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Research Article

DNA mini barcodes vs full-length barcodes of octocoral museum specimens: a case study of the Okinawa Churaumi Aquarium Zoological Collection

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Abstract

Octocorals are noted for their high diversity, with the identification of species and sometimes even genera often difficult based solely on morphological characteristics. Thus, many recent studies have employed DNA barcoding techniques to acquire diversity data. There are many octocoral specimens already deposited within zoological collections that could provide potentially important taxonomic and historical information, but due to their age, often the DNA contained in such specimens has degraded severely. One potential approach to overcome this issue is DNA mini barcoding, amplifying a shorter subset of the original barcode region. This study aimed to utilize a mini barcoding method using novel octocoral-specific primers for validating octocoral species from the Okinawa Churaumi Aquarium Zoological Collection, with specimens dating back to 1982. Based on *in silico* evaluations using reference sequence data including nucleotide variations and match ratio values of species delimitations, using newly designed primers, mini barcodes performed as well as full-length barcodes. Subsequently, mini barcodes were generated by these novel primers, amplifying a 215 base pairs (bp) fragment of the mitochondrial MutS-like protein (mtMutS), successfully amplifying 92.7% of 166 specimens, and sequences could be used to identify to genus level, while full-length barcoding primers amplified only 45.8% of specimens. Moreover, mini barcodes identified 67 different operational taxonomic units (OTUs) of octocorals within the specimens based on the species delimitation method of Assemble Species by Automatic Partitioning (ASAP) and unique sequences. With most octocoral specimens from the Okinawa Churaumi Aquarium Zoological Collection identified only to genus level, these additional molecular data were significantly beneficial in identifying specimens. Our study shows this DNA mini barcoding approach can cheaply and quickly acquire molecular data from comparatively older octocoral specimens, helping to identify such specimens, and adding further value to historical museum specimen collections.

Key words: ASAP, degraded DNA, mtMutS gene, novel primers, Octocorallia, old specimens

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Introduction

The class Octocorallia (phylum Anthozoa), octocorals, consists of more than 3,500 species (WoRMS 2025). As a vital component of many marine ecosystems, octocorals inhabit a wide range of environments in a variety of climate zones, from shallow tropical waters to the cold deep-sea (Fabricius and Alderslade 2001; McFadden et al. 2010). After scleractinian corals, octocorals have been acknowledged as the second-most abundant macro-benthic animal group on many Indo-Pacific coral reefs (Benayahu et al. 2019). Furthermore, octocorals have significant contributions in providing critical habitat that supports feeding and breeding grounds for economically valuable fish (Fujita et al. 1997) and are also a significant source of marine natural products for potential pharmaceutical applications (Cooper et al. 2014; Nurrachma et al. 2021). For these many research purposes, octocorals are regularly collected, and many specimens are stored in various museum collections, with the large majority yet to be thoroughly taxonomically examined (Baena et al. 2024).

For the past three centuries, objects and specimens have been collected and placed in natural history museums around the world (Johnson and Owens 2023). Natural history museum collections are rich repositories of known life forms that form the source of records (Funk 2018), the basis for a wide range of biological studies (Mulcahy et al. 2022), are verifiable by taxonomists (Sampaio et al. 2019), and valuable material for conservation genetics (Nakahama 2021). Museum specimens can also be used for time-series pollutant monitoring across the years, such as microplastic pollution (Ilechukwu et al. 2023; Dettling et al. 2024), and indicators of environmental change (Schmitt et al. 2018). In Okinawa, Japan, Okinawa Churaumi Aquarium is the biggest aquarium in Japan and its largest aquarium tank contains 7,500 cubic meters of seawater. The aquarium also has non-public natural history (zoological) collections built to preserve and study marine animals, particularly those from Okinawa, and one of these collections is of Okinawan octocorals. Most octocoral specimens in this collection have been labeled only to the genus level due to difficulty in species identification (McFadden et al. 2025).

Identifying these specimens and others already existing in natural history museums around the world requires sorting and classification before they are available for final species description and large-scale analyses of biodiversity patterns (Yeo et al. 2020). Sorting methods using traditional approaches involving highly trained taxonomists (museum professionals) are considered time-consuming and expensive; in octocorals, species identification to the species level is highly dependent on sclerite morphology (Vargas et al. 2010; Carlo et al. 2011). Thus, new sorting methods could speed up such work (Krell 2004). Studying poorly understood taxa such as octocorals, and providing a pre-sorting method, at least to the genus level, is thus very useful for further studies. The molecular approach offers a reliable and cost-effective method for pre-sorting large numbers of specimens into their respective taxonomic identities (Yeo et al. 2020).

Due to the long-term storage of specimen collections, the quality of preservation can decrease, which is why museum specimens can experience degradation (Untiedt et al. 2021). Furthermore, preservation protocols often use ethanol, or formalin, or initially fix in formalin and then transfer to ethanol for long-term preservation (Williams and Van Syoc 2007; Zimmermann et al. 2008).

In addition, environmental conditions in storage rooms can contribute to specimen degradation, so providing a stable interior climate, including temperature and humidity, is highly recommended (Janssen and Christensen 2013). This degradation impacts the use of museum specimens for DNA barcoding (Hernández-Triana et al. 2014) and thus hampers molecular phylogenetics and DNA-based species identification (Goodman et al. 2021). For example, it can be challenging to obtain amplicons with a size greater than 250 bp from older specimens, which often have degraded DNA (Moraes-Barros and Morgante 2007). Besides these issues, the presence of old collections that generally provide low quantities and quality of DNA is a limitation for their use (McCormack et al. 2016) as DNA quality is the main limiting aspect in the success of polymerase chain reaction (PCR) amplification reactions (Shokralla et al. 2011).

For molecular analyses, selecting suitable primers when dealing with older specimens is important, and it has been shown that even very short sequences can provide diagnostic information for many taxa if the correct gene region is selected (Hernández-Triana et al. 2014).

These DNA mini barcodes are therefore shorter than the common, full-length barcodes (Yeo et al. 2020), often with a target length of only up to 200 nucleotide base pairs (bp) (Meusnier et al. 2008; Zimmermann et al. 2008). DNA mini barcodes are acquired with primers that target shorter subsets of the original barcode region, amplify comparatively more readily than full barcodes, even when the DNA in the specimen is degraded (Hajibabaei and McKenna 2012), and thus are the preferred choices for barcoding museum specimens stored under suboptimal conditions for decades (Yeo et al. 2020). Thus far, the mini barcoding method has been utilized for museum specimens to identify morphologically similar cryptic species of butterflies (Lepidoptera; Shokralla et al. 2011) and scorpions (Buthidae; Goodman et al. 2021), to confirm first records of sponge species (Geodiidae; Cárdenas and Moore 2019), and haplotype reconstruction for identification and diversity analyses of fruit flies (Tephritidae; Velasco-Cuervo et al. 2019).

Since metabarcoding analyses target short DNA regions, DNA mini barcodes not only have great potential for use with museum specimens, but also as environmental DNA (eDNA) markers (primers) and have become a common choice for metabarcoding projects that rely on DNA with highly variable DNA quality (Deagle et al. 2006; Dopheide et al. 2019; Turanov et al. 2024). Thus far, the study on octocoral metabarcoding by using eDNA samples has been limited only to deep-sea octocorals using the mitochondrial MutS-like protein gene (mtMutS) (Everett and Park 2018) or total anthozoan diversity using the nuclear 28S rRNA gene (28S rDNA) (McCartin et al. 2024). One important difficulty in the study of octocorals through barcoding and metabarcoding techniques is posed by the slow evolution rate of their mitochondrial DNA (Shearer et al. 2002; Huang et al. 2008) due to the presence of a DNA mismatch repair mechanism (Muthye et al. 2022). In the otherwise slowly evolving mitogenome (van der Ham et al. 2009) of octocorals, the mtMutS gene, unique to this group within the cnidaria, is the most rapidly evolving protein-coding region and supports their taxonomic placement (McFadden et al. 2022), although the marker cannot always delineate closely related species. The original mtMutS eDNA primer pair was developed by focusing on deep sea octocorals (Everett and Park 2018), and thus recently we have developed a new primer set for the metabarcoding of all octocorals. This new primer set was successfully used to detect the presence

of soft coral gene sequences (mitochondrial MutS) from the digestive tract of collected copepods using the DNA mini barcoding method (Hakim et al. 2025). In the current study, we utilize these octocoral-specific primers in a mini barcoding method via the examination of 10 to > 40-year-old specimens housed in the Okinawa Churaumi Aquarium Zoological Collection, Japan, in order to validate the utility of this method, and to aid in the identification of octocoral specimens.

Materials and methods

In silico evaluation

First, comparisons between full-length and mini barcodes were conducted *in silico* using computer programs and models following the steps of data set selection, alignment, and excision based on both barcode types and analyses. One to five sequences per species were downloaded from studies focusing on DNA barcoding, but also from broader phylogenetic and marker-development efforts, to increase taxonomic coverage (Meusnier et al. 2008; Lobo et al. 2013). Sequences were downloaded from GenBank accessed through the National Center for Biotechnology Information (NCBI) website (Sayers et al. 2022), incorporated species of family Acanthogorgiidae Gray, 1859 (*Acanthogorgia* Gray, 1857; *Menella* Gray, 1870; and *Paramuricea* Kölliker, 1865), Cladiellidae McFadden, van Ofwegen & Quattrini, 2024 (*Klyxum* Alderslade, 2000), Melithaeidae Gray, 1870 (*Melithaea* Milne Edwards, 1857), Nephtheidae Gray, 1862 (*Dendronephthya* Kükenthal, 1905), Sarcophytidae Gray, 1869 (*Lobophytum* Marenzeller, 1886; *Sarcophyton* Lesson, 1834; and *Sclerophytum* Pratt, 1903), Sinulariidae McFadden, van Ofwegen & Quattrini, 2024 (*Sinularia* May, 1898), and Subergorgiidae Gray, 1859 (*Subergorgia* Gray, 1857). Since the systematics of octocorals have recently undergone a large revision (McFadden et al. 2022), we followed the newest taxonomy as on the World Register of Marine Species (WoRMS) website (Worms 2025, https://www.marinespecies.org). We thus generated an mtMutS alignment with 380 sequences, including 11 genera and 160 species of octocorals, with a length of 685 bp (Suppl. material 1: table S1). Included in the alignment were some GenBank sequences that have not been published in any journal, similar to as in McFadden et al. (2022) and McCartin et al. (2024). While we cannot be completely confident in the identity of such sequences (listed in Suppl. material 1: table S1), we decided to include them in our *in silico* analyses to make as complete an *in silico* analysis as possible. Therefore, care should be taken when referring to the identity of GenBank sequences, as previously referred to in Leray et al. (2019) and Carné et al. (2025). We then aligned all sequences using the MUSCLE algorithm (Edgar 2004) and generated a truncated alignment of 215 bp for mini barcodes. The resulting alignment was inspected manually to identify any discrepancies between taxonomy and species labels before downstream analyses.

Nucleotide variations

To determine the effect of position on nucleotide variation, truncated *in silico* sequences, such as those in full-length and mini barcodes, were explored at variable sites using MEGA v.11 (Tamura et al. 2021). With the aligned

sequences, the number of variable sites was estimated based on the number of sites with at least two different nucleotides observed at the same site (Tamura et al. 2021). Then, the average proportion of nucleotide variation (number of variable sites per total sites) within species was calculated based on sequences with more than one sequence per species. At the same time, the proportion of nucleotide variation within a genus was estimated based on sequences within the same genus for those with more than one species. Proportions of nucleotide variation were tested for normal distribution using the Shapiro-Wilk test (Shapiro and Wilk 1965). We then used a non-parametric test, specifically the Wilcoxon Test (Mann and Whitney 1947), to compare the proportions of nucleotide variation between full-length and mini barcodes. Proportions of nucleotide variation were visualized using ggplot2 (Wickham 2016) in R 4.4.2 (R Core Team 2024).

Species delimitations

Both full-length and mini barcodes were tested to examine the number of expected species by using species delimitation approaches, including two distance-based methods, Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021) and the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012), and two tree-based methods, the Generalized Mixed Yule Coalescent (GMYC) (Pons et al. 2006) and the Bayesian Poisson Tree Processes (bPTP) (Zhang et al. 2013). For the ASAP and ABGD methods, we analyzed separately using three substitution models: Jukes-Cantor (JC69) (Jukes and Cantor 1969), Kimura (K80) (Kimura 1980), and simple-distance (p-distance) (Felsenstein 1988). We examined the threshold distance from both barcode types to select the appropriate model for estimating the number of species using the 30 best ASAP scores in iTaxoTools 0.1 (Vences et al. 2021). For GMYC and bPTP methods, Maximum likelihood (ML) phylogeny trees for both barcode types were constructed following General Time Reversible (GTR) with G (Gamma Distribution) + I (Proportion of Invariable Sites) model as a best-fit model of sequence evolution based on the AIC criterion (Akaike 1974) using MEGA 11 (Tamura et al. 2021). A total of 1,000 bootstrap repetitions were performed to establish the ML tree, and the results were converted into Newick format without annotations on the tree.

The performance of full-length and mini barcodes *in silico* was measured through species-level concordance using the concordance ratio between a species name as annotated in GenBank with the updated taxonomy and species delimitation results as a match ratio, following the method defined by Yeo et al. (2020). From the results of the ASAP and ABGD methods, three values were selected based on the number of subsets that approximated the number of species as annotated in GenBank with the updated taxonomy to be evaluated using the match ratio. To compare the differences in performance by match ratio between the four methods, Kruskal-Wallis tests were used to determine which species delimitation method was responsible for significant differences. The highest match ratio value from each barcode type was selected as the best delimitation methods, and species delimitation results were visualized using Sankey diagrams using RAWGraphs (Mauri et al. 2017).

Specimen acquisition, DNA extraction, amplification, and sequencing

After *in silico* evaluation, we next obtained a total of 166 tissue specimens of octocorals, representing eight putative genera, obtained from ethanol-preserved (70–100%) specimens from the Okinawa Churaumi Aquarium Zoological Collection in Motobu, Okinawa, Japan (Table 1; detailed specimen information in Suppl. material 1: table S2). These octocoral specimens were collected from the shallow water (~2 m) to the deep sea (~350 m), with the oldest specimen collected in 1982, and most specimens collected in the period between 2005 to 2012. In the museum, the specimens were stored in individual plastic or glass

Table 1. List of genera, family, collection year, specimen numbers, depth, and location of provenance of octocoral specimens from Okinawa Churaumi Aquarium Natural History Collections. Note that all identities are museum-label identities provided by the museum and have not been updated regarding recent changes in octocoral systematics.

Museum label identity	Collection year	Number of specimens			Depth (m)	Locations (within Japan)
		Tissue	Successful mini barcode	Successful full-length barcode		
Acanthogorgiidae						
Acanthogorgia	1982	1	1	0	69	Amakusa, Kumamoto
	2005	13	10	4	17–80	Off Kouri Island, Okinawa
	2006	2	2	1	13.5 and 17.9	NA
	2008	2	2	0	150 and 350	Iejima and off Kume Island, Okinawa
	2011	1	1	0	27.5	Nagasaki
Cladiellidae						
Klyxum	2011	4	4	0	2–15	Motobu, Okinawa
	2012	10	9	0	11.8–29.4	Motobu, Okinawa
Melithaeidae						
Melithaea	2004	2	2	0	25 and 220	Iriomote and Motobu, Okinawa
	2005	13	11	1	13–80	Off Kouri Island, Okinawa
	2006	5	5	1	10.6–19.4	NA
	2008	2	2	0	72 and 150–170	Iejima and deep sea, Okinawa
	2009	5	5	0	2–149	Amami Island, Kagoshima, and Miyako and off Kouri island, Okinawa
	2012	2	2	0	91–93 and 300	Higashi, Okinawa
Nephtheidae						
Dendronephthya	2005	1	1	0	80	Off Kouri Island, Okinawa
	2009	1	1	0	73	Off Kume Island, Okinawa
	2011	4	2	0	6–10	Motobu, Okinawa
	2012	9	9	0	7.5–27.9	Motobu, Okinawa
Sarcophytidae						
Lobophytum	2011	11	10	0	2–10	Motobu, Okinawa
	2012	12	12	0	3.3–19.9	Motobu, Okinawa
Sarcophyton	2011	13	11	0	2–10	Motobu, Okinawa
	2012	11	11	0	4.2–23	Motobu, Okinawa
Sinulariidae						
Sinularia	2011	24	24	0	2–15	Motobu, Okinawa
	2012	14	13	0	3.3–16	Motobu, Okinawa
Subergorgiidae						
Subergorgia	2006	2	2	0	NA	Rukan and Kawata, Okinawa
	2008	1	1	0	NA	Deep sea, Okinawa
	2009	1	1	0	NA	Irabujima, Okinawa

jars with a label (including registration number, scientific name (=initial identification made at time of collection), Japanese name, family name, collection date, collector, method, location, depth, preservative, identifier, and remarks). Specimens were stored in cabinets in the museum building with air conditioning running 24 h a day at approximately 21 °C.

A small tissue fragment was used from each specimen to extract DNA using a DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol with a 24 h incubation time during lysis. To avoid a low concentration of DNA from old specimens, the DNA was eluted with 100 µL of AE buffer in the last step of DNA extraction. For mini barcoding with 209–251 bp fragment, amplifications were performed with the primer pair of forward AAH-MutS_F01 (5'-YTRCTTCAAATGGGRTTTCC-3') and reverse AAH-MutS_R250 (5'-ACTATGYCCAYTCATGGCWG-3') following Hakim et al. (2025). Specimens that were successfully amplified were purified using a standard ExoSAP protocol and then sent to Fasmac (Kanagawa) for direct sequencing in both directions.

We also attempted amplification of all examined specimens for a ~800 bp fragment of mtMutS (full-length barcode) using initial standard primer pairs ND42599F and Mut3458R (France and Hoover 2002; Sánchez et al. 2003), following the protocol by Gösser et al. (2025). A semi-nested PCR was performed on specimens that did not produce or only produced a faintly visible PCR products, using the primer ND42625F in place of ND42599F, following the protocol described by McFadden et al. (2006). For these full-length PCRs, to examine the effectiveness of the primers, we only recorded whether PCRs were successful or not based on the appearance of a band without the subsequent sequencing process. Three replications of PCR trials were performed for both mini barcodes and full-length barcodes. For positive control, we used octocoral tissue specimens from the Molecular Invertebrate Systematics and Ecology (MISE) Laboratory collection, which were collected within the last three years, from the same genera as the museum specimen collection, except for *Subergorgia*. Each primer set for full-length barcoding (both first and semi-nested PCR) and mini barcoding was successfully amplified for positive controls of all genera.

Species delimitation and phylogenetic analysis of museum specimens

Both forward and reverse sequences of the mini barcode from museum specimens were edited to remove primer sequences and low-quality base pairs at the end of the sequences. Trimmed sequences were then aligned using the MUSCLE algorithm (Edgar 2004). Acquired sequences were compared to publicly available sequence GenBank data using the Basic Local Alignment Search Tool nucleotide (BLASTn) (Altschul et al. 1990) at the National Center for Biotechnology Information (NCBI) website with program selection optimized for highly similar sequences (megablast). Following the previous results of the *in silico* tests, species delimitation methods were performed to determine the operational taxonomic units (OTUs) numbers for each genus using ASAP with substitution models of JC69, K80, and the p-distance run separately. With the 30 best ASAP scores generated, we applied a threshold distance of ~0.2% to delineate each distinct OTU. In addition, unique sequences were identified using the Biostrings package in R (Pagès et al. 2024).

Maximum likelihood (ML) phylogeny trees with 1000 bootstrap repetitions were constructed following Tamura 3-parameter model (T92) with G (Gamma Distribution) model as a best-fit model of sequence evolution based on the AIC criterion (Akaike 1974). Genetic distances were estimated by establishing a matrix of pairwise genetic distances (Felsenstein 1988) by the p-distance model with 1000 replications of the bootstrap method (Nei and Kumar 2000). Sequence editing, phylogeny trees building, and genetic distance calculating were performed using MEGA v.11 (Tamura et al. 2021) and ASAP was generated with the 30 best scores in iTaxoTools 0.1 (Vences et al. 2021). Some genera had discrepancies between the museum-labeled names and the BLASTn-based molecular results, possibly due to subsequent changes in generic names (taxonomic revision), misidentifications, or short sequences of mini barcodes that incorrectly distinguished the genera. Genetic distances for these genera were visualized into a heatmap and a dendrogram (hierarchical clustering with complete method) in the pheatmap package in R (Kolde 2019).

Morphological analyses

Some specimens with discrepancies between the museum-labeled names and the BLASTn-based molecular results (Suppl. material 1: table S3) were subjected to further morphological analyses. Sclerites were prepared separately from the tissue of the coenenchyma and the polyps for *Acanthogorgia* specimens in question, and also from tissue of the surface and interior of the polypary for *Lobophytum* specimens. Selected tissues were dissolved in 10% sodium hypochlorite (bleach solution) for 5 minutes to extract sclerites and remove fleshy tissues and debris. Subsequently, they were washed with distilled water three times to remove any additional contaminants. Sclerites were examined using a Hitachi Miniscope TM4000PlusII scanning electronic microscope (SEM), and sclerite forms were determined following the definitions of Bayer (1981), Bayer et al. (1983), and Fabricius and Alderslade (2001).

Results

Proportion of nucleotide variation and species delimitation by *in silico* evaluations

The proportions of nucleotide variation within species and within genus for both full-length and mini *in silico* barcodes are shown in Fig. 1. *Acanthogorgia*, *Paramuricea*, and *Klyxum* had identical sequences and thus had no nucleotide variations within species for full-length and mini barcodes. In contrast, mini barcodes had higher proportion of nucleotide variations for *Melithaea* and *Sarcophyton* than full-length barcodes (Fig. 1a). Proportions of nucleotide variation within genus of mini barcodes also had higher variation in four genera (*Acanthogorgia*, *Melithaea*, *Sarcophyton*, and *Subergorgia*) (Fig. 1b). Based on the Wilcoxon test, there were no significant differences in both barcode types regarding proportion of nucleotide variations within species for all genera ($p > 0.05$) (Fig. 1a). On the other hand, the proportion of nucleotide variations within species combined was significantly different for both barcode types ($p < 0.05$), with the proportion of nucleotide variations of full-length barcodes (0.0029) higher than that of mini barcodes

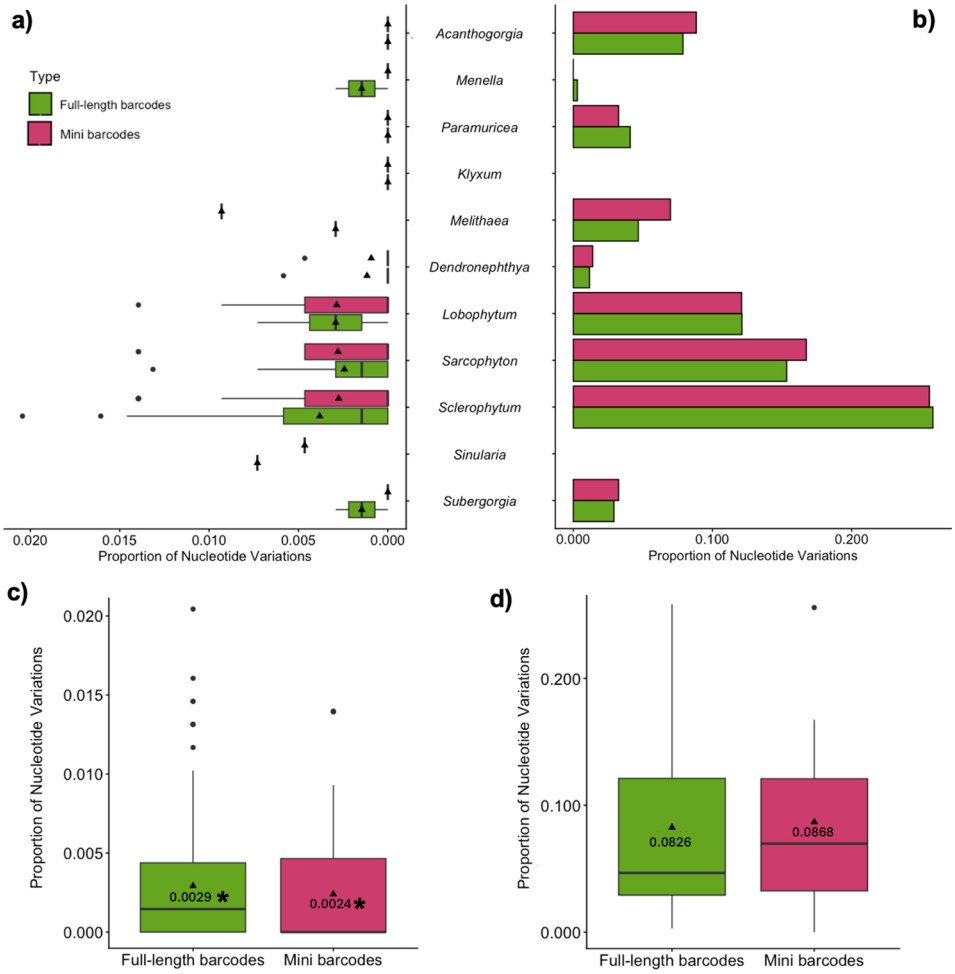


Figure 1. Proportion of nucleotide variations between full-length and mini barcodes for 11 selected genera of Octocorallia. **a.** Within species by each genus; **b.** Within genus; **c.** Within species for all genera (total); **d.** Within genus for all genera (total), asterisk: significant difference ($p < 0.05$), triangle: mean value.

(0.0024) (Fig. 1c). Overall, there were no significant differences in the proportion of nucleotide variation within the genus between the two barcode types (Fig. 1d).

The distance-based species delimitation method showed that almost all prior maximal distances obtained the same number of subsets in each substitution model for ABGD, with the highest number of subsets (ABGD OTUs) at 0.0001 for full-length barcodes. On the other hand, ASAP had a trend with a higher number of subsets (ASAP OTUs) at low threshold distances and vice versa (Fig. 2a). Based on the Kruskal-Wallis test ($p < 0.05$), there was a significant difference in the match ratio values between all methods (ABGD, ASAP, bPTP, and GMYC), with high values for both types of barcodes in the ASAP method. Jukes-Cantor (JC69) and simple distance (p-distance) in the ASAP method were the best substitution models for full-length barcodes with the match ratio value of 0.28. On the other hand, all three substitution models performed well for mini barcodes with the match ratio value 0.25 (Fig. 2b). Those best match ratio values had threshold distances of ~0.2% for both types of barcodes.

We selected the best match ratio values only for those substitution models and the specific threshold distance (~0.2%); there is no significant difference between full-length and mini barcodes based on the Wilcoxon test ($p > 0.05$). The result of ASAP method of full-length barcodes (threshold distance of 0.22%)

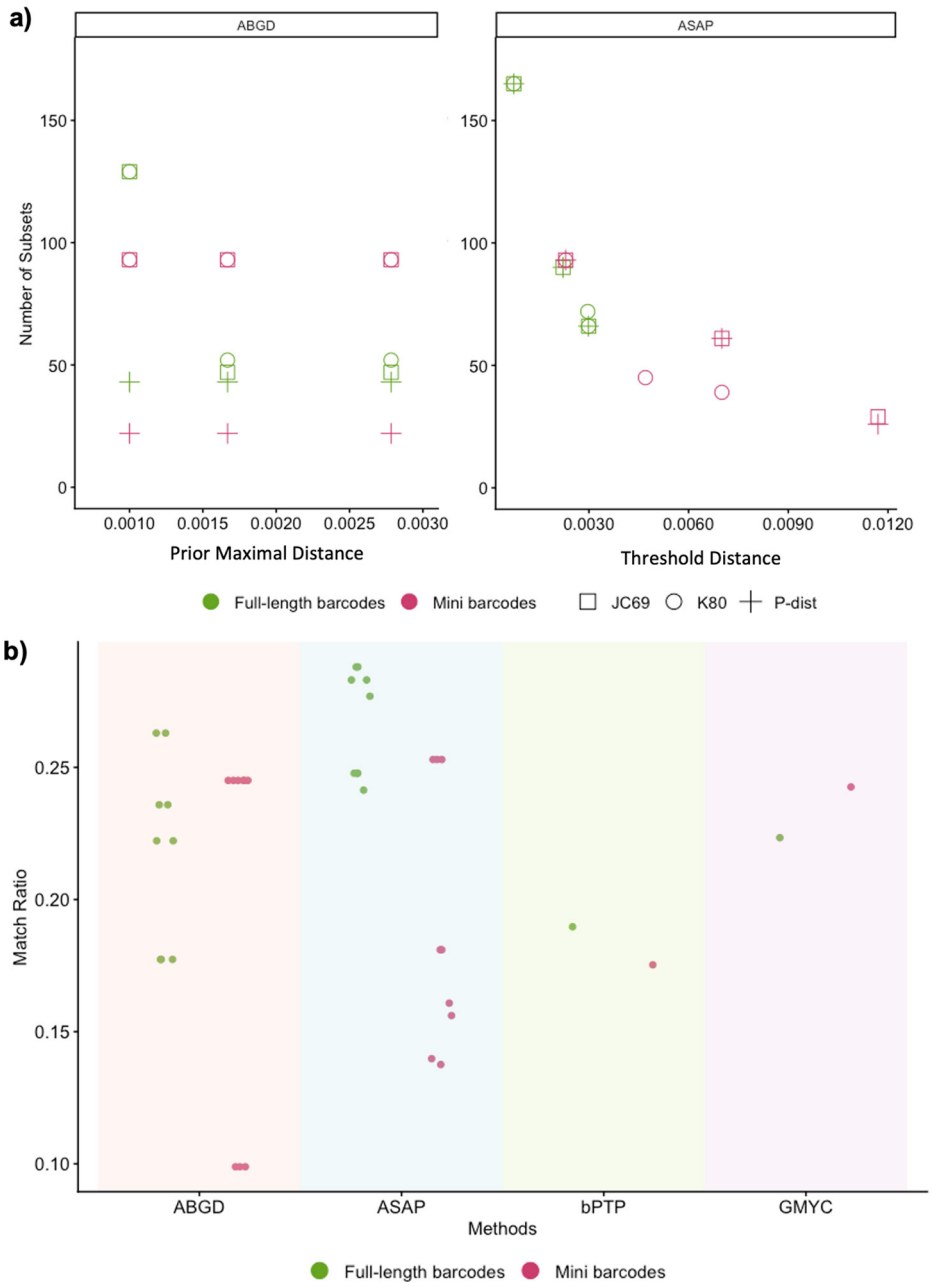


Figure 2. Performance of species delimitation methods for full-length and mini barcodes. **a.** Number of subsets based on prior maximal distance of ABGD and threshold distance of ASAP; **b.** Match ratio distribution from four different methods.

between JC69 and p-distance model showed similar results and were visualized by Sankey diagram (Fig. 3a), as well as three models (including K80) for mini barcodes (threshold distance of 0.23%) (Fig. 3b). Of the 160 octocoral species, the ASAP method was able to group them into 90 subsets (ASAP OTUs) with only 28% of match ratio for full-length barcodes and 93 subsets (ASAP OTUs) with only 25% of match ratio for mini barcodes (Suppl. material 1: table S4).

Amplification of octocoral museum specimens

After *in silico* testing, we performed PCR on museum specimens. Of 166 octocoral specimens from the Okinawa Churaumi Aquarium Zoological Collection, 154 individuals (=92.7%) were successfully amplified using the mini

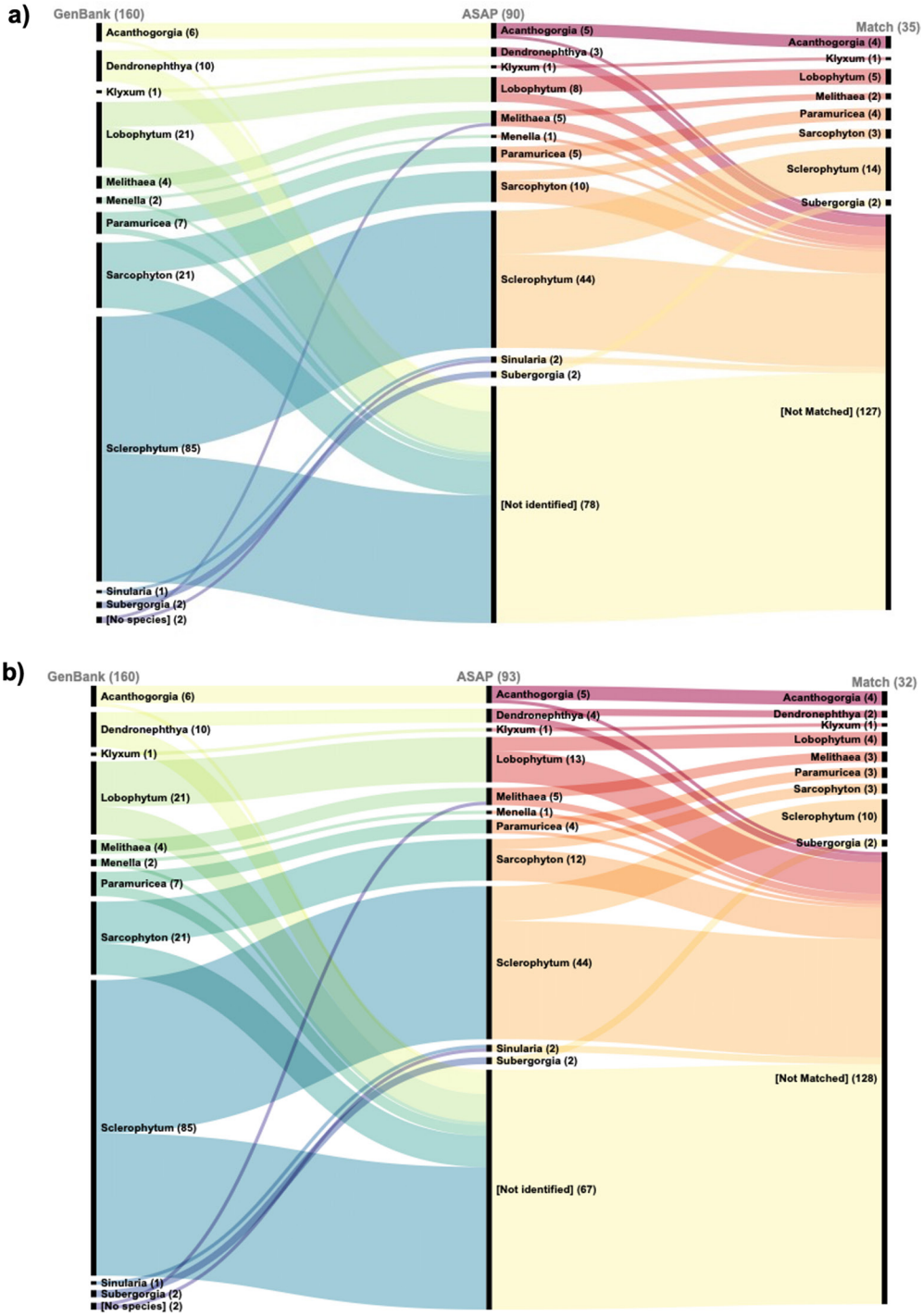
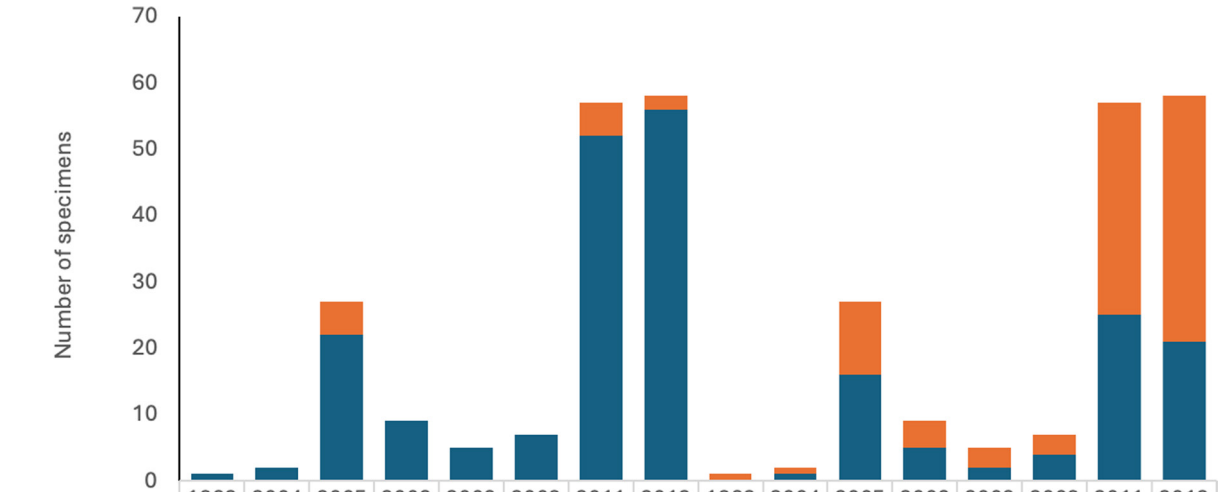


Figure 3. Sankey diagrams showed the number of species from GenBank, ASAP results, and matches between both. **a.** Full-length barcodes; **b.** Mini barcodes.

barcoding primers (Fig. 4). Mini barcode amplification of octocoral specimens did not work for some specimens collected in 2005 (*Acanthogorgia* and *Melithaea*), 2011 (*Dendronephthya*, *Lobophytum*, and *Sarcophyton*), and 2012 (*Klyxum* and *Sinularia*) (all n = 1–3 each). In contrast to the mini barcoding primer, the full-length barcoding primer could only amplify eight of 166 individuals (=4.8%) using the first primer set, and 76 of 166 individuals (45.8%) using the semi-nested primer set. Most specimens that failed to be amplified by the mini barcode primer set also failed to be amplified by the



	1982	2004	2005	2006	2008	2009	2011	2012	1982	2004	2005	2006	2008	2009	2011	2012
Not Amplified	0	0	5	0	0	0	5	2	1	1	11	4	3	3	32	37
Amplified	1	2	22	9	5	7	52	56	0	1	16	5	2	4	25	21

Figure 4. Evaluation of mini barcoding and full-length barcoding amplification on specimens with different collection years.

full-length barcode set, excluding four specimens (Suppl. material 1: table S3). Furthermore, the full-length barcode primers were ineffective on the oldest specimen, which was collected in 1982.

Species delimitation and unique sequences

After sequencing the mini barcodes, we recovered 67 molecular operational taxonomic units (OTUs) using the species delimitation of Assemble Species by Automatic Partitioning (ASAP) method with substitution model of the Jukes-Cantor (JC69), Kimura (K80), and simple distance (p-distance). In addition, we also obtained the same number of unique sequences as the species delimitation results. Based on species delimitation results and the number of unique sequences of mini barcodes, we recovered 67 different OTUs of octocorals in the museum specimens we examined with the marker used.

Mini barcoding confirmed or corrected the generic name on the label of each specimen based on the short fragment of DNA (Suppl. material 1: table S3). *Klyxum*, *Melithaea*, *Dendronephthya*, *Sarcophyton*, and *Subergorgia* specimens were validated as belonging to the same genus, while specimens labeled as *Acanthogorgia* were identified as four different genera: *Acanthogorgia*, *Menella*, *Paramuricea*, and *Villogorgia*. Furthermore, *Sinularia* specimens were correctly validated as *Sinularia* or *Sclerophytum* (the newly accepted genus name of most *Sinularia* species). In addition, most *Lobophytum* specimens were validated as *Lobophytum*, with the remaining *Lobophytum* specimens identified as the genus *Sclerophytum*. Overall, five specimens of *Acanthogorgia* and two specimens of *Lobophytum* did not belong to genus inscribed on their labels, and these specimens were thus flagged to have their sclerites' morphological characteristics examined. *Sclerophytum* was the most speciose genus in our examinations with 20 OTUs, including 19 OTUs from specimens labeled as *Sinularia*, and two OTUs from specimens labeled as *Lobophytum*, with one of the OTUs shared between specimens labeled as two genera of specimens (Table 2).

Table 2. Validation of species based on mini barcodes with the number of Assemble Species by Automatic Partitioning (ASAP) OTUs.

Museum label identity	Number of sequences		Identity via mini barcode	Number of ASAP OTUs
	In family	In genus		
Acanthogorgia	16	11	*Acanthogorgia*	2
		4	Acanthogorgiidae	4
		1	*Villogorgia*	1
Klyxum	13	13	*Klyxum*	1
Melithaea	27	27	*Melithaea*	14
Dendronephthya	13	13	*Dendronephthya*	7
Lobophytum	22	20	*Lobophytum*	4
		2	*Sclerophytum*	2
Sarcophyton	22	22	*Sarcophyton*	11
Sinularia	37	36	*Sclerophytum*	19
		1	*Sinularia*	1
Subergorgia	4	4	*Subergorgia*	2

Sclerite characteristics of specimens with disagreements between museum-labeled names and ASAP OTUs

Two specimens of *Lobophytum* (museum labels; LO16 and LO35) were identified as *Sclerophytum* based on mini barcodes, and this was validated by their morphological characteristics of sclerites. The main characteristics of these two *Sclerophytum* specimens were spindles with complex tubercles or blunt spines, thorn clubs, and leptoclados clubs (Fig. 5a), which are not found in *Lobophytum* (Fig. 5b).

Overall, mini barcode results indicated five specimens labeled as *Acanthogorgia* had different identities (Fig. 6). One specimen (AC35) labeled as *Acanthogorgia* was identified by mini barcode and confirmed by sclerite characteristics to instead belong to *Villogorgia*. The genus *Villogorgia* features calicular thornscales, bow-shaped spindles, and curved scales (Fig. 6a). In addition, one specimen of *Acanthogorgia* was identified by mini barcode as *Paramuricea* (AC06) with a sequence identity of 99.53%. However, upon examination the sclerite characters of this specimen differed from those described for the genus *Paramuricea*. This specimen had elongated spindles with tubercles more developed in the middle-basal portion and tapered tips on both sides, and bow-shaped spindles (Fig. 6b).

In addition, results showed three specimens originally identified as *Acanthogorgia* were instead closely related to *Menella* spp. (AC01, AC08, and AC21) with sequence identities of 99.07–100%, with each sequence having unique differences. However, the sclerite characters of these specimens differed from those reported for the genus *Menella*. The sclerites of two specimens (AC01 and AC21) were slightly curved spindles covered with irregular tubercles or spine-like projections and capstan derivatives (Fig. 6c), while one specimen (AC08) had elongated, curved spiny spindles, and sometimes straight spindles (Fig. 6d). These specimens represent sclerites distinct from those characterized by the genus *Acanthogorgia* (Fig. 6e). We also could not find similar char-

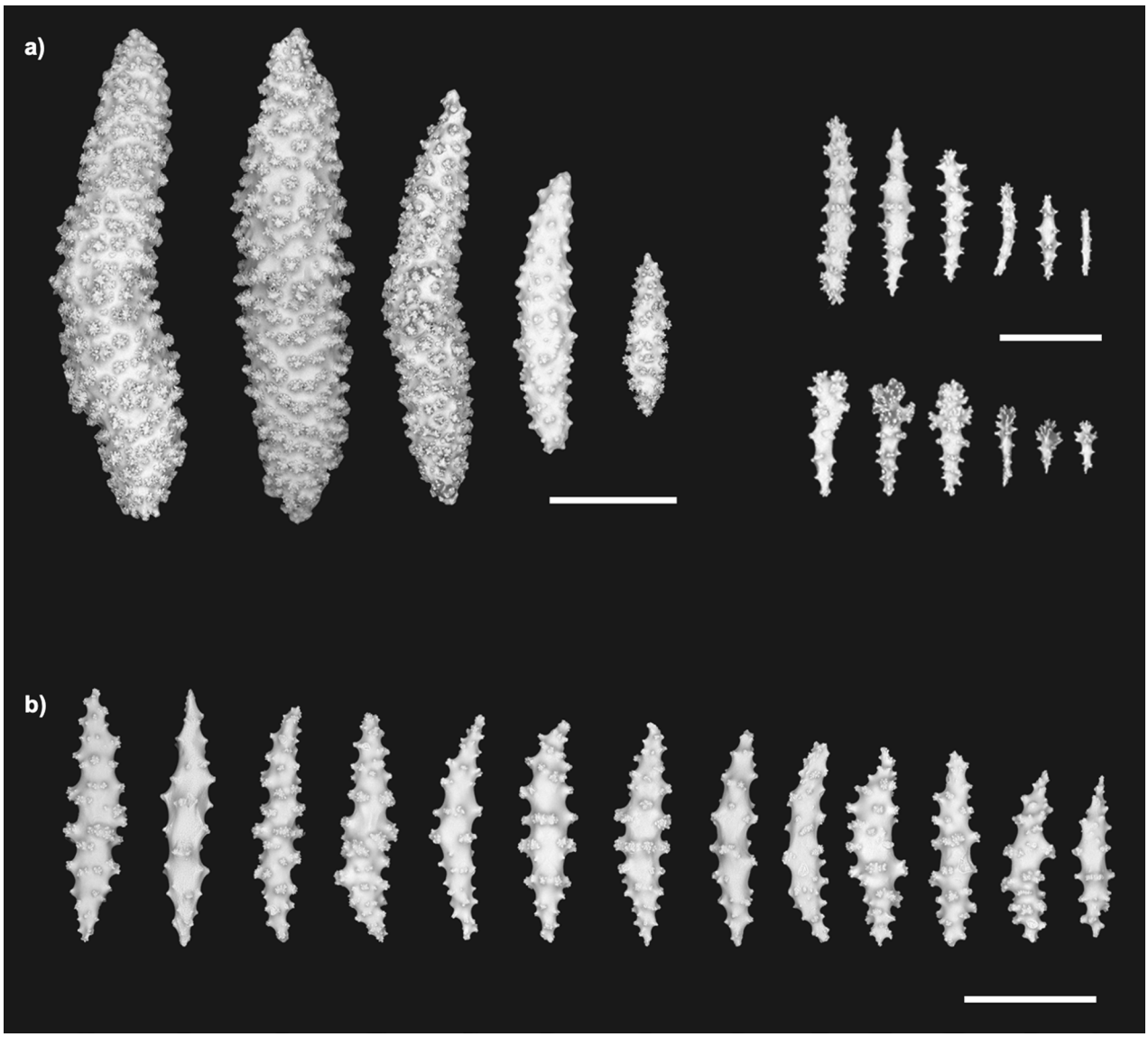


Figure 5. Surface and interior of the polypary sclerites of Sarcophytidae from the Okinawa Churaumi Aquarium Zoological Collection. **a)** *Sclerophytum* sp. 20; **b.** *Lobophytum* sp. 4. Scale bars: left: 0.5 mm, right: 0.2 mm (**a**); 0.2 mm (**b**).

acters described for any of the other genera of the family Acanthogorgiidae in the literature. Thus, based on these results, we decided to identify these four specimens (AC01, AC06, AC08, and AC21) as Acanthogorgiidae sp. 1 to sp. 4 for now. Final identifications will require deeper taxonomic investigations.

Phylogenetic and genetic distances

A maximum likelihood (ML) phylogeny tree was built from the mini barcode sequences with species delimitation based on the ASAP method (Fig. 7). Sequences from most genera were in their own clade. *Lobophytum*, *Sarcophyton*, and *Sclerophytum* formed a single large clade; however, several nodes showed low bootstrap supports, indicating that all three belong to the family Sarcophytidae. In addition, sequences of the family Acanthogorgiidae fell into two clades, with *Acanthogorgia* in one clade and Acanthogorgiidae spp. and *Villogorgia* in the other clade, which was closely related to Nephtheidae.

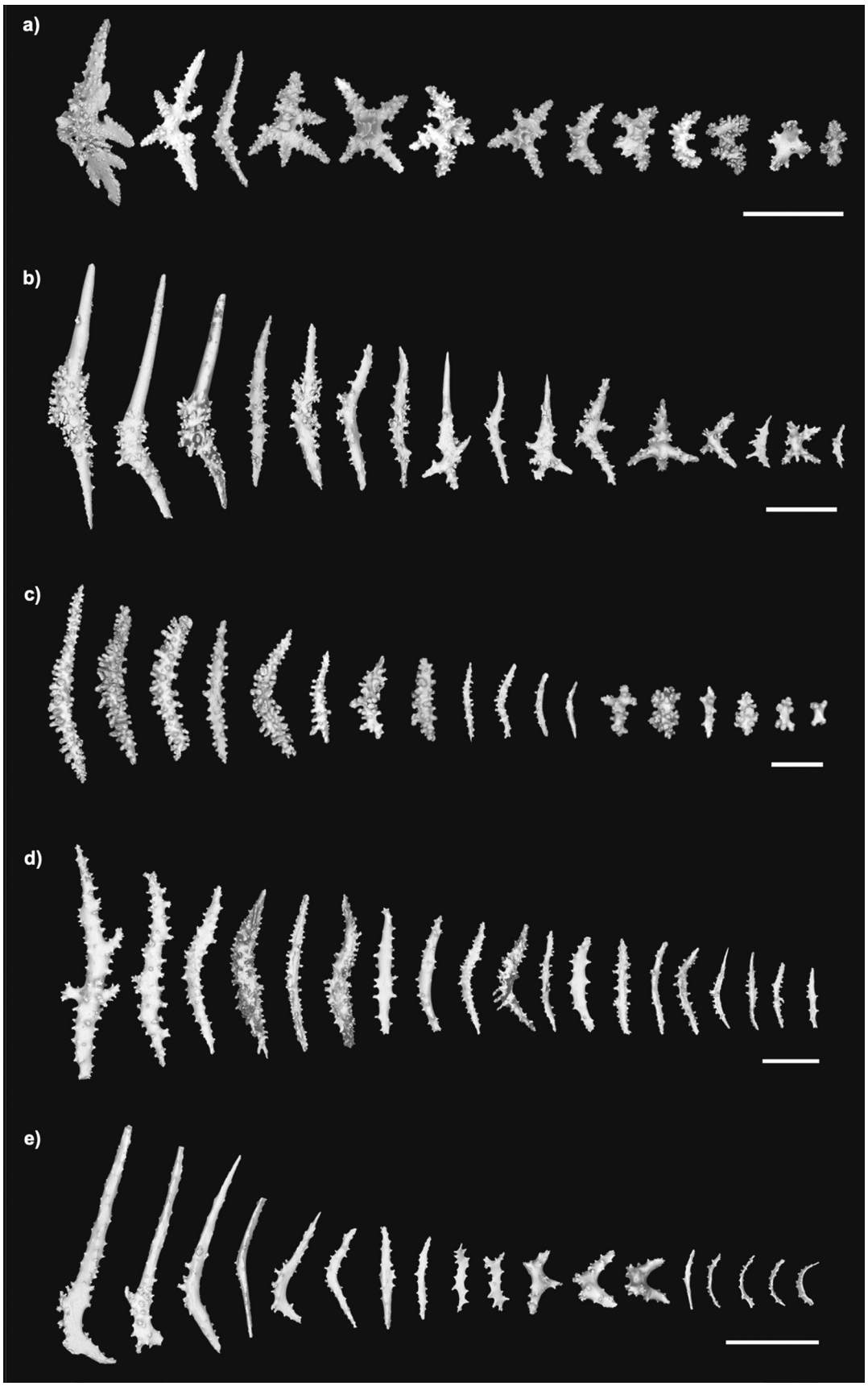


Figure 6. Coenenchyma and polyp sclerites of Acanthogorgiidae from the Okinawa Churaumi Aquarium Zoological Collection. **a)** *Villogorgia* sp.; **b.** Acanthogorgiidae sp. 1; **c.** Acanthogorgiidae sp. 2; **d.** Acanthogorgiidae sp. 3; **e.** *Acanthogorgia* sp. 1. Scale bars: 0.2 mm.

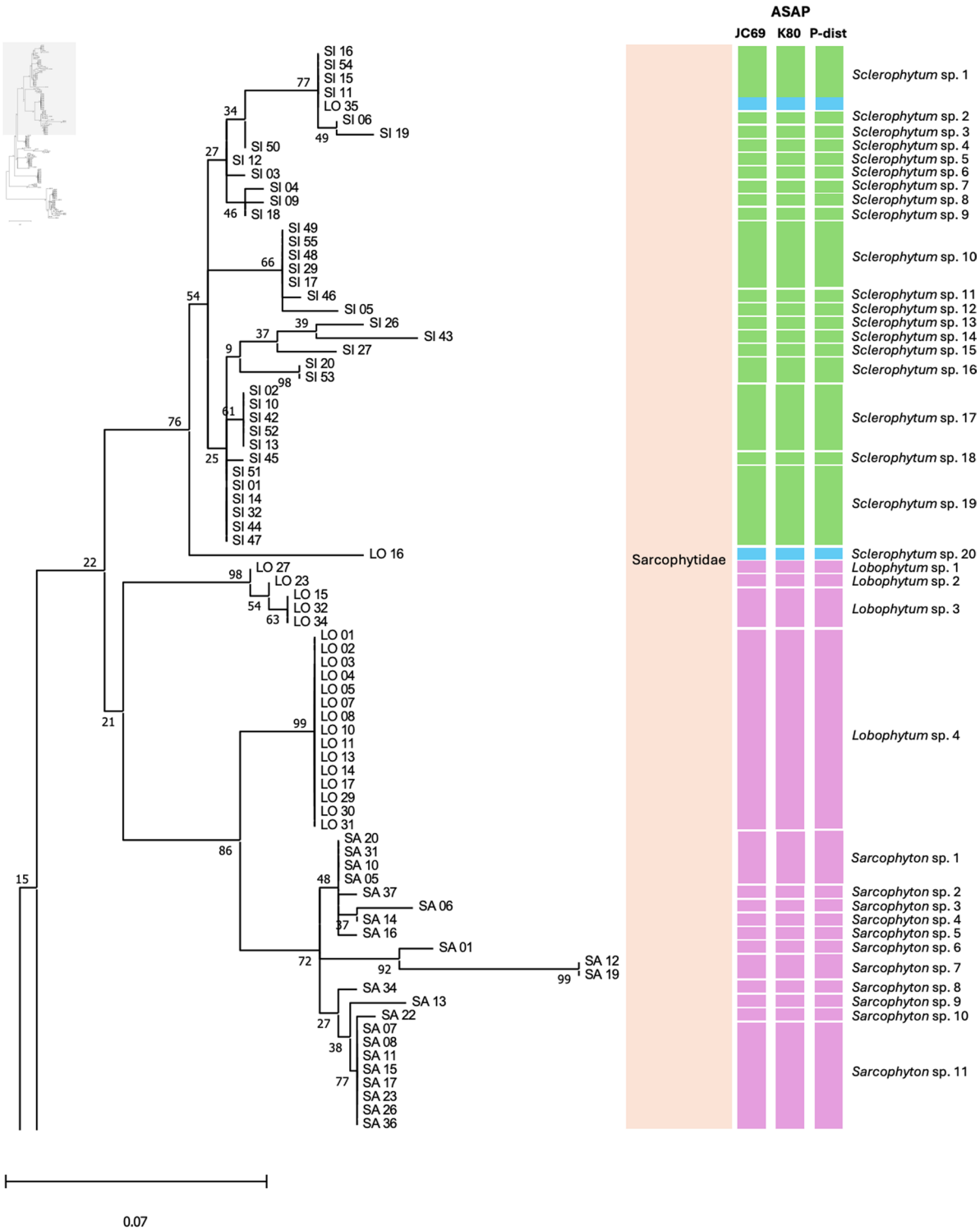


Figure 7. Maximum likelihood tree with results of Assemble Species by Automatic Partitioning (ASAP) using substitution models of Jukes-Cantor (JC69), Kimura (K80), and simple-distance (p-distance) using 0.23% of threshold distance. Green boxes indicate change of genus name due to taxonomic revision; purple boxes indicate agreement between museum-labeled names and ASAP OTUs; blue boxes indicate disagreement.

Acanthogorgia, *Lobophytum*, and *Sinularia* had discrepancies between the museum-labeled names and the BLASTn-based molecular results with the genetic distance visualized in the heatmap (Fig. 8). Specimens labeled as

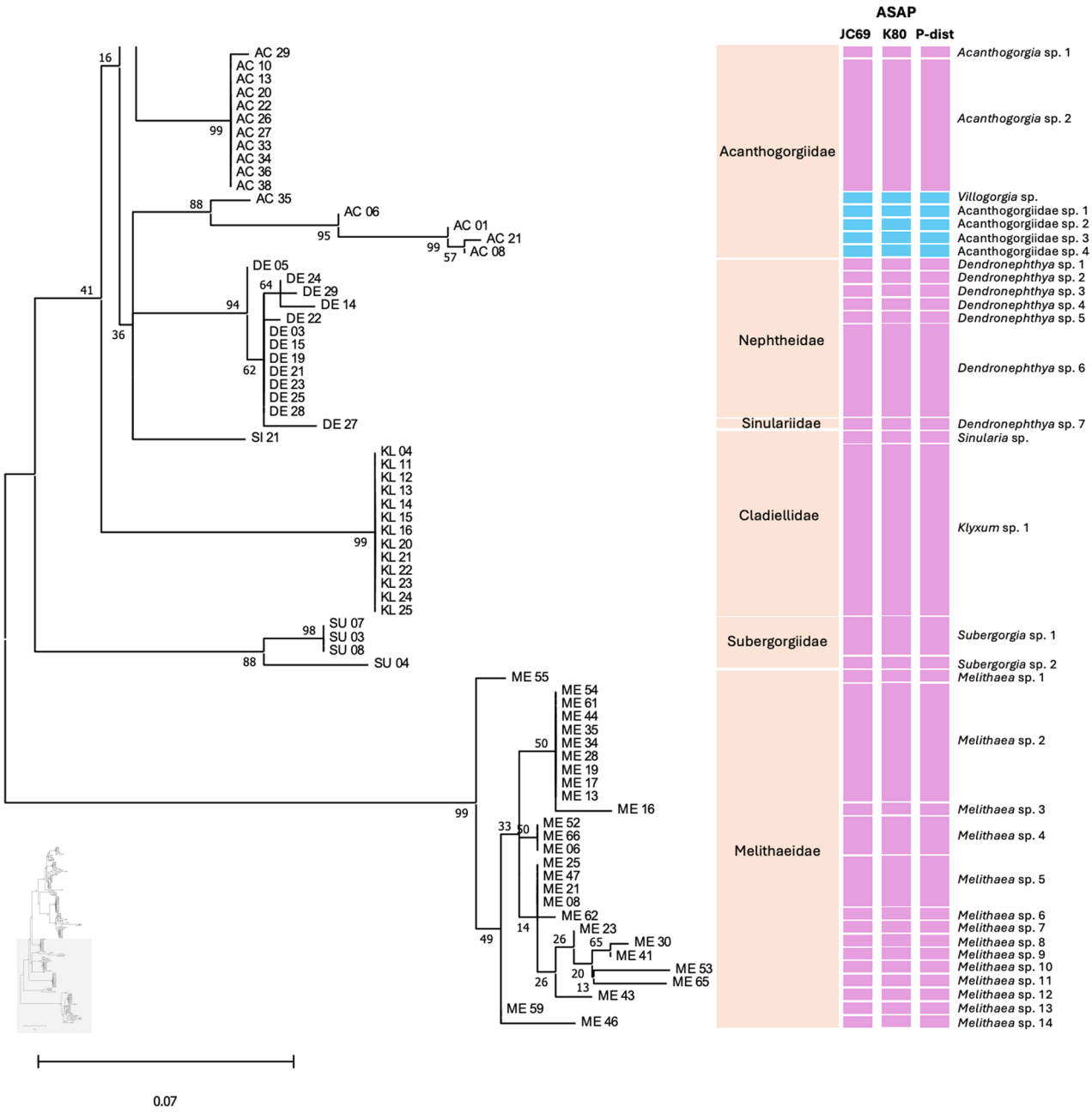


Figure 7. Continued.

Acanthogorgia were confirmed as likely belonging to unidentified Acanthogorgiidae spp. or the genera *Acanthogorgia*, or *Villogorgia*, with similar separation in the heatmap. *Acanthogorgia* had the highest genetic distance from Acanthogorgiidae spp. (0.0744–0.1116) (Fig. 8a). Two *Lobophytum* specimens were assigned to the genus *Sclerophytum* based on the molecular results and had comparatively far distances from those *Lobophytum* with the genetic distance ranging from 0.0837 to 0.0977 (Fig. 8b). Most *Sinularia* species have undergone relatively recent taxonomic revision (McFadden et al. 2022) and are now placed in *Sclerophytum*, leaving one species in the genus *Sinularia* with higher genetic distances (0.0744–0.1116) from all *Sclerophytum* species (Fig. 8c).

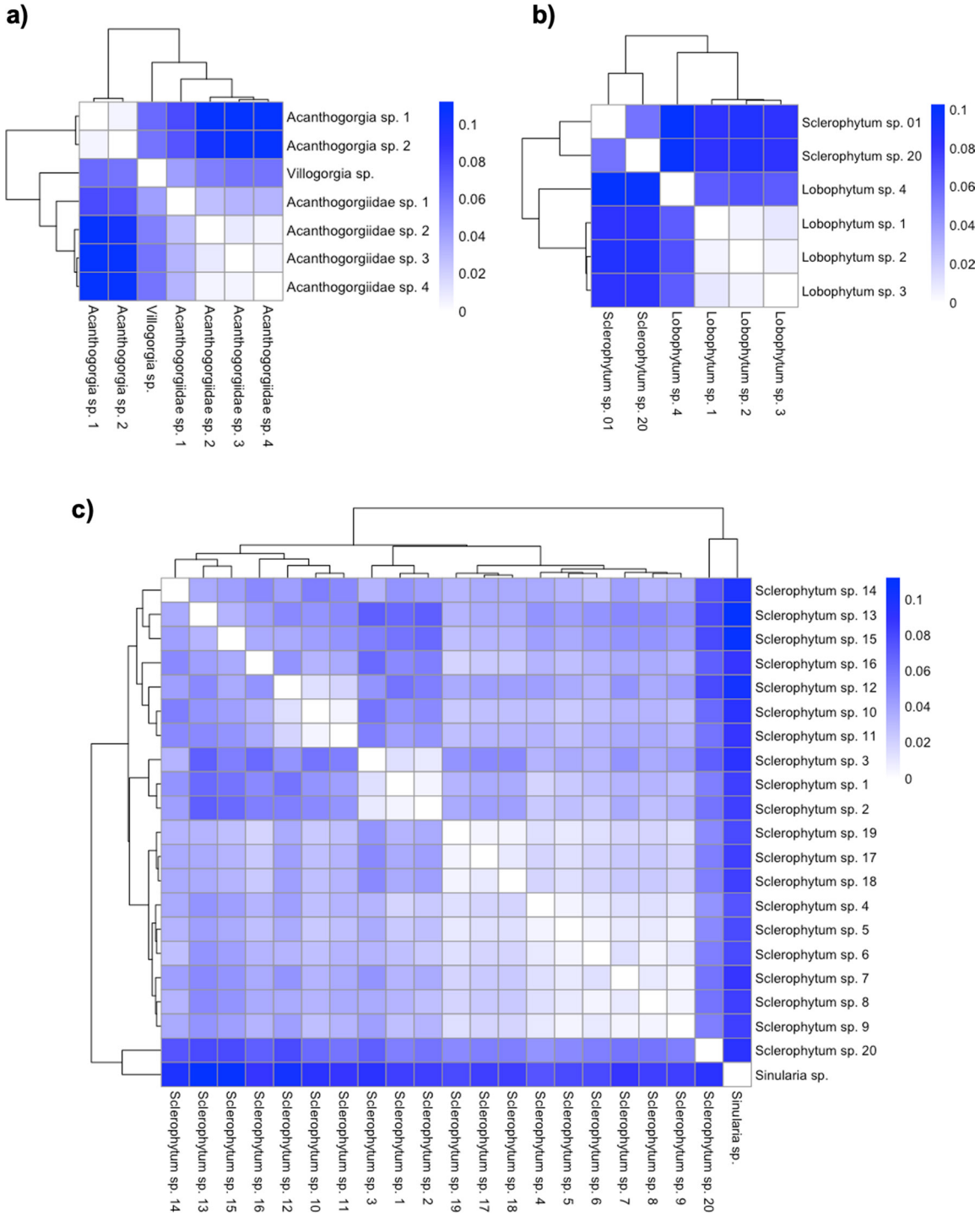


Figure 8. Heatmap depicting pairwise genetic distance between species with dendrogram of 3 selected genera in the specimen label. **a.** *Acanthogorgia*; **b.** *Lobophytum*, and **c.** *Sinularia*. Dark blue indicates higher genetic distances while white indicates lower genetic distances.

Discussion

Species delimitation of octocorals

In Octocorallia, DNA barcoding tools have been widely utilized to identify, validate, and reveal new, cryptic, and complex species (McFadden et al. 2011). Mitochondrial MutS and nuclear 28S rDNA have been the most commonly used DNA

markers (e.g. Quattrini et al. 2019; Frometa et al. 2021), along with mitochondrial cytochrome c oxidase subunit I (COI) and 16S rDNA (Morín et al. 2019). However, in anthozoans, mitochondrial DNA is well known to have a slow rate of evolution (Shearer et al. 2002; Huang et al. 2008; McFadden et al. 2011). DNA barcoding with a single locus in anthozoans, and mini barcoding in particular, may not always provide a species-level identity because the sequences are often similar or identical among closely related species, leaving little room to distinguish between species. Despite this, the mtMutS region has been reported as the most variable region in the mitochondria DNA of octocorals (France and Hoover 2002; McFadden et al. 2011) and is the most commonly used mitochondrial gene for the DNA barcode of octocorals (McFadden et al. 2011). It also has the highest number of sequences available in GenBank, and we utilized this marker for *in silico* evaluations.

In this study, two tree-based methods (GMYC and bPTP) and two distance-based methods (ASAP and ABGD) of species delimitation approaches were unable to discriminate all selected octocoral species from a single locus of the mtMutS gene using both full-length barcodes and mini barcodes. Tree-based methods do not perform well on distinct molecular operational taxonomic units (OTUs) with very small genetic distances, and distance-based methods require barcode gaps that are sometimes not found in octocorals (Morrissey et al. 2022). By comparing all these methods, the ASAP method with JC69, K80, and the p-distance model (run separately), and a threshold distance of 0.23% resulted in the best possible partition; this proved to be the most effective method for delimiting octocoral OTUs in this study. We determined this value as the best threshold distance as it yielded a lower number of subsets (ASAP OTUs) and a lower mismatch ratio (Fig. 2a, b, Suppl. material 1: table S4). The match ratio was then used to assess the performance of the two barcode types, and it was found that mini barcodes were as effective as full-length barcodes for identification purposes (Yeo et al. 2020). On the other hand, a threshold distance higher than 0.3% resulted in a greater decrease in both values. It must be noted that the threshold distances reported by ASAP are not directly comparable to genetic distance thresholds from previous studies. In ASAP, the threshold distance is defined as the midpoint between the lowest distance that triggers the merge and the previous distance in the list (Puillandre et al. 2021). Therefore, the 0.23% threshold in our analysis reflects the shortest possible non-zero genetic distance for the mini barcode (215 bp), which corresponds to a one–base pair difference (0.47%; therefore, midpoint of 0% and 0.47%). In practice, this means that sequences differing by at least once base pair were treated as distinct OTUs. For the full-length barcodes, genetic threshold distances of 0.3% (McFadden et al. 2014; Quattrini et al. 2019, 2022) and 0.2% (Kushida and Reimer 2019) have previously been applied to mtMutS for delimiting species of octocorals.

The ASAP method for species delimitation is becoming an important tool for taxonomists (Puillandre et al. 2021). The distance-based approach using ASAP for single-locus species delimitation has the highest efficiency in recovering species boundaries relative to nominal taxonomy (Guo and Kong 2022). Although ASAP can be considered a robust method, a low concordance ratio between actual species names and species delimitation results (match ratio of < 30%) was obtained for both types of barcodes. However, we found that, given the circumstances, the mini barcodes using our new mtMutS eDNA primers successfully delimited species just as effectively as the full-length barcode marker, with no significant

differences in the match ratio values by the Wilcoxon test. It should still be noted that single-locus barcodes, both full-length barcodes and mini barcodes of individuals from closely related taxa, may have identical DNA sequences. For example, the proportion of the nucleotide variations within genus was lower than 0.100 for *Acanthogorgia* and *Melithaea*, and even less than 0.050 for *Dendronephthya*, *Menella*, *Paramuricea*, and *Subergorgia* (Fig. 1b). With these limitations, we consider that in this study using mini barcodes we cannot confidently identify to the species level. However, mini barcodes are a promising tool for identifying to the genus level and have utility in initial sorting and identification of octocoral species.

DNA mini barcodes of octocorals from the Okinawa Churaumi Aquarium Zoological Collection

We successfully amplified 92.7% (154/166) of the total specimens with our mini barcoding method, but the full-length barcodes only worked on 4.8% of specimens (8/166) with the initial primer set, and on 45.8% of specimens (76/166) with a semi-nested primer set. Ferrari et al. (2022) also observed a high performance of the mini barcoding method while examining fish specimens, retrieving 56.6% of sequences successfully analyzed versus 3% from full-length barcoding. We also successfully applied the DNA mini barcoding method for investigating octocoral species with fragmented and degraded DNA from specimens up to over 40 years old. Mini barcodes are a powerful asset for efficiently sorting old museum materials (Hajibabaei and McKenna 2012), even for 161-year-old dried sponge samples (Cárdenas and Moore 2019).

We failed to amplify mini barcodes from 12 specimens collected in 2005, 2011, and 2012, but the method worked for many older samples collected in 1982 and 2004, as well as for other specimens collected in 2005, 2011, and 2012. Furthermore, no specific genus was observed to have unsuccessful amplifications, and thus we hypothesize that these amplification failures may have been due to poor fixation or specimen conditions. We lack information about fixation during initial handling, preservation, and storage before shipment to the Okinawa Churaumi Aquarium Zoological Collection. However, the collection is currently stored in an air-conditioned room at stable temperature (21 °C). Unlike mini barcodes, full-length barcodes with our initial primer set amplified only sequences from specimens of the genera *Acanthogorgia* and *Melithaea*, suggesting that perhaps gorgonians possess better long-term DNA integrity (see also Baker et al. 2013). The semi-nested primer set we utilized performed better than the initial primer set and was effective on some specimens in all genera. It should also be noted that four specimens that failed to amplify with the mini barcode were amplified with full-length barcode using the semi-nested primer set. We did not test several other primer pairs for full-length barcodes (Williams 1995; Sánchez et al. 2003; France 2007; Brugler and France 2008; Pante et al. 2012), and they also may be successful on some specimens.

Many museum specimens are stored under conditions that accelerate the deterioration of DNA, RNA, and proteins, such as those stored in preservative solutions including ethanol that may have been fixed with formalin before storage (Ruiz-Gartzia et al. 2022). Furthermore, natural history museum specimens preserved in 70%–80% ethanol and stored at room temperature have become the standard for ethanol-preserved museum collections (Derkarabetian et al. 2019).

Higher ethanol concentrations (~ 95%) have a positive effect on long-term DNA preservation compared to 70–80% concentrations, which show faster degradation at lower concentrations (Nagy 2010; Raxworthy and Smith 2021). However, high concentrations of ethanol may cause specimens to become brittle and easily break, so specimen preservation in 70–80% ethanol is often recommended for morphological examination (Marquina et al. 2021). In the Okinawa Churaumi Aquarium Zoological Collection, specimens were preserved in ethanol at concentrations ranging from 70% to 100%, with most *Acanthogorgia* and *Melithaea* specimens using 100% ethanol. We suspect that ethanol concentration significantly impacted the DNA quality, allowing full-length barcode primers to amplify successfully for both genera. In many collections, in addition to preservation in ethanol, some museum specimens are stored in formalin, while other specimens are preserved dry, producing more degraded DNA than in preservation with ethanol.

As well, as most octocoral specimens from the Okinawa Churaumi Aquarium Zoological Collection were labeled only to genus, the additional molecular data might be beneficial in identifying to species level (e.g. sp. 1, sp. 2) of the octocoral museum specimens and will provide baseline information for further studies. Compared to other methods, the Assemble Species by Automatic Partitioning (ASAP) method has been shown to be efficient for the delimitation of octocoral species. In this study, the ASAP method was utilized with a threshold distance of 0.23% for mini barcodes. The acquired sequences of octocoral specimens from the Okinawa Churaumi Aquarium Zoological Collection were delimited into 67 OTUs as a preliminary minimum estimation. This number can be considered conservative, and more taxa are likely to be retrieved using a combination of markers or next-generation sequencing techniques (Liu et al. 2017; Quattrini et al. 2019).

The morphological characters of sclerites confirmed that some mislabeled specimens were successfully assigned to the correct taxonomy by the mini barcodes. In some previous cases, DNA barcoding has helped uncover mislabeling in museum specimens (Chambers and Hebert 2016; Mulcahy et al. 2022; Ren et al. 2022). In the current study, the sequences of three *Acanthogorgia* specimens showed high similarity (99.07–100%) to *Menella* spp., and the sequence of one specimen highly similar to *Paramuricea* (99.53%). However, sclerite characters of these specimens differed from those of the genus *Menella* and *Paramuricea*, and we could not find similar characters in other genera of the family Acanthogorgiidae, thus leading us to suspect these specimens may represent an undescribed genus, but further analyses are needed to confirm this hypothesis. Thus, these four Acanthogorgiidae specimens collected from Amakusa (Kumamoto) and off the coast of Kouri Island (Okinawa) and housed in the Okinawa Churaumi Aquarium Zoological Collection may represent more than one undescribed genus and up to four species.

Limitations, strengths, and appropriate applications of mini barcodes for future octocoral studies

Limitations

Mini barcoding produces short DNA sequences that may fail to contain some regions with variations in full-length sequences. Thus, full-length barcodes with more extended sequences are generally recommended, as they provide increased

confidence in distinguishing species compared to mini barcodes (Hebert et al. 2003; Meusnier et al. 2008), particularly if, full-length primers can amplify old museum specimens. Many other pairs of full-length barcode primers have been developed for octocorals and anthozoans and may potentially yield successful results on some specimens examined in this study. However, different families may require different primer sets or PCR setups, requiring several PCR runs to obtain the correct primer combinations. In this study, we aimed to develop an octocoral-universal mini barcode primer to simplify methodologies for workers.

Based on our results, we determined that the ASAP method with a threshold distance of 0.23% was sufficient for delimiting octocoral OTUs when using our mini barcode mtMutS primers. Note that this value is based on the dataset used in this study only, and the threshold distance may vary for different families (Barraux et al. 2024; Le Cadre et al. 2024). As mentioned above, this ASAP's threshold distance represents a methodological cut-off, and we could not directly interpret this number as a genetic distance threshold. However, we operated under the assumption that a difference of one base pair (bp) (equivalent to ~0.47% divergence) in the short mini barcode sequences (~215 bp) indicated different operational taxonomic units (OTUs). In other words, each unique haplotype was considered as a distinct species using our current method. With a sequence length of ~700 bp in the full-length barcode, one bp difference in species could therefore reflect species boundaries (Kushida and Reimer 2019). However, short barcodes such as ours still can increase the risk of incorrect species assignments (Collins and Cruickshank 2013) and will perform less well among closely related taxa (Hajibabaei et al. 2006). Thus, we cannot conclusively say our mini barcode can identify all octocoral specimens to the species level.

Strengths

Mini barcodes have proven effective for genus or species-level identification of specimens from which full-length barcodes cannot be obtained in many taxonomic groups, with fragment lengths usually of 200–300 base pairs (bp) (Moraes-Barros and Morgante 2007; Meusnier et al. 2008; Zimmermann et al. 2008; Yeo et al. 2020; Goodman et al. 2021), but sometimes even shorter (170 bp in Dhar and Ghosh 2017; only 130 bp in Shokralla et al. 2011). We successfully amplified more specimens (92.7%) with our mini barcoding method compared to full-length barcodes using an initial primer set (ND42599F and Mut3458R; 4.8%) and a semi-nested primer set (ND42625F and Mut3458R; 45.8%). Since some specimens failed to amplify with the mini barcode but were amplified with the full-length barcode using the semi-nested primer set, we could use PCR amplicons from the initial primer set for full-length barcoding in mini barcode analyses in future research.

The availability of standard and universal primers makes mini barcodes a viable option for DNA barcode analyses of museum samples, as well as applied diagnostic and environmental biodiversity analyses (Hajibabaei and McKenna 2012). Even using mini barcodes consisting of short DNA fragments, we helped uncover mislabeling of museum specimens from the Okinawa Churaumi Aquarium Zoological Collection. Combined with sclerite morphology, we confirmed that two *Lobophytum* specimens and five *Acanthogorgia* specimens were originally mislabeled in the museum. Surprisingly, we strongly suspect that four of the five *Ac-*

anthogorgia specimens may belong to undescribed genera and potentially harbor undescribed species. However, we need to conduct further morphological investigations and substantiate this with other genetic information, and descriptions of these new genera and species will likely be presented elsewhere in the future.

Appropriate applications

Most mini barcode research has described new primers for amplification, which requires the skill to develop the primers, and also requires time for testing. However, as metabarcoding utilizes primers with a short target sequence length, there is also the opportunity to use metabarcoding primers in mini barcoding studies. Metabarcoding primers have usually already been field-tested and are thus ready to use for mini barcoding research, making the process easier and more effective. However, established universal metabarcoding primers targeting mitochondrial COI and 16S rRNA, and nuclear 18S rRNA, may have difficulty successfully amplifying in octocorals (e.g. Marquina et al. 2019; Gösser et al. 2023; McCartin et al. 2024), and thus for octocoral specimens we recommend using octocoral-specific primers for mini barcoding as we have done in this study. Another alternative for octocorals is an anthozoan-specific primer set amplifying the 28S rRNA gene (McCartin et al. 2024), which has been shown to successfully distinguish octocoral species as effectively as the full-length 28S barcode (Senofsky et al. 2024).

Furthermore, mini barcode primers may also offer promising applications for formalin-preserved (Appleyard et al. 2021) and/or dry specimens (Gao et al. 2019) in future research. The main challenge lies in extracting DNA from such specimens. Mini barcoding primers targeting short fragments allow for the amplification of degraded DNA and are more suitable given the conditions of these samples.

Our work provides species-OTU-level identifications, similar to as performed in McFadden et al. (2024) in their massive study across the Indo-Pacific Ocean. The obvious next step is linking DNA barcodes with valid species names, but this is an arduous task beyond the scope of this study. Therefore, multilocus or next-generation sequencing approaches, combined with morphological analyses, have been recommended for more accurate identification to the species level (Liu et al. 2017). Despite the aforementioned limitations, DNA barcoding is still widely used because it is fast, objective, and has a comparatively cheaper overall cost (e.g. McFadden et al. 2024). As Octocorallia is comparatively understudied (Santos et al. 2020; Lalas et al. 2022; Lange et al. 2023), additional molecular data from museum specimens can only be beneficial for future studies. Our study also shows that this DNA mini barcoding approach can help discover octocoral diversity, accurately identify specimens, and validate historical museum specimen collections.

Overall conclusions

Specimens for many undescribed species already exist in the world's natural history museums, but these specimens need to be clearly sorted to the species level before they can be available for formal species descriptions. Museum specimens will continue to age as time passes, and additional and continuing DNA damage will occur. The DNA mini barcoding approach can cheaply and quickly acquire molecular data from comparatively older octocoral specimens,

allows for rapid sorting and pre-identification, and adds further value to historical museum specimen collections for supporting future taxonomy, biodiversity monitoring, and conservation of octocorals.

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Supplementary material 1

Supplemental information

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Data type: xlsx

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

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No ethical statement was reported.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.
No AI tools were used in the preparation of this manuscript.

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Author contributions

AAH conceived and designed the research consulting with JDR and MN. AAH and II conducted molecular analyses. AAH and CJLF conducted morphological analyses. JDR and MN provided supervision, funding, and materials. All authors analyzed the data. AAH wrote the manuscript. All authors read, edited, and approved the manuscript.

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Data availability

The data underpinning the analysis reported in this paper are deposited in the Dryad Data Repository at https://doi.org/10.5061/dryad.cvdncjtjr.