Article

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Article

New *Clavelina* (Ascidiacea) species from the Bahamas

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Abstract: The ascidian fauna of the Bahamas remains grossly understudied. Here, we examined specimens of the genus *Clavelina* collected from four Bahamian islands using morphological observations and genetic barcoding. Only 3 species of *Clavelina* have been previously reported in the Caribbean: *C. picta*, *C. oblonga*, and *C. puertosecensis*. Here, we report *C. picta* (Verrill, 1900) and three new species in the Bahamas: *C. rochae*; *C. pawliki*; and *C. erwinorum*. *C. picta* and *C. pawliki* were found in the northernmost island surveyed, while *C. rochae* and *C. erwinorum* were particularly prevalent on the southeastern Bahamian islands. A complete review of genetic barcoding data and morphological characters of accepted *Clavelina* species was performed. The unique combination of *in vivo* coloration, morphological characteristics, haplotypes, and species distribution all supported the establishment of three new species, significantly adding to the diversity of the genus *Clavelina* in the Caribbean.

Keywords: *Clavelina picta*; *Clavelina pawliki*; *Clavelina rochae*; *Clavelina erwinorum*; Clavelinid; Tunicate; Ascidian; COI; Caribbean Sea; phylogenetic analyses

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1. Introduction

The Bahamas islands are located within the Lucayan Archipelago in the Atlantic Ocean, although they are commonly considered part of the Caribbean. The archipelago comprises nearly 700 islands and cays, most of which are uninhabited (Harris et al. 2024). The islands are crossed by the Gulf Stream and Antilles Current, two major warm ocean currents (see Sealey 1994). The Gulf Stream comes from the nearby Gulf of Mexico and circulates between Florida and the northern Bahamian islands. The Antilles Current flows past the outer Bahamas after it has moved westward across the Atlantic Ocean from Africa. The Antilles current moves northward during the summer, causing higher temperatures in the Northern Bahamas (Shaklee 1996). During the winter months, the current shifts southward, bringing warmer temperatures to the southern islands (Shaklee 1996). Both currents influence the marine communities around the islands, with most of the current biota in the Bahamas being of Caribbean origin (Buchan 2000).

The ascidian (Chordata; Tunicata) fauna from the Bahamas is poorly known, with only a handful of studies reporting a few species. Wahl (1995) characterized epibionts on 15 species, seven of which were only identified at the genus level (*Didemnum*). Several years later, López-Legentil et al. (2011) described the cyanobacterial diversity in multiple specimens from three Bahamian didemnids. Sealey & Black (2023) recently listed seven ascidian species from the Exuma Cays Land and Sea Park (the first marine reserve in the Bahamas), including an 'unknown tunicate'. Besides these studies, a few others have occasionally utilized an ascidian species collected from the Bahamas to address specific research topics (e.g., Vervoort et al. 1998, chemical defenses).

The genus *Clavelina* (Aplousobranchia; Clavelinidae) currently comprises 46 species and is globally distributed (Shenkar et al. 2024). In the Caribbean, three species of *Clavelina* have been observed: *C. oblonga* Herdman, 1880, C. *puertosecensis* Millar & Goodbody, 1974, and *C. picta* (Verrill,

1990) (Van Name 1945, Millar 1962, Millar & Goodbody 1974, Goodbody 2000, 2003, Palomino-Alvarez et al. 2019). To our knowledge, only *C. picta* has been reported in the Bahamas. Svane & Young's (1989) study on the ascidian larvae included the first photograph of a colony, and the species was later reported by Wahl (1995) and Sealey & Black (2023).

Clavelina is one of the few ascidian genera where species can be identified in living animals using their external characteristics, such as zooid arrangement and color patterns (Nishikawa & Tokioka 1976; Monniot et al. 1991). After fixation, colors usually fade away, and internal zooid characteristics (often hindered by contraction and preservation) must be observed. Thus, combining *in vivo* images with detailed descriptions of zooid morphological characters is mandatory for taxonomic work in this group. More recently, DNA barcoding and free access to genetic databases have facilitated the unambiguous identification of conspicuous species by a broader range of researchers. Here, we combined field observations, morphological analyses, and DNA barcoding to identify four species of *Clavelina* from the Bahamas, three of which are new to science.

2. Materials and Methods

2.1. Samples

Samples of *Clavelina* species were photographed and collected from Sweeting's Cay (Grand Bahama), Stirrup Cay, Plana Cay, and San Salvador by SCUBA diving in 2008 and 2010 (Table 1, Figure S1) aboard R/V Seward Johnson in 2008 and the R/V Walton Smith in 2010. *In situ* pictures of *Clavelina* species were also taken in the Bahamas in 2007 and were used here to gain a broader knowledge of each species' distribution (Table 1). In 2008 and 2010, after pictures were taken *in situ*, colonies were collected and brought aboard, where one piece of each colony was preserved in absolute ethanol, and another piece was anesthetized by cold exposure in a freezer after the animal was relaxed (Turon 1987). Note that in some cases, relaxation after collection was not achieved. When water was mostly frozen, the animals were killed by adding a small volume of formaldehyde. They were then preserved in 4% formaldehyde for morphological observation and storage. Five to ten zooids per colony were extracted from the tunic and examined for morphological characters. Dissections were performed with micro-scissors and when necessary, structures were stained with Masson's haemalum to increase contrast. Samples were identified based on specialized literature (see Discussion). Voucher specimens were deposited at the Biological Collection Facility of the Center for Advanced Studies of Blanes (CEAB, CSIC).

Table 1. *Clavelina* species observed in the Bahamas. Sampling date, location, GPS position, and species name. Analyzed samples for each species are in bold.

Date	Location	GPS	*Clavelina picta*	*Clavelina pawliki*	*Clavelina erwinorum*	*Clavelina rochae*
June 15, 2007	Sweetings Cay, Grand Bahama	26°34.182'N; 77°53.342'W	X	X		
June 17, 2007	Sweetings Cay, Grand Bahama	26°34.182'N; 77°53.342'W	X	X		
June 19, 2007	Sweetings Cay, Grand Bahama	26°34.326'N; 77°53.733'W	X	X		
June 20, 2007	Stirrup Cay	25°49.600'N; 77°53.970'W	X			
June 25, 2007	Plana Cay	22°36.450'N; 73°37.566''W				X
June 27, 2007	Sweetings Cay, Grand Bahama	26°34.182'N; 77°53.342'W		X		
June 28, 2007	Sweetings Cay, Grand Bahama	26°34.182'N; 77°53.342'W		X		
May 29, 2008	Sweetings Cay, Grand Bahama	26°38.585'N; 77°57.734'W	X			
May 30, 2008	Sweetings Cay, Grand Bahama	26°38.585'N; 77°57.734'W	X	X		
June 5, 2008	San Salvador	24°03.647'N; 74°32.699'W			**X**	**X**
	San Salvador	24°04.161'N; 74°32.684'W				X
June 6, 2008	San Salvador	24°04.703'N; 74°32.797'W			**X**	
June 7, 2008	West Plana Cay	22°36.263'N; 73°37.658'W				X
	West Plana Cay	22°35.834'N; 73°37.764'W				X
June 8, 2008	Sweetings Cay, Grand Bahama	22°36.450'N; 73°33.728'W				**X**
June 10, 2008	Sweetings Cay, Grand Bahama	26°34.070'N; 77°53.206'W	**X**	X		
July 1, 2010	Sweetings Cay, Grand Bahama	26°33.559'N; 77°53.070'W	**X**	X		
July 2, 2010	Sweetings Cay, Grand Bahama	26°34.073'N; 77°53.048'W	X	X		
July 4, 2010	Sweetings Cay, Grand Bahama	26°33.693'N; 77°53.084'W	X	X		

July 5, 2010	Sweetings Cay, Grand Bahama	26°33.693'N; 77°53.084'W		X
July 8, 2010	San Salvador	24°03.515'N; 74°32.474'W	X	X
July 9, 2010	San Salvador	24°04.228'N; 74°32.683'W	X	X

2.2. DNA Extraction, Amplification, and Sequencing

Ethanol-preserved zooids were separated from the tunic under a stereomicroscope and stored in absolute ethanol at -20°C until processed. Total DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen). The primer set LCO1490 and HCO2198 (Folmer et al. 1994) was used to amplify a fragment of the Cytochrome *c* Oxidase subunit I (COI) mitochondrial gene. Total PCR volume was 25 µL, including 1 µL of each primer (10 µM), 10 nmol of each dNTP, 1x Reaction Buffer (Ecogen), 5 units of BIOTAQTM polymerase (Ecogen), and 10 to 20 µg/mL DNA. Reactions were carried in a GenAmp thermocycler (Applied Biosystems), with the following conditions: 5 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 45°C, and 1.5 min at 72°C, and a final extension time of 10 min at 72 °C. PCR products were purified and sequenced at Macrogen, Inc. All sequences have been deposited in GenBank.

2.3. Phylogenetic Analyses

Raw sequence data were processed in Geneious v.R11 (Kearse et al. 2012) and aligned using Clustal X (Thompson et al. 1997) with a gap opening penalty of 28 and a gap extension penalty of 4. All available sequences for *Clavelina* and some sequences from the closely related genus *Pycnoclavella* were retrieved from GenBank to build the phylogenetic trees. The polycitorid species *Cystodytes dellechiajei* (GenBank acc. num. AY523063) was used as an outgroup taxon. Neighbor-joining (NJ) and maximum likelihood (ML) analyses were conducted in MEGA v. 10 (Kumar et al. 2018, Stecher et al. 2020). The Tamura-Nei nucleotide substitution model was used for NJ analyses, and data were re-sampled using 10,000 bootstrap replicates (Felsenstein 1985). For ML analyses, we used the GTR+I+G (Tavaré 1986) model with substitution rates varying among sites according to an invariant and gamma distribution and 1,000 bootstrap replicates.

3. Results

3.1. Morphology and Species Descriptions

3.1.1. Notes on Morphology

We provide some notes on morphological aspects of clavelinids (applicable to other aplousobranch ascidians as well) as, in our experience, they are often misunderstood and not adequately explained or represented in specimens' descriptions. One of these aspects is the twisting of the abdomen region. In a typical clavelinid, the body is divided into a thorax that comprises the branchial sac, and an abdomen that includes the gut and the gonads. The dorsoventral orientation of the zooids (and hence the right-left axis) is determined by the thoracic body part, with the endostyle marking the ventral region and the atrial siphon the dorsal one.

The oesophagus opens near the endostyle at the narrow posterior end of the branchial sac. It then runs downward and ends in a bulbous stomach. From the stomach, the intestine runs downwards to the bottom of the abdomen, turns 180 degrees, and passes anteriorly until opening in an anus placed dorsally at the lower part of the peribranchial cavity. In this configuration, which we call the straight abdomen (Figure 1A), the stomach is ventral. However, in many zooids, the abdomen twists clockwise (as seen from the thorax). In a twisted abdomen, the stomach occupies a dorsal position (Figure 1B). It is unclear whether having a twisted or straight abdomen is a genetically fixed character in all aplousobranchs (as it seems to be in didemnids). Alternatively, the abdomen position may vary within and between species or result from contraction. We have observed twisted abdomens in well-relaxed zooids and straight abdomens in strongly contracted zooids, so we do not believe that different abdomen orientations result solely from contraction. In any case, abdomen orientation affects the distribution of all organs within it and should always be mentioned in species descriptions and figures. For instance, gonads in clavelinids lie on the right-hand side of the gut loop, but if the abdomen is twisted, the gonads lie on the left side. Likewise, the two main fiber bundles that run along the abdomen end in terminal ampullae on the left side of straight abdomens but on the right side of twisted ones.

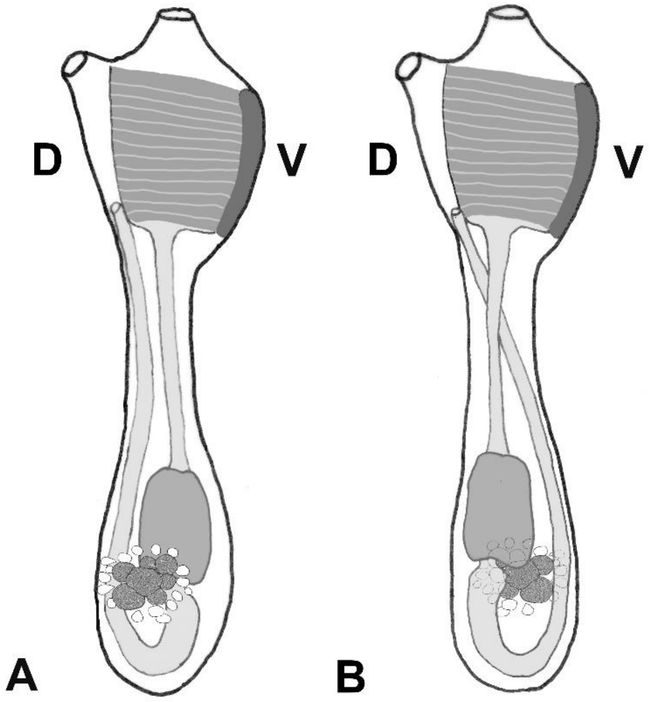

Figure 1. Schematic representation of a zooid with a straight abdomen (A) and with a twisted abdomen (B). D: dorsal side; V: ventral side. Note gonads lie on the right in A and on the left in B.

A second morphological character of taxonomic value in Clavelinids that is often missed or improperly reported is the configuration of the thoracic muscles. They comprise muscular bands (hereafter fibers) of different thicknesses depending on their position, orientation, and contraction degree. Depending on their disposition, the thorax shape is deformed differently when contracted. The most typical pattern is longitudinally oriented fibers (Figure 2A), with some or all of these fibers crossing posteriorly into the abdomen and forming two bundles ending in terminal ampullae in the posterior abdomen. Some of these fibers originate at the anterior region of the endostyle, others close to the oral siphon, and others dorsally around the neural ganglion and the atrial siphon. Formulae have been proposed to describe the origin of these muscles (Tokioka & Nishikawa 1976, Kott 1990). However, in some species, the fibers are oriented transversally (Figure 2B), usually starting at the endostyle and running towards the dorsal branchial side. Some fibers can end there or become inconspicuous, while others merge and move posteriorly close to the mid-dorsal line before entering the abdomen. Yet other species have a pattern where the more ventral fibers run obliquely from the oral siphon to the endostyle (Figure 2C), where they may become indistinct or merge in a few fibers that run posteriorly along both sides of the endostyle. In the species reported here, we have instances of all these configurations.

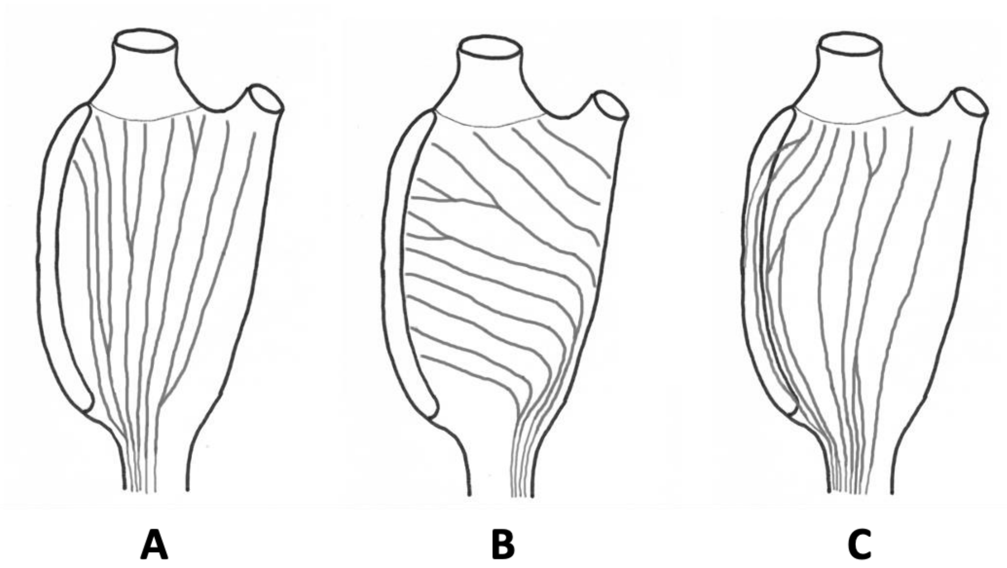

Figure 2. Schemes of the main patterns of thoracic musculature observed. (A) The commonest pattern in *Clavelina* is where fibers run posteriorly from the anterior end of the endostyle and the oral, neural, and atrial zones. (B) Transverse pattern, with fibers originating in the ventral side, running dorsally, and then passing posteriorly. (C) Ventral pattern, where the more ventral fibers run towards the endostyle and then posteriorly. Note that this figure is highly schematic. In the actual zooids, fibers anastomose, bifurcate, and merge over their length.

3.1.2. *Clavelina picta* (Verrill 1900)

References and synonymy:
Diazona picta Verrill (1902), p. 591; *Rhodozona picta* Van Name (1902), p. 335; *Clavelina picta* Berrill (1932), p. 83; *Clavelina picta* Van Name (1945), p. 138.
Zoobank accession number: To be provided upon acceptance
Collection codes: CEAB.ASC.001_A, CEAB.ASC.001_B, CEAB.ASC.001_C
GenBank accession number: PP891404

Colonies can be big, comprising tens of zooids (Figure 3A-B). They are formed by a basal tunic from which digitations with groups of 4-6 zooids emerged. The abdomens are usually embedded in a common tunic, with the thoraces free and enveloped by their own tunic. While alive, the color of the colonies is very distinctive, with an uninterrupted red-carmine ring that surrounds the oral aperture and runs down ventrally, following the endostyle (Figure 3A-B). The whitish neural ganglion is visible just outside the red band. There is a variable amount of scattered pigment of the same reddish color, particularly over the dorsal part of the thoraces, which otherwise have a whitish tinge (Figure 3B). White specks can make the oral siphon appear 8-lobed, but the actual margins of both siphons are smooth. The dorsal rim of the oral siphon is slightly elevated. A shallow dorsal infolding is visible on relaxed zooids along the length of the branchial sac. The visible part of the abdominal area is reddish. The colonies often intermingle with *Clavelina pawliki* (see below; Figure 3B).

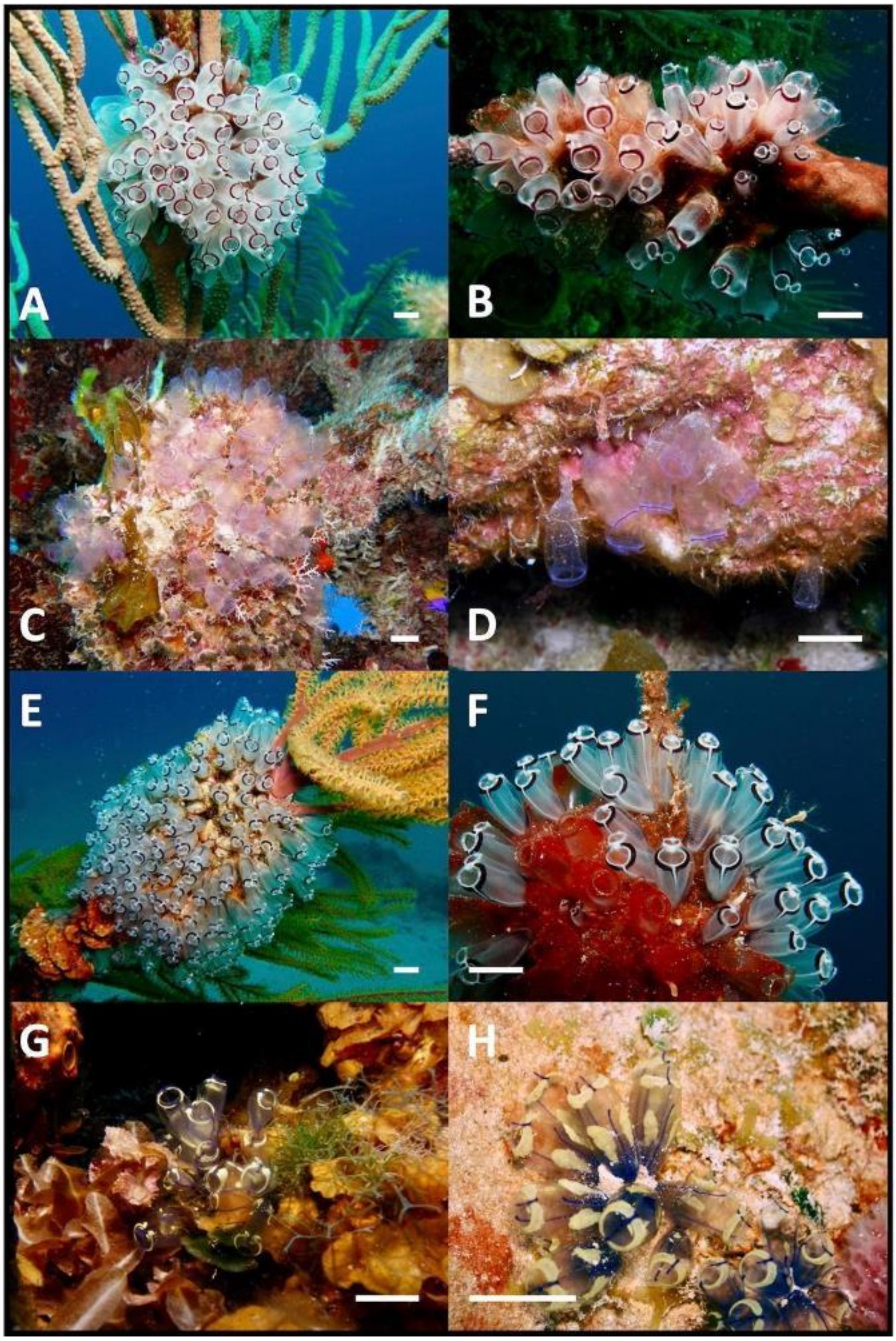

Figure 3. Images of Bahamian *Clavelina* species. (A) Colony of *C. picta* from Sweetings Cay photographed May 30, 2008. (B) *C. picta*, *C. pawliki*, and *Ecteinascidia turbinata* growing together in Sweetings Cay, photo taken June 10, 2008. (C, D) *C. rochae* from Plana Cay, photographed June 25, 2007. (E) Colony of *C. pawliki* photographed at Sweetings Cay on June 17, 2007. (F) *C. pawliki* and *E. turbinata* growing together in Sweetings Cay, June 10, 2008. (G) A colony of *C. erwinorum* from San Salvador photographed June 5, 2008; (H) *C. erwinorum* observed in Key Largo, Florida (USA). Scale bar 1 cm.

The zooids (Figure 4A) are up to 20 mm long (measured from the topmost of the tunic, as the zooids are contracted). The tunic is cartilaginous and consistent in preserved material, while a soft inner layer surrounds the zooids in some colonies. The thorax has smooth-rimmed apertures, the atrial siphon subterminal and close to the oral siphon (Figure 4B-C). The thoracic musculature (Figure 4D-G) is formed by 4-5 fibers that run from the oral siphon towards the endostyle, 4-6 fibers that run from the oral area downwards, and 3-4 fibers that run from the dorsal area downwards. These fibers can be subdivided and anastomosed. The pattern of thoracic musculature corresponds to the ventral type in Figure 2C. The fibers that run towards the endostyle do not end there but instead run posteriorly and parallel to the endostyle before entering the abdomen (Figure 4E). Thus, a right and a left bundle of fibers enter the abdomen and run posteriorly ending on the left side of the digestive loop, forming two papillae posterior to the stomach. The fibers on the right side cross ventrally to the other side (Figure 4G) and end in the papilla closer to the stomach, while the fibers on the left run down vertically and end in the papilla closer to the intestine. This is the arrangement in straight abdomens. In twisted ones, the sides are interchanged.

There are ca. 20 simple tentacles arranged in several rings, with the longer ones occupying a more posterior position. The biggest tentacle lies just above the neural gland, whose aperture is an oblique or vertical slit. There are often copepods in the branchial sac. Depending on the size of the zooid, there are 16 to 24 rows of stigmata with up to 50 stigmata per half row (Figure 4B, 4H). The transverse vessels form a lamina between rows that originates elongated papillae at the dorsal midline (Figure 4H).

Abdomens can be straight, twisted, or half-twisted. The stomach is quadrangular or elongated. Stomachs have marked ridges or only one marked fold (the typhlosole), depending on their fullness level (Figure 4B). The stomach lies some distance away from the bottom of the abdomen and is continued by a gut that loops and turns upward without distinct constrictions or divisions (Figure 4B). The anus opens in the lower part of the peribranchial cavity at the same level as the last rows of stigmata.

Gonads are found only in some of the examined zooids and lie on the right side (if the abdomen is straight). They are formed by a cluster of ova and smaller, whitish male follicles between the stomach and the lower gut loop (Figure 4C). Embryos and larvae (up to 17) are brooded on the right side of the peribranchial cavity. The mature larvae measure up to 1 mm in trunk length, have separated ocellus and statocyte in the sensory vesicle, and have an anterior ventral stalk from which three adhesive papillae emerged: two dorsal and one ventral (Figure 4I). The coiled tail was lodged between the larvae's dorsal and ventral papillae.

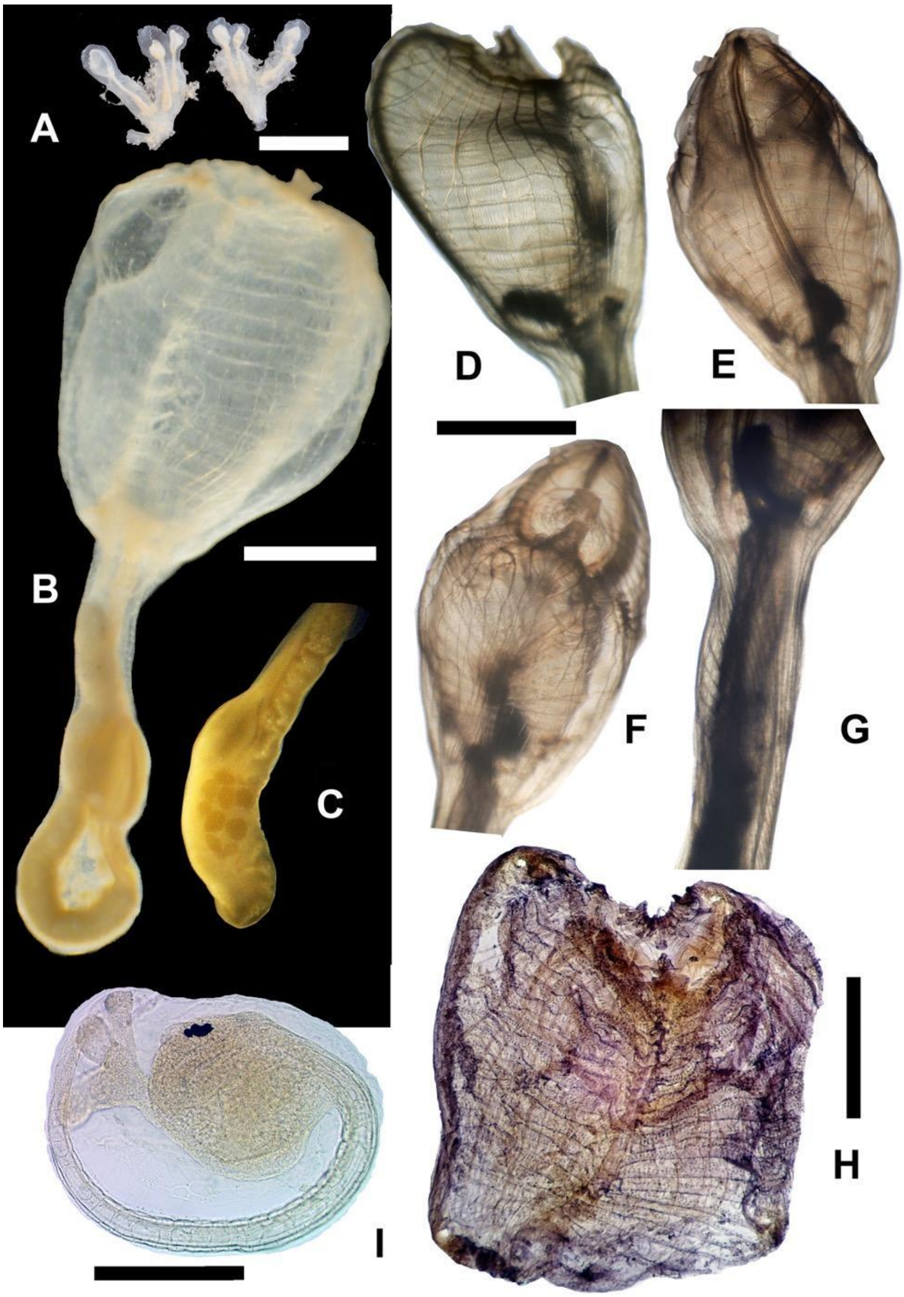

Figure 4. *Clavelina picta*. (A) Image of two zooid clumps; (B) Zooid without gonads, note straight abdomen; (C) Abdomen with gonads. (D-F) Left, ventral, and dorsal views of the thoracic musculature of a zooid. The dark body at the base of the branchial sac is a copepod. (G) Ventral view of the anterior part of the abdomen (straight) of the same zooid, showing muscles passing from right to left. (H) Dissected branchial sac (stained). (I) Larva. Scale bars: A, 15 mm; B-C, 2 mm; D-G, 2 mm; H, 2 mm; I, 0.4 mm.

3.1.3. *Clavelina rochae* sp. nov.

Zoobank accession number: https://www.zoobank.org/42CD0FE3-B7AF-4D99-A45A-5090A3B120B9

Collection codes: holotype: CEAB.ASC.009_A**; paratypes:** CEAB.ASC.009_B, CEAB.ASC.009_C, CEAB.ASC.009_D, CEAB.ASC.009_E

GenBank accession numbers: PP891405-6

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Colonies are not massive and are often formed by only a few scattered zooids (Figure 3C-D). Zooids are mostly independent, sometimes linked by stolons and sometimes with the posterior part of the abdomens embedded in a common tunic (Figure 6A). The transparent tunic has a faint purple ring around the oral siphon (Figure 3C). In some instances, the pigment continues downwards over the endostyle and the dorsal line of the branchial sac. The branchial sac has a dorsal furrow. The thorax has an electric blue tinge of variable intensity, sometimes forming a secondary band posterior to the purple ring (Figure 3D). Other times, the purple ring was indistinct, and the blue hue was the primary color of the thoraces. The oral aperture is large, its diameter close to the whole width of the zooids (Figure 3D).

Zooids (Figure 5B-D) are up to 23 mm long (measured with the tunic). The tunic is soft and sometimes has an internal softer layer that is hard to remove from the zooids. The thoracic musculature (Figure 5C) consists of oblique and vertical fibers: 2-5 going from the oral siphon towards the endostyle, 3-10 originating around the oral siphon area and running down the branchial sac, and from 0 to 3 originating between the neural gland and the atrial siphon and running downwards vertically. The muscular arrangement corresponds to the ventral type in Figure 2C. The fibers reaching the endostyle bent posteriorly and run parallel to both sides of the endostyle before entering the abdomen and ending in two terminal ampullae on the lower gut loop's left side (in straight abdomens). There are 12-14 tentacles in the oral siphon, the largest one placed over the neural gland, whose aperture forms a vertical slit. Fourteen to 19 stigmata rows are observed, with over 50 stigmata per half-row (Figure 5E). An elevated lamina between rows continues dorsally onto the wide, pointed dorsal languets.

There are both straight and twisted abdomens (Figure 5B). The stomach is quadrangular, globular, or elongated, often with conspicuous ridges and a marked typhlosole. The post-stomach has no constrictions, and the gut turns and runs anteriorly (Figure 5D). The anus opens at the posterior end of the peribranchial cavity. The gonads have a few (7-10) ova and numerous male follicles to the right of the posterior part of the gut loop (in "straight" abdomens). Embryos and larvae (up to 16) are brooded on the right-hand side of the peribranchial cavity (Figure 5C). The mature larvae are ca. 1 mm in trunk length, have separate ocellus and statocyte in the sensory vesicle, and a particular arrangement of the adhesive papillae: the two dorsal papillae arise from an anterior outgrowth, while the ventral papilla originates from a separated ventral stalk (Figure 5I). The coiled tail of the unhatched larva is lodged between the dorsal and the ventral papillae.

Etymology. The species name is dedicated to Rosana da Rocha for her numerous and key contributions to ascidian taxonomy and systematics.

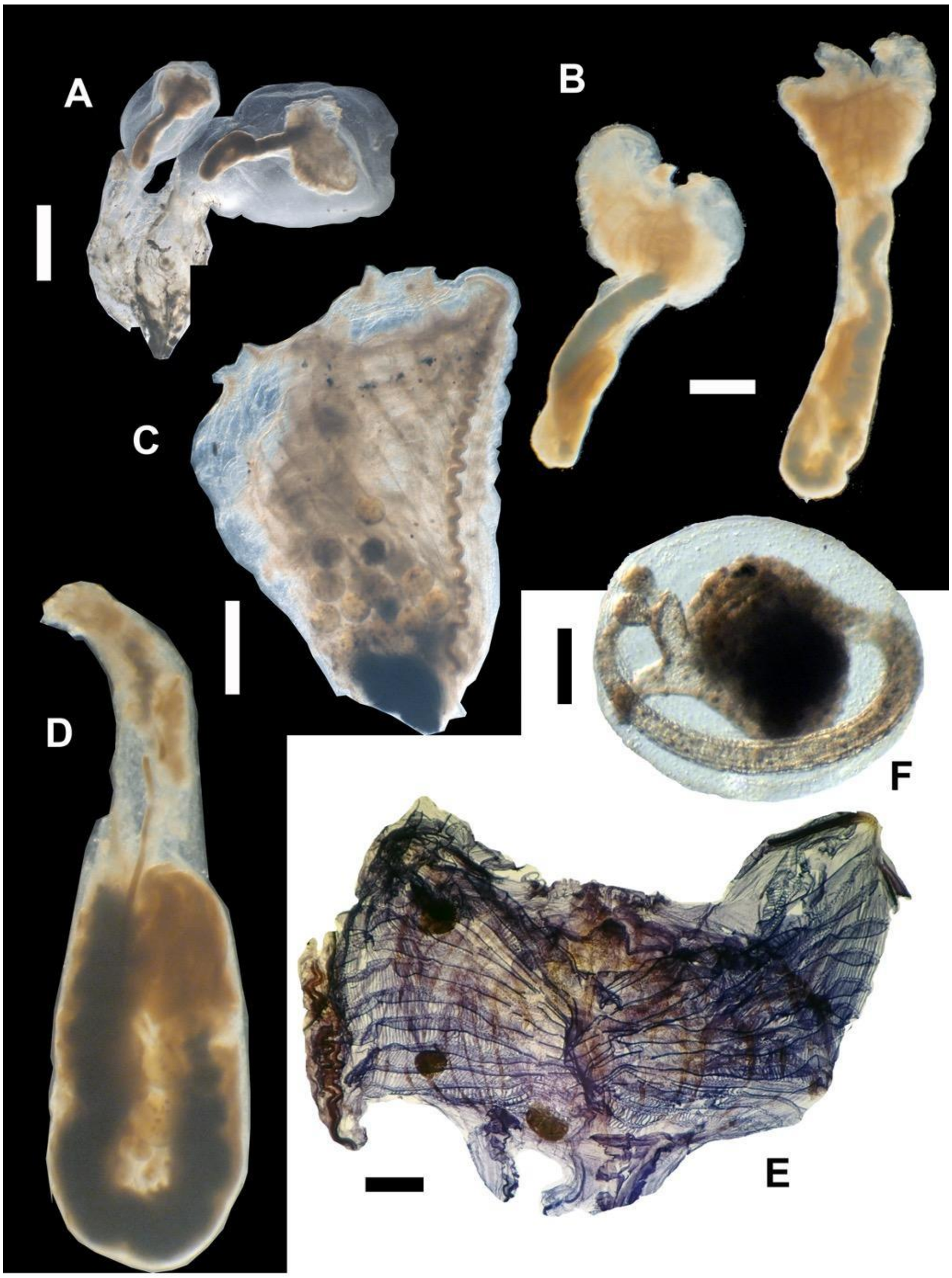

Figure 5. *Clavelina rochae*. (A) Image of two zooids with tunic. (B) Zooids extracted from the tunic. Note the twisted abdomen in the left zooid and the straight abdomen in the right zooid. (C) Branchial sac with brooded embryos. (D) Abdomen with gonads. (H) Dissected branchial sac (stained). (F) Larva. Scale bars: A, 5 mm; B, 2 mm; C-D, 1 mm; E: 1 mm; F, 0.25 mm.

3.1.4. *Clavelina pawliki* sp. nov.

Zoobank accession number: https://www.zoobank.org/CD8E1435-9ACD-438B-A9AB-B4C798EBB730
Collection codes: holotype: CEAB.ASC:004_A; paratype: CEAB.ASC.004_B
GenBank accession numbers: PP891402-3

Colonies of this species can reach tens of zooids that are mostly independent of each other and are united by stolons or a basal lamina (Figure 6A). They are slightly longer and more slender than those of *Clavelina picta*, with which they often intermingle (Figure 3B). The transparent tunic has a well-defined black or dark blue ring around the oral siphon (Figure 3E-F). This ring is interrupted dorsally at the neural ganglion and ventrally at the anterior tip of the endostyle (Figure 3F). The same dark blue or black pigment is more or less scattered over the posterior part of the thorax and may be abundant over the abdomens. Occasionally, the pigment forms a fine strip over the mid-dorsal line of the thorax or a double strip at each side of the endostyle. White pigment accumulates over the two siphonal apertures, on a line between both, and over the endostyle (Figure 3F). In relaxed zooids, a deep furrow is apparent over the dorsal midline of the branchial sac. Dark blue vesicles are scattered throughout the basal lamina and stolons.

The zooids (Figure 6B) are up to 23 mm long (measured with the tunic). The tunic is firm. The apertures are smooth. The oral siphon is somewhat elevated in its dorsal rim, while the posterior rim of the atrial siphon has a small protruding lobe, often with white pigment. The deep-blue color around the oral siphon remains a black ring in preserved animals (Figure 6B). The thoracic musculature is of the transverse type (Figure 2B), with the anterior-most 5 to 7 bands of fibers originating around the siphons and running towards the dorsal midline. followed by 14-15 bands originating at the endostyle and crossing towards the dorsal zone (Figure 6C). The endostylar side of these fibers is often bifurcated (Figure 6D). Dorsally, some fiber bands become indistinct, while others bend posteriorly, forming tracks that run parallel to both sides of the dorsal midline before entering the abdomen (Figure 6E).

Nine to 12 tentacles with a festooned rim are linked by a membrane. The aperture of the neural gland is round or oval. The branchial sac has 20-21 rows of ca. 50 stigmata (per half-row). There are elevated transverse laminae between rows, dorsally forming wide and pointed dorsal languets (Figure 6F).

The abdomens examined for this species are often straight, but instances of twisted abdomens are also seen. The internal structures are hard to observe due to the amount of pigment. The stomach is elongated and quadrangular or irregular in section, with a typhlosole and a few ridges (likely due to contraction). There are no constrictions differentiating sections in the gut loop. The anus opens at the level of the last 2-3 rows of stigmata. Some zooids have incipient or well-developed gonads on the right side of the gut loop (in straight abdomens). The gonads have a few rounded ova and many male follicles. Embryos and larvae are brooded on the posterior-right part of the peribranchial cavity (Figure 6B). When there are just a few embryos, they form a single row (likely in the enlarged end of the oviduct), and if many are present, they form an irregular clump (up to 13 embryos observed). The mature larvae are ca. 1 mm in trunk length, with an ocellus, a statocyte, and a well-marked oral siphon (Figure 6G). A peculiar feature of these larvae is a plate-like anterior outgrowth joined to the trunk by a ventral stalk. The three papillae, two dorsal and one ventral, arise from this plate. Patches of white (in preserved material) pigment occur on the larval trunk, anterior process, and tail.

Etymology. The species is named in recognition of Joseph R. Pawlik, who coordinated and secured funding for the three expeditions to the Bahamas that originated this study, and his work on ascidian chemical ecology.

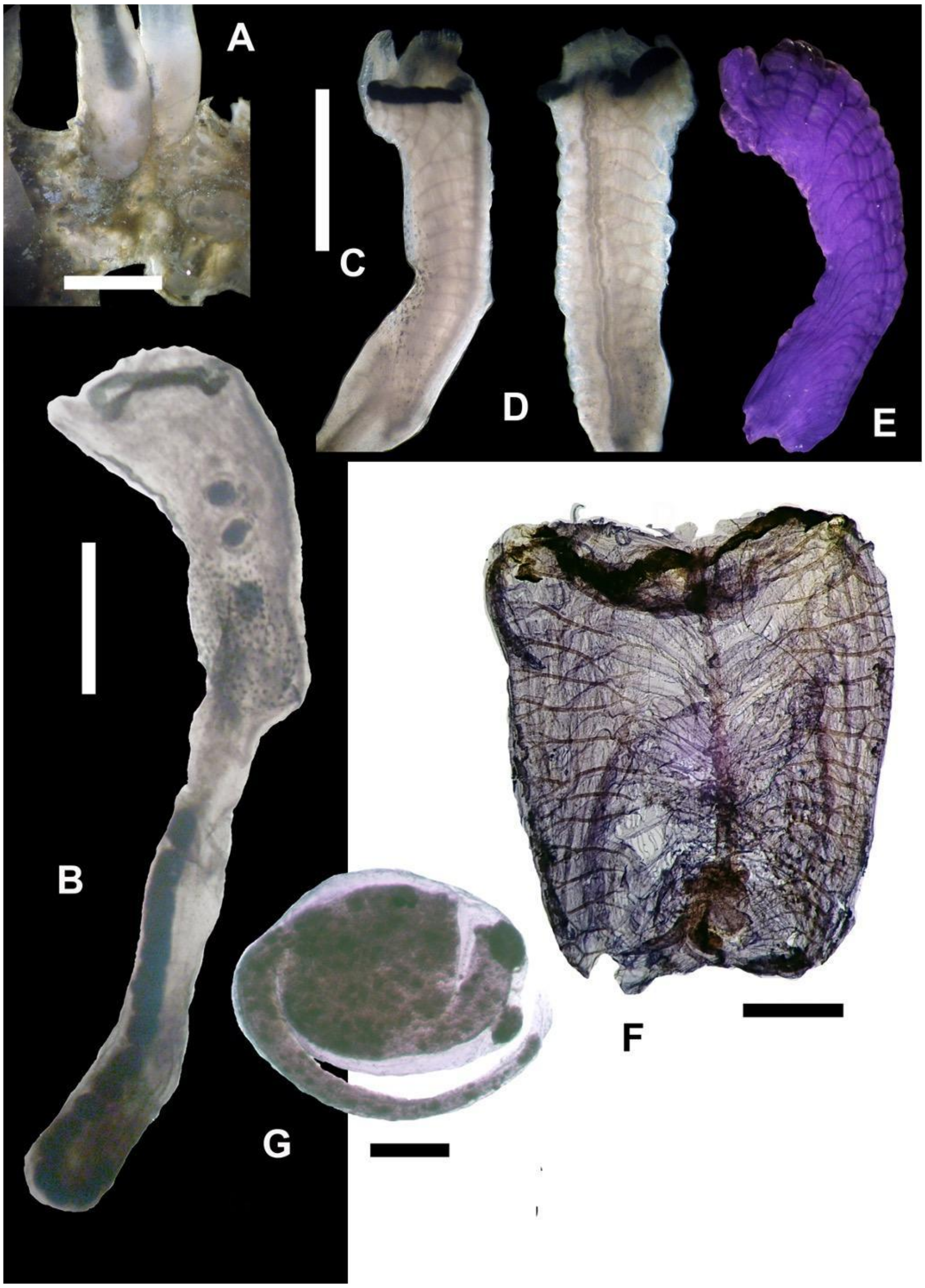

Figure 6. *Clavelina pawliki*. (A) Image of the basal part of two abdomens united to the basal lamina. (B) Zooid, note straight abdomen and some embryos brooded. (C-E) Thoracic musculature from right (C), ventral (D) and dorsolateral (E) views. Image in (E) stained to appreciate the finer bands. (F) Dissected branchial sac (stained). (G) Larva (stained). Note that the tail separated from the body is an artifact of manipulation. Scale bars: A, 2 mm; B, 2 mm; C_R, 2 mm; F, 1 mm; G, 0.3 mm.

3.1.5. *Clavelina erwinorum* sp. nov.

Zoobank accession number: https://zoobank.org/88FBC552-FA0B-4FB1-9271-1FC5DD2DE4A9
Collection codes: holotype: CEAB.ASC.006_A; **paratypes:** CEAB.ASC.006_B, CEAB.ASC.006_C
GenBank accession numbers: PP891398-401

Colonies comprise mostly independent zooids (up to 20), with only the posterior part of the abdomen embedded in a common basal tunic (Figure 7A). The zooid coloration is distinctive (Figure 3G-H). Over an otherwise transparent tunic, there is a yellow ring of pigment around the oral siphon. The ring varies in thickness depending on the colony and is interrupted dorsally (at the neural ganglion area) and ventrally (at the tip of the endostyle). The ring is thickened dorsally, even featuring, in some cases, a second partial ring in this area. Blue lines run down the endostyle and the mid-dorsal line of the thorax in all zooids. Another blue strip goes from the oral to the atrial siphon, enclosing the neural gland. This coloration pattern is complemented with specks of yellow pigment at both sides of the dorsal area of the thorax, sometimes inconspicuous (Figure 3G) but coalescing into two thick parallel bands in some colonies (Figure 3H). Some colonies of the species were previously observed and photographed by SLL in Key Largo, Florida (USA) on November 14, 2006 (Figure 3H).

Zooids measure up to 14 mm in length (Figure 7B). The tunic is soft but consistent. The thoracic muscles are of the longitudinal type (Figure 2A), 3 to 6 originating at the endostyle, 3 to 6 at the oral siphon area, and 2 to 5 originating around the neural ganglion or the atrial siphon (Figures 7C-D). The oral siphon has 15-20 tentacles of different sizes. The aperture of the neural gland is a vertical slit or an oblique oval. There are 16-18 rows of stigmata, with up to 60 stigmata per half-row (Figure 7E).

Most zooids observed have twisted or half-twisted abdomens. A very short post-stomach gives rise to a gut that runs downwards before turning upwards. No clear divisions can be seen in the gut. The anus ends in the peribranchial cavity at the level of the last rows of stigmata. No developed gonads are present, but in some zooids, a sperm duct and incipient testicular lobes are observed on the gut loop's left side (in twisted abdomens). One zooid has 6 embryos incubated in the posterior right side of the branchial sac. Only one of them has developed into a larva (Figure 7F), albeit it is probably not fully developed. The sensory vesicle cannot be observed, and the papillae arise from two ectodermal outgrowths, one ventral and one dorsal (the latter originating the two dorsal papillae). The larval tail runs between the ventral and dorsal papillae.

Etymology. The species' name is in homage to Patrick M. Erwin for his pioneering research on the ascidian microbiome and Elenor Lopez Erwin for the countless hours spent listening to her parents talk about sea squirts and bacteria.

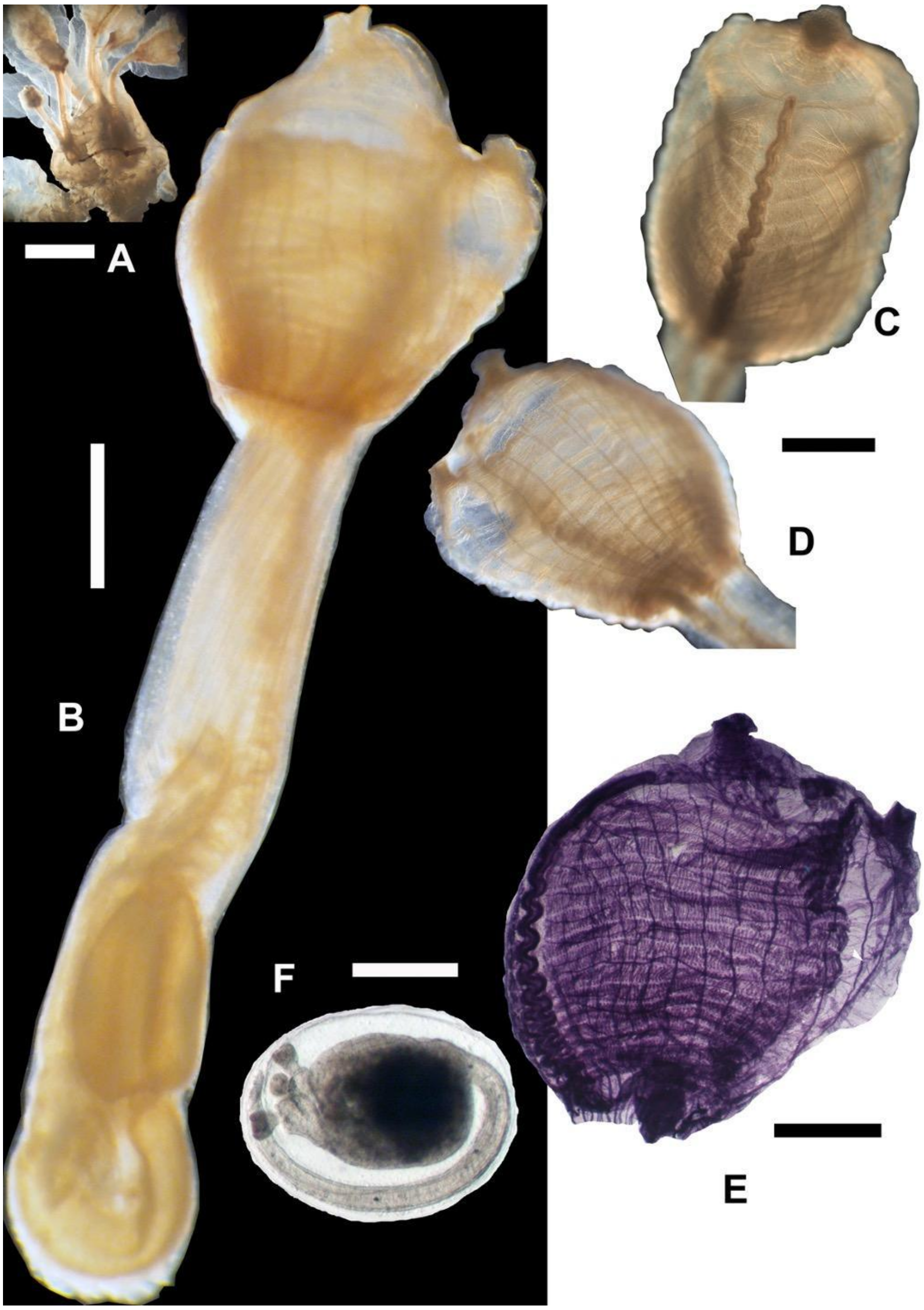

Figure 7. *Clavelina erwinorum.* (A) Image of a clump of zooids basally embedded in a common tunic. (B) Zooid, note the twisted abdomen. (C-D) Thoracic musculature from ventral (C), and laterodorsal (D) views. (E) Branchial sac cut open, the right side is shown (stained). (F) Larva. Scale bars: A, 5 mm; B, 1 mm; C-D, 1 mm; E: 1 mm; F, 0.4 mm.

3.2. Phylogenetic Analyses

Partial COI sequences with a final alignment length of 591 bp were obtained for all four species: one for *Clavelina picta*, two for *C. pawliki*, four for *C. erwinorum*, and two for *C. rochae* (accession numbers PP891398-PP891406). The sequence obtained for *C. picta* formed a well-supported clade with a specimen of the same species collected in Cuba (bootstrap values >99%; Figure 7). *C. picta* was the sister group to a clade formed by the two *C. rochae* sequences (bootstrap values = 100%), and both *C. picta* and *C. rochae* formed a well-supported clade together (bootstrap values = 96%). The two sequences of *C. pawliki* were nearly identical (one-point mutation between them) and formed a strongly supported clade that was not closely related to any other clade. Similarly, the clade formed by all sequences obtained for *C. erwinorum* was not clearly associated with any other *Clavelina* species.

The four sequences of *C. erwinorum* were slightly different, with a % identity ranging from 96.1 to 97.5%. Phylogenetic analyses thus matched morphological observations and supported the taxonomic assignments made.

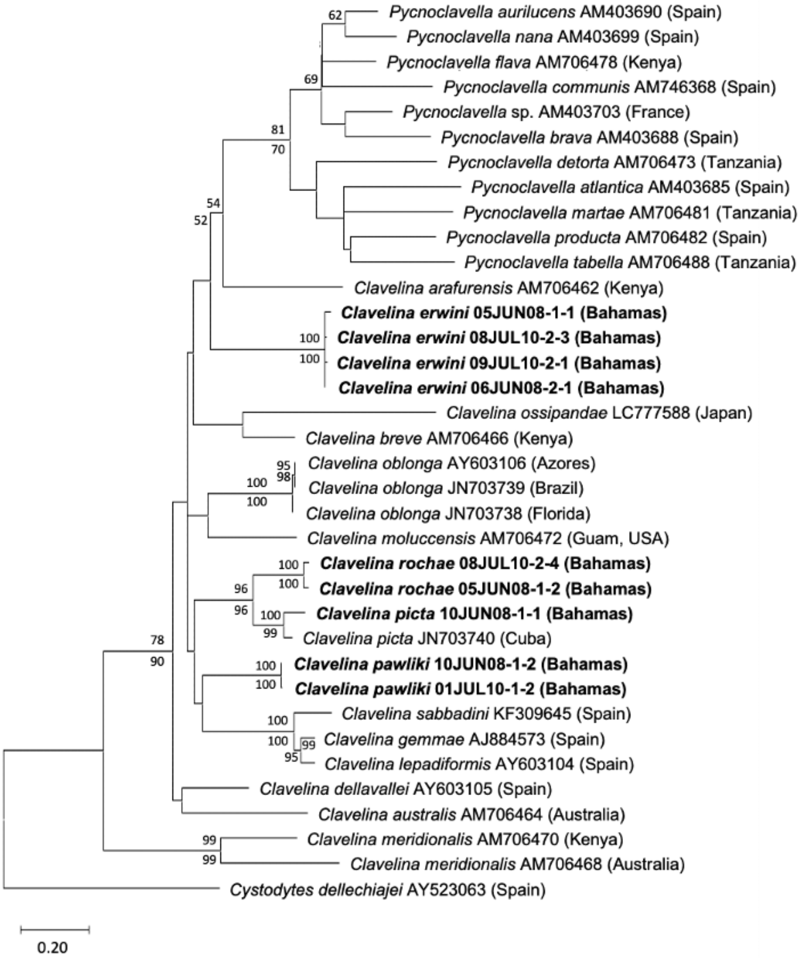

Figure 8. Phylogeny of partial Cytochrome Oxidase I gene sequences from Clavelinidae species. The phylogenetic position of species from this study is highlighted (bold lettering). Labels on terminal nodes of sequences indicate the ascidian species and GenBank accession numbers or sampling code. Collection countries are indicated in parentheses. The tree topology was obtained from maximum likelihood (ML) analysis. Individual bootstrap values from neighbor-joining (NJ) are under the tree nodes and, for ML analyses, above tree nodes when support values are greater than 50%. Scale bar represents number of substitutions per site.

4. Discussion

The four species reported here can be easily distinguished in living colonies because of their distinctive color patterns. While the amount of pigment present varies within species, the overall patterns are constant. In addition, these morphotypes correlate well with groups defined by sequence data of the COI gene. The clusters found set these species apart from all previously barcoded *Clavelina* species. Colony structures ranged from completely embedded abdomens to species with free or almost free zooids, united only by a basal lamina or stolons, in this order: *C. picta* -*C. erwinorum* -*C. pawliki* -*C. rochae*. Note that this character can vary across colonies and even within the same colony, so several specimens must be observed.

In the Caribbean at large (including Bermudas), three species have been found: *Clavelina oblonga, C. picta,* and *C. puertosecensis* (Van Name 1945, Millar & Goodbody 1974, Monniot 1983, Goodbody, 1984, 2000). A fourth species from the northern Brazilian shore has been described: *C. brasiliensis* (Millar 1977). *C. oblonga* was described from the Bermudas, is the most widely reported species in the

Western North Atlantic (Goodbody 2000), and has been introduced in many tropical and temperate areas (Rocha et al. 2012a, Ordóñez et al. 2016). The species is particularly abundant in mangrove roots, harbors, and protected shallow habitats (Monniot 1972, Goodbody 1993). Although we did not observe *C. oblonga* during our surveys, we did not visit the habitats where the species is commonly found and cannot rule out its presence in the Bahamas. *C. oblonga* forms large colonies with easily distinguishable whitish zooids, unlike any of the species reported here. *C. puertosecensis* is characterized by zooids with a uniform purple or deep blue coloration (Goodbody 2000; Rocha et al. 2012b). *C. brasiliensis* zooids are much larger (up to 7.5 cm) than the ones reported here, and the species has a distinct larval type with an anterior swelling (Millar 1977).

Only *Clavelina picta* has been previously reported in the Bahamas (Wahl 1995), and the colonies observed here conform well with previous descriptions, both externally and internally (Van Name 1902, as *Rhodozona picta*; Berrill 1945, Van Name 1945). To our knowledge, no recent description of this species has been published, and the distribution of the muscular bands in the thoraces (with the anterior ones reaching ventrally towards the endostyle and running posteriorly alongside it) has never been reported. The DNA sequence obtained here closely matched (95% identity) a sequence obtained for *C. picta* from La Havana, Cuba (Rocha et al. 2012a).

Clavelina rochae shares some morphological features with *C. picta*, such as the particular disposition of the muscular bands and the general zooid morphology. However, it differs in having colonies with sparse zooids, which are mostly free, instead of having embedded abdomens. The color pattern also differs, with electric blue hues of variable intensity in *C. rochae*. The reddish band around the oral siphon is thinner and broader in diameter than in *C. picta.* Accordingly, the zooids do not appreciably taper anteriorly but instead have a wide oral opening. The zooids of *C. rochae* are "jar-shaped", while those of *C. picta* are more "bottle-shaped". The larvae are also different, with an anterior outgrowth holding the two dorsal papillae in *C. rochae* and three papillae arising from a single anterior stalk in *C. picta* (as was described in Berrill 1932). However, larvae may look similar when not fully mature. Genetically, the new species was well differentiated from *C. picta* (87.5-87.9 % identity) but formed a well-supported clade with the latter, indicating that both species are closely related, as suggested by their similar morphology.

Clavelina pawliki is abundant in the sampled localities, often forming intermingled colonies with *C. picta* and the phlebobranch *Ecteinascidia turbinata*, so it can be easily missed. However, the color pattern with translucent thoraces and a marked dark-blue or black ring interrupted dorsally and ventrally is unlike anything described so far. The colony structure also differs from *C. picta*, with zooids mostly free with separate tunics. Internally, one distinguishing feature is the transverse orientation of the muscle bands, which in contracted zooids cause the thorax to become narrow and elongated. Morphologically, the new species seems close to *C. puertosecensis*, which has a similar pattern of thoracic musculature and colony structure (Millar & Goodbody 1974). However, the coloration is markedly different, and the larvae of *C. puertosecensis* have a wide ampullar anterior process instead of the plate-like process of *C. pawliki*. The infolding of the dorsal wall of the branchial sac in *C. puertosecensis,* highlighted as unusual by Millar & Goodbody (1974) and not reported by Monniot (1983), was present in all species examined here. The infolding was only visible *in vivo* and was lost in preserved zooids due to contraction. In the phylogenetic tree, sequences for *C. pawliki* formed a well-supported clade that was a sister group to a clade comprising Mediterranean sequences for *C. lepadiformis, C. gemmae,* and *C. sabbadini*.

Clavelina erwinorum has a unique color pattern with some degree of intraspecies variation. For instance, the yellow pigment in the posterior part of the thorax coalesced into two thick patches in colonies of this species observed from Key Largo, Florida. In contrast, the pigment was more dispersed in Bahamian specimens. However, the yellow ring surrounding the oral siphon thickened dorsally and the fainter blue bands were always present. This pigment configuration differed from other Caribbean species of *Clavelina* and elsewhere (see below). The arrangement of the muscular bands in this species follows the most typical pattern in *Clavelina* (Kott 1990), with longitudinal bands originating anteriorly and passing to the posterior end of the branchial sac before entering the abdomen. Genetically, the sequences obtained for *C. erwinorum* formed a well-supported clade that was not closely related to any other clade.

Besides *C. picta, C. puertosecensis, C. oblonga,* and *C. brasiliensis*, 42 additional *Clavelina* species are listed in the World Ascidiacea Database (Shenkar et al. 2024). The main morphological characteristics of most of these species were recently listed by Hasegawa and Kajihara (2024). We analyzed these descriptions and looked for shared features (e.g., colony structure, zooid size, number of stigmata rows) with the new species described here. Once look-alike species were identified, we carefully checked their color patterns (when available) and main zooid and larvae characteristics. Some relevant observations are listed below.

There are a few species with yellow rings in the siphonal area reminiscent of the coloration of *C. erwinorum*. Among these, *C. amplexa* Kott, 2002 (described from Darwin, Australia) has a yellow patch at each side of the anterior part of the thorax but with a triangular shape instead of *C. erwinorum*'s ring. *C. amplexa* also has a much lower number of stigmata in the branchial sac (ca. 20 per half-row). The Pacific *C. robusta* Kott, 1990 has a yellowish or fluorescent green band around the margin of the oral siphon, but it is not interrupted ventrally as seen in *C. erwinorum. C. robusta* also has a band around the atrial siphon, and the overall color of the zooids is translucent-dark or bluish (Kott 1990; Monniot & Monniot 1996, 2001). *C. robusta* zooids also have more muscular bands arising from the endostyle and fewer stigmata per half-row (20-24; Monniot & Monniot 1996). *C. cyclus* Tokioka & Nishikawa, 1975 (described from Japan) also has a yellowish band around the oral siphon, but it is not interrupted dorsally, and the color pattern is different. *C. cyclus* also has fewer stigmata per half-row in the branchial sac (up to 50; Nishikawa & Tokioka 1976, Nishikawa 1995).

A few other species of *Clavelina* have the transverse arrangement of the muscular bands seen in *C. pawliki* and *C. puertosecensis*. The morphology of the zooids in *C. coerulea* Oka, 1934 is similar to those reported here, but the color pattern is different and the larvae do not have the plate-like anterior process (Nishikawa & Tokioka 1976). *C. obesa* Nishikawa & Tokioka, 1976 has oblique (rather than transverse) muscle bands, and the colony color is different (Nishikawa & Tokioka 1976, Monniot & Monniot 1996). There are fewer muscles and fewer stigmata rows than in *C. pawliki*. The Indo-Pacific *C. arafurensis* Tokioka, 1952 differs from *C. pawliki* in having almost completely embedded zooids, different color patterns, and the presence of a post-abdomen (Monniot & Monniot 2001). Another Indo-Pacific species, *C. moluccensis* (Sluiter, 1904), has a predominantly transverse muscle arrangement but many more muscle bands and a characteristic pattern of 3 blue spots between the siphons. Unlike *C. pawliki*, *C. moluccensis* has a bulbous pre-stomach, and the intestine is divided into regions (Kott 1990, Monniot C 1997). The Australian species *C. nigra* Kott, 1990 has transverse muscle bands, but those are more numerous, the zooids are throughout dark, and the species has a different gut structure (Kott, 1990).

Finally, the muscular arrangement found in *C. picta* and *C. rochae,* with the more ventral muscles originating from the oral siphon area and extending ventrally to the endostyle, has not yet been reported in other *Clavelina* species. This particular arrangement resembles more what had been described in some Diazonidae (Kott 1990; Monniot & Monniot 2001). In conclusion, the combination of *in vivo* color patterns, morphological characters, and genetic information supports the establishment of the newly described species, thus adding to the known variability of the genus Clavelina, particularly in the Caribbean waters.

As for their distribution, *C. picta* and *C. pawliki* were observed in the northernmost surveyed location (Sweetings Cay, Grand Bahama), while *C. rochae* and *C. erwinorum* were observed on the southeastern Bahamian islands (Stirrup, West Plana, and San Salvador). The distribution of these species appeared to be determined by the short-lived nature of the ascidian larvae (Svane & Young 1989), as well as the two main currents in the region. The Gulf Stream originates in the Gulf of Mexico and flows through the strait of Florida and the northernmost Bahamian islands before continuing its course up the eastern coastline of the United States. The strength and direction of this current would prevent the larvae of *C. picta* and *C. pawliki* from reaching the southern Bahamian islands surveyed here. On the other hand, the Antilles Current flows northward east of the Antilles and joins the Gulf Stream past the outer Bahamas. This current is unlikely to reach Grand Bahama as the island is further

West and partially surrounded by Great Abaco on the East. However, although *C. erwinorum* was not observed in Sweetings Cay, the species was observed in Key Largo, Florida. Thus, some degree of connectivity among populations of the species must exist. Sampling along Central America and the Gulf of Mexico would reveal whether the Caribbean current also plays a role in the species' current distribution.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Map of the Bahamas showing the geographic location of the four surveyed islands and Cays (Sweetings, Stirrup, West Plana, and San Salvador).

Zoobank Registration: This preprint is registered in ZooBank under https://zoobank.org/References/3A916520-9058-42F5-89B0-B76D2C1DF491

Author Contributions: Conceptualization, XT and SLL; methodology, XT and SLL; formal analysis, XT and SLL; resources, XT and SLL; data curation, XT and SLL; writing and editing, XT and SLL; funding acquisition, XT and SLL All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Voucher specimens were deposited at the Center for Advanced Studies of Blanes (CEAB, CSIC) Biological Collection Facility. DNA sequences are accessible through GenBank (accession numbers PP891398-PP891406).

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Conflicts of Interest: The authors declare no conflicts of interest.

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