (Cnidaria: Anthozoa): Staggered alignment of hypervariable sequences improves species tree inference. *Molecular Phylogenetics and Evolution*, *118*, 1-12. https://doi.org/10.1101/161117

Swain, T. D., Schellinger, J. L., Strimaitis, A. M., & Reuter, K. E. (2015). Evolution of anthozoan polyp retraction mechanisms: convergent functional morphology and evolutionary allometry of the marginal musculature in order Zoanthidea (Cnidaria: Anthozoa: Hexacorallia). *BMC Evolutionary Biology*, *15*(1), 1-19. 10.1186/s12862-015-0406-1

Swain, T. D., Strimaitis, A. M., Reuter, K. E., & Boudreau, W. (2017). Towards integrative systematics of Anthozoa (Cnidaria): Evolution of form in the order Zoanthidea. *Zoologica Scripta*, *46*(2), 227-244. https://doi.org/10.1111/zsc.12195

Swain, T. D., & Swain, L. M. (2014). Molecular parataxonomy as taxon description: Examples from recently named Zoanthidea (Cnidaria: Anthozoa) with revision based on serial histology of microanatomy. *Zootaxa*, *3796*(1), 81-107. https://doi.org/10.11646/zootaxa.3796.1.4

Swain, T. D., & Wulff, J. L. (2007). Diversity and specificity of Caribbean sponge–zoanthid symbioses: A foundation for understanding the adaptive significance of symbioses and generating hypotheses about higher-order systematics. *Biological Journal of the Linnean Society*, *92*(4), 695-711. https://doi.org/10.1111/j.1095-8312.2007.00861.x

Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*(22), pp.4673-4680. https://doi.org/10.1093/nar/22.22.4673

Vogler, A.P. & Monaghan, M.T. (2007) Recent advances in DNA taxonomy. *Journal of Zoological Systematics and Evolutionary Research, 45,* 1-10. https://doi.org/10.1111/j.1439-0469.2006.00384.x

Wiley, E. O., & Lieberman, B. S. (2011). *Phylogenetics: Theory and practice of phylogenetic systematics*. John Wiley & Sons.

WoRMS Editorial Board (2023). World register of marine species. Retrieved July 7, 2023 from https://www.marinespecies.org at VLIZ. doi:10.14284/170

Zapalski, M. K., Kise, H., Dohnalik, M., Yoshida, R., Izumi, T., & Reimer, J. D. (2021). Hexacoral-crinoid associations from the modern mesophotic zone: Ecological analogues for Palaeozoic associations. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *572*, 110419. https://doi.org/ARTN 1104190.1016/j.palaeo.2021.110419