

Knots, spoons, and cloches: DNA barcoding unusual larval forms helps document the Diversity of Neotropical Marine Annelids

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Abstract

The morphological diversity of marine annelid larvae is stunning. Although many of the larval forms have been categorized as trochophores or modified trochophores there are a few groups with distinctive larval features that make them easy to distinguish from other annelid larvae. We collected 252 annelid larvae from the plankton, with particular emphasis on oweniids, polygordiids, and thalassematids (i.e., echiurans) and sequenced fragments of their cytochrome c oxidase subunit 1 and 16S ribosomal RNA genes. We found 6 oweniid, 5 polygordiid, and 8 thalassematid OTUs. Thalassematids were found only in samples from the Pacific, while oweniids were found only in Caribbean samples. Among the oweniids we found two distinct morphotypes, one with a narrow, cloche shape and another that had a wider and more rectangular shape with clearly-developed lappets. Among the polygordiids we identified one larva as *Polygordius eschaturus* and several larvae as *Polygordius jenniferae*. All larvae, except for the *Polygordius eschaturus*, which was at a stage too early to make a determination, were endolarvae. Among the thalassematids we identified larvae of *Ochetostoma edax*, and found 7 unidentified OTUs. Finally, 150 miscellaneous polychaete larvae were sequenced, representing ~76 OTUs. 4 rostraria larvae from the Caribbean, whose sequences confirm the long-held assumption that they are amphinomids, could not be identified to species. In total only 5% of these OTUs could be identified to species with known sequences and most could not be identified to genus or even family with reasonable certainty. It is clear that metabarcoding approaches, although useful for documenting the number of OTUs, will be of limited use for identifying them until reference databases have improved their coverage for neotropical marine annelids.

Introduction

The morphological diversity of marine annelid larvae (see Pernet, Qian, Rouse, Young, & Eckelbarger, 2002) has often been summarized as consisting of trochophores or modified trochophores (see review in Rouse, 1999), and most pass through subsequent larval stages where segments are added to the posterior end of the larva. Although many marine annelid larvae look superficially similar, larvae in some families have unique morphologies, which make them easy to distinguish from larvae from other families. These unique morphologies have attracted researchers interested in functional morphology and the evolution of diversity (Emlet & Strathmann, 1994; Miner, Sanford, Strathmann, Pernet, & Emlet, 1999; Pernet et al., 2002).

As part of a study documenting the invertebrate larval diversity in the coastal waters of Panama, we made focused collections of annelid larval forms for three families which occur in soft sediments and are generally poorly documented in the Neotropics. Specifically, the collections focused on the mitraria larvae of oweniids, the endo- and exo-larvae of polygordiids, and the non-segmented larvae of thalassematids (echiurans). Here we describe the genetic and morphological diversity of larvae from the 3 focal groups from the Caribbean and Pacific coasts of Panama. We also describe the DNA barcodes for an additional 150 annelid larvae which were collected incidentally.

Oweniid annelids are characterized by the unusual mitraria larval form (Wilson, 1932; Smart & von Dassow, 2009; Figure 1). Oweniids are small sedentary worms that are most diverse in shallow-water habitats (Nilson & Holthe, 1985) where they can be important ecosystem engineers (Rabaut et al., 2009). There are currently 52-55 accepted species divided among 4 genera: *Owenia* (18 species), *Galathowenia* (12 species), *Myriochele* (20 species) and *Myriowenia* (2 species), according to the WoRMS (World Register of Marine Species) database at <http://www.marinespecies.org>. Many oweniid species have extremely large reported ranges (Nilson & Holthe 1985; Jolly, Viard, Gentil, Thiébaud, & Jollivet, 2006), which, at least in the case of *O. fusiformis*, include numerous divergent clades, suggesting that names are applied to a mix of multiple cryptic species (Jolly et al. 2006). Recent phylogenetic studies confirm that oweniids are one of the earliest branching extant annelid families (Weigert et al. 2014; Helm, Vöcking, Kourtesis, & Hausen, 2016 and references therein), thus they are important in understanding annelid evolution. Mitraria larvae have been subject of various detailed studies on the hydrodynamics of larval dispersal (Barnay et al. 2003; Verdier-Bonnet, Carlotti, Rey, &

Bhaud, 1997; Guizien, Brochier, Duchêne, Koh, & Marsaleix, 2006; Thiébaud, Dauvin, & Lagadeuc, 1992; 1994) although their unique morphology may make them a poor model for the hydrodynamics of other worm larvae.

Polygordiids are thin interstitial worms which can reach impressive densities in shallow soft bottom habitats, where they can sometimes be the dominant benthic organisms (Ramey, 2008). The group is small with only 20 valid species of *Polygordius*, the only genus in the family (Tustison, Ramey-Balci, & Rouse, 2020). 13 of these species have been sequenced for COI or 16S (Lehmacher, Ramey-Balci, Wolff, Fiege, & Purschke, 2016; Ramey-Balci, Fiege, & Struck, 2018; Tustison et al. 2020). The *Polygordius* larva (sometimes referred to as Lovén's larva) is characterized by a large inflated body comprised of a transparent episphere and hyposphere (Cowles, 1903; Ramey-Balci & Ambler, 2014; Figure 2). In exolarvae the definitive worm body extends posterior to the episphere and hyposphere while in endolarvae the body of the developing worm is tucked up inside the episphere and hyposphere (Figured in Ramey-Balci et al. 2014). Exolarvae have been reported for *P. jouinae*, *P. triestinus*, and *P. neapolitanus*, while endolarvae have been reported for *P. appendiculatus*, *P. lacteus* and unidentified *Polygordius* from the east coast of North America (Ramey-Balci et al. 2014) and southern Chile (Cañete et al. 2019). The larval forms of the other species are so far unknown.

Thalassematids (echiurans) were historically considered to belong to their own phylum characterized among other features, by the apparent lack of true segments in the adult, which developmental studies have now shown are present early in development and subsequently lost in the adults (Hou et al. 2019). However molecular analyses place echiurans well inside the annelids (e.g., McHugh 1997; Stuck et al. 2007; Wu et al. 2009) and they are newly considered to belong to the family Thalassematidae (Goto, Monnington, Sciberras, Hirabayashi, & Rouse, 2020). The diversity of the group is poorly known as many species live in the deep sea, and their soft bodies and propensity to shed the proboscis make them difficult to collect intact. Thalassematids are well-known for the occurrence of environmental sex determination in some species (Jaccarini, Agius, Schembri, & Rizzo, 1983) and their burrows are important resources for a number of commensal organisms including fishes, copepods, shrimps and bivalves (Anker et al. 2005; Anker, Hurt, & Knowlton, 2007; Goto, Ishikawa, & Hamamura, 2017; Goto, 2017). They may occasionally be ecologically important, for example fostering diversity in sewage outfall sites (Stull, Haydock, & Montagne, 1986). There are few publications focusing on the

identification and ecology of thalassematid larvae (but see Pilger, 1978; Suer & Phillips 1983) and live larvae are rarely figured. So far there are published reports for only 2 species of echiurans, *Alomasoma belyaevi* and *Anelassorhynchus panamensis*, from the Pacific coast of Panama (Biseswar, 2012; 2016). No species have been reported for the Caribbean coast (Biseswar, 2009). However, unidentified *Echiura* collected from both coasts are included in museum collections indicating that the absence of published records does not reflect an absence of the animals.

Materials and Methods

Sample Collection - Annelid larvae from three distinct sets of samples were photographed and sequenced. Caribbean samples were collected from Bahia Almirante, Bocas del Toro Province, with a 0.5m diameter 125µm mesh plankton net towed horizontally behind a small boat. 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. During the course, 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 and a number of annelids were included.

In 2015-2016, structured sampling involved a campaign of 4 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 a 9-10 day interval, between 7am and 9am. Tows were conducted at a single spot 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 flowmeter was attached to the mouth of the net to determine the volume of water sampled and a depth meter showed that tows generally covered a depth range of 10-20m over a 25m soft bottom.

Pacific samples were taken 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 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 and obtain samples from ~10-40 m over a soft bottom.

Samples were sorted under a stereomicroscope and worm larvae were moved to dishes of filtered sea water. The 2014-2016 samples were sorted exhaustively and all larvae of oweniids, polygordiids, and thalassematids were counted, providing data on larval density. Other unusual or interesting larvae were also selected for sequencing. For morphologically similar larvae, we sequenced no more than 6 of a similar morphotype from each tow. Even when we collected small numbers of larvae, we could not always sequence all of the individuals as animals that were damaged during collection often did not survive long enough to be photographed. For the other samples, taxa of interest were picked out but no effort was made to ensure that all individuals were detected. Larvae were photographed alive, often moving, in a depression slide under a dissecting microscope prior to preservation for DNA sequencing. These fast-moving and changeable larvae required rapid adjustments to the zoom and focus, making concomitant photographs of a scalebar impossible; however, notes on the approximate size of the larvae were made. During the course in 2013 some larvae were relaxed with a solution of MgCl₂ isotonic with seawater prior to being photographed under a compound microscope with differential interference contrast (DIC), resulting in fewer processed samples and more frequent sequencing failures. Notes were taken on the overall appearance, morphological details, and approximate size of each larva before they were preserved for sequencing.

DNA Sequencing – individual larvae were preserved in 150 µl of M2 extraction buffer (AutoGen), frozen and shipped to the Smithsonian's Laboratories of Analytical Biology for extraction and sequencing. Plates with larval samples were extracted using an AutoGenprep 965 phenol-chloroform extraction robot after overnight digestion in the AutoGen buffers with proteinase-K. The suspension volume of extracted DNA was 50 µl. The DNA barcode fragment of the cytochrome oxidase subunit I (COI) was amplified using primarily the primer pair jgLCO1490/jgHCO2198 (Geller, Meyer, Parker, & Hawk, 2013), although the pair dgLCO1490/dgHCO2198 (Meyer 2003) was also used. The 10 µl PCR cocktail included 5 µl GoTaq Hot Start Mix (Promega), 0.1 µl BSA, 0.3 µl each of 10 mM primer. For amplification and sequencing of 16S, the primer pair 16S AR/16S BR (Palumbi et al. 1991) was used. The 10 µL cocktail for 16S used Biolase Taq (Bioline) with the addition of 0.5 µL of 10 mM dNTPs and 0.5 µL of 50 mM MgCl₂. The annealing temperature for all reactions was 50° C.

Data Analyses – Sequences were screened for quality and used to produce contigs of forward and reverse amplicons with Sequencher 5.4.6 (Gene Codes). For COI, only sequences more than 450bp in length and with a Phred quality score ≥ 30 for more than 85% of the bases were combined into contigs and used for analysis. For 16S only sequences greater than 400bp were used. To check for potential contamination, sequences were compared, within the BOLD project workbench (www.boldsystems.org), to all larvae of all phyla sequenced in our project. Likewise, we used BLAST searches to compare our sequences with those available in GenBank. Redundant sequences were removed from trees to aid visualization. Those that produced unusually long branches or were otherwise suspicious were uncommon, and were removed from the dataset. COI alignments were inferred with the BOLD aligner [amino acid based Hidden Markov Model (Ratnasingham & Hebert, 2007)] whereas 16S alignments used ClustalX (gap-opening penalty: 15, gap-extension penalty: 6.66, delay divergent cutoff: 30%, DNA weight matrix: IUB, transition weight: 0.5) followed by manual corrections. Operational Taxonomic Units (OTUs) were identified with the Automatic Barcode Gap Discovery method (Puillandre, Lambert, Brouillet, & Achaz, 2012) [Pmin: 0.001, Pmax: 0.1, Steps: 10, X: 1.5, Distance: Kimura (K80), TS/TV: 2].

For oweniids, neighbor-joining trees (BIONJ, Gascuel 1997) with Kimura 2-parameter distances were constructed independently for each marker from our sequences, plus every oweniid COI or 16S sequence available in GenBank and BoLD as of May 2020. The purpose of this, as with most DNA barcoding, was not to construct a phylogeny, but to visualize the sequence similarity across the dataset to aid in OTU identification. For oweniids in particular the use of COI was the most appropriate approach as 16S failed to amplify for several of the OTUs (see below).

For the analyses of polygordiids and thalassematids we generated Maximum Likelihood trees from a concatenated dataset of the 2 markers, using a GTR model with Gamma distribution and the proportion of invariable sites (GTR+G+I). This model was separately applied to each marker based on the partition scheme suggested by Partition Finder program. Each marker was separately aligned with ClustalX [gap opening penalty: 15, gap extension penalty: 6.66, DNA weight matrix: IUB, transition weight: 0.5, delay divergent cutoff: 30%], followed by manual corrections. Then, each alignment was analyzed with JModelTest (Posada, 2008) and MEGA (Kumar et al., 2008) to find the best model of evolution associated with each marker plus their

gamma and I parameters. The alignments were concatenated and evaluated with PartitionFinder (Lanfear, 2017), which showed that no additional partitions were necessary, before the final analysis. This scheme was then incorporated in the subsequent ML analysis performed with RAxML (Stamatakis, 2014). For the *Polygordius* tree, we used the ingroup species represented by individuals from the Tustison et al. (2020) dataset, and all of our larval specimens that had sequences for both markers. All of the larval OTUs are represented by at least one individual in the resultant phylogeny. For the thalassematid tree, we used all of the Thalassematinae from the dataset in Goto et al. (2020) plus two Bonellinae. We included all of the larval specimens that had sequences for at least one marker, as we did not want to exclude unique OTUs, some of which were only successfully sequenced for one marker.

Results and Discussion

Overall, we successfully obtained sequences from 252 annelid larvae (excluding sipunculans and chaetopterids). 102 of these larvae fell into our focal groups (Table 1) and many of the rest could not be confidently assigned to family based on our level of morphological observation. Oweniids were found only in Bahia Almirante (62 larvae preserved, 56 sequenced successfully), thalassematids were found only in the Bay of Panama (31 larvae preserved, 28 sequenced successfully), and polygordiids were found in both oceans (23 larvae preserved and 18 sequenced successfully). DNA sequences have been deposited in GenBank (Table 1), the datasets are available in BOLD at: DOIxxxxxx. The datasets and tree files used for analyses are available in FigShare at: XXX. The results from each group are described and discussed below.

Oweniids - We obtained 44 COI and 29 16S sequences from the 62 individuals preserved for sequencing. The COI sequences fell into 6 OTUs all of which were from the Caribbean (Figure 4). Three of these were represented by numerous larvae (C1, C5, C6), OTU C2 had 3 larvae, OTU C4 had 2 larvae, and C3 was a singleton. These OTUs all differed from each other and from previously published sequences by $\geq 10\%$ Kimura 2-parameter distance in COI. Sequencing of 16S was successful for OTUs 5 and 6 and failed for the remaining OTUs (Figure 5). GenBank and BOLD include sequences from two COI OTUs identified as *Galathowenia oculata* (from Spain and Canada) and 9 OTUs identified as *Owenia fusiformis* (from Manitoba and Newfoundland, Canada, Alaska, and various places in Europe). None of our sequences fell

into these OTUs. Two of our common OTUs (C5 and C6) grouped most closely with *Galathowenia* while the other 4 OTUs (C1-4) clustered more closely with *Owenia* (Figure 5). Therefore, it appears likely that the success of 16S sequencing with the primers we used is clade specific. As COI sequences from the other two genera in the family, *Myriochele* and *Myriowenia*, are not available we cannot eliminate the possibility that our OTUs belong to one of these genera.

The larvae we collected could be distinguished to some extent based on gross morphology (Figure 1). They could be rapidly sorted into morphotypes based on two characters, the body shape and the color and thickness of the chaetae. The larval body was generally either tall, narrow and cloche-shaped or wider and more rectangular with clearly-developed lappets, similar to the larvae of *O. collaris* (described in Smart & von Dassow, 2009). The chaetae were either uniformly thin and colorless, or wide blade-shaped chaetae with blue birefringence were mixed with thin chaetae. Larvae from OTUs C1-C4, which clustered near *Owenia* were all of the transparent kind. They had thin chaetae and a relatively rectangular body. Unfortunately, we were not able to capture a photograph of the single larva in OTU C3, and 2 of the other OTUs were represented by photographs of one and 2 individuals respectively, insufficient numbers to detect definitive differences between these 4 OTUs. Both OTUs that cluster with *Galathowenia* had a narrow, cloche shape. In OTU C6 the larvae had both blade-shaped and thin chaetae, while in OTU C5 the larvae appeared to only have the thin chaetae (Figure 1). However only 4 individuals of OTU C5 were photographed leaving the possibility that the blade-shaped chaetae were present at another developmental stage or had been shed due to stress. However, this seems unlikely as all of the individuals of OTU C6 for which we have photos do have such chaetae.

Published laboratory studies of the larval development and morphology of mitraria can give some insight into the variation in larval form we discovered. The larvae of *O. collaris* from Oregon and *O. fusiformis* from the UK appear very similar to our OTU C1 with a rounded cross section early in development followed by a slightly triangular dorsal profile, and finally well-developed lappets by the time more than 2-3 pairs of chaetae have developed (Wilson, 1932; Smart & von Dassow, 2009). The development of *O. fusiformis* from France documented by Helm, Vöcking, Kourtesis, & Hausen (2016) show the body of a 1-day old larva as quite triangular before the broadening of the body with the development of the lappets. At this stage the larvae look somewhat similar to our cloche-shaped larvae, but the larvae lack chaetae. Our

cloche-shaped larvae were well-developed, and in some cases the segmented juvenile body could be seen projecting from the larval body, indicating they were not early stages of another form. The larvae of neither *O. collaris* nor *O. fusiformis* develop wide blade-shaped chaetae (Wilson, 1932; Smart & von Dassow 2009; Helm et al. 2016). However, larvae with blade-like chaetae have been reported from England by Wilson (1932), who thought them likely to belong to species of *Myriochele*. Müller (1854) reported something similar from Messina in Italy, suggesting that this morphology is not limited to tropical species. Finally, our mitraria lacked distinctive pigmentation patterns which are often useful in identifications of larvae in other groups. This is not always the case for mitraria, as Fewkes (1889) found mitraria in the Bay of Fundy with red pigment patches on the prototroch, and larvae with similar pigmentation have recently been found in California (<https://bodegahead.blogspot.com/2015/07/laser-light-show.html>). Although not the focus of this study, these observations suggest that sufficient morphological variation exists among mitraria that they could provide useful traits for taxonomic or phylogenetic work, and possibly help identify larva to genus or even to species.

Polygordiids – We obtained 17 COI and 14 16S sequences from the total of 23 individuals preserved for sequencing. These sequences fell into 5 OTUs, 1 from the Bay of Panama and 4 from the Caribbean (Figure 6). These all differed from each other by >10% pairwise distance in COI and >5% in 16S. GenBank and BOLD include COI sequences from 13 other species and 16S sequences for an additional one (reviewed in Tustison et al. 2020). Pairwise COI distances between these species were likewise <10% (Tustison et al. 2020).

Sequences from a single larva from the Bahia Almirante fell within the clade of sequences from *P. eschaturus* Marcus 1948, which were collected in Brazil (Ramey-Balcı et al., 2018), all of which had >95% pairwise similarity in COI. Adults of this species are known only from Brazil and with this result we were able to confirm its presence in Panama. The distributions of marine annelid species are not well-documented, but a number of other taxa including the well-known echinoids *Echinometra lucunter*, *Clypeaster subdepressus*, and *Eucidaris tribuloides* all occur along the tropical Atlantic coast of the Americas, from North America to subtropical Brazil (Collin, Rendina, Goodwin, and McCabe 2018), despite the potential barrier to dispersal created by the low salinity plume generated by the Amazon River. The single specimen we collected was very early in development (Figure 2), suggesting it

originated from nearby and little detail could be observed other than a single pair of red eyes and opaque blue-green spots around the prototroch.

The most abundant OTU clustered with *P. jenniferae* Tustison et al. 2020 which was described from a single specimen collected in Belize. Our samples differed by less than 2% pairwise genetic distance from this sample, and we can therefore confirm that this species also occurs in Panama. The larvae of *P. jenniferae* were endolarvae with yellow pigment spots and a line of orange pigment around the prototroch, yellow spots near the mouth and yellow and black spots on the pygidium. In early stages there were yellow spots on the hyposphere (Figure 2). In the most mature larvae 6 segments extended from the hyposphere.

The other 3 larval OTUs were not conspecific with previously sequenced animals. The OTU from the Pacific appears to be sister to an unidentified species from California (Figure 6). The 2 individuals in this larval OTU from the Bay of Panama had an apical organ flanked by a pair of red eyes. The prototroch had an outermost ring of yellow pigment with a ring of alternating yellow and black dots medial to it and a ring of yellow dots medial to that (Figure 2). In both larvae there were 7 body segments extending out of the episphere and there was a ring of black dots on the pygidium. The gut had a yellowish tint and a ring of anal papillae gave the pygidium a scalloped appearance. The presence of anal papillae can be taxonomically informative for the adults, but it is not known if this carries over from the larval morphology. OTU C1 was a singleton endolarva, with a few green spots along the prototroch. We were not able to obtain an informative photograph of this individual and it seems to be most closely related to *Polygordius* species from Europe (Figure 6). The two larvae from OTU C2 had 10-11 segments extending from the episphere, one pair of red eyes, a yellow ring around the prototroch and red spots medial to that (Figure 2).

Thalassematids – We obtained 28 COI and 15 16S sequences from 31 individuals preserved for sequencing, all from the Bay of Panama. 8 OTUs were detected with the COI dataset and 7 of these were also detected with 16S. Five OTUs were represented by single individuals in the COI dataset and one of these individuals failed to sequence for 16S, thus accounting for the difference in OTUs between markers. Otherwise there was complete concordance between the COI and 16S results. All of our OTUs differed from each other by > 10% pairwise genetic distance.

Maximum likelihood analyses show that all of our larval OTUs fell within the Thalassematini

(Figure 7). For ease of presentation only the Thalassematinae are shown in Figure 7, with the tree rooted between the Thalassematini and the Echiurini following the results of Goto et al. (2020). One of the most common OTUs had >99% sequence similarity with published sequence of *Ochetostoma edax* from the Gulf of California (Figure 7). One singleton larva (OTU P1) nested within a clade of *Arhynchite* species (Figure 7). The remaining larval OTUs are too divergent from the other sequenced species to determine their affinities but OTUs P2, P4 and P5 appear to be closely related to each other (Figure 7).

In this study, we have more than tripled the documented diversity of thalassematids from the tropical eastern Pacific with only moderate sampling effort of planktonic larvae and have added clades that were not previously represented to the molecular phylogeny of the group. We cannot determine if any of our larvae belonged to the two species that have been previously reported from Panama. Sequences are not available for *Anelassorhynchus panamensis* and the available sequence for *Alomasoma belyaevi* is for an animal collected from near South Orkney Islands (Goto et al. 2020), which brings into question the veracity of the record of this species in Panama. Such significant increases in species diversity are typical of barcoding studies focusing on larvae from poorly studied groups of invertebrates (Barber & Boyce, 2006; Mahon, Thornhill, Norenburg, & Halanych, 2010; Collin, Venera-Pontón, Driskell, Macdonald, M. J. Boyle 2019; Collin et al. 2019).

Morphologically the larvae are very difficult to distinguish (Figure 3), and only 3 OTUs were represented by multiple individuals. Superficially these specimens can be divided into the typical larvae that are heavily pigmented with the green spots characteristic of thalassematids (see Goto 2016) and those that are virtually unpigmented. OTUs P5 and P7 were both composed of a mixture of green and pale larvae (Figure 3). In general, the pale larvae appeared to be closer to metamorphosis than the more densely pigmented larvae; they often lacked the mesotroch, showed only fragments of the prototroch, and the body segments were less evident (Figure 3). This is consistent with published observations that thalassematid larvae lose or reduce the green pigment prior to metamorphosis. Many of the larvae we collected had a very obvious pair of hooked chaetae on the anterior ventral part of the body, while in others these chaetae were not visible. They appeared to be more prominent in more mature larvae (Figure 3). We did not observe posterior chaetae in any of the larvae we examined.

Two of the most abundant larval OTUs clearly changed color during development. This has been observed in laboratory-raised larvae of some species (J. Pilger pers. com. 2019) but not others (W.B. Jaeckle pers. com. 2019). Bonellin, the green integumentary pigment expressed by *Bonellia viridis*, develops during early embryonic development (Agius & Jaccarini, 1981). The pigment disappears after settlement but, prior to that, pigment expression can be highly influenced by light exposure (Agius & Jaccarini, 1981). Embryos kept in high light conditions expressed less pigment, if any, and pigment expression was only partially rescued by a shift to the dark (Agius & Jaccarini, 1981). It is therefore possible that the observed variation in pigment among our larvae was due to developmental conditions in addition to developmental stage. Variation in pigmentation could also be due to variation among groups, as Goto (2016) showed that the green pigment common to many thalassematids has a distinct phylogenetic distribution. However, the two OTUs for which we documented multiple larvae, show morphological differences associated with the differences in color; pale larvae often lacked a prototroch, or the prototroch appeared to be reduced or disorganized, the larvae tended to be longer and slimmer and full of lipid droplets, and the segments were less evident. These changes are typical of larvae preparing for settlement and metamorphosis.

Other Annelids – We obtained an additional 130 COI sequences and 113 16S sequences for miscellaneous annelid larvae and small planktonic annelids worms. 70 OTUs were detected with the COI dataset and 6 additional OTUs were detected with 16S in specimens that failed to sequence with COI. The COI dataset was indexed by BOLD into 70 Barcode Index Numbers (BINs; Ratnasingham & Hebert, 2013), of which only 9 included sequences already present in the BOLD database. One of the BINs included sequences identified as *Loimia salazari* and *L. medusa*, one included a sequence identified as *Subadyte* sp. and five other BINs included sequences identified only to the family level (i.e. Sabellariidae, Terebellidae, Pilargidae, Hesionidae, and Pectinariidae). Two other BINs only included sequences identified as “Polychaeta”. Four COI OTUs were not assigned a BIN but one of them had >95% identity with *Alitta acutifolia* (KX537500) and *A. succinea* (JF293301) in GenBank. 5 more COI sequences had >90% identity with GenBank sequences, suggesting a possible congeneric relationship. These were *Scolelepis acuta* (KU900484), *Spiophanes uschakowi* (KM998750) and *Spiophanes norrisi* (MF121352; 3 individuals). In the 16S dataset 2 sequences had > 95% identity with

sequences in GenBank, indicating at least a congeneric relationship with *Bhawania heteroseta* (EU555044) and *Glycera americana* (KT989321). To summarize, 10 of 70 COI OTUs shared >95% identity with previously published sequences but only 2 of these included reference material that had been identified to species, while 2 of 76 16S OTUs shared >95% identity with previously published sequences, both of which were identified to species.

One group we had hoped to identify with this approach were the rostraria larvae (Figure 8). Four of these distinctive larvae were collected. It is widely thought that they belong to amphinomid annelids (Bhaud 1972; Toso et al. 2020), a group that includes the fireworm *Hermodice carnaculata*, an important predator on corals. These worms are abundant on the coral reefs in Bocas del Toro and we speculated that the collected rostraria larvae might belong to this species. However, we found that for 3 of these larvae both the COI and 16S had only ~82% identity with sequences from *H. carnaculata* (COI: KC017629 and MG251653; 16S: KC017560), which was the closest match generated by BLASTn searches of GenBank. The 16S sequence of the fourth larva showed similar sequence identity to another amphinomid, *Cryptonome barbada* (NC037947). Comparison with a large unpublished amphinomid dataset (E. Borda, pers. com. 2019) showed that these larvae are associated with the Amphinomia clade of amphinomids but no greater taxonomic resolution could be obtained. No other larvae blasted to any amphinomid. These results are the first to confirm the long-held supposition that rostraria larvae belong to amphinomid annelids, but are disappointing in that they do not allow us to pinpoint their identity with more taxonomic resolution.

In most cases, we were not able to identify larvae to below super-familial level with any certainty based on larval morphology observed through the stereomicroscope, and coverage of marine annelid diversity in BOLD and GenBank was insufficient to provide assistance. In general, a DNA barcoding approach to identifying organisms is only effective when sequences of close relatives are present in the reference database (Ekrem, Willassen & Stur, 2007; Vences, Thomas, Bonett & Vieites, 2005). Efforts have been made to populate the marine annelid datasets for temperate regions, with significant efforts made in Europe and North America (Carr, Hardy, Brown, Macdonald, & Hebert, 2011; Lobo et al. 2016) as well as Patagonia (Canales-Aguirre Rozbaczylo, & Hernández, 2011; Maturana et al. 2011) and the Southern Ocean (Brasier et al. 2016), but little effort has been spent to generate barcode databases for tropical marine annelids. The result of low sampling effort and high tropical diversity is that currently GenBank

and BOLD databases have little power to identify unknown marine invertebrate sequences. For example, of ~8000 metazoan OTUs sequenced from eDNA in seawater from Bahia Almirante in Bocas del Toro, only ~4% could be identified to species using reference databases (Nguyen et al. 2019). This knowledge gap will become a significant limitation as more studies turn to metabarcoding or eDNA to survey biodiversity. In our dataset more than 50% of OTUs had best matches in GenBank with <85% sequence identity for 16S, and more than 70% of COI OTUs had <85% identity with sequences in GenBank, highlighting how taxonomic affinities could not be determined with any certainty from DNA sequence alone.

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Table 1: Summary of OTUs and sequences generated for the larvae collected in this study.

OTU/Species*#	BIN	Genbank # COI	Genbank # 16S	Location	Collection dates
Oweniidae					
Larval OTU C1		MN662509 MN662497 MN662516 MN662506 MN662515 MN662502 MN662508 MN662512 MN662503	-	Bahia Almirante	November 2015, February 2016, March 2016, June 2016, September 2016
Larval OTU C2		MN662496 MN662518 MN662494	-	Bahia Almirante	March 2016
Larval OTU C3		MN662525	-	Bahia Almirante	March 2016
Larval OTU C4		MN662531 MN662498	-	Bahia Almirante	November 2015, February 2016
Larval OTU C5		MN662513 MN662517 MN662493 MN662500 MN662528 MN662507 MN662491 MN662527 MN662504 MN662501	MN662477 MN662480 MN662463 MN662469 MN662485 MN662472 MN662461 MN662484 MN662471 MN662470 MN662465	Bahia Almirante	March 2016, June 2016, September 2016, December 2016
Larval OTU C6		MN662492 MN662526 MN662490 MN662519 MN662510 MN662530	MN662462 MN662483 MN662474 MN662460 MN662482 MN662459	Bahia Almirante	July 2013, August 2015, November 2015, February 2016, June 2016, September 2016, December 2016

		MN662505 MN662489 MN662523 MN662488 MN662529 MN662522 MN662514 MN662511 MN662499 MN662495 MN662521 MN662524 MN662520	MN662486 MN662479 MN662475 MN662468 MN662466 MN662481 MN662473 MN662478 MN662464 MN662476 MN662467 MN662487		
Polygordiidae					
Larval OTU C1		MN619299	MN619260	Bahia Almirante	February 2016
Larval OTU C2		MN619290 MN619287	MN619251 MN619252	Bahia Almirante	July 2013, November 2015, September 2016
<i>Polygordius jenniferae</i>		MN619300 MN619293 MN619297 MN619289 MN619294 MN619291 MN619301 MN619303 MN619288 MN619292 MN619295	MN619261 MN619255 MN619258 MN619256 MN619253 MN619262 MN619264 MN619254 MN619257	Bahia Almirante	July 2013, August 2015, February 2016, June 2016, September 2016, December 2016
<i>Polygordius eschaturus</i>		MN619296	-	Bahia Almirante	August 2015
Larval OTU P1		MN619302 MN619298	MN619263 MN619259	Bay of Panama	March 2014
Thalassematidae					
Larval OTU P1		MN619329	MN619283	Bay of Panama	March 2014
Larval OTU P2		MN619315	-	Bay of Panama	March 2014

Larval OTU P3		MN619307	MN619272	Bay of Panama	May 2014
Larval OTU P4		MN619313	MN619276	Bay of Panama	April 2014
Larval OTU P5		MN619312 MN619323 MN619327 MN619305 MN619319 MN619328 MN619321 MN619325 MN619322	MN619275 MN619281 MN619282	Bay of Panama	March 2014, April 2014, May 2014, June 2014
Larval OTU P6		MN619331	MN619285	Bay of Panama	November 2014
Larval OTU P7		MN619311 MN619318 MN619324 MN619330 MN619304 MN619317 MN619306 MN619310 MN619314 MN619326	MN619279 MN619284 MN619271 MN619277	Bay of Panama	March 2014, April 2014, May 2014, June 2014
<i>Ochetostoma edax</i>		MN619320 MN619309 MN619308 MN619316	MN619280 MN619274 MN619273 MN619278	Bay of Panama	November 2014

Figure Captions

Figure 1: Plate of the owenid larvae. Top row shows the mitraria larvae that are more closely related to *Owenia* and the bottom row are the mitraria that are more closely related to *Galathowenia*. Larval bodies are typically 200-300 microns in the anterior to posterior axis.

Figure 2: The *Polygordius* larvae collected during this study. The top row shows different stages of the most common Caribbean species – *P. jenniferae*. The bottom row shows the less common C2 and P1 as well as the only larva of *P. eschaturus* that we collected. It was impossible to obtain measurements of these fast swimming larvae, but the spherical part of the larvae ranged from 300 microns to 600 microns and the emergent larval bodies were ~1mm long. We were unable to capture photographs of larvae in OTU C1.

Figure 3: Thalassematid larvae collected during this study. **P2** is shown in ventral view with the neurotroch visible running anterior-posterior between the telotroch and mesotroch. **P4** is shown in ventral view, and the small paired chetae are visible near the mesotroch. **P5** was represented by a number of larvae, the larva figured on the left is shown in ventral view and the neurotroch is clearly visible. The more mature stage on the right has already lost the mesotroch and distinctive larval pigment, and the lateral view shows both chetae. **P6** was represented by a single pale late-stage larva figured here. **P7** is shown with a lateral view (left) with the mouth visible in profile on the right side between the prototroch and mesotroch (~200-300 um) and a dorsal view of a slightly later stage is shown on the right (~500um). *Ochetostoma edax* larvae are marked with very distinct large green spots, which were similar arranged in all 4 larvae we collected. The two photographs show the same larva photographed a few second apart. Scale bars are not shown as it was impossible to obtain single length measurements of these changeable larvae, but these larvae ranged between 300 mm and 1mm.

Figure 4: Neighbor-joining tree of COI sequences from oweniids constructed from sequences generated by this study (bold) and sequences available in GenBank. To the right, the larval OTUs are labelled with their locations (C, Caribbean) and the OTU number (1–6). The length of the branches represents the substitutions per site (see scale), based on the Kimura 2-parameter

model, whereas the numbers under the branches are the bootstrap support values. Numbers in parenthesis indicate the total numbers of individuals, in our dataset, that share the haplotype of the present sequence (if ≥ 2).

Figure 5: Neighbor-joining tree of 16S sequences from oweniids constructed from sequences generated by this study (bold) and sequences available in GenBank. To the right, the larval OTUs are labelled with their location (C, Caribbean) and the OTU number (1–6). The length of the branches represents the substitutions per site (see scale), based on the Kimura 2-parameter model, whereas the numbers under the branches are the bootstrap support values.

Figure 6: Maximum Likelihood tree of cytochrome c oxidase subunit I (COI) and 16S ribosomal DNA sequences from *Polygordius* larvae from this study and from *Polygordius* adults obtained from GenBank. The tree shows only unique haplotypes, with numbers in parenthesis after the sample name indicating the total numbers of individuals that share the haplotype (if ≥ 2). Branch tips for GenBank sequences are labeled with the species name. To the right, the larval OTUs are labelled with their locations (C, Caribbean; P, Pacific) and the OTU number (1–4). Numbers below the branches are bootstrap support values (only values $>60\%$ are shown). Branch lengths represent the substitutions per site (see scale), added from the separate GTR+G+I model of both markers. Specimens lacking one of the markers were excluded from the tree.

Figure 7: Maximum Likelihood tree of cytochrome c oxidase subunit I (COI) and 16S ribosomal DNA sequences from thalassematid larvae from this study and from thalassematid adults obtained from GenBank. The tree shows only unique haplotypes, with numbers in parenthesis after the sample name indicating the total numbers of individuals that share the haplotype (if ≥ 2). Branch tips for GenBank sequences are labeled with the species name. To the right, the larval OTUs are labelled with their locations (C, Caribbean; P, Pacific) and the OTU number (1–4). Numbers below the branches are bootstrap support values (only values >60 are shown). Branch lengths represent the substitutions per site (see scale), added from the separate GTR+G+I models of both markers.

Figure 8: Rostraria larva (A) RCMBAR601. Length: ~450 microns (B) RCMBAR602. Length: ~300 microns.