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# Furthering Ascidian Taxonomy Using Molecular Biology

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by

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**Abstract**

Ascidians are our closest invertebrate relatives and comprise nearly 3,000 species separated into three orders: Aplousobranchia (most speciose), Stolidobranchia, and Phlebobranchia (least speciose). Ascidians can be classified as either solitary or colonial organisms. Species delimitation using morphological characters alone has had varied results. Well known, widely distributed, morphological species have turned out to be catch all species comprised of several cryptic species. Molecular markers can help mitigate some of the issues presented by strictly using morphological observations, including resolving the status of cryptic species, and accessing the expert knowledge required to identify a species. By incorporating molecular markers and pairing them with morphological observations, more species may be correctly identified by the scientific community. This project focuses on comparing the utility of the molecular markers mitochondrial cytochrome oxidase 1 (mtCO1) and 18S rRNA, both commonly used to barcode marine invertebrates, in terms of successfully delimitated species within families. Members of the ascidian families Ascidiidae, Pyuridae, and Styelidae were collected from Belize in July 2022 and July 2023 and were sequenced for CO1 and 18S and identified using morphological techniques. Additional sequences were obtained from GenBank. Species delimitation methods used for this project include Assemble Species by Automatic Partitioning (ASAP) and Bayesian Poisson Tree Process (bPTP). Morphological identifications tended to line up well when using CO1 with ASAP while 18S and ASAP lumped species together. bPTP tended to split species relative to morphological identifications for both genes. Future work includes implementation of the Bayesian input of bPTP into this analysis to see how it compares alongside ASAP. In

addition to this, morphological identification of the Belizean samples down to the species level will also be completed.

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| | |
|---|---|
| ASAP | Assemble Species by Automatic Partitioning |
| BOLD | Barcode of Life Database |
| BPP | Bayesian Phylogenetics and Phylogeography |
| bPTP | Bayesian Poisson Tree Process |
| CBOL | Consortium for the Barcode of Life |
| CO1 | Cytochrome c Oxidase 1 |
| ℃ | Degrees Celsius |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxynucleotide Triphosphate |
| gDNA | Genomic DNA |
| HAB | Harmful Algal Bloom |
| ID | Identification |
| ML | Maximum Likelihood |
| µL | Microliter |
| µM | Micromolar |
| mg | Milligram |
| mL | Milliliter |
| mM | Millimolar |
| ng | Nanograms |
| NCBI | National Center for Biological Information |
| rRNA | Ribosomal Ribonucleic Acid |
| PCR | Polymerase Chain Reaction |
| PTP | Poisson Tree Process |

# 1. Introduction

## 1.1 Ascidian Biology and Ecology

Ascidians, also known as sea squirts or tunicates, are our closest invertebrate relatives within the Phylum Chordata. The Class Ascidiacea is comprised of nearly 3,000 species (Shenkar and Swalla 2011) separated into three orders: Aplousobranchia (most speciose), Stolidobranchia, and Phlebobranchia (least speciose). Ascidians are sessile filter feeders that live on both natural and artificial substrates (Monniot et al. 1991) and feed on microorganisms such as phytoplankton. Ascidians have short larval stages, ranging from as short as 20 minutes to a few days (Monniot et al. 1991). Ascidians also have a natural protection against predators and environmental conditions known as the tunic.

The tunic, which is the outer structure of the animal, can be leathery, gelatinous, or cartilaginous in nature (Burighel and Cloney 1997). Interestingly, the tunic is composed of cellulose, a polysaccharide found normally in plants, making ascidians the only animal capable of producing it (Song et al. 2020). This structure protects the animal, which is connected to the tunic via the internal atrial siphon and oral siphon tissue. Abiotic factors that may endanger the animal include environmental stressors such as temperature, hurricanes, and desiccation. Biotic factors include predation and external parasites. Ascidians are important to their native ecosystems, providing various ecosystem services and functions.

Ascidians provide important ecosystem services such as maintaining water clarity. *Ciona intestinalis* (Linnaeus, 1767), a species in the order Phlebobranchia, has an astonishing filtration rate, and as a population can filter up to the total volume of a shallow fjord (~2 m) daily depending on the time of year (Peterson and Riisgard 1992). Population size of *C. intestinalis* can impact phytoplankton grazing and the lack thereof could lead to harmful algal bloom conditions (Peterson and Riisgard 1992).

Ascidians can also be a food source for predators. Although the tough cellulose tunic helps to protect ascidians, various fish species, crabs, flatworms, sea stars, and nudibranchs feed on ascidians on both natural and artificial substrates. Several of these predators are important to commercial fisheries, including the red rock crab, *Cancer productus* (Randall, 1840) and the dock shrimp, *Pandalus danae* (Stimpson, 1857; Simkanin et al. 2013). Ascidians can also be hosts to various parasites that live within their tunic and digestive tract.

Ascidians are commonly parasitized by various crustacean species (Millar 1971). For example, the parasite *Lankesteria ascidiae* (Lankester, 1872) infects the digestive tract of *C. intestinalis* causing long feces syndrome. This quickly spreads throughout the population during periods of reproduction and often leading to the death of infected individuals over a one-week period (Mita et al. 2012).

Ascidians can also act as a substrate for various sessile epifauna. The solitary species *Microcosmus sabatieri* (Roule, 1885) provides substrate for species within Peracarida, a Superorder of crustaceans, to attach to and filter feed from (Voultsiadou et al. 2007).

## 1.2 Ascidians as Invasive Species

Ascidians have many roles within the habitats they live in. Unfortunately, some species of ascidians have become harmful, both economically and ecologically. For example, the ascidian, *Ciona savignyi* (Herdman, 1882), also has a rapid filtration rate and competes with the Japanese scallop for space via biofouling on aquaculture structures in Japan (Nakai et al. 2018). This has caused negative impacts to the Japanese aquaculture fishery due to greater body size and filtration rate of *C. savignyi* compared to the Japanese scallop, leading to competition with the scallop for food (Nakai et al. 2018). Another well-known ascidian invader, *Didemnum vexillum* (Kott, 2002), has impacted habitats and fisheries in different parts of the world (e.g., Japan and North America). Due to *D. vexillum* forming sheet-like mats along the substrate, native benthic epifauna are replaced and changes to the biodiversity of the habitat occur (Gitten et al. 2012). Macrobenthic organisms (e.g., lobsters) rely on these cobble substrates (Wahle and Steneck 1991), and the smothering caused by *D. vexillum* may result in a demographic bottleneck (Mercer et al. 2009) and negative impacts on commercially important species (Wahle and Steneck 1991).

Commonly, invasive ascidian species are transported anthropogenically via ship hulls (or sea chests) and have the potential to easily spread (Lambert 2009). These invasions can lead to consequences including the alteration of benthic habitats and reduction in native species richness (Lambert and Lambert 2003; Aldred and Clare 2014), such as by occupying space. For example, an ascidian species invasive to The Netherlands, the violet tunicate, *Botrylloides violaceus* (Oka, 1927), outcompeted the native star tunicate, *Botryllus schlosseri* (Pallas, 1766) for space. This was due to the

more successful fouling ability of *Botrylloides violaceus*, overtaking spaces *Botryllus schlosseri* would normally occupy (Gittenberger and Moons, 2011). Unfortunately, this is a common result involving the introduction of nonnative species. There are several causes for this, such as a lack of natural predators, as seen with the invasive and problematic lionfish in the Caribbean (Arias-Gonzalez et al. 2011) and chevron snakehead in Taiwan (Li et al. 2016).

Changes to environmental policy are crucial to prevent the spread of potential invaders. Unfortunately, though many nations have implemented policies to mitigate damage done by invasive species, invaders are still showing up at an increasing rate (Keller et al. 2011; Galil et al. 2019). The easiest solution to prevent invasive species is to stop the invasion before it begins. However, this is easier said than done, as some invasive species have already made their impact over several centuries before being documented as invasive (Keller et al. 2011). The North Atlantic Spider Crab, *Hyas Araneus* (Linnaeus, 1758) is a benthic invertebrate that is normally found only in the North Atlantic and Arctic Oceans. However, the species found its way into the Antarctic unbeknownst to science until the early 2000s, and may have been there for several years. It is unknown exactly how *H. araneus* arrived in the Antarctic, but transport via ship ballast water and the warming of Antarctic waters appear to be the most likely reasons (Tavares and De Melo 2004). The transportation of biofouling ascidians can also bring with them the possibility of Harmful Algal Blooms (HABs). When introducing harmful algal species such as *Karenia brevis* (C.C. Davis, 1948) to biofouling ascidians (e.g., *C. intestinalis*), it was determined that the algae could survive a 48-hour period and could effectively reestablish which could lead to a HAB (Rosa et al. 2013).

## 1.3 Human uses of Ascidians

### 1.3.1 Human Medical Treatments

Advancements in molecular techniques focusing on marine organisms such as ascidians has allowed for the mass production of medications that can combat issues such as cancer or infectious diseases (Thakur et al. 2008). For example, the photo endosymbiont (*Prochloron* sp.) of the colonial ascidian *Lissoclinum patella* (Gottschaldt, 1898), has been extracted and the gene cluster coding for patellamides cloned (Thakur et al. 2008). This has resulted in several human healthcare products such as antitumor medications and anticancer metabolites (Thakur et al. 2008). Stem cell regeneration has also been a topic of interest within ascidians, as they are the only members of the phylum Chordata with this capability (Tiozzo et al. 2008). This could lead to advancements in organ repair/replacement without having to worry about the controversy that comes with using human stem cells in an unborn fetus.

### 1.3.2 Chordates that are Efficient for Laboratory Use

Due to the short, free-living larval stage in many species, ascidians make great lab subjects as recruitment of new individuals is relatively easy to monitor in a controlled setting. It is during this larval phase when many of the regenerative stem cells are observed (Thakur et al. 2008, Tiozzo et al. 2008). For example, *Polycarpa mytiligera* (Savigny, 1816) makes an ideal candidate for biomedical research regarding stem cells and the potential human application for future medical advancements due to the ability to induce year-round spawning while in captivity (Gordon et al. 2020).

### 1.3.3 Natural Products Chemistry

Interestingly, it may not just be the ascidians that provide medical benefits for humans, but the bacteria that live within it. Bacteria found within the gut of *Styela clava* (Herdman, 1881) were shown to provide antimicrobial and antiproliferative effects (Chen et al. 2019). If the bacteria can be cultured, potential new drugs helping to prevent infections and muscle injuries could be developed.

## 1.4 Issues with Morphological Taxonomy

There are two different methods that are used to complement one another in the field of taxonomy. Morphological taxonomy, using an organism's anatomical features to identify it, and molecular taxonomy, using molecular techniques such as DNA barcoding and Phylogenetics to identify it. Before the advent of molecular taxonomy, only morphological methodology was used to identify and classify organisms. However, there are several issues with morphological taxonomy that can lead to incorrect identifications. First, phenotypic plasticity and intraspecific variability can make species delimitation difficult. Second, morphologically cryptic taxa are frequently overlooked when exclusively using morphological taxonomy for species identification. Third, morphological features for some organisms may only be identifiable during certain life stages. Fourth, a high level of expertise is often needed to identify an organism down to the species level, a skill that is exclusive to very few within their field of study (Hebert et al. 2003).

For ascidians in particular, the use of morphological taxonomy alone is a daunting task. Many species of ascidians have various color morphs, with genetically distinct

colonies of the same species appearing to be two separate species (e.g., *Botrylloides giganteus* (Pérès, 1949)). Thus, color is often not an effective diagnostic characteristic when morphologically identifying ascidians. External characteristics are far less important for identification than internal characteristics, and specialist knowledge is required to catalog this interior anatomy (Monniot et al. 1991). Even among the experts, cryptic species of ascidian can cause misidentification in the field or lab when only using morphological taxonomy for identification (e.g., *C. intestinalis*; Rocha et al. 2019), providing a further need to combine morphological identification with molecular identification.

### 1.5 Molecular Techniques in Ecology and Biology

Marine molecular biology has provided insight into questions that may have gone unanswered due to previous limitations in technology. For example, difficulties identifying organisms due to morphological traits (e.g., similarities between species at the egg or larval stage) can be resolved using molecular techniques (Burton 2009). Molecular techniques, such as DNA barcoding and phylogenetics, allow for the identification of organisms based on their DNA sequences/genes. These techniques also allow for the study of organisms too small to study morphologically such as viruses or bacteria. Marine viruses for example, have the potential to be used as cloning vectors within biotechnology and often go understudied due to their small size and difficulty in culturing (Thakur et al. 2008). Phytoplankton blooms and the ecological impacts they have on the environment (e.g., HABs) have been observed using molecular techniques. For example, using immunological and nucleic acid detection probes have been used to estimate phytoplankton growth rate under various environmental conditions (Roche et al. 1999).

Molecular techniques have been applied in conservation as well, allowing for the genetic changes within a population to be observed before events such as overfishing put a species at risk of extinction (Carvalho and Hauser, 1999). Two commonly used molecular techniques, phylogenetics and DNA barcoding, can be used in conjunction to answer questions regarding species relationships.

Phylogenetics allows for the evolutionary history and species relationships of organisms to be studied using molecular markers, a section of an organism's DNA sequence. By comparing similarities of sequences between two or more species, evolutionary relationships between compared species can be defined. For example, a phylogenetic analysis on the colonial ascidian *Botryllus schlosseri* using molecular markers cytochrome c oxidase 1 (commonly referred to as CO1) and 18S rRNA (commonly 18S) resulted in the distinction of three previously cryptogenic species, with only one of these spread globally and all of them morphologically indistinguishable. This was due to the five strongly supported monophyletic clades for CO1 and three for 18S (Bock et al. 2012). Another example involved finding the closest related family to Octacnemidae using *Megalodicopia hians* (Oka, 1918) as a representative deep-sea ascidian. Using the molecular marker 18S rRNA, it was determined that the family Corellidae was the most closely related to Octacnemidae phylogenetically, despite morphological characteristics suggesting both families Cionidae and Corellidae as the most closely related (Kurabayashi et al. 2003). When it comes to molecular taxonomy, DNA barcoding is also an important molecular technique and has assisted in many advancements within taxonomy.

DNA barcoding uses molecular markers such as CO1 to identify organisms using their genomic DNA (gDNA) by annealing to the highly conserved, or unchanged, regions of DNA for each organism. This allows for a unique genetic barcode to be generated for each organism, so long as the molecular marker is compatible with the organisms' DNA (Hebert et al. 2003). Within the last decade, advancements made in DNA barcoding have provided four major improvements to the field of molecular taxonomy. First, museums have built reference collections to serve as a basis for future studies based on pre-existing sequences (Puillandre et al. 2012). Second, by comparing molecular sequences with preserved morphological specimens, species identification errors have become more avoidable (Galimberti et al. 2015). Third, DNA barcoding data is publicly available and allows for sequences to be used in various fields (e.g., species identification by non-experts; Galimberti et al. 2015). Fourth, independent taxonomic characters can be identified. For example, the new ascidian species *Botrylloides conchyliatus* (Ekins, 2019) was identified and taxonomically separated from other members of the same genus due to molecular differences in CO1 between the native species (*Botrylloides giganteus*) and the cryptic species *Botrylloides perspicuus* ((Herdman, 1886); Rocha et al. 2019). However, DNA barcoding does have a limitation, in that it heavily relies on databases (e.g., Barcode of Life Database (BOLD) Bold Systems v4) to compare sequences of organisms for proper identification. If sequences are lacking for a species, it is hard (if not impossible) to properly identify the sequenced organism(s) to the genus or species level.

One of the most important aspects for species identification using molecular taxonomy is determining which marker will be most effective. Identifying which molecular markers are appropriate for species identification can be a daunting task. CO1

and 18S are used in many studies involving molecular taxonomy and phylogenetics. CO1 is often used for two primary reasons: its highly conserved regions support the design of universal PCR primers and its ability to identify and separate taxa in many taxonomic groups (e.g., invasive cryptic European blue mussel, *Mytilus galloprovincialis* (Lamarck, 1819), being separated from native Californian species *Mytilus trossulus* (Gould, 1850); Burton 2009). CO1 has already demonstrated usefulness for ascidian taxonomy. A potentially invasive species, *Eudistoma viride* (Tokioka, 1955), was identified to species using CO1 (Kumaran et al. 2017). Given that *E. viride* is a colonial ascidian that is difficult to identify morphologically due to its few distinguishing characteristics requiring expert knowledge to identify, CO1 can allow for quick and easy to understand results regardless of skill or knowledge level on ascidians (Kumaran et al. 2017). Also, species of ascidians with a poor morphological fossil record have been identified using CO1. Colonial species in India were identified with 99% certainty to be members of the family Didemnidae (Ali et al. 2015), another difficult group of ascidians to identify through morphological taxonomy (Ali et al. 2015). CO1 is not perfect, however, and it has its own limitations within ascidian taxonomy. Previous research suggests that CO1 can be used for species delimitation among members of the order Phlebobranchia and some members of Stolidobranchia, but not the family Styelidae or any members of the order Aplousobranchia (e.g., López-Legentil et al. 2006, Rius et al. 2008, López-Legentil and Turon 2005). For example, the colonial species *Botryllus schlosseri* demonstrated low haplotype diversity despite CO1 being variable at the intraspecies level in other ascidian species. Out of 181 sequences only 16 haplotypes were found, suggesting that there may be a more effective marker when studying this species (López-Legentil et al. 2006). Also,

CO1 heavily relies on available data on sites such as the National Center for Biological Information (NCBI or GenBank National Center for Biotechnology Information (nih.gov)) and because of this, can be fairly limited in its application.

The 18S rRNA gene is highly conserved (unchanging over evolutionary time) at the flanking regions of each DNA sequence therefore allowing the universal (or near universal) primer sites to be identified (Meyer et al. 2010). For example, five species of solitary and three species of colonial ascidians were analyzed using 18S to determine whether their life histories had evolved separately. It was concluded that these life histories had evolved independently after the divergence of the Enterogona and Pleurogona (Wada et al. 1992). 18S has also helped to support changes in ascidian taxonomy. For example, 18S was used to identify a cryptic lineage within a population of the colonial species *Distaplia bermudensis* (Van Name, 1902). Two genetic lineages were identified from 18S sequences and later used in conjunction with morphological traits such as tunic and oral siphon pigmentation (Evans et al. 2021).

There are two main issues with using 18S as a standalone marker. First, 18S cannot be used for certain taxa (e.g., some ascidian species) due to their rapidly evolving genomes (e.g., *C. intestinalis*; Tsagkogeorga et al. 2009). Second, 18S is only as useful as the existing sequences available in databases such as GenBank allow for comparison. This is because 18S is not a Consortium for the Barcode of Life (CBOL)-accepted region (i.e., a region of a DNA sequence that does not meet the global standard for species identification), and therefore has limitations on the quantity of data available within databases (National Center for Biotechnology Information (nih.gov)). If sequence data does not exist for a particular species, it is impossible to determine if the results obtained

from an 18S sequence are useful for molecular taxonomy. This again limits the use of 18S to certain taxa.

### 1.6 Objectives

There are two research objectives within this study. First, to determine the effectives of the markers CO1 and 18S in delimitating the three focal families of this study: Ascidiidae, Pyuridae, and Styelidae. Second, is to determine the effectiveness of two species delimitation methods, Assemble Species by Automatic Partitioning (ASAP) and Bayesian Poisson Tree Process (bPTP), at delimitating the three focal families to species level. Effectiveness is determined based on the three methods of species identification: Species delimitation methods ASAP, bPTP, and morphological taxonomy. The marker will be classified as effective if two out three methods agree.

### 1.7 Why Belizean Ascidians?

The ascidians within Belize have not been documented in nearly 30 years (Goodbody et al. 2000, 2004), providing an opportunity to document any changes that may have occurred since then due to potential invasions, overfishing, or climate change. Additionally, potential misidentifications could be corrected via the combining of morphological and molecular techniques (Hebert et al. 2003). Belize is currently lacking a comprehensive species catalog of native ascidians, allowing for the possibility for invasive species to take hold over the last few decades. In addition to the harbors and marinas of Belize, the barrier reef system may also be at risk due to potential invaders. Endangered coral species could become smothered and outcompeted by ascidians (e.g., *Trididemnum solidum* (Van Name, 1902)), decreasing ecosystem health (Bak et al. 1996).

## 2. Methods

### 2.1 July 2022 Sampling

Ascidians were collected from 21-27 July 2022. Investigated substrates included both natural (mangrove roots) and artificial (docks, pilings). Temperature and salinity data were collected at each sample site via electronic thermometer and refractometer, respectively. Samples were collected via snorkeling, using hand tools to remove ascidians from substrate (e.g., docks, mangrove roots). During collection, samples were held in plastic Ziplock bags filled with seawater. At the end of the sampling period, a handful menthol crystals were added to each bag to relax the samples and allow any feces or undigested food to be expelled. Samples were then left to relax for a period of three to six hours in a cool environment. Sampling sites (Table 1) were clustered around two locations around the Mesoamerican Reef and one mainland location, with each location being investigated for both natural and artificial substrates (Figure 1).

After the samples had sufficiently relaxed, specimens were organized into plastic tubs based on their most likely taxonomic classification. Each sample was given a unique ID and subdivided into two pieces, one preserved in formalin for morphological analysis, the other preserved in ethanol for molecular analysis. For the ethanol samples, an atrial siphon was taken from each solitary ascidian and a small portion of the colony was taken from each colonial specimen. Some smaller specimens were only preserved in formalin or ethanol. A total of 218 ascidians were collected.

## 2.2 July 2023 Sampling

Ascidians were collected from 5-12 July 2023. Investigated substrates included both natural (coral reefs and mangrove roots) and artificial (docks, pilings). Temperature and salinity data was collected via electronic thermometer and refractometer, respectively. Sampling took place at reef and mangrove island sites in the central Belizean Barrier Reef from the Carrier Bow Cay research station (Figure 2). Samples were collected via snorkeling and scuba diving, using hand tools to remove ascidians from substrate (e.g., mangroves and corals). Only locations in which ascidians were found are included in this analysis (Table 2). Samples were contained and relaxed as in 2022.

Specimens were organized and preserved in ethanol and formalin as in 2022. A total of 330 ascidians were collected.

## 2.3 Sample Processing

Each sample was dissected under a microscope. Tissue from the atrial siphon of solitary ascidians was cut in two, with half being placed into 1.5 mL vials filled with ethanol and the other half being placed back into the siphon and stored in a glass vial filled with ethanol. Colonial species had several individual zooids removed from the tunic and placed into 1.5 mL vials filled with ethanol, with the remaining body of the animal placed into a glass vial filled with ethanol.

DNA extractions were done in groups of 6 samples at a time using the DNeasy Blood & Tissue Kit (Qiagen). In brief, ethanol was removed from the sample and samples were then incubated for approximately 10 minutes at 55 °C to evaporate any

remaining ethanol. Next, 180 µL of ATL Buffer and 20 µL of Proteinase K were added to the samples. Samples were then vortexed for approximately 5 seconds, parafilm added, and placed in a water bath for approximately 24 hours at 55 °C. Parafilm was removed from the samples which were then vortexed for approximately 15 seconds and spun down. Next, 20 µL of RNase (10 mg/mL) was added to each sample and left to incubate at room temperature for 5 minutes. The remaining steps followed the manufacturer's protocol (Qiagen). Extracted DNA from each sample was resolved on a 1% agarose gel as a visual confirmation that DNA extraction was successful. DNA quantity (ng/µL) and quality (A260/A280) measurements were taken on the Thermofisher Nanodrop Lite spectrophotometer.

Each PCR reaction contained a 1x ExTaq Buffer, 20 mM each dNTPs, 20 µM of each primer (Table 3), 1 µL of template, and the remaining volume of sterile water for 25 µL or 50 µL reactions. Samples were amplified using a BioRad My Cycler thermocycler. Cycling conditions varied based on primers used (Table 3). Samples and primer sets used are listed below (Table 4). PCR products were resolved on a 1% agarose gel. DNA quantity (ng/µL) and quality (A260/A280) were determined using the Thermofisher Nanodrop Lite spectrophotometer.

PCR products were purified using the DNA Clean & Concentrator kit. DNA quantity (ng/µL) and quality (A260/A280) were determined using the Thermofisher Nanodrop Lite spectrophotometer.

Samples were sent to Eurofins Genomics for Sanger sequencing. Sanger sequencing was performed using proprietary sequencing chemistry. Sequence assembly

and manual checks of ambiguous base calls were done in Sequencher (Sequencher DNA Sequence Analysis Software from Gene Codes Corporation).

### 2.4 Morphological Identification

For Belizean samples, samples were morphologically identified to the genus level by L. Stefaniak. For GenBank samples, though not always reliable, for the purpose of this study morphological identifications were assumed to be reliable.

### 2.5 Data Analysis

To complement sequences generated from Belize, additional sequences were pulled from GenBank (Tables 5 and 6). Two to three sequences were pulled for each representative species if possible. Sequence alignments were done in MEGA X using Clustal W (Kumar et al. 2018). Alignments were then checked by hand and trimmed to equal sequence length (Table 7). Phylogenetic trees were generated in MEGA X using maximum likelihood methods. Substitution model for each tree was inferred from Model Test. Each tree was run at 1000 bootstrap replications. Bayesian trees were attempted but due to format using MrBayes portal CIPRES (phylo.org), but output trees could not be used bPTP portal Species delimitation server (h-its.org) available due to formatting incompatibilities.

CO1 and 18S sequences were analyzed using species delimitation methods ASAP and bPTP (Puillandre et al. 2021, Zhang et al. 2013). Species delimitation results were then compared between CO1 and 18S. ASAP calculates pairwise genetic distances between sequences and identifies a gap between smaller distances (presumed intraspecific) and larger distances (presumed interspecific) to partition the samples into

putative species (Puillandre et al. 2021). The ideal result is a small difference within one species, followed by a gap, and then a small difference within another species. bPTP builds on the existing method PTP (Poisson Tree Process) by adding Bayesian support values to putative species nodes (Zhang et al. 2013). PTP analyzes user-inputted phylogenetic trees and identifies the region of each tree for transition points between branching rates that are consistent with two models, a separation model and a coalescent model (Zhang et al. 2013). Bayesian support values were given as a proportion ranging from zero to one. As with phylogenetic analysis, species or nodes with less than 50% (0.5) support are considered to be unsupported. Due to potential differences in species partitions between ASAP and bPTP, both methods were used (Ducasse et al. 2020). ASAP was run using all three available substitution models: Kimura 2-parameter (Kimura 1980), Jukes-Cantor (Jukes and Cantor 1969), and p-distances. Sequence alignments were uploaded to the ASAP web server: ASAP web (mnhn.fr) (accessed on 24 May 2024). The maximum likelihood (ML) tree generated on MEGA X was analyzed using bPTP on the web server: Species delimitation server (h-its.org) (accessed on 24 May 2024).

## 3. Results

### 3.1 Ascidiidae CO1

ASAP separated sequences into 21 putative species while bPTP separated sequences into 46 putative species when using CO1 gene sequences. A total of 11 sequences (8 species) agreed between the two analyses. When comparing these delimitations to the maximum likelihood (ML) tree (Figure 3), it appears that putative ASAP species generally agreed with species level morphological identifications, whereas bPTP often disagreed with morphological identifications. For bPTP, 32 out of the 46 species had low support values (<0.5), and in several instances, bPTP separated identical or nearly identical sequences into separate species (Figure 3 and Table S1). It should be noted however, that sequences BZ_23-263 and a Genbank sequence of *Ascidia viridina* (Paiva et al. 2015) were grouped together as one species by bPTP and in a well-supported clade on the ML tree but are separate species when using ASAP. bPTP support value for this species is low (0.485), but the branches on the ML tree are relatively long compared to species delimitated by both ASAP and morphology, so this may be inconclusive.

### 3.2 Ascidiidae 18S

ASAP separated sequences into 9 putative species while bPTP separated sequences into 27 putative species when using 18S gene sequences. A total of 4 sequences (3 species) agreed between the two analyses. When comparing these delimitations to the ML tree (Figure 4), neither method generally agrees with species

level morphological identifications. For bPTP, 17 out of the 27 species had low support values (<0.5), and in several instances, bPTP separated identical or nearly identical sequences into separate species (Figure 4 and Table S2).

### 3.3 Pyuridae CO1 (*Herdmania*, *Microcosmus*, and *Pyura*)

For the genus *Herdmania*, ASAP separated sequences into 5 putative species while bPTP separated sequences into 12 putative species when using CO1 gene sequences. A total of 5 sequences (4 species) agreed between the two analyses. When comparing these delimitations to the ML tree (Figure 5), putative ASAP species generally agreed with species level morphological identifications, whereas bPTP often disagreed with morphological identifications. For bPTP, 6 out of the 12 species had low support values (<0.5), and in several instances, bPTP separated identical or nearly identical sequences into separate species (Figure 5 and Table S3).

For the genus *Microcosmus*, ASAP separated sequences into 10 putative species while bPTP separated sequences into 19 putative species when using CO1 gene sequences. A total of 8 sequences (6 species) agreed between the two analyses. When comparing these delimitations to the ML tree (Figure 6), putative ASAP species generally agreed with species level morphological identifications, whereas bPTP often disagreed with morphological identifications. For bPTP, 10 out of the 19 species had low support values (<0.5), and in several instances, bPTP separated identical or nearly identical sequences into separate species (Figure 6 and Table S4). Sequence BZ_23-253 and a Genbank sequence of *Microcosmus curvus* (Tokioka, 1954) were grouped together by bPTP and visually on the ML tree though ASAP separated these two sequences into

different species. The support value for this pairing via bPTP is 0.521 while the ML tree has <50% support, so this may be inconclusive.

For the genus *Pyura*, ASAP separated sequences into 19 putative species while bPTP separated sequences into 34 putative species when using CO1 gene sequences. A total of 12 sequences (10 species) agreed between the two analyses. When comparing these delimitations to the ML tree (Figure 7), putative ASAP species generally agreed with species level morphological identifications, whereas bPTP often disagreed with morphological identifications. For bPTP, 19 out of the 34 species had low support values (<0.5), and in several instances, bPTP separated identical or nearly identical sequences into separate species (Figure 7 and Table S5).

### 3.4 Pyuridae 18S

ASAP separated sequences into 2 putative species while bPTP separated sequences into 29 putative species when using 18S gene sequences. Only 1 sequence (1 species) agreed between the two analyses, which was the outgroup (*Botryllus schlosseri*) and is likely due to the difference in alignment (ASAP) and different family (bPTP) causing this sequence to be split from everything else. When comparing these delimitations to the ML tree (Figure 8), bPTP may have more accurately grouped these sequences into putative species as it is highly unlikely that between field samples and GenBank samples the ASAP grouping of 2 total species is correct. For bPTP, 16 out of the 29 species had low support values (<0.5), and in several instances, bPTP separated identical or nearly identical sequences into separate species (Figure 8 and Table S6). It should be noted, however, that bPTP may still be inadequate for this analysis as it

demonstrates similar problems shown in previous figures, the samples represent at least 25 morphological species and are separated into completely different species.

### 3.5 Styelidae CO1 (Solitary and Colonial Species)

For the solitary styelids, ASAP separated sequences into 11 putative species while bPTP separated sequences into 15 putative species when using CO1 gene sequences. A total of 13 sequences (9 species) agreed between the two analyses. When comparing these delimitations to the ML tree (Figure 9), putative ASAP species generally agreed with species level morphological identifications, whereas bPTP often disagreed with morphological identifications. For bPTP, 9 out of the 15 species had low support values (<0.5), and in several instances, bPTP separated identical or nearly identical sequences into separate species (Figure 9 and Table S7).

For the colonial styelids, ASAP separated sequences into 13 putative species while bPTP separated sequences into 28 putative species when using CO1 gene sequences. A total of 16 sequences (10 species) agreed between the two analyses. When comparing these delimitations to the ML tree (Figure 10), putative ASAP species generally agreed with species level morphological identifications, whereas bPTP often disagreed with morphological identifications. For bPTP, 16 out of the 28 species had low support values (<0.5), and in several instances, bPTP separated identical or nearly identical sequences into separate species (Figure 10 and Table S8).

### 3.6 Styelidae 18S

ASAP separated sequences into 2 putative species while bPTP separated sequences into 28 species when using 18S gene sequences. Only 1 sequence (1 species) agreed between the two analyses, which was the outgroup, *Molgula manhattensis* (De Kay, 1843), and is likely due to the difference in alignment (ASAP) and different family (bPTP) causing this sequence to be split from everything else. When comparing these delimitations to the ML tree (Figure 11), bPTP may have more accurately grouped these sequences into putative species as it is highly unlikely that between field samples and GenBank samples the ASAP grouping of 2 total species is correct. For bPTP, 17 out of the 28 species had low support values (<0.5), and in several instances, bPTP separated identical or nearly identical sequences into separate species (Figure 11 and Table S9). Again, bPTP may still be inadequate for this analysis as it demonstrates similar problems shown in previous figures, the samples represent at least 27 morphological species and are separated into completely different species.

## 4. Discussion

### 4.1 General Trends

This study explored the efficacy of the molecular markers CO1 and 18S and species delimitation methods ASAP and bPTP compared to morphological identifications to separate species in the ascidian families Ascidiidae, Pyuridae, and Styelidae. For six out of six CO1 trees, at least one of the two molecular-based species delimitation methods agreed with available morphological identifications. In general, the species delimitation methods generated with ASAP grouped all CO1 sequences of any particular morphological species, while bPTP divided many of the morphological species into several separate species, even if the members of the morphospecies share identical sequences.

For 18S, with three out of the three trees (Ascidiidae, Pyuridae, and Styelidae Figures 4, 8 and 11, respectively), neither delimitation methods were congruent with the morphological identifications. bPTP rarely agreed with morphological identifications for any of the three taxonomic groups. It compared better with the morphological identifications for Pyuridae (Fig. 8) and Styelidae (Fig. 11) than ASAP, but it still largely disagrees with the morphological identifications in GenBank.

### 4.2 CO1 vs 18S

My first question was are the molecular markers CO1 and 18S effective at delimitating the families Ascidiidae, Pyuridae, and Styelidae? CO1 appears to be more successful at delimitating the species within the 3 focal families: Ascidiidae (Figure 3), *Herdmania* (Figure 5), *Microcosmus* (Figure 6), *Pyura* (Figure 7), Styelidae-solitary (Figure 9), and Styelidae-colonial (Figure 10). The CO1 trees demonstrate higher support at the deeper nodes (values >50) while 18S trees have fewer well supported nodes, generally at the shallower end of the tree (Ascidiidae, Pyuridae, and Styelidae Figures 4, 8, and 11, respectively). The 18S Pyuridae and Styelidae trees also each had a few samples that would group by themselves far away from any closely related species, as seen on the Styelidae tree (Figure 11, sample BZ_23-196). Both markers demonstrated high support at the shallower, species level nodes, which was the primary concern for this study. In general, 18S may be a rather ineffective marker for this study. This is due to various issues such as lower support overall when compared to CO1, the possibility of long branch attraction between samples (e.g., Figure 11 *Asterocarpa humilis* (Heller, 1878) and *Polycarpa pomaria* (Savigny, 1816) GenBank sequences) causing inaccurate and illogical groupings (e.g., Figure 11 BZ_23-196), and the ineffectiveness of both species' delimitation methods on three out of three 18S analyses for this study (Figures 4, 8, and 11).

Another possibility that may influence the success of species delimitation is sequence length. Using 16S as the molecular marker and amphibians as the taxonomic group, it was concluded that sequence length greatly influenced the results of ASAP (Chan et al. 2022). Sequence lengths were divided into 3 groups (short [~500bp], medium

[~800bp], and complete [~1500bp]), with short sequence lengths being practically useless when using ASAP for species delimitation (Chan et al. 2022). The 18S alignments used in this study ranged from 443 bp (Styelidae) to 626 bp (Ascidiidae), placing all of them firmly in the "short" group.

CO1 has demonstrated species delimitation success with both vertebrates and invertebrates, including ascidians. When observing the biodiversity of demersal fish at the community level within the Cosmonaut Sea in the Southern Ocean, CO1 combined with delimitation methods ASAP and Bayesian Phylogenetics and Phylogeography (BPP) were effective at delimitating 98 samples consisting of 24 species down to the species level (Li et al. 2024). Combining molecular and morphological taxonomy for the genus *Botrylloides* generated several conclusions that would have been highly unlikely using morphological taxonomy alone. Using the marker CO1, species thought to be cryptogenic were determined to be two separate species, *Botrylloides giganteus* and *Botrylloides perspicuus*. Additionally, a new species, *Botrylloides conchyliatus*, was described due to molecular taxonomy (Rocha et al. 2019). Looking further into species delimitation using the marker CO1 combined with ASAP and bPTP, 12 new species were described with Family Styelidae (7 in the genus *Botryllus*, 3 in the genus *Botrylloides*, and 2 in the genus *Symplegma*). Styelids are often difficult to identify morphologically due to morphological plasticity, thus requiring the addition of molecular taxonomy (Palomino-Alvarez et al. 2022). The results from this study support our findings, at least at the molecular level when using CO1 and ASAP for now.

### 4.3 ASAP vs bPTP

My second question was are the species delimitation methods ASAP and bPTP effective at the species level for these ascidian families? ASAP appears to do a solid job at effectively delimitating these sequences down to the species level apart from the Ascidiidae, Pyuridae, and Styelidae 18S trees (Figures 4, 8, and 11, respectively). It should be noted that some morphological identifications included in the GenBank sequences, such as *Herdmania momus* (Savigny, 1816) and *Herdmania pallida* (Heller, 1878), are known to be very difficult to distinguish morphologically and therefore could be incorrectly identified (L. Stefaniak, pers. comm.). In addition to this, the amount of variation between the sequences for *H. momus* and *H. pallida* is also incredibly small (<0.02) for all Genbank sequences. Due to this, morphological analysis will need to be performed to confirm the identity of Belizean samples that were grouped together with both *Herdmania* species by the ASAP method (BZ_22-108, BZ_22-110, BZ_22-170, and BZ_23-136; Figure 5).

bPTP does not appear to be effective at species delimitation for these families, at least not when using the maximum likelihood tree input with bPTP. Though it appears that bPTP does a better job at delimitation where ASAP is lacking (Pyuridae (Fig. 8) and Styelidae (Fig. 11)), bPTP still tends to disagree with the morphological IDs from Genbank (e.g., Figure 11 *Botrylloides niger* (Herdman, 1886) sequences). These separations are highly unlikely because of the low levels of genetic distance between sequences, and bPTP can be further scrutinized on other trees in which ASAP was effective at delimitation for these same reasons (Tables S1-S9). Due to these factors,

when used with a maximum likelihood input tree, bPTP appears to be ineffective for all 9 taxonomic groups.

## 5. Future Work

As evidenced by the strong support of ASAP over bPTP within our results, the inclusion of bPTP Bayesian tree results should be implemented to see if these methods will better agree with each other. In addition to this, morphological identifications for our Belizean samples will be important in determining the overall accuracy of our results. While our samples mostly seemed to group appropriately within our trees, it is difficult to determine the efficacy of these results without definitive identifications for these samples. For example, there were a few tentative morphological identifications that grouped with entirely different orders than anticipated. These included a Didemnid in the Styelidae tree comprised entirely of solitary animals (Figure 9) and a sample identified from order Stolidobranchia grouping with a tree comprised entirely of order Phlebobranchia (Figure 4).

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**Table 1**: Sample locations during the July 2022 sampling period.

| Sample Location | GPS Coordinates | Sampled Substrates |
|---|---|---|
| Caye Caulker | 17º44.921'N<br>088º01.499'W | Mangrove roots, docks, pilings |
| Bread and Butter Caye | 16º46.167'N<br>088º09.783'W | Dock |
| Twin Cayes Outer Edge Nearest Ocean | 16º50.011'N<br>088º06.002'W | Mangrove roots |
| Thunderbird Marina | 16º32.587'N<br>088º21.934'W | Docks |
| Cap's Inn Dock | 16º30.629'N<br>088º22.252'W | Dock |
| Placencia Municipal Pier | 16º30.806'N<br>088º21.888'W | Pier |
| Placencia Yacht Club | 16º30.535'N<br>088º21.711'W | Dock |

**Table 2**: Sample locations during the July 2023 sampling period. Only locations in which ascidians were collected are included.

| Sample Location | GPS Coordinates | Sampled Substrate |
|---|---|---|
| Carrie Bow Field Station | 16º47.24'N 088º4.12'W | Docks |
| Patchy Reef Near Carrie Bow | 16º78.946'N 088º08.095'W | Coral Reef |
| Twin Cayes (Smithsonian Old Dock) | 16º49.923'N 088º06.322'W | Mangroves |
| Fore Reef of Carrie Bow | 16º80.2395'N 088º07.8636'W | Coral Reef |
| Pelican Caye | 16º39.932'N 088º10.943'W | Mangroves |
| Tobacco Caye | 16º89.6936'N 088º05.6148'W | Coral Reef |
| Twin Cayes (Boston Bay) | 16º49.564'N 088º06.204'W | Mangroves |
| Earl Reef | 16º75.250'N 088º07.3892'W | Coral Reef |
| Twin Cayes (Lair and Channel) | 16º49.761'N 088º06.075'W | Mangroves |
| South Water Caye | 16º81.8095'N 088º07.8688'W | Coral Reef |
| Twin Cayes (North Main Channel) | Coordinates not taken | Mangroves |
| Blue Ground Range | 16º77.3245'N 088º14.2375'W | Seagrass Meadows/Coral Reefs |
| South Water Caye Docks | Coordinates not taken | Docks |

**Table 3**: List of primers used in this study, 5' to 3' sequences, and PCR cycling conditions. Bolded text represents repeated cycles of PCR.

| Primer Name | 5' to 3' Sequence | Cycling Conditions |
|---|---|---|
| CO1 Folmer | LCO1490: TGTAAAACGACGGCCAGTGGTCAA CAAATCATAAAGATATTGG<br>HC02198: CAGGAAACAGCTATGACTAAACTTCA GGGTGACCAAAAAATCA | 94 ℃ 2min<br>**94 ℃ 45sec**<br>**50 ℃ 45sec**<br>**72 ℃ 50sec**<br>**(30x)**<br>72 ℃ 6min<br>4 ℃ ∞ |
| CO1 Tun | Tun_forward: TCGACTAATCATAAAGATATTA<br>Tun_reverse2: AACTTGTATTTAAATTACGATC | 94 ℃ 1min<br>**94 ℃ 10sec**<br>**50 ℃ 30sec**<br>**72 ℃ 50sec**<br>**72 ℃ 10min**<br>**(60x)**<br>4 ℃ ∞ |
| CO1 Gissi | dinF: CGTTGRTTTATRTCTACWAATCATAARGA<br>Nux1R: GCAGTAAAATAWGCTCGRGARTC<br>cat1F: ATRTCTACWAATCATAARGATATTRG<br>ux1R: ATAAGCTCGWGAATCHACATC | **98 ℃ 10sec**<br>**44-52 ℃ 15sec**<br>**72 ℃ 1.5min**<br>**(30x)**<br>72 ℃ 5min |
| 18S | 18SA: AGCAGCCGCGGTAATTCCAGCTC<br>18SB: AAAGGGCAGGGACGTAATCAACG | 94 ℃ 2min<br>**94 ℃ 20sec**<br>**66 ℃ 20sec**<br>**72 ℃ 2min**<br>**(40x)**<br>72 ℃ 10min<br>4 ℃ ∞ |

**Table 4**: Belize CO1 samples and primer sets used for this study. Primer sets as defined in Table 3.

| Sample ID | CO1 Primer Set |
|---|---|
| BZ-23-263 | Gissi |
| BZ-23-130 | Gissi |
| BZ-23-129 | Gissi |
| BZ-23-127 | Gissi |
| BZ-23-126 | Gissi |
| BZ-23-095 | Gissi |
| BZ-23-094 | Gissi |
| BZ-23-016 | Gissi |
| BZ-22-174 | Gissi |
| BZ-22-115 | Gissi |
| BZ-22-080 | Tun |
| BZ-22-075 | Tun |
| BZ-22-108 | Gissi |
| BZ-22-110 | Tun |
| BZ-22-170 | Tun |
| BZ-23-136 | Gissi |
| BZ-23-276 | Gissi |
| BZ-23-254 | Gissi |
| BZ-23-148 | Gissi |
| BZ-23-125 | Gissi |
| BZ-23-253 | Gissi |
| BZ-23-101 | Gissi |
| BZ-23-102 | Gissi |
| BZ-23-302 | Gissi |
| BZ-23-118 | Gissi |
| BZ-23-274 | Gissi |
| BZ-23-164 | Gissi |
| BZ-23-162 | Gissi |
| BZ-23-138 | Gissi |
| BZ-23-020 | Gissi |
| BZ-23-093 | Gissi |
| BZ-23-134 | Gissi |
| BZ-23-131 | Gissi |
| BZ-23-007 | Gissi |
| BZ-23-012 | Gissi |
| BZ-23-118 | Gissi |
| BZ-23-119 | Gissi |
| BZ-23-275 | Gissi |
| BZ-23-277 | Gissi |
| BZ-23-303 | Gissi |
| BZ-23-288 | Gissi |

**Table 4 continued**

| | |
|---|---|
| BZ-23-145 | Gissi |
| BZ-23-091 | Gissi |
| BZ-23-163 | Gissi |
| BZ-23-169 | Gissi |
| BZ-22-196 | Gissi |
| BZ-22-213 | Gissi |
| BZ-22-094-096 | Folmer |
| BZ-22-111 | Folmer |
| BZ-22-214 | Gissi |
| BZ-22-194 | Tun |
| BZ-22-011 | Gissi |
| BZ-23-089 | Gissi |
| BZ-23-155 | Gissi |
| BZ-23-293 | Gissi |
| BZ-23-115 | Gissi |
| BZ-23-113 | Gissi |
| BZ-23-196 | Gissi |
| BZ-23-096 | Gissi |
| BZ-23-274 | Gissi |

**Table 5**: 18S GenBank sequences used in analysis

| Scientific Name | Accession Number | Source |
|---|---|---|
| *Ascidia ahodori* | AB104871.1 | Kurabayashi et al. 2003 |
| *Ascidia ceratodes* | L12378.2 | Hadfield et al. 1995 |
| *Ascidia ceratodes* | KJ720729.1 | Tianero et al. 2015 |
| *Ascidia sydneiensis* | AF165819.1 | Wada et al. 1992 |
| *Ascidia zara* | LC547325.1 | Shito et al. 2020 |
| *Ascidia zara* | AB811926.1 | Nishikawa et al. 2014 |
| *Ascidiella aspersa* | LC547322.1 | Shito et al. 2020 |
| *Ascidiella aspersa* | LC547321.1 | Shito et al. 2020 |
| *Ascidiella aspersa* | AB811920.1 | Nishikawa et al. 2014 |
| *Ascidiella scabra* | AB811932.1 | Nishikawa et al. 2014 |
| *Ascidiella scabra* | AB811931.1 | Nishikawa et al. 2014 |
| *Ascidiella scabra* | AB811928.1 | Nishikawa et al. 2014 |
| *Ascidiella sp.* | FM244843.1 | Tsagkogeorga et al. 2009 |
| *Phallusia fumigata* | KF268454.1 | Vandepas et al. 2015 |
| *Phallusia fumigata* | FM244844.1 | Tsagkogeorga et al. 2009 |
| *Phallusia mammilata* | AF236803.2 | Cameron et al. 2000 |
| *Phallusia nigra* | KJ875973.1 | Vandepas et al. 2015 |
| *Phallusia nigra* | KJ875972.1 | Vandepas et al. 2015 |
| *Phallusia nigra* | KJ875971.1 | Vandepas et al. 2015 |
| *Phallusia philippinensis* | KF268462.1 | Vandepas et al. 2015 |
| *Phallusia philippinensis* | KF268461.1 | Vandepas et al. 2015 |
| *Phallusia philippinensis* | KF268460.1 | Vandepas et al. 2015 |
| *Ciona intestinalis* | JN573244.1 | Lee and Shin 2011(Unpublished) |
| *Halocynthia spinosa* | FM244851.1 | Tsagkogeorga et al. 2009 |
| *Herdmania mirabilis* | AJ250773.1 | Won et al. 1999 |
| *Herdmania momus* | KY807049.1 | Yi 2017 (Unpublished) |
| *Herdmania momus* | AF165827.1 | Swalla et al. 2000 |
| *Herdmania sp.* | FM897329.1 | Perez-Portela et al. 2009 |
| *Herdmania sp.* | FM897330.1 | Perez-Portela et al. 2009 |
| *Herdmania sp.* | FM244852.1 | Tsagkogeorga et al. 2009 |
| *Herdmania sp.* | LC547315.1 | Shito et al. 2020 |
| *Microcosmus exasperatus* | KT387603.1 | Gewing et al. 2015 |
| *Microcosmus exasperatus* | KT387604.1 | Gewing et al. 2015 |
| *Pyura dura* | FM244856.1 | Tsagkogeorga et al. 2009 |
| *Pyura dura* | FM897337.1 | Perez-Portela et al. 2009 |
| *Pyura gangelion* | FM244857.1 | Tsagkogeorga et al. 2009 |
| *Pyura vittata* | AJ250772.1 | Won et al. 1999 |

**Table 5 continued**

| | | |
|---|---|---|
| *Botryllus schlosseri* | JN573239.1 | Lee and Shin 2011 (Unpublished) |
| *Asterocarpa humilis* | MG800796.1 | Alié et al. 2018 |
| *Botrylloides niger* | OQ255573.1 | Temiz et al. 2023 |
| *Cnemidocarpa clara* | AJ250775.1 | Won et al. 1999 |
| *Polycarpa cryptocarpa* | LC547316.1 | Shito et al. 2020 |
| *Polycarpa pomeria* | MG800799.1 | Alié et al. 2018 |
| *Polycarpa pomeria* | L12441.2 | Hadfield et al. 1995 |
| *Stolonica socialis* | MG800801.1 | Alié et al. 2018 |
| *Styela plicata* | LC432328.1 | Hasegawa and Kajihara 2019 |
| *Styela plicata* | LC547313.1 | Shito et al. 2020 |
| *Styela plicata* | KJ818250.1 | Liu 2014 (Unpublished) |
| *Molgula manhattensis* | AB921975.1 | Kanamori and Kawasaki 2014 |

**Table 6:** Cytochrome oxidase I (CO1) GenBank sequences used in analysis

| Scientific Name | Accession Number | Source |
|---|---|---|
| *Ascidia ceratodes* | MZ782796.1 | Nichols et al. 2023 |
| *Ascidia ceratodes* | MZ782795.1 | Nichols et al. 2023 |
| *Ascidia ceratodes* | MZ782794.1 | Nichols et al. 2023 |
| *Ascidia colleta* | OM912774.1 | Virgili et al. 2022 |
| *Ascidia colleta* | OM912773.1 | Virgili et al. 2022 |
| *Ascidia colleta* | OM912772.1 | Virgili et al. 2022 |
| *Ascidia columbiana* | MH242676.1 | Leray and Paulay 2018 (Unpublished) |
| *Ascidia conchilega* | MN064597.1 | Couton et al. 2019 |
| *Ascidia conchilega* | MN064596.1 | Couton et al. 2019 |
| *Ascidia gemmata* | KX650763.1 | Jaffarali and Sobon 2016 (Unpublished) |
| *Ascidia interrupta* | KY111416.1 | Villalobos et al. 2017 |
| *Ascidia interrupta* | KY111415.1 | Villalobos et al. 2017 |
| *Ascidia malaca* | OM912753.1 | Virgili et al. 2022 |
| *Ascidia paratropa* | MH242677.1 | Leray and Paulay 2018 (Unpublished) |
| *Ascidia virginea* | ON062302.1 | Nydam and Lambert 2022 (Unpublished) |
| *Ascidia virginea* | ON062301.1 | Nydam and Lambert 2022 (Unpublished) |
| *Ascidia viridina* | KR604726.1 | Paiva et al. 2015 |
| *Ascidia zara* | MZ782792.1 | Nichols et al. 2023 |
| *Ascidia zara* | MZ782791.1 | Nichols et al. 2023 |
| *Ascidia zara* | MZ782787.1 | Nichols et al. 2023 |
| *Ascidiella aspersa* | MZ782798.1 | Nichols et al. 2023 |
| *Ascidiella aspersa* | MZ782797.1 | Nichols et al. 2023 |
| *Ascidiella aspersa* | MW872314.1 | Nichols et al. 2023 |
| *Ascidiella scabra* | KF309650.1 | López-Legentil et al. 2015 |
| *Ascidiella scabra* | KF309572.1 | López-Legentil et al. 2015 |
| *Ascidiella scabra* | KF309560.1 | López-Legentil et al. 2015 |
| *Phallusia arabica* | KP779903.1 | Stalin et al. 2015 (Unpublished) |
| *Phallusia arabica* | KF414706.1 | Selva and Ananthan 2013 (Unpublished) |
| *Phallusia fumigata* | OM912038.1 | Virgili et al. 2022 |
| *Phallusia fumigata* | OM912037.1 | Virgili et al. 2022 |
| *Phallusia fumigata* | OM912036.1 | Virgili et al. 2022 |
| *Phallusia julinea* | KC017431.1 | Erwin et al. 2014 |

**Table 6 continued**

| | | |
|---|---|---|
| *Phallusia mammillata* | OM912040.1 | Virgili et al. 2022 |
| *Phallusia mammillata* | OM912039.1 | Virgili et al. 2022 |
| *Phallusia mammillata* | KF309607.1 | López-Legentil et al. 2015 |
| *Phallusia nigra* | MW858365.1 | Nydam et al. 2022 |
| *Phallusia nigra* | MT637958.1 | Streit et al. 2021 |
| *Phallusia nigra* | KX650762.1 | Jaffarali et al. 2016 (Unpublished) |
| *Ciona intestinalis* | KU647848.1 | Schreiber et al. 2016 (Unpublished) |
| *Herdmania pallida* | MW278777.1 | Paulay et al. 2020 (Unpublished) |
| *Herdmania momus* | KM411616.1 | Jaffar et al. 2014 (Unpublished |
| *Herdmania momus* | MH720940.1 | Ahmed and Jaffar 2018 (Unpublished) |
| *Herdmania momus* | MH720939.1 | Ahmed and Jaffar 2018 (Unpublished) |
| *Herdmania sp.* | LC546999.1 | Shito et al. 2020 |
| *Herdmania sp.* | MW278689.1 | Paulay et al. 2020 (Unpublished) |
| *Herdmania sp.* | MW278787.1 | Paulay et al. 2020 (Unpublished) |
| *Herdmania grandis* | FJ528630.1 | Perez-Portela et al. 2009 |
| *Microcosmus polymorphus* | OM912472.1 | Virgili et al. 2022 |
| *Microcosmus polymorphus* | OM912473.1 | Virgili et al. 2022 |
| *Microcosmus polymorphus* | OM912475.1 | Virgili et al. 2022 |
| *Microcosmus curvus* | KT693194.1 | Jaffarali et al. 2015 (Unpublished) |
| *Microcosmus claudicans* | FJ528605.1 | Perez-Portela et al. 2009 |
| *Microcosmus sulcatus* | GQ294471.1 | De Luca and Fulgione 2009 (Unpublished) |
| *Microcosmus squamiger* | OM912583.1 | Virgili et al. 2022 |
| *Microcosmus squamiger* | OM912585.1 | Virgili et al. 2022 |
| *Microcosmus squamiger* | OM912587.1 | Virgili et al. 2022 |
| *Microcosmus helleri* | KX650803.1 | Jaffarali et al. 2016 (Unpublished) |
| *Microcosmus helleri* | KX650804.1 | Jaffarali et al. 2016 (Unpublished) |

**Table 6 continued**

| | | |
|---|---|---|
| *Microcosmus exasperatus* | MW858357.1 | Nydam et al. 2022 |
| *Microcosmus exasperatus* | MT637987.1 | Streit et al. 2021 |
| *Microcosmus exasperatus* | MT637985.1 | Streit et al. 2021 |
| *Pyura squamulosa* | FJ528625.1 | Perez-Portela et al. 2009 |
| *Pyura chilensis* | MW785988.1 | Haye et al. 2021 |
| *Pyura chilensis* | MW786587.1 | Haye et al. 2021 |
| *Pyura haustor* | MH242956.1 | Leray and Paulay 2018 (Unpublished) |
| *Pyura dura* | FJ528618.1 | Perez-Portela et al. 2009 |
| *Pyura dura* | OM912461.1 | Virgili et al. 2022 |
| *Pyura dura* | OM912465.1 | Virgili et al. 2022 |
| *Pyura vannamei* | MH258880.1 | Counts et al. 2018 (Unpublished) |
| *Pyura vittata* | MT637976.1 | Streit et al. 2021 |
| *Pyura australis* | FJ528617.1 | Perez-Portela et al. 2009 |
| *Pyura gibbosa* | FJ528614.1 | Perez-Portela et al. 2009 |
| *Pyura praeputialis* | JF961983.1 | Teske et al. 2011 |
| *Pyura praeputialis* | JF961969.1 | Teske et al. 2011 |
| *Pyura praeputialis* | JF961937.1 | Teske et al. 2011 |
| *Pyura stolonifera* | JF961845.1 | Teske et al. 2011 |
| *Pyura stolonifera* | JF961839.1 | Teske et al. 2011 |
| *Pyura stolonifera* | JF961830.1 | Teske et al. 2011 |
| *Pyura herdmani* | JF961853.1 | Teske et al. 2011 |
| *Pyura herdmani* | JF961874.1 | Teske et al. 2011 |
| *Pyura spinifera* | FJ528611.1 | Perez-Portela et al. 2009 |
| *Pyura spinifera* | FJ528612.1 | Perez-Portela et al. 2009 |
| *Pyura dalbyi* | JF962200.1 | Teske et al. 2011 |
| *Pyura dalbyi* | JF962223.1 | Teske et al. 2011 |
| *Pyura dalbyi* | JF962215.1 | Teske et al. 2011 |
| *Botryllus schlosseri* | KU647843.1 | Schreiber et al. 2016 (Unpublished) |
| *Styela plicata* | OQ323204.1 | Aguilar et al. 2022 (Unpublished) |
| *Styela plicata* | OQ323194.1 | Aguilar et al. 2022 (Unpublished) |
| *Styela plicata* | OQ322828.1 | Aguilar et al. 2022 (Unpublished) |
| *Polycarpa spongiabilis* | MT637949.1 | Streit et al. 2021 |

**Table 6 continued**

| | | |
|---|---|---|
| *Polycarpa spongiabilis* | MH258879.1 | Counts et al. 2018 (Unpublished) |
| *Polycarpa spongiabilis* | MH258878.1 | Counts et al. 2018 (Unpublished) |
| *Botrylloides niger* | OQ211499.1 | Karahan et al. 2023 (Unpublished) |
| *Botrylloides sp.* | LS992552.1 | Gissi 2018 (Unpublished) |
| *Botrylloides sp.* | LS992550.1 | Gissi 2018 (Unpublished) |
| *Botrylloides niger* | OQ211501.1 | Karahan et al. 2023 (Unpublished) |
| *Botrylloides niger* | OQ211500.1 | Karahan et al. 2023 (Unpublished) |
| *Botrylloides niger* | OQ211499.1 | Karahan et al. 2023 (Unpublished) |
| *Botrylloides nigrum* | MW278779.1 | Paulay et al. 2020 (Unpublished) |
| *Botrylloides nigrum* | MH367290.1 | Kaleemullah and Abdul 2018 (Unpublished) |
| *Botryllus sp.* | LR743465.1 | Gissi 2019 (Unpublished) |
| *Botryllus sp.* | LR743464.1 | Gissi 2019 (Unpublished) |
| *Botryllus sp.* | LR743463.1 | Gissi 2019 (Unpublished) |
| *Botryllus schlosseri* | OQ323341.1 | Aguilar et al. 2022 (Unpublished) |
| *Botryllus schlosseri* | AY600987.1 | Turon and López-Legentil 2004 |
| *Botryllus schlosseri* | JN248377.1 | Bock et al. 2012 |
| *Symplegma brakenhielmi* | OM912790.1 | Virgili et al. 2022 |
| *Symplegma brakenhielmi* | OM912789.1 | Virgili et al. 2022 |
| *Symplegma brakenhielmi* | OM912788.1 | Virgili et al. 2022 |
| *Symplegma reptans* | ON076054.1 | Nydam 2022 (Unpublished) |
| *Symplegma reptans* | ON076053.1 | Nydam 2022 (Unpublished) |
| *Symplegma reptans* | OM816672.1 | Lee 2022 (Unpublished) |

**Table 7:** List of genes, substitution models, and alignment lengths for each family/group used in this study.

| **Family/Group** | **Gene** | **Substitution Model for Each Tree** | **Alignment Length** |
|---|---|---|---|
| Family Ascidiidae | CO1<br>18S | Tamura-Nei<br>Kimura 2-parameter | 390bp<br>626bp |
| Genus *Herdmania*<br>Genus *Microcosmus*<br>Genus *Pyura*<br>Family Pyuridae | CO1<br>CO1<br>CO1<br>18S | Tamura-Nei<br>Tamura-Nei<br>Tamura-Nei<br>Kimura 2-parameter | 538bp<br>546bp<br>428bp<br>519bp |
| Solitary Species<br>Colonial Species<br>Family Styelidae | CO1<br>CO1<br>18S | Tamura-Nei<br>Tamura-Nei<br>Jukes-Cantor | 484bp<br>458bp<br>443bp |


**Figure 1**: Map of sample sites in Belize for summer of 2022. (A): Caye Caulker (1), Bread and Butter Caye (2), Twin Cayes (3), and Placencia (4-7). (B) Placencia sample sites: Thunderbird Marina (4), Cap's Inn Dock (5), Placencia Municipal Pier (6), and Placencia Yacht Club (7). See Table 1 for GPS coordinates. Image taken from Google Earth.


**Figure 2**: Map of sample sites for Belize Summer of 2023. Carrie Bow Field Station (1), patchy reef near South Water Caye (2), Twin Cayes (3), Pelican Caye (4), Tobacco Caye (5), and Earl Reef (6). See Table 2 for GPS coordinates. Image taken from Google Earth.

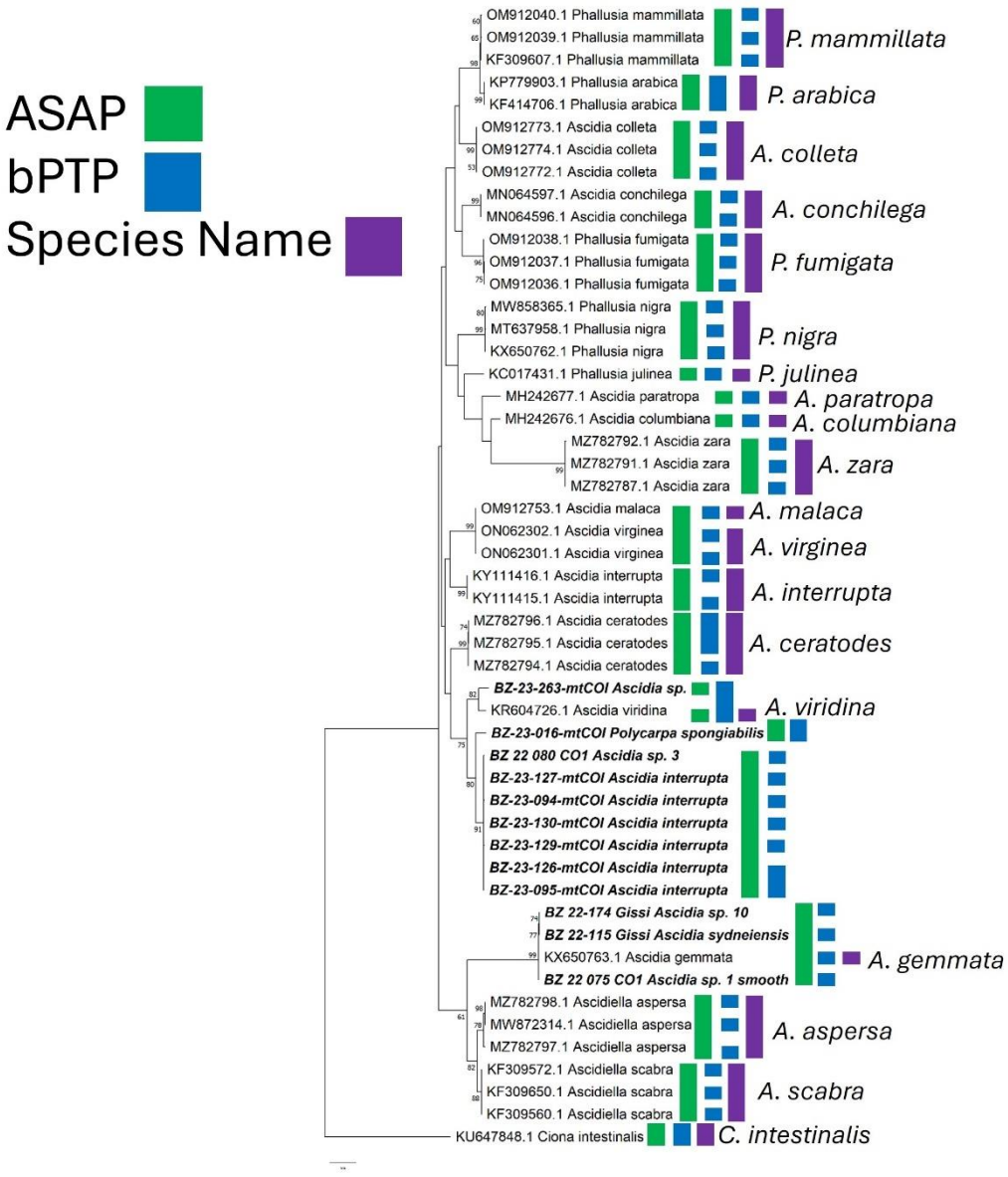


**Figure 3**: A 1000 bootstrap replicate species delimitation tree for the family Ascidiidae constructed in a Maximum Likelihood framework using the marker CO1. Support values greater than 50% are listed at each node. Green vertical bars represent ASAP delimitation method. Blue vertical bars represent bPTP delimitation method. Purple vertical bars represent morphological identifications identified to the species level. Bolded fonts represent tentative morphological identifications of Belizean samples. Maximum Likelihood tree was generated in MEGA X using the Tamura-Nei substitution model.

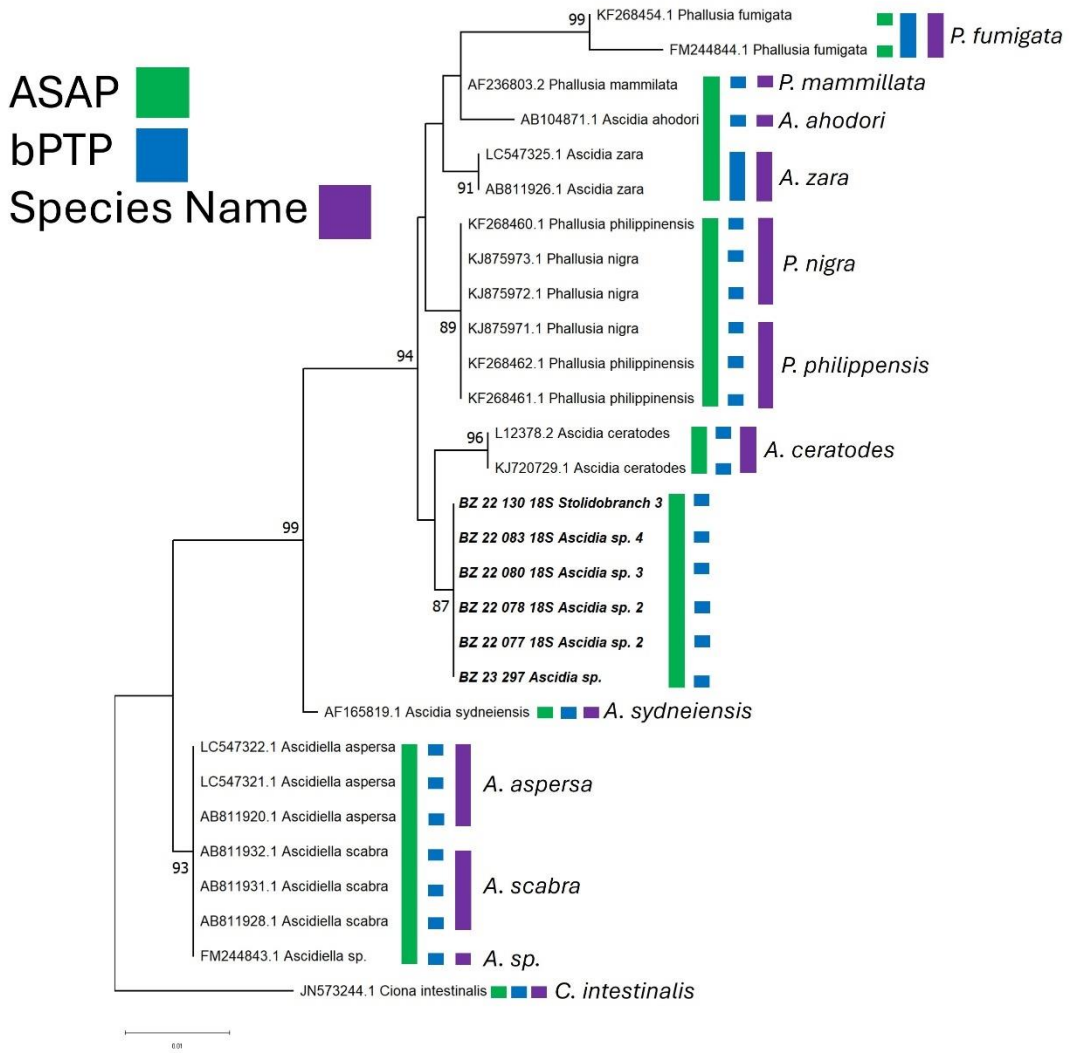


**Figure 4**: A 1000 bootstrap replicate species delimitation tree for the family Ascidiidae constructed in a Maximum Likelihood framework using the marker 18S. Support values greater than 50% are listed at each node. Green vertical bars represent ASAP delimitation method. Blue vertical bars represent bPTP delimitation method. Purple vertical bars represent morphological identifications identified to the species level. Bolded fonts represent tentative morphological identifications of Belizean samples. Maximum Likelihood tree was generated in MEGA X using the Kimura 2-parameter substitution model.

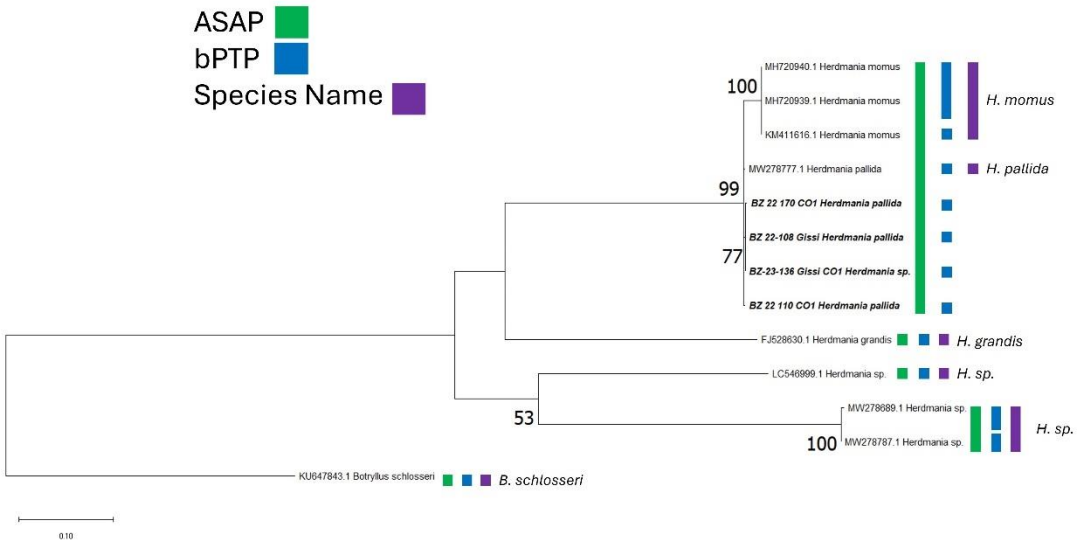


**Figure 5**: A 1000 bootstrap replicate species delimitation tree for the genus *Herdmania* constructed in a Maximum Likelihood framework using the marker CO1. Support values greater than 50% are listed at each node. Green vertical bars represent ASAP delimitation method. Blue vertical bars represent bPTP delimitation method. Purple vertical bars represent morphological identifications identified to the species level. Bolded fonts represent tentative morphological identifications of Belizean samples. Maximum Likelihood tree was generated in MEGA X using the Tamura-Nei substitution model.

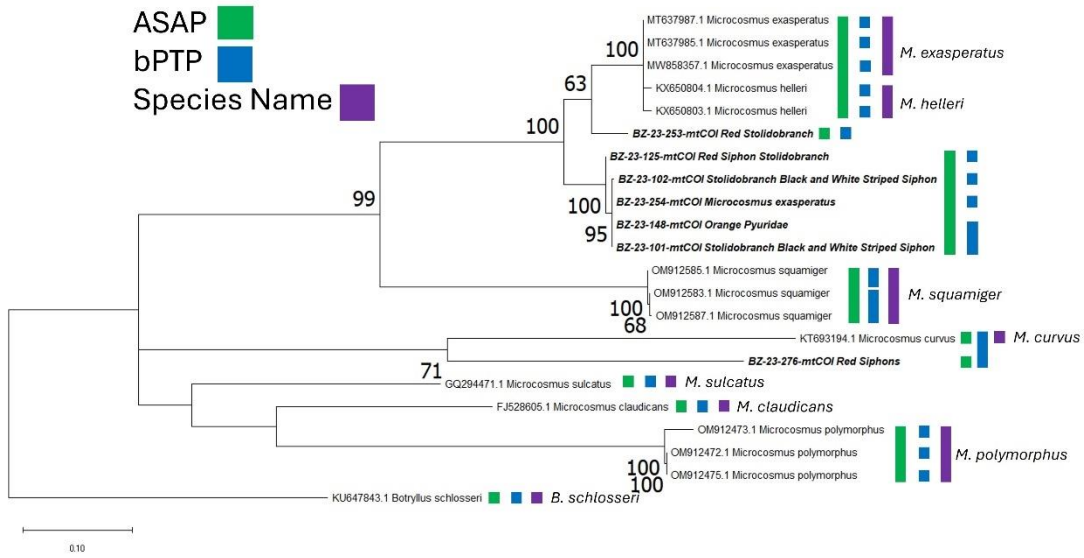


**Figure 6**: A 1000 bootstrap replicate species delimitation tree for the genus *Microcosmus* constructed in a Maximum Likelihood framework using the marker CO1. Support values greater than 50% are listed at each node. Green vertical bars represent ASAP delimitation method. Blue vertical bars represent bPTP delimitation method. Purple vertical bars represent morphological identifications identified to the species level. Bolded fonts represent tentative morphological identifications of Belizean samples. Maximum Likelihood tree was generated in MEGA X using the Tamura-Nei substitution model.

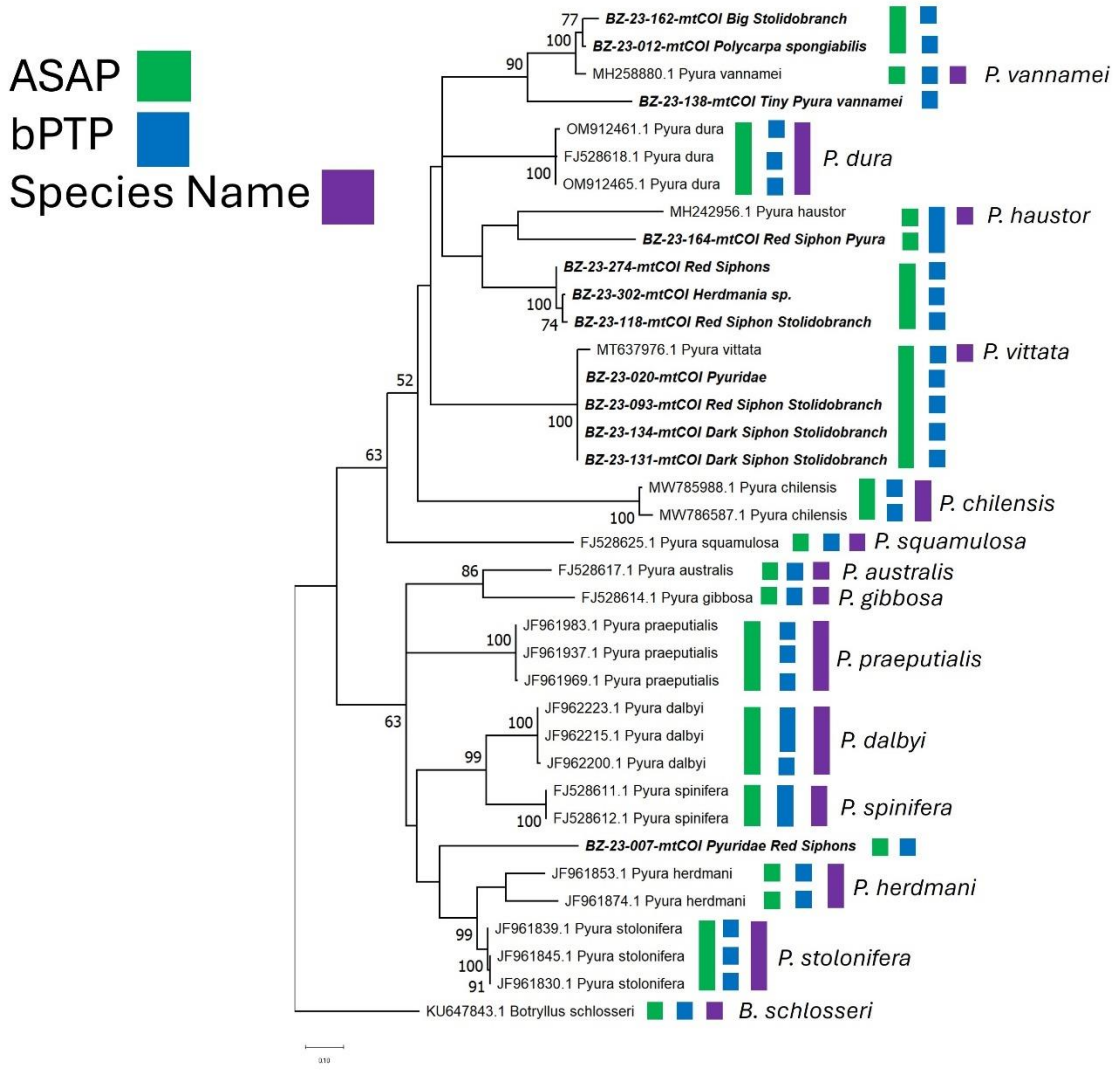


**Figure 7**: A 1000 bootstrap replicate species delimitation tree for the genus *Pyura* constructed in a Maximum Likelihood framework using the marker CO1. Support values greater than 50% are listed at each node. Green vertical bars represent ASAP delimitation method. Blue vertical bars represent bPTP delimitation method. Purple vertical bars represent morphological identifications identified to the species level. Bolded fonts represent tentative morphological identifications of Belizean samples. Maximum Likelihood tree was generated in MEGA X using the Tamura-Nei substitution model.

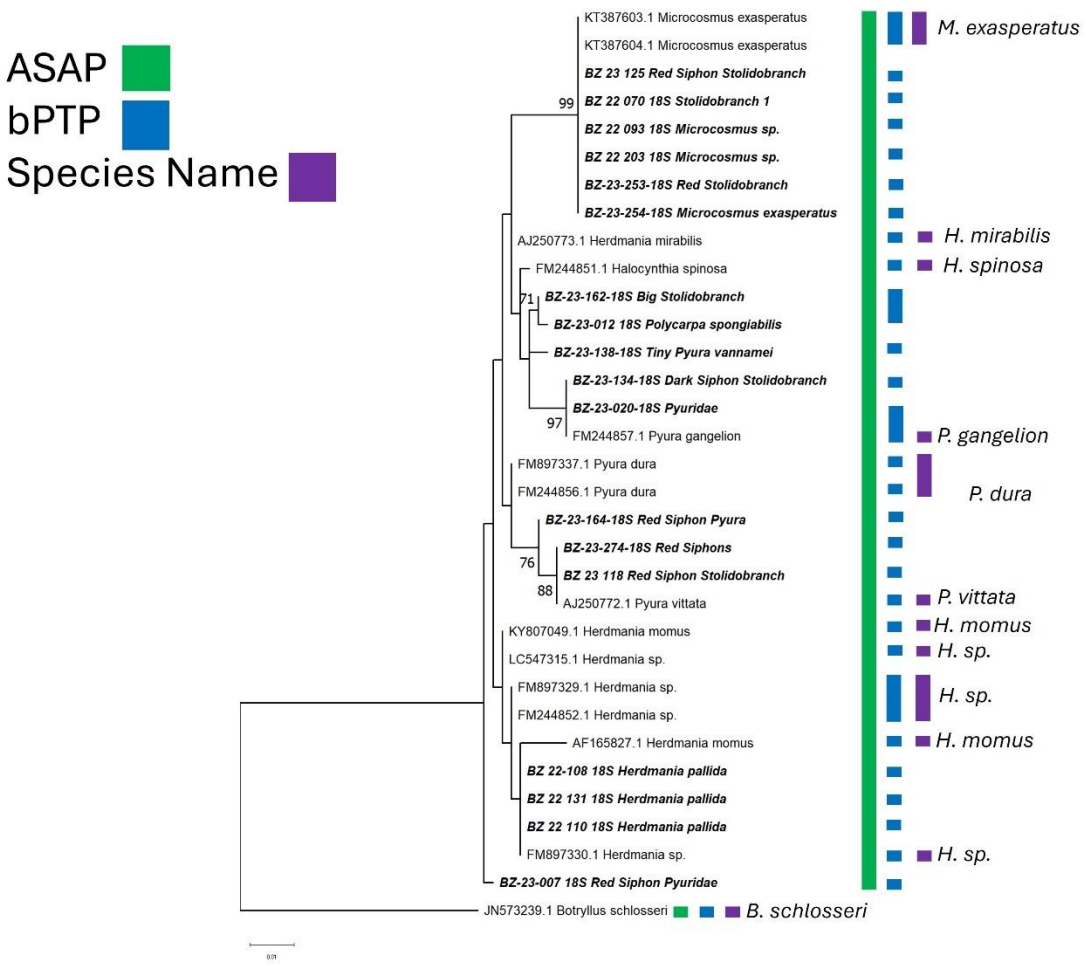


**Figure 8**: A 1000 bootstrap replicate species delimitation tree for the family Pyuridae constructed in a Maximum Likelihood framework using the marker 18S. Support values greater than 50% are listed at each node. Green vertical bars represent ASAP delimitation method. Blue vertical bars represent bPTP delimitation method. Purple vertical bars represent morphological identifications identified to the species level. Bolded fonts represent tentative morphological identifications of Belizean samples. Maximum Likelihood tree was generated in MEGA X using the Kimura 2-parameter substitution model.

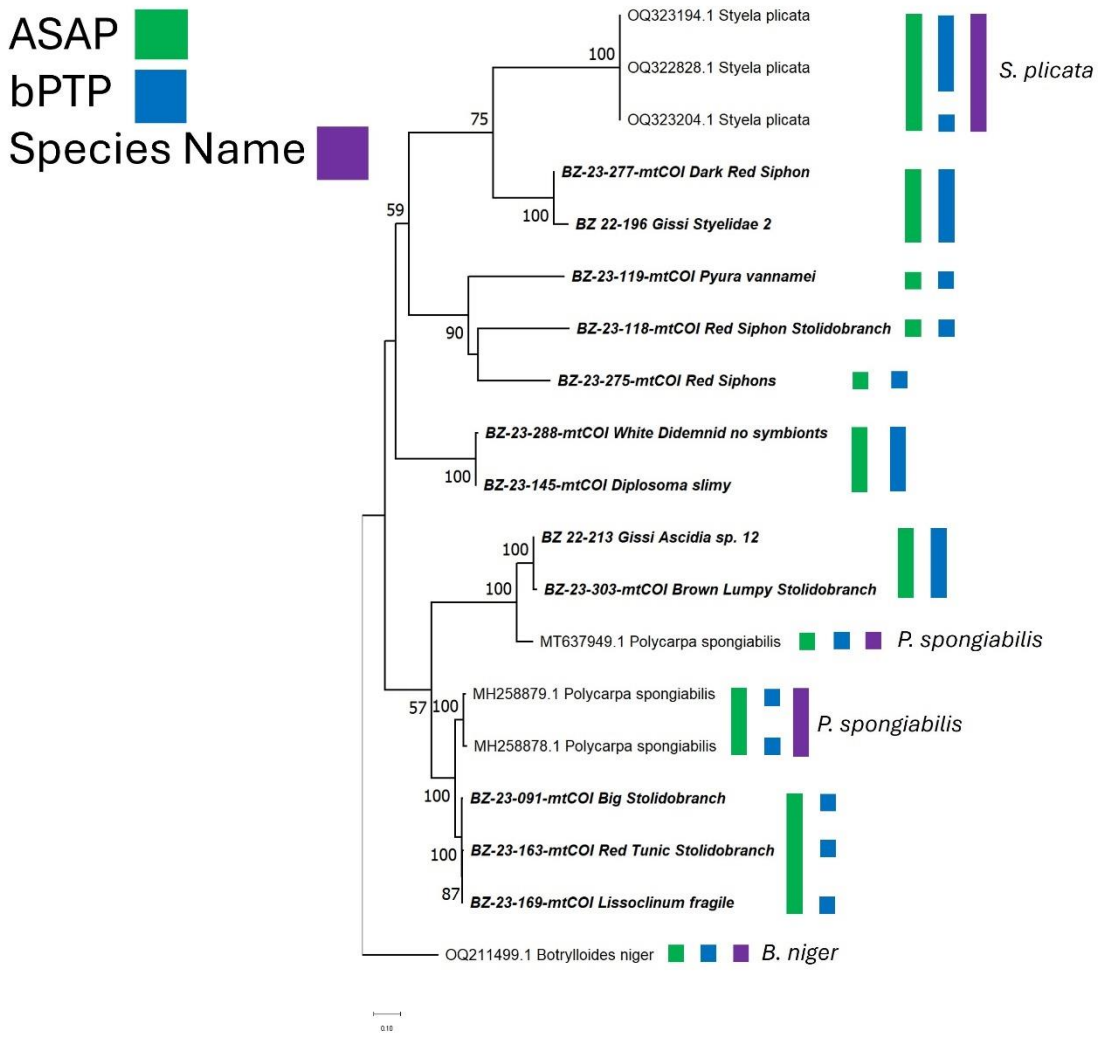


**Figure 9**: A 1000 bootstrap replicate species delimitation tree for the solitary members of the family Styelidae constructed in a Maximum Likelihood framework using the marker CO1. Support values greater than 50% are listed at each node. Green vertical bars represent ASAP delimitation method. Blue vertical bars represent bPTP delimitation method. Purple vertical bars represent morphological identifications identified to the species level. Bolded fonts represent tentative morphological identifications of Belizean samples. Maximum Likelihood tree was generated in MEGA X using the Tamura-Nei substitution model.

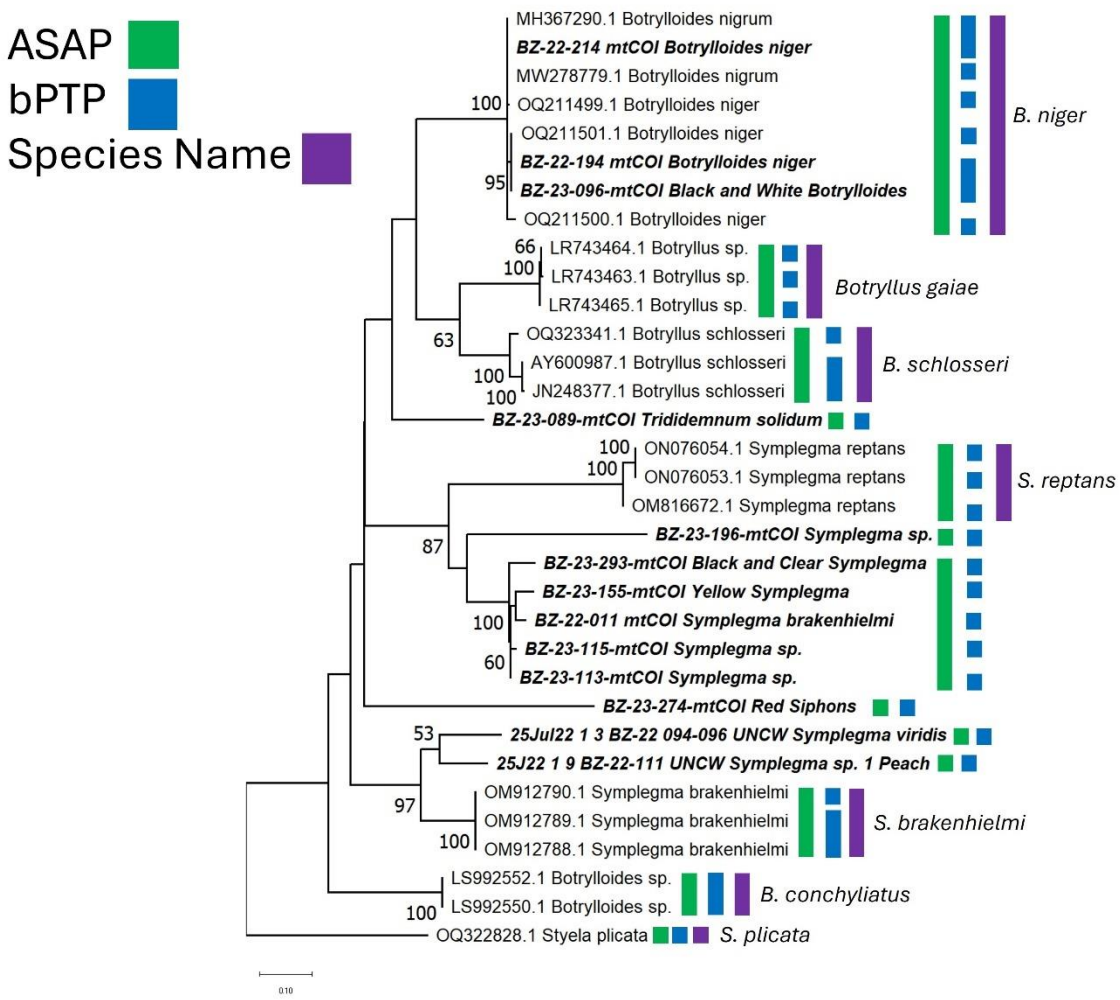


**Figure 10**: A 1000 bootstrap replicate species delimitation tree for the colonial members of the family Styelidae constructed in a Maximum Likelihood framework using the marker CO1. Support values greater than 50% are listed at each node. Green vertical bars represent ASAP delimitation method. Blue vertical bars represent bPTP delimitation method. Purple vertical bars represent morphological identifications identified to the species level. Bolded fonts represent tentative morphological identifications of Belizean samples. Maximum Likelihood tree was generated in MEGA X using the Tamura-Nei substitution model.

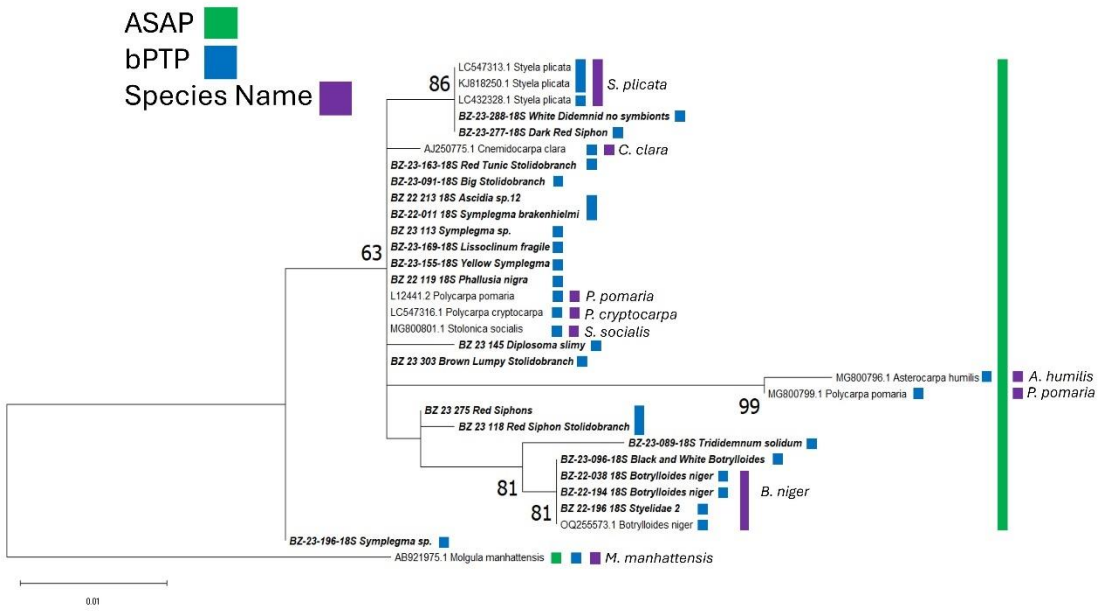


**Figure 11**: A 1000 bootstrap replicate species delimitation tree for the family Styelidae constructed in a Maximum Likelihood framework using the marker 18S. Support values greater than 50% are listed at each node. Green vertical bars represent ASAP delimitation method. Blue vertical bars represent bPTP delimitation method. Purple vertical bars represent morphological identifications identified to the species level. Bolded fonts represent tentative morphological identifications of Belizean samples. Maximum Likelihood tree was generated in MEGA X using the Jukes-Cantor substitution model.

## Appendix

Table S1: Genetic Distance of Ascidiidae CO1

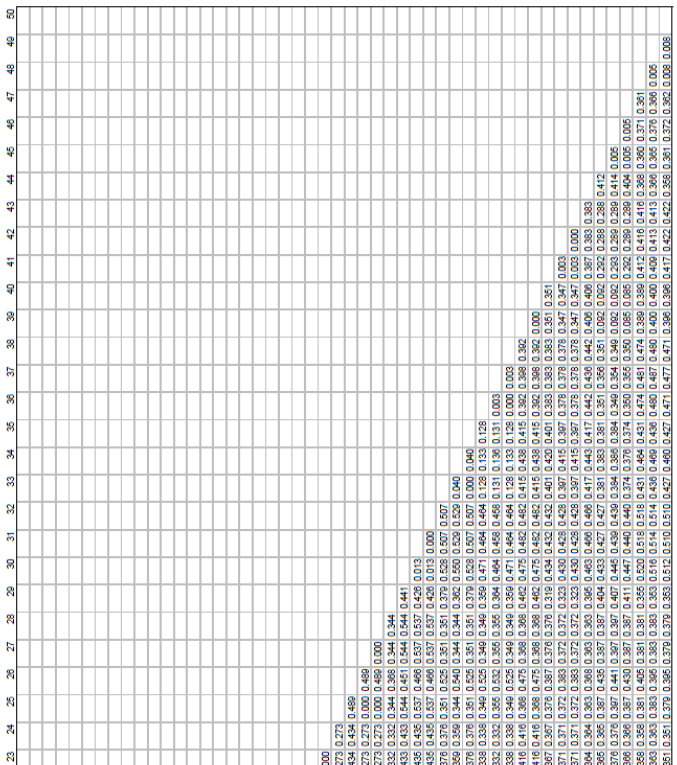

Table S2: Genetic Distance of Ascidiidae 18S

| Ascidiidae 18S | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 BZ_22_130_18S_Stolidobranch_3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 BZ_22_083_18S_Ascidia_4 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 BZ_22_080_18S_Ascidia_3 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 BZ_22_078_18S_Ascidia_2 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 BZ_22_077_18S_Ascidia_2 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 BZ_23_297 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | |
| 7 AB104871.1_Ascidia_ahodori | 0.011 | 0.011 | 0.011 | 0.011 | 0.011 | 0.011 | | | | | | | | | | | | | | | | | | | | | | |
| 8 L12378.2_Ascidia_ceratodes | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.008 | | | | | | | | | | | | | | | | | | | | | |
| 9 KJ720729.1_Ascidia_ceratodes | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.009 | 0.000 | | | | | | | | | | | | | | | | | | | | |
| 10 AF165819.1_Ascidia_sydneiensis | 0.015 | 0.015 | 0.015 | 0.015 | 0.015 | 0.015 | 0.018 | 0.013 | 0.013 | | | | | | | | | | | | | | | | | | | |
| 11 LC547325.1_Ascidia_zara | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 | 0.006 | 0.008 | 0.008 | 0.016 | | | | | | | | | | | | | | | | | | |
| 12 AB811926.1_Ascidia_zara | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 | 0.006 | 0.008 | 0.008 | 0.016 | 0.000 | | | | | | | | | | | | | | | | | |
| 13 LC547322.1_Ascidiella_aspersa | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.021 | 0.021 | 0.015 | 0.021 | 0.021 | | | | | | | | | | | | | | | | |
| 14 LC547321.1_Ascidiella_aspersa | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.021 | 0.021 | 0.015 | 0.021 | 0.021 | 0.000 | | | | | | | | | | | | | | | |
| 15 AB811920.1_Ascidiella_aspersa | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.021 | 0.021 | 0.015 | 0.021 | 0.021 | 0.000 | 0.000 | | | | | | | | | | | | | | |
| 16 AB811932.1_Ascidiella_scabra | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.021 | 0.021 | 0.015 | 0.021 | 0.021 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | |
| 17 AB811931.1_Ascidiella_scabra | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.021 | 0.021 | 0.015 | 0.021 | 0.021 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | |
| 18 AB811928.1_Ascidiella_scabra | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.021 | 0.021 | 0.015 | 0.021 | 0.021 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | |
| 19 FM244843.1_Ascidiella_sp. | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.021 | 0.021 | 0.015 | 0.021 | 0.021 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | |
| 20 KF268454.1_Phallusia_fumigata | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.021 | 0.021 | 0.026 | 0.016 | 0.016 | 0.035 | 0.035 | 0.035 | 0.035 | 0.035 | 0.035 | 0.035 | | | | | | | | | |
| 21 FM244844.1_Phallusia_fumigata | 0.023 | 0.023 | 0.023 | 0.023 | 0.023 | 0.023 | 0.023 | 0.028 | 0.028 | 0.033 | 0.023 | 0.023 | 0.042 | 0.042 | 0.042 | 0.042 | 0.042 | 0.042 | 0.042 | 0.007 | | | | | | | | |
| 22 AF236803.2_Phallusia_mammilata | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.005 | 0.010 | 0.010 | 0.015 | 0.005 | 0.005 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.011 | 0.018 | | | | | | | |
| 23 KJ875973.1_Phallusia_nigra | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.011 | 0.010 | 0.010 | 0.015 | 0.008 | 0.008 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.016 | 0.023 | 0.006 | | | | | | |
| 24 KJ875972.1_Phallusia_nigra | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.011 | 0.010 | 0.010 | 0.015 | 0.008 | 0.008 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.016 | 0.023 | 0.006 | 0.000 | | | | | |
| 25 KJ875971.1_Phallusia_nigra | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.011 | 0.010 | 0.010 | 0.015 | 0.008 | 0.008 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.016 | 0.023 | 0.006 | 0.000 | 0.000 | | | | |
| 26 KF268462.1_Phallusia_philippinensis | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.011 | 0.010 | 0.010 | 0.015 | 0.008 | 0.008 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.016 | 0.023 | 0.006 | 0.000 | 0.000 | 0.000 | | | |
| 27 KF268461.1_Phallusia_philippinensis | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.011 | 0.010 | 0.010 | 0.015 | 0.008 | 0.008 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.016 | 0.023 | 0.006 | 0.000 | 0.000 | 0.000 | 0.000 | | |
| 28 KF268460.1_Phallusia_philippinensis | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.011 | 0.010 | 0.010 | 0.015 | 0.008 | 0.008 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.016 | 0.023 | 0.006 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | |

Table S3: Genetic Distance of Pyuridae (*Herdmania*) CO1

| **Herdmania COI** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 MW278777.1_Herdmania_pallida | | | | | | | | | | | | | |
| 2 KM411616.1_Herdmania_momus | 0.019 | | | | | | | | | | | | |
| 3 MH720940.1_Herdmania_momus | 0.019 | 0.000 | | | | | | | | | | | |
| 4 MH720939.1_Herdmania_momus | 0.019 | 0.000 | 0.000 | | | | | | | | | | |
| 5 LC546999.1_Herdmania_sp. | 0.215 | 0.234 | 0.234 | 0.234 | | | | | | | | | |
| 6 MW278689.1_Herdmania_sp. | 0.238 | 0.252 | 0.252 | 0.252 | 0.229 | | | | | | | | |
| 7 MW278787.1_Herdmania_sp. | 0.233 | 0.247 | 0.247 | 0.247 | 0.229 | 0.004 | | | | | | | |
| 8 FJ528630.1_Herdmania_grandis | 0.197 | 0.213 | 0.213 | 0.213 | 0.229 | 0.235 | 0.230 | | | | | | |
| 9 KU647843.1_Botryllus_schlosseri | 0.298 | 0.310 | 0.310 | 0.310 | 0.307 | 0.329 | 0.323 | 0.294 | | | | | |
| 10 BZ_22-108_Gissi | 0.004 | 0.019 | 0.019 | 0.019 | 0.220 | 0.238 | 0.233 | 0.202 | 0.301 | | | | |
| 11 BZ-23-136-mtCOI_1 | 0.004 | 0.019 | 0.019 | 0.019 | 0.220 | 0.238 | 0.233 | 0.202 | 0.301 | 0.000 | | | |
| 12 BZ_22_170_CO1_Herdmania_pallida | 0.006 | 0.021 | 0.021 | 0.021 | 0.224 | 0.242 | 0.236 | 0.206 | 0.305 | 0.002 | 0.002 | | |
| 13 BZ_22_110_CO1_Herdmania | 0.004 | 0.019 | 0.019 | 0.019 | 0.219 | 0.242 | 0.237 | 0.203 | 0.296 | 0.004 | 0.004 | 0.006 | |

Table S4: Genetic Distance of Pyuridae (*Microcosmus*) CO1

| Microcosmus COI | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 OM912472.1_Microcosmus_polymorphus | | | | | | | | | | | | | | | | | | | | | | |
| 2 OM912473.1_Microcosmus_polymorphus | 0.021 | | | | | | | | | | | | | | | | | | | | | |
| 3 OM912475.1_Microcosmus_polymorphus | 0.000 | 0.021 | | | | | | | | | | | | | | | | | | | | |
| 4 KT693194.1_Microcosmus_curvus | 0.235 | 0.234 | 0.235 | | | | | | | | | | | | | | | | | | | |
| 5 FJ528605.1_Microcosmus_claudicans | 0.192 | 0.199 | 0.192 | 0.230 | | | | | | | | | | | | | | | | | | |
| 6 GQ294471.1_Microcosmus_sulcatus | 0.203 | 0.210 | 0.203 | 0.210 | 0.168 | | | | | | | | | | | | | | | | | |
| 7 OM912583.1_Microcosmus_squamiger | 0.234 | 0.238 | 0.234 | 0.247 | 0.199 | 0.214 | | | | | | | | | | | | | | | | |
| 8 OM912585.1_Microcosmus_squamiger | 0.232 | 0.236 | 0.232 | 0.244 | 0.197 | 0.212 | 0.001 | | | | | | | | | | | | | | | |
| 9 OM912587.1_Microcosmus_squamiger | 0.234 | 0.238 | 0.234 | 0.247 | 0.199 | 0.214 | 0.001 | 0.003 | | | | | | | | | | | | | | |
| 10 KX650803.1_Microcosmus_helleri | 0.246 | 0.248 | 0.246 | 0.246 | 0.197 | 0.211 | 0.160 | 0.159 | 0.162 | | | | | | | | | | | | | |
| 11 KX650804.1_Microcosmus_helleri | 0.253 | 0.255 | 0.253 | 0.243 | 0.199 | 0.210 | 0.160 | 0.158 | 0.162 | 0.011 | | | | | | | | | | | | |
| 12 MW858357.1_Microcosmus_exasperatus | 0.244 | 0.246 | 0.244 | 0.236 | 0.190 | 0.202 | 0.152 | 0.151 | 0.154 | 0.006 | 0.006 | | | | | | | | | | | |
| 13 MT637987.1_Microcosmus_exasperatus | 0.244 | 0.246 | 0.244 | 0.236 | 0.190 | 0.202 | 0.152 | 0.151 | 0.154 | 0.006 | 0.006 | 0.000 | | | | | | | | | | |
| 14 MT637985.1_Microcosmus_exasperatus | 0.244 | 0.246 | 0.244 | 0.236 | 0.190 | 0.202 | 0.152 | 0.151 | 0.154 | 0.006 | 0.006 | 0.000 | 0.000 | | | | | | | | | |
| 15 KU647843.1_Botryllus_schlosseri | 0.251 | 0.253 | 0.251 | 0.236 | 0.204 | 0.206 | 0.231 | 0.229 | 0.229 | 0.221 | 0.222 | 0.214 | 0.214 | 0.214 | | | | | | | | |
| 16 BZ-23-276-mtCOI_1 | 0.235 | 0.252 | 0.235 | 0.206 | 0.239 | 0.227 | 0.250 | 0.248 | 0.250 | 0.249 | 0.246 | 0.239 | 0.239 | 0.239 | 0.246 | | | | | | | |
| 17 BZ-23-254-mtCOI_1 | 0.237 | 0.232 | 0.237 | 0.228 | 0.193 | 0.206 | 0.165 | 0.163 | 0.167 | 0.069 | 0.069 | 0.063 | 0.063 | 0.063 | 0.221 | 0.236 | | | | | | |
| 18 BZ-23-148-mtCOI_1 | 0.237 | 0.232 | 0.237 | 0.228 | 0.193 | 0.206 | 0.165 | 0.163 | 0.167 | 0.069 | 0.069 | 0.063 | 0.063 | 0.063 | 0.221 | 0.236 | 0.000 | | | | | |
| 19 BZ-23-125-mtCOI_1 | 0.232 | 0.230 | 0.232 | 0.226 | 0.191 | 0.207 | 0.161 | 0.159 | 0.163 | 0.066 | 0.066 | 0.060 | 0.060 | 0.060 | 0.223 | 0.235 | 0.004 | 0.004 | | | | |
| 20 BZ-23-253-mtCOI_1 | 0.243 | 0.240 | 0.243 | 0.247 | 0.210 | 0.194 | 0.166 | 0.168 | 0.168 | 0.053 | 0.053 | 0.047 | 0.047 | 0.047 | 0.220 | 0.225 | 0.056 | 0.056 | 0.053 | | | |
| 21 BZ-23-101-mtCOI | 0.237 | 0.232 | 0.237 | 0.228 | 0.193 | 0.206 | 0.165 | 0.163 | 0.167 | 0.069 | 0.069 | 0.063 | 0.063 | 0.063 | 0.221 | 0.236 | 0.000 | 0.000 | 0.004 | 0.056 | | |
| 22 BZ-23-102-mtCOI | 0.240 | 0.234 | 0.240 | 0.225 | 0.195 | 0.204 | 0.165 | 0.163 | 0.167 | 0.071 | 0.071 | 0.064 | 0.064 | 0.064 | 0.223 | 0.235 | 0.001 | 0.001 | 0.006 | 0.058 | 0.001 | |

Table S5: Genetic Distance of Pyuridae (*Pyura*) CO1

| Pyura COI | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 FJ528625.1_Pyura_squamulosa | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 MW785988.1_Pyura_chilensis | 0.367 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 MW786587.1_Pyura_chilensis | 0.366 | 0.036 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 MH242956.1_Pyura_haustor | 0.400 | 0.375 | 0.343 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 FJ528618.1_Pyura_dura | 0.310 | 0.348 | 0.345 | 0.276 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 OM912461.1_Pyura_dura | 0.319 | 0.353 | 0.350 | 0.288 | 0.012 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 7 OM912465.1_Pyura_dura | 0.315 | 0.353 | 0.350 | 0.276 | 0.002 | 0.009 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 8 MH258880.1_Pyura_vannamei | 0.297 | 0.366 | 0.363 | 0.335 | 0.233 | 0.240 | 0.236 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 9 MT637976.1_Pyura_vittata | 0.307 | 0.368 | 0.380 | 0.340 | 0.273 | 0.277 | 0.277 | 0.277 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 FJ528617.1_Pyura_australis | 0.330 | 0.406 | 0.403 | 0.336 | 0.309 | 0.322 | 0.313 | 0.372 | 0.339 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 11 FJ528614.1_Pyura_gibbosa | 0.287 | 0.392 | 0.409 | 0.369 | 0.259 | 0.279 | 0.263 | 0.310 | 0.313 | 0.180 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 12 JF961983.1_Pyura_praeputalis | 0.341 | 0.396 | 0.370 | 0.390 | 0.295 | 0.312 | 0.299 | 0.338 | 0.332 | 0.240 | 0.240 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 13 JF961969.1_Pyura_praeputalis | 0.345 | 0.391 | 0.365 | 0.395 | 0.299 | 0.308 | 0.303 | 0.342 | 0.336 | 0.244 | 0.243 | 0.002 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 14 JF961937.1_Pyura_praeputalis | 0.341 | 0.396 | 0.370 | 0.390 | 0.295 | 0.312 | 0.299 | 0.338 | 0.332 | 0.240 | 0.240 | 0.000 | 0.002 | | | | | | | | | | | | | | | | | | | | | | | | |
| 15 JF961845.1_Pyura_stolonifera | 0.320 | 0.348 | 0.340 | 0.382 | 0.286 | 0.302 | 0.290 | 0.328 | 0.302 | 0.232 | 0.228 | 0.192 | 0.195 | 0.192 | | | | | | | | | | | | | | | | | | | | | | | |
| 16 JF961839.1_Pyura_stolonifera | 0.323 | 0.349 | 0.342 | 0.393 | 0.295 | 0.311 | 0.299 | 0.338 | 0.304 | 0.236 | 0.236 | 0.194 | 0.198 | 0.194 | 0.005 | | | | | | | | | | | | | | | | | | | | | | |
| 17 JF961830.1_Pyura_stolonifera | 0.320 | 0.348 | 0.340 | 0.382 | 0.286 | 0.302 | 0.290 | 0.328 | 0.302 | 0.232 | 0.228 | 0.192 | 0.195 | 0.192 | 0.000 | 0.005 | | | | | | | | | | | | | | | | | | | | | |
| 18 JF961853.1_Pyura_herdmani | 0.320 | 0.347 | 0.330 | 0.448 | 0.272 | 0.280 | 0.268 | 0.347 | 0.309 | 0.213 | 0.250 | 0.229 | 0.233 | 0.229 | 0.108 | 0.111 | 0.108 | | | | | | | | | | | | | | | | | | | | |
| 19 JF961874.1_Pyura_herdmani | 0.336 | 0.304 | 0.318 | 0.397 | 0.326 | 0.334 | 0.321 | 0.354 | 0.335 | 0.235 | 0.254 | 0.254 | 0.258 | 0.254 | 0.132 | 0.132 | 0.132 | 0.132 | | | | | | | | | | | | | | | | | | | |
| 20 FJ528611.1_Pyura_spinifera | 0.324 | 0.386 | 0.417 | 0.362 | 0.300 | 0.313 | 0.300 | 0.362 | 0.347 | 0.255 | 0.242 | 0.236 | 0.239 | 0.236 | 0.220 | 0.219 | 0.220 | 0.253 | 0.247 | | | | | | | | | | | | | | | | | | |
| 21 FJ528612.1_Pyura_spinifera | 0.324 | 0.386 | 0.417 | 0.362 | 0.300 | 0.313 | 0.300 | 0.362 | 0.347 | 0.255 | 0.242 | 0.236 | 0.239 | 0.236 | 0.220 | 0.219 | 0.220 | 0.253 | 0.247 | 0.000 | | | | | | | | | | | | | | | | | |
| 22 JF962200.1_Pyura_dalbyi | 0.290 | 0.373 | 0.384 | 0.407 | 0.310 | 0.323 | 0.306 | 0.373 | 0.362 | 0.260 | 0.301 | 0.253 | 0.257 | 0.253 | 0.208 | 0.211 | 0.208 | 0.251 | 0.252 | 0.149 | 0.149 | | | | | | | | | | | | | | | | |
| 23 JF962223.1_Pyura_dalbyi | 0.282 | 0.364 | 0.375 | 0.407 | 0.302 | 0.314 | 0.298 | 0.363 | 0.353 | 0.253 | 0.293 | 0.253 | 0.257 | 0.253 | 0.201 | 0.204 | 0.201 | 0.244 | 0.252 | 0.143 | 0.143 | 0.005 | | | | | | | | | | | | | | | |
| 24 JF962215.1_Pyura_dalbyi | 0.282 | 0.364 | 0.375 | 0.407 | 0.302 | 0.314 | 0.298 | 0.363 | 0.353 | 0.253 | 0.293 | 0.253 | 0.257 | 0.253 | 0.201 | 0.204 | 0.201 | 0.244 | 0.252 | 0.143 | 0.143 | 0.005 | 0.000 | | | | | | | | | | | | | | |
| 25 KU647843.1_Botrylloides_schlosseri | 0.346 | 0.400 | 0.391 | 0.391 | 0.285 | 0.301 | 0.289 | 0.292 | 0.380 | 0.275 | 0.271 | 0.287 | 0.291 | 0.287 | 0.270 | 0.272 | 0.270 | 0.270 | 0.303 | 0.321 | 0.321 | 0.349 | 0.340 | 0.340 | | | | | | | | | | | | | |
| 26 BZ-23-302-mtCOI_1 | 0.339 | 0.335 | 0.337 | 0.268 | 0.230 | 0.237 | 0.233 | 0.284 | 0.272 | 0.332 | 0.318 | 0.303 | 0.307 | 0.303 | 0.265 | 0.274 | 0.265 | 0.314 | 0.339 | 0.334 | 0.334 | 0.340 | 0.332 | 0.332 | 0.332 | | | | | | | | | | | | |
| 27 BZ-23-118-mtCOI_1 | 0.339 | 0.335 | 0.337 | 0.260 | 0.219 | 0.226 | 0.222 | 0.268 | 0.268 | 0.333 | 0.323 | 0.295 | 0.299 | 0.295 | 0.250 | 0.258 | 0.250 | 0.309 | 0.330 | 0.339 | 0.339 | 0.345 | 0.336 | 0.336 | 0.332 | 0.014 | | | | | | | | | | | |
| 28 BZ-23-274-mtCOI_1 | 0.320 | 0.330 | 0.331 | 0.236 | 0.220 | 0.227 | 0.223 | 0.271 | 0.273 | 0.329 | 0.324 | 0.292 | 0.296 | 0.292 | 0.264 | 0.273 | 0.264 | 0.319 | 0.336 | 0.320 | 0.320 | 0.344 | 0.335 | 0.335 | 0.310 | 0.019 | 0.024 | | | | | | | | | | |
| 29 BZ-23-164-mtCOI_1 | 0.303 | 0.333 | 0.337 | 0.260 | 0.273 | 0.288 | 0.277 | 0.302 | 0.317 | 0.335 | 0.321 | 0.331 | 0.335 | 0.331 | 0.302 | 0.296 | 0.302 | 0.333 | 0.368 | 0.361 | 0.361 | 0.365 | 0.365 | 0.365 | 0.341 | 0.247 | 0.251 | 0.245 | | | | | | | | | |
| 30 BZ-23-162-mtCOI_1 | 0.323 | 0.393 | 0.414 | 0.368 | 0.236 | 0.240 | 0.240 | 0.064 | 0.280 | 0.404 | 0.337 | 0.393 | 0.397 | 0.393 | 0.334 | 0.344 | 0.334 | 0.341 | 0.369 | 0.405 | 0.405 | 0.399 | 0.389 | 0.389 | 0.315 | 0.285 | 0.277 | 0.272 | 0.334 | | | | | | | | |
| 31 BZ-23-138-mtCOI_1 | 0.382 | 0.387 | 0.355 | 0.350 | 0.252 | 0.267 | 0.255 | 0.193 | 0.312 | 0.392 | 0.355 | 0.341 | 0.346 | 0.341 | 0.288 | 0.294 | 0.288 | 0.309 | 0.366 | 0.354 | 0.354 | 0.364 | 0.359 | 0.359 | 0.304 | 0.246 | 0.257 | 0.241 | 0.265 | 0.212 | | | | | | | |
| 32 BZ-23-020-mtCOI_1 | 0.301 | 0.329 | 0.336 | 0.312 | 0.267 | 0.271 | 0.271 | 0.264 | 0.031 | 0.323 | 0.282 | 0.312 | 0.316 | 0.312 | 0.287 | 0.290 | 0.287 | 0.294 | 0.311 | 0.339 | 0.339 | 0.368 | 0.359 | 0.359 | 0.362 | 0.254 | 0.251 | 0.255 | 0.290 | 0.266 | 0.289 | | | | | | |
| 33 BZ-23-093-mtCOI_1 | 0.297 | 0.333 | 0.341 | 0.316 | 0.263 | 0.267 | 0.267 | 0.260 | 0.029 | 0.319 | 0.286 | 0.316 | 0.320 | 0.316 | 0.291 | 0.294 | 0.291 | 0.290 | 0.315 | 0.335 | 0.335 | 0.363 | 0.354 | 0.354 | 0.357 | 0.251 | 0.247 | 0.252 | 0.286 | 0.262 | 0.285 | 0.002 | | | | | |
| 34 BZ-23-134-mtCOI_1 | 0.297 | 0.333 | 0.341 | 0.316 | 0.263 | 0.267 | 0.267 | 0.260 | 0.029 | 0.319 | 0.286 | 0.316 | 0.320 | 0.316 | 0.291 | 0.294 | 0.291 | 0.290 | 0.315 | 0.335 | 0.335 | 0.363 | 0.354 | 0.354 | 0.357 | 0.251 | 0.247 | 0.252 | 0.286 | 0.262 | 0.285 | 0.002 | 0.000 | | | | |
| 35 BZ-23-131-mtCOI_1 | 0.297 | 0.333 | 0.341 | 0.316 | 0.263 | 0.267 | 0.267 | 0.260 | 0.029 | 0.319 | 0.286 | 0.316 | 0.320 | 0.316 | 0.291 | 0.294 | 0.291 | 0.290 | 0.315 | 0.335 | 0.335 | 0.363 | 0.354 | 0.354 | 0.357 | 0.251 | 0.247 | 0.252 | 0.286 | 0.262 | 0.285 | 0.002 | 0.000 | 0.000 | | | |
| 36 BZ-23-007-mtCOI_2 | 0.318 | 0.383 | 0.376 | 0.423 | 0.355 | 0.364 | 0.355 | 0.356 | 0.367 | 0.253 | 0.292 | 0.254 | 0.258 | 0.254 | 0.228 | 0.223 | 0.228 | 0.210 | 0.249 | 0.258 | 0.258 | 0.265 | 0.257 | 0.257 | 0.331 | 0.372 | 0.382 | 0.366 | 0.342 | 0.383 | 0.372 | 0.354 | 0.349 | 0.349 | 0.349 | | |
| 37 BZ-23-012-mtCOI | 0.283 | 0.334 | 0.340 | 0.336 | 0.230 | 0.241 | 0.234 | 0.042 | 0.256 | 0.369 | 0.310 | 0.332 | 0.337 | 0.332 | 0.297 | 0.306 | 0.297 | 0.298 | 0.333 | 0.342 | 0.342 | 0.360 | 0.351 | 0.351 | 0.281 | 0.277 | 0.269 | 0.264 | 0.288 | 0.040 | 0.186 | 0.251 | 0.247 | 0.247 | 0.247 | 0.351 | |

Table S6: Genetic Distance of Pyuridae 18S

| Pyuridae 18S | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 BZ-23-274-18S | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 BZ-23-254-18S | 0.026 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 BZ-23-253-18S | 0.026 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 BZ-23-164-18S | 0.004 | 0.022 | 0.022 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 BZ-23-162-18S | 0.014 | 0.020 | 0.020 | 0.010 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 BZ-23-138-18S | 0.016 | 0.020 | 0.020 | 0.012 | 0.006 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 7 BZ-23-134-18S | 0.012 | 0.024 | 0.024 | 0.008 | 0.010 | 0.012 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 8 BZ-23-020-18S | 0.012 | 0.024 | 0.024 | 0.008 | 0.010 | 0.012 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 9 BZ-23-012_18S | 0.016 | 0.022 | 0.022 | 0.012 | 0.002 | 0.008 | 0.012 | 0.012 | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 BZ-23-007_18S | 0.012 | 0.020 | 0.020 | 0.008 | 0.012 | 0.014 | 0.016 | 0.016 | 0.014 | | | | | | | | | | | | | | | | | | | | | | | |
| 11 BZ_22_203_18S_Microcosmus | 0.026 | 0.000 | 0.000 | 0.022 | 0.020 | 0.020 | 0.024 | 0.024 | 0.022 | 0.020 | | | | | | | | | | | | | | | | | | | | | | |
| 12 BZ_22-108_16S | 0.014 | 0.022 | 0.022 | 0.010 | 0.016 | 0.018 | 0.018 | 0.018 | 0.018 | 0.006 | 0.022 | | | | | | | | | | | | | | | | | | | | | |
| 12 BZ_22_131_18S_Herdmania_pallida | 0.014 | 0.022 | 0.022 | 0.010 | 0.016 | 0.018 | 0.018 | 0.018 | 0.018 | 0.006 | 0.022 | 0.000 | | | | | | | | | | | | | | | | | | | | |
| 13 BZ_22_110_18S_Herdmania | 0.014 | 0.022 | 0.022 | 0.010 | 0.016 | 0.018 | 0.018 | 0.018 | 0.018 | 0.006 | 0.022 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | |
| 14 BZ_22_093_18S_Microcosmus | 0.026 | 0.000 | 0.000 | 0.022 | 0.020 | 0.020 | 0.024 | 0.024 | 0.022 | 0.020 | 0.000 | 0.022 | 0.022 | 0.022 | | | | | | | | | | | | | | | | | | |
| 15 BZ_22_070_18S_Stolidobranch_1 | 0.026 | 0.000 | 0.000 | 0.022 | 0.020 | 0.020 | 0.024 | 0.024 | 0.022 | 0.020 | 0.000 | 0.022 | 0.022 | 0.022 | 0.000 | | | | | | | | | | | | | | | | | |
| 16 BZ_23_125 | 0.026 | 0.000 | 0.000 | 0.022 | 0.020 | 0.020 | 0.024 | 0.024 | 0.022 | 0.020 | 0.000 | 0.022 | 0.022 | 0.022 | 0.000 | 0.000 | | | | | | | | | | | | | | | | |
| 17 BZ_23_118 | 0.000 | 0.026 | 0.026 | 0.004 | 0.014 | 0.016 | 0.012 | 0.012 | 0.016 | 0.012 | 0.026 | 0.014 | 0.014 | 0.014 | 0.014 | 0.026 | 0.026 | | | | | | | | | | | | | | | |
| 18 FM244851.1_Halocynthia_spinosa | 0.014 | 0.018 | 0.018 | 0.010 | 0.006 | 0.008 | 0.010 | 0.010 | 0.008 | 0.012 | 0.018 | 0.014 | 0.014 | 0.014 | 0.018 | 0.018 | 0.018 | 0.014 | | | | | | | | | | | | | | |
| 19 AJ250773.1_Herdmania_mirabilis | 0.014 | 0.012 | 0.012 | 0.010 | 0.006 | 0.006 | 0.010 | 0.010 | 0.008 | 0.008 | 0.012 | 0.010 | 0.010 | 0.010 | 0.012 | 0.012 | 0.012 | 0.014 | 0.004 | | | | | | | | | | | | | |
| 20 KY807049.1_Herdmania_momus | 0.010 | 0.018 | 0.018 | 0.006 | 0.012 | 0.014 | 0.014 | 0.014 | 0.014 | 0.006 | 0.018 | 0.004 | 0.004 | 0.004 | 0.018 | 0.018 | 0.018 | 0.010 | 0.010 | 0.006 | | | | | | | | | | | | |
| 21 AF165827.1_Herdmania_momus | 0.022 | 0.032 | 0.032 | 0.018 | 0.020 | 0.026 | 0.022 | 0.022 | 0.022 | 0.012 | 0.032 | 0.010 | 0.010 | 0.010 | 0.032 | 0.032 | 0.032 | 0.022 | 0.024 | 0.020 | 0.014 | | | | | | | | | | | |
| 22 FM897329.1_Herdmania_sp. | 0.012 | 0.020 | 0.020 | 0.008 | 0.014 | 0.016 | 0.016 | 0.016 | 0.016 | 0.008 | 0.020 | 0.002 | 0.002 | 0.002 | 0.020 | 0.020 | 0.020 | 0.012 | 0.012 | 0.008 | 0.002 | 0.012 | | | | | | | | | | |
| 23 FM897330.1_Herdmania_sp. | 0.014 | 0.022 | 0.022 | 0.010 | 0.016 | 0.018 | 0.018 | 0.018 | 0.018 | 0.006 | 0.022 | 0.000 | 0.000 | 0.000 | 0.022 | 0.022 | 0.022 | 0.014 | 0.014 | 0.010 | 0.004 | 0.010 | 0.002 | | | | | | | | | |
| 24 FM244852.1_Herdmania_sp. | 0.012 | 0.020 | 0.020 | 0.008 | 0.014 | 0.016 | 0.016 | 0.016 | 0.016 | 0.008 | 0.020 | 0.002 | 0.002 | 0.002 | 0.020 | 0.020 | 0.020 | 0.012 | 0.012 | 0.008 | 0.002 | 0.012 | 0.000 | 0.002 | | | | | | | | |
| 25 LC547315.1_Herdmania_sp. | 0.010 | 0.018 | 0.018 | 0.006 | 0.012 | 0.014 | 0.014 | 0.014 | 0.014 | 0.006 | 0.018 | 0.004 | 0.004 | 0.004 | 0.018 | 0.018 | 0.018 | 0.010 | 0.010 | 0.006 | 0.000 | 0.014 | 0.002 | 0.004 | 0.002 | | | | | | | |
| 26 KT387603.1_Microcosmus_exasperatus | 0.026 | 0.000 | 0.000 | 0.022 | 0.020 | 0.020 | 0.024 | 0.024 | 0.022 | 0.020 | 0.000 | 0.022 | 0.022 | 0.022 | 0.000 | 0.000 | 0.000 | 0.026 | 0.018 | 0.012 | 0.018 | 0.032 | 0.020 | 0.022 | 0.020 | 0.018 | | | | | | |
| 27 KT387604.1_Microcosmus_exasperatus | 0.026 | 0.000 | 0.000 | 0.022 | 0.020 | 0.020 | 0.024 | 0.024 | 0.022 | 0.020 | 0.000 | 0.022 | 0.022 | 0.022 | 0.000 | 0.000 | 0.000 | 0.026 | 0.018 | 0.012 | 0.018 | 0.032 | 0.020 | 0.022 | 0.020 | 0.018 | 0.000 | | | | | |
| 28 FM244856.1_Pyura_dura | 0.010 | 0.018 | 0.018 | 0.006 | 0.008 | 0.010 | 0.010 | 0.010 | 0.010 | 0.006 | 0.018 | 0.010 | 0.010 | 0.010 | 0.018 | 0.018 | 0.018 | 0.010 | 0.008 | 0.004 | 0.006 | 0.018 | 0.008 | 0.010 | 0.008 | 0.006 | 0.018 | 0.018 | | | | |
| 29 FM897337.1_Pyura_dura | 0.010 | 0.018 | 0.018 | 0.006 | 0.008 | 0.010 | 0.010 | 0.010 | 0.010 | 0.006 | 0.018 | 0.010 | 0.010 | 0.010 | 0.018 | 0.018 | 0.018 | 0.010 | 0.008 | 0.004 | 0.006 | 0.018 | 0.008 | 0.010 | 0.008 | 0.006 | 0.018 | 0.018 | 0.000 | | | |
| 30 FM244857.1_Pyura_gangelion | 0.012 | 0.024 | 0.024 | 0.008 | 0.010 | 0.012 | 0.000 | 0.000 | 0.012 | 0.016 | 0.024 | 0.018 | 0.018 | 0.018 | 0.024 | 0.024 | 0.024 | 0.012 | 0.010 | 0.010 | 0.014 | 0.022 | 0.016 | 0.018 | 0.016 | 0.014 | 0.024 | 0.024 | 0.010 | 0.010 | | |
| 31 AJ250772.1_Pyura_vittata | 0.000 | 0.024 | 0.024 | 0.004 | 0.014 | 0.014 | 0.012 | 0.012 | 0.016 | 0.012 | 0.024 | 0.014 | 0.014 | 0.014 | 0.024 | 0.024 | 0.024 | 0.000 | 0.014 | 0.014 | 0.010 | 0.022 | 0.012 | 0.014 | 0.012 | 0.010 | 0.024 | 0.024 | 0.010 | 0.010 | 0.012 | |

Table S7: Genetic Distance of Styelidae (Solitary) CO1

| Solitary Styelidae COI | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 OQ323204.1_Styela_plicata | | | | | | | | | | | | | | | | | | | |
| 2 OQ323194.1_Styela_plicata | 0.000 | | | | | | | | | | | | | | | | | | |
| 3 OQ322828.1_Styela_plicata | 0.000 | 0.000 | | | | | | | | | | | | | | | | | |
| 4 MT637949.1_Polycarpa_spongiabilis | 0.302 | 0.302 | 0.302 | | | | | | | | | | | | | | | | |
| 5 MH258879.1_Polycarpa_spongiabilis | 0.366 | 0.366 | 0.366 | 0.246 | | | | | | | | | | | | | | | |
| 6 MH258878.1_Polycarpa_spongiabilis | 0.356 | 0.356 | 0.356 | 0.229 | 0.015 | | | | | | | | | | | | | | |
| 7 OQ211499.1_Botrylloides_niger | 0.393 | 0.393 | 0.393 | 0.316 | 0.262 | 0.261 | | | | | | | | | | | | | |
| 8 BZ-23-118-mtCOI_1 | 0.407 | 0.407 | 0.407 | 0.351 | 0.348 | 0.338 | 0.342 | | | | | | | | | | | | |
| 9 BZ-23-275-mtCOI_1 | 0.374 | 0.374 | 0.374 | 0.370 | 0.303 | 0.310 | 0.343 | 0.266 | | | | | | | | | | | |
| 10 BZ-23-119-mtCOI_1 | 0.397 | 0.397 | 0.397 | 0.381 | 0.325 | 0.342 | 0.336 | 0.272 | 0.262 | | | | | | | | | | |
| 11 BZ-23-277-mtCOI_1 | 0.309 | 0.309 | 0.309 | 0.390 | 0.334 | 0.334 | 0.332 | 0.375 | 0.313 | 0.383 | | | | | | | | | |
| 12 BZ_22-196_Gissi | 0.311 | 0.311 | 0.311 | 0.406 | 0.332 | 0.332 | 0.327 | 0.381 | 0.297 | 0.391 | 0.046 | | | | | | | | |
| 13 BZ_22-213_Gissi | 0.329 | 0.329 | 0.329 | 0.090 | 0.250 | 0.239 | 0.309 | 0.363 | 0.354 | 0.349 | 0.374 | 0.370 | | | | | | | |
| 14 BZ-23-303-mtCOI_1 | 0.345 | 0.345 | 0.345 | 0.100 | 0.254 | 0.243 | 0.316 | 0.370 | 0.355 | 0.353 | 0.391 | 0.374 | 0.010 | | | | | | |
| 15 BZ-23-288-mtCOI | 0.336 | 0.336 | 0.336 | 0.278 | 0.264 | 0.272 | 0.257 | 0.329 | 0.290 | 0.282 | 0.334 | 0.314 | 0.262 | 0.272 | | | | | |
| 16 BZ-23-145-mtCOI | 0.340 | 0.340 | 0.340 | 0.267 | 0.264 | 0.272 | 0.253 | 0.333 | 0.287 | 0.286 | 0.330 | 0.310 | 0.251 | 0.261 | 0.006 | | | | |
| 17 BZ-23-091-mtCOI_1 | 0.381 | 0.381 | 0.381 | 0.243 | 0.051 | 0.053 | 0.242 | 0.331 | 0.310 | 0.323 | 0.340 | 0.342 | 0.238 | 0.241 | 0.259 | 0.259 | | | |
| 18 BZ-23-163-mtCOI_2 | 0.385 | 0.385 | 0.385 | 0.240 | 0.053 | 0.055 | 0.249 | 0.339 | 0.318 | 0.330 | 0.348 | 0.350 | 0.234 | 0.238 | 0.266 | 0.266 | 0.006 | | |
| 19 BZ-23-169-mtCOI | 0.381 | 0.381 | 0.381 | 0.237 | 0.051 | 0.053 | 0.249 | 0.339 | 0.314 | 0.330 | 0.345 | 0.346 | 0.237 | 0.241 | 0.262 | 0.262 | 0.004 | 0.002 | |

Table S8: Genetic Distance of Styelidae (Colonial) CO1

| Colonial Styelidae COI | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 LS992552.1_Botrylloides_sp. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 LS992550.1_Botrylloides_sp. | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 OQ211501.1_Botrylloides_niger | 0.231 | 0.231 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 OQ211500.1_Botrylloides_niger | 0.238 | 0.238 | 0.022 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 OQ211499.1_Botrylloides_niger | 0.235 | 0.235 | 0.011 | 0.016 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 MW278779.1_Botrylloides_nigrum | 0.231 | 0.231 | 0.009 | 0.013 | 0.002 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 7 MH367290.1_Botrylloides_nigrum | 0.235 | 0.235 | 0.009 | 0.014 | 0.002 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 8 LR743465.1_Botryllus_sp. | 0.255 | 0.255 | 0.199 | 0.205 | 0.194 | 0.193 | 0.199 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 9 LR743464.1_Botryllus_sp. | 0.258 | 0.258 | 0.202 | 0.208 | 0.197 | 0.196 | 0.203 | 0.002 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 LR743463.1_Botryllus_sp. | 0.259 | 0.259 | 0.205 | 0.212 | 0.200 | 0.199 | 0.206 | 0.004 | 0.002 | | | | | | | | | | | | | | | | | | | | | | | | |
| 11 OQ323341.1_Botryllus_schlosseri | 0.244 | 0.244 | 0.200 | 0.195 | 0.193 | 0.189 | 0.193 | 0.160 | 0.163 | 0.163 | | | | | | | | | | | | | | | | | | | | | | | |
| 12 AY600987.1_Botryllus_schlosseri | 0.237 | 0.237 | 0.193 | 0.188 | 0.186 | 0.182 | 0.185 | 0.163 | 0.166 | 0.166 | 0.034 | | | | | | | | | | | | | | | | | | | | | | |
| 13 JN248377.1_Botryllus_schlosseri | 0.240 | 0.240 | 0.196 | 0.191 | 0.189 | 0.185 | 0.188 | 0.166 | 0.169 | 0.169 | 0.036 | 0.002 | | | | | | | | | | | | | | | | | | | | | |
| 14 OM912790.1_Symplegma_brakenhielmi | 0.201 | 0.201 | 0.237 | 0.246 | 0.241 | 0.237 | 0.245 | 0.233 | 0.236 | 0.237 | 0.210 | 0.212 | 0.215 | | | | | | | | | | | | | | | | | | | | |
| 15 OM912789.1_Symplegma_brakenhielmi | 0.201 | 0.201 | 0.237 | 0.246 | 0.241 | 0.237 | 0.245 | 0.233 | 0.236 | 0.237 | 0.210 | 0.212 | 0.215 | 0.000 | | | | | | | | | | | | | | | | | | | |
| 16 OM912788.1_Symplegma_brakenhielmi | 0.201 | 0.201 | 0.237 | 0.246 | 0.241 | 0.237 | 0.245 | 0.233 | 0.236 | 0.237 | 0.210 | 0.212 | 0.215 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | |
| 17 ON076054.1_Symplegma_reptans | 0.341 | 0.341 | 0.306 | 0.321 | 0.308 | 0.306 | 0.318 | 0.267 | 0.271 | 0.275 | 0.343 | 0.333 | 0.336 | 0.280 | 0.280 | 0.280 | | | | | | | | | | | | | | | | | |
| 18 ON076053.1_Symplegma_reptans | 0.341 | 0.341 | 0.306 | 0.321 | 0.308 | 0.306 | 0.318 | 0.267 | 0.271 | 0.275 | 0.343 | 0.333 | 0.336 | 0.280 | 0.280 | 0.280 | 0.000 | | | | | | | | | | | | | | | | |
| 19 OM816672.1_Symplegma_reptans | 0.334 | 0.334 | 0.287 | 0.300 | 0.288 | 0.287 | 0.294 | 0.262 | 0.266 | 0.270 | 0.323 | 0.306 | 0.309 | 0.288 | 0.288 | 0.288 | 0.022 | 0.022 | | | | | | | | | | | | | | | |
| 20 OQ322828.1_Styela_plicata | 0.301 | 0.301 | 0.355 | 0.359 | 0.350 | 0.348 | 0.360 | 0.296 | 0.300 | 0.295 | 0.307 | 0.329 | 0.332 | 0.315 | 0.315 | 0.315 | 0.390 | 0.390 | 0.382 | | | | | | | | | | | | | | |
| 21 25Jul22_1_3_BZ-22_094-096_UNCW | 0.246 | 0.246 | 0.262 | 0.262 | 0.254 | 0.256 | 0.266 | 0.210 | 0.213 | 0.217 | 0.231 | 0.215 | 0.218 | 0.163 | 0.163 | 0.163 | 0.279 | 0.279 | 0.281 | 0.323 | | | | | | | | | | | | | |
| 22 25J22_1_9_BZ-22-111_UNCW | 0.237 | 0.237 | 0.274 | 0.285 | 0.279 | 0.275 | 0.280 | 0.217 | 0.220 | 0.217 | 0.264 | 0.248 | 0.251 | 0.139 | 0.139 | 0.139 | 0.277 | 0.277 | 0.282 | 0.326 | 0.142 | | | | | | | | | | | | |
| 23 BZ-23-089-mtCOI | 0.219 | 0.219 | 0.187 | 0.193 | 0.190 | 0.187 | 0.196 | 0.223 | 0.226 | 0.230 | 0.216 | 0.199 | 0.202 | 0.223 | 0.223 | 0.223 | 0.297 | 0.297 | 0.286 | 0.302 | 0.193 | 0.262 | | | | | | | | | | | |
| 24 BZ-22-214_mtCOI | 0.231 | 0.231 | 0.009 | 0.013 | 0.002 | 0.000 | 0.000 | 0.193 | 0.196 | 0.199 | 0.189 | 0.182 | 0.185 | 0.237 | 0.237 | 0.237 | 0.306 | 0.306 | 0.287 | 0.348 | 0.256 | 0.275 | 0.187 | | | | | | | | | | |
| 25 BZ-22-194_mtCOI | 0.228 | 0.228 | 0.002 | 0.020 | 0.009 | 0.007 | 0.007 | 0.195 | 0.199 | 0.202 | 0.197 | 0.190 | 0.193 | 0.234 | 0.234 | 0.234 | 0.306 | 0.306 | 0.287 | 0.351 | 0.258 | 0.278 | 0.184 | 0.007 | | | | | | | | | |
| 26 BZ-23-155-mtCOI_1 | 0.251 | 0.251 | 0.235 | 0.248 | 0.240 | 0.241 | 0.243 | 0.229 | 0.232 | 0.229 | 0.252 | 0.233 | 0.236 | 0.257 | 0.257 | 0.257 | 0.210 | 0.210 | 0.211 | 0.346 | 0.254 | 0.262 | 0.248 | 0.241 | 0.239 | | | | | | | | |
| 27 BZ-23-293-mtCOI_1 | 0.247 | 0.247 | 0.227 | 0.242 | 0.231 | 0.233 | 0.233 | 0.243 | 0.246 | 0.250 | 0.265 | 0.239 | 0.241 | 0.262 | 0.262 | 0.262 | 0.239 | 0.239 | 0.239 | 0.231 | 0.356 | 0.260 | 0.268 | 0.256 | 0.233 | 0.224 | 0.080 | | | | | | |
| 28 BZ-23-115-mtCOI_1 | 0.223 | 0.223 | 0.205 | 0.210 | 0.202 | 0.204 | 0.207 | 0.206 | 0.210 | 0.206 | 0.224 | 0.199 | 0.202 | 0.244 | 0.244 | 0.244 | 0.222 | 0.222 | 0.214 | 0.338 | 0.245 | 0.250 | 0.227 | 0.204 | 0.201 | 0.045 | 0.051 | | | | | | |
| 29 BZ-22-011_mtCOI | 0.236 | 0.236 | 0.215 | 0.221 | 0.213 | 0.215 | 0.218 | 0.212 | 0.215 | 0.212 | 0.237 | 0.218 | 0.220 | 0.232 | 0.232 | 0.232 | 0.225 | 0.225 | 0.222 | 0.352 | 0.239 | 0.246 | 0.239 | 0.215 | 0.212 | 0.043 | 0.067 | 0.033 | | | | | |
| 30 BZ-23-113-mtCOI_1 | 0.227 | 0.227 | 0.217 | 0.223 | 0.215 | 0.217 | 0.220 | 0.212 | 0.215 | 0.212 | 0.236 | 0.211 | 0.214 | 0.237 | 0.237 | 0.237 | 0.215 | 0.215 | 0.207 | 0.335 | 0.238 | 0.246 | 0.232 | 0.217 | 0.214 | 0.036 | 0.041 | 0.009 | 0.024 | | | | |
| 31 BZ-23-196-mtCOI_1 | 0.314 | 0.314 | 0.291 | 0.299 | 0.289 | 0.284 | 0.293 | 0.272 | 0.276 | 0.280 | 0.291 | 0.282 | 0.285 | 0.313 | 0.313 | 0.313 | 0.286 | 0.286 | 0.272 | 0.376 | 0.275 | 0.293 | 0.270 | 0.284 | 0.287 | 0.202 | 0.228 | 0.196 | 0.201 | 0.189 | | | |
| 32 BZ-23-096-mtCOI | 0.228 | 0.228 | 0.002 | 0.020 | 0.009 | 0.007 | 0.007 | 0.195 | 0.199 | 0.202 | 0.197 | 0.190 | 0.193 | 0.234 | 0.234 | 0.234 | 0.306 | 0.306 | 0.287 | 0.351 | 0.258 | 0.278 | 0.184 | 0.007 | 0.000 | 0.239 | 0.224 | 0.201 | 0.212 | 0.214 | 0.287 | | |
| 33 BZ-23-274-mtCOI | 0.297 | 0.297 | 0.289 | 0.292 | 0.284 | 0.285 | 0.296 | 0.292 | 0.295 | 0.296 | 0.290 | 0.279 | 0.276 | 0.277 | 0.277 | 0.277 | 0.319 | 0.319 | 0.330 | 0.352 | 0.288 | 0.304 | 0.255 | 0.285 | 0.285 | 0.290 | 0.292 | 0.266 | 0.260 | 0.269 | 0.349 | 0.285 | |

Table S9: Genetic Distance of Styelidae 18S

| Styelidae 18S | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 BZ-23-277-18S | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 BZ-23-163-18S | 0.005 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 BZ-23-091-18S | 0.005 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 BZ-23-096-18S | 0.016 | 0.012 | 0.012 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 BZ_22_213_18S_Ascidia_12 | 0.005 | 0.000 | 0.000 | 0.012 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 BZ_22-196_18S | 0.016 | 0.012 | 0.012 | 0.000 | 0.012 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 7 BZ-22-194_18S | 0.016 | 0.012 | 0.012 | 0.000 | 0.012 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | |
| 8 BZ-22-038_18S | 0.016 | 0.012 | 0.012 | 0.000 | 0.012 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | |
| 9 BZ-22-011_18S | 0.005 | 0.000 | 0.000 | 0.012 | 0.000 | 0.012 | 0.012 | 0.012 | | | | | | | | | | | | | | | | | | | | | | |
| 10 BZ-23-089-18S | 0.021 | 0.016 | 0.016 | 0.009 | 0.016 | 0.009 | 0.009 | 0.009 | 0.016 | | | | | | | | | | | | | | | | | | | | | |
| 11 BZ-23-288-18S | 0.000 | 0.005 | 0.005 | 0.016 | 0.005 | 0.016 | 0.016 | 0.016 | 0.005 | 0.021 | | | | | | | | | | | | | | | | | | | | |
| 12 BZ_22_119_18S_Phallusia_nigra | 0.005 | 0.000 | 0.000 | 0.012 | 0.000 | 0.012 | 0.012 | 0.012 | 0.000 | 0.016 | 0.005 | | | | | | | | | | | | | | | | | | | |
| 13 BZ_23_303 | 0.005 | 0.000 | 0.000 | 0.012 | 0.000 | 0.012 | 0.012 | 0.012 | 0.000 | 0.016 | 0.005 | 0.000 | | | | | | | | | | | | | | | | | | |
| 14 BZ_23_275 | 0.007 | 0.002 | 0.002 | 0.009 | 0.002 | 0.009 | 0.009 | 0.009 | 0.002 | 0.014 | 0.007 | 0.002 | 0.002 | | | | | | | | | | | | | | | | | |
| 15 BZ_23_145 | 0.009 | 0.005 | 0.005 | 0.016 | 0.005 | 0.016 | 0.016 | 0.016 | 0.005 | 0.016 | 0.009 | 0.005 | 0.005 | 0.007 | | | | | | | | | | | | | | | | |
| 16 BZ_23_118 | 0.009 | 0.005 | 0.005 | 0.012 | 0.005 | 0.012 | 0.012 | 0.012 | 0.005 | 0.016 | 0.009 | 0.005 | 0.005 | 0.002 | 0.009 | | | | | | | | | | | | | | | |
| 17 BZ_23_113 | 0.005 | 0.000 | 0.000 | 0.012 | 0.000 | 0.012 | 0.012 | 0.012 | 0.000 | 0.016 | 0.005 | 0.000 | 0.000 | 0.002 | 0.005 | 0.005 | | | | | | | | | | | | | | |
| 18 BZ-23-196-18S | 0.012 | 0.007 | 0.007 | 0.012 | 0.007 | 0.012 | 0.012 | 0.012 | 0.007 | 0.016 | 0.012 | 0.007 | 0.007 | 0.009 | 0.012 | 0.012 | 0.007 | | | | | | | | | | | | | |
| 19 BZ-23-169-18S | 0.005 | 0.000 | 0.000 | 0.012 | 0.000 | 0.012 | 0.012 | 0.012 | 0.000 | 0.016 | 0.005 | 0.000 | 0.000 | 0.002 | 0.005 | 0.005 | 0.000 | 0.007 | | | | | | | | | | | | |
| 20 BZ-23-155-18S | 0.005 | 0.000 | 0.000 | 0.012 | 0.000 | 0.012 | 0.012 | 0.012 | 0.000 | 0.016 | 0.005 | 0.000 | 0.000 | 0.002 | 0.005 | 0.005 | 0.000 | 0.007 | 0.000 | | | | | | | | | | | |
| 21 MG800796.1_Asterocarpa_humilis | 0.031 | 0.031 | 0.031 | 0.043 | 0.031 | 0.043 | 0.043 | 0.043 | 0.031 | 0.048 | 0.031 | 0.031 | 0.031 | 0.033 | 0.036 | 0.036 | 0.031 | 0.038 | 0.031 | 0.031 | | | | | | | | | | |
| 22 OQ255573.1_Botryllioides_niger | 0.016 | 0.012 | 0.012 | 0.000 | 0.012 | 0.000 | 0.000 | 0.000 | 0.012 | 0.009 | 0.016 | 0.012 | 0.012 | 0.009 | 0.016 | 0.012 | 0.012 | 0.012 | 0.012 | 0.012 | 0.043 | | | | | | | | | |
| 23 AJ250775.1_Cnemidocarpa_clara | 0.007 | 0.002 | 0.002 | 0.014 | 0.002 | 0.014 | 0.014 | 0.014 | 0.002 | 0.019 | 0.007 | 0.002 | 0.002 | 0.005 | 0.007 | 0.007 | 0.002 | 0.009 | 0.002 | 0.002 | 0.033 | 0.014 | | | | | | | | |
| 24 LC547316.1_Polycarpa_cryptocarpa | 0.005 | 0.000 | 0.000 | 0.012 | 0.000 | 0.012 | 0.012 | 0.012 | 0.000 | 0.016 | 0.005 | 0.000 | 0.000 | 0.002 | 0.005 | 0.005 | 0.000 | 0.007 | 0.000 | 0.000 | 0.031 | 0.012 | 0.002 | | | | | | | |
| 25 MG800799.1_Polycarpa_pomaria | 0.031 | 0.026 | 0.026 | 0.038 | 0.026 | 0.038 | 0.038 | 0.038 | 0.026 | 0.043 | 0.031 | 0.026 | 0.026 | 0.028 | 0.031 | 0.031 | 0.026 | 0.033 | 0.026 | 0.026 | 0.005 | 0.038 | 0.028 | 0.026 | | | | | | |
| 26 LI2441.2_Polycarpa_pomaria | 0.005 | 0.000 | 0.000 | 0.012 | 0.000 | 0.012 | 0.012 | 0.012 | 0.000 | 0.016 | 0.005 | 0.000 | 0.000 | 0.002 | 0.005 | 0.005 | 0.000 | 0.007 | 0.000 | 0.000 | 0.031 | 0.012 | 0.002 | 0.000 | 0.026 | | | | | |
| 27 MG800801.1_Stolonica_socialis | 0.005 | 0.000 | 0.000 | 0.012 | 0.000 | 0.012 | 0.012 | 0.012 | 0.000 | 0.016 | 0.005 | 0.000 | 0.000 | 0.002 | 0.005 | 0.005 | 0.000 | 0.007 | 0.000 | 0.000 | 0.031 | 0.012 | 0.002 | 0.000 | 0.026 | 0.000 | | | | |
| 28 LC432328.1_Styela_plicata | 0.000 | 0.005 | 0.005 | 0.016 | 0.005 | 0.016 | 0.016 | 0.016 | 0.005 | 0.021 | 0.000 | 0.005 | 0.005 | 0.007 | 0.009 | 0.009 | 0.005 | 0.012 | 0.005 | 0.005 | 0.031 | 0.016 | 0.007 | 0.005 | 0.031 | 0.005 | 0.005 | | | |
| 29 LC547313.1_Styela_plicata | 0.000 | 0.005 | 0.005 | 0.016 | 0.005 | 0.016 | 0.016 | 0.016 | 0.005 | 0.021 | 0.000 | 0.005 | 0.005 | 0.007 | 0.009 | 0.009 | 0.005 | 0.012 | 0.005 | 0.005 | 0.031 | 0.016 | 0.007 | 0.005 | 0.031 | 0.005 | 0.005 | 0.000 | | |
| 30 KJ818250.1_Styela_plicata | 0.000 | 0.005 | 0.005 | 0.016 | 0.005 | 0.016 | 0.016 | 0.016 | 0.005 | 0.021 | 0.000 | 0.005 | 0.005 | 0.007 | 0.009 | 0.009 | 0.005 | 0.012 | 0.005 | 0.005 | 0.031 | 0.016 | 0.007 | 0.005 | 0.031 | 0.005 | 0.005 | 0.000 | 0.000 | |