



Two new species of *Amphiglana* (Sabellidae, Annelida), with an assessment of hidden diversity in the Mediterranean

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Abstract

Amphiglana is a clade of sabellid annelids that has 12 named species from around the world. New COI and 18S sequences were combined with some available data to generate a molecular phylogeny for *Amphiglana*. Two new species of *Amphiglana* are described as a result, using an integrative approach combining molecular evidence with morphological descriptions using histology, 3D reconstructions and electron microscopy. *Amphiglana seaveriae* n. sp. is described from Florida, USA and *Amphiglana joyceae* n. sp. from Edithburgh, South Australia. Our analyses also reveal a previously underemphasized species complex in the Mediterranean, with up to six undescribed species. This highlights the potential diversity of these minute annelid worms yet to be named.

Key words: morphology, taxonomy, DNA, polychaete, sabellid, feather-duster worm, cryptic species

Introduction

Amphiglana Claparède, 1864 is a clade of small sabellid worms with twelve accepted species, described from temperate and tropical latitudes around the globe (Capa & Rouse 2007; Rouse 1993; Rouse & Gambi 1997). All described *Amphiglana* species are known from shallow waters associated with coralline algae, calcareous substrates or coarse sand. Rouse & Gambi (1997; 1998a, b) and Capa & Rouse (2007) assessed the monophyly of *Amphiglana* and investigated the phylogenetic relationships within this taxon via cladistic analyses using morphological data. Capa & Rouse (2007) described six new *Amphiglana* species, doubling the number of accepted *Amphiglana* species. To date no molecular sequence data has been published or analyzed in a phylogenetic context for the majority of known *Amphiglana* spp. As DNA sequencing becomes an essential part of taxonomist's toolkit, it is a primary concern to link the available species names from morphological species descriptions with sequence data (Rouse *et al.* 2018).

In this paper we analyze all currently available genetic data for *Amphiglana*, providing new sequences for *A. terebro* Rouse, 1993, *A. bondi* Capa & Rouse 2007 and *A. lindae* Rouse & Gambi 1997. Our findings led to the discovery of two new species, *Amphiglana joyceae* n. sp. and *Amphiglana seaveriae* n. sp., from Australia and Florida respectively. The description of *A. joyceae* n. sp. is further complemented with histological sections and includes details of reproductive anatomy and spermathecal structure. Several species of *Amphiglana* have previously been subject to studies on their reproductive anatomy (Rouse 1993; Rouse & Gambi 1997, 1998a; b), allowing a detailed comparison of microanatomical structures.

The type species of *Amphiglana* is *Amphicora mediterranea* Leydig, 1851, which was described from Nice, France and is the most commonly known *Amphiglana* species, with a proposed pan-Mediterranean distribution (Sarda 1991). Calosi *et al.* (2013) extensively sampled and sequenced DNA for *A. mediterranea* from Italy as part of an ecological study investigating adaptation and acclimatization to ocean acidification in annelids. This sampling was included in our analyses, which revealed a surprising genetic diversity of *A. mediterranea*. Cryptic speciation in annelids is not uncommon (Nygren 2014) and we discuss the implications of our results for the overall known diversity of this genus.

Materials and methods

Specimens. Specimens of *Amphiglena joyceae* **n. sp.** were collected from Edithburgh, South Australia (35°05'03.8"S 137°44'55.3"E). Two specimens of *Amphiglena seaveræ* **n. sp.** were collected from a small marine pond at Whitney Marine Lab in St. Augustine, Florida (29.6703° N, 81.2145° W). Three specimens of *A. joyceae* **n. sp.** were preserved in OsO₄, eight in seawater/formalin and one directly in ethanol for molecular study. Of the two specimens of *A. seaveræ* **n. sp.** one was preserved in ethanol for molecular study, while the other specimen was preserved in seawater/formalin. Type and voucher specimens are deposited in the Benthic Invertebrate Collection at Scripps Institution of Oceanography (SIO-BIC), La Jolla, California (USA). Table 1 summarizes the collection details, voucher information and the source of analyzed *Amphiglena* sequences.

TABLE 1. Origin of taxa included in this study, vouchers, and GenBank accession numbers. New sequences in bold. * Voucher information from Calosi *et al.* 2013

Specimen	Voucher	Origin	18S	COI
<i>Amphiglena terebro</i>	SIO-BIC A9469	Australia	MK810446	—
<i>Amphiglena lindae</i>	SIO-BIC A9489	Belize	MK810447	MK813354
<i>Amphiglena bondi</i>	SIO-BIC A9477	Australia	MK810443	MK813350
<i>Amphiglena joyceae</i> n. sp.	SIO-BIC A9479	Edithburgh, South Australia	MK810448	MK813353
<i>Amphiglena seaveræ</i> n. sp.	SIO-BIC A9470	Florida, USA	MK810444	MK813355
<i>Eudistylia vancouveri</i>	SIO-BIC A10039	Friday Harbor, USA	MK810445	MK813356
<i>Amphiglena</i> cf. <i>mediterranea</i> III	SIO-BIC A9478	Nisida, Italy	MK810450	MK813351
<i>Amphiglena</i> cf. <i>mediterranea</i> V	SIO-BIC A9472	Madeira	MK810448	MK813352
<i>Amphiglena</i> cf. <i>mediterranea</i> I	100580 *	Nisida, Italy	—	KC591798
<i>Amphiglena</i> cf. <i>mediterranea</i> I	100573 *	Nisida, Italy	—	KC591797
<i>Amphiglena</i> cf. <i>mediterranea</i> II	100572 *	Nisida, Italy	—	KC591796
<i>Amphiglena</i> cf. <i>mediterranea</i> III	100591 *	Ischia, Italy	—	KC591801
<i>Amphiglena</i> cf. <i>mediterranea</i> III	100570 *	Nisida, Italy	—	KC591795
<i>Amphiglena</i> cf. <i>mediterranea</i> III	100586 *	Nisida, Italy	—	KC591799
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100561 *	Ischia, Italy	—	KC591792
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100560 *	Ischia, Italy	—	KC591791
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100557 *	Ischia, Italy	—	KC591790
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100563 *	Ischia, Italy	—	KC591793
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100596 *	Ischia, Italy	—	KC591803
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100564 *	Ischia, Italy	—	KC591794
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100597 *	Ischia, Italy	—	KC591804
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100594 *	Ischia, Italy	—	KC591802
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100590 *	Ischia, Italy	—	KC591800
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100545 *	Ischia, Italy	—	KC591786
<i>Amphiglena</i> cf. <i>mediterranea</i> IV	100553 *	Ischia, Italy	—	KC591789
<i>Amphiglena</i> cf. <i>mediterranea</i> VI	100544 *	Ischia, Italy	—	KC591785
<i>Amphiglena</i> cf. <i>mediterranea</i> VI	100549 *	Ischia, Italy	—	KC591788
<i>Amphiglena</i> cf. <i>mediterranea</i> VI	100547 *	Ischia, Italy	—	KC591787
<i>Amphiglena</i> cf. <i>mediterranea</i> VI	100543 *	Ischia, Italy	—	KC591784
<i>Amphiglena</i> cf. <i>mediterranea</i> VI	100541 *	Ischia, Italy	—	KC591782
<i>Amphiglena</i> cf. <i>mediterranea</i> VI	100542 *	Ischia, Italy	—	KC591783
<i>Amphiglena</i> cf. <i>mediterranea</i> VII	100612 *	S. Caterina, Italy	—	KC591805
<i>Amphiglena</i> cf. <i>mediterranea</i> VII	100617 *	S. Caterina, Italy	—	KC591810
<i>Amphiglena</i> cf. <i>mediterranea</i> VII	100615 *	S. Caterina, Italy	—	KC591808
<i>Amphiglena</i> cf. <i>mediterranea</i> VII	100613 *	S. Caterina, Italy	—	KC591806
<i>Amphiglena</i> cf. <i>mediterranea</i> VII	100614 *	S. Caterina, Italy	—	KC591807
<i>Amphiglena</i> cf. <i>mediterranea</i> VII	100616 *	S. Caterina, Italy	—	KC591809

DNA Sequences. Whole specimens were used for DNA extraction due to the small size of the organism. DNA extraction was conducted using the Zymo Research *Quick-DNA*TM MicroPrep kit. Up to 645bp of mitochondrial cytochrome c oxidase subunit I (COI) were amplified using the HCO2198/LCO1490 primer set (Folmer *et al.* 1994) or dgHCO/dgLCO (Poupin & Malay 2009). Up to 1,858 bp of ribosomal 18S rRNA (18S) was amplified in three fragments using the primer sets; 18S1F/18S5R, 18Sa2.0/18S9R, and 18S3F/18Sbi (Giribet *et al.* 1996). Amplification was carried out using 8.5µl of ddH₂O, 12.5µl of ApexTM 2.0x Taq RED DNA Polymerase Master Mix (Genesee Scientific) or Lambda Biotech Conquest 2X Master Mix Taq (Lambda Biotech), 1µl each of the forward and reverse primers (10µM), and 2µl of eluted DNA. The reactions were carried out in an Eppendorf thermal cycler. The COI reaction protocol was as follows: HCO/LCO; 94°C/180s – (94°C/30s – 47°C/45s – 72°C/60s) * 5 cycles – (94°C/30s – 52°C/45s – 72°C/60s) * 30 cycles – 72°C/300s. dgHCO/dgLCO ; 95°C/120s – (95°C/40s – 47°C/40s – 72°C/60s) * 35 cycles – 72°C/420s. The 18S reaction protocols were as follows. 1F/5R and a2.0/9R: 95°C/180s – (95°C/30s – 50°C/30s – 72°C/90s) * 40 cycles – 72°C/480s. 3F/bi: 95°C/180s – (95°C/30s – 52°C/30s – 72°C/90s) * 40 cycles – 72°C/480s. Successfully amplified products were purified using 2µl of ExoSAP-IT PCR product cleaning reagent. The cleaned products were then sequenced by Eurofins Genomics (Louisville, KY) and assembled with Geneious v.11.0.2 (<https://www.geneious.com>).

Phylogenetic Analyses. In addition to the specimens sequenced for this study (Table 1), all available *A. mediterranea* COI sequences on GenBank were used along with a *Eudistylia vancouveri* (Kinberg, 1866) COI sequence that was used as an outgroup for the phylogenetic analyses (Table 1). While there was a single COI sequence of *Amphiglena terebro* available on GenBank (AF342670), this sequence was excluded from this study, since the BLAST results indicates it is not a sabellid sequence. 18S sequences were generated for the outgroup and a terminal from six of the *Amphiglena* species, including a specimen that falls within the *A. mediterranea* complex (Table1). These were aligned using MAFFT (G-INS-i) (Katoh & Standley 2013). For the concatenated COI and 18S dataset, most *A. mediterranea* COI sequences were excluded, only leaving one representative of each clade identified via COI alone (I-VII). Three phylogenetic analyses were then performed on the concatenated dataset. Maximum likelihood (ML) analyses were executed using RAxML v.8.2.10 (Stamatakis 2014). Best-fyt models for the partitions were selected with ModelTest-NG (<https://github.com/ddarriba/modeltest>), and for 18S TrNef+I, for COI HKY+I+G was selected using the Bayesian information criterion (BIC). Node support was assessed through bootstrapping (1000 pseudoreplicates). Maximum parsimony (MP) analyses were conducted using PAUP* v.4.0a161 (Swofford 2002), using heuristic searches with the tree-bisection-reconnection branch-swapping algorithm and 1000 random addition replicates. Support values were then determined via 100 bootstrap replicates. Bayesian Inference (BI) analyses were performed using MrBayes v.3.2.6 (Ronquist *et al.* 2012). Four Markov chains run over 10M generations, sampled every 1000th step, and with a burnin of 0.25. The complete COI dataset, including all available sequences was analyzed using RAxML, using the model HKY+I+G. Uncorrected pairwise distances were calculated for the COI dataset with PAUP* v.4.0a161 (Swofford 2002).

Morphology. Live *A. joyceae* **n. sp.** and *A. seaverae* **n. sp.** were photographed in the field with a Leica MZ8 stereomicroscope and digital camera. The preserved holotypes of *A. joyceae* **n. sp.** and *A. seaverae* **n. sp.** were imaged under a Leica S8APO stereomicroscope mounted with a Canon EOS Rebel T3i digital camera to document the whole body, and all externally visible structures. In *A. joyceae* **n. sp.**, a separate specimen was cut at the thorax/abdomen junction and a single chaetiger was removed from both the thorax and the abdomen. These chaetigers were placed on separate microscope slides and covered in 50% bleach to dissolve the tissue. The tissue was slightly teased away from the chaetae, examined, and photographed with a Leica DMR HC compound microscope. The same procedure was conducted on the *A. seaverae* **n. sp.** holotype. Two OsO₄ fixed specimens of *A. joyceae* **n. sp.** were prepared for SEM via critical point drying and coating with platinum. Three formalin-fixed *A. joyceae* **n. sp.** were cleared using Murray's Clear (1:2 benzyl alcohol, benzyl benzoate) after dehydration in an ascending series of ethanol and inspected using a compound microscope to determine the distribution of gametes. One formalin-fixed specimen of *A. joyceae* **n. sp.** was embedded in Spurr's Resin. Semi-thin sections (1µm) were prepared using a "Diatome Histo Jumbo" diamond knife on an RMC PowerTome X ultramicrotome. Sections were stained with toluidine blue (1% toluidine, 1% sodium tetraborate and 20% sucrose) and mounted on slides using Spurr's resin.

Results

The concatenated COI+18S dataset comprised 2472 bases and the results are summarized in Figure 1A with the

topologies of the ML, MP and BI analyses being largely congruent, though the ML topology is shown here. The maximum parsimony analysis showed there were 328 parsimony-informative characters and gave a single most parsimonious tree of length 1180 steps. A well-supported clade of all Mediterranean *Amphiglena* species was recovered in ML, MP and BI analyses. *Amphiglena joyceae* n. sp. and *A. seaveriae* n. sp. formed a clade with the *A. mediterranea* terminals and these new species either formed a grade in ML or BI (Fig. 1A) or a poorly supported clade (MP, not shown). In both the ML and BI results, *A. bondi*, *A. lindae* and *A. terebro* formed a clade with high support (80% and 1.0). In the MP analysis *A. bondi* instead was the sister group to *A. joyceae*, *A. seaveriae*, and the *A. mediterranea* terminals. In all analyses, *A. lindae* and *A. terebro* were sister taxa with high support (Fig. 1A). Within the *A. mediterranea* species complex the only two nodes with high support were clade II and III as sister species and the clade of V with VI and VII.

The complete ML tree topology recovered from the COI-only analysis corresponds to the concatenated analyses and is not shown here, as the focus is on the *A. mediterranea* complex (Fig. 1B). The analysis of COI sequences (Fig. 1B) included all *Amphiglena mediterranea* sequences published in Calosi *et al.* (2013). As with the concatenated analyses, a well-supported clade of all *Amphiglena* species from the Mediterranean, including one specimen from Madeira (Atlantic Ocean) was recovered. Within the *A. mediterranea* clade there were seven distinct lineages among which were relatively high divergences (Fig. 1B). The heatmap next to the COI phylogeny clearly shows the high p-distances in among these seven *Amphiglena mediterranea* clades and the low p-distances within each clade. The maximum p-distance within a clade was 0.019 and was between two individuals of clade IV. The lowest p-distances were between two clades were 0.064 (clade II and III), and 0.085 (clade V and VI). The COI sequences of the two new *Amphiglena* species differed markedly from other available *Amphiglena* sequences. For instance, *A. joyceae* n. sp. had a minimum p-distance of 0.238 to *A. mediterranea* III and this distance was higher against all other *Amphiglena* terminals. For *A. seaveriae* n. sp. the smallest p-distance was 0.209 to *A. mediterranea* I.

Discussion

Phylogenetic relationships within *Amphiglena*. Capa & Rouse (2007) published the most recent phylogenetic analysis on *Amphiglena*, building on Rouse & Gambi (1998b) and reassessing its monophyly and sister group. This cladistic analysis was based on 37 morphological characters and included all 12 of the then accepted species of *Amphiglena*. Given the difference in taxonomic sampling, the 18S+COI phylogeny shown here (Fig. 1A) was largely similar, except for the position of *Amphiglena bondi*. However, as noted above the current sampling of *Amphiglena* does not allow to properly assess and resolve the phylogeny and until more genetic data is generated across *Amphiglena* the internal phylogeny of *Amphiglena* and its sister group within Sabellidae remains unresolved.

Hidden Diversity of *Amphiglena* in the Mediterranean. Leydig (1851) published the first description of *Amphiglena mediterranea* (as *Amphicora*) from Nice, France. Leydig's description was brief and he did not leave a type specimen. Claparède (1864) described *Amphiglena armandi* from Port-Vendres, France (pp. 492-496). However, in later text (page 588 and 594) he wrote a "Rectification" where he synonymized *Amphiglena armandi* with *Amphicora mediterranea* Leydig, 1851. Other junior synonyms of *A. mediterranea* described from the Mediterranean coast of France include *Fabricia gracilis* Grube, 1855 and *Amphicorina desiderata* Quatrefages 1866 (see Banse 1957; Fitzhugh 1990; Rouse 1994).

Subsequently, St. Joseph (1894) published more detailed descriptions of *A. mediterranea* from the Mediterranean coast of France and Rioja (1923) found *A. mediterranea* on both the Mediterranean and Atlantic coasts of Spain. Rouse & Gambi (1997) published a detailed redescription of *A. mediterranea* from individuals collected in the Bay of Naples, Italy, though they did not designate a neotype, stating: "The similarity of all the available descriptions of *A. mediterranea* suggests that a single species is present in the Mediterranean and neighboring Atlantic waters. For this reason, no neotype material is designated in this study. More detailed examination of collections made from a variety of regions within this area may show that further division is necessary and then collection of material from the type locality in Nice, France and nomination of a neotype may be necessary."

As the type species of the genus, *Amphiglena mediterranea* is possibly the most common name used in publications from around the world. Historically, the name *mediterranea* has been used for various *Amphiglena* species across the globe (Dauvin *et al.* 2003; Day 1967; Dorgham *et al.* 2013; Faulwetter *et al.* 2017; Hartmann-Schröder 1990; Hartmann-Schröder & Rosenfeldt 1989; Knight-Jones *et al.* 1991; Knight-Jones *et al.* 2017). For many

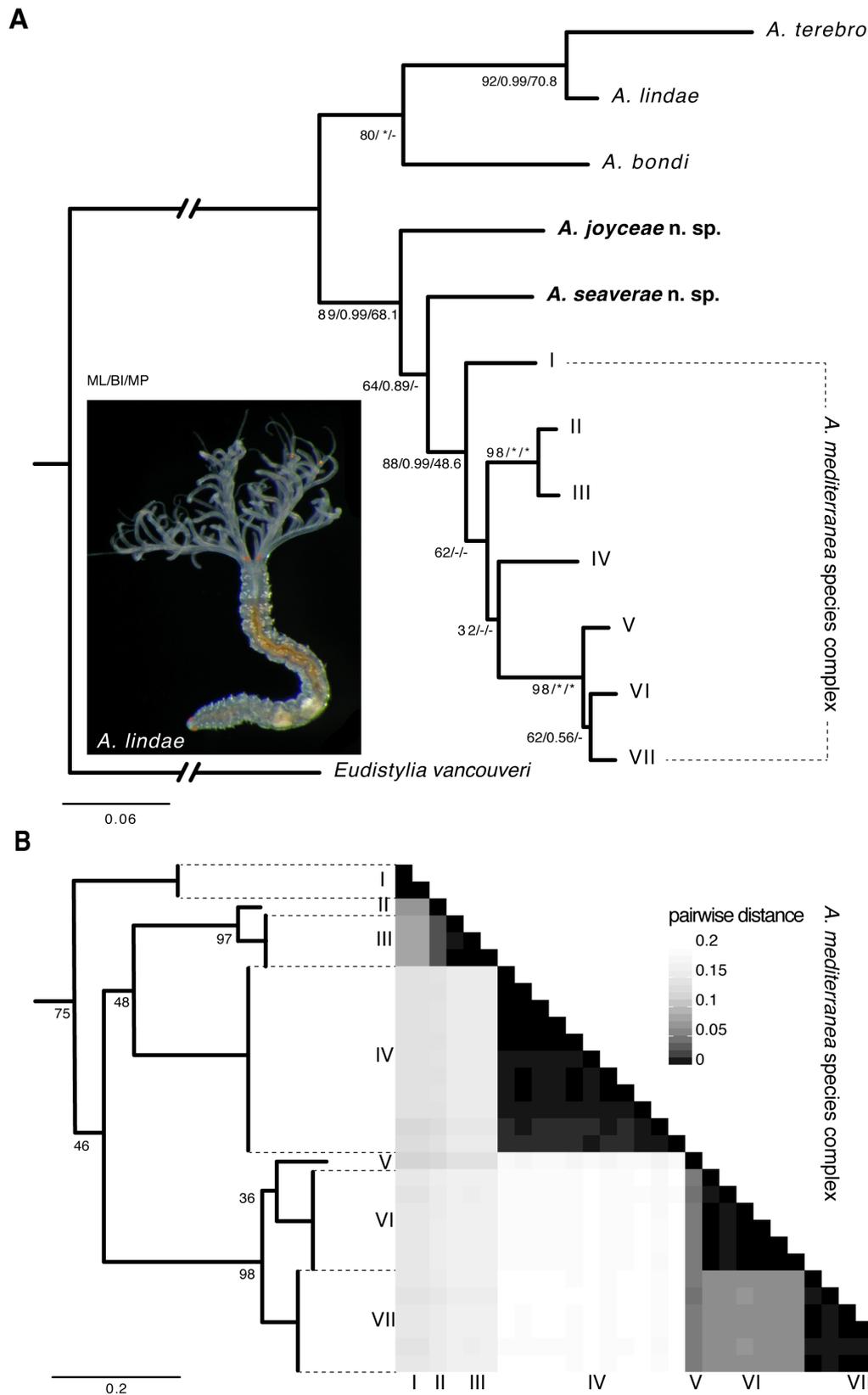


FIGURE 1. A, Maximum likelihood tree of the combined analysis from two genes (18S, COI) aligned with MAFFT and then concatenated. Support values at nodes are bootstrap support percentages from RAxML and Maximum Parsimony analyses, followed by Bayesian posterior probabilities; **B**, Maximum likelihood tree of all COI sequences for Mediterranean *Amphiglena* species, with the corresponding heatmap showing inter- and intraspecific uncorrected p-distances within the *Amphiglena mediterranea* species complex. The other *Amphiglena* terminals and the outgroup are not shown here.

taxonomists it is apparent that real cosmopolitan distributions are rare. However, species names like *A. mediterranea* are still often used in ecological and biodiversity studies, indicating that many non-taxonomists take the so-called cosmopolitan distribution of such species for granted (Hutchings & Kupriyanova 2018).

Calosi *et al.* (2013) published the COI sequences of 29 *Amphiglena mediterranea* specimens collected from both the western and eastern coast of Italy (Fig. 9). In that study they only briefly address the high divergence amongst these sequences, and notably do not discuss that they are possibly from multiple species (Fig. 1B). The main focus of their paper is to identify whether there is a genetic differentiation between populations that live under acidified conditions. Interestingly, they show that there is no genetic separation between acidified and non-acidified sites.

We analyzed these COI sequences together with the remaining *Amphiglena* sequences and they form a clade together with an *Amphiglena* specimen collected from Madeira (clade V in Figs 1, 9). Five out of the seven identified Mediterranean *Amphiglena mediterranea* species were collected within a 15 km radius. Considering the large bias in sampling and lack of representation from other geographic regions it is hard to draw conclusions on the genetic connectivity between different localities. In addition to the results of our molecular analysis, (Fig. 1) a comparison of the historic *Amphiglena mediterranea* descriptions also suggests that there are even possible morphological differences that need further exploration. Specimens examined by Rouse & Gambi (1997) had between five and eight thoracic chaetigers regardless of body size. Claparède (1864) described his specimens as having six thoracic chaetigers while St Joseph (1894) observed eight. Both Rioja (1923) and Fauvel (1927) describe *A. mediterranea* as having up to 9 or 10 thoracic chaetigers, though it is unclear whether or not these authors were counting the peristomium as a thoracic segment. Furthermore, reported body sizes of *A. mediterranea* also vary. Specimens studied by Rouse & Gambi (1997) were from 1.52 mm to 3.8 mm (excluding crown) and had 22–33 chaetigerous segments. Other authors, *e.g.*, Claparède (1864), described finding specimens with a length of up to 18 mm, though most were 7–8 mm long. St Joseph (1894) and Rioja (1923) found specimens between 4–8 mm. Again, it is unclear whether the crown was included in these measurements or not.

All this shows that a more thorough analysis of *Amphiglena mediterranea* is clearly necessary; linking molecular data with a clear unambiguous and well-documented morphological description of a neotype from Nice, France (Fig. 9). Until then naming the remaining *Amphiglena* species in the Mediterranean remains impossible. It may also be that some of the junior synonyms of *A. mediterranea*, outlined above, could be reinstated.

While we currently classify the *A. mediterranea* complex as cryptic, we have no morphological information on the majority of these sequences, and as indicated above further investigation could reveal overlooked morphological differences. Cryptic speciation is not uncommon in annelids, and it has been shown repeatedly that DNA sequencing can reveal species that are morphologically similar (Álvarez-Campos *et al.* 2017; Capa *et al.* 2010, 2013; Halt *et al.* 2009; Nygren 2014; Nygren & Pleijel 2011; Pleijel *et al.* 2009). Cryptic species constitute an important part of biodiversity. Especially in small animals like *Amphiglena*, it is clear that morphology-based identifications alone can significantly underestimate the number of species. Our results demonstrate this for the *Amphiglena mediterranea* ‘species complex’, but also with the description of *Amphiglena seaveriae* **n. sp.** which is morphologically indistinguishable from *Amphiglena lindae* (Rouse & Gambi 1997). It is important to focus future research in the formal naming of cryptic species, since this can be of crucial importance for biodiversity assessment and conservation efforts (Delić *et al.* 2017).

Taxonomy

SABELLIDAE Johnston, 1846

Amphiglena Claparède, 1864

Amphiglena joyceae **n. sp.**

(Figures 2–6)

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Type locality Edithburgh, South Australia (35°05'03.8"S 137°44'55.3"E)

Material examined. Holotype: SIO-BIC A9479 (GenBank COI sequence MK813353), collected in the shal-

low intertidal, from the algal turf next to a jetty at Edithburgh, South Australia (35°05'03.8"S 137°44'55.3"E), May 21, 2006, G. Rouse. Fixed in formalin, preserved in ethanol. **Paratypes:** All collected in the same locality and date as the holotype; six fixed in formalin and preserved in ethanol. **SIO-BIC A9480–A9484;** two fixed in OsO4 and preserved in ethanol; two on SEM stub **SIO-BIC A9486.** Serial plastic sections of 1 paratype **SIO-BIC A10038.** One specimen destroyed for DNA sequencing.

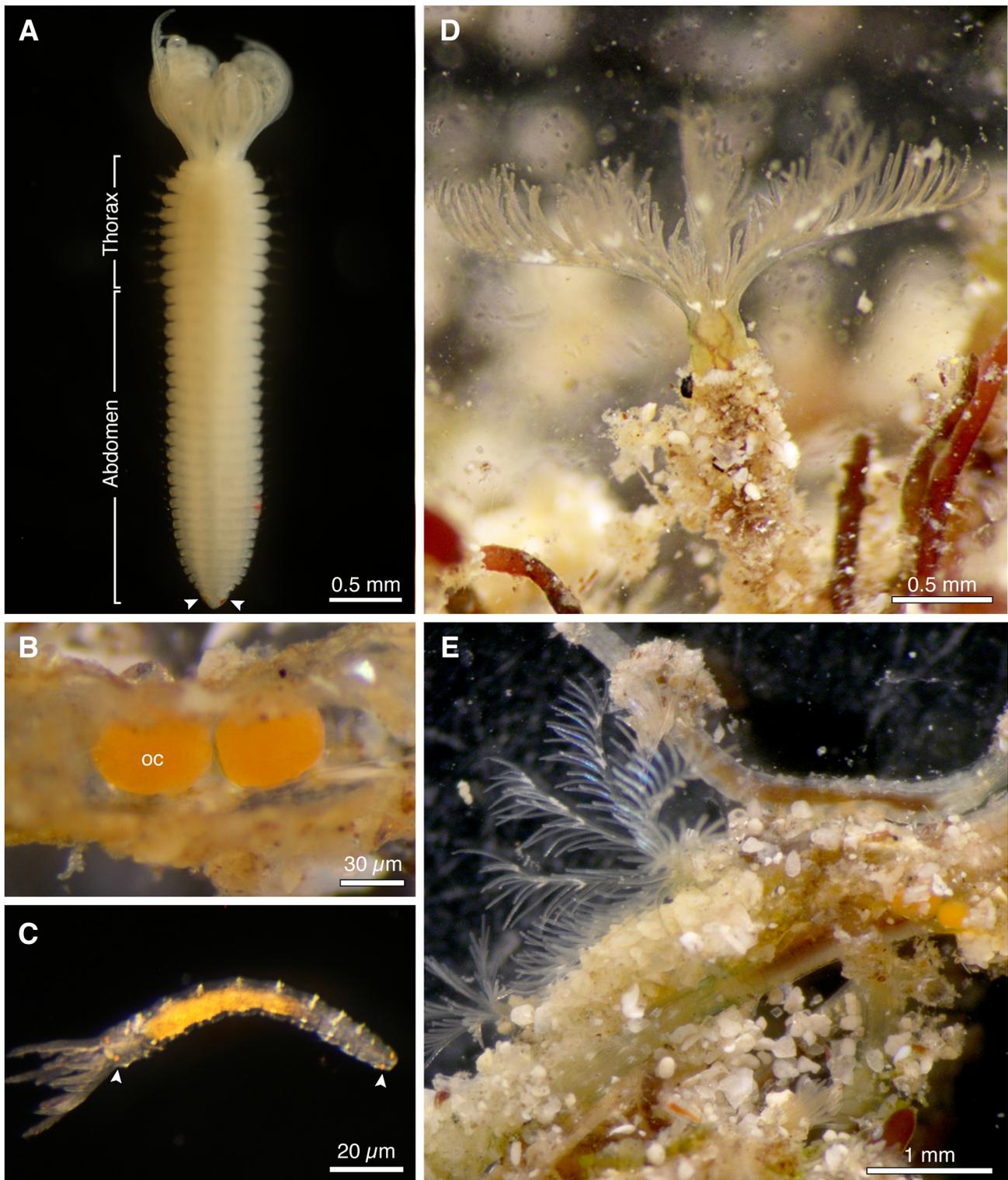


FIGURE 2. *Amphiglena joyceae* n. sp. **SIO-BIC A9479** **A**, Micrographs showing the habitus of the preserved holotype; **B–E**, Micrographs showing live specimens; **B**, Oocytes (*oc*) visible through the translucent body wall of a worm inside the tube; **C**, juvenile specimen, *arrow heads* mark peristomial and pygidial eyes; **D and E**, live specimens with crowns sticking out of the sediment covered tubes. Note the white pigmentation of spermathecae in **D**.

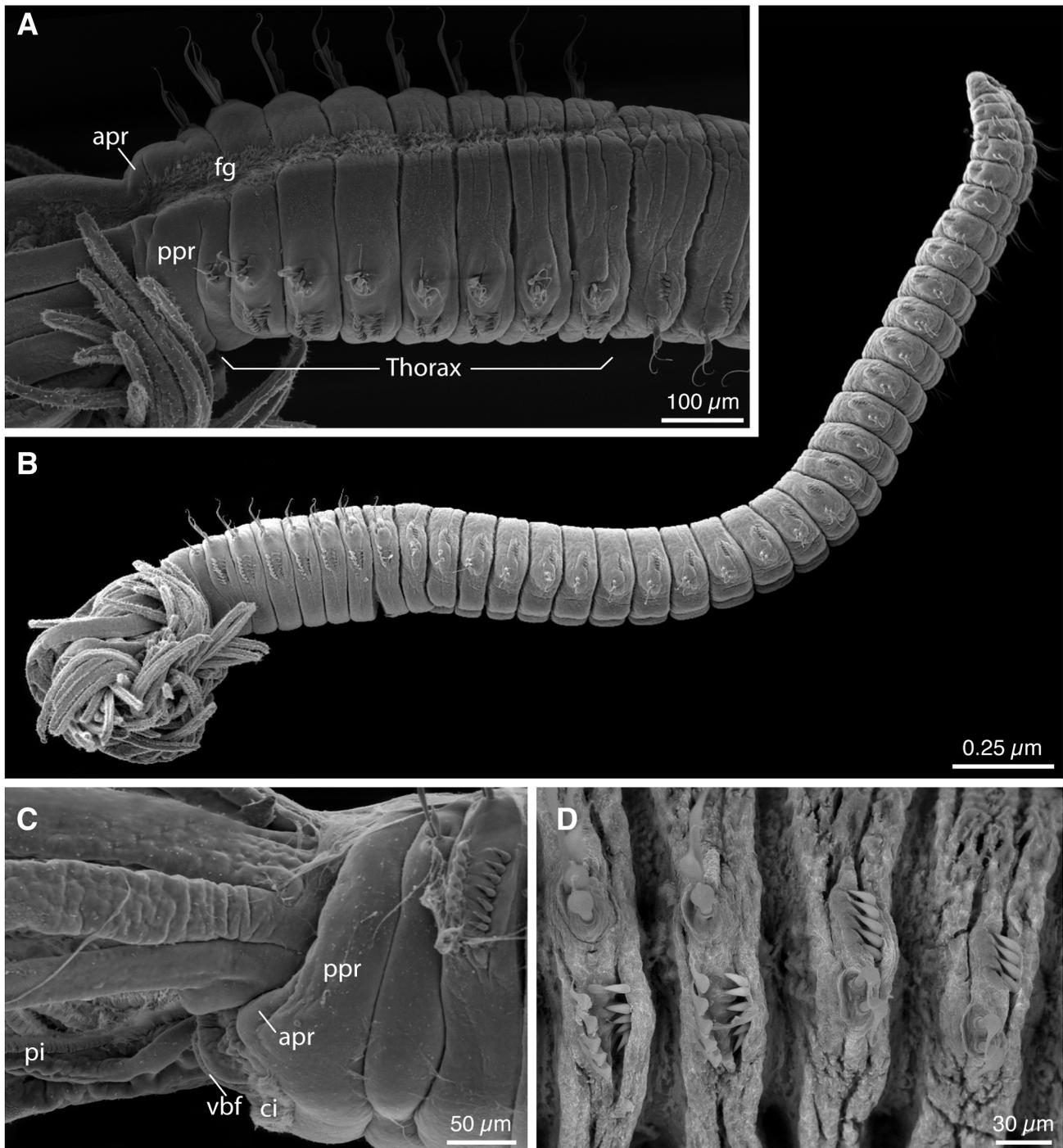


FIGURE 3. SEM micrographs of *Amphiglana joyceae* n. sp. (SIO-BIC A9486) **A**, dorsal view of the thorax, showing the faecal groove (*fg*), anterior (*apr*) and posterior peristomial rings (*ppr*); **B**, overview of the complete body; **C**, junction of the crown and peristomium, short ventral basal flanges (*vbf*) not connected to the ventralmost radioles, ciliated patch of the anterior peristomial ring (*ci*), and the densely ciliated pinnules (*pi*); **D**, lateral view showing the transition from thorax and abdomen, showing the details of chaetae and uncini.

Description. Holotype complete, with eight thoracic and 31 abdominal chaetigers (Fig. 2A). Total body length 2.8 mm (branchial crown 0.75 mm); maximum body width 0.6 mm (Fig. 2A). Crown holds five pairs of radioles with nine pairs of pinnules in two alternating rows. Pinnules similar in length along radiole. Radiolar skeleton with two rows of cells. Dorsal lips with short dorsal radiolar appendages. Posterior peristomial ring even in height all around (Fig. 3A, C). Anterior peristomial ring short and not visible laterally (Fig. 3C). No posterior peristomial ring collar. Ventral basal flanges present, short and not connected to crown (Fig. 3C). Red peristomial eyes present

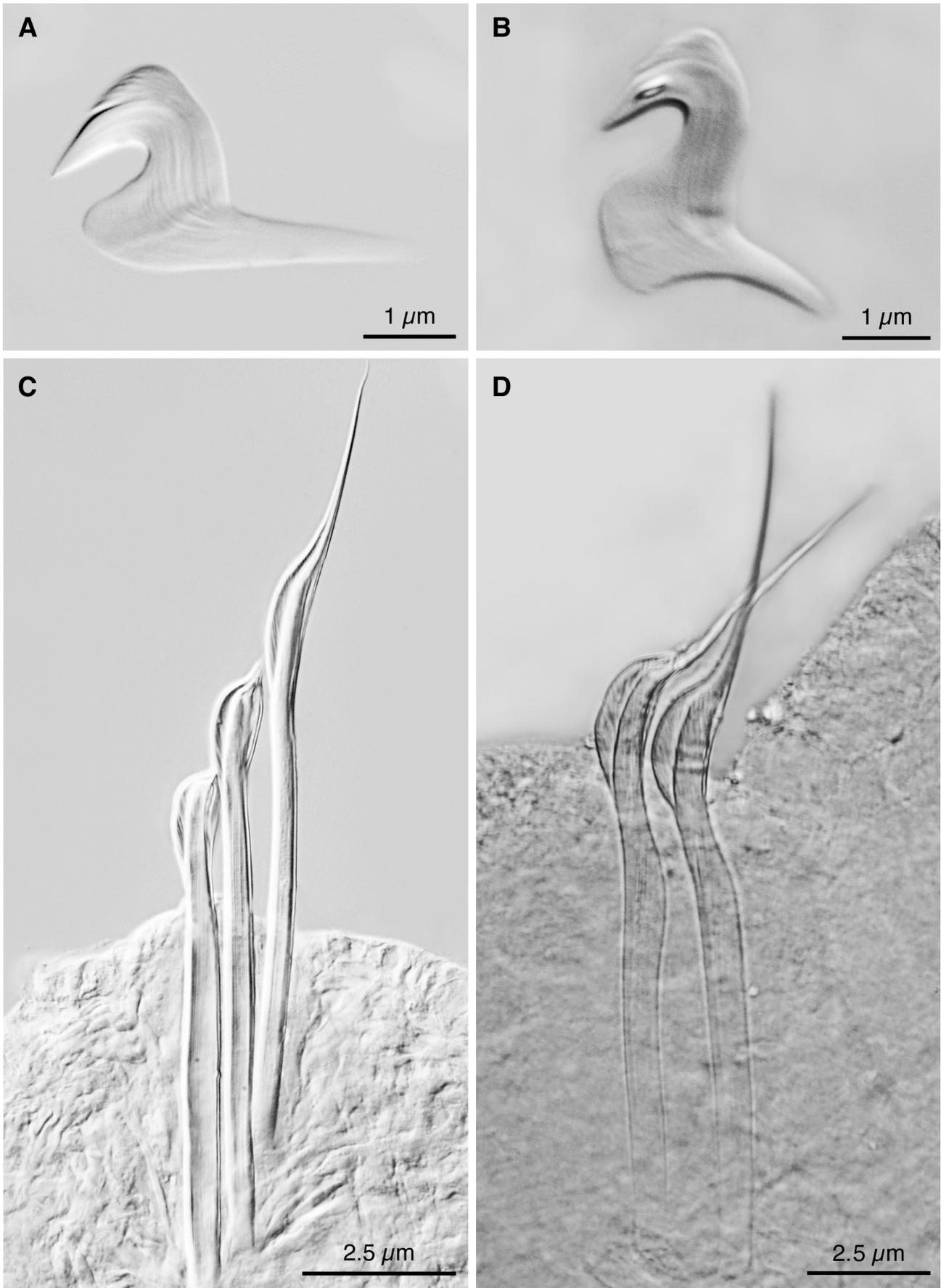


FIGURE 4. Chaetae and uncini of *Amphiglena joyceae* n. sp. **A**, thoracic uncinus; **B**, abdominal uncinus; **C**, Thoracic chaetae; **D**, abdominal chaetae.

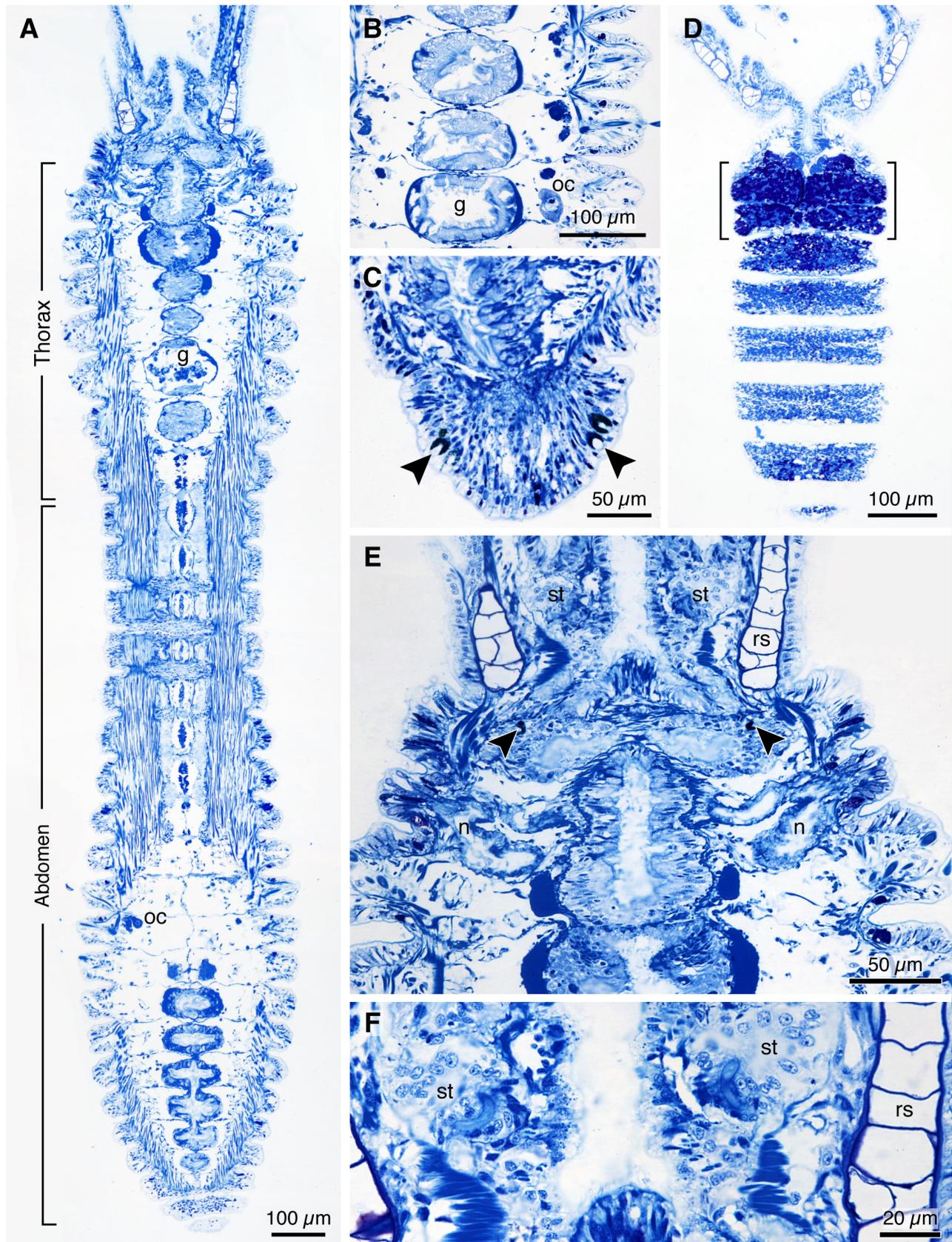


FIGURE 5. Histology of *Amphiglena joyceae* n. sp. (SIO-BIC A10038). **A**, Horizontal section of the entire body; **B**, Abdominal segments 11–13 showing details of early oocytes (*oc*) and the gut (*g*); **C**, pygidium, *arrow heads* mark the pygidial eyes; **D**, section through the epidermis, *brackets* mark the anterior glandular ring; **E**, anterior segments, *arrow heads* mark the peristomial eyes. Section shows the nephridia (*n*), vacuolated cells of the radiolar skeleton (*rs*) and spermathecae (*st*); **F**, detail of the spermathecae.

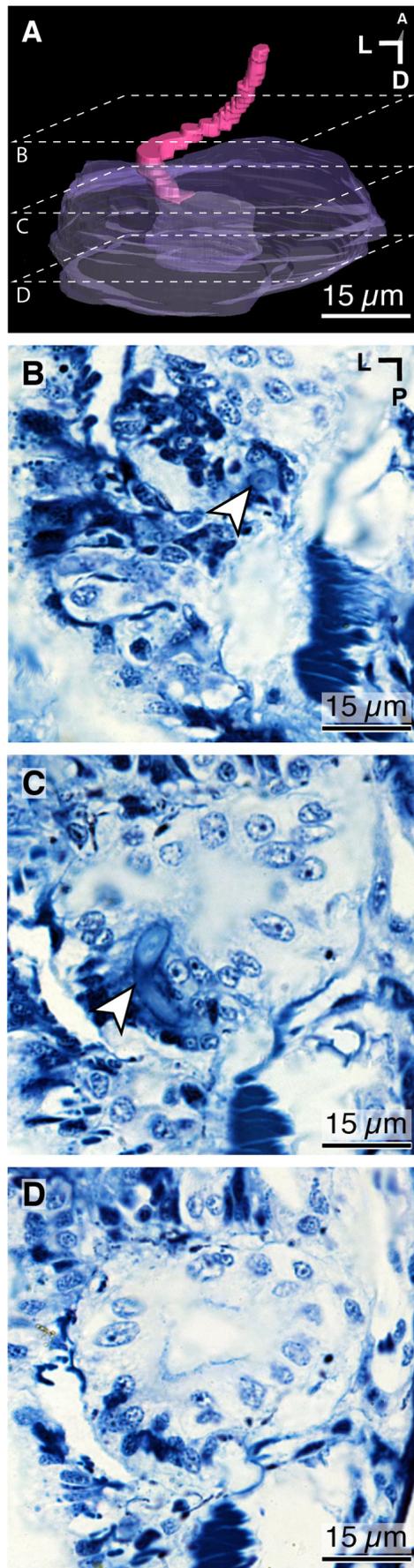


FIGURE 6. A, 3D Reconstruction of a spermatheca; B–D, serial semi-thin sections used for 3D reconstruction. Position of each section on the z-axis is marked as planes in A. Arrow heads mark the narrow spermathecal tube.

(Figs 2D, 5E). Thorax longer than wide. Second to eighth thoracic chaetigers with 4–7 uncini on each torus (Fig. 3A, B). Thoracic uncini with broad, well-developed breast double the space to the main fang (Fig. 4A). Approximately five rows of long teeth above main fang spanning half its length. Handles of thoracic uncini long (Fig. 4A). Companion chaetae present, with straight shaft and long mucro (Fig. 3C, D). Abdominal neurochaetae thin and broadly hooded (Fig. 4C). Uncini, with similar-sized small teeth above the main fang, well-developed broad breast, and medium length handles (Fig. 4B). Pygidial eyes present, red spots on lateral margins of pygidium (Fig. 2A). Faecal groove visible mid-dorsally along thorax (Fig. 3A). Pair of spermathecae present in base of dorsal lips, white pigmentation visible in live material (Fig. 2D). Eggs present in abdominal chaetigers, orange in live specimens (Fig. 2B, E). Intratubular brooding of juveniles (Fig. 2C). Tube transparent with sediment incorporated (Fig. 2B, D, E).

Etymology: Named for Kathryn Feerst's grandmother Joyce Beck.

Remarks: Distinct morphologically and molecularly from all known species of *Amphiglana* including those described from Australia. The lack of connectivity of the ventral basal flanges and radioles is described in *A. gracilis*, *A. magna* and *A. terebro*. *Amphiglana joyceae* n. sp. can be differentiated from *A. gracilis* by its larger body size and the lack of elongated distal pinnules. Furthermore, the companion chaetae in *A. joyceae* n. sp. are much more broadly hooded and finely toothed than in *A. gracilis*. *Amphiglana magna* is much larger than *A. joyceae* n. sp. and has 10 to 15 pairs of pinnules.

Histology: A paratype of *Amphiglana joyceae* n. sp. was serially sectioned for histology (Fig. 5), allowing the investigation of the internal anatomy and the reconstruction of the 3D-structure of a spermatheca (Fig. 6). The spermathecae in *A. joyceae* n. sp. are paired and lie at the base of the radiolar crown (Fig. 5E, F). They consist of a coiled narrow duct that opens inside a blind sac-like lumen. The total length of the duct was $\pm 40 \mu\text{m}$ and the empty lumen was $\pm 10 \mu\text{m}$ wide. The epithelial lining of the lumen was $\pm 15 \mu\text{m}$ thick and was densely ciliated (Fig. 6). No musculature was observed associated with the spermathecae and there were no sperm in the spermathecal lumen. The spermathecal structure is similar to that described in other *Amphiglana* spp. (Rouse & Gambi 1998b). *Amphiglana terebro* is the only species with a simple cavity like spermathecae, lacking the convoluted duct.

The prominent glandular ring of the posterior-most thoracic segments can be seen in the sections through the epidermis (Fig. 5D). The peristomial eyes are directly associated with the central nervous system and are located lateral to the cerebral ganglion (Fig. 5E). The single pair of nephridia, with a U-shaped duct, can be seen in the first thoracic segment (Fig. 5E).

The sectioned specimen only possessed early developing oocytes in abdominal segments 11–13 (Fig. 5D) no spermatids or spermatozoa were observed. Interestingly, no sperm was observed in the three cleared mature specimens either. While all known *Amphiglana* species are simultaneous hermaphrodites, this raises the question as to whether all collected specimens of *A. joyceae* n. sp. were female.

Amphiglana seaverae n. sp.

(Figures 7 and 8)

urn:lsid:zoobank.org:act:DD3BDAF5-56E9-4AC4-A6E1-D4913CDDEC59

Type locality: St. Augustine, Florida (29°40'13.8"N 81°12'54.0"W)

Material examined. Holotype: SIO-BIC A9470, a simultaneous hermaphrodite, fixed in formalin and preserved in ethanol, collected from the edge of a small artificial seawater pond at Whitney Marine Lab in St. Augustine, Florida (29°40'13.8"N 81°12'54.0"W), G. Rouse and E. Tilic, April 16, 2018. A second individual was used completely for DNA sequencing (GenBank COI sequence MK813355).

Description: Holotype 3.5 mm long with a 1.3 mm long branchial crown and maximum body width of 0.45 mm (Fig. 7A). Crown with 4 pairs of radioles. (Fig. 7A, B). Pinnules appear in alternating longitudinal rows of 6–10 on each radiole, all similar in length (Fig. 7B). Radioles with palmate membrane. No radiolar flanges (Fig. 7B). Body with eight thoracic and 25 abdominal chaetigers (Fig. 7A). Ventral basal flanges present. Dorsal pinnular appendages absent. Anterior peristomial ring even in height all around. Posterior peristomial ring collar absent. Red peristomial eyes present (Fig. 7A). Thorax longer than wide. Thoracic chaetigers with a single broadly hooded superior chaeta and two paleate inferior chaetae (Fig. 8A). Thoracic uncini 4–5 per torus (Fig. 8C). Thoracic uncini with a broad breast, shorter than space from breast to main fang (Fig. 8C). Thoracic uncini with long handles (Fig. 8C). Companion chaetae present, geniculated with a straight shaft and very long mucro. Abdominal neurochaetae in a single row, broadly hooded (Fig. 8B). Abdominal uncini breast broad and handles medium. (Fig. 8D). Pygidial

eyes present. Eggs seen in lower mid-abdomen. Pair of spermathecae present, located at base of dorsal lips with red pigmentation (Fig. 7A, B). Simultaneous hermaphrodite. Oocytes present in abdominal segments 10–15 (Fig 7C). Sperm present in the posterior abdominal segments.

Etymology: We name this species for Dr. Elaine Seaver in recognition of her contributions to annelid biology. Dr. Seaver is a Professor at the Whitney Laboratory for Marine Bioscience (UFL), the type locality of this species.

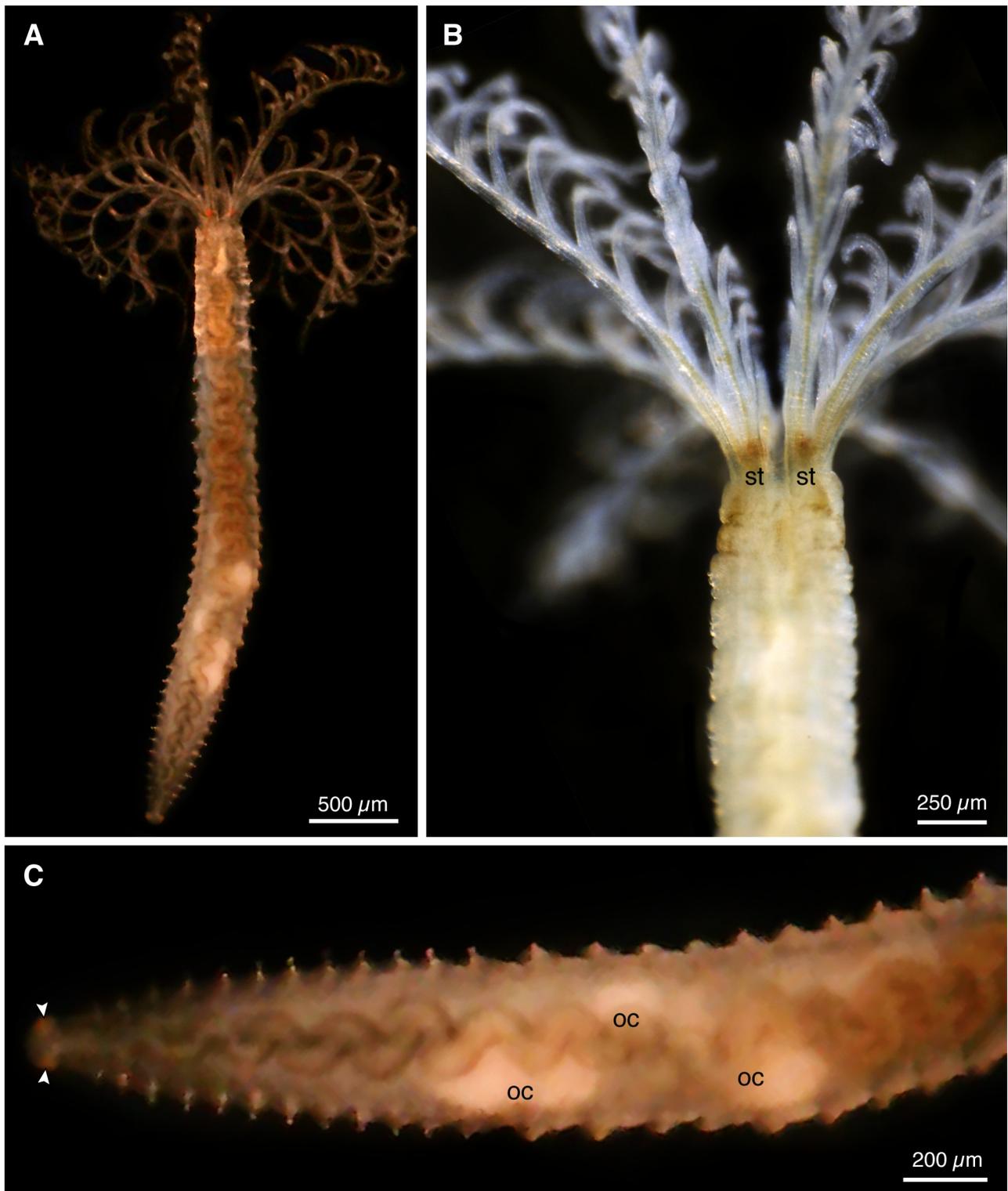


FIGURE 7. *Amphiglena seaverae* n. sp. (SIO-BIC A9470) Micrographs showing the live holotype. **A**, habitus, note the bright red pigmentation of the spermathecae in the live specimen; **B**, Detail of the crown and anterior thorax in the preserved holotype, spermathecae (*st*) with redish-brown pigmentation; **C**, posterior abdomen with oocytes (*oc*) and red pygidial eye spots (*arrow heads*).

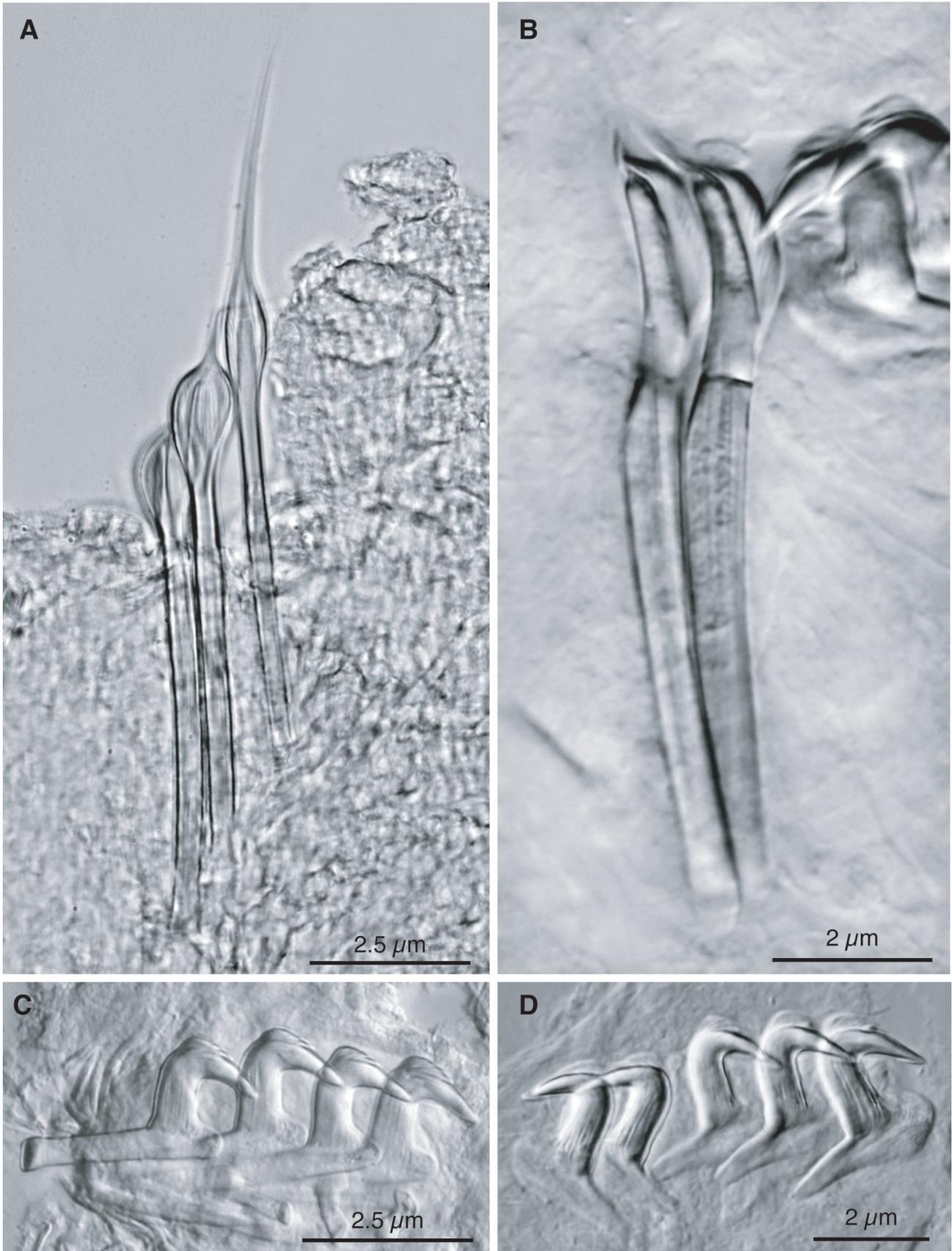


FIGURE 8. Chaetae and uncini of *Amphiglena seaveræ* n. sp. **A**, thoracic chaetae; **B**, abdominal chaetae; **C**, thoracic uncini; **D**, abdominal uncini.

Remarks: *A. seaverae* n. sp. appears morphologically very similar to *A. lindae* Rouse & Gambi 1997 from Belize, yet with a p-distance of 0.27 the COI sequences are so diverse that we can clearly identify *A. seaverae* as a distinct species. This indicates another potential cryptic species complex of *Amphiglena* in the Caribbean and western Atlantic. *A. lindae* is described from the shallow intertidal, with coarse sand, filamentous algae and coral rubble. *A. seaverae* n. sp. is so far only found in an artificial habitat. Future collections from its natural environment will make a comparison of their habitats possible.



FIGURE 9. Map of the Mediterranean showing where each of the seven different *Amphiglena mediterranea* clades were collected. Nice, France, the type locality of *A. mediterranea* is labeled with a question mark. No COI sequence was available from here making it impossible to assign either of the seven clades as the true *A. mediterranea*.

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