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How I wonder what you are: Can DNA barcoding identify the larval asteroids of Panama?

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Abstract

As part of a project to document the diversity of larval invertebrates on both coasts of Panama, we collected and photographed 141 larval asteroids and sequenced fragments of their mitochondrial *cytochrome c oxidase subunit I* (*COI*) and *16S* ribosomal DNA. We uncovered 10 Caribbean operational taxonomic units (OTUs) and five Pacific OTUs. We could identify six of the 15 OTUs based on >99% similarity with reference sequences in GenBank: The Pacific species *Astropecten verrilli* and *Pentaceraster cumingi* and the Caribbean species *Astropecten marginatus*, *Astropecten antillensis*, *Oreaster reticulatus*, and *Mithrodia clavigera*. Two other OTUs were placed in BINs in the Barcode of Life Database (BOLD) with unpublished sequences that were identified as *Pharia pyramidata* from the Pacific and *Valvaster striatus*, now known from the Caribbean as well as the Indo-West Pacific. The remaining seven species appear likely to belong to *Luidia*, as *16S* sequences from each have 87%–95% identity with various species of *Luidia*, and the sequences nest among species of *Luidia* in neighbor-joining trees. The low diversity of asteroid larvae reflects ~10% of the diversity of adult sea stars reported from Panama, and highlights the need for broader collection efforts and improved coverage of DNA barcode reference sequences for *Luidia* and other soft-bottom species.

KEYWORDS
brachiolaria, Caribbean, meroplankton, Panama

1 | INTRODUCTION

Surveys of marine invertebrate larvae are a powerful tool for the detection of hidden diversity (Barber & Boyce, 2006; Collin, et al., 2019; Collin et al., 2019a, 2019b; Mahon et al., 2010). They may be particularly effective at capturing deep-water, small, or infaunal species that are difficult to collect as adults by use of standard sampling techniques, and thus provide an independent assessment of diversity that complements species lists based solely on studies of

adults (Collin, et al., 2020; Mahon et al., 2010; Sewell & Jury, 2011). In some cases, larval sampling can uncover unexpectedly high diversity (Collin, et al., 2019; Mahon et al., 2010).

Here we apply this approach to compare the diversity of adult and larval asteroids from the coastal waters of Panama. A review of Caribbean biodiversity from the Census of Marine Life documented 116 species of adult asteroids in the wider Caribbean (Miloslavich et al., 2010: supplemental data table S8). Twenty-five species were reported for the Caribbean coast of Panama, less

than the diversity reported for Cuba (35), Mexico (44), Honduras (30), Nicaragua (29), Colombia (51), and Venezuela (36), and similar to the diversity reported for Puerto Rico (22), Dominican Republic (22), Haiti (19), and Jamaica (20) using the same methodology (Miloslavich et al., 2010). Panama has been a center of marine biology research for over 50 years (Robertson et al., 2009), and it seems unlikely that the low reported diversity along the Caribbean coast of Panama is the result of less sampling effort than is typical for the region. A similar methodology was used to report 45 asteroid species for the Tropical Eastern Pacific (Miloslavich et al., 2011). Coppard and Alvarado (2013) reported 56 species for the Pacific coast of Panama alone. Unfortunately, neither of these publications on the Pacific fauna includes a list of the species reported to belong to the fauna. An earlier paper reported 51 asteroid species (Alvarado et al., 2010), but included North Pacific cold-temperate species such as *Luidia foliolata* GRUBE 1866 and *Pisaster ochraceus* (BRANDT 1835), whose presence in Panama would result in an extraordinary disjunct geographic distribution for each species. Regardless of these details, it seems clear that, unlike the situation in a number of other taxa, the Pacific coast supports a greater diversity of asteroids than the Caribbean coast.

As part of a survey of the diversity of marine invertebrate larvae in the coastal waters of Panama, we documented asteroid larvae from Bocas del Toro on the Caribbean coast and from the Bay of Panama on the Pacific coast. We applied a DNA barcoding approach to identify asteroid larvae with the objectives of (a) determining whether surveying larvae uncovers unexpected asteroid diversity as it has for other groups (Barber & Boyce, 2006; Collin, et al., 2019; Mahon et al., 2010); (b) determining whether larval asteroids are more diverse on the Pacific coast than the Caribbean coast, as are the adults; and (c) determining whether variation in larval morphology can be used to distinguish species at these sites.

2 | METHODS

2.1 | Sample Collection

Asteroid larvae from three distinct sets of samples were photographed and sequenced. Caribbean samples were collected from Bahia Almirante in Bocas del Toro Province with a 0.5-m diameter 125-μm mesh plankton net towed horizontally behind a small boat drifting in the current, with the engine alternating in and out of gear, and sampling the water column by gently bouncing the net through the middle third of the water column (from 10 to 20 m depth). In 2013, larvae were collected incidentally as part of the short-course on Larval Invertebrate Diversity, Form and Function at the Bocas del Toro Research Station (BRS) of the Smithsonian Tropical Research Institute. Samples were collected over 2 weeks from various sites around Bahia Almirante in July 2013 and sorted by a team of 12 students. Larvae were then selected for barcoding based on student interests. In 2015–2016, structured sampling involved a campaign of four plankton surveys evenly spaced throughout a year (August

2015, November 2015, February–March 2016, and June 2016). Each survey consisted of 3–5 tows conducted on different days over 9 days, between 7 a.m. and 9 a.m. Tows were conducted at a single location in the channel between Isla Colon and Isla Cristobal (latitude, 09°20′8.9″N to 09°20′36.3″N; longitude, 82°15′41.0″W to 82°15′50.0″W). A flow meter was attached to the mouth of the net to determine the volume of water sampled, and a depth meter recoded that tows did not drop below 20 m. The depth at this site is ~25 m.

Pacific samples were collected in the northern part of the Bay of Panama, between Taboga and Contadora Islands, from 2013 to 2016 (August 2013, March 2014, April 2014, May 2014, June 2014, November 2014, December 2015, March 2016). Pacific surveys were exploratory, not quantitative, and included multiple locations on each sampling date. Pacific samples were collected by towing the net horizontally, but small changes in speed were used to induce a bouncing depth profile to obtain samples from ~10 to 40 m.

Samples were sorted using a stereomicroscope and asteroid larvae were moved to dishes of filtered seawater. The 2015–2016 Caribbean samples were sorted exhaustively, providing data on larval density; however, for morphologically similar larvae we prepared no more than eight individuals of a similar morphotype from each tow for sequencing. Even when there were small numbers of larvae collected, we could not always sequence all of the individuals, because animals that were damaged during collection often did not survive long enough to be photographed. Therefore, larval counts did not always match the number of individuals sequenced from the same tow. Individual larvae were photographed alive, often moving, in a depression slide under a dissecting microscope prior to preservation for DNA sequencing. During the course in 2013, larvae were relaxed with $MgCl_2$ isotonic with seawater prior to photographing under a compound microscope with differential interference contrast, resulting in fewer processed samples with higher resolution photographs. Notes were taken on the overall appearance, morphological details, and approximate size of each larva before they were preserved for sequencing.

2.2 | DNA Extraction and Sequencing

Individual larvae were preserved in 150 μl of M2 extraction buffer (AutoGen), frozen and shipped to the Smithsonian's Laboratories of Analytical Biology (LAB) for extraction and sequencing. Plates with larval samples were extracted using an AutoGenprep 965 extraction robot after overnight digestion in the AutoGen buffer with proteinase-K. The resuspension volume of extracted DNA was 50 μl. The ~600 bp DNA barcode fragment of the *cytochrome c oxidase subunit I* (*COI*) was amplified using primarily the primer pair jgLCO1490/jgHCO2198 (Geller et al., 2013), although the pairs dgLCO1490/dgHCO2198 (Meyer, 2003) and COIceF/COIceR (Hoareau & Boissin, 2010) were also used. The 10-μl PCR cocktail for *COI* included 5 μl GoTaq Hot Start Mix (Promega), 0.1 μl BSA,

TABLE 1 Summary of identifications of the sequenced asteroid larvae from Panama and the new GenBank sequences resulting from this study

Species or OTU (number of larvae sequenced)	GenBank match Sequence identity	New COI sequences[a]	New 16S sequences[a]	BIN#
Caribbean sea				
Valvaster striatus (46)	"Oreasteridae sp." **16S:** >99%; **COI:** > 99%	MN730656, MN730659, MN730664, MN730665, MN730673	MN730780, MN730783, MN730788, MN730789, MN730799	ABA3725
Oreaster reticulatus (32)	Oreaster reticulatus **16S:** >99% **COI:** >99%	MN730660, MN730061, MN730666, MN73067, MN730675	MN730777, MN730784, MN730785, MN730790, MN730791	ABA6961
Luidia sp. C3 (3)	Luidia sp. **16S:** 87% **COI:** 84%	MN730704, MN730728, MN730776	MN730832, MN730861, MN730916	ADE1546
Luidia sp. C4 (2)	Luidia sp. **16S:** 87%	MN730700, MN730743	MN730828, MN730877	ADQ9336
Mithrodia clavigera (4)	Mithrodia clavigera **16S:** >99% **COI:** >99%	MN730668, MN730690, MN730696, MN730751	MN730792, MN730817, MN730823, MN730888	ABA2469
Luidia sp. C6 (8)	Luidia sp. **16S:** 88%	MN730674, MN730685, MN730689, MN730736, MN730752	MN730794, MN730800, MN730811, MN730816, MN730869	ADE1365
Luidia sp. C7 (4)	Luidia sp. **16S:** 92% **COI:** 90%	MN730669, MN730764	MN730793, MN730812, MN730855, MN730902	ADF0173
Astropecten marginatus (4)	Astropecten marginatus **16S:** >99%	MN730658, MN730662, MN730705	MN730782, MN730786, MN730833, MN730837	ADQ7491
Luidia sp. C9 (3)	Luidia sp. **16S:** 94%	MN730744	MN730878, MN730883, MN730913	–[b]
Astropecten antillensis (20)	Astropecten antillensis **16S:** >99% **COI:** > 99%	MN730657, MN730663, MN730670, MN730671, MN730677	MN730781, MN730787, MN730795, MN730796, MN730803	ADQ9567
Pacific Ocean				
Pentaceraster cumingi (6)	Pentaceraster cumingi **16S:** >99% **COI:** >99%	MN730654, MN730655, MN730676, MN730681, MN730767	MN730778, MN730779, MN730802, MN730807, MN730905	ABA3605

TABLE 1 (Continued)

Species or OTU (number of larvae sequenced)	GenBank match Sequence identity	New COI sequences[a]	New 16S sequences[a]	BIN#
Astropecten verrilli (6)	Astropecten verrilli 16S: >99%	MN730672, MN730679, MN730735, MN730738, MN730745	MN730797, MN730805, MN730868, MN730871, MN730879	–
Luidia sp. P3 (1)	Luidia sp. 16S: 88%	MN730761	MN730899	–
Luidia sp. P4 (1)	Luidia sp. 16S: 90%	MN730684	MN730810	–
Pharia pyramidata (1)	No match in GenBank	MN730746	MN730882	ADK8826

[a]Only the first five GenBank sequences are listed. The complete datasets can be found with the BOLD DOI: dx.doi.org/10.5883/DS-ASTEROID.

[b]BINs are not generated by BOLD if too few sequences are available or if other record quality criteria are not met for enough sequences in the putative BIN.

and 0.3 μl of each 10-mM primer. For amplification and sequencing of a ~490 bp fragment of *16S*, the primer pairs 16SAR/16SBR (Palumbi et al., 1991) or 16SL2/16SH2 (Arnedo et al., 2001; Schubart et al., 2002) were used. The cocktail for *16S* used Biolase Taq (Bioline) with the addition of 0.5 μl 50 mM $MgCl_2$. The annealing temperature was 48°C for *COI* and 50°C for *16S*.

2.3 | Analysis

Sequences were screened for quality and used to generate contigs of forward and reverse amplicons with Sequencher 5.4.6 (Gene Codes). Only sequences with more than 90% of the expected length and with a Phred quality score of at least 30 for more than 85% of the bases were combined into contigs and used for analyses. To check for potential contamination, sequences were compared internally with all larvae sequenced in our project within the BOLD project workbench (www.boldsystems.org) and to sequences available in GenBank using BLASTn searches. For *COI* we followed the suggestions of Song et al. (2008) to check for the presence of pseudogenes in our datasets. No gaps were identified, nor were there any stop codons in the *COI* sequences.

Neighbor-joining trees (BIONJ, Gascuel, 1997) with Kimura two-parameter distances were constructed from our sequences, trimmed to the length of the shortest sequence to aid in OTU visualization. *COI* alignments were inferred with the BOLD aligner (amino acid based Hidden Markov Model; Ratnasingham & Hebert 2007) using the default settings; *16S* was aligned using ClustalX (www.clustal.org) followed by manual editing in McClade (www.mcclade.org). OTUs were identified with the Automatic Barcode Gap Discovery (ABGD) method ($P_{min} = 0.001$; $P_{max} = 0.1$; Steps = 10; X (relative gap width) = 1.5; Kimura (K80) distance; TS/TV = 2) (Puillandre et al., 2012). To try to confirm the genus or family identity of the unidentified larval OTUs, they were analyzed with all available *16S* sequence data for species of *Luidia* as above.

DNA sequences generated by this project have been deposited in GenBank (accession numbers: MN730654–MN730776 for *COI*; MN730777–MN730916 for *16S*), and the *COI* dataset can be found at dx.doi.org/10.5883/DS-ASTEROID. Alignments are available at FigShare: 10.25573/data.c.5084306.

3 | RESULTS AND DISCUSSION

Asteroid larvae were fairly abundant in Bocas del Toro, with densities from 0.4 to 8.8 larvae/m^3 (mean = 2.7; SD = 2.9 larvae/m^3) in the sampled seawater. Overall, we preserved 154 larvae for DNA sequencing and obtained 123 *COI* and 140 *16S* sequences from them. Asteroid larvae were uncommon in the Pacific, and only 16 larvae were collected for sequencing.

Using ABGD, we detected 15 larval OTUs, with 5 from the Pacific and 10 from the Caribbean (Table 1; Figure 1). They are
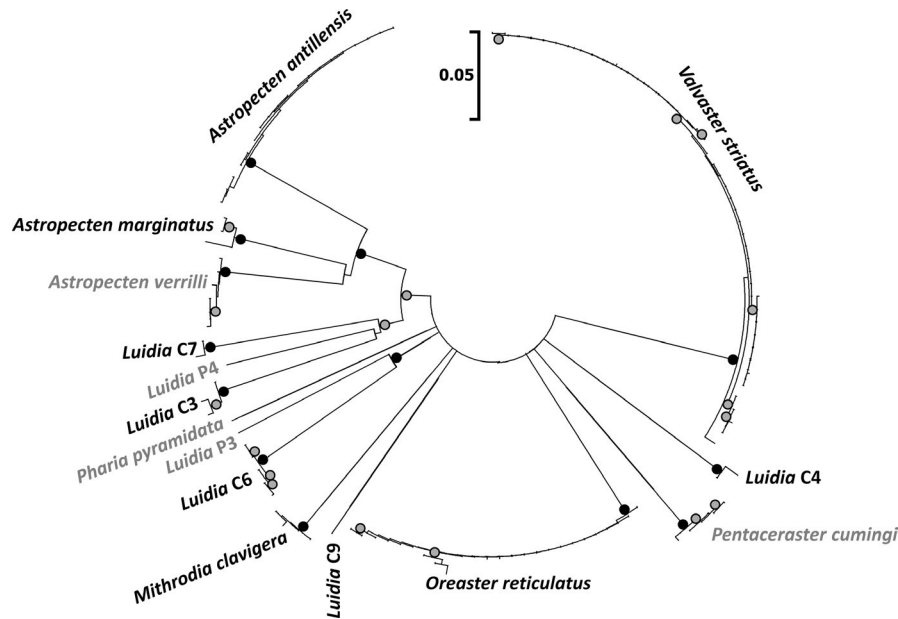
FIGURE 1 Phylogram constructed from the Kimura two-parameter distances of *COI* barcode sequences for larvae collected during this study. Black circles indicate clades with >95% support, grey circles indicate clades with >70% support. Species names (when they could be determined by comparison with BINS or GenBank samples) or OTU (operational taxonomic unit) names (when we could not find a match for our sequences in a repository) from the Caribbean are indicated in black, species and OTU names from the Pacific are in grey

easily visualized as distinct in a neighbor-joining tree, and the OTUs identified both ways were concordant and the same OTUs were obtained with *16S* and *COI*. The most abundant OTUs were found in the Caribbean and had 46, 32, and 20 larvae, respectively. The remaining OTUs contained between 1 and 8 larvae (Figure 1). Each *COI* OTU corresponded to a distinct Barcode Index Number (BIN) in BOLD (Ratnasingham & Herbert, 2013). OTUs differed from each other by at least 10% divergence in *COI* and with no more that 2% intra-OTU divergence (Figure 1). Thus, we infer that each of these OTUs corresponds to a different species.

We identified 6 of the 15 OTUs based on >99% similarity with reference sequences in GenBank using BLASTn searches of the *COI* and *16S* sequences (Table 1). These were the Pacific species *Astropecten verrilli* DE LORIOL 1899 and *Pentaceraster cumingi* (GRAY 1840) and the Caribbean species *Astropecten marginatus* GRAY 1840, *Astropecten antillensis* LÜTKEN 1859, *Oreaster reticulatus* (LINNAEUS 1758), and *Mithrodia clavigera* (LAMARCK 1816) (Table 1). Two other OTUs were placed in BINs with unpublished sequences. One of these was identified as *Pharia pyramidata* (GRAY 1840) from Pacific samples (Table 1). The other OTU, collected in the Caribbean, was identified as *Valvaster striatus* (LAMARCK 1816), a species that was previously thought to be endemic to the Indo-West Pacific (IWP) (see Collin et al., 2020 for details). The remaining seven OTUs, which could not be identified to species, are likely to belong to *Luidia*, as they each have 87%–95% identity in *16S* with various species of *Luidia* (Table 1; Figure 2), and neighbor-joining trees constructed with our *16S* sequences and these sequences from GenBank place them closer to species of *Luidia* than to species of *Astropecten*. Unfortunately, there are too few *COI* barcodes for species of *Luidia* available from the Neotropics to adequately perform a similar comparison with our *COI* sequences.

Although Knott et al. (2003) indicate that asteroid larvae cannot be identified to species with any certainty based on morphology, we present some of our observations of the larval morphology from our Pacific (Figure 3) and Caribbean (Figures 4 and 5) OTUs in the hopes of illustrating the variation among species and shining light on potentially useful features that may help to distinguish the major groups of Neotropical asteroid larvae. For each group, we provide a discussion of their molecular identification and any relevant information on their reproduction and development in the Neotropics.

3.1 | *Astropecten*

We found three species of *Astropecten* in our larval samples and identified them primarily by comparisons with sequences generated as part of a global molecular phylogeny of 117 specimens representing ~40 of the ~150 known species of *Astropecten* (Zulliger & Lessios, 2010). These sequences include (following their taxonomy) *A. antillensis*, *A. articulatus* (SAY 1825), *A. cingulatus* SLADEN 1883, *A. marginatus*, and *A. oerstedi* LUTEKEN 1859 collected from the Caribbean of Panama, and *Astropecten alligator* PERRIER 1881, *A. americanus* (VERRILL 1880), *A. comptus* VERRILL 1915, *A. duplicatus* GRAY 1840, and *A. nitidus* VERRILL 1915 from other sites in the Caribbean and Gulf of Mexico. Of these 10 Caribbean species, only 2, *A. antillensis* and *A. marginatus*, were identified among our larval OTUs. Specimens of *A. articulatus* were collected from Bocas del Toro as adults (Zulliger & Lessios, 2010), but surprisingly, we did not collect larvae with corresponding sequences. On the Pacific coast, *A. erinaceus* GRAY 1840, *A. siderialis* VERRILL 1914, and *A. verrilli* were previously sequenced
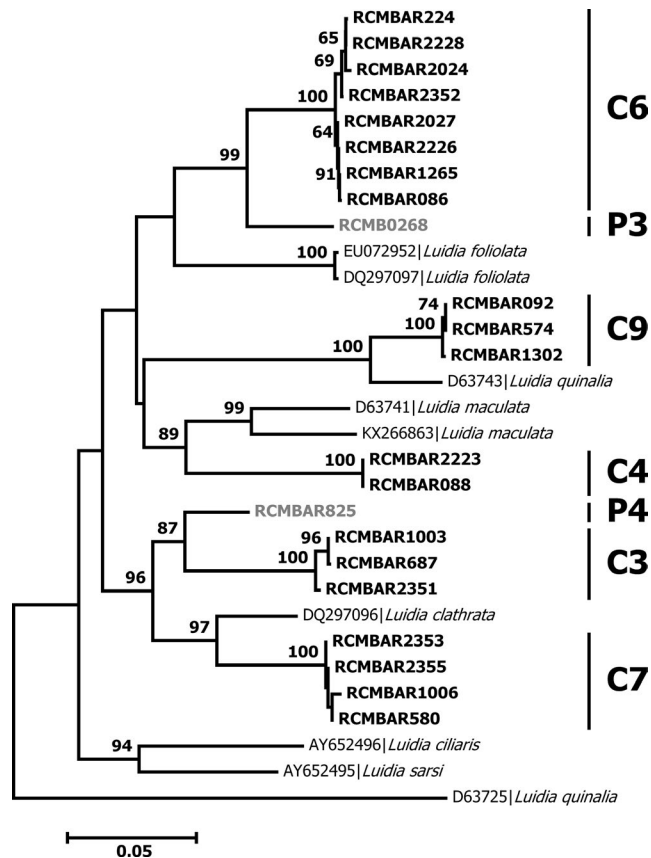
FIGURE 2 Neighbor-joining tree of *16S* sequences from species of *Luidia* represented in GenBank and larval sequences attributed to *Luidia* collected during this study. Species names follow the names attached to the sequences from GenBank. Our samples are indicated in bold and labelled with our sample numbers, with Caribbean samples indicated in black and Pacific samples indicated in grey. Bootstrap values >60% are indicated by the nodes. The OTU (operational taxonomic unit) names are indicated to the right

from Panama, and *A. regalis* GRAY 1840 from the Pacific of Costa Rica (Zulliger & Lessios, 2010). Our larval samples were all attributable to *A. verilli* (distinct from *A. californicus* FISHER 1906; see Zulliger & Lessios, 2010 for discussion of the taxonomy of these species).

The development and larvae of *Astropecten armatus* GRAY 1840 from California has been recently described in detail (Pernet et al., 2017). The larvae from the three OTUs we collected were similar to larvae of *A. armatus*. All had five pairs of bipinnarial arms with orange pigment on their tips and the juvenile rudiment, when present, was white. We did not observe any indication of cloning in the larvae we collected. As expected for this genus, no larvae had brachiolarial arms. None of the larvae we collected had bipinnarial arms as long as those figured by Pernet et al. (2017), who indicated that arm length may reflect egg size.

Larvae of *A. verrilli* were notable as their bipinnarial arms were very short, even in larvae with a developing juvenile (Figure 3C,D), and their orange pigment was less distinct than it was in the other two species of *Astropecten* we identified (Figure 5C,D,F–H). All six of the larvae of *A. verrilli* were collected in the Bay of Panama on December

7, 2015, with the largest larva measuring 1.2-mm long. The largest of the 20 larvae of *A. antillensis* we collected were 1.1 mm, and, like the larvae of *A. armatus*, larvae of *A. verrilli*, *A. marginatus*, and *A. antillensis* each had a noticeable blue tint to their stomach and/or intestine (Figures 3C,D and 5C,D,F,G). The four larvae of *A. marginatus* we collected were similar to the larvae of *A. antillensis*, although two of them showed a more pronounced scattering of mesenchyme cells across the epithelium (Figure 5C). This was not as evident in the other larvae of the genus. Additionally, the preoral lobes and bipinnarial arms of *A. antillensis* were more defined and protruded further than in their congeneric species, which may reflect species-specific or developmental differences (Figure 5F,G).

3.2 | *Luidia*

Seven OTUs were assigned to *Luidia* based on larval morphology and the fact that the closest BLASTn matches were species of *Luidia*. In addition, in the neighbor-joining trees we generated that included sequences of *Luidia* from GenBank, our sequences occurred mixed among the other species, without generating particularly long branches (Figure 2). There were two OTUs from the Pacific (e.g., Figure 3E,F) and five from the Caribbean (e.g., Figures 4E–G and 5A,B,E). The Caribbean fauna is widely considered to be composed of six species of *Luidia*, many of which are attributed to subspecies of species with extraordinarily wide latitudinal ranges. These species include records of adults of *Luidia clathrata* (SAY 1825), *L. alternata* (SAY 1825), *L. barbadensis* PERRIER 1881, *L. ludwigi* FISHER 1906, *L. senegalensis* (LAMARCK 1816), and *L. sarsii* DÜBEN & KOREN in DÜBEN 1844, which range from the southeastern US, through the Gulf of Mexico and the Caribbean, and south to Brazil (Lawrence Duran-Gonzalez et al., 2013). Two other species, *L. lawrencei* HOPKINS & KNOTT 2010 and *L. sagamina* DÖDERLEIN 1920, occur along the east coast of the US and the Gulf of Mexico but are not known to extend southward into the Caribbean. Of these eight species, Milaslovich et al. (2010) reported only *L. alternata*, *L. clathrata*, and *L. senegalensis* for the Caribbean coast of Panama. Recently collected vouchers at the Florida Museum of Natural History, University of Florida (UF), confirmed the presence of *L. alternata*, and one of us (RC) has observed *L. senegalensis* in Bahia Almirante. Regarding this Caribbean fauna, GenBank contains sequence data only for *L. clathrata* (*COI* and *16S*) and *L. sarsii* (*COI*; from the North Sea). In addition, UF has unpublished *COI* sequences from *L. senegalensis*, *L. lawrencei*, and *L. alternata* from the Caribbean (G. Paulay, unpublished data; pers. com.). None of the five OTUs corresponding to *Luidia* we identified from our Caribbean samples showed high sequence identity (>95%) with any sequences from these five species of *Luidia*. These results suggest that larvae from our collections either belong to other species reported from the region, which lack available sequence data, or that regional subspecies within these widespread taxa are genetically dissimilar enough to be considered distinct species.
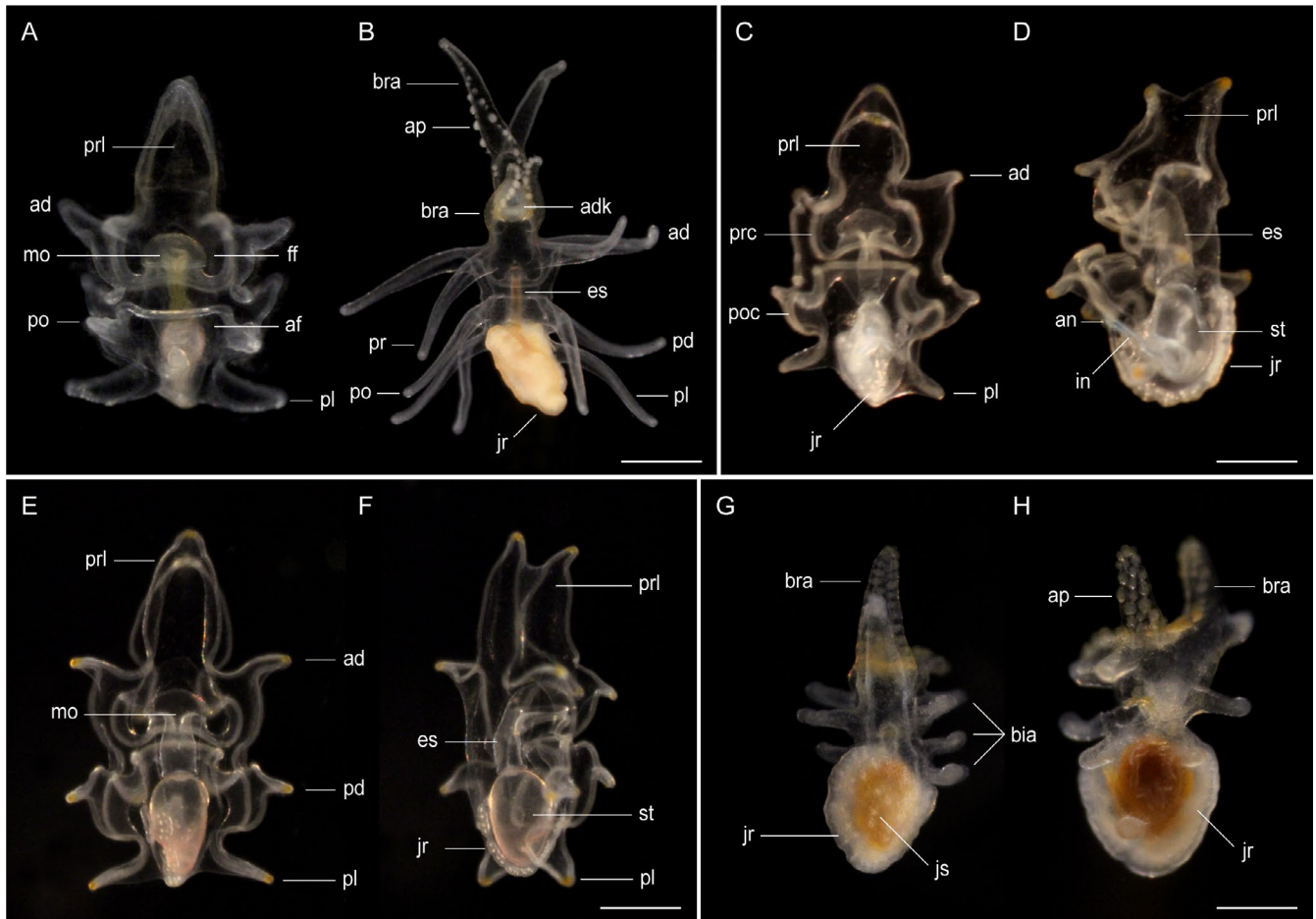
FIGURE 3 Asteroid larval representatives of four DNA barcode operational taxonomic units (OTUs) from the Bay of Panama. All larvae are oriented with anterior to the top. A,B. Ventral views of a bipinnaria (**A**) and brachiolaria (**B**) of *Pentaceraster cumingi*. **C,D.** Ventral and left-lateral views of two bipinnaria of *Astropecten verrilli*, with juvenile rudiments. **E,F.** Dorsal and right-lateral views of the unidentified bipinnaria of *Luidia* sp. P3. **G,H.** Dorsolateral and lateral views of the brachiolaria larva of *Pharia pyramidata*, with juvenile rudiment. All images were captured from live specimens. ad, anterodorsal arm; adk, adhesive disk; af, anal field; an, anus; ap, adhesive papilla; bia, bipinnaria arm; bra, brachiolar arm; es, esophagus; ff, frontal field; in, intestine; jr, juvenile rudiment; js, juvenile skeletal element; mo, mouth; pd, posterodorsal arm; pl, posterolateral arm; po, postoral arm; poc, postoral ciliary band; pr, preoral arm; prc, preoral ciliary band; prl, preoral lobe; st, stomach. Scale bars ~250 μm

The Tropical Eastern Pacific (TEP) fauna is composed of eight species of *Luidia* (Alvarado et al., 2010; excluding *L. foliolata*, which is clearly in error), although none of these have sequences in GenBank. *Luidia armata* Ludwig 1905 and *L. asthenosoma* Fisher 1906 from Southern California have sequences in BOLD, but neither one shares a BIN with our larvae (i.e., are not conspecific with our larvae). In addition, the *COI* of *L. latiradiata* (Gray 1871) has been sequenced by UF and does not match our larvae (G. Paulay, unpublished data; pers. com.). We can therefore eliminate the possibility that these three species are present in our larval samples, but the other five species reported to occur in the Pacific fauna have not been sequenced and therefore remain as possible matches to our larvae.

Little is known about the reproduction and development of *Luidia* in the Caribbean, and nothing has been published on the life histories of *Luidia* from the TEP. In the Caribbean, *L. clathrata* and *L. senegalensis* both show a single seasonal peak in gonad index, which occurs either in the fall or very early spring in Florida (Dehn, 1980;

Miller & Lawrence, 1999). All of the larvae we assigned to *Luidia* were morphologically consistent with descriptions by Komatsu et al. (1991) of three species from Florida: typical bipinnaria larvae that did not exceed 1.5 mm, with five pairs of bipinnarial arms, and two anterior processes of the anterior lobe. None of our larvae were brachiolaria (a larval stage that is not known to occur in species of *Luidia*). In *L. sarsi*, larvae are highly derived and giant, sometimes >3 cm in length (Domanski, 1984). We did not find this type or size of larvae in our samples. *Luidia* sp. C3 (Figure 4E) was represented by three larvae, the largest of which was 1.1 mm and had orange tips on the bipinnarial arms and the early development of a rudiment. *Luidia* sp. C4 (Figure 4F,G) was represented by two larvae, the largest of which was a bipinnaria with an orange esophagus and pink gut. This larva was notable because there was some orange pigment on the body (Figure 4G). *Luidia* sp. C6 was represented by eight larvae, which also had orange-tipped bipinnarial arms and a clear gut (Figure 5A,B). *Luidia* sp. C7 (Figure 5E) was represented
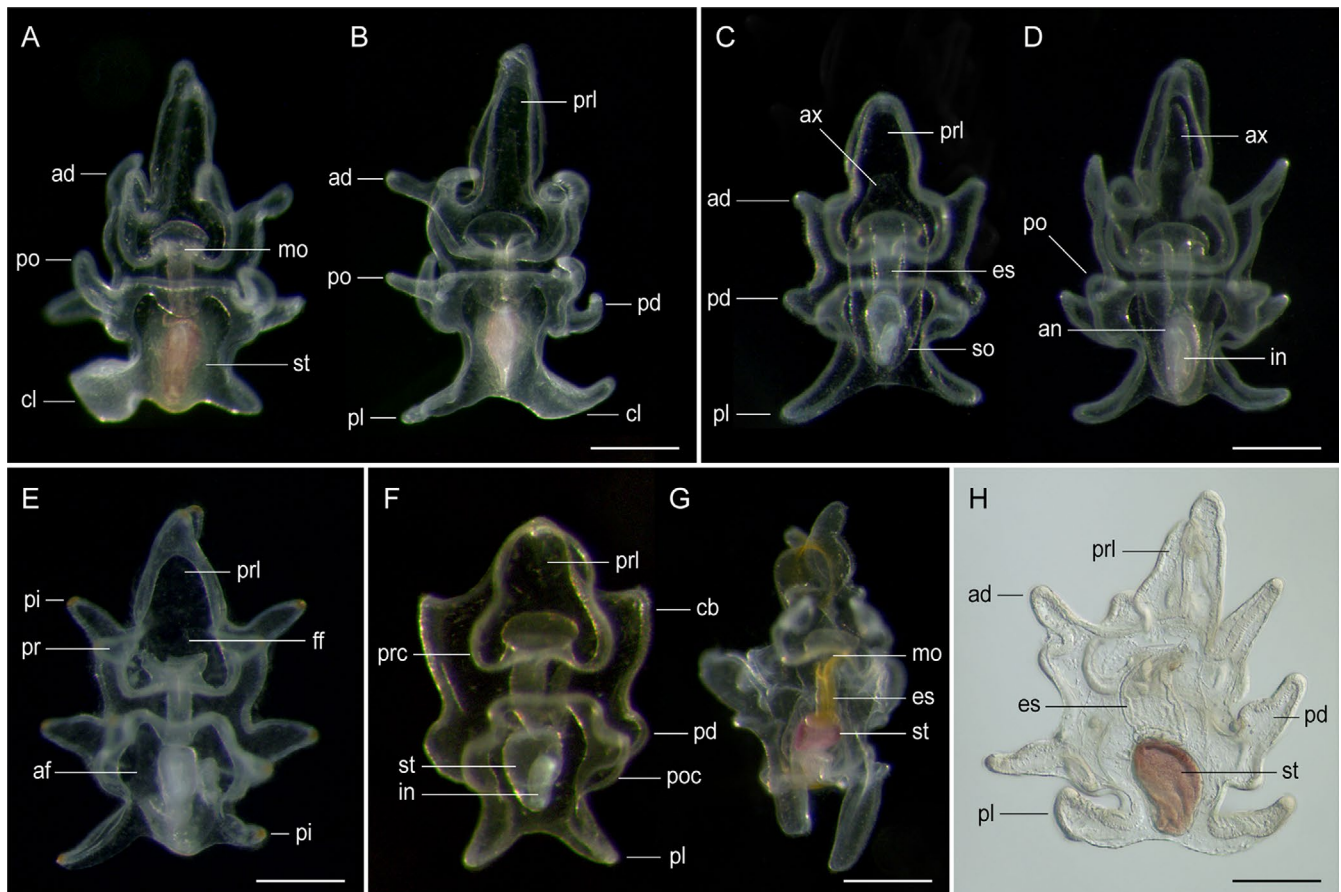
FIGURE 4 Asteroid larval representatives of five DNA barcode operational taxonomic units (OTUs) from Bahia Almirante in Bocas del Toro Province. **A,B.** Ventral views of two bipinnaria of *Valvaster striatus* with clonal development. **C,D.** Ventral views of two bipinnaria of *Oreaster reticulatus*. **E.** Bipinnaria of *Luidia* sp. C3. **F,G.** Ventral views of two bipinnaria of *Luidia* sp. C4. **H.** Dorsal view of a bipinnaria of *Mithrodia clavigera* (specimen is compressed). All images were captured from live specimens. ad, anterodorsal arm; af, anal field; an, anus; ax, axocoel; cb, ciliary band; cl, clone of larva; es, esophagus; ff, frontal field; in, intestine; mo, mouth; pd, posterodorsal arm; pi, pigment; pl, posterolateral arm; po, postoral arm; poc, postoral ciliary band; pr, preoral arm; prc, preoral ciliary band; prl, preoral lobe; so, somatocoel; st, stomach. Scale bars ~250 μm

by four larvae. Three of these showed evidence of cloning, with at least one missing posterolateral arm tip on each larva. These may be the same species as the cloning larvae of *Luidia* reported by Knott et al. (2003), which were placed close to *L. clathrata* in the phylogeny of Galac et al. (2016). BOLD records indicate that the nearest BIN to our OTU is an unpublished BIN identified as *L. clathrata*. Unfortunately, the sequence fragment used by Knott et al. (2003) does not overlap sufficiently with either of our sequences to support direct comparisons between our datasets. *Luidia* sp. C9 (not shown) was represented by three larvae, one of which was a large bipinnaria with orange tips on the arms. There were two OTUs that corresponded to species of *Luidia* in our Pacific samples, each represented by a single larva. *Luidia* sp. P3 was a large bipinnaria with orange-tipped arms, a pinkish gut, and a white-tipped rudiment flanking the stomach (Figure 3E,F). *Luidia* sp. P4 was an unpigmented bipinnaria with a white rudiment (not shown). None of these larvae of *Luidia* are easily distinguishable from each other, and the possibility of larval plasticity in response to different food rations, which has been demonstrated in the North Pacific species

L. foliolata (George, 1994), further complicates the interpretation of any subtle differences in shape or allometry.

3.3 | Valvatacea

The remaining five larval OTUs could all be identified based on DNA sequence data. In the Caribbean, these included the well-documented larvae of three shallow-water species. The most common larva we encountered was *V. striatus* (LAMARCK 1816) (46 larvae; Figure 4A,B). These cloning larvae have been discussed in detail elsewhere (Collin, et al., 2020; Galac et al., 2016; Janies et al., 2019; Knott et al., 2003). *Valvaster striatus* were the most abundant larvae overall and included small early stages as well as brachiolaria with developing rudiments. There may be a seasonal pattern to their appearance in the plankton, with this species representing the majority of asteroid larvae collected in August and November, but with few individuals collected in March and June. The other most abundant larvae belonged to *O. reticulatus* (LINNAEUS
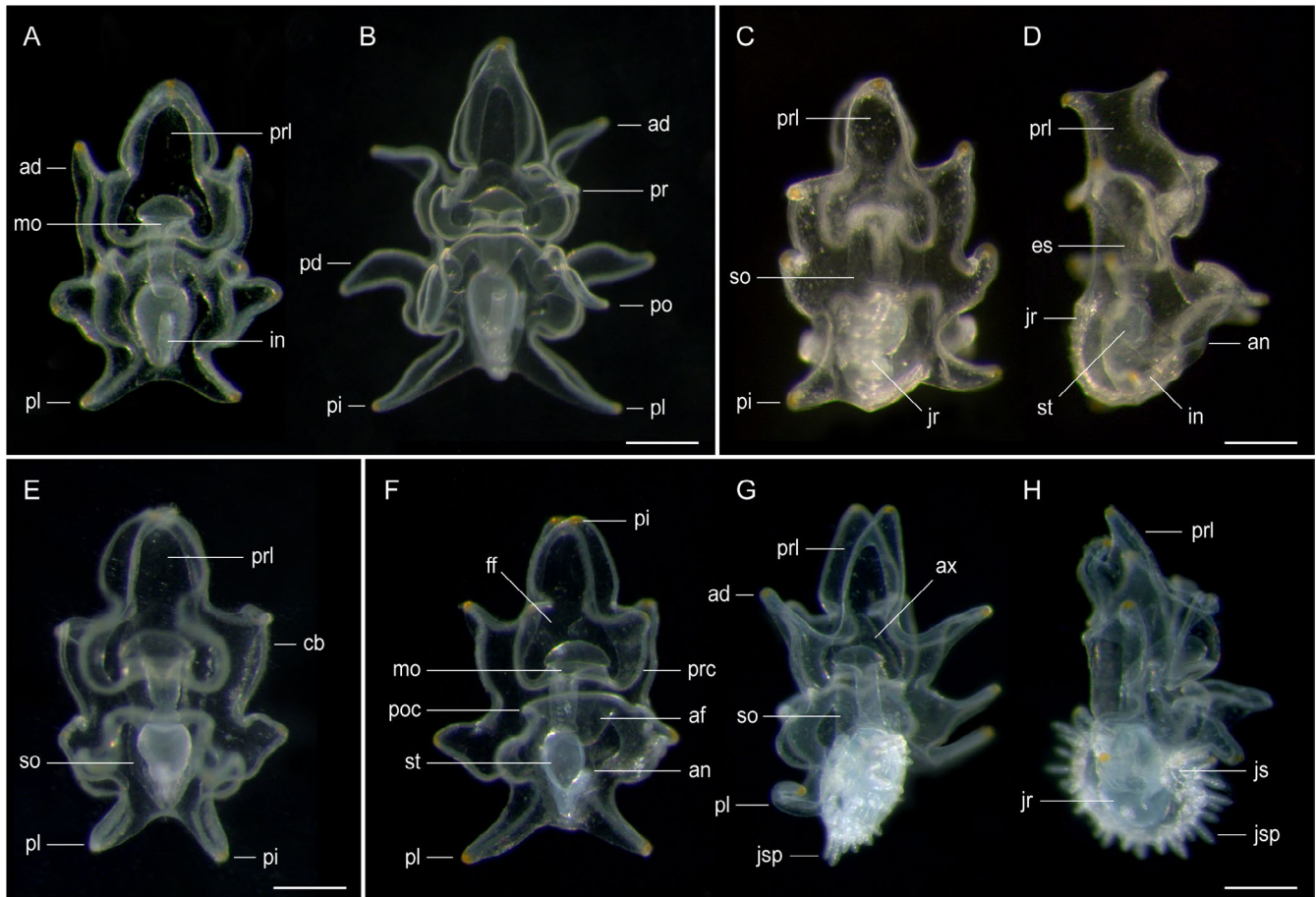
FIGURE 5 Asteroid larval representatives of four DNA barcode operational taxonomic units (OTUs) from Bahia Almirante in Bocas del Toro Province. **A,B.** Ventral views of two bipinnaria of *Luidia* sp. C6. **C,D.** Dorsal and right-lateral views of a bipinnaria of *Astropecten marginatus*. **E.** Ventral view of a bipinnaria of *Luidia* sp. C7. F–H. Ventral view (**F**) and paired ventral and right-lateral views (**G,H**) of bipinnaria larvae of *Astropecten antillensis*. All images were captured from live specimens. ad, anterodorsal arm; af, anal field; an, anus; ax, axocoel; cb, ciliary band; es, esophagus; ff, frontal field; in, intestine; jr, juvenile rudiment; js, juvenile skeletal element; jsp, juvenile spine; mo, mouth; pd, posterodorsal arm; pi, pigment; pl, posterolateral arm; po, postoral arm; poc, postoral ciliary band; pr, preoral arm; prc, preoral ciliary band; prl, preoral lobe; so, somatocoel; st, stomach. Scale bars ~250 μm

1758) (32 larvae; Figure 4C,D), an abundant species found in benthic habitats of Bocas del Toro. These specimens included both young bipinnaria and more mature forms with large rudiments and brachiolarial arms. Our observations of these larvae during each of the four sampling periods supports Guzmán and Guevara's (2002) conclusion that this species does not exhibit seasonal reproduction in Panama, unlike the late summer spawning activity reported by Scheibling (1981) in the U.S. Virgin Islands. The larvae we collected had morphologies consistent with those described by Metaxas et al. (2008).

We also collected four larvae identified as *M. clavigera* (LAMARCK 1816) (Figure 4H). As described by Galac et al. (2016; referencing Knott et al., 2003), these larvae also have a colored gut, similar in color to, but darker than, the larvae of *V. striatus*. Galac et al. (2016) were able to identify specimens of "larval group 4" from Knott et al. (2003) as cloning larvae belonging to this species, although they observed that cloning was uncommon, and none of the four larvae of *M. clavigera* that we collected showed evidence of cloning.

It should be noted that, like *V. striatus*, *M. clavigera* is primarily an IWP species, but has been recently reported from the Caribbean (Bribiesca-Contreras et al., 2013; Clark & Downey, 1992). Our *COI* and *16S* sequences revealed high-percent identity with sequences of adults of *M. clavigera* collected from the Caribbean and with some sequences from the Indian Ocean (G. Paulay, pers. com.). Specimens of *M. clavigera* collected from other parts of the IWP fall into different clades based on *COI* barcode data (G. Paulay, unpublished data; pers. com.).

Only two valvatacean OTUs were collected from the Pacific coast of Panama. The most abundant larvae were identified as *P. cumingi* (GRAY 1840), which was represented by six specimens. This species, which has been found in Aztec ceremonial sites (Martín-Cao-Romero et al., 2017), is abundant throughout the TEP, and may form dense spawning aggregations (Reyes-Bonilla et al., 2018). Of the six larvae recovered, one was a brachiolaria with 10 long bipinnarial arms and 3 brachiolarial arms, with a yellow adhesive disk at the ventral base of the preoral lobe (Figure 3A,B). The elongate

rudiment was yellow-orange. These larvae were all collected during the non-upwelling season in the Bay of Panama (May, June, August, and November). The other Pacific valvatacean OTU was represented by a single brachiolaria stage larva of *P. pyramidata* (Gray 1840) (Figure 3G,H). The bipinnarial arms were stubby and the round rudiment was a rusty brown color. This larva was collected in November, which is consistent with the spawning season of July–September reported from the Mexican tropical Pacific (Benítez-Villalobos & Martinez-Garcia, 2012). There is no other published information on the reproduction of this species.

4 | CONCLUSIONS

The larval asteroid fauna of Panama collected during this investigation was not particularly diverse, with only 15 OTUs detected, compared to a total of ~80 species reported to occur as adults in Panamanian waters. From the Caribbean our intense sampling documented 10 OTUs from ~130 samples compared to an adult fauna of 25 species. In the Pacific, we documented five OTUs from 16 samples, compared to the adult fauna of 56 species. This strongly suggests that additional plankton sampling efforts, with broader spatial and temporal coverage, would likely increase the larval diversity reported for the Pacific. It is interesting to note that we found no forcipulatacean larvae in our samples, yet several forcipulatacean species have been reported in Panamanian waters. Additionally, half of the larval diversity reported in our study belong to a single genus, *Luidia*. These soft-bottom specialists are in need of taxonomic revision and improved representation in genetic databases such as GenBank and BOLD. Importantly, our efforts succeeded in collecting and characterizing larval stages of 15 distinct asteroids, providing evidence, for the first time for most of them, of their indirect life history patterns, their respective larval morphologies, and by extension, their reproductive populations along Pacific and Caribbean coasts of Panama.

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REFERENCES

Alvarado, J. J., Solís-Marín, F. A., & Ahearn, C. G. (2010). Echinoderm (Echinodermata) diversity in the Pacific coast of Central America. *Marine Biodiversity, 40*(1), 45–56.

Arnedo, M. A., Orom, P., & Ribera, C. (2001). Radiation of the spider genus *Dysdera* (Araneae, Dysderidae) in the Canary Islands: Cladistic assessment based on multiple data sets. *Cladistics, 17*, 313–353.

Barber, P., & Boyce, S. L. (2006). Estimating diversity of Indo-Pacific coral reef stomatopods through DNA barcoding of stomatopod larvae. *Proceedings of the Royal Society of London Series B: Biological Sciences, 273*, 2053–2061.

Benítez-Villalobos, F., & Martinez-Garcia, M. (2012). Reproductive biology of the starfish *Pharia pyramidatus* (Echinodermata: Asteroidea) from the Mexican tropical Pacific. *Journal of the Marine Biological Association of the United Kingdom, 92*(6), 1409–1418.

Bribiesca-Contreras, G., Solís-Marín, F. A., Laguarda-Figueras, A., & Zaldívar-Riverón, A. (2013). Identification of echinoderms (Echinodermata) from an anchialine cave in Cozumel Island, Mexico, using DNA barcodes. *Molecular Ecology Research, 13*(6), 1137–1145.

Clark, A. M., & Downey, M. E. (1992). *Starfishes of the Atlantic*. Chapman & Hall.

Collin, R., Venera-Pontón, D. E., Driskell, A. C., Chan, K.-Y.-K., Macdonald, K. S., & Boyle, M. J. (2019). Documenting Neotropical diversity of phoronids with DNA barcoding of planktonic larvae. *Invertebrate Biology, 138*, e12242. https://doi.org/10.1111/ivb.12242

Collin, R., Venera-Pontón, D. E., Driskell, A. C., Macdonald, K. S., & Boyle, M. J. (2019a). Planktotrophic brachiopod larvae from the Pacific and Caribbean of Panama. *Diversity, 11*, 2.

Collin, R., Venera-Pontón, D. E., Driskell, A. C., Macdonald, K. S., & Boyle, M. J. (2019b). Unexpected molecular and morphological diversity of hemichordate larvae from the neotropics. *Invertebrate Biology, 138*, e12273. https://doi.org/10.1111/ivb.12273

Collin, R., Venera-Pontón, D. E., Driskell, A. C., Macdonald, K. S., Geyer, L., Lessios, H. A., & Boyle, M. J. (2020). DNA barcoding of echinopluteus larvae uncovers cryptic diversity in neotropical echinoids. *Invertebrate Biology, 139*(2), e12292.

Collin, R., Venera-Pontón, D. E., Paulay, G., & Boyle, M. J. (2020). World travelers: DNA barcoding unmasks the origin of cloning asteroid larvae from the Caribbean. *Biological Bulletin, 239*(2), 73–79.

Coppard, S. E., & Alvarado, J. J. (2013). Echinoderm diversity in Panama: 144 years of research across the Isthmus. In J. J. Alvarado, & F. A. Solís-Marín (Eds.), *Echinoderm research and diversity in Latin America* (pp. 107–144). Springer.

Dehn, P. F. (1980). The annual reproductive cycle of two populations of *Luidia clathrata* (Asteroidea). I. Organ indices and occurrence of larvae. In M. Jangoux (Ed.), *Echinoderms: Present and past* (pp. 361–367). CRC Press.

Domanski, P. A. (1984). Giant larvae: Prolonged planktonic larval phase in the asteroid *Luidia sarsi*. *Marine Biology, 80*(2), 189–195.

Galac, M. R., Bosch, I., & Janies, D. A. (2016). Bacterial communities of oceanic sea star (Asteroidea: Echinodermata) larvae. *Marine Biology, 163*(7), 162. https://doi.org/10.1007/s00227-016-2938-3

Gascuel, O. (1997). BIONJ: An improved version of the NJ algorithm based on a simple model of sequence data. *Molecular Biology and*

Evolution, *14*(7), 685–695. https://doi.org/10.1093/oxfordjournals.molbev.a025808

Geller, J., Meyer, C. P., Parker, M., & Hawk, H. (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Research*, *13*(5), 851–861.

George, S. B. (1994). Phenotypic plasticity in the larvae of *Luidia foliolata* (Echinodermata: Asteroidea). In B. David, A. Guille, J. P. Feral, & M. Roux (Eds.), *Echinoderms through time* (pp. 297–307). A. A. Balkema.

Guzmán, H., & Guevara, C. (2002). Annual reproductive cycle, spatial distribution, abundance, and size structure of *Oreaster reticulatus* (Echinodermata: Asteroidea) in Bocas del Toro, Panama. *Marine Biology*, *141*(6), 1077–1084.

Hoareau, T. B., & Boissin, E. (2010). Design of phylum-specific hybrid primers for DNA barcoding: Addressing the need for efficient *COI* amplification in the Echinodermata. *Molecular Ecology Resources*, *10*, 960–967. https://doi.org/10.1111/j.1755-0998.2010.02848.x

Janies, D., Hernández-Díaz, Y. Q., Solís-Marín, F. A., Lopez, K., Alexandrov, B., Galac, M., Herrera, J., Cobb, J., Ebert, T. A., & Bosch, I. (2019). Discovery of adults linked to cloning oceanic starfish larvae (*Oreaster*, Asteroidea: Echinodermata). *Biological Bulletin*, *236*, 174–185.

Knott, K. E., Balser, E. J., Jaeckle, W. B., & Wray, G. A. (2003). Identification of asteroid genera with species capable of larval cloning. *The Biological Bulletin*, *204*(3), 246–255. https://doi.org/10.2307/1543596

Komatsu, M., Oguro, C., & Lawrence, J. M. (1991). A comparison of development in three species of the genus, *Luidia* (Echinodermata: Asteroidea) from Florida. In T. Yanagisawa, I. Yasumasu, C. Oguro, N. Suzuki, & T. Motokawa (Eds.), *Biology of Echinodermata* (pp. 489–498). A.A. Balkema.

Lawrence, J. M., Duran-Gonzalez, A., Solis-Marin, F. A., Herrera, J., & Ventura, C. R. (2013). Distribution of *Luidia clathrata* and *Luidia lawrencei* (Echinodermata: Asteroidea) along the coast of the western Atlantic Ocean, the Gulf of Mexico and the Caribbean Sea. *Cahiers De Biologie Marine*, *54*, 525–529.

Mahon, A. R., Thornhill, D. J., Norenburg, J. L., & Halanych, K. M. (2010). DNA uncovers Antarctic nemertean biodiversity and exposes a decades-old cold case of asymmetric inventory. *Polar Biology*, *33*(2), 193–202. https://doi.org/10.1007/s00300-009-0696-0

Martín-Cao-Romero, C., Solís-Marín, F. A., Caballero-Ochoa, A. A., Hernández-Díaz, Y. Q., Luján, L. L., & Zúñiga-Arellano, B. (2017). New echinoderm remains in the buried offerings of the Templo Mayor of Tenochtitlan, Mexico City. *Revista De Biología Tropical*, *65*(1–1), S168–S179. https://doi.org/10.15517/rbt.v65i1-1.31686

Metaxas, A., Scheibling, R. E., Robinson, M. C., & Young, C. M. (2008). Larval development, settlement, and early post-settlement behavior of the tropical sea star *Oreaster reticulatus*. *Bulletin of Marine Science*, *83*(3), 471–480.

Meyer, C. P. (2003). Molecular systematics of cowries (Gastropoda: Cypraeidae) and diversification patterns in the tropics. *Biological Journal of the Linnean Society*, *79*, 401–459. https://doi.org/10.1046/j.1095-8312.2003.00197.x

Miller, S. R., & Lawrence, J. M. (1999). Gonad and pyloric caeca production in the nine-armed starfish *Luidia senegalensis* off the southwest Florida Gulf coast during the annual reproductive cycle. *Bulletin of Marine Science*, *65*(1), 175–184.

Miloslavich, P., Díaz, J. M., Klein, E., Alvarado, J. J., Díaz, C., Gobin, J., Escobar-Briones, E., Cruz-Motta, J. J., Weil, E., Cortés, J., Bastidas, A. C., Robertson, R., Zapata, F., Martín, A., Castillo, J., Kazandjian, A., & Ortiz, M. (2010). Marine biodiversity in the Caribbean: Regional estimates and distribution patterns. *PLoS One*, *5*(8), e11916. https://doi.org/10.1371/journal.pone.0011916

Miloslavich, P., Klein, E., Díaz, J. M., Hernández, C. E., Bigatti, G., Campos, L., Artigas, F., Castillo, J., Penchaszadeh, P. E., Neill, P. E., Carranza, A., Retana, M. V., Díaz de Astarloa, J. M., Lewis, M., Yorio, P., Piriz, M. L., Rodríguez, D., Yoneshigue-Valentin, Y., Gamboa, L., & Martín, A.

(2011). Marine biodiversity in the Atlantic and Pacific coasts of South America: Knowledge and gaps. *PLoS One*, *6*(1), e14631. https://doi.org/10.1371/journal.pone.0014631

Palumbi, S., Martin, A., Romano, S., McMillan, W. O., Stice, L., & Grabowski, G. (1991). *The Simple Fool's Guide to PCR*, *Version 2*. Department of Zoology and Kewalo Marine Laboratory, University of Hawaii.

Pernet, B., Livingston, B. T., Sojka, C., & Lizárraga, D. (2017). Embryogenesis and larval development of the seastar *Astropecten armatus*. *Invertebrate Biology*, *136*(2), 121–133.

Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology*, *21*, 1864–1877. https://doi.org/10.1111/j.1365-294X.2011.05239.x

Ratnasingham, S., & Hebert, P. D. (2007). BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). *Molecular Ecology Notes*, *7*(3), 355–364.

Ratnasingham, S., & Hebert, P. D. (2013). A DNA-based registry for all animal species: The Barcode Index Number (BIN) system. *PLoS One*, *8*(7), e66213. https://doi.org/10.1371/journal.pone.0066213

Reyes-Bonilla, H., Vázquez-Arce, D., González-Cuéllar, O. T., Herrero-Pérezrul, M. D., & Weaver, A. H. (2018). Mass aggregation of the cushion starfish *Pentaceraster cumingi* in the southern Gulf of California. *Marine Biodiversity*, *48*(2), 949–950. https://doi.org/10.1007/s12526-016-0529-7

Robertson, D. R., Christy, J. H., Collin, R., Cooke, R. G., D'Croz, L., Kaufmann, K. W., Heckadon Moreno, S., Mate, J. L., O'Dea, A., & Torchin, M. (2009). The Smithsonian Tropical Research Institute: Marine research, education, and conservation in Panama. *Smithsonian Contributions to Marine Sciences*, *38*, 73–93. https://doi.org/10.5479/si.01960768.38.73

Scheibling, R. E. (1981). The annual reproductive cycle of *Oreaster reticulatus* (L.) (Echinodermata: Asteroidea) and interpopulation differences in reproductive capacity. *Journal of Experimental Marine Biology and Ecology*, *54*(1), 39–54.

Schubart, C. D., Cuesta, J. A., & Felder, D. L. (2002). Glyptograpsidae, a new brachyuran family from Central America: Larval and adult morphology, and a molecular phylogeny of the Grapsoidea. *Journal of Crustacean Biology*, *22*(1), 28–44. 10.1651/0278-0372(2002)022[0028:GANBFF]2.0.CO;2

Sewell, M. A., & Jury, J. A. (2011). Seasonal patterns in diversity and abundance of the high Antarctic meroplankton: Plankton sampling using a Ross Sea desalination plant. *Limnology and Oceanography*, *56*(5), 1667–1681. https://doi.org/10.4319/lo.2011.56.5.1667

Song, H., Buhay, J. E., Whiting, M. F., & Crandall, K. A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(36), 13486–13491. https://doi.org/10.1073/pnas.0803076105

Zulliger, D. E., & Lessios, H. A. (2010). Phylogenetic relationships in the genus *Astropecten* Gray (Paxillosida: Astropectinidae) on a global scale: Molecular evidence for morphological convergence, species-complexes and possible cryptic speciation. *Zootaxa*, *2504*, 1–19. https://doi.org/10.11646/zootaxa.2504.1.1