Unexpected Molecular and Morphological Diversity of Hemichordate Larvae from the Neotropics

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Abstract

The diversity of tropical marine invertebrates is poorly documented, especially those groups for which collecting adults is difficult. We collected the planktonic tornaria larvae of hemichordates (acorn worms) to assess their hidden diversity in the Neotropics. Larvae were retrieved in plankton tows from waters of the Pacific and Caribbean coasts of Panama, followed by DNA barcoding of mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S ribosomal DNA to estimate their diversity in the region. With moderate sampling efforts, we discovered 6 operational taxonomic units (OTUs) in the Bay of Panama on the Pacific coast, in contrast to the single species previously recorded for the entire Tropical Eastern Pacific. We found 8 OTUs in Bocas del Toro Province on the Caribbean coast, compared to 7 species documented from adults in the entire Caribbean. All OTUs differed from each other and from named acorn worm sequences in GenBank by >10% pairwise distance in COI and >2% in 16S. Two of our OTUs matched 16S hemichordate sequences in GenBank, one was an unidentified or unnamed *Balanoglossus* from the Caribbean of Panama, and the other was an unidentified ptychoderid larva from the Bahamas. The species accumulation curves suggest that nearly all the species have been collected and only one more species might still remain undetected in the Pacfic. In contrast, the Caribbean species accumulation curve suggests that further sampling could yield more than 10 additional OTUs. Tornaria from the 14 OTUs exhibited typical planktotrophic morphologies, and in some cases, may be distinguished by differences in pigmentation and the number of telotrochal ciliary bands, but in general, few diagnostic differences were detected.

Introduction

Marine biodiversity is generally poorly documented and it has been estimated that 30-60% (Appeltans et al., 2012) or even 90% (Mora et al. 2011) of eukaryotic species remain to be described or discovered. Groups that are particularly understudied include most of the smaller marine invertebrate phyla, with tropical regions usually less studied than temperate areas. In many cases, rapid surveys of adult habitats by experts can result in numerous new records of species for a region, as well as new species or even genera of tropical marine invertebrates (e.g., Diaz 2005; Goodheart et al. 2016; Miglietta et al. 2018; Rocha, Faria and Moreno 2005). Although surveys of adults are typically used for documenting local diversity, they may be of limited use for taxa that are particularly difficult to collect, or which occur at depths not easily reached by SCUBA diving. In the few cases where they have been undertaken, surveys of larval diversity have resulted in the discovery of species not documented by studies of adults from the same location (Barber & Boyce 2006; Collin, Venera-Pontón, Driskell, MacDonald, Chan & Boyle 2019; Collin, Venera-Pontón, Driskell, MacDonald & Boyle 2019; Mahon, Thornhill, Norenburg, & Halanych 2010; Maslakova et al. unpublished).

The development of hemichordates, especially enteropneusts or acorn worms, has received significant research attention, as it provides information on the evolution of development in the animal lineage that includes chordates (Freeman et al. 2012; Gonzalez, Uhlinger & Lowe 2017; Gonzalez, Jiang & Lowe 2018; Green, Norris, Terasaki & Lowe 2013; Ikuta et al. 2009; Kaul-Strehlow & Stach 2013; Röttinger & Lowe 2012; Tagawa, Humphreys and Satoh 1998; Tagawa, Nishino, Humphreys, and Sato 1998; Tagawa 2016). As a close relative of chordates, and as a group which may be similar to the deuterostome ancestor (Gerhart, Lowe, & Kirschner 2005; Tassia et al. 2016), these organisms offer us the chance to better understand the origins of chordates and help us to refine our ideas about evolutionary transitions in development. Nevertheless, the diversity of this group as a whole is poorly documented (Tassia et al. 2016), and the larval development of most species is largely unknown.

A recent review of hemichordate diversity showed unusual global patterns across biogeographic regions (Tassia et al. 2016). Along the Pacific coast of the Americas, the lowest recorded diversity was found in the tropical region, with only 1 enteropneust species, *Saxipendium coronatum* Woodwick & Sensenbaugh 1985, reported in the literature for the tropical eastern Pacific compared to 16 in the northeastern Pacific (Tassia et al. 2016). This

pattern is likely the result of relative differences in sampling effort, and it remains to be seen whether it also reflects species diversity. Along the Atlantic coast of the Americas, 10 hemichordates (3 pterobranchs and 7 enteropneusts) have been reported for the greater Caribbean, with far fewer in the adjacent temperate regions (Tassia et al. 2016). The enteropneust species reported for the Caribbean include: *Meioglossus psammophilus* Worsaae, Sterrer, Kaul-Strehlow, Hay-Schmidt, 2012, *Mesoglossus caraibicus* (van der Horst 1924), *Balanoglossus gigas* Müller in Spengel 1893, *Balanoglossus jamaicensis* Wiley 1899, *Glossobalanus crozieri* van der Horst 1924, *Ptychodera bahamensis* Spengel 1893, and *Schizocardium brasiliense* Spengel 1893. Only three of these have DNA sequences in GenBank (Table 1).

As part of a larger effort to document the diversity of marine invertebrates through surveys of larval diversity on both coasts of Panama, we collected, photographed, and DNA barcoded enteropneust (acorn worm) hemichordate larvae from the Bay of Panama (Pacific coast) and Bocas del Toro Province (Caribbean coast). Our sampling strategies differed between the two oceans, following a structured protocol at the Caribbean site while being more exploratory in the Pacific. We used morphological observations, photographs and DNA sequence data to determine whether COI and 16S barcodes can be reliably amplified for hemichordates and to ultimately determine how many operational taxonomic units (OTUs) are present on each coast. We also evaluated whether these larval OTUs can be identified by comparing their DNA with previously identified sequences deposited in GenBank. Our structured sampling in the Caribbean also provided information on the density and seasonality of these larvae.

Materials and Methods

Sample Collection - Tornaria and metamorphosing 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 0.5m diameter 125μm mesh plankton net towed horizontally behind a small boat drifting in the current with the engine alternating in and out of gear to sample the water column by gently bouncing the net through the middle third of the water column (from 10-20m 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 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 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 flowmeter 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 20m. The depth at this site is ~25m.

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 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 approximately 10-40 m.

Samples were sorted using a stereomicroscope and tornaria were moved to dishes of filtered sea water. The 2015-2016 Caribbean samples were sorted exhaustively, providing data on larval density; however, for morphologically similar larvae we prepared no more than 8 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 as 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. For the other samples, taxa of interest were picked out but no effort was made to ensure that all hemichordate larvae were detected. 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 (DIC), resulting in fewer processed samples with higher resolution. 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) in 96-well plates, frozen and shipped to the Smithsonian's Laboratories of Analytical Biology (LAB) for extraction and sequencing. DNA was extracted from plates containing larval samples from a variety of taxa using an AutoGenprep 965 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 (Gellar et al. 2013), although dgLCO1490/dgHCO2198 (Meyer et al. 2003) were also used. The 10 µl PCR cocktail included 5 µl GoTaq Hot Start Mix (Promega), 0.1 µl BSA, and 0.3 µl of each 10 mM primer. For amplification and sequencing of 16S, the primer pair 16S AR/16S BR (Palumbi, Martin, Romano, McMillan, Stice et al. 1991) was used. The cocktail for 16S included Biolase Taq (Bioline) with the addition of 0.5 µL 50 mM $MgCl_2$. The annealing temperature for all reactions was 50° C.

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 BLAST searches. Sequences that were more than 95% similar to other phyla (<2) were eliminated from subsequent analyses. Close similarity to the sparse published sequences for hemichordates was not necessarily expected and was therefore not used as a criterion to retain sequences. We followed the suggestions of Song, Buhay, Whiting, and Crandall (2008) to check for the presence of pseudogenes in our data sets. Briefly, COI sequences were checked and no gaps were indentified, nor were there any stop codons. The AT% content for both COI and 16S was similar to other hemichordate sequences in GenBank (Table 2).

Neighbor joining trees (BIONJ, Gascuel 1997) with Jukes-Cantor distances were constructed from our sequences with every COI or 16S sequence available in GenBank for enteropneust hemichordates as of March 10, 2018. 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 method (Pmin: 0.001, Pmax: 0.1, Steps: 10, X: 1.5, Distance: JC69) (Puillandre, Lambert, Brouillet & Achaz, 2012). Then species accumulation curves, based on hemichordate OTUs, were separately calculated for the Pacific and Caribbean coasts of Panama, using number of individuals as sampling units. Each curve was replicated 1000 times by randomizing the order of individuals and analyzed with asymptotic regression models to estimate the total number of OTUs predicted to be present in each site (richness) and the sampling effort required to detect them.

DNA sequences generated by this project have been deposited in GenBank (accession numbers: MK091956-MK092032 and MN134671-MN134686 for COI; MK073490-MK073588 for 16S), and the COI dataset has been assigned the BoLD DOI dx.doi.org/10.5883/DS-HEMICHOR.

Results

A total of 109 hemichordate larvae (54 from the Bahia Almirante and 55 from the Bay of Panama) were collected for sequencing (Figures 1-5). Structured samples from Bahia Almirante showed 0 to 36 tornaria per tow with an average density of 1.5 (s.d. = 2.2) individuals per m^3 of seawater sampled. Some larvae were collected during all 4 sampling periods but particular peaks were observed on August 22, 2015 and June 12, 2016. However the previous and subsequent sampling dates in the same sampling period had typically low counts. In the Pacific, a particular peak in abundance occurred in November, 2014 when a single tow netted >100 larvae. All of the larvae we collected had morphologies reflecting typical planktotrophic development ranging from small early stage tornaria to metamorphic stage larvae that are competent to settle (Figures 1-5). We did not find any *Planctosphaera* or any other very large tornaria, or any larvae with elaborate tertiary tentaculate extensions of the ciliary band (e.g., Hadfield, 1975; 2002 Hart, Miller & Madin, 1994; Scheltema, 1970).

Of the 109 larvae, 99 were sequenced for both genes successfully. These sequences have been deposited in GenBank, and represent a significant increase over the 10 previously published unique COI sequences for five species of enteropneusts (Table 1). Automatic Barcode Gap

Discovery (ABGD) analysis for COI found a very conspicuous gap extending between 2.5% and 9.6% pairwise distances. Using this gap the COI sequences from Panama grouped into 13 distinct OTUs (Figure 6) which differed from each other by more than 10% Jukes-Cantor distance. ABGD analysis for 16S detected a barcode gap between 0.7% and 2.4% (Figure 7). The 16S sequences grouped into 14 OTUs (Figure 7), the same 13 OTUs as were discovered in the COI analysis plus an additional OTU comprised of 3 individuals none of which amplified for COI. None of our OTUs clustered in or near any harrimaniids and we therefore eliminated that family (which is not known to have species with feeding larvae) from our Figure 7 for brevity.

Overall, GenBank sequences from the same enteropneust family clustered together in the 16S Neighbor Joining tree (Figure 7). Therefore, the location of our larval OTUs in this topology may indicate a family-level affinity. However this should be treated with caution since mitochondrial genes are not ideal for assessing deep phylogenetic relationships and many of the nodes in the tree lack bootstrap support. We were also able to match 2 of our larvae to previously reported sequences: one larva matched an unidentified or un-named species of *Balanoglossus* that was collected as an adult from the Bahia Almirante only a few kilometers from our sampling site; the other individual matched an unidentified ptychoderid tornaria collected in the Bahamas. None of the other sequences from Panama were sufficiently similar to any GenBank sequences to be considered conspecific, nor did they appear to be close relatives.

Our descriptions of the larvae assigned to each OTU follow the staging described in Hadfield (2002). Briefly, very early larvae that have a telotroch but have not yet developed the longitudinal ciliary band with lobes and saddles are **Heider** stage. The **Metschnikoff** stage has simple lobes and saddles followed by the **Krohn** stage in which secondary lobes and saddles are evident, and which may have tentacles. These mature Krohn larvae have been characterized as "defying description except by reference to figures" (Hadfield, 2002) and no adequate descriptive terminology has been developed for these larvae. As the larva approaches metamorphosis, the anterior lobes start to smooth out in the **Spengel** stage and gill pouches may be visible. Finally, the **Agassiz** and metamorphic stages have a fully developed proboscis, but retain the telotroch on the posterior end. We endeavor to describe the larvae attributed to each OTU below.

Larvae from the Pacific

In the Pacific, COI data showed OTUs P1 and P6 were each represented by a single individual. OTUs P2, P3 and P4 had 6, 11, and 21 individuals respectively. The same 6 OTUs were identified with 16S data (Figures 6 & 7).

P1 – This OTU is represented by a single small individual collected on June 23, 2014. The small tornaria had no pigment and was, unfortunately, not photographed. The 16S sequence clustered with the Ptychoderids and most closely with *Glossobalanus berkeleyi* at around 11% divergence (Figure 5).

P2 – Samples of this OTU were all collected on March 13, 2014. They clustered close to *Schizocardium* cf. *brasiliense* in the 16S tree but the 3.5% divergence from this species indicates that they are not conspecific but may be congeners (Figure 7). The tornaria ranged from 1.0 – 1.5 mm in length and included Krohn and Spengel stages (Figure 1A-D). The largest Krohn stage tornaria show the development of small lobes but no tentacles. The cells along the telotroch, and the area between the stomach and the esophagus were yellowish, and burgundy spots followed the ciliated bands around the body lobes and around the telotroch (Figure 1b-d). There was no second telotroch. Spengel stages had visible gill development and regressed cilia on the body lobes.

P3 – This OTU was abundant in tows from August 2013, and March and June 2014, and clustered most closely with other larval OTUs from both coasts of Panama. The closest identified adults are 3 spengelids which are ~20% divergent in the 16S tree, which is too distant to assign any particular affinity with confidence (Figure 7). Samples of this OTU included Krohn stage tornaria and metamorphic stages (Figure 1E-K). The tornaria ranged from 700 µm to 1.1mm. All but the smallest larvae from this OTU could be distinguished from others based on the opaque and colorful stomachs. The anterior stomach is yellowish and the color changes to green and then turquoise at the posterior end of the stomach (Figure 1F-I). There are red-burgundy spots on all of the ciliary bands, in the area between the bands and on the posterior end of the larva. The midventral ciliary band (neurotroch) can be seen running from the mouth to the anus. The metamorphic stage was ~900 µm in length, still had the colorful stomach, and had yellow pigment along the telotroch (Figure 1J-K). The body had a scattering of red pigment concentrated just anterior and posterior to the telotroch in later stages. The proboscis is also covered with a scattering of pigment spots and has a pair of red eyes flanking the apical tuft and apical organ.

P4 – This was the most abundant OTU in the Pacific and occurred in March, April, and June 2014. In the 16S tree this species clustered most closely with, but was >3% divergent from, the GenBank sequence for *Schizocardium* cf. *brasiliense*, and from P2, suggesting that they are not conspecific but may be congeners (Figure 7). This OTU included tornaria in multiple stages, including Krohn, Spengel and Agassiz stages, as well as metamorphic stages (Figure 2). Small 300-400 micron larvae already have red pigment spots along the ciliary bands (Figure 2A-B) and some larger tornaria have red pigment spots along all ciliary bands, with later stages showing visible gill bars (Figure 2C-D). Metamorphic stages also had red pigment spots on the ciliary band, and often had a transparent blue or yellow tint to the gut. Larvae do not have a second telotroch. The Agassiz stages are ~1mm and have red pigment on the anterior of the proboscis, around the telotroch, and in the collar region. Gill slits are visible in the collar region (Figure 2G). Metamorphic stages about 1.5-2mm retain the telotroch but actively crawl on the bottom of the dish.

P5 – This OTU includes only a single small individual collected on November 18, 2014. In the 16S tree this OTU was nested well within a clade of *Balanoglossus* species (Figure 7). This transparent Krohn stage tornaria had scalloped ciliary bands, a typical pair of cup-shaped red eye spots and a few red pigment spots on the anterior end. These larvae could be distinguished from other larvae by a ring of bright green pigment on the telotroch and a ring of bright red spots just posterior to the telotroch (Figure 3A-C). A second ring of red spots occurs on the anal field on a ridge, concentric with the telotroch. This is probably a second telotroch, although we could not detect cilia associated with the ridge, which may have been lost prior to metamorphosis. The gut was transparent, gills pouches were not visible, but a protocoel was distinct, and metacoels flanking the stomach were distinct (Figure 3A-C).

P6 – Three larvae were in this OTU, all of which were collected on November 18, 2014. These 16S sequences separated this OTU from the others and placed it most closely with several other larval OTUs. The closest identified adults are 3 spengelids which were ~20% divergent in the 16S tree, too distant to assign affinity with confidence (Figure 7). A 1mm long Agassiz stage larva had smooth primary lobes and saddles and its opaque stomach was bright turquoise and green (Figure 3D-E) with more blue compared to the colorful larvae of P3 (Figure 1F-I). There were scattered spots of red pigment over all body regions and the ciliary bands. No apical tuft was visible near the two large red eyes on the apical area. Gill

pouches were not visible. Two other individuals were metamorphic stage larvae with similar colorful stomachs, changing gradually from yellow anteriorly, through green to blue posteriorly (Figure 3F-G), similar to P3 (Figure 1J-K). These 1 mm larvae had 2 red eye spots and some red pigment scattered on anterior and posterior sides of the telotroch. There was some distinct orange pigment under the ciliary band of the telotroch. A second telotroch was not visible.

Larvae from the Caribbean

In the Caribbean, COI sequences identified 8 OTUs; 5 OTUs were represented by single individuals, one by 4 individuals, one by 5 individuals and one by 23 individuals. The 16S results were similar, although the distinction between C1 and C2 was not as conspicuous as it was with the COI data. Nevertheless, the distance between these OTUs was larger than the barcode gap.

C1 – This OTU was collected only once in July 2013. It is represented by a single small (~400 micron) Heider stage tornaria which was distinct from all of the others as it had yellow spots along the ciliary band (Figure 4A). We do not know if the spots may darken to the typical red found in many of the other Panamanian larvae as the tornaria matures. This larva clustered most closely with other larval OTUs, and the closest identified adults are 3 spengelids which are ~20% divergent in the 16S tree; too distant to assign affinity with confidence (Figure 7). A conspicuous apical strand was visible (Figure 4A), although this detection may be due to imaging with a compound microscope under DIC optics, unlike most larvae, that we photographed with a stereoscope.

C2 - This OTU is represented by 8 larvae that were all collected in February and June 2016. These include only small (300-400 micron) tornaria (not figured) with a clear midventral neurotroch in addition to the typical ciliary bands and no clear distinguishing features. This OTU clustered most closely with other larval OTUs. The closest identified adults are 3 spengelids (the same as for C1) which are ~20% divergent in the 16S tree, again too distant to assign affinity with confidence (Figure 7).

C3 – This OTU, represented by a single Krohn stage tornaria collected in July 2013, matches GenBank EU728430, an unidentified ptychoderid larva from the Bahamas (Canon et al. 2009). The larva had a regular pattern of orange and red pigment spots on its ciliary bands,

and a flat anal field. The single specimen showed a lateral saddle and groove, and some early ruffling along the pre- and postoral siliary bands (Figure 4B).

C4 – This OTU is represented by a single early stage tornaria with an apical tuft, simple lobes, no pigment on ciliary bands, and flat anal field. Two red eye spots are present. The larva was collected in August 2015 and the 16S fragment failed to amplify.

C5 - This OTU is represented by a single tornaria collected in November 2015. This larva clusters most closely to C6 and the GenBank sample KF683557 from an adult *Balanoglossus* sp. The 4.1% divergence suggests this OTU is not conspecific with C6, but the 16S sequence place it within a *Balanoglossus* clade (Figure 7). There are small pigment spots along the ciliary band, and the larva had a second telotroch within the anal field. KF683557 was collected in Bahia Almirante less than 5 km from our sampling site.

C6 – Sequences of this OTU, collected in March 2016, were closely related but distinct from C5 (~4% divergent in the 16S tree; Figure 7) and matched (i.e., distance is less then the barcode gap) GenBank sample KF683557, an unidentified *Balanoglossus* species. In our study this OTU was represented by a single large (900 micron) tornaria which had a second telotroch, a pair of eye spots near the apical organ and red spots along the ciliary bands. The perianal field is flat and the larva appeared very similar to that of C5 (Figure 4D).

C7 - This OTU was represented by several large (1.1 mm) Krohn stage larvae which had ruffled ciliary bands, but did not have distinct tentacles (Figure 4E-H). These tornaria were taller and narrower than the larvae from the other OTUs, and had a convex anal field. Burgundy red pigment dots lined the ciliary bands, the telotroch, and a second telotroch (Figures 4E, G). One large red eye on each side of the apical organ was clearly visible in each larva. We also collected one metamorphic stage. This larva had red spots on the telotroch and around the junction of the "head and body". This OTU was collected during the 2013 class on larval diversity and during all the sampling periods except November. This larva clustered far (>20%) from any other adult and larval OTU (Figure 7).

C8 – This was the most abundant OTU (29 individuals) and was collected during all 4 sampling periods. All samples from February were early in development, larger tornaria were collected in June and November, and a mix of stages was collected in August. These tornaria ranged from ~350 μm to 1.5 mm. They had a single telotroch with no second telotroch (Figure 5). The gut was transparent. In early stages (~400 micron larvae) the red spots were

already evident on the ciliary bands and a neurotroch was visible (Figure 5F). At 800 µm, the Metschnikoff stage larva often had a distinct morphology with the posterior part being narrower than the anterior part of the larval body. There were sparse, widely spaced, red spots along the ciliary band Figure 5E). At 1.2-1.5mm the Krohn stage tornaria had a transparent bluish tint between esophagus and stomach. At this stage, there was no evidence of tentacles or ruffles on the ciliary bands, and the gill pouches were not yet visible (Figure 5F-H). The 1mm Spengel stage larva (Figure 5I-J) had red pigment only on the posterior side of the telotroch and on the ciliary bands, and the gill bars were clearly visible in some larvae (not figured). No metamorphic stages of this OTU were collected. This OTU was most closely related to P2, P4, and *Schizocardium* cf. *brasiliense* (~4-5% divergent from each) again suggesting a congeneric relationship.

Species accumulation curves

The regression model with the best goodness of fit for both (Pacific and Caribbean) accumulation curves was Biexponential 5P: a combination of two exponential models with a total of five parameters including the asymptote. This model showed the lowest Akaike and Bayesian information criteria as well as the lowest differences between actual vs. interpolated values. The Pacific curve was fairly flat and the 95% range predicted for the asymptote was 6.6-6.9 OTUs (Figure 8: gray), suggesting that nearly all the OTUs have been collected, and only one more species might still remain undetected. In contrast, the Caribbean curve was steeply increasing and the 95% range for the asymptote was 16.8-25.7 OTUs (Figure 8: black), suggesting that further sampling could yield 9-18 additional Caribbean OTUs. Extrapolations of the Caribbean curve indicated that a sample of 315 individuals would yield, approximately, 90% of the Caribbean diversity.

Discussion

There are numerous and diverse examples of how DNA barcoding can bring hidden diversity to light. Studies of ‘cosmopolitan’ marine invertebrate species commonly uncover cryptic taxa (e.g., Barroso, Klautau, Solé-Cava & Paiva 2010; Collin 2005; Cornils Wend-Heckmann & Held 2017; Kawaguchi, & Giribet 2014; Pérez-Portela, Arranz, Rius & Turon, 2013; Jossart, Sands & Sewell 2019), and previous barcoding studies of larvae have discovered local diversity that was

not appreciated from studies of adults (Barber & Boyce 2006; Collin et al. 2019a; Mahon et al. 2010; Maslakova et al. unpublished). Here we sextupled the documented species diversity of acorn worms reported for the tropical eastern Pacific with only moderate sampling effort of planktonic larvae. We also demonstrated that the local diversity of hemichordate larvae at one site on the Caribbean coast of Panama exceeds the diversity of adults previously reported for the entire Caribbean. This may not be surprising as adult acorn worms are difficult to sample; they live in soft-bottom habitats and can often withdraw into deep tubes or burrows, and they fragment easily during collection. The poor knowledge of hemichordate diversity is widely acknowledged (Cameron & Perez 2012; Tassia et al. 2016); however, surveys of larvae might help to identify hotspots where taxonomists can focus additional efforts to collect adults.

Species accumulation curves can be used to predict the number of species in a group which may be present in a fauna (Gotelli & Colwell 2011; Thompson, Withers, Pianka & Thompson 2003; Thompson, Thompson, Withers and Fraser 2007; Ugland, Gray & Ellingsen 2003) and are often used to determine if sampling effort has been adequate to document diversity. Our results suggest that diversity might be fairly well assessed in the Bay of Panama with 6 OTUs detected in our surveys and 7 OTUs predicted, while in Bocas del Toro our 8 documented OTUs are not close to including the 17-26 species predicted by the species accumulation curves. The difference between the predicted diversity on the two coasts could be a real reflection of the diversity, but it may also reflect our sampling strategy. The structured sampling in Bocas del Toro was designed to ensure that all larvae were picked out of each tow, and that all larvae that survived were sequenced, while our strategy in the Pacific resulted in only a subset of larvae in each tow being picked out and sequenced. Most of the sampling in Bocas del Toro focused on a single site while in the Pacific we sampled a larger area. With the latter strategy, it is possible that sampling was biased towards the larger or showier larvae that dominated some tows. Because many larvae that resembled others were not sequenced, rarer species could have been missed, although we sequenced similar numbers of larvae from each coast. Such a large number of singletons in the Caribbean may seem surprising, however, intensive sampling of biodiversity in other groups often find that a significant number of all the collected species are rare. For example, the intensive documentation of the molluscs of French Polynesia, shows that 20% of 2,700 species of marine molluscs in New Caledonia are represented by singletons and that ~30% of species were represented by dead shells only (Bouchet, Lozouet, Maestrati & Heros 2002). Similarly,

massive, intensive sampling of 23,000 specimens of triphorids from Vanuatu showed that 13% of the species were represented by singletons and most were represented by fewer than 20 individuals (Albano, Sabelli & Bouchet 2011).

One of the major limitations of the interpretation of our data, and of any barcoding study of hemichordates, is the lack of identified sequences in GenBank. This is also likely to be an impediment to the use of NextGen sequencing approaches to metabarcoding, as Blast search queries of several of our hemichordate COI sequences did not generate close matches to anything published in GenBank, and could not be attributed to phylum with any certainty. COI is ideal for barcoding as it evolves quickly and can distinguish closely related species (Hebert, Cywinska, Ball & deWaard 2003); however, this trait makes it difficult to identify unknowns to a family, order or phylum level when close relatives are not in the reference database (Ekrem, Willassen & Stur 2007; Vences, Thomas, Bonett & Vieites 2005), as COI reaches saturation at low levels of phylogenetic divergence. Therefore, COI can be used most effectively only when reference databases offer dense taxonomic coverage. The 16S gene may be a more useful marker as there are currently more identified hemichordate sequences available. However, there is still a shortage of sequences from the Spengelidae and many of the 22 recognized genera in WoRMS are not represented by any sequences in BoLD.

Morphological identification of tornaria larvae

The morphology of tornaria larvae is complex, especially the convolutions of the ciliary band, and it is not clear how these and other characteristics can be used to identify unknown larvae. Authors from the 19th century were primarily focused on understanding the overall morphology and development of the tornaria form (e.g., Agassiz 1873; Bateson 1886; Bourne 1889; Morgan 1894;) and they provided few comments on the comparative morphology of the species they described, as hemichordate development was considered particularly relevant to understanding the relationships of chordates. In the early 20th century, significant effort was made to identify morphotypes of larvae primarily collected from the plankton (e.g., Björnberg1953; Damas & Stiasny 1961; Stiasny-Wijnhoff & Stiasny 1926; 1927). The current inaccessibility of some of this literature (due to language barriers as well as difficulty of obtaining the papers) is presumably the reason why these classic references are not more widely cited in modern descriptions of tornaria (e.g., Gonzalez et al. 2017; Hadfield 2002; Miyamoto &

Saito 2007; Nezlin & Yushin 2004; Urata & Yamaguchi 2004). As many of the tornaria described in the older literature were not linked to enteropneust species with any certainty, their utility is limited in matching larval morphotypes to specific clades of hemichordates.

One of the most distinct and easily observable features in our samples was the presence of the second telotroch (also referred to as the anal ring in the older literature). A second telotroch has been reported in the ptychoderids *Balanoglossus simodensis, Balanoglossus misakiensis* and *Balanoglossus proterogonius* (Miyamoto & Saito 2007; Nezlin & Yushin 2004; Urata & Yamaguchi 2004), while the spengelids *Glandiceps hacksi* and *Schizocardium californicum* seem to have only one telotroch (Gonzalez et al. 2017; 2018 Urata et al. 2014). In our 16S phylogram, our samples with a secondary telotrochs (C5, C6, C7 and possibly P5) all appear to be more closely related to ptychoderids than to spengelids.

Pigmentation is often considered to be a variable trait that could be useful to distinguish species rapidly. This appears to be partially true for OTUs P3 and P6 which appear to be fairly closely related based on our 16S sequences, and which can be distinguished from other Pacific hemichordate larvae by their opaque colorful stomachs, which are evident from the early Metschnikoff stage to the metamorphic stage. It is curious, however, that the related species from the Caribbean (C1 and C2) did not have obviously colorful guts, although the samples from these OTUs were all of early-stage larvae. It would be interesting to know if later stages of these larvae had colorful stomachs and to test if coloration is related to diet. Other obvious pigmentation patterns we observed are the rows of dark red spots that are commonly evident along the ciliary bands in most of our OTUs, and the yellow/brown pigment along the telotroch in some larvae. It might seem that the red spots along the ciliary bands are almost ubiquitous, however, descriptions and photographs of the development of *Balanoglossus simodensis* and *Balanoglossus misakiensis* do not include red spots (Miyamoto & Saito 2007; Urata & Yamaguchi 2004). Red spots can be seen on the photo of *Schizocardium californicum* (Gonzalez et al. 2017), which suggests that there may be some useful information in this feature. Finally, our OTUs P3, P5, and P6 had some yellow, orange or green pigment along the telotroch in later stages and similar brownish pigmentation has also been observed on *Balanoglossus misakiensis* (Urata & Yamaguchi 2004).

Other characteristics that could be useful for tornaria identification based on the observations that species in the genus *Ptychodera* (Hadfield 1975; Peterson, Cameron, Tagawa,

Satoh & Davidson 1999) or possibly all ptychoderid Krohn larvae (Hadfield 2002) have tentacles, while Krohn stage *Glandiceps* and possibly all Spengelidae do not develop tentacles (Hadfield 2002). It is interesting to note that none of our Krohn stage larvae had distinct tentacles, although several had numerous small lobes which gave a ruffled appearance. These ruffles could be incipient or degrading tentacles, but it seems unlikely that if a tentaculate stage was present we would have missed it in the abundant species. Finally, the developmental timing of, and number of, gill pores have also been suggested as useful characters, as Agassiz stage larvae may differ in the number of gill bar anlagen (Agassiz 1873; Kaul-Strehlow & Röttinger 2015; Morgan 1894). We found very few tornaria larvae with clear gill anlagen, or even distinct rows of gill bars. Without further information on the taxonomic distribution of this character we cannot use it to infer much about the identity of our larvae.

Clearly, careful descriptions of larvae from more species are necessary to determine which morphological characters are useful for identification of field-collected larvae. This study represents a first step in documenting neoptropical diversity of hemichordates and we hope that the high diversity of larvae collected will stimulate further study or enteropneust diversity in the region.

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Table 1: Summary of DNA sequences from our OTUs and GenBank sequences analyzed in this study.

OTU/Species[a]	Genbank # COI	Genbank # 16S	Location[b]	Collection dates
New Data				
Larval OTU C1	MK092006	MK073556	Bahia Almirante	July, 2013
Larval OTU C2	MK091977; MK091956; MK091966; MK092028	MK073585; MK073521; MK073492; MK073508; MK073561; MK073509; MK073554; MK073536	Bahia Almirante	February and June, 2016
Larval OTU C3	MK091980	MK073526; EU728430	Bahia Almirante	July, 2013
Larval OTU C4	MK092023		Bahia Almirante	August, 2015
Larval OTU C5	MK091994	MK073541	Bahia Almirante	November, 2015
Larval OTU C6	MK091984	MK073530; KF683557	Bahia Almirante	March, 2016
Larval OTU C7	MK091996; MK091990; MK091976; MK091960; MK091978	MK073543; MK073495; MK073537; MK073520; MK073498; MK073523; MK073553	Bahia Almirante	July 2013, August 2015, February-March and June 2016
Larval OTU C8	MK092007-9; MK091975; MK092022; MK091958-59; MK091993; MK092013-14; MK091967; MK091981-82; MK092025; MK091965; MK091989; MK092002; MK091999; MK091971; MK091995; MK092011; MK092001; MK092029; MN134683-4; MN134675; MN134681	MK073558; MK073519; MK073580; MK073494; MK073497; MK073540; MK073566; MK073510; MK073577; MK073559; MK073567; MK073527; MK073557; MK073582; MK073506; MK073535; MK073549; MK073546; MK073514; MK073542; MK073529; MK073563; MK073528; MK073573; MK073503; MK073560; MK073548; MK073569	Bahia Almirante	July 2013, August and November 2015, February and June 2016
Larval OTU P1	MK092000	MK073547	Bay of Panama	June, 2014

Larval OTU P2	MK092032; MK091972; MK091998; MK091985; MK092021; MK091983	MK073588; MK073516; MK073545; MK073531; MK073576	Bay of Panama	March, 2014
Larval OTU P3	MK091974; MK091969-70; MK091979; MK092027; MK092018; MK091962; MK091961; MK091964; MK091992; MK091986; MN134686; MN134682; MN134678; MN134674	MK073518; MK073512-13; MK073524; MK073579; MK073584; MK073572; MK073552; MK073515; MK073499-501; MK073504; MK073539; MK073532;	Bay of Panama	August 2013, March, June and November 2014
Larval OTU P4	MK092003-04; MK091957; MK092026; MK092010; MK092017; MK092019; MK092005; MK091968; MK092012; MK091988; MK092015-16; MK092020; MK091997; MK091991; MK092031; MK091973; MK092024; MK091987; MK091963; MN134680; MN134673; MN134676-77; MN134671	MK073551; MK073525; MK073493; MK073583; MK073562; MK073496; MK073507; MK073574-5; MK073555; MK073511; MK073565; MK073570-71; MK073564; MK073534; MK073550; MK073568; MK073544; MK073505; MK073538; MK073587; MK073490; MK073517; MK073581; MK073533; MK073502	Bay of Panama	March, April and June 2014
Larval OTU P5	MK092030	MK073586	Bay of Panama	November, 2014
Larval OTU P6	MN134672; MN134685; MN134679	MK073491; MK073578; MK073522	Bay of Panama	November, 2014
Published Data				**Reference**
Ptychoderidae				
Balanoglossus carnosus	AF051097; NC001887; LC018636	AF051097; LC018638; NC001887; LC120732-45;	Japan; Papua New Guinea	Cartresana et al. 1998a & 1998b, Urata 2015, Perseke et al. 2010, Urata Unpublished
Balanoglossus clavigerus	FN56279; NC013877	EU728425; FN562579; NC013877	France, Northern Coast	Cannon et al. 2009, Perseke et al. 2010
Balanoglossus numeensis		LC120747-9; LC120754	Papua New Guinea; Vietnam	Urata Unpublished

Balanoglossus sp.		LC120746	Australia, Pacific Coast	Urata Unpublished
Balanoglossus cf. *aurantica*		KF683555-6; EU728427	USA, Gulf Coast	Canon et al. 2009 & 2013
Ptychodera bahamensis		KF683560	Bermuda	Cannon et al. 2013
Ptychodera flava	LC018631-5; KC706836	EU728428-9; LC018637	Hawaii; Moorea; Japan	Cannon et al. 2009, Urata 2015, Leray et al. 2013
Ptychoderidae sp.	KC706837		Moorea	Leray et al. 2013
Glossobalanus berkeleyi		EU728426; KF683554	Washington State	Cannon et al. 2009 & 2013
Glossobalanus marginatus		KF683559	Norway	Canon et al. 2013
Torquaratoridae				
Enteropneusta sp.		KF683558	Antarctica	Canon et al. 2013
Torquaratoridae sp. 1		EU520500-1; JN886743-6	USA, Pacific coast (Oregon), and Gulf of California?	Holland et al. 2009
Torquaratoridae sp. 2		EU520502-3; JN886747	USA, Pacific coast (Oregon), and Gulf of California?	Holland et al. 2009, Osborn et al. 2012
Torquaratoridae sp. 3		EU728431	13°N on the east Pacific Rise	Canon et al. 2009
Torquaratoridae sp. 4		JN886740-42	NE Pacific	Osborn et al. 2012
Tergivelum baldwinae		EU520494-7	USA, Pacific coast (Oregon)	Holland et al. 2009
Tergivelum sp.		JN886752-4	NE Pacific	Osborn et al. 2012
Torquaratoridae sp. 5		JN886748; JN886750	NE Pacific	Osborn et al. 2012
Torquaratoridae sp. 6		JN886749	NE Pacific	Osborn et al. 2012
Torquaratoridae sp. 7		JN886751; EU520498-9;	USA, NE Pacific	Holland et al. 2009, Osborn et al. 2012
Coleodesmium karaensis		KF683553; KC907711	Iceland; NE Pacific	Cannon et al. 2013, Osborn et al. 2012

Torquaratoridae sp. 8		KF683552	Antarctica	Cannon et al. 2013
Spengelidae				
Glandiceps hacksi		JN886755	NE Pacific	Osborn et al. 2012
Glandiceps abyssicola		KC776732	Romanche Trench, Atlantic	Holland et al. 2013
Schizocardium cf. brasiliense		KF683561	USA, Gulf Coast	Cannon et al. 2013
Harrimaniidae				
Saxipendium coronatum		EU520493; EU728423	37 47.5 S on SEPR—Alvin dive # 4090; USA, Pacific Coast	Cannon et al. 2009, Holland et al. 2009
Saxipendium sp.		JN886756		Osborn et al. 2012
Protoglossus koehleri		EU728420	South Australia	Cannon et al. 2009
Harrimania planktophilus		EU728421	British Columbia, Canada	Cannon et al. 2009
Stereobalanus candensis		EU728424	Maine	Cannon et al. 2009
Saccoglossus kowalevskii	AY336131; NC_007438	AY336131; NC007438; L19302	???	Freeman et al. 2008, Holland et al. 2009, Perseke et al. 2010
Saccoglossus sp.		KF683546	Norway	Cannon et al. 2013
Saccoglossus mereschkowskii		KF683544-5	Washington State; Russia, White Sea	Cannon et al. 2013
Saccoglossus pusillus		EU728422	British Columbia, Canada	Cannon et al. 2009
Saccoglossus bromophenolosus		L26348	Washington State	Cannon et al. 2009
Harrimaniidae spp. *various species*		KF683532-43; KF683547-51	Oregon State, Antarctica, Iceland, Norway	Cannon et al. 2013

b Locality for the GenBank sequences from GenBank records or the published papers that cite them.
a Species names follow those given by the authors of the GenBank records, although if there are multiple un-named species in the same genus we added species numbers.

Table 2: AT% content of the 16S and COI sequences for hemichordates from this study and from GenBank.

	16S mean (s.d.)	**COI mean (s.d.)**
This study	49.9 (2.3)	52 (3)
GenBank	52.6 (3)	51 (5)

Figure Legends

Figure 1 Hemichordate larvae collected from the Bay of Panama, showing representatives of genetic haplotypes from two distinct Operational Taxonomic Units (OTUs P2 and P3). Tornaria larvae in A-I are oriented with anterior to the top; metamorphic stages in J-K with anterior to the left. **A-D.** P2; three individual larvae in ventral (A), ventral and right-lateral (B-C) and left-lateral (D) views at progressively later stages. **E-I.** P3; early tornaria larva in ventral view (E), and paired ventral and lateral views of two tornaria larvae (F-G and H-I). **J-K.** P3; metamorphic-stage individuals. All photographs were produced from live animals. Scale bars: A-D, E, F-G, H-I and J-K = 300 µm. af, anal field; an, anus; ao, apical organ; ey, eye; gb, gill bar; in, intestine; mo, mouth; nt, neurotroch; of, oral field; pc, proboscis coelom; pds, primary dorsal saddle; pof, postoral field; pos, postoral loop of circumoral ciliary band; pr, proboscis; pre, preoral loop of circumoral ciliary band; prf, preoral field; ps, pigment spots; pvl, primary ventral lobe; st, stomach; tt, telotroch; vs, ventral saddle. Terminology follows Gonzalez et al. (2018).

Figure 2 Hemichordate larvae collected from the Bay of Panama, showing representatives of genetic haplotypes from one distinct Operational Taxonomic Unit (OTU P4). All specimens oriented with anterior to the top. **A-B.** ventral and left-lateral views of a tornaria larva. **C-D.** ventral and left-lateral views of a late-stage tornaria larva with gill bars. **E-F.** ventral and left-lateral views of an early metamorphic stage. **G.** lateral view of a metamorphic stage. **H.** Juvenile worm. All photographs were produced from live animals. Scale bars: A-D = 200 µm; E-F = 300 µm; G-H = 500 µm. af, anal field; an, anus; ao, apical organ; co, collar; ey, eye; gb, gill bar; in, intestine; ls, lateral saddle; mo, mouth; pds, primary dorsal saddle; pof, postoral field; pos, postoral loop of circumoral ciliary band; pr, proboscis; pre, preoral loop of circumoral ciliary band; pro, protocoel; ps, pigment spots; pvl, primary ventral lobe; st, stomach; tt, telotroch; vs, ventral saddle. Terminology follows Gonzalez et al. (2018).

Figure 3 Hemichordate larvae collected from the Bay of Panama, showing representatives of genetic haplotypes from two distinct Operational Taxonomic Units (OTUs P5 and P6). Tornaria larvae in A-E are oriented with anterior to the top; metamorphic stages in F-G with anterior to the left. **A-C.** P5; ventral, ventro-lateral and right-lateral views of a late tornaria larva. **D-E.** P6; ventral and left-lateral views of a tornaria larva. **F-G.** P6; metamorphic-stage individuals. Scale

bars: A-C, D-E, F and G = 500 µm. af, anal field; an, anus; ao, apical organ; ey, eye; gb, gill bar; in, intestine; met, metacoel; mo, mouth; of, oral field; pc, proboscis coelom; pos, postoral loop of circumoral ciliary band; pr, proboscis; pre, preoral loop of circumoral ciliary band; pro, protocoel; pvl, primary ventral lobe; st, stomach; ts, second telotroch; tt, telotroch. Terminology follows Gonzalez et al. (2018).

Figure 4 Hemichordate larvae collected from Bocas del Toro Province, Panama, showing representatives of genetic haplotypes from five distinct Operational Taxonomic Units (OTUs C1, C3, C5, C6 and C7). All specimens oriented with anterior to the top. **A.** C1; ventral view of early tornaria. **B.** C3; left-lateral view of early tornaria. **C.** C5; ventral view of tornaria. **D.** C6; ventral view of tornaria. **E-H.** C7; ventral views at surface and gut levels of a tornaria (E-F), and ventral and left-lateral views of a tornaria (G-H). Scale bars: A-B = 100 µm; C, D, E-H = 300 µm. af, anal field; an, anus; ao, apical organ; ey, eye; in, intestine; ldl, lower dorsal lobe; ll, lateral lobe; ls, lateral saddle; mo, mouth; nt, neurotroch; pds, primary dorsal saddle; pos, postoral loop of circumoral ciliary band; pre, preoral loop of circumoral ciliary band; ph, pharynx; prf, preoral field; pro, protocoel; pvl, primary ventral lobe; pvs, primary ventral saddle; st, stomach; tt, telotroch; vs, ventral saddle. Terminology follows Gonzalez et al. (2018).

Figure 5 Hemichordate larvae collected from Bocas del Toro Province, Panama, showing representatives of genetic haplotypes from one distinct Operational Taxonomic Unit (OTU C8). All specimens oriented with anterior to the top. **A.** ventral view of early tornaria. **B-C.** ventral and left-lateral views of an early tornaria. **D-E.** dorsal and left-lateral views of a tornaria. **F-H.** ventral, left-lateral and dorsal views of a tornaria. **I-J.** ventral and left-lateral views of a late-stage tornaria. Scale bars: A, B-C = 300 µm; D-E, F-H and I-J = 500 µm. af, anal field; an, anus; ao, apical organ; as, apical strand; at, apical tuft; ey, eye; in, intestine; lg, lateral groove; ll, lateral lobe; ls, lateral saddle; mc, mesenchymal cell; mo, mouth; nt, neurotroch; of, oral field; pds, primary dorsal saddle; ph, pharynx; pos, postoral loop of circumoral ciliary band; pre, preoral loop of circumoral ciliary band; prf, preoral field; ps, pigment spots; pro, protocoel; pvl, primary ventral lobe; pvs, primary ventral saddle; st, stomach; ts, second telotroch; tt, telotroch; vs, ventral saddle. Terminology follows Gonzalez et al. (2018).

Figure 6: Neighbor-Joining tree of cytochrome c oxidase subunit I (COI) DNA sequences of tornaria from Panama and other Enteropneusta available in GenBank. The tree shows only unique haplotypes, followed by their frequency in number of individuals if ≥2. The Jukes-Cantor distance (substitutions per site) between haplotypes is proportional to the length of the branches separating them. Bootstrap values >60% are indicated on the corresponding branches. Caribbean sequences are indicated with bold black letters and Pacific sequences are indicated with bold gray. OTU labels are indicated in the far right side with P# format for Pacific OTUs and C# for Caribbean OTUs.

Figure 7: Neighbor-Joining tree of the 16S ribosomal DNA sequences of tornaria from Panama and other Enteropneusta available in GenBank. The Harrimaniidae, which are thought to be exclusively direct developers, were recovered as monophyletic and did not include any of our larval samples; therefore, they were removed from the tree for easier visualization. The tree shows only haplotypes that differ by three or more nucleotides, followed by an integer indicating the frequency of similar haplotypes that differ by one or two nucleotides, if >1. The Jukes-Cantor distance (substitutions per site) between haplotypes is proportional to the length of the branches separating them. Bootstrap values >60% are indicated on the corresponding branches. Caribbean sequences are indicated with bold black letters and Pacific sequences are indicated with bold gray. OTU labels are indicated in the far right side with P# format for Pacific OTUs and C# for Caribbean OTUs.

Figure 8: Species accumulation curves showing the number of Operational Taxonomic Units (OTUs) detected versus the number of individuals sequenced for the Pacific (grey) and Caribbean (black) coasts of Panama. The Y-values (average OTUs for a given number of individuals) were calculated by averaging 1000 plots with individuals in random order.