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Documenting neotropical diversity of phoronids with DNA barcoding of planktonic larvae

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Abstract

Phoronid larvae, actinotrochs, are beautiful and complicated organisms which have attracted as much, if not more, attention than their adult forms. We collected actinotrochs from the waters of the Pacific and Caribbean coasts of Panama, and used DNA barcoding of mtCOI, as well as 16S and 18S sequences, to estimate the diversity of phoronids in the region. We discovered three operational taxonomic units (OTUs) in the Bay of Panama on the Pacific coast and four OTUs in Bocas del Toro on the Caribbean coast. Not only did all OTUs differ from each other by >10% pairwise distance in COI, but they also differed from all phoronid sequences in GenBank, including the four species for which adults have been reported for the Pacific of Panama, *Phoronopsis harmeri*, *Phoronis psammophila*, *Phoronis muelleri*, and *Phoronis hippocrepia*. In each ocean region, one common OTU was more abundant and occurred more frequently than other OTUs in our samples. The other five OTUs were relatively rare, with only one to three individuals collected during the entire project. Species accumulation curves were relatively flat but suggest that at least one more species is likely to be present at each site. Actinotrochs from the seven sequenced OTUs had morphologies typical of species with non-brooded planktotrophic development and, in some cases, may be distinguished by differences in pigmentation and the arrangement of blood masses. We found one larva with morphology typical of brooded planktotrophic larvae for which sequencing failed, bringing the total number of potential species detected to eight and representing >50% of the adult species currently recognized globally.

KEYWORDS

actinotroch, Caribbean, lophophorate, meroplankton, Panama, tropical East Pacific

1 | INTRODUCTION

Phoronids are unusual among marine invertebrates in that the morphologies and distributions of the larvae are as well documented as those of the adults. The distinctive larval form for the phylum, the actinotroch, has attracted attention from scientists sorting plankton since the 1800s, when many larvae were described as distinct species belonging to the family Actinotrochidae and genus *Actinotrocha* MÜLLER 1846, prior to the recognition of their adult forms as a distinct phylum. These larvae have a cylindrical body topped with a ring of tentacles and a large oral hood (reviewed in Emig, 1982a; Temereva, 2009). The larvae are often decorated with a number of yellow pigment spots, and when present, the blood corpuscles or blood masses are visible as internal patches of red or pinkish pigment. Both larval morphology and the process of metamorphosis have been described in detail

(Emig, 1977, 1982b; Hay-Schmidt, 1987, 1989; Santagata, 2002, 2004, 2015; Santagata & Zimmer, 2002; Temereva, 2009, 2010; Temereva & Malakhov, 2015; Temereva, Neretina, & Stupnikova, 2016a, 2016b; Temereva & Tsitrin, 2014).

As part of a larger effort to document the diversity of marine invertebrate larvae on both coasts of Panama, we collected, photographed, and obtained DNA barcode data from actinotroch larvae from the Bay of Panama on the Pacific coast, and Bocas del Toro on the Caribbean coast. The objective of this study was to use DNA barcoding (Bucklin, Steinke, & Blanco-Bercial, 2011; Hajibabaei, Singer, Hebert, & Hickey, 2007; Herbert & Gregory 2004) to identify larvae from Panamanian waters. DNA barcoding is an approach designed to facilitate the documentation of diversity, by using a database of DNA sequences from material that has been identified by experts to aid in identification of unknown samples, as well to aid in identification of material that is not morphologically definitive for some reason. Rapidly evolving sequences are used to maximize our ability to distinguish among species for identification purposes, and DNA barcoding approaches should not be confused with molecular phylogenetics, which uses different methods and has different goals (Bucklin et al., 2011; Hajibabaei et al., 2007).

Phoronids are an ideal group to use as a test of the DNA barcoding approach for identifying invertebrate larvae. Phoronida is a very small group: the World Register of Marine Species (WoRMs; 2017) provides a list of 11 species of named adults, with three species in the genus *Phoronopsis* and eight species in the genus *Phoronis*. However, recent and ongoing taxonomic work continues to add to this diversity, with an updated worldwide count of 15 species (13 in Temereva et al., 2016a, 2016b plus *Phoronis savinkini* and *Phoronis embryolabi* described in Temereva & Neklyudov, 2017 and Temereva & Chichvarkhin, 2017). Currently DNA barcode sequences are available in GenBank for 75% of recognized adult species (Table 1). Such high taxonomic coverage of rapidly evolving genes suggests that there should be a high probability of identifying wild-caught planktonic larvae using this approach.

However, recent application of DNA sequence data for actinotroch identification by matching larvae to previously identified adults suggests that phoronid diversity is greater than currently recognized. For example, sequences of the slowly evolving 18S and 28S ribosomal genes, combined with morphological data, have led to the documentation of two new adult forms and three new larvae from the South China Sea (Temereva et al., 2016b). In addition, at least 25 larval forms have been reported (Temereva, 2009; Temereva et al., 2016b). Since particular larvae are not always definitively linked to an adult form, the convention is to refer to them as belonging to the genus *Actinotrocha*. This nomenclatural issue is further complicated by the fact that only competent larvae can be identified with any certainty (Santagata & Cohen, 2009; Temereva, 2009; Zimmer, 1991). Overall, four of the known adult species have not been matched to any larval form, and a large number of larval forms have not been matched to adults. There is a general consensus that phoronid diversity is underestimated (Hirose, Fukiage, Katoh, & Kajihara, 2014; Santagata & Zimmer, 2002; Temereva & Chichvarkhin, 2017), indicating that larval barcoding might also be an effective way to detect the presence of previously undocumented species.

Our sampling strategies differed in the two ocean regions, with a structured protocol in the Caribbean and a more exploratory approach in the Pacific. We used DNA sequences to address the following questions: (a) How many operational taxonomic units (OTUs) are present in each ocean region? (b) Can DNA sequences be used to identify any of these OTUs by comparison with GenBank sequences of identified adults? We also report our morphological observations of the larvae we collected. Our structured sampling approach in the Caribbean further provides information on the density and seasonality of these larvae.

2 | METHODS

2.1 | Sample collection

Actinotrochs from three distinct sets of samples were photographed and sequenced. The Caribbean larvae were collected from Bahia Almirante in Bocas del Toro Province, with a 0.5-m diameter, 125-μm mesh plankton net towed horizontally at 10–20-m depth behind a small boat that was drifting with the engine primarily in neutral, or engaged briefly, enabling the net to move vertically upward in order to sample different levels of the water column. In 2013, larvae were collected as part of the Larval Invertebrate Diversity, Form and Function short-course at the Bocas del Toro Research Station of the Smithsonian Tropical Research Institute (STRI). Samples were collected over 2 weeks from various sites within Bahia Almirante in July 2013 and sorted by a team of 12 students. Larvae were selected for barcoding based on participant interests. During 2015–2016, structured sampling involved a regular campaign of four sampling periods over a year (August 2015, November 2015, February/March 2016, and June 2016). Each period included three or four collections spread over a 9–10-day interval. Every tow was conducted between 7 a.m. and 9 a.m. in the channel between Isla Colon and Isla Cristobal. Longitude ranged from 09°20′8.9″N to 09°20′36.3″N, and latitude ranged from 82°15′41.0″W to 82°15′50.0″W. A flowmeter (General Oceanics) was attached to the mouth of the net. In the 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, and March 2016). Pacific tows were primarily exploratory and performed by towing the net at a variety of depths between 5 and 20 m. Variation in the depth of each tow was obtained by changing the length of line deployed or by moving the engine in or out of gear.

Live plankton samples were sorted under Nikon SMZ645 stereomicroscopes, and actinotrochs were moved into dishes of filtered seawater. For the 2015–2016 Caribbean samples, the entire tow was sorted exhaustively to provide data on larval density. For the other samples, larval taxa of interest were picked from the sample but no effort was made to ensure all actinotrochs were found. Individual larvae were photographed live in a depression slide under a stereomicroscope prior to preservation for DNA sequencing. During the short-course

TABLE 1 Summary of phoronid larval operational taxonomic units (OTUs) detected in this study (and GenBank accession numbers for new sequence data), and published sequence data (GenBank accession numbers) from adult phoronids

OTU or species[a]	COI	16S	18S	Location[d]	Collection dates
New data					
Larval OTU P1	MK028633	MK260069	MK260160	Bay of Panama	June 2014
Larval OTU P2	MK028634 MK028620	MK260161 MK260147	MK260070 MK260056	Bay of Panama	June 2014
Larval OTU P3	MK028621 MK028628-9 MK028638 MK028640 MK028642-3 MK028645 MK028647-9 MK028651 MK028656 MK028659-63 MK028665	MK260057 MK260064-5 MK260074 MK260076-78 MK260080 MK260082-4 MK260086 MK260091 MK260094-8 MK260101	MK260148 MK260155-6 MK260165 MK260167 MK260169-70 MK260172 MK260174-6 MK260178 MK260182 MK260185-89 MK260191	Bay of Panama	March, April, and June 2014
Larval OTU C1	MK02862-5 MK028631-2 MK028635-7 MK028644 MK028646 MK028650 MK028652-3 MK028655 MK028657-8 MK028664 MK028666	MK260058-61 MK260067-8 MK260071-3 MK260079 MK260081 MK260085 MK260088 MK260090 MK260092-3 MK260102	MK260149-52 MK260158-9 MK260162-4 MK260171 MK260173 MK260177 MK260179 MK260181 MK260183-4 MK260190 MK260192	Bahia Almirante	August and November 2015; February–March and June 2016
Larval OTU C2	MK028630	MK260157	MK260066	Bahia Almirante	March 2016
Larval OTU C3	MK028627 MK028654	MK260063 MK260089	MK260154 MK260180	Bahia Almirante	July 2013
Larval OTU C4	MK028626 MK028639 MK028641	MK260062 MK260075	MK260153 MK260166 MK260168	Bahia Almirante	August and November 2015
Published data					**Reference**
Phoronis					
Phoronis australis	EU484457-58	—	AF202111 EU334122	New Caledonia and Japan	Santagata and Cohen (2009)
	—	—	EU334123	Pacific Coast of Australia	Santagata and Cohen (2009)
	—	—	AF119079	Mediterranean Coast of Spain	Santagata and Cohen (2009)
	—	—	KT030908-10	South China Sea	Temereva et al. (2016a)
Phoronis emigi	AB621915	—	AB621913	Japan	Hirose et al. (2014)
Phoronis hippocrepia	EU484459	—	AF202112	Mediterranean Coast of France	Santagata and Cohen (2009)
	JF509717	—	JF509726	Sweden	Andrade et al. (2012)
	—	—	U08325	Mediterranean Coast of France	Temereva and Neretina (2013)
			KT030907	South China Sea	Temereva et al. (2016b)
Phoronis ijimai	AB752305	—	AB752304	Japan	Hirose et al. (2014)
	KY643692-93	—	—	Bering Sea and Sea of Japan	Temereva and Chichvarkhin (2017)
	FJ196088	—	FJ196118	Washington State	Fuchs, Obst, and Sundberg (2009)
	EU484462	—	AF202113	California	Santagata and Cohen (2009)

(Continues)

TABLE 1 (Continued)

Published data					Reference
Phoronis muelleri	EU484460	—	EU334125	Sweden	Santagata and Cohen (2009)
	—	—	KJ193748	Germany	Mohrbeck, Raupach, Arbizu, Knebelsberger, and Laakmann (2015)
Phoronis ovalis	EU484461	—	EU334126	Irish Sea	Santagata and Cohen (2009)
	GU125773 (excluded)[e]	—	GU125758	Sweden	Fuchs, Iseto, Hirose, Sundberg, and Obst (2010)
Phoronis pallida	—	—	EU334127	Washington State	Santagata and Cohen (2009)
Phoronis psammophila[b]	AY368231	AY368231	AF025946 (excluded)	Gulf Coast of Florida	Helfenbein and Boore (2004)
	—	—	U36271	Gulf Coast of Florida	Temereva and Neretina (2013)
			AF025946	Gulf Coast of Florida	Cohen, Gawthrop, and Cavalier-Smith (1998)
	KU905825 KU905982 KU905732 KU906085 KU906050 KU905924 KU905900 KU905751 KU905854 KU905741	—	—	Chesapeake Bay	Aguilar et al. (2017)
Phoronis embryolabi	KY643690-91	—	—	Sea of Japan	Temereva and Chichvarkhin (2017)
Phoronis architecta			AF025946	Gulf Coast of Florida	Cohen et al. (1998)
Phoronis sp.	KC706882-83	—	—	French Polynesia (from fish gut contents)	Leray et al. (2013)
Phoronis sp.	—	—	AB106269		Hall, Hutchings, and Colgan (2004)
Phoronis sp. 1	—		KT030906	South China Sea	Temereva et al. (2016b)
Phoronis sp. 2	—		KT030901-02	South China Sea	Temereva et al. (2016b)
Phoronis sp. 3	—		KT030903-05	South China Sea	Temereva et al. (2016b)
Phoronopsis					
Phoronopsis californica	EU484463		EU334129	California	Santagata and Cohen (2009)
Phoronopsis harmeri	EU484464		EU334130	Japan	Santagata and Cohen (2009)
	KY643694			Sea of Japan	Temereva and Chichvarkhin (2017)
	—		KC161253-54	Sea of Japan	Temereva and Neretina (2013)
	JX136706-11		—	Unknown	E. N. Temereva and V. V. Malahov unpublished data
	NC018761	NC018761	—	Unknown	P. Lesny et al. unpublished data
	JN832704	JN832704	—	Unknown	Podsiadlowski, Mwinyi, Lesný, and Bartolomaeus (2014)
Phoronopsis viridis	EU484465		AF123308	California	Santagata and Cohen (2009)
Actinotrocha sp.[a]	JX136712-14		—	Unknown	E. N. Temereva and V. V. Malahov unpublished data
	—	—	KC161255	Sea of Japan	Temereva and Neretina (2013)

[a]Refers to the larval stage which are placed in the larval genus *Actinotrocha* when they cannot be definitively linked to an adult. [b]Species name disputed by Santagata and Cohen (2009). [c]Species names following those in GenBank provided by the researchers who submitted the sequences. [d]Locality for the Genbank sequences from Genbank records or the published papers that cite them. [e]Sequences that produce unusually long branches or show other potential indications of incorrect identification or contamination are excluded from our analyses.

in 2013, larvae were relaxed with 7.5% $MgCl_2$ prior to photographing through a Nikon E600 compound microscope under DIC optics, resulting in fewer processed samples and lower success in subsequent sequencing, but higher quality photographs. Notes were recorded on the overall appearance, morphological details, and approximate size of each larva before they were preserved for sequencing.

2.2 | DNA sequencing

We sequenced two mitochondrial genes commonly used for DNA barcoding of marine invertebrates, cytochrome c oxidase subunit I (COI) and large subunit ribosomal RNA (16S) (Bucklin et al., 2011; Moura, Cunha, Porteiro, & Rogers, 2011; Zheng, He, Lin, Cao, & Zhang, 2014). COI sequences for phoronids are well-represented in global sequence databases like GenBank, but 16S has been sequenced for few phoronids. However, 16S is commonly used in metabarcoding, and increased representation of this marker for uncommon phyla in databases will be useful for such analyses. We also sequenced the nuclear small subunit ribosomal RNA(18S) because this marker has good taxon coverage for phoronids in GenBank. Individual larvae were preserved in 150 µl of M2 extraction buffer (AutoGen), stored frozen at −20°C, and shipped to the Smithsonian's Laboratories of Analytical Biology for DNA extraction and Sanger sequencing. Plates with larval samples from a variety of taxa were extracted using an AutoGenprep 965 extraction robot after overnight digestion in AutoGen buffers with proteinase-K. Resuspension volume of extracted DNA was 50 µl. The DNA barcode fragment (654 bp) of COI, sometimes referred to as the "Folmer fragment," was amplified (Table 2). The PCR cocktail included 5 µl GoTaq Hot Start Mix (Promega), 0.1 µl 20 µg/µl BSA, and 0.3 µl each 10-mM primer in a total volume of 10 µl with an annealing temperature of 50°C. Attempts to amplify and sequence 16S using the 16Sar/16Sbr primers (Palumbi et al., 1991) were largely unsuccessful. 16S is a useful barcode marker in several animal phyla and is preferred to COI in some taxa (Moura et al., 2011; Zheng et al., 2014) but has not been commonly used for phoronids. Therefore, phoronid-specific primers 16Sar_Phor and 16Sbr_Phor (Table 2) were designed to obtain a 498 bp amplicon of 16S for this project. A fragment (604 bp) of 18S was amplified using the primers EukF (modified from primer A in Medlin, Elwood, Stickel, & Sogin, 1988) and SR7 (Vilgalys & Sun, 1994). 18S is a slowly evolving gene that is not generally used for DNA barcoding, but is used in metabarcoding samples that might include phoronids, and it has previously contributed to the discovery of new phoronid species (Temereva et al., 2016b). DNA sequences generated by this project have been deposited in GenBank (accession numbers: MK028620–MK028666 for COI; MK260056–MK260102 for 16S; and MK260147–MK260192 for 18S), and the dataset has been assigned the BoLD doi dx.doi.org/10.5883/DS-PHORONID.

2.3 | Analysis

Sequences were screened for quality, and contigs of forward and reverse sequences were produced using Sequencher 5.4.6 (Gene Codes). Only sequences of more than 90% of the total expected length of the fragment, and with a Phred quality score of at least 30 for more than 85% of the bases, were combined into contigs and used for analysis. To check for potential contamination, sequences were compared internally to all larvae sequenced in our project within the BoLD project workbench database tool (www.boldsystems.org; Ratnasingham & Hebert, 2007), and they were compared to other available sequences using BLAST searches in GenBank. COI sequences were also checked with the methods of Song, Buhay, Whiting, and Crandall (2008), and none of the sequences showed traits of nuclear mitochondrial pseudogenes. Briefly, there were no gaps in the sequences, nor were there any stop codons. The AT/CG bias was similar to other phoronid COI sequences in GenBank (AT% = 63%; Table 2). Because so few published sequences are available for most marine invertebrate taxa, close similarity to published phoronid species was not necessarily expected and was therefore not used as a criterion to retain sequences. Sequences that matched taxa in other phyla (<5 sequences) were excluded from the analysis.

Because DNA barcoding seeks to match unknown samples with known sequences, similarity criteria are used to make this comparison. Therefore, neighbor-joining trees (BIONJ, Gascuel, 1997) with Jukes–Cantor distances were constructed from our sequence data and from every phoronid COI, 16S, or 18S sequence available in GenBank as of March 10, 2018. COI alignments were inferred with the BoLD aligner (amino acid-based Hidden Markov Model; Ratnasingham & Hebert, 2007), whereas 16S and 18S alignments were inferred with the Kalign algorithm (Lassmann & Sonnhammer, 2005) using the default settings of the BoLD workbench. Species names used here follow the name attached to the sequence in GenBank accessions, because our aim was to compare with previously published sequences and not to revise the taxonomy of phoronids. OTUs were identified with the Automatic Barcode Gap Discovery (ABGD) method (Puillandre, Lambert, Brouillet, & Achaz, 2012). Species accumulation curves based on COI OTUs were separately calculated for the Pacific and Caribbean coasts of Panama and used number of individuals as sampling units. Each curve was replicated 1,000 times by randomizing the order of individuals and

TABLE 2 Summary of DNA fragments and primers used in this study

Gene	Primers	Fragment length	Compositional bias, AT%Mean (range)	References
COI "Folmer" barcode fragment	jgLCO1490/jgHCO2198 or dgLCO1490/dgHCO2198	654	63 (61–65)	Geller, Meyer, Parker, and Hawk (2013); Meyer et al. (2005)
16S "Palumbi" fragment	16Sar_Phor:TCATCTGTTTAATAAAAACATAG 16Sbr_Phor: CACCGGTTTAAACTCAGATCATGTAAG	~498	71 (69–72)	This study
18S	EukF SR7	~604	53 (52–54)	Medlin et al. (1988); Vilgalys and Sun (1994)

analyzed with asymptotic regression models to estimate the total number of OTUs present at each site (richness).

3 | RESULTS

A total of 52 phoronid larvae (29 from the Bahia Almirante and 23 from the Bay of Panama) were collected (Figures 1–3). Quantitative samples from Bahia Almirante in the Caribbean showed 0–3 actinotrochs per tow, with an average density of 0.21 (*SD* = 0.28) individuals per m^3 of seawater sampled. Although larvae were collected during all four sampling periods, they were consistently present during February/March and only present in some tows during June, August, and November; however, the small sample sizes make it difficult to statistically test this pattern. All of the larvae had morphologies typical of large, long-lived, non-brooded planktotrophic actinotrochs, except one with a small compact body, which was similar to the morphology of typically brooded planktonic larvae (Temereva, 2009; Temereva & Chichvarkhin, 2017; Figure 2).

Of the 52 larvae, 47 (25 from the Bahia Almirante and 22 from the Bay of Panama) were sequenced successfully for COI, 44 for 16S, and 46 for 18S. These sequences have been deposited in GenBank, doubling the number of COI sequences published for phoronids. None of our sequences matched those already in GenBank, either by using an informal criterion (two sequences are conspecific if they show >95% nucleotide similarity in COI), or by using a formal criterion (two sequences are conspecific if they show a Jukes–Cantor pairwise distance that is smaller than the barcode gap; Puillandre et al., 2012). ABGD analyses found a conspicuous gap between 2% and 13% divergence for COI and between 0.32% and 5% for 16S. There was an inconspicuous gap between 0.1% and 0.31% sequence divergence for 18S. The smaller divergence and less conspicuous gap are expected for the more slowly evolving 18S gene, which is not particularly useful to distinguish among species within a genus.

Phoronid sequences from Panama grouped into seven distinct OTUs that differed from each other by >13% COI Jukes–Cantor distance, and each of which had >95% bootstrap support when more than one haplotype was present in the neighbor-joining analysis (Figure 4). These larval OTUs differed from OTUs formed by sequences from adults in GenBank by as much as these adult species differed from each other. Results were also very similar for 16S and 18S (Figures 5 and 6), in which the same seven OTUs differed from each other by more than the minimum barcode gap distance. One OTU (P1) was not found with 18S (it fell within the dominant OTU P3), but 18S is only expected to resolve differences between quite divergent taxa. One specimen (RCMBAR714) was assigned to different OTUs with different genetic markers, moving from OTU C4 (with COI and 18S) to the dominant OTU C1 (with 16S). As this larva had a similar morphology to the other larvae in OTU C4, the 16S sequence was likely in error. Finally, since none of our phoronid larvae

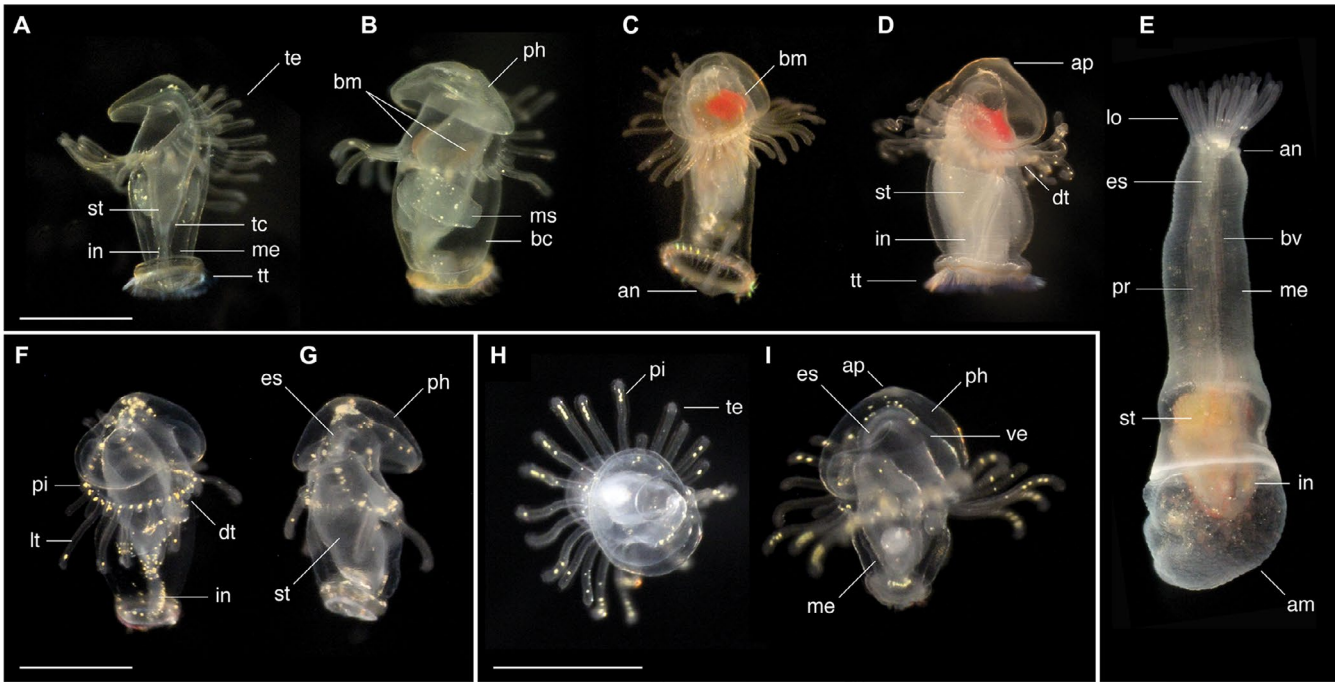

FIGURE 1 Actinotroch larvae of phoronids collected from the Bay of Panama, including representative individuals from which we obtained cytochrome oxidase subunit I (COI) haplotypes from three distinct Operational Taxonomic Units (OTUs P1, P2, and P3). Each larva is oriented with anterior to the top, except for H. **A–E.** P3; left-to-right series of larvae at different stages of growth, with juvenile worm at far right. **F,G.** P2; dorsolateral views of two different specimens. **H,I.** P1; anterior (left) and lateral (right) views of the same specimen. All photographs were produced from live animals. Scale bars: A–D, F–I = 500 μm; no scale bar for juvenile worm in E. am, ampulla; an, anus; ap, apical plate; bc, blastocoel; bm, blood mass; bv, blood vessel; dt, definitive tentacle; es, esophagus; in, intestine; lo, lophophore; lt, larval tentacle; me, metacoel; ms, metasomal sac; ph, preoral hood; pi, pigment; pr, prestomach; st, stomach; tc, trunk coelom; te, tentacle; tt, telotroch; ve, vestibule

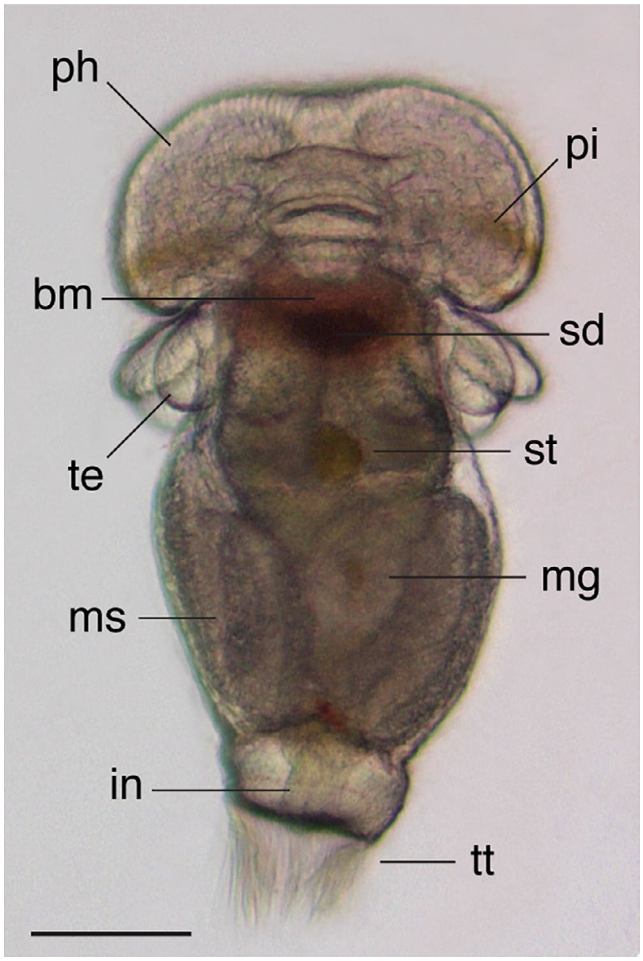

FIGURE 2 Actinotroch larva of a phoronid collected from the Bay of Panama. The short, stubby tentacles and opaque body suggest this larva is of the brooded type of development (Temereva, 2009). Details include cilia of the telotroch extending posteriorly, preoral hood with pigment, and blood mass between the hood and trunk. Scale bar = 100 μm. bm, blood mass; in, intestine; mg, midgut; ms, metasomal sac; ph, preoral hood; pi, pigment; sd, pigmented stomach diverticulum; st, stomach; te, tentacle; tt, telotroch

matched, or were even similar to, sequences already published in GenBank, we could not identify any of them as named species.

Species accumulation curves based on the number of individuals sequenced were relatively flat (Figure 7), as expected for the low diversity of phoronids. However, all analyses predict that there is at least one additional species that has not yet been sampled in each of the two ocean regions (Table 3). They also predict that a sample of 57 individuals would allow the detection of all the Atlantic OTUs, whereas Pacific OTUs would require sampling 87 individuals. Such predictions are based on the Biexponential 5P model which showed the best goodness of fit (lowest Akaike and Bayesian information criteria [AIC_c and BIC] and lowest differences between actual vs. interpolated values; Figure 7).

In the Pacific, OTUs P1 and P2 were rare, with one and two individuals collected, respectively, whereas OTU P3 was most abundant, with 18 individuals collected during five of the seven sampling dates. This species accounted for more than 85% of the phoronids sequenced from the Bay of Panama.

The single specimen of P1 was collected in June 2014 and had the overall appearance typical of non-brooded planktotrophic actinotroch larvae. The larva was transparent (Figure 1H,I). At the stage with 22–24 tentacles, the larva had a body length of 700 μm. It did not have visible blood corpuscles, indicating that it was precompetent. The metasomal sac was not visible. Yellow pigment spots were located in the distal end of the tentacles, near the apical plate and around the anus (Figure 1H,I).

The two specimens of P2 (Figure 1F,G) were collected on the same day in June 2014 and had the overall appearance typical of non-brooded planktotrophic actinotroch larvae. Larvae were transparent. At the stage with 22 tentacles, larvae were 1.1 mm long and did not have evident blood corpuscles (Figure 1F,G). There were definitive tentacles under the bases of the larval tentacles (Figure 1F,G). Yellow pigment spots were located at the base of tentacles, around the apical plate, midgut, and intestine. Both larvae were ~1.1 mm in length; one had at least 22 tentacles total, but the other seemed to be damaged, with only seven larval tentacles and some short definitive tentacles.

Larvae of P3 were by far the most abundant and were collected in March, April, and June 2014. The overall appearance was typical of non-brooded planktotrophic actinotroch larvae (Figure 1A–E). The smallest larvae were ~200 μm and had 10–14 tentacles; larger larvae of 1.0 mm had 28–30 tentacles (Figure 1B–D). We also collected a 1.2-mm competent larva that began to metamorphose in the dish, and a juvenile with ~30 juvenile tentacles (Figure 1E). The competent larva was transparent. There was some dark pigmentation on the telotroch epithelium. In mature larvae, there was an unpaired ventral stomach diverticulum (Figure 1B). A single large blood mass was located ventrally above the stomach diverticulum in the blastocoel of the collar region. The single blood mass formed before metamorphosis (Figure 1D) by the fusion of two lateral blood masses (Figure 1B). No larvae smaller than 600 μm had visible blood masses. There were definitive tentacles under the bases of the larval tentacles (Figure 1D). These larvae had a sparse scattering of small pale yellow spots on the tentacles, on the trunk area, and under the apical organ, which increased in number during development (Figure 1A–D).

The larva of PB was morphologically distinct from all the other larvae we collected but was similar to the typical morphology of the planktonic phase of brooded larvae (Temereva, 2009), or to the morphology of *Phoronis pallida* (Santagata, 2004). A single competent opaque larva ~500 μm long was collected during August 2013 (Figure 2). At this stage it had few, possibly eight, short tentacles. Brown pigmentation was present as two horizontal lines along the lateral sides of the edge of the preoral lobe. The darkly pigmented ventral stomach diverticulum was visible through the integument. There was one ventral blood mass, which was located between the body wall and stomach diverticulum. Unfortunately, we could not obtain sequences from this larva.

In the Caribbean, OTU C1 was most abundant, representing >75% of the actinotrochs sequenced from Bocas del Toro, with 19 individuals collected during 6 of the 15 quantified tows and during

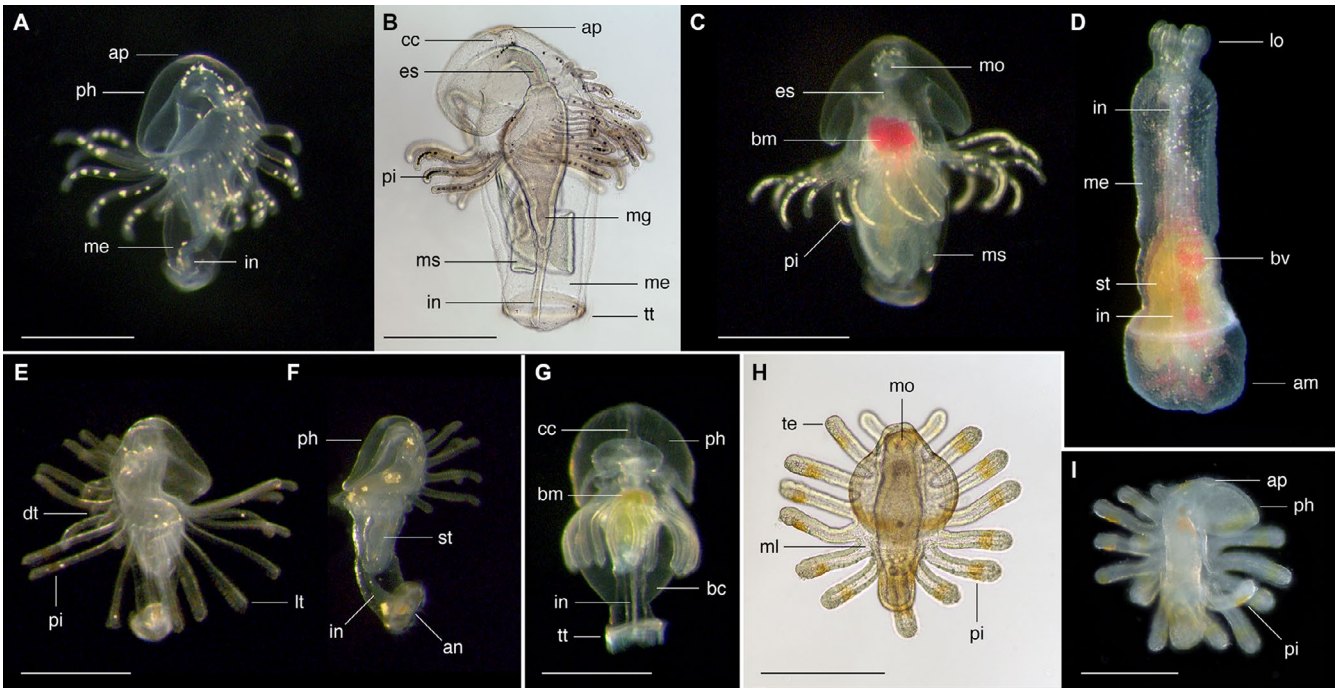

FIGURE 3 Actinotroch larvae of phoronids collected from Bocas del Toro Province, Panama, including representative individuals from which we obtained cytochrome oxidase subunit I (COI) haplotypes from four distinct Operational Taxonomic Units (OTUs C1, C2, C3, and C4). Each larva is oriented with anterior to the top. **A-D.** C1; left-to-right series of larvae in different stages of growth, with juvenile worm at far right. **E,F.** C4; dorsal (left) and lateral (right) views of two different specimens. **G.** C2; dorsal view. **H,I.** C3; anterior-ventral (left) and lateral (right) views of two different specimens. All photographs were produced from live animals. Scale bars: A = 300 μm, B-C = 500 μm; no scale bar for juvenile worm in D; E-G = 500 μm; H-I = 300 μm. am, ampulla; an, anus; ap, apical plate; bc, blastocoel; bm, blood mass; bv, blood vessel; cc, coelomic cylinder; dt, definitive tentacle; es, esophagus; in, intestine; lo, lophophore; lt, larval tentacle; me, metacoel; ml, mesocoel; mo, mouth; ms, metasomal sac; ph, preoral hood; pi, pigment; st, stomach; te, tentacle, tt, telotroch

the 2013 short-course. These larvae were all large and had morphologies typical of species with non-brooded planktotrophic development described in Temereva (2009). Larvae from this OTU occurred during all four sampling periods. These larvae ranged from 300 μm with 10–12 tentacles to 1.1 mm with 24–28 tentacles (Figure 3A–D). Most of these larvae had one large, vivid red blood mass, located ventrally near the stomach diverticulum, and well-developed metasomal sacs (Figure 3B,C). However, in the largest actinotroch we collected (1.4 mm) the blood corpuscle mass was almost transparent. Emig (1982b) indicates that in some species this character is variable and may be less obvious in older larvae, which appears to be the case in this species. In younger larvae, a pair or scattering of blood masses were visible ventrally around the stomach diverticulum (Figure 3B). There was a coelomic cylinder from the apical plate to the esophagus (Figure 3B), as is typical of *Phoronopsis* larvae. Definitive tentacles were not visible. Yellow pigment was present on the trunk, in the lumen of each tentacle, and there were a few spots on the oral hood (Figure 3A–C). The telotroch was slightly pinkish or brownish. One larva metamorphosed into a juvenile (Figure 3D).

OTU C2 was represented by a single larva, collected in March 2016. This typical actinotroch was ~1 mm long (Figure 3G). The most distinctive feature of this larva was the colorful gut with a stomach that ranged from yellow-green to blue, which we did not observe in any of the other larvae. This is unlikely to be gut contents as larvae were kept in filtered water overnight before photographing. Overall, the body of this young actinotroch had a distinctly large blastocoel and a coelomic cylinder under the apical plate, a feature of *Phoronopsis* larvae (Figure 3G). The tentacles had a soft peach tint and a pair of orange blood masses could be seen in the collar region, near the base of the tentacles (Figure 3G). At this stage, there were no yellow pigment spots and the metasomal sac was not evident.

OTU C3 was represented by only two larvae that were both collected during July 2013. These small ~500-μm larvae had 15 tentacles (Figure 3H,I). They were unusual among the actinotrochs we collected in that they did not have any yellow pigment spots and they were less transparent than the other larvae. Clusters of pink corpuscles were evident along the stomach diverticulum (Figure 3I). There was a band of orange pigment just proximal to the distal tip of each tentacle, and along the anterior margin of the oral hood and at the apical plate (Figure 3H,I).

OTU C4 was represented by three larvae, including two collected in August and one in November 2015. The mature actinotroch of this species is a typical transparent non-brooded planktotrophic larva (Figure 3E,F). At a length of 900 μm, the larva had 14 larval tentacles and a number of smaller definitive tentacles emerging below the larval tentacles. At this stage there were three sets of very pale blood corpuscular masses: one pair located dorsolaterally, under the hood; one unpaired mass located ventrally,

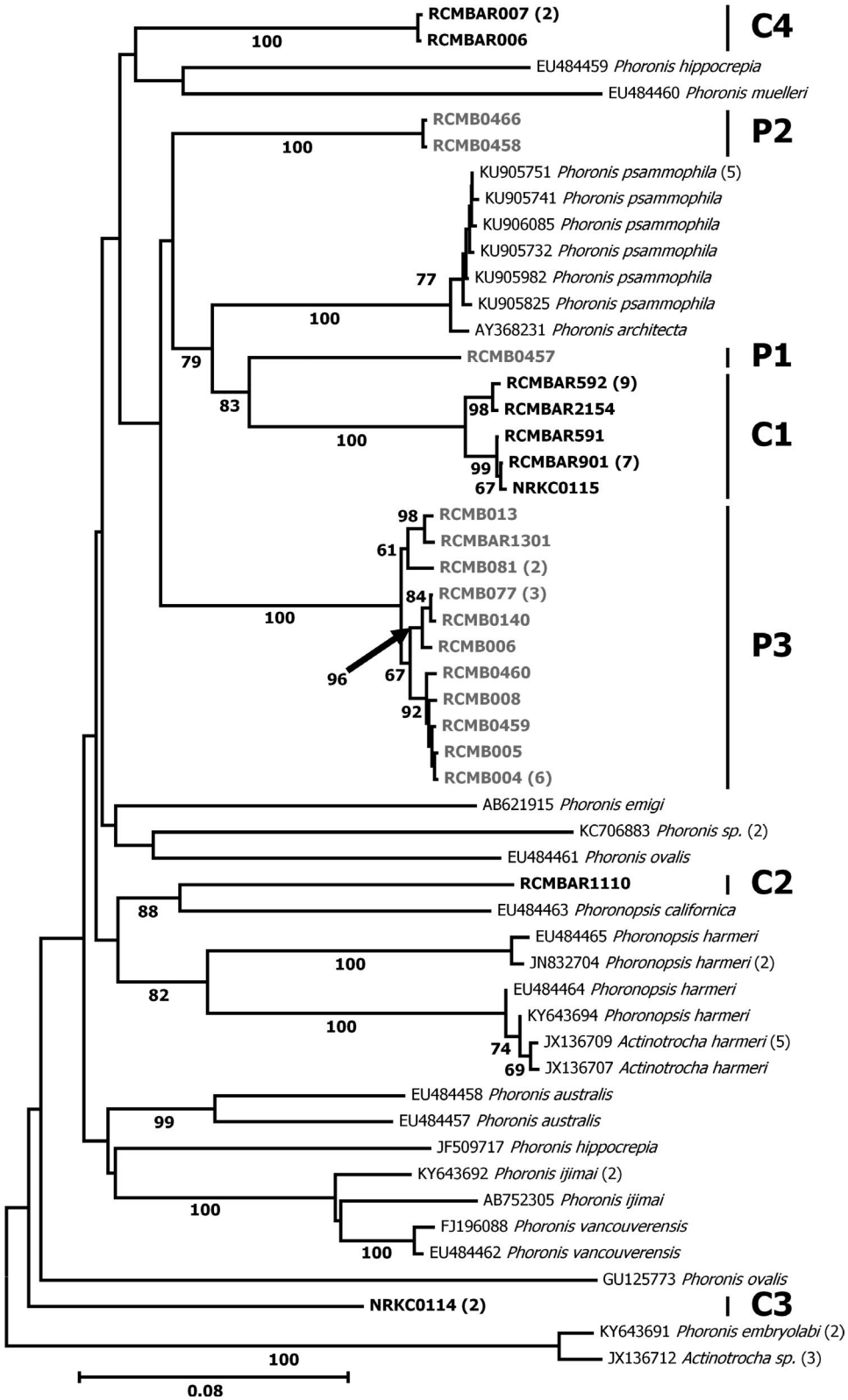

FIGURE 4 Neighbor-joining tree of cytochrome c oxidase subunit I (COI) sequences from phoronid actinotroch larvae from this study and from phoronid adults obtained from GenBank. The tree shows only unique haplotypes. Branch tips for sequences from larvae are labeled with the sample identification number of a representative individual, followed in parentheses by the number of individuals (if ≥2) sharing an identical haplotype. Branch tips for sequences from adults are labeled with a representative GenBank Accession number and the species name (followed by the number of individuals in parentheses). To the right, the OTUs are labeled with their locations (C, Caribbean; P, Pacific) and the OTU number (1–4). Numbers below the branches are bootstrap support values (only values >70 are shown). The scale below shows the Jukes–Cantor substitutions per site

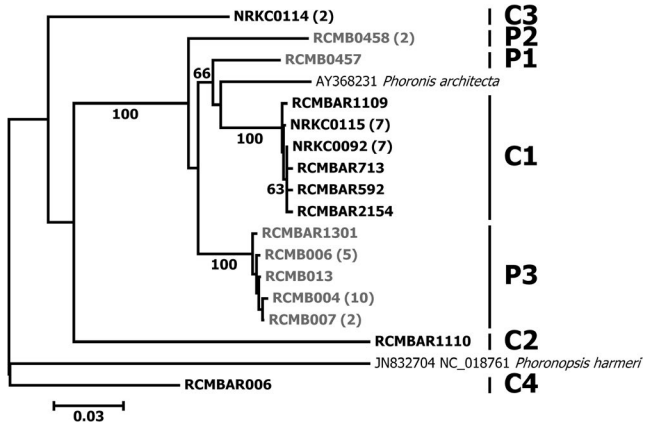

FIGURE 5 Neighbor-joining tree of 16S ribosomal DNA sequences from phoronid actinotroch larvae from this study and from phoronid adults obtained from GenBank. The tree shows only unique haplotypes. Branch tips for sequences from larvae are labeled with the sample identification number of a representative individual, followed in parentheses by the number of individuals (if ≥2) sharing an identical haplotype. Branch tips for sequences from adults are labeled with a representative GenBank Accession number and the species name (followed by the number of individuals in parentheses). To the right, the 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 >70 are shown). The scale below shows the Jukes–Cantor substitutions per site

near the gut; and one pair located dorsally, in the mid-trunk. The metasomal sac was not visible at this stage. Another larva, ~1.0 mm, and a smaller larva, ~700 μm, had 20 tentacles but no visible blood masses, although the larger individual had definitive tentacles (Figure 3E). These individuals also had some very slight red pigmentation towards the ends of the tentacles (Figure 3E), which was not visible in the more mature larva. One of these individuals had a particularly large oral hood that reached almost halfway down the trunk, with a beak-like profile. All three larvae had a few yellow pigment spots on the oral hood and on the trunk, and very few, if any, on the tentacles (Figure 3E,F).

4 | DISCUSSION

It is common for DNA barcoding studies of larvae to show more species than have been detected in surveys of adult diversity (e.g., Barber & Boyce, 2006; Mahon, Thornhill, Norenburg, & Halanych, 2010). This study of phoronid larvae is no exception. Our major result, that none of our samples match published sequences from previous studies, and that they were as different from these other species as the known species are from each other, further supports the idea that the global diversity of phoronids is significantly underestimated (Hirose et al., 2014; Santagata & Zimmer, 2002; Temereva & Chichvarkhin, 2017). Five species of adult phoronids have been previously reported for the Bay of Panama (Emig, 2017). Four of these species, *Phoronopsis harmeri*, *Phoronis psammophila*, *Phoronis muelleri*, and *Phoronis hippocrepia*, have been sequenced from other places in the world (Table 1), and yet none of them matched (i.e., divergence less than the barcode gap) the larvae we collected.

Likewise, although no phoronids have been reported as adults or larvae along the Caribbean coast of Panama, three species have been reported for other parts of the Caribbean (*P. psammophila*, *P. muelleri*, and *P. hippocrepia*; Emig, 2009). None of the sequences for adults of those three species are similar to our larval sequences from the Caribbean coast. Because phoronid larvae can spend an estimated 2–3 months in the plankton (Santagata, 2004; Temereva, 2009), it seems unlikely that small-scale patchiness of the adults along the Panamanian coast could result in the complete absence in our samples of the species previously documented as adults occurring in the region, while seven other OTUs are present in our samples. It is more likely that the low number of clearly diagnostic morphological features that can be easily observed in both adults and larvae limits our ability to distinguish biological species, resulting in poor species-level resolution and taxonomic lumping of distinct taxa (Emig, 1982a; Hirose et al., 2014; Santagata & Zimmer, 2002; Temereva, 2009).

As currently understood, the geographic distributions of adults of many phoronid species include most of the world's oceans, including both temperate and tropical locations. For example, *Phoronis ovalis*, a species with lecithotrophic development, has been reported from the Arctic, northern temperate, tropical, southern temperate, and Antarctic regions, and is present in the Pacific, Indian, and Atlantic Oceans (Emig, 2017; Temereva, Malakhov, Yakovis, & Fokin, 2000). *Phoronis albomaculata*, a species with non-brooded planktotrophic development, has a similarly broad distribution including South Africa, the Pacific coasts of Panama and Costa Rica, Madagascar, Hawaii, and Australia (Dean, Sibaja-Cordero, & Cortés, 2010; Emig, 1982a, 2017), further supporting the conclusion that taxonomic lumping has resulted in significant underestimates of global diversity and overestimates of species range sizes.

Our neighbor-joining analysis of all available COI sequences for phoronids highlights a few particular points of taxonomic uncertainty (Figure 4). For most species, only a single sequence is available, but for those with multiple sequences, the following observations can be made: (a) specimens from the east coast of the United States (Chesapeake Bay and Florida) currently attributed to *P. psammophila* in GenBank form a coherent and well-supported clade with *Phoronis architecta*. It should be noted that none of the *P. psammophila* samples that have been sequenced come from near the type locality of this species in Europe, suggesting that this clade is comprised entirely of *P. architecta* (Santagata & Cohen, 2009). (b) *Phoronopsis harmeri* is comprised of two clades which differ by 18% sequence divergence. One clade includes samples from the eastern Pacific, some of which have been identified as *Phoronopsis viridis*. This clade includes material from near the species' type locality close to Vancouver Island, Canada. The second clade is from the western Pacific. Unfortunately, some of the sequences are not associated with locality information. (c) The

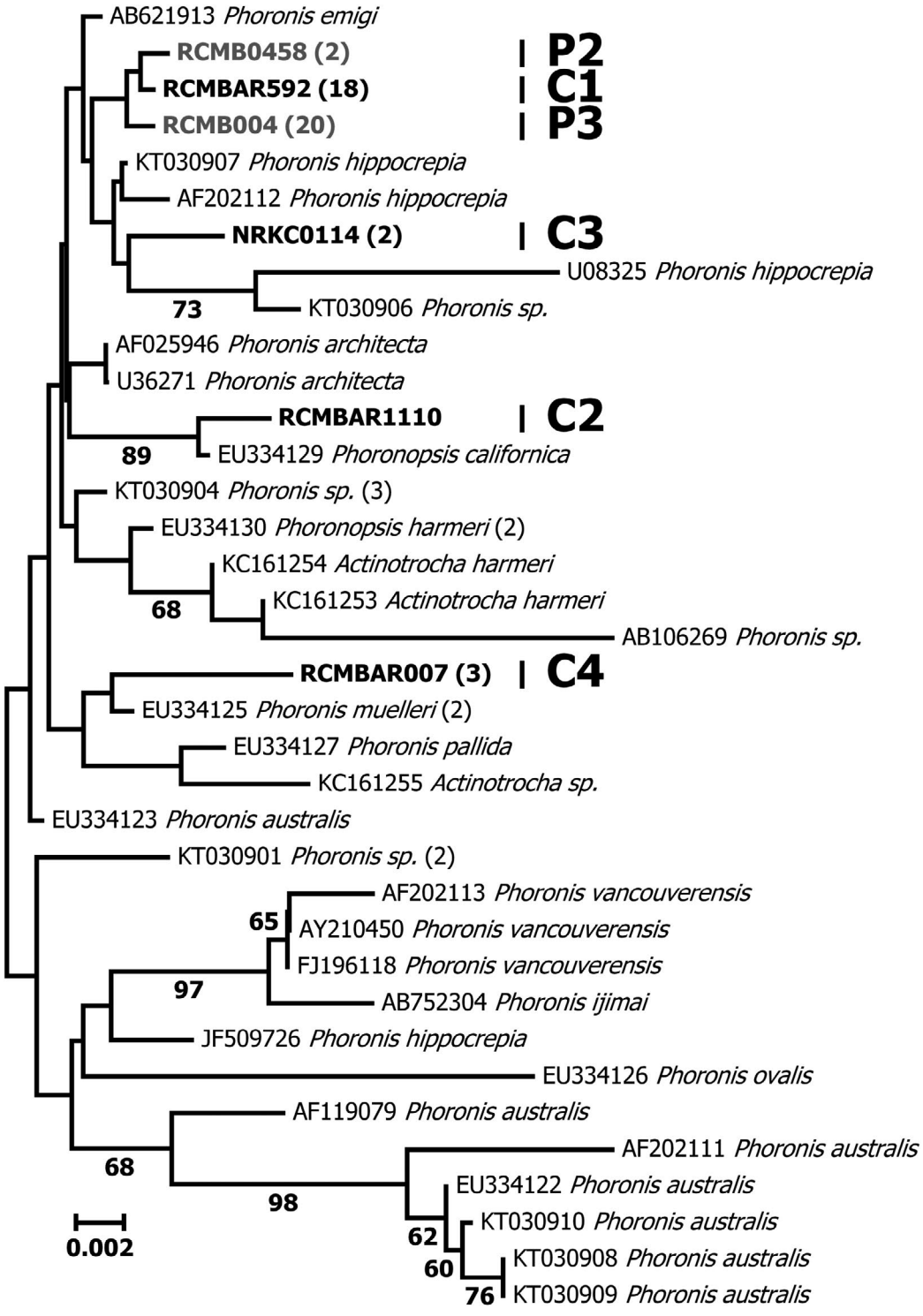

FIGURE 6 Neighbor-joining tree of 18S ribosomal DNA sequences from phoronid actinotroch larvae from this study and from phoronid adults obtained from GenBank. The tree shows only unique haplotypes. Branch tips for sequences from larvae are labeled with the sample identification number of a representative individual, followed in parentheses by the number of individuals (if ≥2) sharing an identical haplotype. Branch tips for sequences from adults are labeled with a representative GenBank Accession number and the species name (followed by the number of individuals in parentheses). To the right, the 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 >70 are shown). The scale below shows the Jukes–Cantor substitutions per site

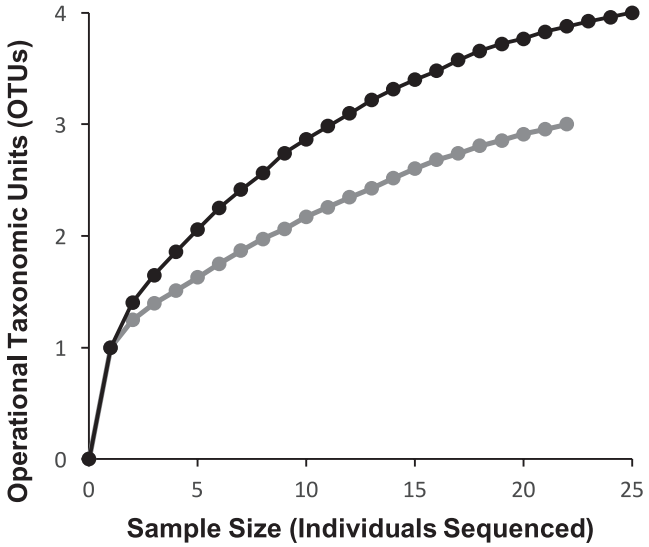

FIGURE 7 Species accumulation curves showing the number of Operational Taxonomic Units (OTUs) detected versus the number of individuals sequenced for the Pacific (grey) and Caribbean (black) coasts of Panama

consistent placement of OTU C2 as sister to *Phoronopsis californica* and near the other *Phoronopsis* species, as well as the presence of the preoral cylinder in these larvae, strongly indicate that this is a *Phoronopsis* species. (d) The two sequences attributed to *P. ovalis* are as different from each other as they are from any other phoronid, although they were collected relatively near to each other in Sweden and in the Irish Sea. It is possible that one of these sequences is incorrect, as the 18S sequence from the same paper was excluded from our analysis because it generated an extraordinarily long branch compared to all of the other 18S sequences. (5) The two sequences of *P. hippocrepia* are also highly divergent and were both collected in Europe (one from Sweden and one from France). These results are not surprising because, in virtually all phyla of marine invertebrates, studies have reported numerous cryptic species in what were once thought to be cosmopolitan species, through detection by DNA barcoding (e.g., Barroso, Klautau, Solé-Cava, & Paiva, 2010; Collin, 2005; Cornils, Wend-Heckmann, & Held, 2017; Kawauchi & Giribet, 2014; Pérez-Portela, Arranz, Rius, & Turon, 2013). However, our analysis of GenBank sequences suggest that increased sampling effort, even in places like Europe with a well-known fauna, is likely to significantly alter our estimates of phoronid diversity and their corresponding geographic ranges. It is also clear that a thorough taxonomic revision of the group, including morphological data from adults and larvae, as well as molecular data and material from the type localities, will be necessary before phoronids can be identified by name with any certainty.

TABLE 3 Total number of operational taxonomic units (OTUs) estimated for each ocean site by fitting species accumulation curves (averaged across 1,000 replicates) to non-linear asymptotic regression models

	Atlantic	Pacific
OTUs found	4	3
OTUs estimated by Biexponential 5P – Model	4.53 (4.49–4.57)	4.05 (3.92–4.18)
OTUs estimated by Michaelis–Menten – Model	5.04 (4.80–5.27)	3.58 (3.28–3.88)

Our quantitative sampling in Bocas del Toro revealed actinotroch densities of ~0.2 individuals per m^3. This is significantly lower than the high maximum densities of 3,940 individuals per m^3 reported for Vostok Bay, Sea of Japan, where actinotrochs represent a significant proportion (10.5%) of the total zooplankton at certain times of the year (Omelyanenko & Kulikova, 2011). Our impressions are that in both the Caribbean and the Pacific it is typical to obtain 1–3 actinotrochs per 10- to 15-min plankton tow, when towing with a small boat primarily in neutral gear and with a 0.5-m-diameter net. None of our tows appeared to have hit a patch of particularly concentrated larvae, suggesting either that we were not towing at the time, depth, or location where larvae concentrate, or that larvae generally occur at lower densities in Panama than in some other locations. Unfortunately, this low abundance made it difficult to assess seasonality with certainty for most of the OTUs. In our quarterly Caribbean samples, the relatively abundant OTU C1 was present in 47% of the tows and occurred during each quarter that we sampled, suggesting that its occurrence is not seasonal. In the Pacific, the most abundant OTU P3 was collected during March, April, and June suggesting that the occurrence of this larva might be associated with upwelling (January–mid May).

The results of this study confirm the idea that invertebrate larvae can be used as a unique and independent method for documenting biodiversity. On the Pacific coast, where four species of phoronid adults had been previously reported, we collected four distinctive, previously undocumented larval OTUs, suggesting that a reexamination of the adults could be a fruitful line of future endeavor. On the Caribbean coast of Panama, where there are no previous records of adult or larval phoronids, we showed that at least four species are present, based on the occurrence of larvae. Years of STRI research on the Caribbean coast of Panama, including in the San Blas Archipelago and at Galeta Point near the city of Colon (Robertson et al., 2009), and more recent intensive study by invertebrate taxonomists (although not phoronid specialists) of the small and cryptic subtidal fauna of Bocas del Toro, have yielded no records of adult phoronids from the Caribbean coast of Panama.

Finally, this study developed phoronid-specific 16S primers which will allow the use of 16S as a barcode marker for phoronids, in addition to COI, and which may also help provide phylogenetic resolution among closely related species in the context of multi-gene phylogenetic datasets. Photographic documentation of larvae linked to COI, 16S, and 18S sequences will enable our unnamed Panamanian taxa to be placed in a broader, comparative context as the phylogeny of phoronids gains resolution with future sampling worldwide.

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