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Applying Integrative Systematics to Long-Standing Species Boundary Questions in the Zoanthidea

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HALMOS COLLEGE OF ARTS AND SCIENCES

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By
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Submitted to the Faculty of Halmos College of Arts and Sciences in partial fulfillment of the requirements for the degree of Master of Science with a specialty in:

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Abstract

The study of systematics has been around for over 200 years. Some recent systematic studies have shifted to the idea of genetic barcoding for identification and classification. This shift is notably present in the cnidarian order Zoanthidea. Systematists have employed genetic barcoding exclusively for the classification of these animals resulting in taxonomic uncertainty. A prominent Mediterranean species complex (*Parazoanthus axinellae*) is at the center of these taxonomic issues. Recently two color-morpho-types: “slender” yellow and “stocky” orange have been used to differentiate modern *P. axinellae* specimens, advancing the uncertainty associated with this complex. This lack of taxonomic identity becomes a prevalent issue now that *P. axinellae* is increasing in modern ecological publications. Using integrative systematics, the complete historical and modern boundary of the *P. axinellae* species complex was investigated. Based on a multi-gene phylogeny there are three subclades in the *P. axinellae* species complex designated here as: *P. axinellae* (restricted), *P. aff. juan-fernandezii* (Mediteranean-1), and *P. aff. juan-fernandezii* (Mediteranean-2). These clades appear to follow the idea of the color-morpho-types to some degree, where the “slender” yellow can be used to distinguish *P. axinellae* (restricted) from the “stocky” orange *P. aff. juan-fernandezii* (Mediterranean-1) and (Mediteranean-2). Microanatomical differences in column mesoglea as well as microneme length and thickness were detected between *P. axinellae* (restricted) and *P. aff. juan-fernandezii* (Mediterranean-1). Unfortunately, due to the historical ambiguity, we were unable to match these clades to any historical boundaries. The *P. axinellae* (restricted) distribution can be expanded to include the East Coast of the United States.

Keywords: *Parazoanthus axinellae*, Zoanthidea, Phylogenetics, Systematics, Species Complex, Histology

Acknowledgments

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Introduction

Invertebrates make up about 97 percent of the total described animal species on earth (Brusca, 2016). We organize and attempt to understand this diversity using systematics. This field provides scientific names, classifications, organism descriptions, establishes keys for identification, data on species distributions, preserves collections, and finally investigates the evolutionary histories and environmental adaptations of organisms (Mayr, 1991; Michener et al., 1970). Although systematics is one of the oldest scientific disciplines, it has only recently shifted from utilitarian classification to proposing and testing hypotheses (De Queiroz, 2007). Even more recently systematics has shifted from delimiting species and organizing higher taxa around single data sources to integrating multiple and complementary data streams (Dayrat, 2005; Padial, Miralles, De la Riva, & Vences, 2010; Will, Mishler, & Wheeler, 2005).

A popular modern shortcut to time-intensive integrative systematics is molecular parataxonomy. This approach focuses solely on DNA barcoding to detect, delimit, and describe species and higher taxa. DNA barcodes are short, easily amplified, and sequenced gene segments that must be evolutionarily conserved across vast portions of the tree of life. Relying on barcodes to detect, delimit, and describe species and higher taxa has several distinct disadvantages compared to integrative systematics (Swain, 2018; Swain & Swain, 2014). The primary disadvantage is that molecular parataxonomy creates a competing novel system that does not communicate with the existing taxonomic system because it shares no characters. This has multiple deleterious effects, including (1) the inability to detect if targeted species or higher taxa are novel (i.e. if they have already been described or are new to science), (2) inability to convincingly place new taxa within existing taxa, (3) inability to place existing species or genera within new higher taxa, (4) inability to identify specimens for which DNA sequencing is impossible (e.g. nearly all museum specimens), (5) inability to use generated characters in higher analyses (e.g. DNA barcodes are insufficient for molecular phylogenetics seeking to recover species rather than gene trees) and therefore molecular parataxonomic taxa must be further examined before they are discredited, confirmed, or are otherwise useful and should be considered a preliminary assessment of taxon delimitation (Brower, 2006; Collins & Cruickshank, 2013; Meier, 2008). While molecular parataxonomy seems like an efficient way to classify organisms that have historically been challenging to work with, it ignores nearly all previous research and generates largely useless hypotheses and data. Integrative systematics

seeks to create more robust hypotheses and bridge the gap between disparate approaches. This effort seeks to integrate rich analyses from multiple and complementary data streams such as biogeography, comparative morphology, population genetics, ecology, development, behavior, cytology, immunology, phylogenetics, etc. and can resolve the disjuncture between the existing traditional taxonomic system and recent barcode-based molecular parataxonomy (Swain, 2018; Swain & Swain, 2014). This methodology will result in the most accurate systematics currently possible.

There is a relatively understudied order in the phylum Cnidaria, the Zoanthidea. Zoanthideans are colonial or solitary soft-bodied polyps that differ from other Anthozoa hexacorallian orders in the arrangement of tentacles (two alternating cycles) and the development (added to the initial 12 only within the exocoels of ventral directives) and arrangement of their mesenteries (coupled dimorphic pairs). Zoanthideans have a single siphonoglyph (ciliated groove in the actinopharynx) located between the ventral directives, and along with the mesenteries and their associated retractor muscles, define the bilateral symmetry of the polyp. Zoanthideans do not build their own skeletons, rather they incorporate various sediments from the surrounding environment and attach themselves to other invertebrates to gain access to water flow above the benthos. Zoanthidea are abundant due to their ability to form symbiotic attachments with almost any invertebrate with a hard body part (Burnett, Benzie, Beardmore, & Ryland, 1997; Reimer, Ono, Takishita, Tsukahara, & Maruyama, 2006). Zoanthidea can be found in a wide range of benthic habitats, from the intertidal to the deep sea and the tropics to the poles (Appeltans et al., 2012). Having such a wide distribution the Zoanthidea can drastically alter the marine systems they find themselves in (Villamor, Signorini, Costantini, Terzin, & Abbiati, 2020). Zoanthidea are often associated with sponges, and as such are a principal group in benthic habitats (Coll et al., 2010; Swain & Wulff, 2007)

These organisms, which are likely the third most speciose order of hexacorallians, only have three current taxonomic experts making them a neglected group (Appeltans et al., 2012). Even with their importance, the paucity of Zoanthidea taxonomists and systematists leaves the true diversity of species, genera, and families in this order largely unknown (Reimer et al., 2006). Overall, there are approximately 100 described species of Zoanthidea, but an estimated 270–1,170 undescribed species (Appeltans et al., 2012). These undescribed species include cryptic and morphologically plastic species that are difficult to detect and classify (Reimer et al., 2006).

Previous taxonomic studies with Zoanthidea were done using classic systematic methods (Abel, 1959; Pax, 1937, 1957; Schmidt, 1862). These efforts were focused on microanatomy and anatomical characters that separated the species within the Zoanthidea (Haddon & Shackleton, 1891). With greater access to genetic sequencing, challenges in data collection from classical characters, and difficulties with historical systematics hypotheses, current systematics work on Zoanthidea has become almost entirely dependent upon molecular parataxonomy. This move towards molecular parataxonomy has created numerous new species, genera, and families that are completely disconnected from the existing taxonomic system and the previous two centuries of research on Zoanthidea. Therefore, it is important to revisit these taxa to discredit or confirm their hypotheses and reunite them with the existing taxonomic system.

While there are ample targets for taxonomic research in Zoanthidea, the *Parazoanthus axinellae* (Schmidt, 1862) species complex with its four daughter subspecies, *P. a. adriaticus* (Pax, 1937), *P. a. linguricus* (Pax, 1937), *P. a. mülleri* (Pax, 1957), and *P. a. brevitentacularis* (Abel, 1959) (Table 1) is simultaneously neglected while emerging as a target of modern ecological research and is therefore, a priority for zoanthidean systematists. *P. axinellae* and its daughter subspecies can be considered a species complex since they are a group of apparently closely related organisms that are so similar that the boundaries between them are unclear (Table 1). The *P. axinellae* complex is known to be ecologically important throughout the Mediterranean Sea, northeastern Atlantic Ocean, and has been hypothesized to be transatlantic to the Gulf of Mexico and Southeast Atlantic Coasts of the United States (Swain, 2009). In some habitats, like caves, *P. axinellae* is considered a keystone species for benthic invertebrate communities (Enrichetti et al., 2019). On the Mediterranean deep continental shelf, the symbiotic relationship with sponge hosts is both the most abundant and most widely distributed of benthic organisms (Enrichetti et al., 2019; Pax, 1937). Currently, within the Mediterranean Sea, *P. axinellae* is thought to be the most common zoanthidean dominating the benthos and cave systems (Villamor et al., 2020). Due to the dominant nature of this species complex it is imperative to understand how many species are involved and what their individual ecological roles may be.

P. axinellae was originally discovered in the Adriatic Sea region of the Mediterranean Sea, and was described in 1862 as *Palythoa axinellae* (Schmidt, 1862). The genus was reassigned in 1891 when genus *Parazoanthus* was erected by Haddon and Shackleton and was assigned as the

type species for the genus (Haddon & Shackleton, 1891). Since then *Parazoanthus axinellae* has been expanded to include four subspecies based upon biogeography, symbiotic host specificity, cytology (nematocyst length), and lime content (Abel, 1959; Pax, 1937, 1957). While some of these morphological characters have been accepted as part of modern *P. axinellae*, most have been ignored due to a lack of research effort (Herberts, 1972). In the literature between the 1970s to the early 2000s, *P. axinellae* is not commonly found and few studies have exclusively targeted it. The literature from this time that mentions *P. axinellae* was mainly interested in zoanthidean reproduction and life-history traits but did not attempt to identify the subspecies included in the research nor clarify any of their descriptions or delimitations (Joaquim, 1999; Ryland, 1997; Won, Rho, & Song, 2001). In 2015, *P. axinellae* started to appear in the literature again as a modern research target. Cachet 2015 acknowledges the uncertainty in species and subspecies identities within the *P. axinellae* species complex. However, instead of attempting to clarify, Cachet splits the parent species into new "slender" and "stocky" morphotypes, which are also color morphs yellow and orange (respectively), none of which can be determined from previously collected samples as preserved polyps are usually retracted, and their color has been dissolved by preservative (Cachet et al., 2015). Adding to the general confusion, Ocana, 2019 wrote cryptically about potential new species or subspecies based on ecological characters within the complex without defining them; shortly after in 2020, Villamore introduced DNA sequences for the morphotypes without linking them to definitive anatomical features that could be connected to the existing taxonomic system (Ocaña, 2019; Villamor et al., 2020). This is yet another example of the disconnect between a DNA barcode-based taxonomy and the existing anatomy-based taxonomic system.

Within the context of the historical definition of *P. axinellae* and its four daughter subspecies, modern researchers have adopted a concept of *P. axinellae* and two morpho-color-types without a robust analysis of the relationship between historical and modern concepts. Therefore, we are uncertain if modern *P. axinellae* and historical *P. axinellae* are the same species, any of the four named subspecies, or if either of the modern *P. axinellae* morpho-color-types correspond to historical *P. axinellae* or any of the four named subspecies. Ultimately this may only be accomplished through an integrative approach applied to modern and historical specimens (preferably types), however, it remains unclear if this can be accomplished *in toto*.

The primary focus of the research presented here is to apply an integrative approach to examine specimens of modern *P. axinellae* to decern potential species boundaries and what their relationship is with historical concepts as a first step in unraveling the *P. axinellae* species complex. Molecular and anatomical data from a transatlantic collection of modern *P. axinellae* specimens were extracted and combined with published molecular data on modern *P. axinellae* morpho-color-types and anatomical data extracted from unidentified *Parazoanthus* sponge-symbiont museum specimens to answer the following questions. (1) Is there complementary evidence across multiple data streams consistent with species boundaries within modern *P. axinellae*? (2) Does the molecular data from modern *P. axinellae* morpho-color-types fall within the detected integrative boundaries? (3) Do the detected integrative boundaries appear to correspond to the historical definition of *P. axinellae* or any of its four daughter subspecies? (4) Does the anatomical data from unidentified *Parazoanthus* sponge-symbiont museum specimens fall within the detected integrative boundaries? (5) Do the detected integrative boundaries support transatlantic distributions of *P. axinellae*?

Table 1. A compiled collection from classic and modern literature of important morphological characters, symbiotic associations, and sampling location for *P. axinellae* and its daughter subspecies.

Name	**Number of Mesenteries**	**Tentacle Length (mm)**	**Holotrich length (µm)**	**Calcium Content (%)**	**Nematocyst Length (µm)**	**Depth found (m)**	**Symbiosis**	**Location**
P. axinellae (Schmidt, 1862)	28-39	5-10	NA	NA	NA	30-100	*Axinellae verrucosa*	Adriatic Sea
P. a. adriaticus Pax, 1937	32	NA	~20-26	15	10-16	36-89	*Axinellae sp.*	Adriatic Sea
P. a. linguricus Pax, 1937	36-38	NA	24-32	25-50	15-17	200-350	*Thenea sp.*	Ligurian Sea
P. a. mülleri Pax, 1957	32	NA	24-36	~50	18-21	NA	*Axinellae damicornis*	Gulf of Naples
P. a. brevi-tentacularis Abel, 1959	34-38	NA	32-35	NA	20-21	NA	Rocky substrate	Gulf of Lion

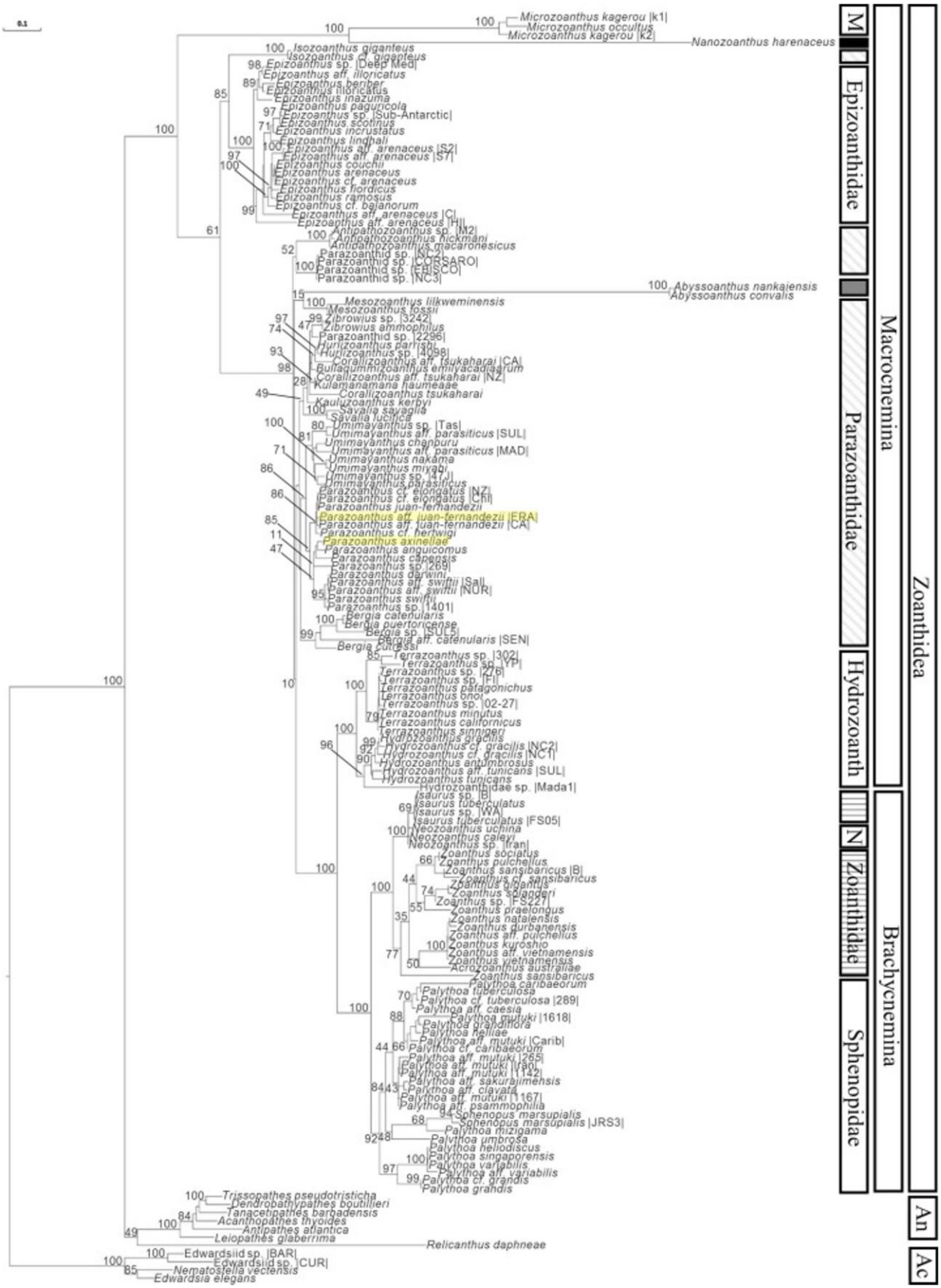
0.1
Microzoanthus kagerou |k1|
Microzoanthus occultus
Microzoanthus kagerou |k2|
Nanozoanthus harenaceus
Isozoanthus giganteus
Isozoanthus cf. giganteus
Epizoanthus sp. |Deep Med|
Epizoanthus aff. illoricatus
Epizoanthus berberi
Epizoanthus illoricatus
Epizoanthus inazuma
Epizoanthus paguricola
Epizoanthus sp. |Sub-Antarctic|
Epizoanthus scotinus
Epizoanthus incrustatus
Epizoanthus lindahli
Epizoanthus aff. arenaceus |S2|
Epizoanthus aff. arenaceus |S7|
Epizoanthus couchii
Epizoanthus arenaceus
Epizoanthus cf. arenaceus
Epizoanthus fiordicus
Epizoanthus ramosus
Epizoanthus cf. bajanorum
Epizoanthus aff. arenaceus |C|
Epizoanthus aff. arenaceus |H|
Antipathozoanthus sp. |M2|
Antipathozoanthus hickmani
Antipathozoanthus macaronesicus
Parazoanthid sp. |NC2|
Parazoanthid sp. |CORSARO|
Parazoanthid sp. |ERISCO|
Parazoanthid sp. |NC3|
Abyssoanthus nankaiensis
Abyssoanthus convalis
Mesozoanthus lilkweminensis
Mesozoanthus fossii
Zibrowius sp. |3242|
Zibrowius ammophilus
Parazoanthid sp. |2296|
Hurlizoanthus parksi |Sh|
Hurlizoanthus sp. |4098|
Corallizoanthus aff. tsukaharai |CA|
Bullaqummizoanthus emilyacadiaarum
Corallizoanthus aff. tsukaharai |NZ|
Kulamanamana haumeaae
Corallizoanthus tsukaharai
Kauluzoanthus kerbyi
Savalia savaglia
Savalia lucifica
Umimayanthus sp. |Tas|
Umimayanthus aff. parasiticus |SUL|
Umimayanthus chonburu
Umimayanthus aff. parasiticus |MAD|
Umimayanthus nakama
Umimayanthus miyagi
Umimayanthus sp. |47|
Umimayanthus parasiticus
Parazoanthus cf. elongatus |NZ|
Parazoanthus cf. elongatus |Chl|
Parazoanthus juan-fernandezii
Parazoanthus aff. juan-fernandezii |ERA|
Parazoanthus aff. juan-fernandezii |CA|
Parazoanthus cf. herdwigi
Parazoanthus axinellae
Parazoanthus anguicomus
Parazoanthus capensis
Parazoanthus sp. |269|
Parazoanthus darwini
Parazoanthus aff. swiftii |Sal|
Parazoanthus aff. swiftii |NOR|
Parazoanthus swiftii
Parazoanthus sp. |1401|
Bergia catenularis
Bergia puertoricense
Bergia sp. |SUL5|
Bergia aff. catenularis |SEN|
Bergia cuneiss
Terrazoanthus sp. |302|
Terrazoanthus sp. |YP|
Terrazoanthus sp. |276|
Terrazoanthus sp. |F|
Terrazoanthus patagonichus
Terrazoanthus ono|
Terrazoanthus sp. |02-27|
Terrazoanthus minutus
Terrazoanthus californicus
Terrazoanthus sinnigeri
Hydrozoanthus gracilis
Hydrozoanthus cf. gracilis |NC2|
Hydrozoanthus cf. gracilis |NC1|
Hydrozoanthus antumbrosus
Hydrozoanthus aff. tunicans |SUL|
Hydrozoanthus tunicans
Hydrozoanthidae sp. |Mada1|
Isaurus sp. |B|
Isaurus tuberculatus
Isaurus sp. |WA|
Isaurus tuberculatus |FS05|
Neozoanthus uchina
Neozoanthus caleri
Neozoanthus sp. |Iran|
Zoanthus sociatus
Zoanthus pulchellus
Zoanthus sansibaricus |B|
Zoanthus cf. sansibaricus
Zoanthus gigantus
Zoanthus solanderi
Zoanthus sp. |FS221|
Zoanthus praelongus
Zoanthus natalensis
Zoanthus durbanensis
Zoanthus aff. pulchellus
Zoanthus kuroshio
Zoanthus aff. vietnamensis
Zoanthus vietnamensis
Acrozoanthus australiae
Zoanthus sansibaricus
Palythoa caribaeorum
Palythoa tuberculosa
Palythoa cf. tuberculosa |289|
Palythoa aff. caesia
Palythoa mutuki |1618|
Palythoa grandiflora
Palythoa helliae
Palythoa aff. mutuki |Carib|
Palythoa cf. caribaeorum
Palythoa aff. mutuki |265|
Palythoa aff. mutuki |Iran|
Palythoa aff. mutuki |1142|
Palythoa aff. sakurajimensis
Palythoa aff. clavata
Palythoa aff. mutuki |1167|
Palythoa aff. psammophilia
Sphenopus marsupialis
Sphenopus marsupialis |JRS3|
Palythoa mizigama
Palythoa umbrosa
Palythoa heliodiscus
Palythoa singaporensis
Palythoa variabilis
Palythoa aff. variabilis
Palythoa cf. grandis
Palythoa grandis
Trissopathes pseudotristicha
Dendrobathypathes boutillieri
Tanacetipathes barbadensis
Acanthopathes thyoides
Antipathes atlantica
Leiopathes glaberrima
Relicanthus daphneae
Edwardsiid sp. |BAR|
Edwardsiid sp. |CUR|
Nematostella vectensis
Edwardsia elegans
M
Epizoanthidae
Parazoanthidae
Hydrozoanth
N
Zoanthidae
Sphenopidae
Macrocnemina
Brachycnemina
Zoanthidea
An
Ac

Figure 1. Complete-data tree from Swain 2018. Maximum likelihood phylogeny of Zoanthidea based on a staggered alignment of concatenated nuclear (18S, ITS1, 5.8S, ITS2, & 28S) and mitochondrial (12S & 16S) ribosomal RNA and mitochondrial protein-coding (COI) nucleotide sequences. Support indicated by 1000 pseudoreplicate maximum likelihood bootstrap values. Taxonomic notations: order, Ac= Actiniaria, An =Antipatharia; family, M =Microzoanthidae, black bar =Nanozoanthidae, gray diagonal lines =Parazoanthidae, gray bar = Abyssoanthidae, Hydrozoanth = Hydrozoanthidae, gray horizontal line= Zoanthidae, N= Neozoanthidae. *P. axinellae* can be seen highlighted in the *Parazoanthus* group and is near *P. aff. juan-fernandezii* (also highlighted), a species considered to be closely related to the *P. axinellae* group and found within the Mediterranean Sea. These two species were found in two separate clades meaning they are not as closely related as once thought, or even sister taxa (Swain, 2018).

Materials and Methods

Integrative systematic methods use disparate data streams to increase confidence in the resulting conclusions. Here we used two main data streams, phylogenetics and histological analyses of microanatomy, to answer the research questions. Phylogenetics used a targeted region of evolutionarily conserved and variable genes (ITS region) to infer species-level relationships. The histological analyses were used to assess any morphological differences in microanatomy between the closely related potential species. When both data were combined, the most robust analysis of *P. axinellae* species was compiled and analyzed.

Sample Acquisition:

Specimens of *Parazoanthus axinellae* were obtained from multiple collaborators and the United States National Museum of Natural History (USNM). Additionally, original species descriptions and indicative scientific literature were translated from German to attempt to locate any historic specimens or species defining characters. Museums thought to have type, holotype, paratype, or authoritative specimens were contacted to request access for inclusion in this study. Specimens included in this analysis originated from the Mediterranean Sea (Spain, Italy, Croatia, and France), Ireland, Gulf of Mexico, South Carolina, North Carolina, and Chile (Figure 2a-d). Specimens were stored at -80° C in 95 percent ethanol in the collection of Dr. Timothy Swain at the Nova Southeastern University Guy Harvey Oceanographic Center. Each sample was given a specific ID that correlated to the sample location and either the known or expected species identification.

Phylogenetics:

DNA from each sample was extracted and sequenced for phylogenetic inference. Complete genomic DNA was extracted using the cetyl-trimethyl-ammonium bromide (CTAB) technique (Doyle & Doyle, 1987). Polymerase chain reaction (PCR) selectively amplified the

ITS rRNA nuclear genes using primers ITSf, 5′-CTAGTAAGCGCGA GTCATCAGC-3′ and ITSr, 5′-GGTAGCCTTGCCTGATC TGA-3′ (Swain, 2009). This targeted the complete ribosomal RNA internal transcribed spacer region (5 genes, 3 complete and 2 incomplete), including partial 18S, complete ITS1, complete 5.8S, complete ITS2, and partial 28S genes (Swain, 2009). The rRNA ITS nuclear genes have previously been demonstrated to contain multiple zoanthidean species-level markers (Swain, 2009). The thermal protocol used was: 94 °C for 3 minutes, 32 cycles of 94 °C for 30 seconds, 50°C for 60 s, 72 °C for 90 seconds, with a final extension step of 72 °C for 10 minutes. Product from the PCR was purified through enzymatic removal of excess nucleotides and primers using exonuclease and shrimp alkaline phosphatase (ExoSAP-IT®; USB Corporation). The purified PCR product was detected with standard agarose gel electrophoresis to verify amplicon size and concentration. PCR product was sent for sanger sequencing in the forward and reverse directions using BigDye™ Terminator chemistry at the Florida State University Sequencing Facility using the amplification primers. DNA sequences collected from in-hand specimens were combined with sequence data publicly available in GenBank from Villamor (2020), and Singer (2009) into a staggered multi-gene alignment using BioEdit (Sinniger & Häussermann, 2009; Villamor et al., 2020). Staggering hypervariable sequences allows retention of every nucleotide sequenced into a single phylogenetic inference rather than eliminating ambiguously homologous sequence positions (Swain, 2018). The hypervariable regions that differ in sequence identity and length will be aligned among closely related species and designated as unknown between distantly related species, forming a phylogeny-informed alignment that is a combination of local (among closely related specimens) and universal alignments.

The analysis of molecular data followed a model inferential type of analysis after a multi-gene alignment had been created. The staggered alignment was partitioned following the boundaries of the five ITS region genes for independent fitting to a unique model of molecular evolution for each partition. Molecular evolution model-fitting and phylogenetic tree inference were performed in a maximum-likelihood analysis of RAxML v8.2.8 in the CIPRES Science Gateway v3.3 using a General Time Reversible (GTR) with gamma (+C) model on the partitioned sequence data. Node bootstrap support was estimated in RAxML using GTR, a categorical per site rate heterogeneity approximation (CAT) from 1000 pseuoreplicates (Stamatakis, 2014).

Histology:

Four polyps per specimen, two for cross-sections and two for longitudinal sections, were selected (when possible) using a dissecting scope for histological analysis of microanatomy. The four polyps were selected based on their size and shape to ensure they would be best suited for histological sectioning. From 37 colony specimens, 148 polyps were dissected ($n_{colonies}$=37, n_{polyps}=148). Individual polyps were treated with acids outlined below to dissolve any non-organic material taken up by the polyps to make samples suitable for sectioning. About 2 ml of a formic acid formalin mixture (Formical-4) was manually pipetted into each tube and incubated for four hours, then removed and repeated to ensure complete dissolution of calcium carbonate and proper fixation of tissues. Then a wash of reverse osmosis (RO) water was added to rinse out any remaining acid. The water was replaced with hydrofluoric acid to dissolve silicon dioxide (sponge spicules or radiolarian tests) from the polyps. The polyps incubated in hydrofluoric acid for 12 hours before being rinsed again with RO water and stored in 70% ethanol to await embedding in paraffin wax.

Before embedding, polyps were dehydrated in ethanol and then cleared in xylene. Samples were incubated with 2ml of 80% ethanol in their tubes for 10 minutes. This was replaced with 2ml of 90% ethanol for 15 minutes each for two changes. Then replaced with 100% ethanol for 20 minutes each for three changes. After the ethanol incubations (6 ethanol changes total), the ethanol was replaced with xylene. Each polyp was incubated with 2 ml of 100% xylene four times for 25 minutes each change to replace all ethanol within the tissues. After the four xylene changes, a 50% xylene 50% paraffin mixture was added to begin the process of impregnating tissues with wax. Polyps were incubated in the 50/50 mixture overnight at 30°C so that the wax did not solidify but instead was absorbed by the polyp tissue to prevent the polyps from collapsing when sectioned. After the 50/50 mixture was removed, two changes of pure paraffin were added to the samples in their tubes to sit overnight in the incubator before they were transferred into pure paraffin blocks designed for histology.

Histological sectioning was done using a Leica RM2125 RTS microtome. The polyps were cut in 10 µm thick sections and were mounted to poly-L-lysine glass slides which allowed tissue to stick to slides and avoid the loss of sections. Tissues were then deparaffinized with xylene and stained using Harris' hematoxylin and eosin Y stain (H & E staining) and covered with a glass slip to be photographed. The full staining procedure can be found in Swain, 2009

(Swain, 2009). Sections that captured the targeted regions (at the height of the actinopharynx in cross-sections and at the height of the marginal muscle in longitudinal sections) were photographed using a Leica DM2500 LED compound microscope and micrographs were measured.

Measurements were taken following Swain 2009. Ten serial sections (when applicable) were photographed for each specimen and selected characters were measured in micrometers (µm) using Leica LASX software. The characters measured in cross-sections were ventral directive length and thickness, microneme length and thickness, siphonoglyph mesoglea thickness, siphonoglyph endoderm thickness, siphonoglyph ectoderm thickness, column mesoglea thickness, column endoderm thickness, and column ectoderm thickness (Figure 3a). From longitudinal sections, characters measured were length of the marginal muscle, length of the longest muscle attachment site, diameter of the marginal muscle and surface area of the muscle (Figure 3b). Other characters observed were whether the siphonoglyph is prominent, depth of encrustation penetration in the column wall, if the ventral directive was hetero or homomorphic, and if the actinopharynx lining was furrowed or smooth. To analyze the morphological characters after the genetic analysis, polyps that had morphological data and a confirmed genetic identity were grouped. Basic scatterplots were generated for any characters that could be related (ex: microneme length vs. microneme width). Any characters that appeared to have a mean difference between the genetically different samples were further investigated using a two tailed t-test. The statistically significant characters, which had a p-value less than 0.05, were then used to identify the genetically unknown samples. Microanatomical characters of the genetically unknown samples were placed on scatterplots of the significant characters to indicate morphological affinity with genetically identified specimens.

Sample Site Locations

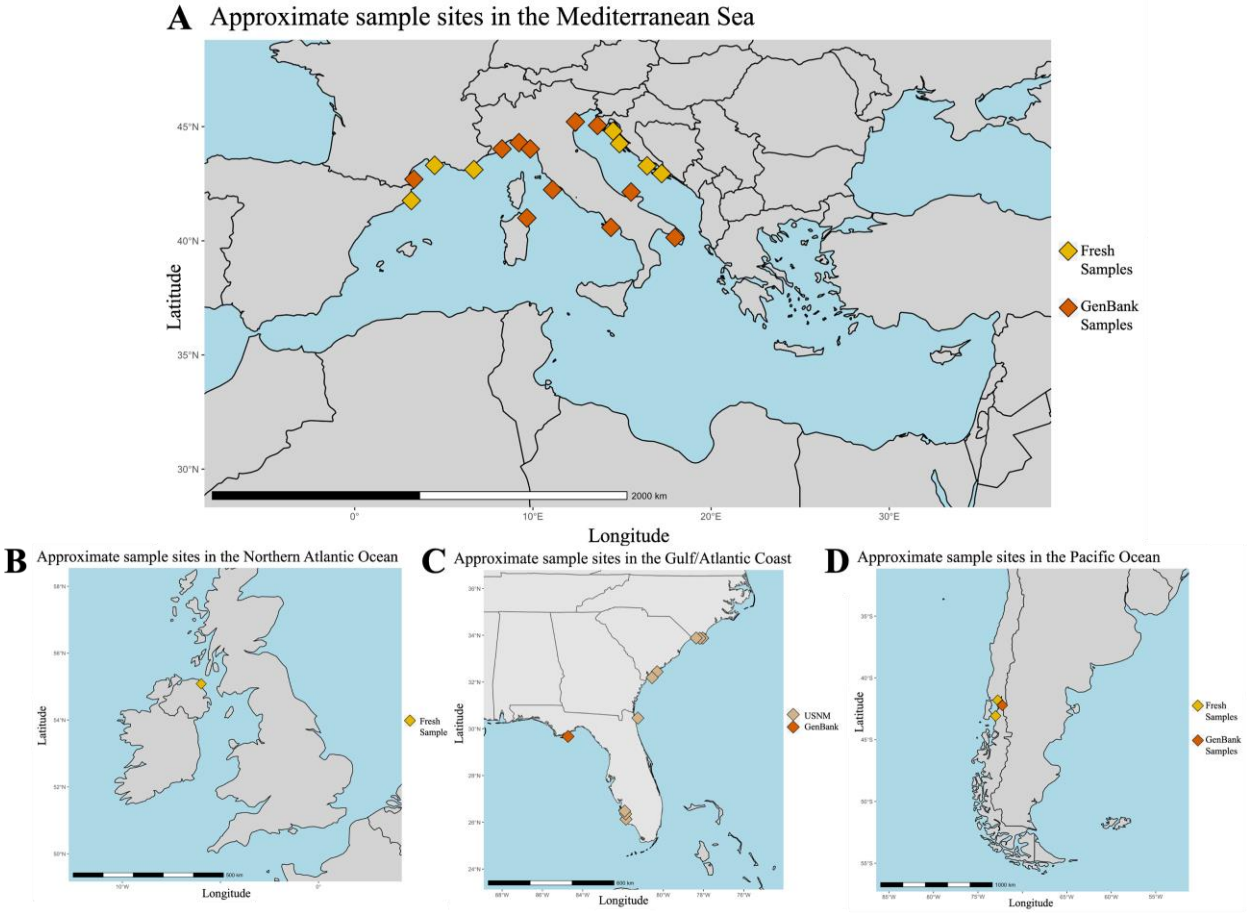

Figure 2. Approximate sample locations of the 37 samples collected, loaned from the USNM, and the samples pulled from GenBank. (A) Locations of samples from the Mediterranean (Adriatic Sea, Ligurian Sea, Gulf of Lion, and Tyrrhenian Sea). (B) Location of samples obtained from Northern Ireland. (C) GenBank and USNM samples from the Gulf Coast and east coast of the United States. (D) Samples from the Pacific Ocean near Chile.

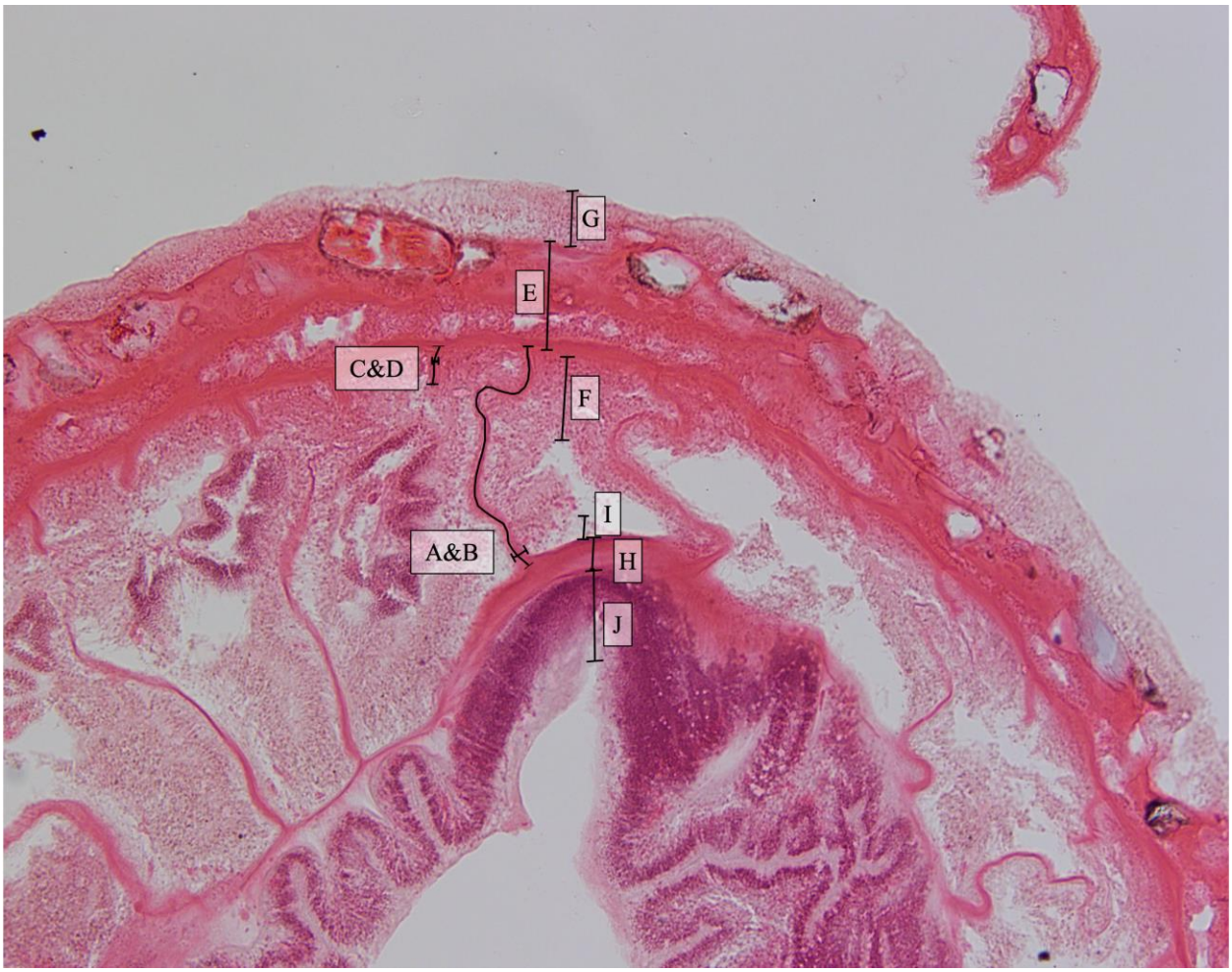

Figure 3a. Specimen M1 as a cross-section taken from the actinopharynx region. Labeled features are, length of the ventral directive (A), width of the ventral directive (B), length of a microneme (C), width of the microneme (D), thickness of the column mesoglea (E), thickness of the column endoderm (F), thickness of the column ectoderm (G) thickness of the siphonoglyph mesoglea (H) thickness of the siphonoglyph endoderm (I), and thickness of the siphonoglyph ectoderm (J). Photograph taken by Dr. Timothy Swain.

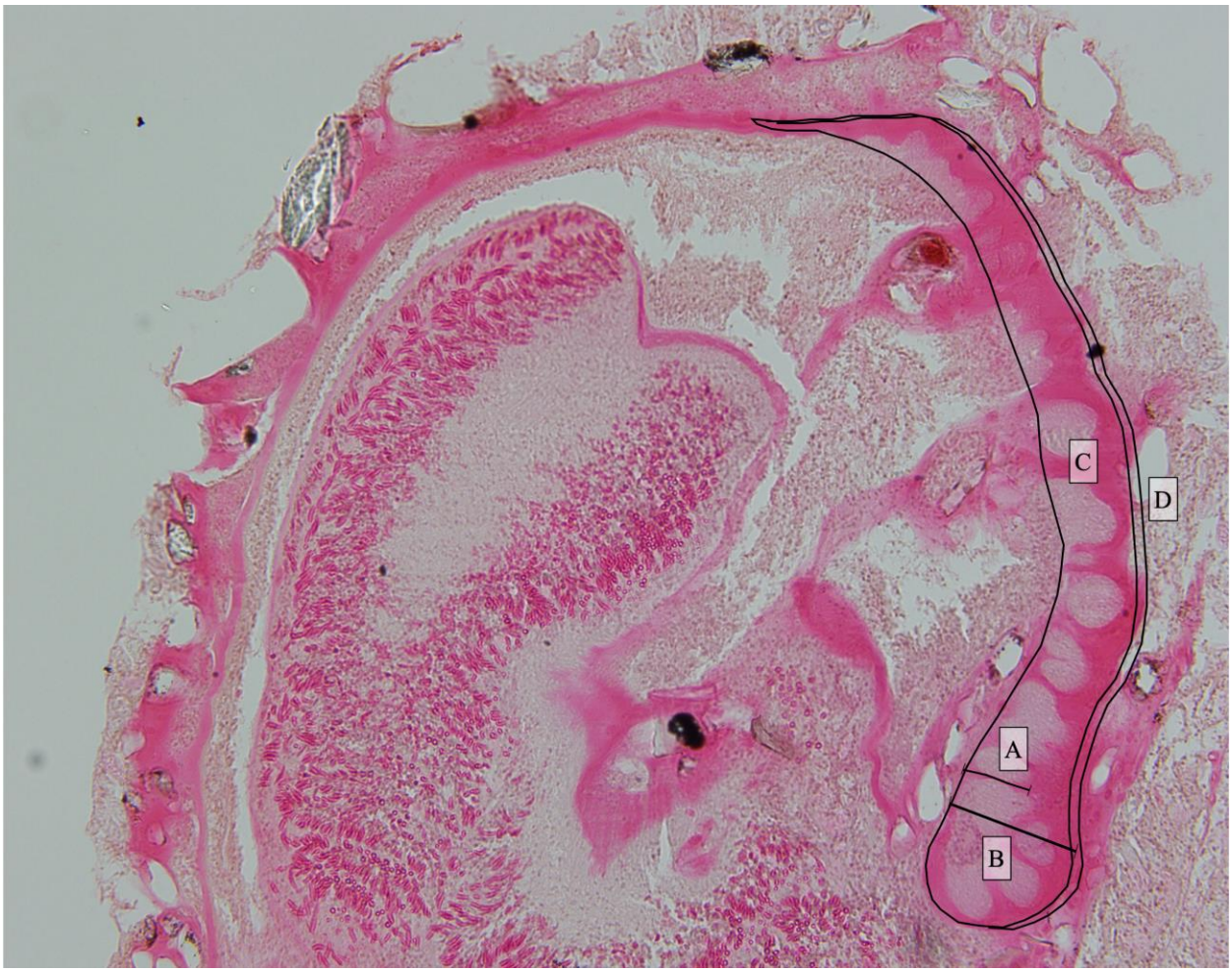

Figure 3b. M1 as a longitudinal histological section. The labeled features are the longest attachment site (A), diameter of the muscle (B), muscle surface area (C), and length of the marginal muscle (D). Photograph taken by Dr. Timothy Swain.

Results

Sample Acquisition:

The search for holotypes or authoritative specimens of *P. axinellae* and subspecies, *P. a. adriaticus*, *P. a. linguricus*, and *P. a. brevitentacularis* yielded no confirmed specimens in museum or university collections. A type specimen of the subspecies *P. a. mülleri* was confirmed to be in the collection at the Naples Zoological Station in Italy but was inaccessible at the time of this research. Researchers who have worked with modern specimens of *P. axinellae* were contacted to try and obtain more modern samples to increase the sample size and validity of this project. These potential collaborators were all located in areas of Europe, during the pandemic of 2020, most of Europe was under lockdown, and these samples were never obtained.

Phylogenetics:

The staggered alignment phylogeny recovered seven distinct ingroup clades of species affiliated with *Parazoanthus axinellae* and supported the conclusion that with the current sampling that there are at least three genetically distinguishable sibling species within modern *P. axinellae* (Figure 4).

Most basal of the ingroup clades is the Caribbean *Parazoanthus swiftii* (Duchassaing & Michelotti, 1860) and eastern Pacific *Parazoanthus darwini* (Reimer & Fujii, 2010) clade and affiliated specimens (Figure 4). These species share similar shallow water demosponge hosts and are closely related to modern *P. axinellae*. The next ingroup clade is comprised of specimens of *Parazoanthus atlanticus* (Montenegro, Hoeksema, Santos, Kise, & Reimer, 2020)(Figure 4), a transatlantic species of shallow water demosponge symbiotic zoanthideans (Montenegro et al., 2020).

A clade described here as *P. axinellae* (restricted) included physical specimens 64, 65, F1.1, F1.2, F2, GS-1, M1 through M5, R1 through R4, V1 through V4, as well as the ID groups SRO, CAM, SRY, PFY, BAY, and PVO from Villamor 2020. *P. axinellae* (restricted) is sister to a clade comprised of *Parazoanthus capensis* (Duerden, 1907) from South Africa and *Parazoanthus anguicomus* (Norman, 1869) from the northeastern Atlantic and Mediterranean Sea (Figure 4). All these species are symbionts of shallow water demosponges. Specimens of *P. axinellae* (restricted) are from Italy, Spain, France, Croatia, Ireland, and the northern Gulf of Mexico. The Villamore 2020 GenBank data showed all samples included in this clade were identified as yellow and "slender", except for one individual, SRO1, which was designated as orange and "stocky."

The *P. axinellae* (restricted), *P. capensis*, *P. anguicomus* clade is sister to a monophyly comprised of three sibling clades of specimens closely affiliated with *Parazoanthus juan-fernandezii* (Carlgren, 1922)(Figure 4). Many of these specimens had been previously published as morpho-color-types of modern *P. axinellae* but are genetically quite different from the specimens of modern *P. axinellae* (restricted) discussed above. The specimens in the first *P. juan-fernandezii* associated clade are PC1 through PC5, and ID groups PVO, SRO, and SRY from the Villamore 2020 GenBank data. These samples were all collected from Mediterranean France. Both PVO and SRO were identified as "stocky" orange specimens, but again, one nonconforming "slender" yellow specimen, SRY2, was in this clade. This first clade has a

bootstrap support value of 95 (Figure 4). Specimens PC1 through PC5 were previously designated as *Parazoanthus aff. juan-fernandezii* (Mediterranean) by Swain in 2018 and the Villamor sequences were initially reported as *P. axinellae* (Swain, 2018; Villamor et al., 2020). They are designated here as *Parazoanthus aff. juan-fernandezii* (Mediterranean-1). The phylogeny supports the interpretation that these specimens are likely a separate species within what modern authors refer to as *P. axinellae.*

A second sibling clade is comprised of specimens 219, 187, as well as sample N and FS222 of *Parazoanthus juan-fernandezii* which were collected from the coast of Chile and had a bootstrap support value of 74 (Sinniger & Häussermann, 2009)(Figure 4). *P. juan-fernandezii* is a symbiont of Pacific shallow water demosponges.

A third sibling clade is comprised of two apparent species. The first is a specimen previously designated as *Parazoanthus aff. juan-fernandezii* (California) by Swain in 2018 from the eastern Pacific and is likely to be a species that is new to science (Swain, 2018). The second is comprised of specimens from the ID groups PVO, ALO, and PFO from the Villamore 2020 data set designated here as *Parazoanthus aff. juan-fernandezii* (Mediterranean-2) and were designated as "stocky" orange specimens from Mediterranean France and Italy (Figure 4). This clade had a bootstrap support value of 65. The phylogeny supports the interpretation that these Mediterranean specimens are likely a separate species within what modern authors refer to as *P. axinellae.*

For Mediterranean *P. axinellae* affiliates, there seem to be three species distinguishable by complete ITS rRNA gene region: *Parazoanthus axinellae* (restricted), *Parazoanthus aff. juan-fernandezii* (Mediterranean-1), and *Parazoanthus aff. juan-fernandezii* (Mediterranean-2). From the genetic data alone, it is impossible to know which, if any, of these specimens correspond to the historical definition of *P. axinellae* or any of the named subspecies, except that they are unlikely to be *P. a. linguricus* as it is not known from less than 200 m depth.

Histology:

Although all specimens assessed through histology are considered by other authors to be within the modern concept of *P. axinellae*, there are clear genetic differences that support two potential species (no tissue specimens of the third potential species detected by phylogenetics, *Parazoanthus aff. juan-fernandezii* (Mediterranean-2), were available). The sampled characters that appeared to have different sample means were the siphonoglyph ectoderm, siphonoglyph

endoderm, column ectoderm, column endoderm, microneme length and width, ventral directive length and width, length of the marginal muscle, height of the marginal muscle attachment sites, and the mean number of marginal muscle attachment pleats (Table 3). Two-tailed t-tests revealed which microanatomical characters are significantly different between the two species (Table 4). The significant characters were the microneme width, the microneme length, and the thickness of the column mesoglea (Table 4, Figure 5a-d). The microneme length in the *P. axinellae* (restricted) polyps ranged from 27.51-88.84 µm, where for *P. aff. juanfernedezii* (Mediterranean-1) the range was 54.4-135.24 µm. The microneme width range for *P. axinellae* (restricted) was 3.42-8.47 µm and for *P. aff. juanfernedezii* (Mediterranean-1) was 7.2-21.97 µm (Table 3; Figure 6). The column mesoglea for *P. axinellae* (restricted) ranged from 12.65-118.33 µm and for *P. aff. juan-fernandezii* 46.81-111.83 µm (Table 4). Additionally, the height of the attachment sites of the marginal muscle had a marginally significant p-value but was unusable for identification purposes.

Using the statistically significant microanatomical characters, the shallow water demosponge-symbiotic museum specimens from the United States east, southeast coast that were either designated as *Parazoanthus sp*. or unknown Zoanthidea were compared to phylogenetically differentiated specimens. When placed on a microneme length vs width scatterplot and within a table of measured characters the unknown specimens generally fell within the range of the *P. axinellae* (restricted) parameters (Table 4; Figure 7).

Table 2. 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	1-190	0.2482	0.2395	0.2768	0.2356	1.0740	118.1601	0.0001	0.0001	1.2380	0.0200
ITS1	191-936	0.2107	0.2390	0.2582	0.2921	0.9864	1.9890	0.7594	1.5414	2.3081	0.7680
5.8S	937-1093	0.2356	0.2038	0.2803	0.2803	0.0001	0.0092	0.0001	0.0001	0.0107	0.0200
ITS2	1094-1454	0.1852	0.2847	0.2926	0.2376	1.5351	5.3897	1.3866	1.6908	4.2423	0.6911
28S	1455-1512	0.2413	0.2878	0.2129	0.2581	5.4151	0.0001	3.9929	2.0667	54.5511	0.2314

Table 3. Complete list of characters and anatomical observations for *P. axinellae* (restricted), *P. aff. juan-fernandezii* (Mediterranean-1), and the USNM specimens. Statistically significant characters are in bold. Observational characters were given values to describe the variation: Encrusting penetration (0 = ectoderm surface 1 = through ectoderm 2 = outer mesoglea 3 = center mesoglea 4 = through mesoglea), Encircling sinus (0 = absent 1 = discontinuous 2 = continuous), Ventral directive hetero or homomorph (0 = homo 1 = hetero).

Character	*P. axinellae (restricted)*		*P. aff. juan-fernandezii* (Mediterranean-1)		USNM	
	Mean	Range	Mean	Range	Mean	Range
Microneme length (µm)	**49.84**	**27.51-88.84**	**103.06**	**54.4-135.24**	**41.09**	**25.05-88.57**
Microneme width (µm)	**5.25**	**3.42-8.47**	**13.71**	**7.2-21.97**	**4.13**	**2.54-6.62**
Ventral Directive length (µm)	293.41	147.8-478.42	460.98	246.1-638.26	148.31	53.57-247.55
Ventral Directive width (µm)	7.29	4.00-17.16	11.17	7.00-25.62	6.49	2.92-10.75
Siphonoglyph ectoderm (µm)	41.32	14.17-79.80	57.32	40.00-92.9	28.16	10.21-47.28
Siphonoglyph mesoglea (µm)	25.54	12.99-56.35	41.68	16.33-106.4	11.06	7.31-23.50
Siphonoglyph endoderm (µm)	32.06	12.30-72.12	19.32	9.40-26.00	11.06	7.31-12.52
Column ectoderm (µm)	32.73	9.37-73.18	55.82	12.75-92.2	19.33	6.74-30.81
Column mesoglea (µm)	**63.77**	**12.65-118.33**	**104.93**	**46.81-111.83**	**49.46**	**4.59-81.25**
Column ectoderm (µm)	24.96	7.15-76.89	39.16	9.49-75.20	19.15	8.27-49.53
Marginal muscle area (μm^2)	28585.63	2311.92-44159.91	23484.06	6217.38-41454.98	11207.99	5981.28-19122.55
Marginal muscle length (µm)	656.39	185.77-874.77	630.61	387.84-1146.40	382.24	292.04-532.50
Marginal muscle diameter (µm)	72.00	15.11-103.67	97.32	56.57-247.30	52.75	26.29-100.85
Marginal muscle attachment site height (µm)	34.68	9.39-63.79	24.93	16.16-42.30	16.54	12.95-26.59
Number of attachment sites	20.01	15.40-21.88	18.66	16.6-22.20	17.53	15.80-20.33
Encrustation penetration	2.5	2-3	2.2	2-3	3.1	3-4
Encircling sinus	1.2	0-2	1.3	0-2	0.6	0-1
Max number of mesenteries	29.79	26-34	33.00	26-48	27.00	26-31
Ventral directive hetero or homomorph	0.06	0-1	0.2	0-1	0	0
Actinopharynx lining	1.33	0-2	0.4	0-1	0.63	0-2
Prominent siphonoglyph	0.63	0-1	1	1	0.25	0-1

Table 4. The resulting p- values from two-tailed t-tests for each related morphological character. Statistically significant characteristics are in bold. Characters listed are (SM) siphonoglyph mesoglea, (SE) siphonoglyph ectoderm, (SEN) siphonoglyph endoderm, (CM) column mesoglea, (CE) column ectoderm, (CEN) column endoderm, (ML) microneme length, (MW) microneme width, (VDL) ventral directive length, (VDW) ventral directive width, (MMA) marginal muscle area, (MMD) marginal muscle diameter, (LMM) length of the marginal muscle, (HAS) height of the attachment sites, and (MP) mean number of pleats.

	SM	SE	SEN	**CM**	CE	CEN	**ML**	**MW**
P-value	0.495	0.1777	0.0911	**0.0463**	0.1477	0.259	**0.0106**	**0.0036**
	VDL	VDW	MMA	MMD	LMM	HAS	MP	
P-value	0.1024	0.1466	0.3912	0.8366	0.4069	0.0653	0.1793	

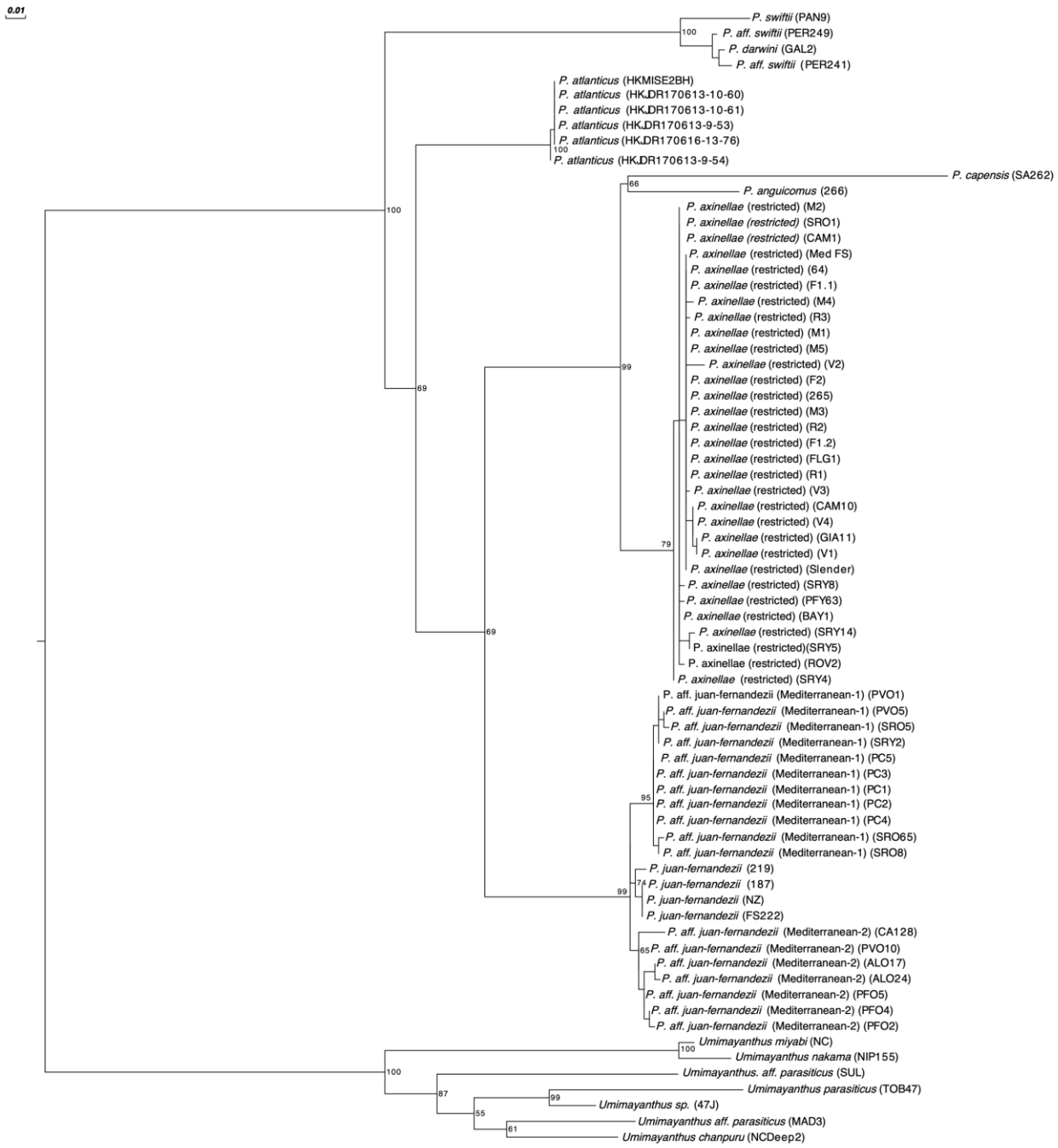

Figure 4. Phylogeny inferred from complete ITS rRNA nuclear data showing seven distinguishable *P. axinellae* affiliated species: *P. capensis*, *P. anguicomus*, *P. axinellae* (restricted), *P. aff. juanfernandezii* (Mediterranean-1), *P. juan-fernandezii*, *P. aff. juan-fernandezii* (Mediterranean-2), and *P. aff. juan-fernandezii* (California).

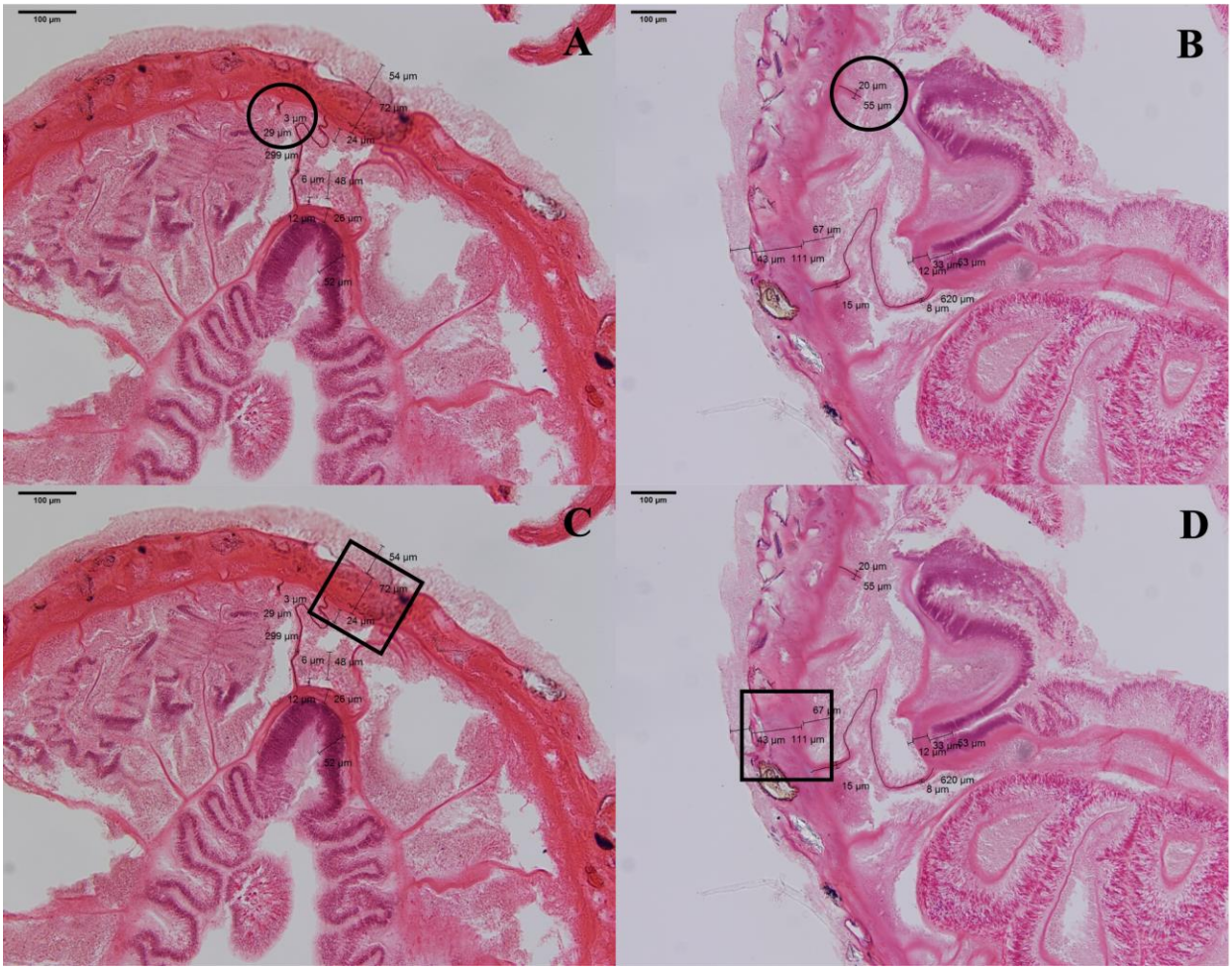

Figure 5a-d. (A) *P. axinellae* (restricted) microneme with a length of 29 µm and a width of 3 µm (black circle). (B) *P. aff. juan-fernandezii* (Mediterranean-1) microneme with a length of 55 µm and a width of 20 µm (black circle). (C) *P. axinellae* (restricted) column mesoglea, 72 µm long (black square). (D) *P. aff. juan-fernandezii* (Mediterranean-1) column mesoglea (black square) with a length of 111 µm. The comparison between figures A and B, as well as C and D, show the significant differences between these microanatomical characters between species.

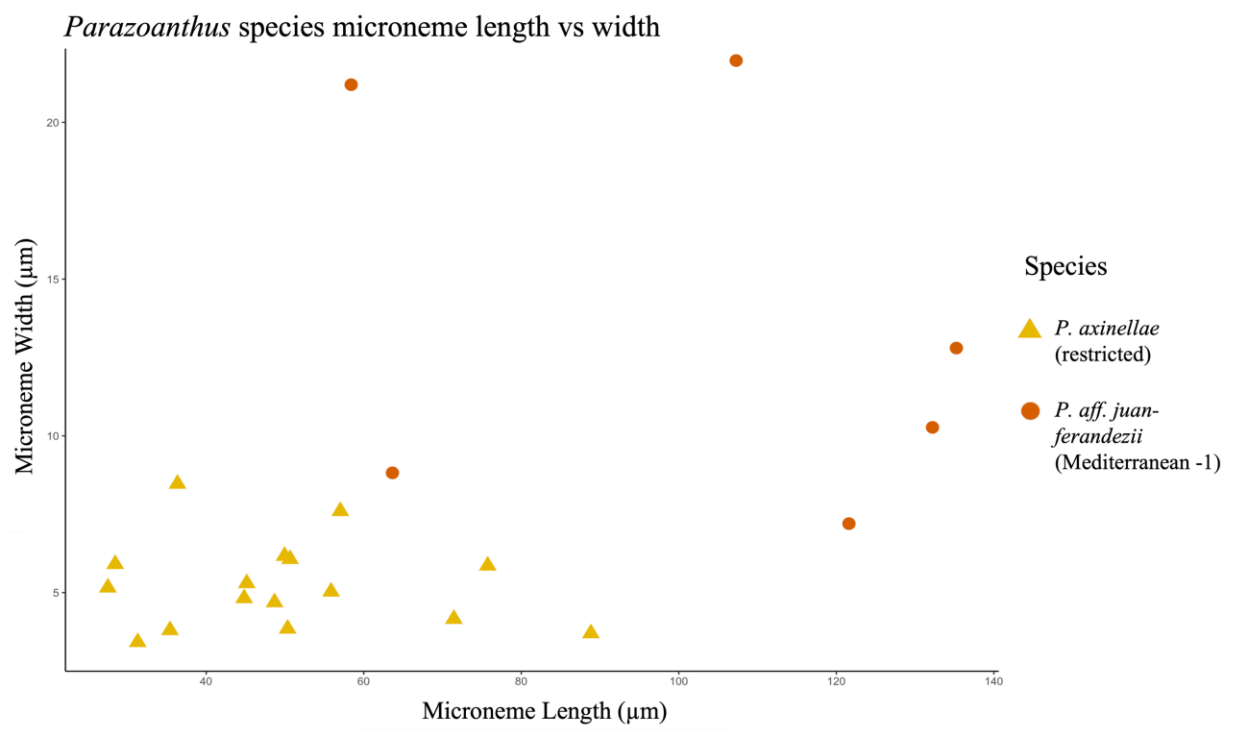

Figure 6. Graph showing the difference in the microneme length for two *Parazoanthus* species. *P. axinellae* (restricted) (yellow triangles) has a smaller microneme in both length and width than the *P. aff. juanfernandezii* (Mediterranean-1) samples (orange circles).

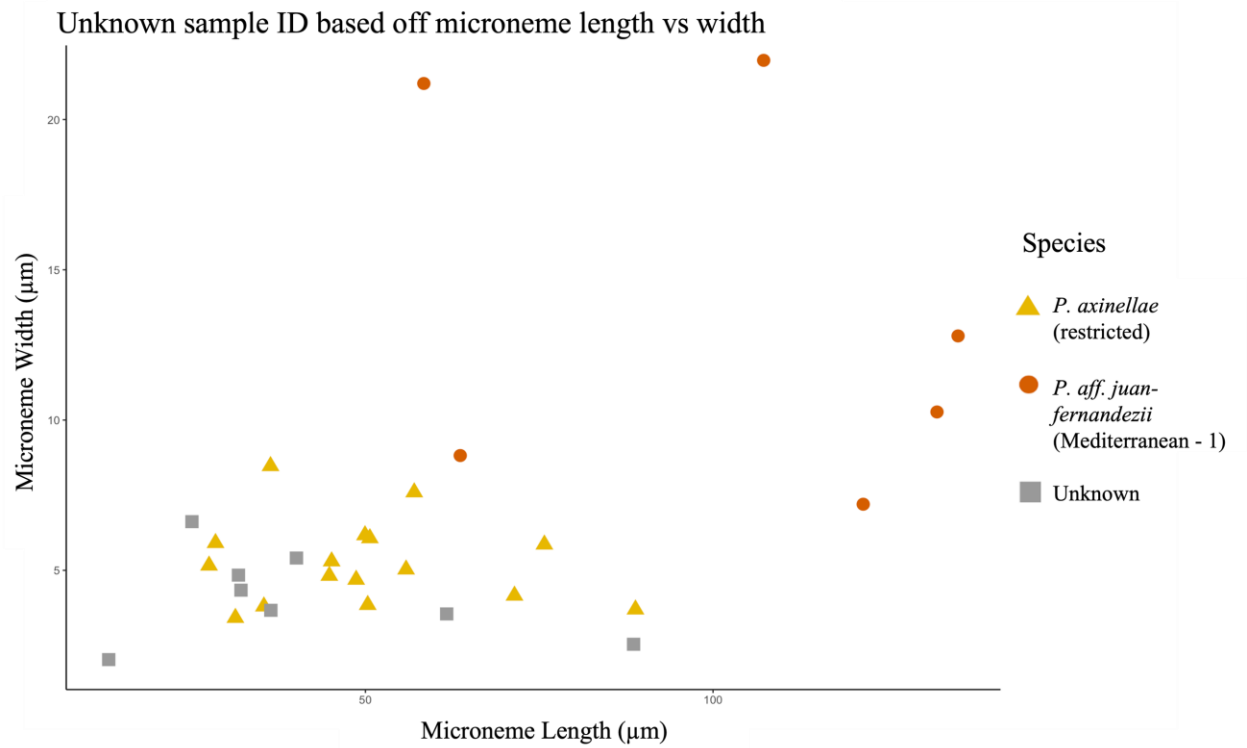

Figure 7. The unknown samples mixed in with the known *P. axinellae* (restricted) and *P. aff juan-fernandezii* (Mediterranean-1) using the statistically significant microneme characters. The unknown USNM specimens (grey square) fall within the range of the *P. axinellae* measurements.

Discussion

Research Question 1: Is there complementary evidence across multiple data streams consistent with species boundaries within modern *P. axinellae*?

Both phylogenetic and microanatomical datasets were able to differentiate two apparent species within specimens considered by other authors to fall within the modern concept of *P. axinellae*, designated here as *P. axinellae* (restricted) and *P. aff. juan-fernandezii* (Mediterranean-1). Tissue specimens of a third potential species, *P. aff. juan-fernandezii* (Mediterranean-2) could not be obtained at the present time and therefore only genetic differences are detected here. Clear genetic differences within the complete ITS gene region (Figure 4) corresponded to significant differences in the size and width of micronemes as well as the size of the column mesoglea observed at the level of the actinopharynx (Figure 5; Figure 6).

ITS has been previously demonstrated to be a species-level marker within the Zoanthidea, and its hypervariable nature results in some genetic structure even within species (Aguilar & Reimer, 2010; Hillis & Dixon, 1991; Swain, 2009, 2018). The *P. axinellae* (restricted) and *P. aff. juan-fernandezii* (Mediterranean-1) clades recovered in the phylogeny presented here show little or no genetic differentiation within clades even though specimens were collected from disparate regions of the Mediterranean and even from the Gulf of Mexico. Therefore, the clades are not genetically distinguishable by location or depth. These two clades are separated by branch lengths that are orders of magnitude longer than within clade branch lengths and are significantly supported by bootstrapping (Figure 4).

Similarly, the third potential species *P. aff. juan-fernandezii* (Mediterranean-2) is well differentiated phylogenetically, has little or no genetic differentiation with the clade, and is separated from other clades by branch lengths that are orders of magnitude longer. Its closest relative is an apparently new species collected from California and is the only potential species of the three that is consistent with morpho-color-types (all specimens are “stocky” and orange) and apparently geographically isolated (all specimens are from the Ligurian sea). This would seem to make these specimens a good candidate for representatives of *P. a. linguricus*; however, the original description stipulates that *P. a. linguricus* is only to be found at depths of 200-350 meters (Table 1) and these specimens are from shallow waters.

An additional data stream of nematocyst identity and size distribution across multiple tissue regions within polyps remains as an unexplored potential source of differentiating characters for integrative systematics of the *P. axinellae* species complex.

Research Question 2: Does the molecular data from modern *P. axinellae* morpho-color-types fall within the detected integrative boundaries?

Villamor proposed that modern *P. axinellae* has morphotypes based on polyp gestalt ("stocky" versus "slender") and matching color variations (orange versus yellow) that are genetically differentiated (Villamor et al., 2020). The ITS DNA sequences collected by Villamor were included in the analyses presented here with the result that these morpho-color-types are reflected in the monophylies recovered by the phylogenetic inference however, they are imperfect. The analysis presented here split modern *P. axinellae* into three Mediterranean clades designated as *P. axinellae* (restricted), *P. aff. juan-fernandezii* (Mediterranean-1), and *P. aff. juan-fernandezii* (Mediterranean-2). The *P. axinellae* (restricted) clade is made up of "slender" yellow polyps, except for sample SRO1 which was "stocky" orange, while the *P. aff. juan-fernandezii* (Mediterranean-1) clade comprised of "stocky" orange specimens except for specimen SRY2 which was "slender" yellow (Figure 4). *P. aff. juan-fernandezii* (Mediterranean-2) only includes "stocky" orange (Figure 4). Meaning that "stocky" orange morpho-color-types could be used to represent *P. aff. juan-fernandezii* (Mediterranean-1) and (Mediterranean-2) while the "slender" yellow morpho-color-types could represent *P. axinellae* (restricted); however, a small amount of error is involved. Villamor also stated that the "stocky" orange morpho-color-type could be closely related to the Pacific species *P. juan-fernandezii* or *P. elongatus* (Villamor et al., 2020), but it was also a component of a more distant clade. Early experts in this group speculated that *Parazoanthus* polyp colors resulted from the amount of light available to them, not based on the species themselves, and as previously discussed, zoanthidean species are highly variable, so general size and shape may be poor determinants (Abel, 1959; Reimer et al., 2006).

Research Question 3: Do the detected integrative boundaries appear to correspond to the historical definition of *P. axinellae* or any of its four daughter subspecies?

To understand species boundaries for the *P. axinellae* species complex, historical definitions and type or authoritative specimens should be the primary sources of information. *P. axinellae* was described over 150 years ago in Europe, and the history of documenting specimens

in taxonomic works and historical events in Europe have blurred the existence and chains of possession of these important specimens (Harclerode & Pittaway, 1999; Schmidt, 1862; Warsaw, 2020). Most notably, the two World Wars fought in Europe destroyed buildings, collections, institutional knowledge, and generally increased uncertainty around the location and existence of any authoritative specimens (Bothwell, Hansen, & MacMillan, 2008; Dean, 2009; Harclerode & Pittaway, 1999). When Schmidt described *P. axinellae* in 1862, he failed to list a location of a type specimen (Schmidt, 1862). Later in 1891, Haddon and Shackleton assigned *P. axinellae* as the type species for the *Parazoanthus* genus without listing a location for a type specimen (Haddon & Shackleton, 1891). In 1937 when Pax described two new subspecies he listed the type specimen for *P. axinellae* to be at the University of Graz, and the co-type to be at the University of Strasbourg (Pax, 1937). However, communication with the curators from these universities yielded little to no results (Ludes-Fraulob, 2020; Sturmbauer, 2020). Additionally Schmidt worked with museums like the University of Wroclaw across Poland (Pax, 1937, 1957; Warsaw, 2020). Unfortunately, during World War II, the German army occupied Poland (1939-45) and destroyed museum collections for storage space (Warsaw, 2020). In these collections, the specimen jars contained alcohol for organism preservation. The soldiers removing the collections discovered this and drank the remaining alcohol which destroyed the preserved organisms (Warsaw, 2020). The Museum of Natural History at the University of Wroclaw confirmed in May of 2020 that there are no specimens of *P. axinellae* or any *P. axinellae* subspecies submitted by Schmidt (Jurkowska, 2020). Schmidt also worked as the head of the zoological collection at the Joanneum Museum while he taught at the University of Graz (Schmidt, 1862; Ulrike, 2021). Just like the University of Wroclaw, the Joanneum Museum has no samples of *P. axinellae* in their collection, as confirmed by the museum's curator, Ulrike Hausl-Hofstätter in July of 2021 (Ulrike, 2021). At the present, it must be concluded that the type specimen for *P. axinellae* has been lost over time.

Additionally, two of the subspecies described by Pax (*P. a. adriaticus* and *P. a. linguricus*) had no type specimen location listed with the original descriptions (Pax, 1937). Pax worked for the University of Wroclaw during the time these samples would have been submitted (Pax, 1937). As previously stated, the University confirmed they have no *P. axinellae* specimens in their collection (Jurkowska, 2020). However, Pax also described a third subspecies, *P. a. mülleri,* which did have a type specimen designated in the original description and was submitted

to the Naples Zoological Station in Italy, near where the specimen was collected (Pax, 1957). The published online invertebrate collection catalog confirmed that both the holotype and a paratype for *P. a. mülleri*, as well as other *P. axinellae* samples submitted by Pax, are currently housed in the collection at the Naples Zoological Station (Travaglini, 2020; "Zoological Collection Database @ Stazione Zoologica 'Anton Dorn' di Napoli," 2007). The zoological station is dedicated to upholding and preserving type specimens, but extended an invitation in May of 2020 to visit and work with the *P. a. mülleri* types and *P. axinellae* specimens only if it could be done at the zoological station (Travaglini, 2020). Unfortunately, due to the global pandemic, the lockdown in Italy, and travel restrictions, it was not possible to travel to work with these specimens.

The last subspecies, *P. a. brevitentacularis*, also had no type specimen designated in the original species description (Abel, 1959). Likely collections in Europe where Abel worked at the time, such as the University of Vienna, were contacted to verify if any specimens were submitted by Abel or under the name *P. a. brevitentacularis*. Unfortunately, there were no specimens to be found in the collections of the University of Vienna and we must assume that they have been lost or never existed (Ludes-Fraulob, 2020). Further investigation into *P. a. brevitentacularis* revealed that the identity of the subspecies has often been debated. Scientists have wavered on whether or not to dissolve *P. a. brevitentacularis* as a viable subspecies due to the lack of information (Abel, 1959; Maria & Sardà, 1982; Riedl, 1966). Therefore, due to the subspecies' ambiguity the search for any usable type specimens within the species complex was concluded. There were no usable type specimens or other authoritative specimens associated with this species complex (other than *P. a. mülleri*), meaning that there was no way to confirm the historical definition or taxonomic boundaries of the *P. axinellae* species complex at this time. However, as three of the four named subspecies were described by Pax, and the type of *P. a. mülleri* and specimens of *P. axinellae* identified by Pax exist, finding additional Pax specimens and treating them as authoritative specimens to clarify *P. axinellae* and daughter subspecies hypotheses (and potentially designating them as new types) may be the only remaining viable solution to assess the historical boundaries of the complex (Pax, 1937, 1957; Travaglini, 2020).

Without access to historical specimens, this study had to rely on the integrative systematics of modern *P. axinellae* specimens to investigate the species boundaries associated with this complex. *P. axinellae* and its subspecies were described solely on their microanatomy

and sample locations (Abel, 1959; Pax, 1937, 1957; Schmidt, 1862). The characters that were used to describe all five of these species/subspecies were the tentacle length, the holotriche length, the number of mesenteries, sample depth, and sample location (Table 1) (Abel, 1959; Pax, 1937, 1957; Schmidt, 1862). As previously stated, Zoanthidea species are morphologically plastic, and as such, a wide variation of morphological characters is to be expected (Reimer et al., 2006). The polyps used in this study were all preserved and retracted, so it was impossible to collect tentacle length data, and due to time constraints, we were unable to collect data to analyze the holotrich nematocysts. The historical *P. axinellae* species boundary for the number of mesenteries is between 28 and 39 (Schmidt, 1862). In modern specimens of *P. axinellae,* described as *P. axinellae* (restricted), the number of mesenteries ranged between 26 and 34 but, none of the subspecies' descriptions had the number of mesenteries lower than 32 (Table 1) (Abel, 1959; Pax, 1937, 1957). Due to the variability possible in this order it can be assumed that the lower number of mesenteries could be caused by plasticity. *P. axinellae* and its subspecies were historically described through sample depth and location. *P. axinellae* can be found at depths of 30-100 meters (Abel, 1959; Pax, 1937, 1957; Schmidt, 1862). Again, this is a highly variable range, and the subspecies have similar overlapping bathymetries, except in the case of *P. a. linguricus* which is found at depths of 200-350 meters (Table 1)(Pax, 1937). With an integrative systematic approach, there are other data streams possible to explore these species and subspecies hypotheses.

Given the lack of type or authoritative specimens and the low quality of the original species descriptions, it is currently impossible to place any of the specimens used in this research within the historical concepts of *P. axinellae* or any of the daughter species, with the possible exception of excluding them from assignment to *P. a. linguricus* because this is historically defined as a deep-water species.

Research Question 4: Does the anatomical data from unidentified *Parazoanthus* sponge-symbiont museum specimens fall within the detected integrative boundaries?

To identify unidentified museum specimens unsuitable for DNA amplification and sequencing, consistent morphological differences had to be identified that corresponded to potential species boundaries. This research revealed consistent microanatomical differences between two genetically different potential species, designated as *P. axinellae* (restricted) and *P. aff. juan-fernandezii* (Mediterranean-1) (Table 4). Histology revealed that *P. axinellae*

(restricted) polyps have a smaller microneme length and thickness and a smaller column mesoglea than *P. aff. juan-fernandezii* (Mediterranean-1) (Table 4; Figure 5). Using a scatterplot of the microneme length vs width collected from specimens in these two clades, previously unidentified museum specimens were plotted. It was confirmed that their microneme dimensions fell within the range of *P. axinellae* (restricted) specimens (Figure 6). These specimens from the USNM were collected from shallow water demosponge hosts along the southeast coast of the United States and their microanatomy is consistent with *P. axinellae* (restricted) (Table 4). These identifications show that integrative systematics is a viable way to include older samples from museums that are thought to be 'lost to time' in future studies.

Research Question 5: Do the detected integrative boundaries support transatlantic distributions of *P. axinellae*?

The distribution of *P. axinellae* was considered limited to the Mediterranean Sea and northern eastern Atlantic (Horton et al., 2021). However, a specimen collected from the Gulf Coast of Florida was previously identified through phylogenetics as *P. axinellae* (Swain), and again here as *P. axinellae* (restricted) (Swain, 2009). Additionally, nine previously unidentified USNM museum specimens collected from along the southeast coast of the United States (Figure 2c) appear to belong to the *P. axinellae* (restricted) clade (Table 4; Figure 6). These observations support the conclusion that *P. axinellae* (restricted) is transatlantic, similar to the closely related *P. atlanticus*, another transatlantic species of shallow water demosponge symbiotic zoanthidean (Figure 8) (Montenegro et al., 2020).

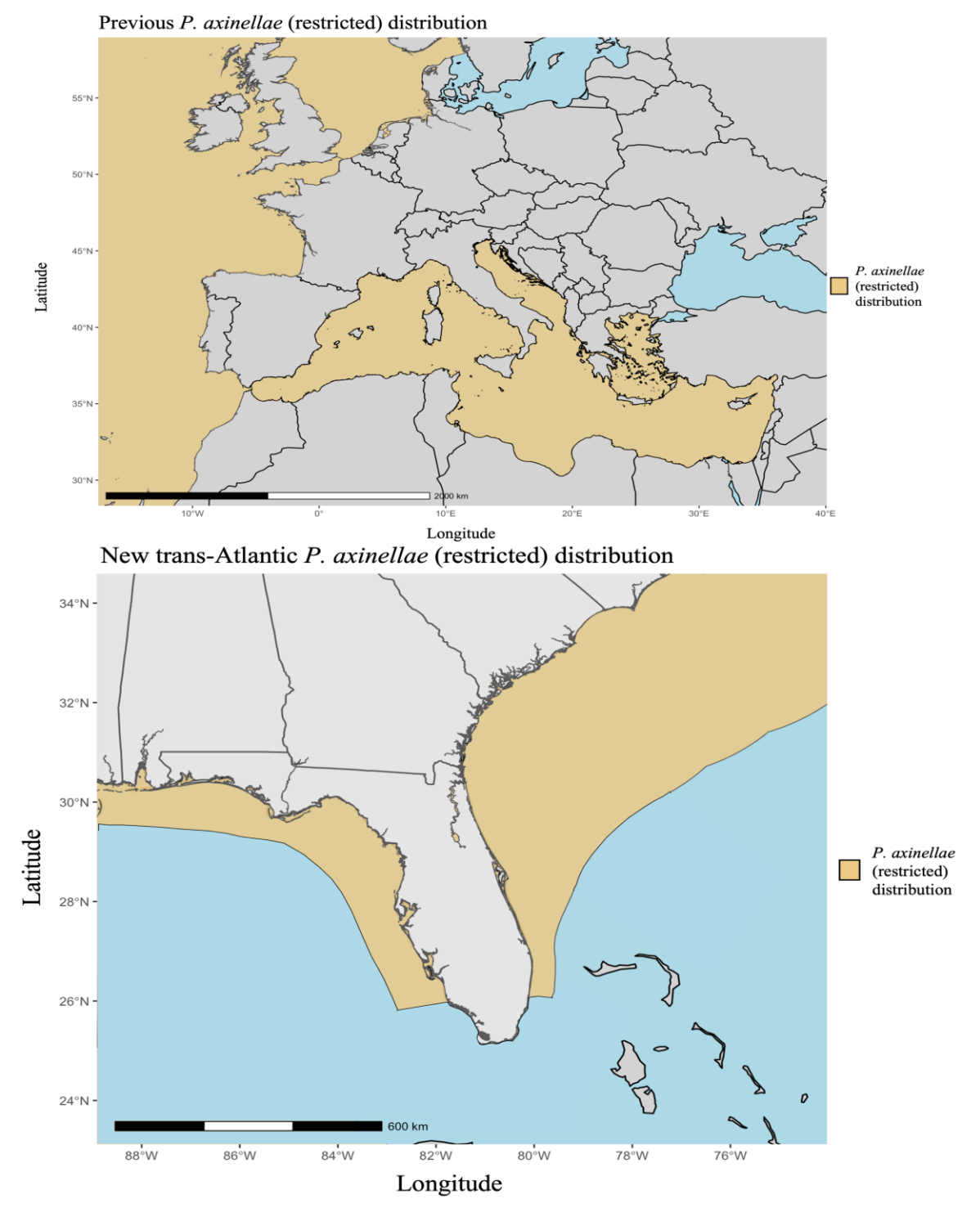

Figure 8. Top map shows the current accepted distribution for *P. axinellae* (restricted) which spans the Mediterranean Sea and the Northern Atlantic Ocean (Horton et al., 2021). The bottom map shows the expanded *P. axinellae* (restricted) distribution that now includes the gulf coast of Florida and part of the Atlantic coast.

Conclusion

This thesis research had five questions it aimed to answer using integrative systematic techniques. The first question, if the modern *P. axinellae* species concept contains sufficient variation to suggest multiple species. The samples in the Mediterranean Sea and the northern Atlantic showed significant genetic variation to suggest that there are at least three Mediterranean species, two of which have consistent microanatomical differences supporting the genetic species boundaries. The second question — if the genetic boundaries of the newly erected morpho-color-types fall within the detected integrative boundaries — was also addressed and clarified. The morpho-color-types "stocky" orange and "slender" yellow almost consistently correspond to the three reconstructed specimen clades in the phylogenetic analysis but are imperfect and should be examined closer. The third question from this research was if the integrative boundaries correspond with the historical idea of species and subspecies in this complex. The attempt to corroborate the boundaries of historical concepts of *P. axinellae* and its four daughter subspecies largely failed because of the inability to locate nearly all of the type specimens and the generally poor quality of the original species descriptions. The only conclusion that can be offered is that none of the specimens examined are likely to fit within the historical concept of *P. a. linguricus* as it is only to be found at depths of 200-350m. The fourth question sought to use all the data collected here to identify unidentified museum specimens. By integrating microanatomical and phylogenetic data of known specimens, patterns in the microanatomical characters may align with characters collected from the unidentified museum specimens and support preliminary identifications. By using the length and width of micronemes and all the characters listed in Table 4, the 12 unidentified museum specimens were identified as *P. axinellae* (restricted). The last question asks if the totality of the data collected support the hypothesis that *P. axinellae* has a transatlantic distribution. One specimen collected from the Gulf Coast was genetically identified as *P. axinellae* (restricted), and the USNM specimens collected from the southeast coast of the United States were also identified as *P. axinellae* (restricted) based on microanatomy, supporting the transatlantic hypothesis.

Integrative systematics applied to the *P. axinellae* species complex was able to resolve species boundaries of three cryptic species, expand previous morpho-color-types, identify museum specimens for which only partial data could be recovered, and detect enormous distribution expansions. Reconnecting modern concepts with historical definitions would have

also been successful if type specimens could have been located or accessed to supplement written species descriptions. Although this research significantly advances our understanding of species diversity within the *P. axinellae* species complex, a great amount of work remains to be done before all outstanding issues are resolved.

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