Molecular and morphological congruence of three new cryptic *Neopetrosia* spp. in the Caribbean

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ABSTRACT

Neopetrosia proxima (Porifera: Demospongiae: Haplosclerida) is described as a morphologically variable sponge common on shallow reefs of the Caribbean. However, the range of morphological and reproductive variation within putative *N. proxima* led us to hypothesize that such variability may be indicative of cryptic species rather than plasticity. Using DNA sequences and morphological characters we confirmed the presence of three previously undescribed species of *Neopetrosia*. Morphological differences of each new congener were best resolved by partial gene sequences of the mitochondrial cytochrome oxidase subunit 1 over nuclear ones (18S rRNA and 28S rRNA). Several new characters for *Neopetrosia* were revealed by each new species. For example, *N. dendrocrevacea* sp. nov. and *N. cristata* sp. nov. showed the presence of grooves on the surface of the sponge body that converge at the oscula, and a more disorganized skeleton than previously defined for the genus. *N. sigmafera* sp. nov. adds the (1) presence of sigma microscleres, (2) significantly wider/longer oxeas (>200 µm), and (3) the presence of parenchymella larvae. Sampling of conspecifics throughout several locations in the Caribbean revealed larger spicules in habitats closer to the continental shelf than those in remote island locations. Our study highlights the importance of integrating molecular and morphological systematics for the discrimination of new *Neopetrosia* spp. despite belonging to one of several polyphyletic groups (families, genera) within the current definition of the order Haplosclerida.

Subjects Biodiversity, Marine Biology, Molecular Biology, Taxonomy, Zoology
Keywords Demosponges, Haplosclerida, Neopetrosia, Molecular systematics, Caribbean

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INTRODUCTION

Cryptic species have posed a challenge to taxonomy and biodiversity studies for over 300 years, but access to DNA sequencing has provided relatively simple tools to resolve species boundaries among morphologically similar species (*Bickford et al., 2007*; *Stat et al., 2012*). Particularly for taxa belonging to highly diverse orders with variable growth forms and limited morphological characters, such as corals and sponges, the integration of molecular and morphological approaches can be invaluable (*Wörheide & Erpenbeck, 2007*;

Concepcion et al., 2008; Forsman et al., 2009). In sponges, congruence of molecular and morphological datasets have been successful at the subclass level and have reclassified Demospongiae into subclasses Verongimorpha, Keratosa, and the Heteroscleromorpha (Borchiellini et al., 2004; Sperling, Peterson & Pisani, 2009; Cárdenas, Pérez & Boury-Esnault, 2012; Morrow & Cárdenas, 2015). The presence of siliceous megascleres (monaxons and/or tetraxons) and highly diversified microscleres as synapomorphic characters in Heteroscleromorpha were substantiated by partial nuclear gene sequences (28S rRNA and 18S rRNA) and mitochondrial gene sequence (Holmes & Blanch, 2007; Lavrov, Wang & Kelly, 2008). However, congruence of morphological and molecular datasets for lower taxonomic classifications within Heteroscleromorpha (>6,800 species) have been unsuccessful. Most species within Heteroscleromorpha belong to the order Haplosclerida (1,101 species) (Morrow & Cárdenas, 2015; Van Soest et al., 2018). Although mitochondrial and nuclear genes show Haplosclerida to form a well-supported divergent clade from Heteroscleromorpha (Lavrov, Wang & Kelly, 2008; Thacker et al., 2013), almost every family within Haplosclerida is polyphyletic (Redmond et al., 2011, 2013).

Among these polyphyletic families is the Petrosiidae, which currently consists of 212 species with most of these belonging to *Petrosia* Vosmaer, 1885 (120 species), followed by *Xestospongia* De Laubenfels, 1932 (57 species), *Neopetrosia* De Laubenfels 1949 (33 species), and *Acanthostrongylophora* Hooper, 1984 (two species) (Van Soest et al., 2018). *Xestospongia* and *Neopetrosia* are mainly distinguished on the basis of spicule size, the former usually having spicules larger than 200 µm and the latter shorter. *Neopetrosia* congeners are distributed worldwide and nine are found in the Tropical Western Atlantic. These include *Neopetrosia carbonaria* Lamarck, 1814, *N. subtriangularis* Duchassaing De Fonbressin, 1850, *N. proxima* Duchassaing De Fonbressin & Michelotti, 1864, *N. rosariensis* Zea & Rützler, 1983, *N. dominicana* Pulitzer-Finali, 1986, and *N. sulcata* Santos, Sandes, Cabral & Pinheiro, 2016, which are found in shallow to deep reefs; and *N. dutchi* Van Soest, Meesters & Becking, 2014, *N. eurystomata* Van Soest, Meesters & Becking, 2014, and *N. ovata* Van Soest, Meesters & Becking, 2014, which are recently discovered mesophotic reef species. Mitochondrial and nuclear sequence data have been published for eight congeners which deeply diverge from one another and are polyphyletic (Redmond et al., 2011; Thacker et al., 2013; Redmond et al., 2013). Mindful of the polyphyletic nature of *Neopetrosia*, our purpose for this study was not to find markers that resolve the monophyly for this genus but rather use a pairwise comparison of mitochondrial and nuclear DNA sequences of our material with those from GenBank to confirm molecular and morphological separation for new congeners in the Caribbean.

Among tropical W. Atlantic *Neopetrosia*, *N. proxima* is a rather widespread species, distributed from the Bahamas to Northern Brazil and shows considerable habitat and geographical variability (Zea, 1987; Zea, Henkel & Pawlik, 2014). In fact, detailed morphological revision of material previously considered to belong to this species has yielded new species (Santos et al., 2016). In this study, Santos and colleagues distinguished *N. sulcata* from *N. proxima* by noticing a digitate morphology, lighter color tones with no differentiation between the ectosome and choanosome. While reviewing material

of what was believed to be *N. proxima* or close relatives from Colombia, Panamá, and Martinique, we found several morphologically distinct morphotypes. After detailed molecular barcoding with partial sequence of the cytochrome oxidase subunit 1 (COI), 28S rRNA, and 18S rRNA and morphological comparisons, we were able to distinguish three new species from morphologically similar *N. proxima*, which we describe and compare here.

MATERIALS AND METHODS

Specimen collection

Sponges were photographed in situ and collected in Bocas del Toro-Panamá, Colombia and Martinique at depths ranging between 4 and 36 m. Specimens from Colombia were collected at Golfo de Urabá, Cartagena and Santa Marta on the South American coast, and the San Andrés/Old Providence Archipelago in the SW Caribbean. Field observations (in vivo) of each specimen's morphology, color, consistency, surface, oscules, exudates, and odors were recorded. Samples were preserved in 95% ethanol, and 4% paraformaldehyde (PFA) for histological examination. Samples preserved in PFA for 2–3 days were later transferred to 70% ethanol.

Type and other specimens were deposited in the Florida Museum of Natural History (catalogue number beginning with acronym UF) in Florida, USA, the Makuriwa Museum of Marine Natural History of Colombia at the Institute of Marine and Coastal Research—INVEMAR in Santa Marta (acronym INV POR) and the Natural Science Institute at the National University of Colombia in Bogota (acronym ICN-MHN(Po)). Fragments were also deposited in the Zoological Museum of Amsterdam at the Naturalis Biodiversity Center in Leiden, The Netherlands (acronym ZMA.POR). Fragments of specimens collected in Panamá were deposited in the Museum of Marine Biology and Limnology at the University of Panamá as required by the collection permit of fauna Nr. 5 issued by the "Autoridad Nacional del Ambiente." Collecting in Colombia was carried out under Decree 309–2003 of the Ministry of the Environment and Sustainable Development as part of the ongoing project "Sponges of the Colombian Caribbean" of INVEMAR's Makuriwa Museum. Some uncatalogued samples were studied during the "Porifera Tree of Life Project Workshop" in Bocas del Toro, Panamá, August 2012. Uncatalogued samples from Martinique were studied during the "2013 Training Course on the sponge biodiversity of the Caribbean Sea, workshop of La Martinique" and the "Kick-off meeting of the Associated International Laboratory MARRIO" in December 2013 (see also *Pérez et al., 2017*).

DNA extraction, sequencing, and phylogenetic analysis

Sponge pieces (30 mg) were removed from type material (preserved in 95% ethanol) collected in Panamá (UF 3854, UF 3856–3860) and were used for DNA extractions. DNA was extracted using the Promega E.Z.N.A. Tissue DNA Kit, following the manufacturer's instructions. DNA concentrations were checked by absorbance ratios using a UV–visible spectrophotometer (Thermo Scientific NanoDrop,

Wilmington, DE, USA). DNA from the first elution was diluted to a working stock concentration of 35 ng μL^{-1}.

A list of primers for polymerase chain reaction (PCR) amplification targeting fragments of the COI, the D1–D2 region of the 28S rRNA gene sequence, and the 18S rRNA gene sequence are provided in Table S1. Partial sequences of the different *Neopetrosia* spp. were made possible using previously reported primer combinations in our PCR reactions (*Folmer et al., 1994*; *Kelly-Borges & Pomponi, 1994*; *Geller et al., 2013*; *Chombard, Boury-Esnault & Tillier, 1998*). Specific primers were then designed using NetPrimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) when sequence data was missing to complete the gene sequence region of interest.

Polymerase chain reactions were carried out in 25 µL total volume including the following: nine µL of H_2O, 12.5 µL of BioMix™ Red (Bioline, Taunton, MA, USA) PCR Mastermix, 0.5 µL of each primer (10 mM), two µL of BSA (100 µg/mL), and 0.5 µL of template DNA. The PCR program consisted of an initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, annealing temperatures ranged between 45 and 60 °C for 30 s to 1 min 30 s depending on the primer combination and gene product of interest, and 1 min extension at 72 °C. A final extension at 72 °C for 8 min finished the reaction. Primer combinations and annealing temperature for each PCR product is listed in Table S2. PCR products were all ran on a 1% agarose gel stained with GelRed and purified using EXOFAP (EXO1 and FastAP). Sequencing reactions were performed using the BigDye TM terminator v. 3.1, and sequencing was done with an ABI Prism 3730XL automated sequencer.

Forward and reverse reads were sequenced to achieve the greatest base calling accuracy for each species and targeted gene fragment. Sequence chromatograms in forward and reverse directions were trimmed (at an error probability limit of 0.05). Chromatograms were then assembled and edited by eye using Geneious 10 (*Kearse et al., 2012*). Base calling while editing was made using the highest confidence score for any given base on one of the two chromatograms. All assembled chromatograms resulted in >90% high quality base pair reads with a mean Phred quality score $\geq$40. Assembled sequences were saved and exported as a fasta file. Each fasta file from targeted gene sequences and each *Neopetrosia* spp. were checked for contamination using the BLAST (*Altschul et al., 1990*) function from GenBank. BLAST results that showed >85% sequence identity and a query cover of >60% to those belonging to Porifera were exported to Geneious 10 and aligned using the ClustalW function with default parameters. Alignments were generated using 439 bp of the COI gene sequence, 821 bp of the D1–D2 region of the 28S rRNA and 638 bp for the 18S rRNA gene sequence. Phylogenetic trees were rooted on outgroups *Baikalospongia intermedia* DAQ167168.1, *Axinella corrugata* KC869523.1 and EF092264.1 for COI, 28S rRNA and 18S rRNA, respectively. A plugin for MrBayes version 3.2.1 (*Huelsenbeck & Ronquist, 2001*) for Bayesian inference (BI) and RaxML (*Stamatakis, 2006*) was added for phylogenetic analyses using a maximum likelihood (ML) framework in Geneious 10. Both analyses were implemented using the GTRGAMMA model with 1,000 bootstrap replicates. The BI was run using

5 million generations sampled every 200 generations. The analysis was stopped when the standard deviation (SD) of split frequencies fell below 0.01. At this point convergence was assumed and the burnin value was determined. Phylogenetic trees were generated in Mega7 (*Kumar, Stecher & Tamura, 2016*). Resulting bootstrap values of >50 from the ML and Bayesian posterior probabilities >0.50 from the BI analysis were incorporated to the tree. Sequences of holotypes and other specimens for each species collected in Panamá were deposited in GenBank and assigned accession numbers reported in Table S3.

Sectioning and spicule preparation

Permanent slides with clean spicules and thick (~1 mm) histological sections (tangential and perpendicular) were prepared for each specimen following the methods in *Zea (1987)*. Spicules were digested from small (20 mg) sponge pieces soaked in commercial sodium hypochlorite and shaken for 12 h. Spicules were subsequently washed and centrifuged three times with DI and resuspended in ethanol; a few drops of spicule suspensions were added to microscope slides, dried on a warm plate, and mounted on Permount®. Tissue sections were either dried on a warm plate or dehydrated and stained in successively stronger ethanol solutions (96%, 100%), and then cleared in xylene; then sections were mounted on Permount®. Individual spicule types and skeletal framework were photographed with a Zeiss AxioCam ERc5s mounted on a Zeiss AxioLabA.1 light microscope. Photographs were processed in Photoshop and measurements carried out from photos with AxioVision SE64 Rel.4.9.1 and ImageJ® (*Abràmoff, Magalhães & Ram, 2005*) (http://imagej.nih.gov/ij/). The lengths and widths of 50 spicules per specimen and spicule types are presented as (minimum–mean (±1 SD)–maximum length/width in μm). A few drops of the spicule suspension from Panamanian specimens were added to a stub, air dried, and imaged under high vacuum with a JEOL 5600 SEM scanning electron microscope (SEM) at the Nano Imaging Facility, University of Maryland Baltimore County. Spicule suspension from Colombian and Martinique specimens were carbon coated with a Quorum Q150R and photographed under a QUANTA 200 FEI SEM. Measurements of spicule tracts, skeletal arrangement of fibers, and meshes were compared across species and specimens from different collection sites.

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RESULTS

Phylogenetic analysis

The phylogenetic relationship between novel *Neopetrosia* spp. using mitochondrial (COI) and nuclear genes (28S rRNA/18S rRNA) reconfirmed the polyphyletic nature of this genus (*Erpenbeck et al., 2007*; *Redmond et al., 2011*; *Setiawan, 2014*; *Setiawan et al., 2018*) (Fig. 1). Nevertheless, the use of different markers allowed us to detect enough genetic differences across all *N. proxima* paratypes and new *Neopetrosia* spp. In particular, COI showed the highest resolution of sequence dissimilarity between all new congeners and confirmed our hypothesis that morphological variability was indicative of cryptic species (Fig. 1A). For example, *N. proxima*, "*N. dendrocrevacea* sp. nov.," and "*N. cristata* sp. nov." were all closely related and formed a divergent clade that was closely related (87% identical) to *Amphimedon queenslandica Hooper & Van Soest, 2006* sequence EU237474.1 (*Kayal & Lavrov, 2008*). Within this clade all *N. proxima* morphotypes were 100% identical to each other, 96% identical to "*N. dendrocrevacea* sp. nov.," 95% identical to "*N. cristata* sp. nov.," and 81% identical to "*N. sigmafera* sp. nov.". "*N. sigmafera* sp. nov." was the most distantly related (<85% sequence similarity) congener with a well-supported and deeply divergent clade. The closest relative to "*N. sigmafera* sp. nov." was *Gelliodes wilsoni Carballo, Aquilar-Camacho, Knapp & Bell, 2013* with 99% identity. Additional congeners like *N. exigua Kirkpatrick, 1900* sequence KX454496.1 and *N. seriata Hentschel, 1912* sequence JN242213.1, were distantly related (<85%) from all new congeners in this study.

The phylogenetic tree of the 28S rRNA gene showed a very similar topology to COI. For example, as in COI, a well-supported clade (1/100) with all specimens of *N. proxima*, "*N. dendrocrevacea* sp. nov.," and "*N. cristata* sp. nov.," were also deeply divergent from the rest of the congeners in the tree (Fig. 1B). Nonetheless, 28S rRNA did show a lower resolution of sequence dissimilarity, with no sequence differences between "*N. cristata* sp. nov." and "*N. dendrocrevacea* sp. nov." Both sequences of these species were 97% identical, with strong support (1/100), to all *N. proxima* morphotypes in the clade. All congeneric sequences in this clade were 93–94% identical to *Petrosia lignosa Wilson, 1925* KC869595.1. In addition, "*N. sigmafera* sp. nov." showed <85% sequence identity to all congeners in this clade. The closest relatives to "*N. sigmafera* sp. nov." were *Gelliodes callista De Laubenfels, 1954* KC869562.1 (89% identical) and *Xestospongia deweerdtae Lehnert & Van Soest, 1999* KX668524.1 (90% identical). Additional sequences from congeners like *N. rosariensis Zea & Rützler, 1983* KC869457.1 and *N. subtriangularis* KC869591.1, were <85% identical to all new species. The closest congener to "*N. sigmafera* sp. nov." appears to be *N. carbonaria* with 88% sequence identity.

The phylogeny of *Neopetrosia* spp. using 18S rRNA resulted in the lowest resolution of sequence dissimilarity with four congeners being 100% identical and grouping into Clade C (*Redmond et al., 2011*) (Fig. 1C). Identical congeners include "*N. cristata* sp. nov.," *N. proxima*, *N. exigua*, and "*N. dendrocrevacea* sp. nov.". The sequence from "*N. sigmafera* sp. nov." was 93% identical to congeners in Clade C, and

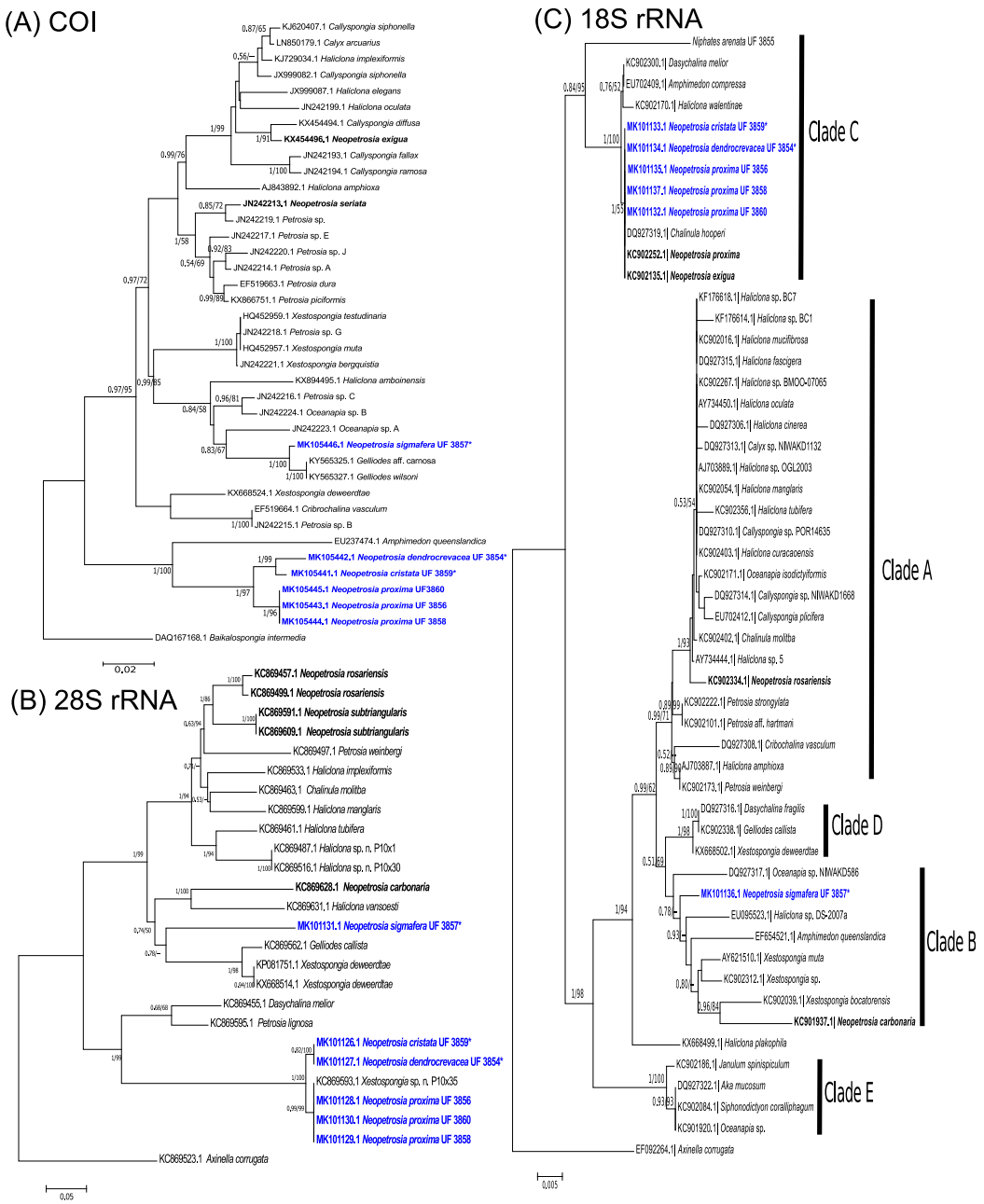

Figure 1 Phylogenetic trees. Bayesian and maximum likelihood topology generated from partial sequences spanning the (A) Folmer (5′) region of the cox1 gene, (B) D1–D2 region of the 28S rRNA gene, and (C) 18S rRNA gene, from Haplosclerida taxa generated in this study (blue) and sequences downloaded from GenBank. Clades in (C) correspond to clades assigned by *Redmond et al. (2013)*. Sequences in bold highlight other *Neopetrosia* species. Bootstrap values less than 50% have been omitted from the trees. Numerical values at nodes show Bayesian posterior probabilities followed by RAxML bootstrap values. Nodes with "—" refer to the absence of the node generated by RAxML. Type specimens are indicated by an asterisk at the end of the specimen's catalogue number. Full-size DOI: 10.7717/peerj.6371/fig-1

grouped into Clade B, which also included *N. carbonaria*. *N. rosariensis* grouped into Clade A and was 95% identical to congeners in Clade C, and 97% identical to "*N. sigmafera* sp. nov.".

SYSTEMATICS

Class Demospongiae *Sollas, 1885*

Subclass Heteroscleromorpha *Cárdenas, Pérez & Boury-Esnault, 2012*

Order Haplosclerida *Topsent, 1928*

Family Petrosiidae *Van Soest, 1980*

Definition. Haplosclerida with an ectosomal skeleton consisting of an isotropic reticulation of single spicules or spicule tracts and choanosomal skeleton verging towards an isotropic reticulation of spicule tracts, in which primary and secondary tracts are indistinct (*Van Soest, 1980*).

Genus *Neopetrosia* *De Laubenfels, 1949*

Definition. Petrosiidae with finely hispid surface produced by fine brushes of oxeas issued from subectosomal tracts, and a compact choanosomal network combining rounded meshes with a superimposed anisotropic reticulation. Megascleres oxeas <200 µm long (*Desqueyroux-Faúndez & Valentine, 2002*).

Neopetrosia proxima* *Duchassaing De Fonbressin & Michelotti, 1864

(Fig. 2; Fig. S1; Table 1)

Thalysias proxima *Duchassaing De Fonbressin & Michelotti, 1864*: 84, Pl. VIII, Figs. 2 and 3.
Densa araminta *De Laubenfels, 1934*: 14.
Neofibularia proxima; *Wiedenmayer, 1977*: 147, 255.
Xestospongia proxima; *Van Soest et al. 1983*: 198; *Van Soest, 1984*: 143; *Zea, 1987*: 116, Fig. 34, pl. IX, Figs. 3 and 4; *Van Soest & Stentoft 1988*: 132, pl. XII, Fig. 4, text, fig. 64; *Lehnert & Van Soest 1996*: 77, Fig. 29; *Díaz, 2005*: 470; *Collin et al. 2005*: 648; *Rützler et al. 2000*: 278.
Neopetrosia proxima; *Campos et al. 2005*: 13, Figs. 8A–D; *Muricy et al. 2011*: 106 (with further synonyms from Brazil); *Zea, Henkel & Pawlik, 2014* (field guide); *Santos et al. 2016*: 336, Fig. 4; *Van Soest, 2017*: 35, Fig. 21a–d; *Pérez et al. 2017*: 10.

Material examined. Bocas del Toro, Panamá: UF 3856, Punta Caracol (9.3777° N, 82.1265° W) eight m in depth, coll. Jan Vicente, May 8, 2015; UF 3858 and UF 3860 Dolphin Rock (9.35076° N, 82.1863° W), 14 m in depth coll. Jan Vicente and Arcadio Castillo May 20, 2015. Uncatalogued fragments PPA 35, 37 and 38, Isla Colón, Aeropuerto (9.3339° N, 82.2548° W), on rubble and sand, fringing reef, seven m in depth, coll. Sven Zea, August 9, 2012. Colombia: INV POR1304, Bahía de Santa Marta, El Morro (11.2494° N, 74.2302° W) reef base, 30–36 m in depth, coll. S. Zea, February 10, 1988. INV POR1306, San Andrés Archipelago Old Providence, north of Low Cay (Pallat Bank, 13.5525° N, 81.3245° W), fore reef terrace, 25 m in depth, coll. Sven Zea, October 19, 1994. Further Colombian material is described in *Zea (1987)*.

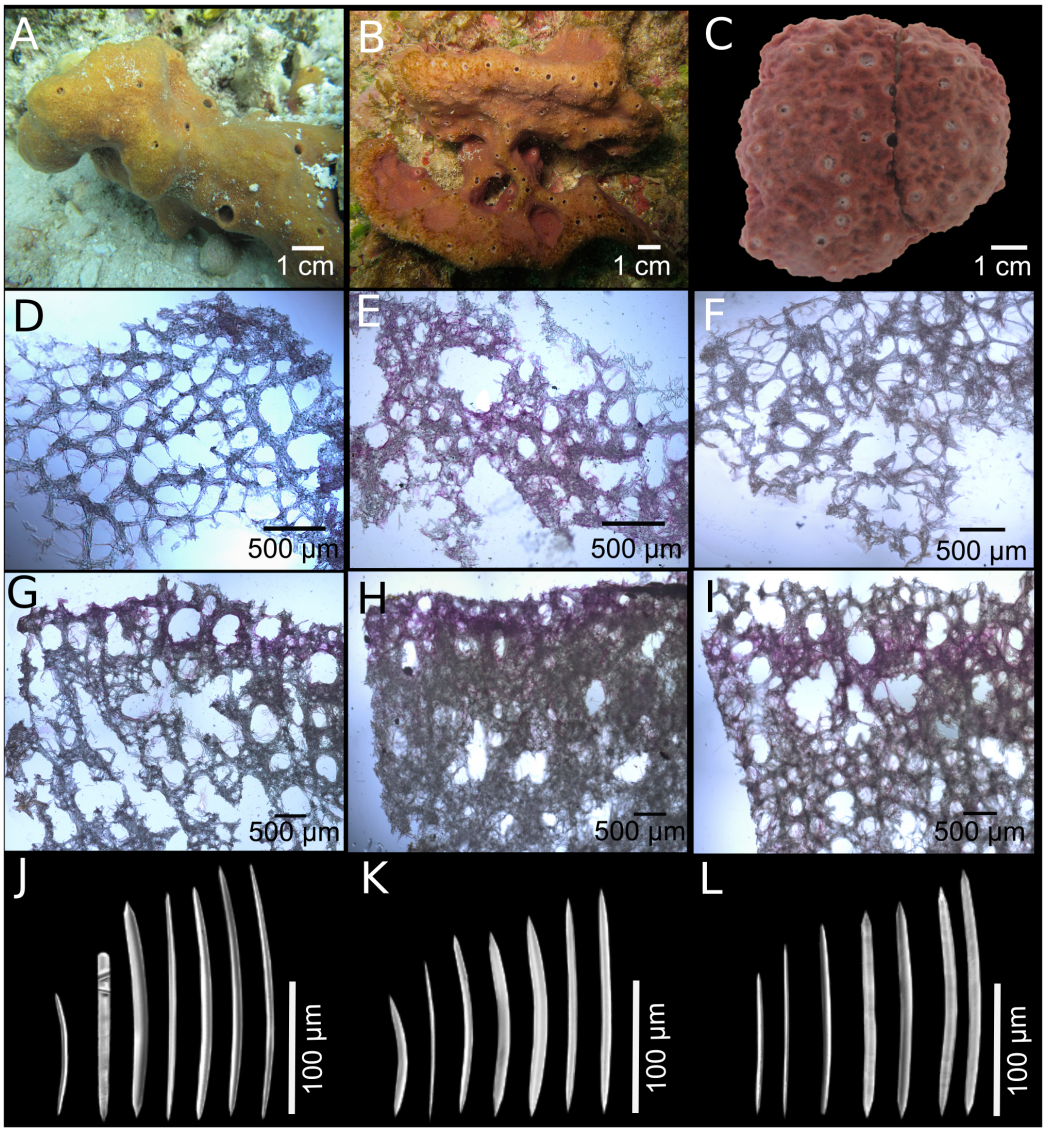

Figure 2 ***Neopetrosia proxima*** ***Duchassaing De Fonbressin & Michelotti, 1864*****.** In situ images of Panama specimens (A) UF 3856, (B) UF 3858, and ex situ image of (C) UF 3860, with corresponding images of (D–F) tangential sections of the ectosome (LM); (G–I) perpendicular sections through the ectosome and choanosome (LM); (J–L) size and morphological variations of oxeas (SEM).

Full-size DOI: 10.7717/peerj.6371/fig-2

Description (Fig. 1; Table 1). The external morphology varies from cylindrical (Fig. 2A) or flat branching individuals (from 5 × 15 cm by 5 cm thick), to thickly encrusting (two cm thick) mounds (Figs. 2B and 2C); encrusting specimens often fill cavities and appear level with the substratum. Oscule size varies between two and seven mm in diameter and are either randomly scattered along the body of the sponge (Figs. 2A and 2C), or aligned along elevated ridges (Fig. 2B). A white membrane collar surrounding the oscules was observed in some individuals (Fig. 2C). Consistency is toughly compressible but difficult to cut with a scalpel or a knife. The surface texture is velvety, from even and smooth (Fig. 2A) to rugged (Figs. 2B and 2C), often knobby from conical

Table 1 Spicule measurements of oxeas (length and width) of *Neopetrosia* spp. described in this study.

Species	Specimen	Location	Length (μm)	Width (μm)
Neopetrosia proxima *Duchassaing De Fonbressin & Michelotti (1864)*	UF 3856	Bocas del Toro, Panama	98–*158.7* (±19.9)–193	3–*10.9* (±1.7)–11
	UF 3858	Bocas del Toro, Panama	92–*146.6* (±14.0)–168	3–*9.0* (±1.7)–12
	UF 3860	Bocas del Toro, Panama	117–*159.3* (±14.0)–181	6–*9.3* (±1.1)–12
	Uncat. PPA 35	Bocas del Toro, Panama	75–*NA* (± NA)–205	2.7–*NA* (± NA)–10.7
	Uncat. PPA 37	Bocas del Toro, Panama	85–*NA* (± NA)–167	1.7–*NA* (± NA)–12.7
	INV POR1306	Old Providence, Colombia	110–*NA* (± NA)–150	2.5–*NA* (± NA)–5
Neopetrosia dendrocrevacea sp. nov.	UF 3854 Holotype	Bocas del Toro, Panama	91–*165.2* (±15.9)–188	2.8–*7.4* (±1.5)–10.5
	Uncat. PPA 07	Bocas del Toro, Panama	111—*156.5* (±14.6)—181	4.5—*6.6* (±0.9)—8.9
	INV POR0535	Urabá, Colombia	134–*171.4* (±12.9)–198	4.4–*7.4* (±1.0)–9.4
	INV POR 1336	Cartagena, Colombia	133–*165.1* (±12.9)–189	4.6–*6.4* (±0.6)–7.7
	INV POR1337	Cartagena, Colombia	139–*164.4* (±12.9)–192	4.1–*7.3* (±1.3)–9.8
	ICN-MHN(Po) 0269	Santa Marta, Colombia	130–*151.6* (±9.4)–168	5.0–*6.5* (±0.9)–9
	INV POR1335	Santa Marta, Colombia	103–*147.8* (±12.3)–169	4–*6.5* (±1.1)–9
	INV POR1333	San Andrés, Colombia	86–*119.3* (±11.3)–150	3.5–*4.8* (±0.6)–6
	INV POR1334	San Andrés, Colombia	114–*130.1* (±7.7)–149	5.1–*3.8* (±0.6)–7
Neopetrosia cristata sp. nov.	UF 3859 Holotype	Bocas del Toro, Panama	121–*142.1* (±9.8)–163.2	2.1–7.2 (±1.7)–9.6
Neopetrosia sigmafera sp. nov.	UF 3857 Holotype	Bocas del Toro, Panama	173–*235.9* (±14.1)–259	6.5–*13.6* (±1.5)–15.9
	Uncat. PPA 36	Bocas del Toro, Panama	174–*226.5* (±12.4)–248	6.5–*14.2* (±2.4)–17.6
	Uncat. PPA 48	Bocas del Toro, Panama	196–*232.7* (±10.4)–233	6.9–*13.5* (±1.9)–15.5
	INV POR 1338	Cartagena, Colombia	203.5–*237.4* (±11.8)–255	5.8–*14.0* (±2.8)–14.0
	INV POR 1339	Cartagena, Colombia	201.9–*240.2* (±14.5)–260	5.0–*17.0* (±2.9)–17.7
	ICN-MHN(Po) 270	Cartagena, Colombia	201.9–*240.2* (±14.5)–260	5.0–*17.0* (±2.9)–17.7
	Uncat. SZ-20	Martinique	153–*197.2* (±10.6)–219	6.9–*8.2* (±0.6)–9.3
	Uncat. SZ-21	Martinique	115–*204.6* (±18.6)–230	6.8–*8.8* (±0.7)–10.3
	Uncat. SZ-23	Martinique	130–*190.4* (±13.2)–190	4.9–*6.8* (±1.0)–8.6

Notes:
Measurements are expressed as minimum–*mean* (±1 standard deviation)–maximum. *N* = 50.
NA, not available; Uncat, uncatalogued sample.

or blunt elevations around oscules; massive specimens often have keyhole to irregular grooves. All individuals produced a sticky substance when cut or squeezed in situ. Surface color across individuals from Panamá varied from yellow (Fig. 2A), dark brown (Fig. 2B), to light purple (Fig. 2C); Santa Marta specimens in Colombia are characteristically violet to pink (see *Zea, 1987*); in other areas color is predominantly yellowish to purplish dark brown. Internal coloration across all specimens is light-yellow.

Skeleton. The skeleton consists of a fasciculated reticulation of isotropic multispicular tracts that form circular to irregularly elongated meshes. In the ectosome, a paratangential reticulation of tracts (20–200 μm) makes meshes that vary between 120 and 400 μm in diameter (Figs. 2D–2F; Figs. S1A–S1C) depending on the individual (180–300 μm (Fig. 2D), 80–240 μm (Fig. 2E), and 280–390 μm (Fig. 2F)). Smaller circular meshes in the ectosome seem to be the result of thicker spicule tracts (80–170 μm, Fig. 2E; Fig. S1B), when compared to individuals with thinner spicule tracts (50–100 μm, Figs. 2D–2F;

Figs. S1A–S1C). Dark purple pigments from cyanobacteria penetrate about 750 µm into the choanosome (Figs. 2G and 2H; Figs. S1D and S1E). In some individuals, pigments were not observed from the surface but 500 µm below the ectosome (Fig. 2I; Fig. S1F). The ectosome can also be distinguished by the presence of large (500 µm) subectosomal spaces, clearly visible in some individuals (Figs. 2G and 2I; Figs. S1D and S1F), but in others it forms smaller (250 µm) openings (Fig. 2H; Fig. S1E) as a result of denser and thicker spicule tracts. Erect ascending spicule brushes radiate at the ectosome surface. The choanosome also shows a large number of circular meshes that vary in abundance and size (200–700 µm) according to the thickness of spicule tracts (Figs. 2G–2I; Figs. S1G–S1I).

Spicules. Most spicules are slightly curved, symmetric oxeas with very few strongyloxeas present (Figs. 2J–2L); some are more curved and there is variation in size with developmental stage. Oxea endings vary between hastate and conical shapes. Size 92–205 µm long by 1.7–12 µm wide (Table 1).

Habitat and ecology. This species is found living from shallow rocky shores and reefs, to deep reef habitats in a variety of wave-exposures (*Zea, 1987*; *Zea, Henkel & Pawlik, 2014*); also, in caves (*Pérez et al., 2017*). Specimens UF 3858 and UF 3860 were collected in a highly exposed reef (Dolphin Rock) with strong wave energy, while specimen UF 3856 was collected inside Almirante Bay (Punta Caracol) with very low wave exposure. Strong wave energy is known to influence the appearance of aligned oscula (observed in *X. deweerdtae* collected in the same site, see Fig. 7B of *Vicente, Zea & Hill, 2016*) and is apparent in specimen UF 3856. Brooding larvae were not observed in any specimens; zoanthids were also absent.

Distribution. Bahamas (*Zea, Henkel & Pawlik, 2014*). Caribbean: Puerto Rico, US Virgin Islands, Jamaica, Martinique, Barbados, Panamá, Colombia, Belize (*Zea, 1987*; *Van Soest & Stentoft, 1988*; *Lehnert & Van Soest, 1996*; *Rützler et al., 2000*; *Díaz, 2005*; *Collin et al., 2005*; *Zea, Henkel & Pawlik, 2014*; *Pérez et al., 2017*). Guyana (*Van Soest, 2017*). Brazil: North to North East Regions (Amapa, Maranhão, Rio Grande do Norte and Sergipe states) (*Campos et al., 2005*; *Muricy et al., 2011*; *Santos et al., 2016*).

Taxonomic remarks. All *N. proxima* specimens collected in this study exhibited varied morphologies (physical appearance, color, thickness of fiber tracts, circular meshes). These differences initially lead us to think that these were heterospecific. However, these variations showed no nuclear or mitochondrial genetic differences, and seem to be plastic characters within this species. Upon closer examination, spicule sizes, spicule shapes, the skeletal arrangement of the choanosome and ectosome are all in agreement with previous descriptions (*Zea, 1987*; *Díaz, 2005*; *Zea, Henkel & Pawlik, 2014*).

***Neopetrosia dendrocrevacea* sp. nov.**
(Fig. 3; Fig. S2; Table 1)

Haplosclerida unident. sp. 1; *Zea 2001*, Table 1.
Neopetrosia sp. –"soft"; *Zea, Henkel & Pawlik, 2014* (field guide).

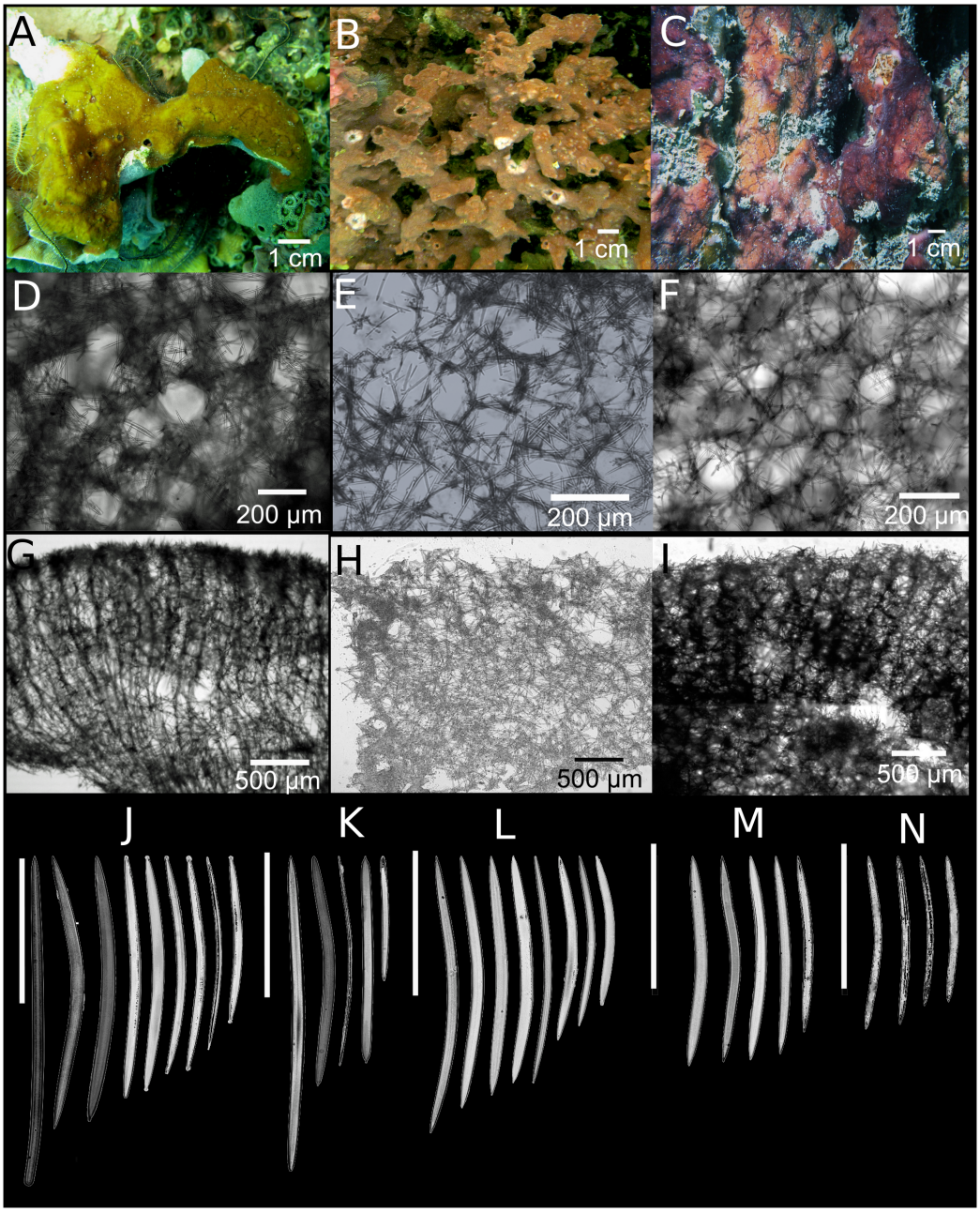

Figure 3 ***Neopetrosia dendrocrevacea* sp. nov.** In situ images of individual PPA 07 (A) and the holotype UF 3854 (B), both from Panama, and of the paratype ICN-MHN(Po) 0269 (C) from Santa Marta, Colombia, with corresponding (second and third rows) images of (D–F) tangential sections of the ectosome (LM); (G–I) perpendicular sections through the ectosome and choanosome (LM); size and morphological variations of oxeas from specimens collected in (J) Uraba, (K) Panama, (L) Cartagena, (M) Santa Marta, and (N) San Andrés Archipelago (LM). Scale bar of (D) is 100 µm.

Full-size DOI: 10.7717/peerj.6371/fig-3

?*Neopetrosia proxima*; *Zea, Henkel & Pawlik, 2014* (field guide, in part, only two images of partly branching and knobby individuals, taken in Panamá, Bocas del Toro, Isla Solarte, Punta Hospital, March 3, 2012, identified from fresh spicule preparations).

Type material and type locality. *Holotype*: UF 3854, Panamá, Bocas del Toro, STRI point (9.3429° N, 82.1258° W), two m in depth, coll. Jan Vicente, June 10, 2015. *Paratypes*: Colombia: ICN-MHN(Po) 0269, Bahía de Nenguange, playa del Manglar, Santa Marta (11.2494° N, 74.2301° W), 1.5 m in depth, coll. Sven Zea, March 18, 1999. INV POR1335, Bahía de Chengue (11.3200° N, 74.1267° W), 1.5 m in depth, coll. Sven Zea, May 19, 1982. INV POR1336, Bancos de Salmedina, Cartagena (10.3735° N, 75.6663° W), 24 m in depth, coll. Sven Zea, August 19, 1980. INV POR1337, Islas del Rosario, Isla Rosario (10.1583° N, 75.8050° W), eight m in depth, coll. Sven Zea, March 7, 1998. INV POR0535, Cabo Tiburón, Golfo de Urabá (8.6840° N, 77.3710° W), nine m in depth, coll. Sven Zea, September 28, 1995. INV POR1333, Isla de Providencia, San Andrés Archipelago (13.5058° N, 81.3558° W), 16 m in depth, coll. Sven Zea, October 21, 1994. INV POR 1334, Banco Serrana, leeward terrace, San Andrés Archipelago (14.4592° N, 80.2740° W), 16 m in depth, coll. Sven Zea, May 14, 1995.

Additional material. Bocas del Toro, Panamá: uncatalogued sample PPA 07, Isla Bastimentos, Adriana's reef (9.2419° N, 82.1736° W), five m in depth, coll. Sven Zea, March 2, 2012.

Description. Thin to thick (one cm) encrustations growing up to 30 cm in diameter; or made up of coalescing, one to two mm thick branches, elevating to 10–15 cm from the base (Fig. 3B). The surface has densely reticulated or scattered characteristic grooves that converge at the rim of the oscules, cutting through them and making them appear lumpy or incomplete (Figs. 3A–3C); sometimes the grooves surround smooth knobs of varied sizes. Oscular diameter range from one to two mm in encrusting individuals to 0.5 cm in branching ones. A translucent membrane surrounds the oscules, sometimes closing them. Consistency from slightly soft to firm, but crumbly. Texture is particularly velvety and when squeezed in situ the sponge produces a sticky substance. External color is golden yellow to reddish brown to dark purple with ochre yellow tinges; light-yellow in ethanol. Interior color light-yellow.

Skeleton. Ectosome as a paratangential reticulation, composed of rather confused, loose, uni to paucispicular tracts, up to 4–10 spicules and 25–70 µm across, forming polygonal meshes 100–200 µm in diameter (Figs. 3D–3F; Fig. S2A–S2C). Single spicules and spicule brushes from the end of choanosomal ascending tracts pierce the surface. Pigments from cyanobacteria penetrate about 600 µm inside the choanosome. The choanosome consists of an anisotropic reticulation with distinguishable, but loose primary tracts, 6–13 spicules and 10–50 µm across, separated by 50–200 µm (Figs. 3G–3I; Figs. S2D–S2F). Tracts are interconnected by solitary spicules or loose paucispicular tracts, forming confused meshes measuring 80–300 µm in diameter (Figs. 3G–3I; Figs. S2G–S2I).

Spicules. Symmetric oxeas, curved, with hastate endings (short but thick pointed ends, 86–198 µm long by 2.8–10.5 µm wide (Table 1). Spicule sizes vary by geographic location. For example, spicules from specimens collected closer to the continental shelf (i.e., Urabá) measured 171.4 ± 12.9 µm × 7.4 ± 1.0 µm while those collected

on the insular shelf (i.e., San Andrés Archipelago) were smaller and measured 130.1 ± 7.7 µm × 3.8 ± 0.6 µm (Table 1; Figs. 3J–3N).

Habitat and ecology. This species is found on shallow rocky substrates (1.5 m) and deep reefs (16 m), living on dead coral rubble or over other sponges. This species is a common sponge of the leeward fore reef terrace of Banco Serrana in the San Andrés Archipelago with an average density of 0.56 individuals per 20 m^2 (*Zea, 2001*).

Distribution. Panamá (Bocas del Toro), Colombia (Urabá, Cartagena, Santa Marta, San Andrés Archipelago, cf. *Zea, 2001*), Puerto Rico (*Zea, Henkel & Pawlik, 2014*). S.Z. examined a dried fragment from the Bay of Honduras which belongs to this species (courtesy of J.C. Lang).

Taxonomic remarks. Although some specimens initially analyzed showed different characteristics from *N. proxima*, like *Haplosclerida* unident. sp. 1 (*Zea, 2001*), or as *Neopetrosia* sp.-"soft" (*Zea, Henkel & Pawlik, 2014*), others were thought to be *N. proxima* (e.g., ICN-MHN(Po) 0269 and INV POR1335). Accordingly, a more detailed molecular and morphological analysis was pursued to detect less obvious differences. COI sequence data of *N. dendrocrevacea* sp. nov. was 96% identical to *N. proxima* and confirmed heterospecificity to *N. proxima* (Fig. 1). Some obvious morphological differences between these species lie in the consistency of individuals, where *N. proxima* is generally firmer and tougher to cut than *N. dendrocrevacea* sp. nov. *N. proxima* also exudes a stickier mucus when cut. Oscules are larger in *N. proxima* and the surface lacks the grooves that seem to be a diagnostic morphological character of *N. dendrocrevacea* sp. nov. The arrangement of the choanosomal and ectosomal skeleton shows very distinct morphologies from *N. proxima*, with reticulation being more isotropic in *N. proxima*. Meshes are also larger in diameter and better organized in *N. proxima*; multispicular tracts are thicker, more dense and fasciculated as described by *Campos et al. (2005)* and *Zea (1987)*. In the field, *N. dendrocrevacea* sp. nov. can be easily confused with *Svenzea cristinae Alvarez, Van Soest & Rützler, 2002* which is also a crumbly, thin to thicker encrustation with yellow tinges, but its spicules are long styles (*Zea, Henkel & Pawlik, 2014*). *N. dendrocrevacea* sp. nov. also shares some similar external features with *Haliclona* (*Soestella*) *walentinae Díaz, Thacker, Rützler, Piantoni, 2007* including the sometimes bumpy surface between shallow grooves, and the similar oxea (100–180 × 3–9 µm). The latter are more thinly encrusting and soft, has a looser and more unispicular skeleton, and the tissue is crisscrossed by purple filamentous cyanobacteria.

Etymology. The given species name is an adjective derived from the Greek word *dendron* that refers to tree, and *crevace* from the old French word referring to groove (*Brown, 1956*) which denotes the presence of branching and meandering grooves along the surface of the sponge. We use the feminine *dendrocrevacea* assuming that *Neopetrosia* is feminine, following Article 31.2 of the International Code for Zoological Nomenclature (http://www.iczn.org/, accessed on October 1, 2018).

Neopetrosia cristata sp. nov.

(Fig. 4; Table 1)

Type material and type locality. *Holotype*: UF 3859, Panamá, Bocas del Toro, Dolphin Rock (9.35076° N, 82.1863° W), 14 m in depth, coll. Jan Vicente and Arcadio Castillo, May 20, 2015.

Description. The holotype is a thickly (up to one cm) encrusting sponge with an irregular shape, 10 cm in diameter. Surface with scattered pointy conulose ends or smooth ridges. Oscules aligned on ridges along the sponge body, sometimes on top of conical elevations, <1 mm in diameter. There are also sometimes narrow grooves that converge around oscules (Fig. 4A, arrow). Consistency is firm but crumbly when torn. Surface texture is smooth and velvety. Specimens exude a sticky substance when squeezed in situ. External color is reddish brown to dark purple and the interior is light-yellow. Interior and exterior tissues turned to a light-yellow color in ethanol.

Skeleton. The ectosome is composed of a rather confused reticulation of loose multispicular tracts, 3–15 spicules and 40–120 µm across, forming circular to polygonal meshes, 150–250 µm in diameter (Fig. 4B). Cyanobacterial pigments penetrate to 700 µm inside the choanosome (Fig. 4C). The choanosome consists of a confused reticulation of loose multispicular tracts, 5–20 spicules and 60–100 µm across (Fig. 4D), forming circular meshes, 100–150 µm in diameter (Fig. 3E).

Spicules. Slightly curved oxeas, 121–160 × 2.1–9.6 µm (Fig. 3F; Table 1).

Habitat and ecology. The holotype was found in a spur and groove, high wave energy environment, growing on a dead coral skeleton.

Distribution. Bocas del Toro, Panamá.

Taxonomic remarks. As predicted by similarities in their morphological characters, the COI phylogeny showed *N. cristata* sp. nov. to be more closely related to *N. dendrocrevacea* sp. nov. than any other congeneric, with 98% identity, and diverged from *N. proxima* with strong bootstrap support (Fig. 1A). Additional sequence data spanning the COI I3-M11 extension (700 bp product alignment) showed that *N. dendrocrevacea* sp. nov. and *N. cristata* sp. nov. were 96% identical which further supports their heterospecificity. This species shares many external morphological characters with *N. dendrocrevacea* sp. nov. These characters are (1) the appearance of grooves along the sponge's surface that converge at the oscules, (2) the velvety texture of the sponge surface, and (3) the disorganized reticulation of the choanosome and ectosome. Nevertheless, both of these species are distinguishable based on the morphology of the grooves along the surface of the sponge which are a lot less pronounced and fewer in number in *N. cristata* sp. nov. (Fig. 4A). In *N. dendrocrevacea* sp. nov. up to seven grooves converge around the oscules in both branching (Fig. 3B) and encrusting (Figs. 3A and 3C) individuals, forming a star-like pattern around the oscules. The appearance of a crown or irregular mounds around

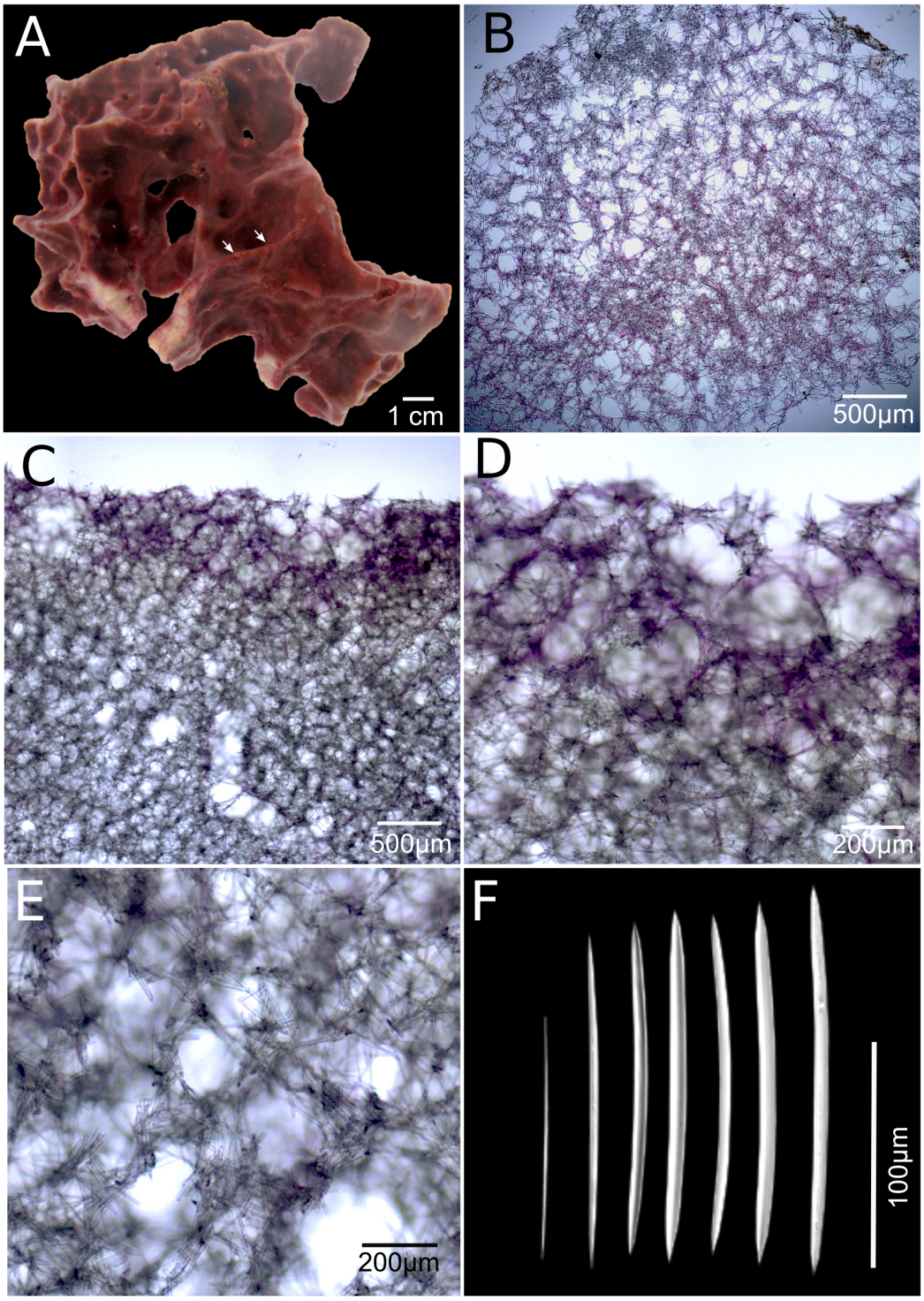

Figure 4 ***Neopetrosia cristata*** **sp. nov.** Holotype (UF3859) (A) ex situ image of live sponge specimen; (B) tangential section of the ectosome (LM); (C) perpendicular section through the ectosome and choanosome (LM); (D) close-up of perpendicular section through the ectosome (LM); (E) close-up of perpendicular section through the choanosome (LM); (F) variation of oxeas (SEM).

Full-size DOI: 10.7717/peerj.6371/fig-4

the oscules is also missing in *N. cristata* sp. nov. The diameter of the oscules is <1 mm in *N. cristata* sp. nov., being larger than one mm in *N. dendrocrevacea* sp. nov. The surface of *N. cristata* sp. nov. is also smoother and lacks the rounded knobs surrounded by grooves found in *N. dendrocrevacea* sp. nov., while those are pointed and dispersed in *N. cristata* sp. nov. Spicules in Panamá are also somewhat smaller and straighter in *N. cristata* sp. nov. (holotype UF 3859: 121–*142.1* (±9.8)–163.2) than in *N. dendrocrevacea* sp. nov. (PPA 07: 111–*156.5* (±14.6)–181; holotype UF 3854: 91–*165.2* (± 15.9)–188).

Etymology. The given species name is an adjective derived from the Latin word *crista*, referring to the surface ridges of the holotype (*Brown, 1956*). We use the feminine *cristata*, assuming that *Neopetrosia* is feminine, following Article 31.2 of the International Code for Zoological Nomenclature (http://www.iczn.org/, accessed on October 1, 2018).

***Neopetrosia sigmafera* sp. nov.**

(Fig. 5; Fig. S3; Tables 1 and 2)

Type material and type locality. *Holotype*: UF 3857, Bocas del Toro, Panamá, Punta Caracol (9.3777° N, 82.1863° W), three m in depth, coll. Jan Vicente, May 8, 2015. *Paratypes:* Cartagena, Colombia: ICN-MHN(Po) 270, Islas del Rosario, Pajarales, close to Yohmara islet (10.1779° N, 75.7750° W), five m in depth, coll. Sven Zea, March 10, 2002. INV POR1338, 1339, Isla del del Rosario, Pajarales (lagoon) (10.1780° N, 75.7750° W), four to five m in depth coll. Sven Zea, August 13, 2014.

Additional material. Bocas del Toro, Panamá: uncatalogued samples PPA 36, Isla Colón, Aeropuerto (9.3339° N, 82.2548° W), seven m in depth, coll. Sven Zea, August 9, 2012; PPA 48, Isla Cristobal, Buoy 19 (9.3018° N, 82.2943° W), eight m in depth, coll. Sven Zea, August 15, 2012. Martinique: uncatalogued samples SZ-20, SZ-21, Les Anses d´Arlet, Le Grande Anse, Salomon´s Garden, Northeast point (14.5053° N, 61.0947° W), 11–18 m, coll. Sven Zea, December 5, 2013. Uncatalogued fragment SZ-23, Les Anses d´Arlet, Le Grande Anse, Pointe Legarde, Southeast point (14.4969° N; 65.0897° W), 24 m, coll. Sven Zea, December, 2013.

Description. Group of tubes or chimneys or ramified, erect, anastomosed mounds, reaching 10–30 cm in width and 10–30 cm in height (Figs. 5A–5C). Sponge surface is smooth, but sometimes with horizontal crests (sinuous channels) (Fig. 5C). Surface is also quite porous (0.5–1 mm diameter pores) and in some specimens can be reticulated. Oscules are generally apical and measure two to five mm in some individuals (Fig. 5A) and up to a one to two cm in others (Fig. 5C). A translucent membrane surrounding the oscules was obvious in some individuals. Consistency is firm, rigid and tough to cut with scalpel but brittle once squeezed with considerable force. Unlike the other congeneric species described in this study, the sponge did not exude a sticky substance when squeezed in situ. The exterior color varies between brownish amber, yellow with sporadic brownish-green blotches (Fig. 5A), to crimson with light and dark tones (Fig. 5C). Color at the base and in the interior of the sponge is light-yellow.

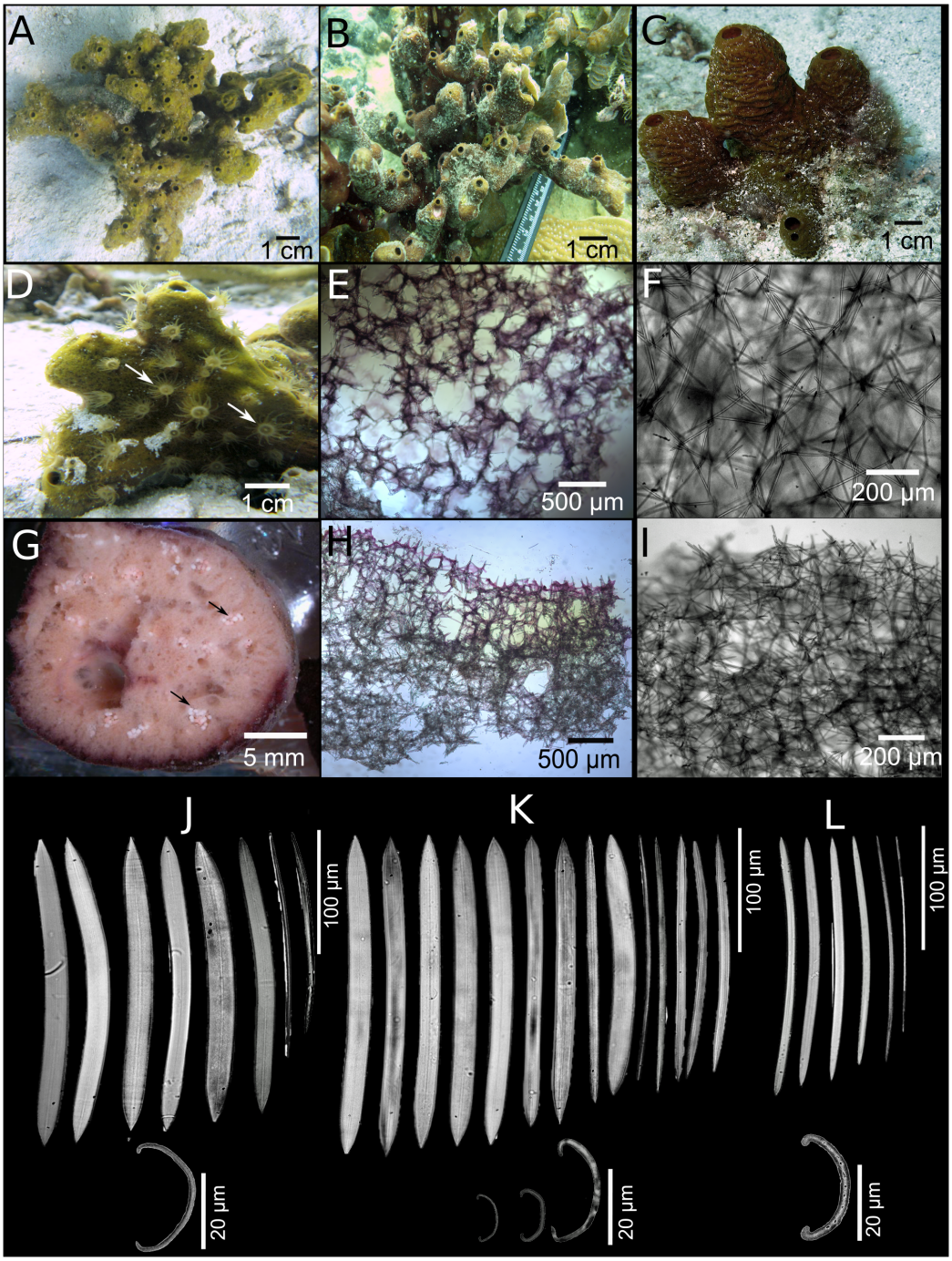

Figure 5 ***Neopetrosia sigmafera*** **sp. nov.** In situ images of the holotype UF 3857 (A) and individuals PPA 38 (B), both from Panama, and SZ-21 from Martinique (C), with corresponding images (D) of zoanthids; (E and F) tangential sections of the ectosome (LM); (G) brooding larvae (arrows) (H and I) perpendicular sections through the ectosome and choanosome (LM); Size and morphological variations of oxeas and sigmas from (J) Panama, (K) Cartagena, (L) Martinique (LM). Full-size DOI: 10.7717/peerj.6371/fig-5

Skeleton. The ectosome is partially tangential, isodictyal, with unispicular or paucispicular tracts (one to six spicules and 13–75 µm across) (Figs. 5E and 5F; Figs. S3A–S3C). Spongin was detected in some nodal points in the ectosome where ascending choanosomal

Table 2 Lengths of sigma of *Neopetrosia sigmafera* sp. nov.

Specimen	Location	Number of sigmas	Length (µm)
UF 3857, holotype	Bocas del Toro, Panama	20	7.6–*22.0*–27.0
Uncat PPA 36	Bocas del Toro, Panama	15	12.7–*22.6*–29.3
Uncat PPA 38	Bocas del Toro, Panama	13	20.6–*24.4*–29.5
INV POR 1338	Cartagena, Colombia	10	11.0–*20.2*–28.7
INV POR 1339	Cartagena, Colombia	10	8.7–*15.1*–30.2
ICN-MHN(Po) 270	Cartagena, Colombia	10	11.2–*19.2*–31.4
Uncat SZ-20	Martinique	10	20.6–*24.4*–29.5
Uncat SZ-21	Martinique	6	10.0–*21.2*–28.7
Uncat SZ-23	Martinique	10	9.3–*19.8*–31.0

Note:
Measurements are expressed as minimum–*mean*–maximum.

tracts connect with perpendicular spicules along the ectosome (Fig. 5E; Fig. S3A). Pigments from cyanobacteria penetrate about one mm into the choanosome (Figs. 5H and 5I; Fig. S3D). The choanosome is an anisotropic reticulation with ascending multispicular tracts (four to eight spicules and 1–80 µm across) and occasional 130–250 µm openings, interconnected by single or loosely arranged spicules (Figs. 5H and 5I; Figs. S3G–S3I). The choanosomal tracts have a larger number of free spicules, are thicker, more confused, and become harder to depict deeper into the choanosome. Tracts become thinner as they ascend towards the ectosome, eventually becoming almost unispicular. Channels in the choanosome have a diameter 0.3–2 mm.

Spicules. Slightly curved oxeas, hastate, with conical to sharp ends, 130–260 × 5–18 µm (Table 1). The mean of oxea sizes vary according to the location where specimens were collected (Figs. 5J–5L). C-shaped sigmas are present in the ectosome and choanosome, 8–*21*–33 µm in length, showing no variation in size among geographic locations but varied abundance across specimens (Table 2).

Habitat and ecology. This species is found on shallow patch reefs and sand flats (three m) in Bocas del Toro and Islas del Rosario (Cartagena), and deeper reef habitats in Martinique (11–24 m). This species is viviparous, being the only congener observed to brood larvae in the summer months in Bocas del Toro, Panamá (Fig. 5G). A detailed description of the morphology and phototactic swimming behavior of *N. sigmafera* sp. nov. larvae are described by *Collin et al. (2010)*. At the time, *N. sigmafera* sp. nov. was misidentified as *N. proxima*, but the morphological assessment in this study clearly shows a different spicule composition, and skeletal arrangement of the choanosome and ectosome from *N. proxima*. The presence of larvae seems to be a diagnostic character that also helps distinguish it from *N. proxima* when both are found living in the same habitat. Zoanthids are occasionally found growing on *N. sigmafera* sp. nov. but not on *N. proxima* in shallow reef habitats of Bocas del Toro (Fig. 5D). This sponge is known to harbor a host specific community of the cyanobacteria species *Synechococcus spongiarum* which produce high amounts of chlorophyll-a (*Erwin & Thacker, 2007*, *2008*).

Distribution. Panamá (Bocas del Toro), Colombia (Cartagena), Martinique (Les Anses d' Arlet). S. Zea observed specimens in Belize (Carrie Bow Cay and Pelican Cays).

Taxonomic remarks. Similar in situ characters shared between *N. sigmafera* sp. nov. and *N. proxima* have made their classification difficult over the last decade; particularly in Bocas del Toro, Panamá, where they are sympatric (Figs. 5A and 2A, respectively). Nevertheless, the phylogenetic analysis of both nuclear and mitochondrial sequence data placed *N. sigmafera* sp. nov. in a well-supported and deeply divergent clade (<85% sequence similarity) from all other new congeners and *N. proxima* (Fig. 1). These results were further supported by several morphological differences. For example, a closer look at the spicules of each species revealed that oxeas are longer, thicker, and have more hastate endings in *N. sigmafera* sp. nov. (130–260 × 5–18 μm) compared to *N. proxima* (85–223 × 2.4–10 μm). *N. sigmafera* sp. nov. also has sigmas as microscleres, which are never present in *N. proxima* or any other *Neopetrosia* spp. The skeleton is also less dense, with less massive spicule tracts in *N. sigmafera* sp. nov. Oxeas are also smaller and thinner than observed in *N. dendrocrevacea* sp. nov. and *N. cristata* sp. nov. Grooves, which are a diagnostic character of *N. dendrocrevacea* sp. nov. are also absent in *N. sigmafera* sp. nov. From all congeners, larvae were only found in *N. sigmafera* sp. nov., suggesting that viviparity seems to be a diagnostic character of this species.

The external morphology of this species is also similar to *N. dominicana*, but the ladder has strongyles instead of oxeas, and also lacks sigmas. There are also some similarities with *X. caminata* (*Pulitzer-Finali, 1986*), although the oscules are much larger (5–10 mm) and spicules are larger (200–260 × 5–14 μm) in the latter species. In addition to oxeas, the spicule composition of *X. caminata* also includes strongyles, while sigmas are absent. Additionally, although *N. sigmafera* sp. nov. also shares a similar branching morphology with *N. subtriangularis*, the skeleton of *N. subtriangularis* is much more neatly reticulated with numerous circular channels, denser multispicular tracts, and smaller oxeas (131–181 × 1.6–11.7 μm). *X. bocatorensis* *Díaz, Thacker, Rützler & Piantoni, 2007* also has hastate oxeas and sigmas, but oxeas reach greater lengths (230–320 × 8–15 μm), and sigmas have a smaller length range (10–26 vs. 8–33 μm in *N. sigmafera* sp. nov.). In addition, *X. bocatorensis* is a thinly encrusting sponge with a purple signature color from associated *Oscillatoria* filamentous cyanobacteria dispersed throughout the ectosome and choanosome (*Díaz et al., 2007*). In contrast, color patterns in *N. sigmafera* sp. nov. are similar to other congeners, having two distinct colors across the body, brown ectosome from cyanobacteria and light-yellow choanosome.

Etymology. The given species name is an adjective that combines the name of the *sigma* microsclere with the Greek suffix *phero*, which translates to "carrying" or "bearing" (*Brown, 1956*). We use the feminine *sigmafera*, assuming that *Neopetrosia* is feminine, following Article 31.2 of the International Code for Zoological Nomenclature (http://www.iczn.org/, accessed on October 1, 2018).

DISCUSSION

Molecular and morphological assessments of putative *N. proxima* and close relatives sampled from Martinique, and the Southern Caribbean, revealed three new species with a variety of new morphological characters, and a new reproductive strategy for the genus. Differences in morphological characters were mostly resolved by partial sequences of the mitochondrial (COI) gene but less so by nuclear genes (28S rRNA and 18S rRNA). *Neopetrosia* is defined by having the presence of a hispid surface produced by the rise of subectosomal tracts above a compact choanosomal skeleton composed of circular meshes with anisotropic reticulation of oxeas that are <200 µm in length (*Desqueyroux-Faúndez & Valentine, 2002*). These characters were well supported by COI, 28S and 18S rRNA sequences in three variable specimens of *N. proxima* which were 100% identical. Identical sequences across the three *N. proxima* paratypes support plasticity of morphological variations in color, oscula alignment, the size of circular meshes throughout the choanosome and ectosome, and the thickness of spicule tracts. In a closer examination of the new congeners we have found that *N. dendrocrevacea* sp. nov. and *N. cristata* sp. nov. have a more confused ectosomal and choanosomal skeleton, with less obvious circular meshes. These morphological differences were supported by a 4–5% divergence in COI sequences of *N. cristata* sp. nov. and *N. dendrocrevacea* sp. nov. to *N. proxima*. The recently discovered congener from mesophotic reefs in Curacao, *N. ovata,* also shows a similar confused skeleton organization as *N. dendrocrevacea* sp. nov. and *N. cristata* sp. nov. (*Van Soest, Meesters & Becking, 2014*). In addition, *N. sigmafera* sp. nov. further deviates from this definition by the presence of sigmas (microscleres), oxeas >200 µm in length, and being the only congener so far known to brood larvae. *N. sigmafera* sp. nov. was also the most distantly related congener to *N. proxima* based on mitochondrial and nuclear sequences (<85% sequence identity).

Improved resolution of the COI gene over nuclear genes are in agreement with the phylogeny of other Haplosclerida where mitochondrial genes (including the COI I3–M11 extension) resolved up to 12 well supported subclades of *Haliclona* spp., while ribosomal sequences only resolve six (*Knapp et al., 2015*). Similar results were also observed in *Tethya* spp. where mitochondrial genes resolved up to five supported subclades, while ribosomal sequences supported four (*Schaffer, Davy & Bell, 2018*). In all phylogenetic trees, *N. proxima*, *N. dendrocrevacea* sp. nov., and *N. cristata* sp. nov. formed a well-supported clade with deep divergence from *N. sigmafera* sp. nov. These results are congruent with multiple diagnostic morphological characters present in *N. sigmafera* sp. nov. that are absent in all other congeners (i.e., presence of sigmas and brooding larvae).

Despite these striking differences, and distant genetic relatedness to other congeners, it is difficult to place *N. sigmafera* sp. nov. in a different genus on the basis of its viviparous nature or presence of sigmas. Other than *X. bocatorensis, N. sigmafera* sp. nov. is the only other larval brooding Petrosiidae (*Collin et al., 2010*), which rejects the hypothesis that all Petrosiidae are oviparous (*Fromont & Bergquist, 1994*; *Maldonado & Riesgo, 2009*), and shows that viviparity is not a good synapomorphic character (*Van Soest & Hooper, 2002*). In addition, the only other Petrosiidae with sigmas is also *X. bocatorensis*

(*Díaz et al., 2007*), which shows that sigmas can be shared across different genera within Petrosiidae. Although being closely related to the genus *Gelliodes* based on mitochondrial and nuclear markers, *N. sigmafera* sp. nov. shares no morphological characters with this genus other than the presence of sigmas and oxeas.

Shared morphological characters between *N. sigmafera* sp. nov. and other *Neopetrosia,* are the pauci- to multispicular ascending and interconnecting tracts, often ending in spicule brushes which support, when present, a tangential uni- to paucispicular reticulation. The firm consistency, the presence of brown- purple pigments in the ectosome with a light-yellow-colored interior, plus the overall size of the spicules are all characters that support a generic morphological classification for *N. sigmafera* sp. nov. within *Neopetrosia*. The presence of sigmoid microscleres is generally not a diagnostic character at the generic level in this family or other Haplosclerid families, and together with viviparity, they are not monophyletic across different taxa by mitochondrial or ribosomal molecular markers (*Redmond et al., 2011*). Thus, given the current lack of congruence between morphological and molecular classifications of Haplosclerida, we are hesitant to erect a new genus for *N. sigmafera* sp. nov. Its placement should be considered temporary while more suitable molecular markers showing monophyly for its unique characters are discovered.

Our study also highlights the effect that environmental factors may have on the size of oxeas. Previous studies have shown that spicule morphology can be influenced by hypersilicification as a result of high silica concentrations (*Maldonado et al., 1999*). Sponges can also produce smaller spicules by living in association with other sponges (*Vicente et al., 2014*). In this study, higher silica concentrations from terrestrial runoff in habitats closer to the continental shelfs are likely the cause of larger oxeas in both *N. dendrocrevacea* sp. nov. and *N. sigmafera* sp. nov. collected in Bocas del Toro, Urabá, Cartagena, and Santa Marta (continental shelf), than in specimens collected in San Andres or Martinique (oceanic islands). Similar variations in spicule sizes have been reported for other species collected in sites with low/high terrestrial runoff (*Zea, 1987*; *DeBiasse & Hellberg, 2015*; *Vicente, Zea & Hill, 2016*; *Silva & Zea, 2017*).

Despite highlighting the polyphyletic nature of Haplosclerida, applying a multilocus based approach using ribosomal and mitochondrial markers continues to prove as a useful tool in resolving the taxonomy between congeneric species. Recently this approach has been used across a wide taxonomic range of sponges (*Erpenbeck et al., 2016*; *Yang et al., 2017*). These methods are useful as a first pass assessment of classification for a wide range diversity of sponges, to be subsequently integrated with morphological systematics. However, in order to understand the evolutionary relationship within Haplosclerida we must continue to focus our research efforts toward finding monophyletic markers by sequencing more genomes from species within different families of Haplosclerida.

CONCLUSIONS

We report molecular and morphological congruence of three new *Neopetrosia* spp. in the Caribbean. Molecular congruence was mostly revealed at the highest resolution by partial sequences of the mitochondrial COI and less by nuclear ones (18S rRNA and

28S rRNA). The most distantly related new congener based on partial COI sequences was *N. sigmafera* sp. nov., which adds the presence of sigma microscleres, significantly wider/longer oxeas (>200 µm), and the presence of parenchymella larvae to the genus. *N. dendrocrevacea* sp. nov. and *N. cristata* sp. nov. were confirmed as sister species based on partial COI sequences and by the shared appearance of a more confused skeletal arrangement, and the presence of grooves on the surface of the sponge body converging to its oscula. Differences in morphological characters from *N. proxima* were also confirmed by differences in COI sequences. Despite being a polyphyletic genetic marker in *Neopetrosia* spp., our study shows that the partial COI gene fragment continues to be a useful marker in resolving cryptic species belonging to highly diverse orders with variable growth forms.

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Competing Interests

Robert J. Toonen serves as an Academic Editor for PeerJ.

Author Contributions

- Jan Vicente conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Jaime Andrés Ríos conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Sven Zea conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Robert J. Toonen contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft, provided additional references and key points to the introduction and discussion of the paper.

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The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

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Data Availability

The following information was supplied regarding data availability:

Raw Sequences and data of spicule measurements are available as Supplementary Files.

All accession numbers are available in Table S3. All sequences were deposited in GenBank under accession numbers: MK105442, MK101127, MK101134, MK105443, MK101128, MK101135, MK105446, MK101131, MK101136, MK105444, MK101129, MK101137, MK105441, MK101126, MK101133, MK105445, MK101130, MK101132.

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Neopetrosia dendrocrevacea LSID: urn:lsid:zoobank.org:act:EEDD5E08-036B-4834-808B-DB68A6B0856B;
Neopetrosia sigmafera LSID: urn:lsid:zoobank.org:act:6B6539BD-029E-4FE5-AECD-447AE7ADE95E;
Neopetrosia cristata LSID: urn:lsid:zoobank.org:act:09FF04FC-F830-43A5-B014-138026C6863C.

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