

## New symbiotic association in marine annelids: ectoparasites of comb jellies

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A new genus of ectoparasitic marine annelids living on ctenophores, *Ctenophoricola* **gen. nov.**, is described and its feeding behaviour, reproduction and developmental stages are discussed. Its unusual morphology challenged its placement within the known marine families. However, analyses of mitochondrial and nuclear sequence data showed the new genus as member of the Alciopini, a group of holopelagic annelids included within the Phyllodocidae. *Ctenophoricola masanorii* **sp. nov.** from Japan and *Ctenophoricola rousei* **sp. nov.** from the Canary Islands (Spain) are described. A third species from the Gulf of California is not formally described because the specimens are in poor condition. The new genus is characterized by having: 1) two distinctive body regions, the anterior with reduced parapodia lacking chaetae, and the posterior with long parapodia and chaetae and 2) a pair of large, elongate lensed eyes. These eyes are here described using histology and 3D reconstruction based on a Californian specimen. The two new species mainly differ in colour pattern, shape of parapodia, number of chaetae and body ciliation.

ADDITIONAL KEYWORDS: Annelida – Alciopini – ctenophores – *Ctenophoricola* – new genus – new species – parasite – Phyllodocidae.

### INTRODUCTION

Annelids, or segmented worms, are an important group of animals that include around 25 000 species (Read & Fauchald, 2019). They show a huge morphological and ecological diversity, inhabiting marine, freshwater and terrestrial environments (Rouse & Pleijel, 2001). Within the marine realm, most annelids are benthic,

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inhabiting the pelagic realm only as larvae or during a short reproductive phase (epitoky). However, the Typhloscolecidae, Lopadorhynchiidae, Tomopteridae and Alciopini are all holopelagic (Rouse & Pleijel, 2001), showing important morphological adaptations to this different type of life, and thus they have substantially distinct morphologies compared to other annelids. Therefore, their phylogenetic positions within the Annelida have been a matter of debate during the last decade (e.g. Struck & Halanych, 2010; Nygren & Pleijel, 2011).

Marine annelids are typically considered free living, although a large number of species are involved in more or less close symbiotic associations (mainly ectosymbiotic) with other annelids, sponges, cnidarians, molluscs, crustaceans, echinoderms and fishes (e.g. Martin & Britayev, 1998, 2018; Rouse & Pleijel, 2001; Britayev & Antokhina, 2012; Andrews *et al.*, 2015; Hernández-Alcántara *et al.*, 2015; Molodtsova *et al.*, 2016; Igawa *et al.*, 2017; Goto *et al.*, 2016, 2017). Less commonly, marine annelids are also found as endoparasites of other animals, such as the recently described *Proceraea exoryxae* Martin, Nygren & Cruz-Rivera, 2017 tunnelling within the tunic of the ascidian *Phallusia nigra* Savigny, 1816 (Martin *et al.*, 2017) or the holopelagic *Alciopina parasitica* Claparède & Pancer, 1867 reported in the gastrovascular canals of the ctenophore *Hormiphora plumosa* M. Sars, 1859 (Claparède & Panceri, 1869; Fauvel, 1923; see Clark (1956) and Martin & Britayev (1998) for detailed revisions). Marine annelids can also be hosts of different organisms, including other annelids, invertebrates, vertebrates (Martin & Britayev, 1998, 2018; Britayev *et al.*, 2017; Britayev & Martin, 2019) and, obviously, microorganisms (Álvarez-Campos *et al.*, 2014; Turón *et al.*, 2019). Among the last, some particular associations involving bacteria are highly interesting, as they allow annelids to occupy specialized habitats, such as whale bones or hydrothermal vents (e.g. Rouse *et al.*, 2004; Goffredi *et al.*, 2005; Thornill *et al.*, 2008). Many of these symbioses involve large anatomical modifications that obscure their phylogenetic affinities. For instance, the highly modified metamerism of siboglinids resulted in the clade being considered closely related to annelids, but in separate phyla for many years (e.g. Jones, 1985), or the Nautiniliidae Miura & Laubier, 1989 and Calamyzidae Arwidsson, 1932, which are in fact a clade modified by its symbiotic life of another family, the Chrysopetalidae Ehlers, 1864 (Aguado *et al.*, 2013). Given the broad, often lifestyle-driven, morphological variation within the Annelida, in many cases high discordance between morphology-based and genetics-based phylogenies has been found, leaving several questions of annelid

evolutionary history unanswered (e.g. Weigert *et al.*, 2014; Andrade *et al.*, 2015).

The present study formally describes the morphology, feeding mode and developmental stages of a group of unusual, minute annelids found on the external surface of various ctenophores as a new ectoparasitic genus, *Ctenophoricola*. These worms appear to spend their entire life siphoning up ctenophore tissue with their eversible pharynx. They were reported from off El Hierro and between Tenerife and Gran Canaria (Canary Islands, Spain), as an “Unidentified family” (Collazo *et al.*, 2017) or as a “Family? Genus? Species?” (Núñez *et al.*, 1993) and are herein described as the new species *C. rousei*. Additional species have been found in Japan and in the Gulf of California. However, only the Japanese and the Canarian ones are described here as new species, because the poor condition of the Californian specimens prevents us from formally naming them. Additionally, histology and 3D reconstruction analyses based on Californian material allows us to reveal the exact nature of the internal, pigmented lobes located posterior to the prostomium, which are in fact lensed eyes.

## MATERIAL AND METHODS

### SPECIMEN HANDLING

The specimens from the Canary Islands were collected south-east of Tenerife (Radazul) and south-west of El Hierro (Las Lapillas) by L. Moro and J. Escatillar (Biodiversity Service, Government of the Canary Islands) between March 1994 and December 1997. The host ctenophores were captured by SCUBA diving, with the help of containers with hermetic caps at both ends, which allows capturing of gelatinous organisms without damaging them. At the laboratory, they were kept alive in refrigerated native sea water to allow observing and photographing over several days. The parasitic worms were separated from their ctenophore hosts, fixed in 4% formaldehyde buffered in seawater and later transferred to 70% ethanol.

The specimens from Japan were collected alive with their ctenophore hosts using a scoop net by members of the staff of Enoshima Aquarium in September 2013. Specimens were photographed, filmed alive, fixed in 4% formaldehyde buffered in seawater and later transferred to 70% ethanol for taxonomic examination, or directly preserved in 96% ethanol for molecular investigation.

Drawings were made to scale with a camera lucida attached to a Nikon Optiphot microscope. Live specimens were photographed using a Sony Handycam HDR-CX550 digital video camera attached to an Olympus SZ60 dissecting microscope, and with

a Nikon D300 reflex camera and a Nikon 60 mm f / 2.8G Ed AF-s Micro Nikkor lens, attaching KENKO extension tubes of 12, 22 and 32 mm. We used a fish tank designed for macrophotography that allowed backlighting with two wireless Nikon SB-R200 flashes.

For scanning electron microscopy (SEM), selected specimens were prepared on an Emitech K850 Critical Point Dryer, gold-coated with a Q150T-S Turbo-Pumper Sputter Coater and examined with a Hitachi S-3000N SEM at the Servicio Interdepartamental de Investigación de the Universidad Autónoma de Madrid (SIDI).

The holotypes and paratypes of the new species were deposited at the Museum of Comparative Zoology, Harvard University, Cambridge, USA (MCZ) and at the Museo Nacional de Ciencias Naturales, Madrid, Spain (MNCN) (Table 1). Comparative specimens used for the phylogenetic work were collected by authors F. Pleijel, A. Nygren and K. Osborn throughout various expeditions and collecting trips. Tissues were preserved in chilled 95% ethanol and vouchers deposited in different collections (Table 1).

**Table 1.** Origin of sequenced terminals, specification of vouchers and GenBank accession numbers (new sequences in bold). All vouchers are hologenophores (Pleijel *et al.*, 2008), unless otherwise stated. The following abbreviations are used for geographic origins of specimens: BA: Banyuls, France; BR: Brittany, Easter Island, France; EIJ: Enoshima Island, Japan; KO: Koster area, Sweden; LJ: La Jolla, California, USA; MA: Madeira, Portugal; MO: Monterey Canyon, California, USA; NAZ: Nazare, Portugal; NCA: North Carolina, USA; PLY: Plymouth, UK; PS: Point Sur, California; SCI: Scilly Islands, UK; SHE: Shetland, UK; SSH: South Shetland Islands, Antarctica; SVA: Svalbard; TRO: Trondheim, Norway; VF: Villefranche, France; WE: Weddell Sea, Antarctica; YO: Yokohama, Japan. More detailed collection data are deposited with the specimens. MNCN: Museo Nacional de Ciencias Naturales de Madrid, Spain; SMNH: Swedish Museum of Natural History, Stockholm; MCZ: Museum of Comparative Zoology, Harvard University, Boston; SIO-BIC: Scripps Institution of Oceanography Benthic Invertebrate Collection, San Diego

	Geographic origin	Voucher	COI	16S	18S	28S
<b>Ingroups</b>						
<i>Alciopina</i> sp.	-	-	-	-	DQ790073	DQ790021
<i>Chaetoparia nilssoni</i>	KO	SMNH 90970	AY996125	AY996069	AY996090	<b>MG254447</b>
<i>Ctenophoricola masonorii</i> .	EIJ	MCZ 25325	<b>MG254493</b>	<b>MG254417</b>	<b>MG254392</b>	<b>MG254448</b>
<i>Eteone foliosa</i>	KO	SMNH 124052	<b>MG254494</b>	<b>MG254418</b>	<b>MG254393</b>	<b>MG254449</b>
<i>Eteone pacifica</i>	LJ	SMNH 124053	<b>MG254495</b>	<b>MG254419</b>	<b>MG254394</b>	<b>MG254450</b>
<i>Eteone picta</i>	BR	SMNH 90971	AY996124	AY996068	AY996089	<b>MG254451</b>
<i>Eulalia aurea</i>	PLY	SMNH 124056	<b>MG254496</b>	<b>MG254420</b>	<b>MG254395</b>	<b>MG254452</b>
<i>Eulalia bilineata</i>	TRO	SMNH 90972	-	AY996067	AY996088	<b>MG254453</b>
<i>Eulalia expusilla</i>	TRO	SMNH 90973	-	AY996066	AY996087	<b>MG254454</b>
<i>Eulalia hanssoni</i>	TRO	SMNH 124057	<b>MG254497</b>	<b>MG254421</b>	<b>MG254396</b>	<b>MG254455</b>
<i>Eulalia mustela</i>	KO	SMNH 90974	AY996123	AY996065	AY996086	<b>MG254456</b>
<i>Eulalia tjalfiensis</i>	TRO	SMNH 124054	<b>MG254498</b>	<b>MG254422</b>	<b>MG254397</b>	<b>MG254457</b>
<i>Eulalia viridis</i>	KO	SMNH 90975	<b>MG254499</b>	AY340455	AY996085	<b>MG254458</b>
<i>Eumida arctica</i>	SVA	SMNH 90976	<b>MG254500</b>	AY996063	AY996084	<b>MG254459</b>
<i>Eumida bahusiensis</i>	KO	SMNH 110638	HM358649	<b>MG254423</b>	<b>MG254398</b>	<b>MG254460</b>
<i>Eumida kelaino</i>	KO	SMNH T-7982 (COI, 28S); SMNH 90977 (16S, 18S)	HM358686	AY996062	AY996083	<b>MG254461</b>
<i>Eumida longicornuta</i>	LJ	SIO-BIC A26121	<b>MG254501</b>	<b>MG254424</b>	<b>MG254399</b>	<b>MG254462</b>
<i>Eumida ockelmanni</i>	KO	SMNH 110635	HM358646	<b>MG254425</b>	<b>MG254400</b>	<b>MG254463</b>
<i>Hesionura elongata</i>	SHE	SMNH 124059	<b>MG254502</b>	<b>MG254426</b>	<b>MG254401</b>	<b>MG254464</b>
<i>Mystides caeca</i>	TRO (COI, 16S, 18S); MA (28S)	SMNH 90978; SMNH 124061	AY996119	AY996060	AY996081	<b>MG254465</b>
<i>Nereiphylla castanea</i>	YO	SMNH 124063	<b>MG254503</b>	<b>MG254427</b>	<b>MG254402</b>	-
<i>Nereiphylla lutea</i>	TRO	SMNH 90979	AY996118	AY996059	AY996080	<b>MG254466</b>

Table 1. Continued

	Geographic origin	Voucher	COI	16S	18S	28S
<i>Nereiphylla rubiginosa</i>	SCI	SMNH 124065	<b>MG254504</b>	<b>MG254428</b>	<b>MG254403</b>	<b>MG254467</b>
<i>Notophyllum foliosum</i>	KO (COI, 16S); TRO (18S, 28S)	SMNH 90980; SMNH 106062	GQ464335	<b>MG254429</b>	AY996079	<b>MG254468</b>
<i>Notophyllum japonicum</i>	YO	SMNH 124067	<b>MG254505</b>	<b>MG254430</b>	<b>MG254404</b>	-
<i>Paranaitis katoi</i>	KO (COI, 16S); TRO (18S, 28S)	SMNH 90981; SMNH 97335	EU431178	EU431123	AY996077	<b>MG254469</b>
<i>Paranaitis kosteriensis</i>	TRO	SMNH 90083	<b>MG254506</b>	<b>MG254431</b>	AY996078	<b>MG254470</b>
<i>Paranaitis speciosa</i>	NCA	SMNH 124069	<b>MG254507</b>	<b>MG254432</b>	<b>MG254405</b>	<b>MG254471</b>
<i>Phyllodoce citrina</i>	SVA	SMNH 124074;	<b>MG254508</b>	<b>MG254433</b>	-	<b>MG254472</b>
<i>Phyllodoce groenlandica</i>	SVA	SMNH 90982	AY996114	EU431118	AY996076	<b>MG254473</b>
<i>Phyllodoce laminosa</i>	SCI	SMNH 124076	<b>MG254509</b>	<b>MG254434</b>	-	<b>MG254474</b>
<i>Phyllodoce lineata</i>	BR	SMNH 124078	<b>MG254510</b>	<b>MG254435</b>	<b>MG254406</b>	<b>MG254475</b>
<i>Phyllodoce longipes</i>	KO	SMNH 90983	AY996113	AY996056	AY996075	<b>MG254476</b>
<i>Phyllodoce maculata</i>	KO	SMNH 124081	AY839586	<b>MG254436</b>	AY176302	<b>MG254477</b>
<i>Phyllodoce mucosa</i>	SCI	SMNH 124080	<b>MG254511</b>	<b>MG254437</b>	<b>MG254407</b>	<b>MG254478</b>
<i>Phyllodoce rosea</i>	KO	SMNH 124079	<b>MG254512</b>	<b>MG254438</b>	<b>MG254408</b>	<b>MG254479</b>
<i>Protomystides exigua</i>	TRO	SMNH 90084	-	AY996055	AY996074	<b>MG254480</b>
<i>Pseudomystides limbata</i>	KO	SMNH 90984	AY996112	AY996054	AY996073	<b>MG254481</b>
<i>Pseudomystides spinachia</i>	KO	SMNH 124082	<b>MG254513</b>	<b>MG254439</b>	<b>MG254409</b>	<b>MG254482</b>
<i>Pterocirrus macroceros</i>	BA	SMNH 90985	<b>MG254514</b>	AY996053	AY996072	<b>MG254483</b>
<i>Pterocirrus montereyensis</i>	LJ	SMNH 124084	<b>MG254515</b>	<b>MG254440</b>	<b>MG254410</b>	<b>MG254484</b>
<i>Pterocirrus nidarosiensis</i>	TRO	SMNH 124083	<b>MG254391</b>	<b>MG254441</b>	AY996053	<b>MG254485</b>
<i>Rhynchonerella gracilis</i>	WE	SMNH 124085	<b>MG254516</b>	<b>MG254442</b>	<b>MG254411</b>	<b>MG254486</b>
<i>Sige fusigera</i>	KO	SMNH 110630 (COI, 28S); SMNH 90986 (16S, 18S)	HM35864	AY996052	AY996071	<b>MG254487</b>
<i>Sige oliveri</i>	KO	SMNH 124086	<b>MG254517</b>	<b>MG254443</b>	<b>MG254412</b>	<b>MG254488</b>
<i>Torrea</i> sp.	-	-	-	-	DQ790096	DQ790068
<i>Vanadis antarctica</i>	WE	SMNH 124090	-	<b>MG254445</b>	<b>MG254415</b>	<b>MG254490</b>
<i>Vanadis formosa</i>	VF	SMNH 124089	<b>MG254518</b>	<b>MG254446</b>	<b>MG254414</b>	<b>MG254491</b>
<b>Outgroups</b>						
<i>Aglaophamus pulcher/circinata</i>	NAZ/unknown	-	GU179413	GU179360	DQ790072	DQ790020
<i>Ancistroyllis groenlandica</i>	-	-	-	-	DQ790075	DQ790023
<i>Glycera dibranchiata</i>	-	-	AY995210	AY995209	AY995208	AY995207
<i>Lacydonia eliasoni</i>	TRO	SMNH 909871	AY996120	AY996061	<b>MG254416</b>	<b>MG254492</b>
<i>Lepidonotus sublevis</i>	-	-	AY894317	-	AY894301	DQ790039
<i>Lopadorrhynchus</i> sp.	SSH	-	-	-	GU230894	GU230896
<i>Lumbrinereis latreilli</i>	-	-	AY364855	AY838833	AY525623	AY366512
<i>Nereis pelagica</i>	KO	-	-	AY340470	AF474279	AY340407

Table 1. Continued

	Geographic origin	Voucher	COI	16S	18S	28S
<i>Oxydromus pugettensis</i>	LJ (COI, 16S); unknown (18S, 28S)	-	KJ855074	KJ855069	DQ790086	DQ790046
<i>Paralacydonia paradoxa</i>	BA (COI, 16S); unknown (18S, 28S)	-	GQ426684	GQ426619	DQ790088	DQ790050
<i>Pelagobia longicirrata</i>	VF	SMNH 124071	-	-	<b>MG254390</b>	<b>MG254389</b>
<i>Sigalion spinosus</i>	PS (COI, 18S); unknown (28S)	-	AY894319	-	AY894304	DQ790062
<i>Travisopsis coniceps</i>	WE	SMNH 124088	-	<b>MG254444</b>	<b>MG254413</b>	<b>MG254489</b>
<i>Typhloscolex</i> sp.	MO	-	-	-	GU230895	GU230897

## HISTOLOGY

One Californian specimen was washed three times in 0.1 M sodium cacodylate buffer (pH 7.4) (Electron Microscopy Science [EMS]) with 0.2 M sodium chloride (wash buffer). The specimen was postfixed in 0.1 M sodium cacodylate buffer (pH 7.4) with 0.2 M sodium chloride and 1% osmium tetroxide overnight in the dark, washed the next day three times in wash buffer, then through the following 15-min dehydration steps: 30%, 50%, 70%, 85%, 95%, 2 × 100% ethanol, 2 × 100% dehydrated acetone, 2 × propylene oxide, overnight in 50:50 mixture of propylene oxide and Spurr's Low Viscosity resin firmness (Polysciences Inc., Warrington, Pennsylvania). The next day, the specimen was transferred twice for 2 h to fresh 100% Spurr's Low Viscosity resin firmness A, then embedded at 70 °C overnight. The entire first five segments were sectioned to 1 µm thickness using a HistoJumbo diamond knife from Diatome with a RMC MT6000 Ultramicrotome (Ruthensteiner, 2008), then stained with Richardson's Blue (1% methylene blue, 1% azure II, 1% sodium borate mixed in distilled water then diluted 1:1 with distilled water and filtered prior to use), for 15 s at 80 °C, then rinsed with DI water for 2 min at room temperature, followed by a final rinse. After drying, the sections were mounted on a cover slipped slide using Permount diluted with a mixture of 1% terpineol in toluene. Each of the 547 sections was photographed with an Olympus BX63 compound fluorescent microscope equipped with a DP80 camera and Cell Sens software, then aligned using TrakEM2 in Fiji (Cardona *et al.*, 2012).

## MOLECULAR STUDY

*C. masonorii*, together with 47 newly sequenced species of Phyllocodidae and 14 Aciculata outgroups (*sensu* Andrade *et al.*, 2015) (Table 1) were included in the phylogenetic analyses. As this was the only species for which tissue

was preserved appropriately for genetic work, it has been chosen as the type species for the new genus.

Genomic DNA was extracted using a DNAeasy Tissue Kit (Qiagen) following the manufacturer's protocol. We amplified 658 and approximately 500 base pairs (bp) of the mitochondrial genes *COI* and 16S rDNA, respectively, and approximately 1650 and 1750 bp of the nuclear 28S rDNA and 18S rDNA, respectively (Table 2). PCR reactions contained 21 µL double-distilled H<sub>2</sub>O, 1 µL of each primer (10 µM), 2 µL DNA template, and puReTaq Ready-To-Go PCR Beads (Amersham Biosciences). The temperature profile was as follows: 96 °C for 240 s – (94 °C for 30 s, 48–58 °C for 30 s, 72 °C for 60 s) × 45 cycles with 72 °C for 480 s at the end. PCR products were purified with 5 µL mixture of exonuclease I and FastAP thermosensitive alkaline phosphatase (Fermentas) (Werle *et al.*, 1994). Sequencing of all the specimens, except for the *Ctenophoricola* sample, was performed at Macrogen Inc. facilities (Seoul, Korea). *C. masonorii* was sequenced at MCZ (Harvard University), following the same protocol described in Álvarez-Campos *et al.* (2017). Overlapping sequence fragments were merged into consensus sequences using Geneious v.7.06 (Kearse *et al.*, 2012). The protein coding *COI* was trivial to align, whereas the ribosomal genes were aligned with MAFFT v.7.017 (Katoh *et al.*, 2002) within Geneious v.7.06 with the following settings: algorithm = E-INS-i, scoring matrix = 200 PAM / k = 2, gap open penalty = 1.53. Each gene and each positions in *COI* (except for the first and second ones that were combined into a single partition) were unlinked. Best-fit models were selected for the partitions using the Akaike information criterion in jModelTest 2.1.4 (Darrriba *et al.*, 2012). The GTR+I+G model was selected for all partitions except for the third positions in *COI* where instead a GTR+I model was selected. We assumed a Dirichlet distribution for base frequencies and an uninformative prior topology.

**Table 2.** Primers used for amplification and cycle sequencing

Gene	Primer	Sequence	Author
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> , 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer <i>et al.</i> , 1994
	COI-E	TATACTTCTGGGTGCCGAAGAATCA	Bely & Wray, 2004
	jgLCO1490	TITCIACIAAYCAYAARGAYATTGG	Geller <i>et al.</i> , 2013
	jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA	Geller <i>et al.</i> , 2013
16S	16SarL	CGCCTGTTTATCAAAAACAT	Palumbi, 1996
	16SbrH	CCGGTCTGAACTCAGATCACGT	Palumbi, 1996
	16SANNF	GCGGTATCCTGACCGTRCWAAGGTA	Sjölin <i>et al.</i> , 2005
28S	28SC1	ACCCGCTGAATTTAAGCAT	Lê <i>et al.</i> , 1993
	28S900F	CCGTCTTGAAACACGGACCAAG	Lockyer <i>et al.</i> , 2003
	28S1100R	AGGCATAGTTCACCATCTTTTCG	This study
	28S1900R	CCATGTTCAACTGCTGTTCCACATG	This study
18S	PCR1F	AYCTGGTTGATCCTGCCAGT	Nygren & Sundberg, 2003
	PCR2F	TAAAGYTYGTGCAGTTAAA	Nygren & Sundberg, 2003
	PCR1R	TASGACGGTATCTGATCGTCTT	Nygren & Sundberg, 2003
	PCR2R	ACCTTGTTACGACTTTTACTTCCTC	Nygren & Sundberg, 2003

We ran four chains simultaneously, three heated and one cold. The number of generations for the combined analysis was set to 30 million. The chains were sampled every 1000 generations and one quarter of the samples was discarded as burn-in. The Maximum Likelihood (ML) analyses were performed in raxmlGUI (Silvestro & Michalak, 2012). In RAxML, the analyses were run using GTRGAMMAI for the molecular partitions and GAMMA for the morphological partition, and clade support was assessed with the option Bootstrap + consensus with 2000 repetitions. Bayesian Inference (BI) was implemented in MrBayes 3.2.1 (Ronquist *et al.*, 2012) and run with four Markov chains that started from a random tree and run simultaneously for 20 million generations, with trees sampled every 2000 generations (samplefreq = 2000). The initial 25% of trees were discarded as burn-in (burninfrac = 0.25), after assessing for convergence with Tracer v.1.6 (Rambaut *et al.*, 2014) and AWTY (Wilgenbusch *et al.*, 2004).

## RESULTS

### PHYLOGENETICS

The final alignment was 4832 bp long and composed of partial sequences of the nuclear 28S (1822 bp) and 18S (1886 bp) and the mitochondrial 16S (464 bp) and COI (657 bp) from 62 specimens. Both ML and BI analyses of the concatenated matrix of the four loci recovered similar topologies but varied in node supports, which are typically higher in BI (Fig. 1 and Supporting Information, Fig. S1). Results for both analyses show the tiny annelids living in association with ctenophores consistently in a well-supported clade within the

Phyllococidae, more specifically within the Alciopini and being sister to *Alciopina*, *Rhynchonereella*, *Torrea* and *Vanadis* (Fig. 1 and Supporting Information, Fig. S1). These results, combined with their unique morphology allowed us consider them a new genus (see *Taxonomic account*).

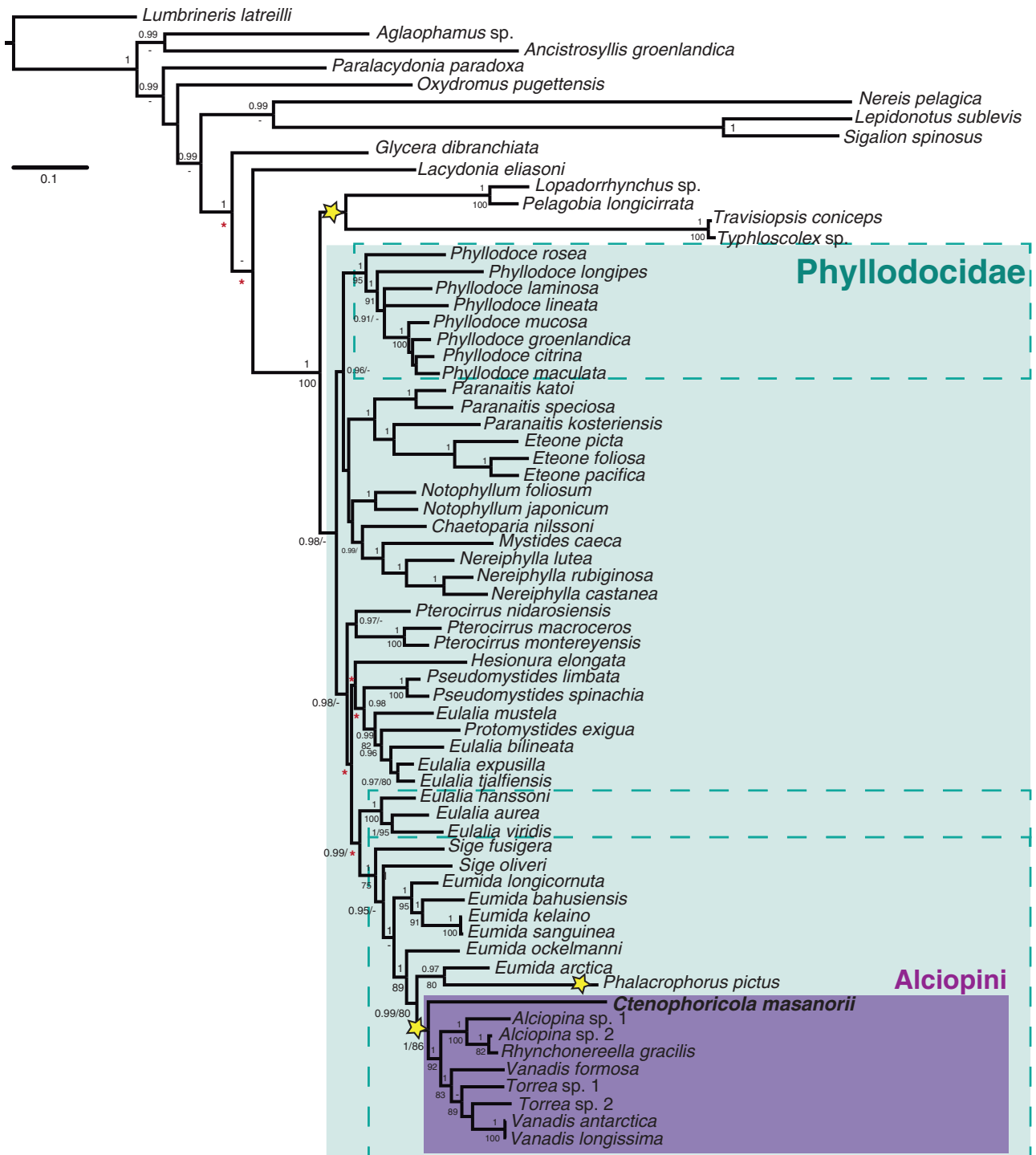
In addition, both the BI and ML results support the monophyly of the pelagic Typhloscolecidae and Lopadorrhynchidae (although their relationships are not well resolved) and some Phyllococidae, such as *Eteone*, *Nereiphylla*, *Notophyllum*, *Phyllococe* and *Pseudomystides* (Fig. 1 and Supporting Information, Fig. S1). *Pterocirrus* also appear as a well-supported genus in the BI results (Fig. 1), while *Eulalia*, *Eumida*, *Paranaitis*, *Pterocirrus* and *Sige*, as currently defined, are paraphyletic and polyphyletic in all analyses. We also recovered some other well-supported clades such as those containing *Phyllococe* (subclade B1), *Eulalia viridis* (the type species of the genus, subclade A4) and the clade containing species of the genera *Alciopina*, *Eumida*, *Rhynchonereella*, *Sige* and *Torrea* (subclade A3) (Leiva *et al.*, 2018), together with *Phalacrophorus*, *Vanadis* and the new genus *Ctenophoricola* (Fig. 1 and Supporting Information, Fig. S1).

### TAXONOMIC ACCOUNT

#### CTENOPHORICOLA GEN. NOV.

Isid: zoobank.org:act:60F0CA86-D905-469D-8B6B-65326EA195F9

*Diagnosis*: Alciopini with body divided in two distinctly different regions, densely covered by cilia. Anterior region highly contractile, with small, acute parapodia, with indistinct dorsal and ventral lobes,



**Figure 1.** Phylogenetic tree obtained from BI analysis of concatenated data set (18S, 28S, 16S and *COI*) showing *C. masanorii* clearly within the Phyllodocidae and specifically as part of the Alciopini. Holopelagic clades are indicated with a star. Dotted lines point out supported clades that agree with recent studies in the family Phyllodocidae (Leiva *et al.*, 2018). Numbers above branches indicate posterior probability (PP) support values (only PP  $\geq 0.95$  are indicated); numbers below branches indicate bootstrap support (BS) values (only BS  $> 75\%$  are indicated); red asterisks indicate discrepancy between BI and ML analyses (see also Supporting Information, Fig. S1). The scale bar represents the number of nucleotide substitutions per site.

without chaetae but with acicula. Posterior region with distinctly larger segments; parapodia with distinctly developed and elongated dorsal and ventral lobes and capillary chaetae. Parapodia all uniramous, with thin, single acicula. Prostomium small, with two tiny retractile palps, without antennae. Pharynx eversible, unarmed, without papillae. Two distinctly pigmented, elongate, lensed eyes, extending through several anterior segments.

*Remarks:* Reproduction unknown. Due to its peculiar morphology, we hypothesize that some of the specimens with different sizes could either be regenerating forms after fragmentation (i.e. asexual reproduction) or predation, or juveniles descending directly from the most developed specimens.

*Type-species:* *Ctenophoricola masanorii* by present designation.

*Etymology:* The name refers to the habitat of the new genus, combining Ctenophora, the scientific name for comb jellies (from Ancient Greek κτείς, a comb, and φέρω, to carry) with the Latin root *-cola* meaning “living on” (from *incola*, dweller). The gender of the name is masculine.

#### **CTENOPHORICOLA MASANORII SP. NOV.**

**Figs 2–7, Supporting Information (Movies S1–S3)**

lsid: zoobank.org:act:4E29046B-899F-410C-8A46-C1E99F5E50E9

*Material examined:* Holotype: MNCN 16.01/17896, off south-east Enoshima Island (Kanagawa Prefecture, Japan) on *Beroe campana* Komai, 1918, fixed in 4% formaldehyde buffered in seawater, preserved in 70% ethanol, 9 September 2013. Paratypes: MCZ 25325 & 25326 (two adults) and MNCN 16.01/17894 (two juveniles), on *Bolinopsis mikado* Moser, 1908, 96% ethanol; MNCN 16.01/17895 (six adults, one juvenile) on *Bo. mikado*, fixed in 4% formaldehyde buffered with seawater, preserved in 70% ethanol; MNCNM 16.01/17898 (six adults, three juveniles) on *Be. campana*, 96% ethanol; MNCN 16.01/17897 (four adults, two juveniles) and MNCN 16.01/15342 (two additional adults used for SEM), fixed in 4% formaldehyde buffered with seawater. All collected off south-east Enoshima Island, Kanagawa Prefecture, Japan, 9 September 2013.

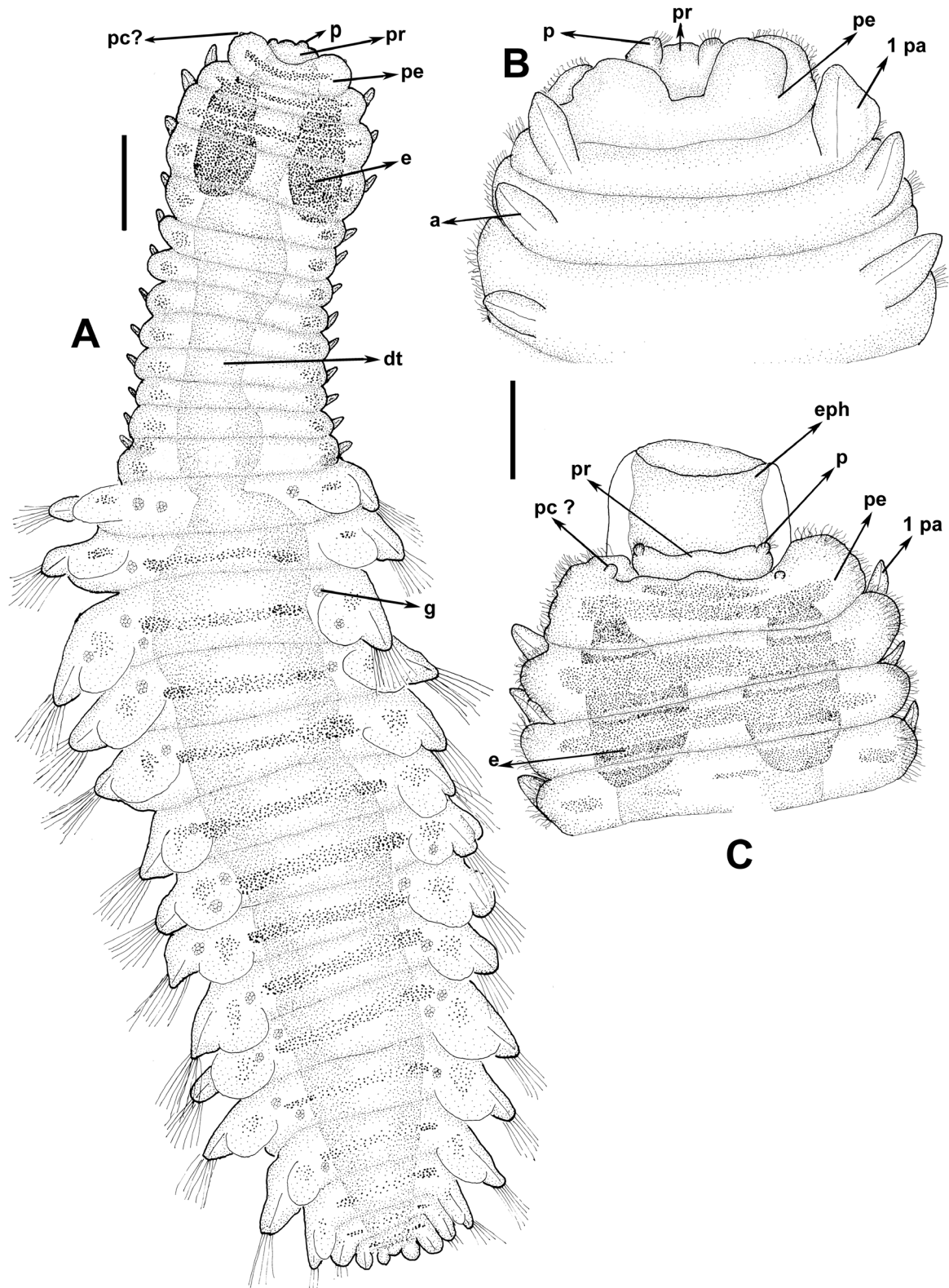
*Diagnosis:* Species of *Ctenophoricola* with transverse pigmented bands, lightly coloured on dorsum and lacking marked caeca in gut.

*Description of largest individuals (adults?):* Holotype (Figs 2A, B) 2.6 mm long (live, relaxed specimens up

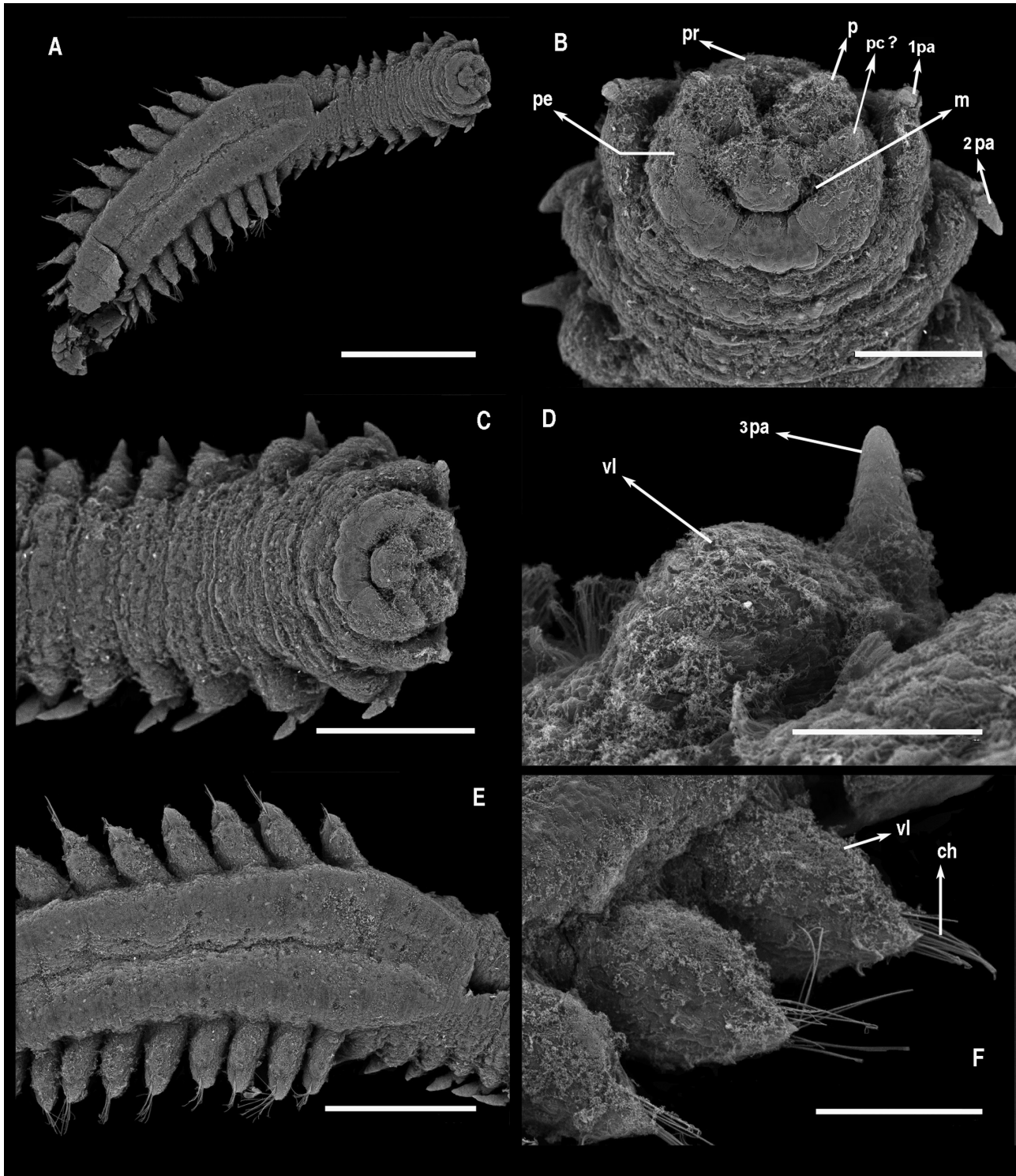
to 3 mm long), anterior and posterior regions 0.4 mm and 0.6 mm wide, respectively, excluding parapodia. Body small, cylindrical, somewhat dorsoventrally flattened on posterior half, with peristomium and 27 segments with parapodia, divided in two distinctly different regions (Figs 2A, 3A, 5C–D), densely covered by short cilia (Figs 2B–C, 3, 4). Translucent to yellowish *in vivo* (Fig. 5), with one reddish narrow transverse band dorsally on peristomium, sometimes with a pigmented band on each of the two most anterior segments (Fig. 2A, C) and on posterior region (Fig. 2A) segments; some reddish areas on laterals of some segments; other specimens without colour pattern (Fig. 5). Prostomium small, semicircular, without external eyes, with two minute, retractile palps distally ciliated, partially retracted inside peristomium (Figs 2A–C, 3B–C, 4B–D). Peristomium similar in length to subsequent segments, but narrower than chaetigers; a median, small, dorsal lobe on some specimens (Figs 2A, C, 4B–C) often with two minute papillae (Figs 2A, C, 3B, 4D). Mouth covered by one dorsal and two ventral lips (Fig. 3B–C). Pharynx cylindrical, everted in some specimens (Fig. 4B, D), unarmed, without papillae. Two conspicuous, anterior, interior lensed eyes, either dark reddish-brown or yellowish (e.g. in juveniles) (Fig. 5), internally reaching to segment 4–5 in preserved specimens (2–3 *in vivo*). Anterior region with 12–13 segments. Parapodia conical, with thin, internal acicula, without chaetae (Figs 2, 3A–D, 4B, D–E), with a small, indistinct, rounded, dorsal lobe, densely covered by cilia (Fig. 4E); ventral lobe similar (Fig. 3D); most anterior parapodia laterofrontally directed (Figs 2, 4B, D). Posterior region with 15 segments, distinctly wider than those of anterior region; sometimes with distinct clusters of large cells in larger individuals (Fig. 2A); parapodia larger and longer with internal slender acicula and a fascicle of few, thin capillary chaetae; dorsal and ventral lobes distinctly enlarged (Figs 2A, 3A, E–F, 4A) and lateroposteriorly directed. Gut straight, visible through body wall, distinctly wider in posterior region (Figs 1A, 5C–D). Pygidium small with two rounded, short anal cirri.

*Description of smallest individuals:* Minute specimens (*c.* 0.33 mm long), showing slightly bilobed prostomium, non-perceptible palps and a well-defined peristomium, with no sign of peristomial cirri. Anterior region with *c.* seven segments, with parapodia similar to those of adults; posterior region as a triangular bud showing traces of segments, but lacking parapodia. Gut is fully developed. External eyes not detectable (Fig. 6A). Specimens of *c.* 0.5 mm are similar to the smallest ones (i.e. 0.33 mm), with a more developed posterior region and distinct

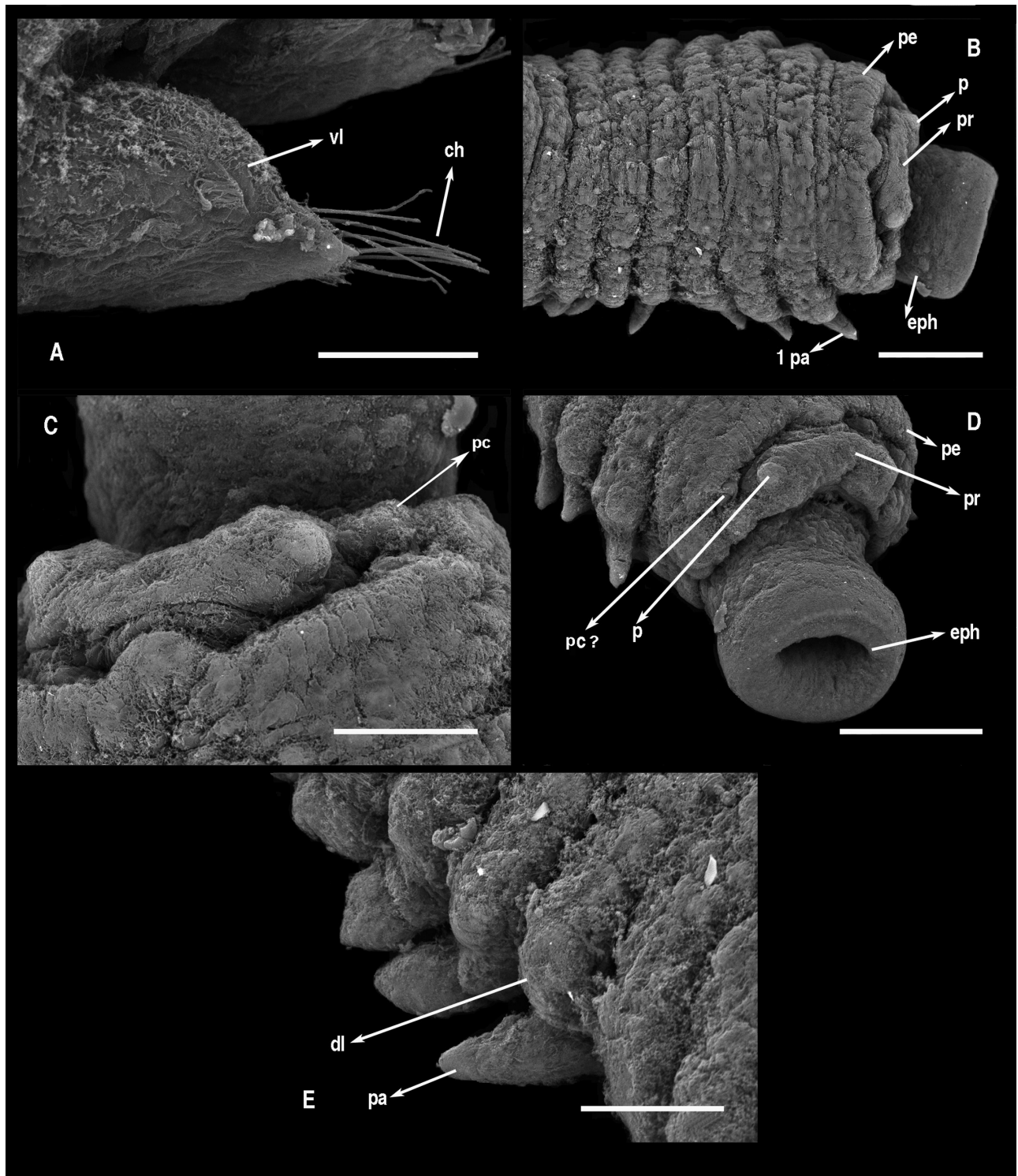




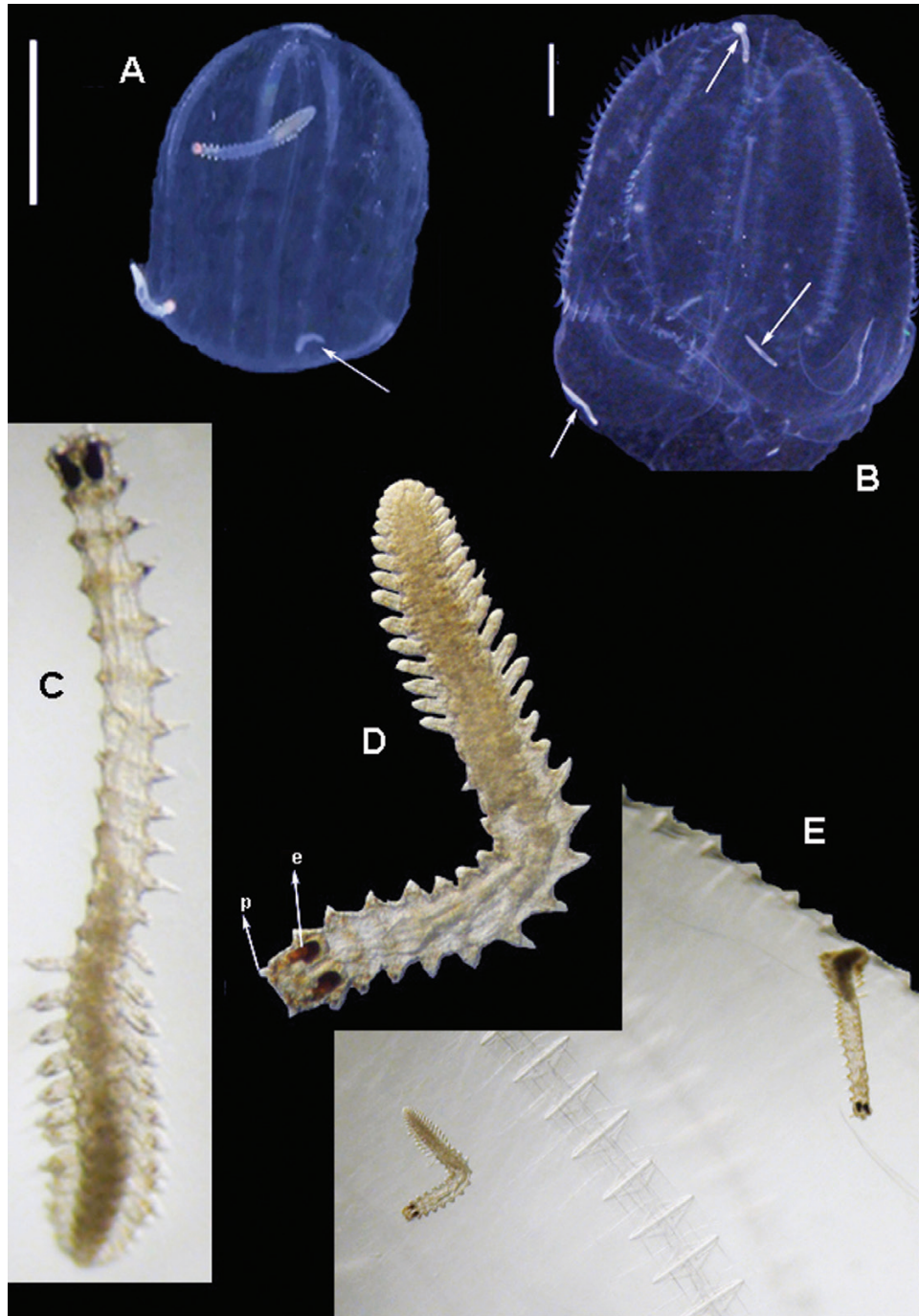
**Figure 2.** *Ctenophoricola masanorii* A, holotype, complete specimen, dorsal view. B, holotype, anterior end detail, ventral view. C, paratype, anterior end detail, dorsal view. Abbreviations: 1pa, first parapodium; a, acicula; dt, digestive tract; e, eye; eph, everted pharynx; g, embryos or gonads; p, palps; pc, peristomial cirri; pe, peristomium; pr, prostomium. Scale bars: A, 0.2 mm; B, C, 0.1 mm.



**Figure 3.** *Ctenophoricola masanorii* Paratype SEM. A, complete specimen, ventral view. B, anterior end, frontal view. C, anterior end, ventral view. D, parapodium of anterior region, ventral view. E, posterior region, ventral view. F, parapodia of posterior region, ventral view. Abbreviations: 1, 2 or 3pa, first, second or third parapodium; ch, chaetae; m, mouth; p, palp; pc, peristomial cirri; pe, peristomium; pr, prostomium; vl, ventral lobe. Scale bars: A, 500  $\mu$ m; B, F, 100  $\mu$ m; C, 200  $\mu$ m; D, 50  $\mu$ m; E, 300  $\mu$ m.



**Figure 4.** *Ctenophoricola masanorii* Paratype SEM. A, parapodium of posterior region, ventral view. B, anterior end, dorsal view. C, detail of prostomium and peristomium, dorsal view. D, anterior end, frontal view. E, parapodia, anterior region, dorsal view. Abbreviations: 1 pa, first parapodium; ch, chaetae; dl, dorsal lobe; eph, everted pharynx; p, palp; pa, parapodium; pc, peristomial cirri; pe, peristomium; pr, prostomium; vl, ventral lobe. Scale bars: A, C, E, 50  $\mu$ m; B, D, 100  $\mu$ m.

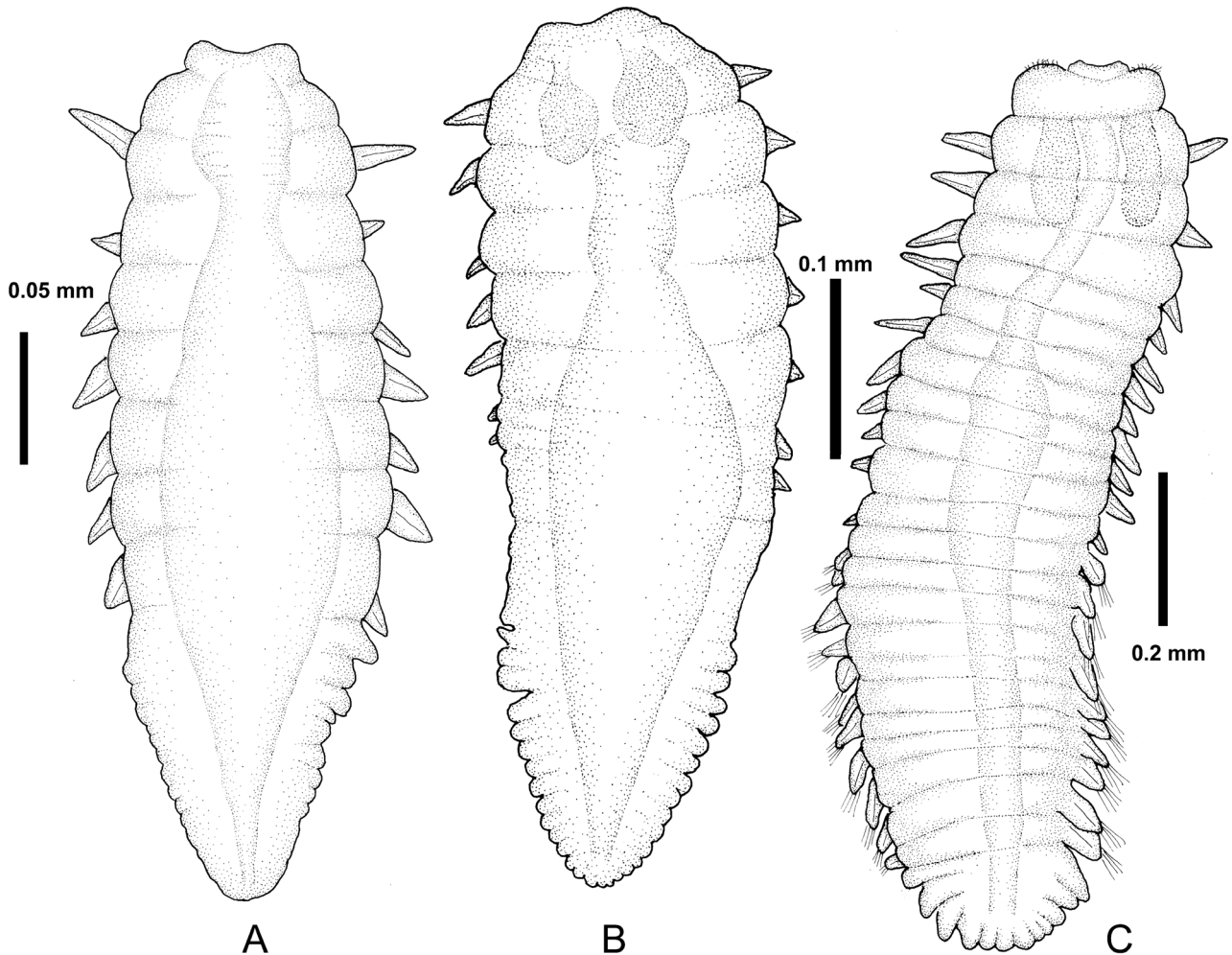


**Figure 5.** *Ctenophoricola masanorii*, live specimen micrographs. Three specimens on a ctenophore: (A) on *Be. campana*, (B) on *Bo. mikado*. C-D, complete specimens, dorsal view. E, detail of two specimens on a comb jelly. Abbreviations: e, eye; p, palp. Scale bars: A, B, 5 mm.

segments, but still lacking parapodia and traces of internal eyes (Figs 6B, 7 middle). Specimens of c. 1.2 mm, are more similar to the largest ones (adults?), with well-developed posterior parapodia, and yellowish internal eyes, but with posterior region (Figs 6C, 7 right, Supporting Information, Movie S3) not

as distinct as in the largest specimens (adults?) parapodia.

*Behaviour:* *Ctenophoricola masanorii* were observed feeding directly on the surface of the host by everting their pharynx and sucking from the epithelia and



**Figure 6.** *Ctenophoricola masanorii* specimens, dorsal view. A, smallest specimen. B, larger specimen developing eyes. C, large complete specimen. Scale bars: A, 0.05 mm; B, 0.1 mm; C, 0.2 mm.



**Figure 7.** *Ctenophoricola masanorii* collected from a single host arranged from small (left) to large (right) specimens. Scale bar 0.1 mm.

underlying mesoglea. They triggered a contraction of the affected area ([Supporting Information, Movie S3](#)) similar to that caused by the movement of other

parasites ([Supporting Information, Movie S2](#)), suggesting they disturb the host.

Locomotion of *C. masanorii* results from accordion-like contractions and extensions in which the anterior region extends, attaches to the ctenophore surface by parapodia and then is slowly contracted pulling the posterior region forward ([Supporting Information, Movies S1 and S3](#)). The worms were observed extending the anterior region out away from the host while the posterior region was still attached ([Supporting Information, Movies S1 and S3](#)), either to explore all of the available host surface before choosing a direction to head in or to find new hosts. Three specimens that may be regenerating or juvenile and subadult forms, appeared on the same ctenophore as adults ([Figs 6–7](#)); early ([Figs 6A, 7 left](#)) and later ([Fig. 6B, 7 middle](#)) regenerating/juveniles were found together with a specimen almost identical to the adult (i.e. subadult, [Figs 6C, 7 right](#), [Supporting Information, Movie S2](#)). These small specimens seemed to have

limited capacities either to maintain their attachment to the host or to float in the water column (Supporting Information, Movie S2). Thus, we suggest that some of these small specimens are able to hold on the same as the largest forms, whereas others detach and drift either until they encounter another host or they die in the water column.

*Type locality:* South-east Enoshima Island, Japan.

*Habitat and distribution:* On the surface of the ctenophores *Bo. mikado* and *Be. campana*. Known only from the type locality in Japan.

*Etymology:* The species is named after Dr Masanori Sato, from Kagoshima University, a well-known and enthusiastic polychaetologist, who sent the specimens

of the new species, together with the pictures and videos, and also provided the co-first authors with numerous and valuable syllids from Japan.

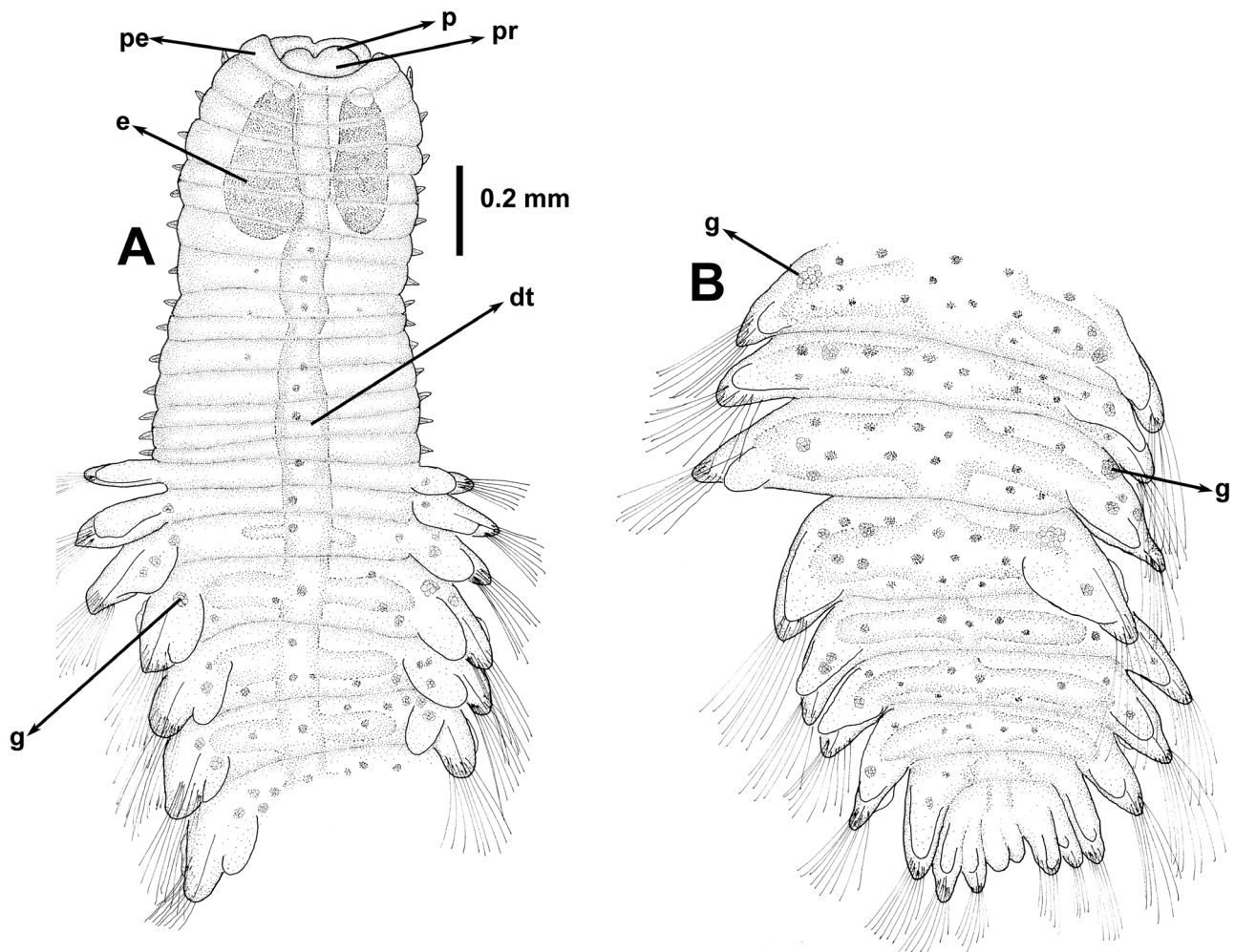
***CTENOPHORICOLA ROUSEI* SP. NOV.**

**Figs 8–11**

Isid: zoobank.org:act:55C8FC03-98BA-4590-987A-2C92217B4A88

*Holotype:* MNCN 16.01/17900. Las Lapillas (Mar de las Calmas), south-west of El Hierro Island, on *Leucothea multicornis* (Quoy & Gaimard, 1824), fixed in 4% formaldehyde buffered in seawater, preserved in 70% ethanol, March 1994.

*Paratypes:* MNCN 16.01/17898 (six adults, two juveniles) Radazul, south-east of Tenerife Island,



**Figure 8.** *Ctenophoricola rousei* Holotype, dorsal view. A, anterior end. B, posterior end. Abbreviations: p, palps; pr, prostomium; pe, peristomium; e, eye; dt, digestive tract; g, gonads or embryos. Scale bar 0.2 mm.

on *Eurhamphaea vexilligera* Gegenbaur, 1856 and *Cestum veneris* Lesueur, 1813, December 1996; MNCN 16.01/17899 (one adult used for SEM) from same locality; MNCN 16.01/17901 (three specimens) January 1997; MNCN 16.01/17902 (two specimens), same data.

**Additional material:** One specimen, Cape Spartel, Tangier, Morocco, 35°47.534'N, 5°55.630'W, 18 October 2010, on *E. vexilligera*.

**Diagnosis:** Species of *Ctenophoricola* with minute size, having scattered red spots on dorsum. Posterior gut wall with one pair of lateral caeca per segment protruding up to twice width of gut, sometimes extending into parapodia.

**Description:** Holotype (Fig. 8) 3 mm long, longest complete specimen, anterior region 0.5 mm wide excluding parapodia, posterior region 0.65 mm wide excluding parapodia. Body cylindrical, somewhat flattened in posterior region, divided in two distinctly different regions (Figs 8, 9A–D, 10A–B), sparsely ciliate. Translucent yellowish, with reddish spots, more numerous on posterior segments (Figs 8, 9A–D) two white spots distally on parapodial lobes of posterior region (Fig. 9A–D). Prostomium small, semicircular, without external eyes, with two minute, retractile palps, not ciliate (Fig. 10C), partially retracted inside peristomium (Fig. 8A). Peristomium similar in length to subsequent segments; peristomial (tentacular) cirri not found. Two conspicuous, anterior, lensed eyes, internally reaching to segment 4–5 in preserved (2–3 *in vivo*) specimens (Figs 8A, 9A–C, E–F), strongly pigmented dark reddish-brown in adults, paler orange to yellow in juveniles. Anterior region with 12–16 segments, with parapodia conical, triangular, acute, with a thin, internal acicula, without chaetae (Fig. 8A), with minute dorsal and ventral lobes; anteriormost parapodia of anterior region anterolaterally directed (Fig. 8A). Posterior region with 17 segments, distinctly wider than those of anterior region (Figs 8A, 9A–D, 10A–B), with larger and longer, acute parapodia (Fig. 10B), with internal slender acicula and a fascicle of several, thin capillary chaetae (Fig. 9B, D), thin dorsal and ventral lobes (Fig. 10B, D) and groups of cells (likely gonads, gametes or maybe developing embryos) on posterior parapodia (Fig. 11). Pharynx cylindrical, everted in some specimens (Fig. 9F), unarmed, without papillae. Gut straight in anterior region, visible through body wall, distinctly wider in posterior region, with one pair of lateral caeca per segment, individual caeca length to at least twice width of gut, sometimes

protruding into parapodia (Figs 8, 9A–D). Living specimens with bright yellow material inside gut and caeca of posterior region. Pygidium small with two rounded, short anal cirri. Juveniles similar, with less-developed posterior region.

**Remarks:** *Ctenophoricola rousei* resembles *C. masanorii*, except in having scattered spots instead transversal bands, more acute parapodia carrying more numerous chaetae, a less ciliated body and distinct laterally projecting caecae in the gut wall.

**Type locality:** Las Lapillas, Mar de las Calmas, El Hierro, Canary Islands, Spain.

**Habitat and distribution:** Canary Islands and Cape Spartel (Tangier), Atlantic coast of Spain and Morocco, on the surface of the ctenophores, *E. vexilligera*, *L. multicornis* and *C. veneris*.

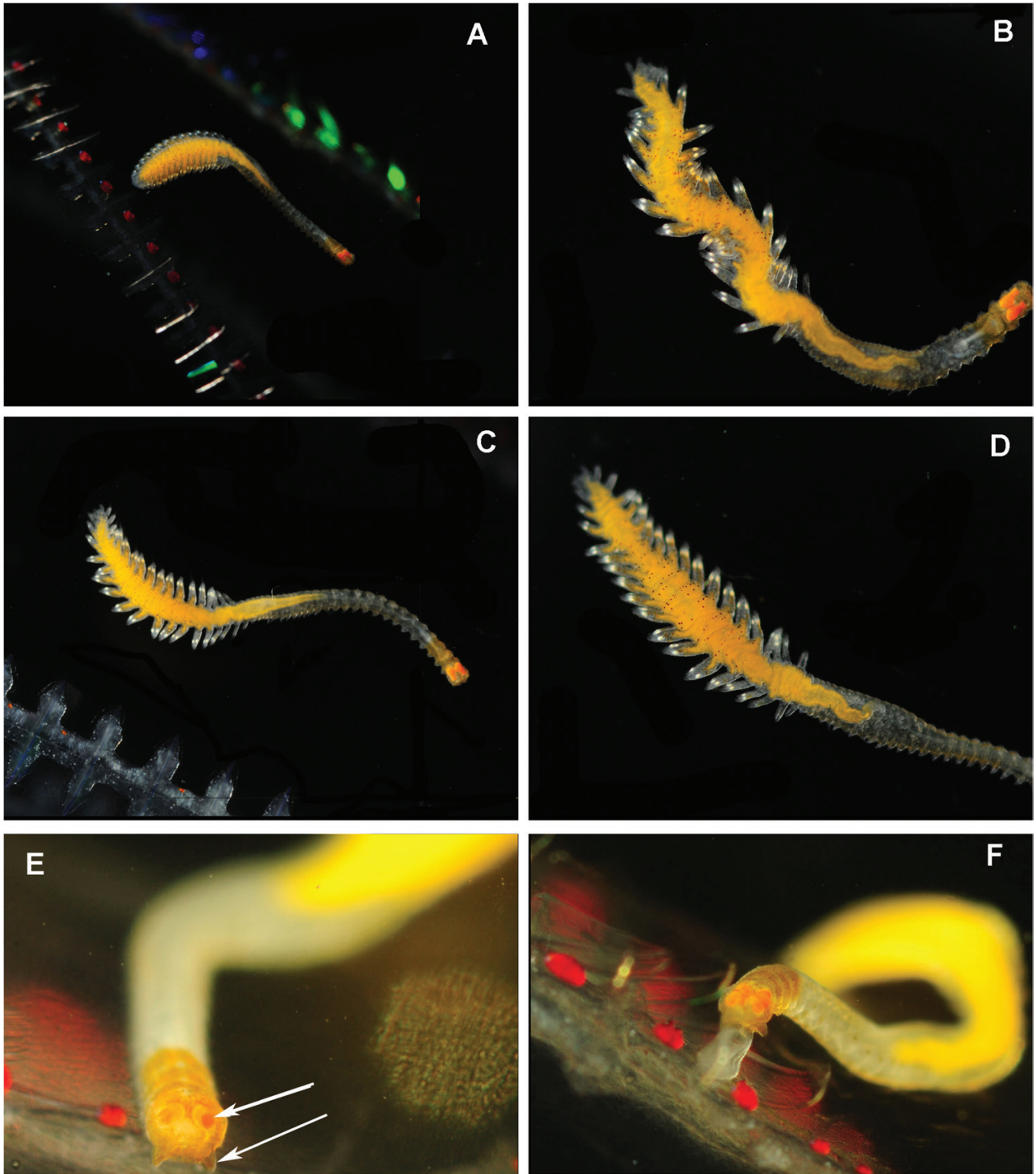
**Etymology:** The species is named after Dr Greg Rouse, renowned polychaetologist, colleague and friend, for his invaluable contributions to the knowledge of annelid morphology and evolution.

#### CTENOPHORICOLA SP.

##### Figs 12, 13, Supporting Information, Movies S4 and S5

**Material:** Gulf of California, near the mouth, between 26 ° and 23 ° 50' N, Mexico. Single complete specimen, 29 anterior fragments and numerous posterior fragments; zooplankton samples from *GOLCA 8404* (20 March–7 April 1984) and *El Golfo I* expeditions.

**Description:** Single complete specimen a mature female with developing gonads/oocytes in anterior and posterior parapodia, 5 mm long, 1.7 mm wide, 26 chaetigers (Figs 12–13). Other specimens only with anterior region and few posterior region segments or with only posterior region and pygidium. Body yellowish, with small pigment spots in rows on dorsum and ventrum of anterior segments and on posteriormost anterior region parapodia and all parapodia of posterior region. Prostomium usually contracted. Single pair of short, indistinct palps. Mouth with one ventral and two lateral lips. One pair conspicuous, internal lensed eyes with pigments visible posteriorly (Fig. 13). Everted proboscis smooth, cylindrical, unarmed. Anterior parapodia reduced compared to posterior, lacking ventral cirri and having short chaetal lobes; acicula not exceeding lobe length. Parapodia of posterior region stout,

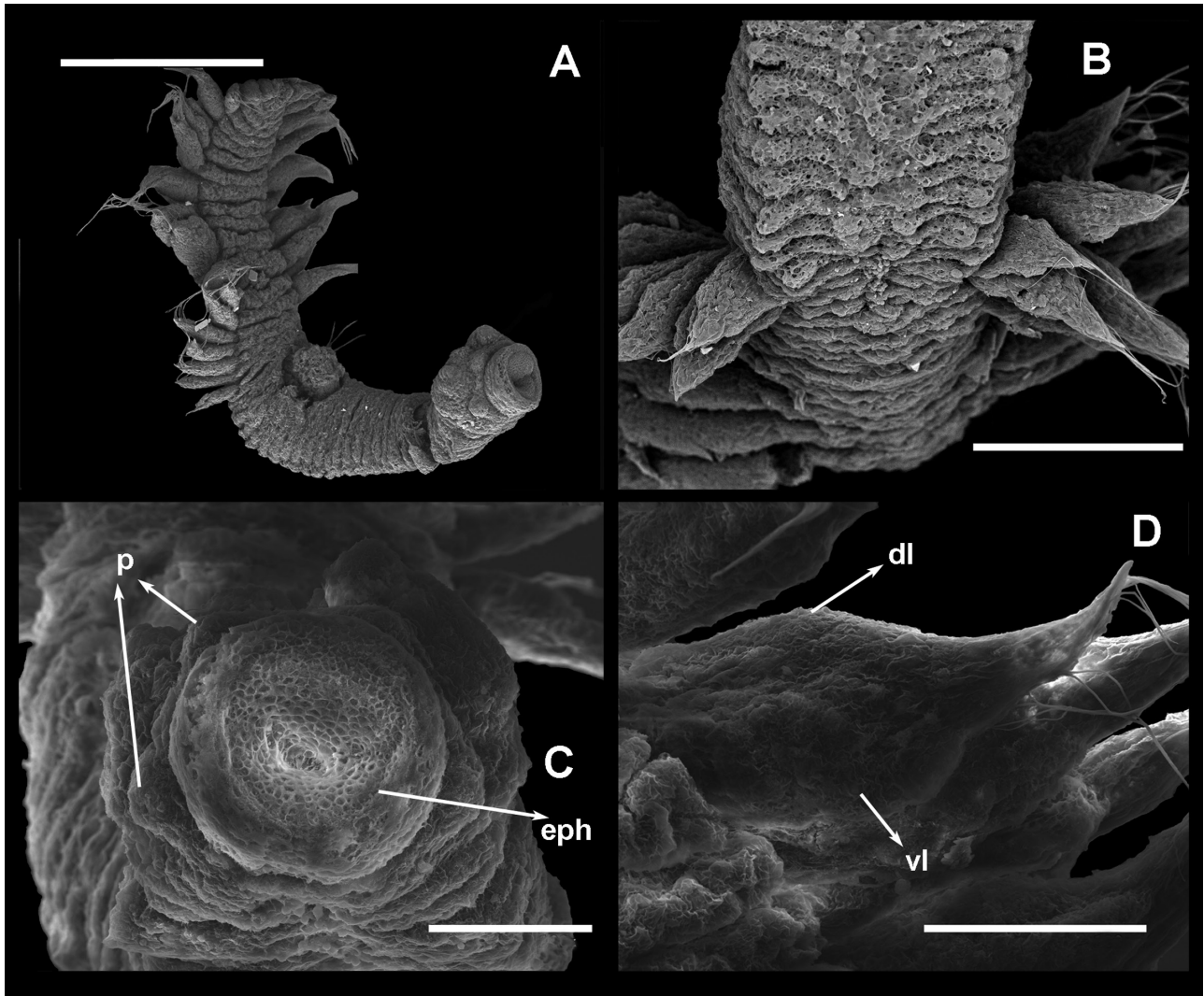


**Figure 9.** *Ctenophoricola rousei*, live specimen micrographs. A–C, complete specimens. D, dorsal view showing pigmentation pattern. E, frontal view with arrow indicating eye and palps clearly visible. F, the same individual with pharynx everted siphoning tissue.

trilobed, with laminar ventral and dorsal cirri fused with parapodia (Fig. 12C), with acicula and simple capillary chaetae arranged in fan, surrounded basally

by prechaetal and postchaetal lamella. Pygidium with a pair of elongated cylindrical cirri. Hosts are unknown.



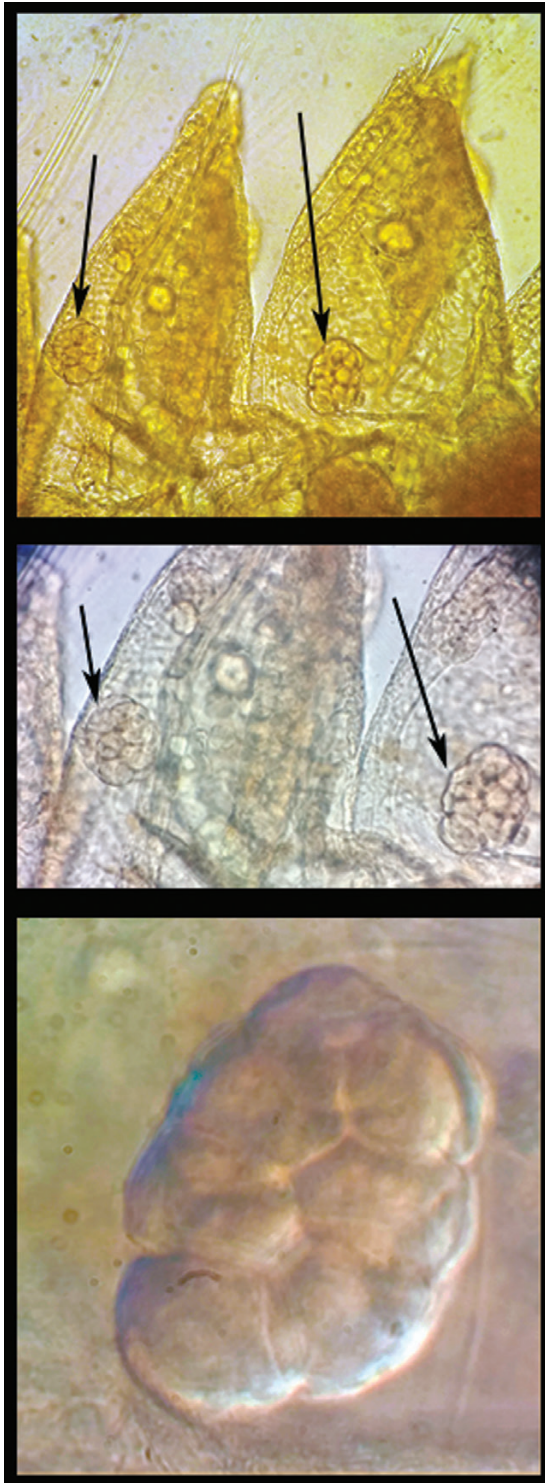


**Figure 10.** *Ctenophoricola rousei* SEM. A, complete specimen, dorsolateral view. B, ventral view of the end of the anterior region and the beginning of the posterior one. C, everted pharynx and prostomium. D, posterior region parapodium. Abbreviations: dl, dorsal lobe; eph, everted pharynx; vl, ventral lobe; p, palp. Scale bars: A, 500  $\mu\text{m}$ ; B, 200  $\mu\text{m}$ ; C, D, 100  $\mu\text{m}$ .

*Histology of eyes:* The eyes are large, from just posterior to the central brain through the second chaetiger (Fig. 13 and Supporting Information, Movie S4). The eyes are approximately cylindrical, with a diameter slightly smaller in the anterior half (possibly a handling artefact); the width/length ratio is 0.4. The lenses are approximately spherical, slightly elongate (again, possibly a handling artefact) and apparently denser in the centre and outer margin (Fig. 13B–C, Supporting Information, Movie S4). This type of pigmented receptor with nuclear and plexiform layers were also found in the eyes of the Alciopini *Vanadis tagensis* Dales, 1955 by Hermans and Eakin (1974), but only in the posterior half of the eye. Additionally, there are extensive microvilli extending from the

photoreceptor cells that fill the back half of the eye (Fig. 13F). The pigment granules are not numerous. There may be a small secondary retina ventrolateral to the lens and anterior to the optic nerve (Supporting Information, Movie S5; Fig. 13B). The optic nerve is large and exits the eye ventrally, just anterior to its midpoint (Supporting Information, Movies S4 and S5, Fig. 13D). A distinct, continuous basal lamina encapsulates the eyes, optic nerves and brain (Fig. 13B).

*Remarks:* Entire specimen minute, likely damaged by crushing together with other organisms during net collection, pierced by several spines from other planktonic organisms (Supporting Information, Movie S5).



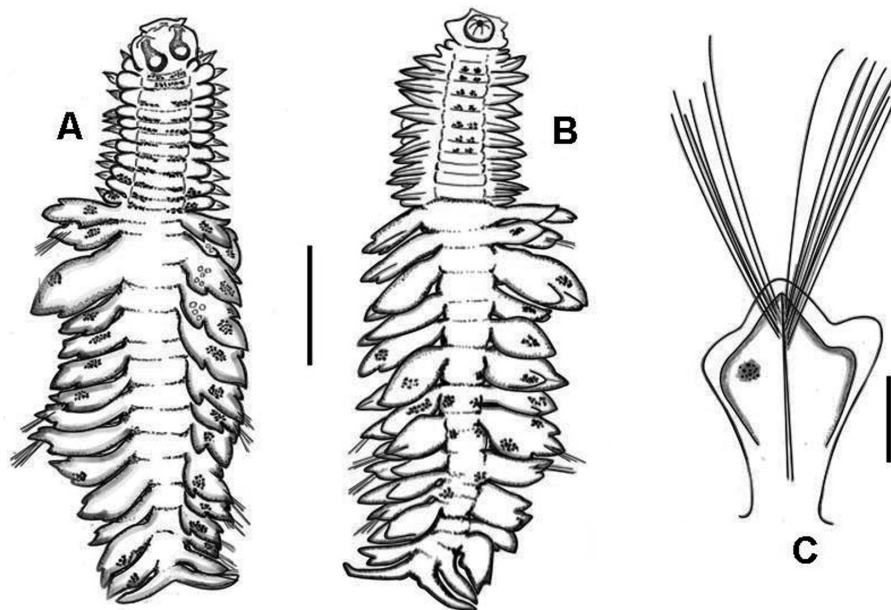
**Figure 11.** *Ctenophoricola rousei* Developing gonads, gametes or embryos inside parapodia of posterior region (arrows). A, showing 2 parapodia with developing gonads, gametes or embryos inside. B, closer view of the same. C, a unique developing gonad, gamete or embryo.

All this damage and the extreme delicate nature of the specimen prevent us to describe many relevant morphological structures.

## DISCUSSION

### MORPHOLOGY AND SYSTEMATICS

The presence of both palps (although small and retractile) and intraparapodial aciculae, placed the new genus within the Palpata, Aciculata (Rouse & Fauchald, 1997; Rouse & Pleijel, 2001). Our molecular and morphological results together with their holopelagic mode of life, allow us to consider *Ctenophoricola* within the Alciopini (*sensu* Rouse & Pleijel, 2001; Eklöf *et al.*, 2007). Although this well-supported group is still considered a family (WoRMS 2020; Collazo *et al.*, 2017; Fernández-Álamo, 2018), our results firmly support alciopids as a derived lineage of the Phyllodocidae, in agreement with Rouse & Pleijel (2001), Halanych *et al.* (2007) and Leiva *et al.* (2018). The Alciopini present five antennae, compound or simple chaetae and two highly developed lensed eyes. These eyes, unique among annelids, cover most of (and often protrude from) the prostomium, passing in some cases the lateral margins of even the widest parts of the body (Fauvel, 1923; Rouse & Pleijel, 2001). The structure and composition of the eyes of *Ctenophoricola* are similar to those of other Alciopini, although they slightly differ in elongation location and orientation. In addition, they are within the prostomium and first few segments (Figs 2, 6–9), instead perched on the prostomium as in other Alciopini. Beyond the differences in eyes, *Ctenophoricola* also differs from other genera of Alciopini in lacking antennae and being tiny (other genera may be up to 1 m long), probably as an adaptation to living symbiotically on relatively small hosts. The Tomopteridae, Iospilidae and Lopadorrhynchidae also have transparent bodies or modified parapodia/chaetae resulting from an holopelagic and parasitic mode of life (Rouse & Pleijel, 2001). The Typhloscolecidae parasites (or highly specialized predators) of chaetognaths have a siphoning pharynx similar that in *Ctenophoricola* (see Martin & Britayev (2018) and references herein). Both the Iospilidae and *Ctenophoricola* also lack the antennae and have small prostomiums and reduced anterior parapodia; however, in contrast, iospilids have chaetae in all their parapodia, simple eyes and chitinous jaws in their eversible pharynx (at least in *Phalacrophorus*) (see Rouse & Pleijel, 2001; Halanych *et al.*, 2007). *Phalacrophorus pictus* Greeff, 1879, the unique sequenced iospilid was found as a sister clade to *C. masanorii*, and is actually more related to *Eumida arctica* (Annenkova, 1946) (Fig. 1 and Supporting



**Figure 12.** *Ctenophoricola* sp. from the Gulf of California. A, dorsal view of complete specimen. B, ventral view of complete specimen. C, posterior region parapodia, lateral view. Scale bars: A, B, 1 mm; C, 0.1 mm.

Information, Fig. S1). In fact, the genus *Eumida* is undoubtedly polyphyletic, with a well-supported monophyletic clade including the type species (*Eumida* s. s.) and some other species apparently more closely related to the Alciopini, even though they are not holopelagic and clearly differ morphologically. The persistently found polyphyly and paraphyly in many genera within the Phyllodocidae (Fig. 1 and Supporting Information, Fig. S1; Leiva *et al.*, 2018), certainly requires further molecular and morphological analyses (including type specimens and/or localities) to resolve the taxonomical status of some of their clades.

#### ECOLOGY

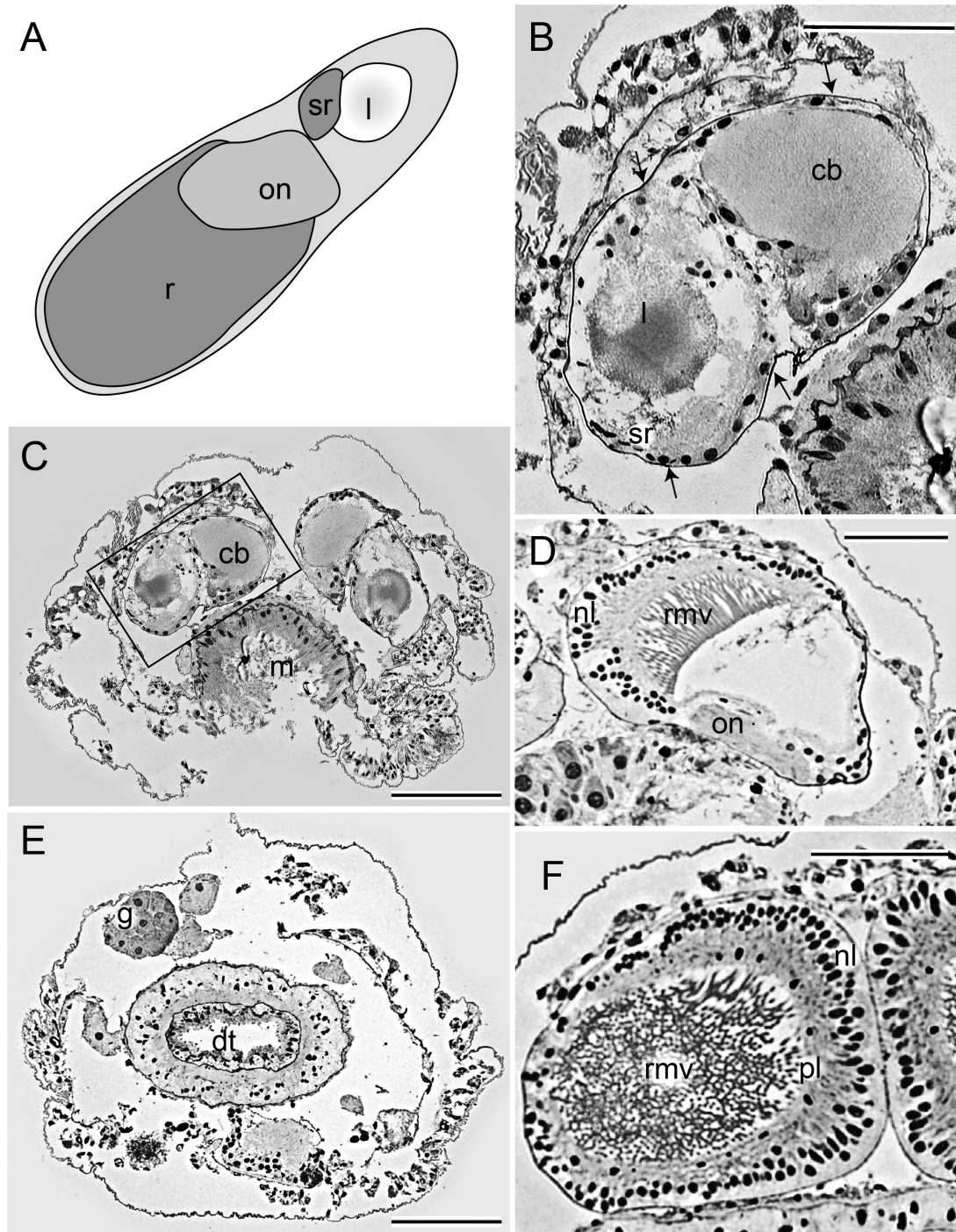
Given the apparent annoyance observed on the host ctenophores (Supporting Information, Movies S2 and S3), we consider *Ctenophoricola* as an ectoparasite. However, other type of relationships cannot be excluded, such as a specialized predator-prey one, suggested also for Typhloscolecidae associated to chaetognaths (Britayev and Martin, 2019), since we could not check if the ctenophores were totally damaged by the annelids or not. Contrary to *A. parasitica*, which is only known from the gastrovascular canals of the ctenophore *H. plumosa*, the species of *Ctenophoricola* appear to be less selective, since we have found *C. masanorii* and *C. rousei* on two and three different species of ctenophores, respectively. We thus expect that further careful studies on ctenophores from different regions would result in new findings that

increase the current knowledge on the life history and behaviour of these rare symbionts.

#### LIFE CYCLE AND REPRODUCTION

We have observed several individuals of *C. masanorii* with different sizes, apparently showing traces of regenerating their anterior ends (Fig. 7). This likely suggests asexual reproduction as a way to quickly colonize a host, previously reported in symbiotic spionids or syllids (e.g. Tzetlin & Britayev, 1985; López *et al.*, 2001; Lattig & Martin, 2011; David & Williams, 2012).

On the other hand, the presence of cell clusters in the posterior segments of all species of *Ctenophoricola* could be related with a sexual reproduction mode adapted to the pelagic life and to a presumable low frequency of sexual encounters, as occurs in other alciopids (Rice, 1984, 1987; Eckelbarger & Rice, 1988). For instance, in *Rhynchonerella*, *Torrea* and *Vanadis* sperm storage in females has been proposed as one of the main reproductive strategies, allowing compensation of their low population densities (Eckelbarger & Rice, 1988). Sperm storage cannot be confirmed for the species of *Ctenophoricola*; however, we suggest that the cell clusters could be developing embryos, and point to a similar strategy that involves direct development and permanence of juveniles, at least for some time, on the same host as the progenitors. In this latter case, the development would be direct



**Figure 13.** *Ctenophorocola* sp. from the Gulf of California. A, ventral view of a schematic of a single eye drawn from the 3D reconstruction (Supporting Information, Movie S4). B-C, transverse section through the peristomium showing the central brain, lens, part of the secondary retina of eye, and basal lamina surrounding eye and central brain; B is detail of the boxed area in C. D, transverse section through central region of eye showing the beginnings of the retina microvilli and the optic nerve. E, transverse section through chaetiger 2 showing digestive tract, posterior margin of parapodia, and cluster of developing gonads. F, transverse section through posterior portion of eye showing receptor layer microvilli, pigment layer with sparse pigment granules, and nuclear layer. arrows, basal lamina. Abbreviations: cb, central brain; dt, digestive tract; g, gonad; l, lens; m, mouth; nl, nuclear layer; on, optic nerve; pl, pigment layer; r, retina; rmv, receptor microvilli; sr, secondary retina. Scale bars: B, D, F 50  $\mu\text{m}$ ; C, E, 100  $\mu\text{m}$ .

with the juvenile forms retained, at least during some time, on the same host as the parental individuals. These two reproductive modes are not exclusive so that they may both constitute intrinsic phases of the life cycle of these species supporting an adaptation to life on their host ctenophores. However, further data are necessary to validate our hypotheses and to increase the current knowledge of these animals. Moreover, accounting for the present lack of knowledge, we could not discard that the symbiotic individuals could just be phases of the complex life cycles of still undiscovered (or still unsequenced) Alcopini, showing what has been traditionally accepted as a “normal” planktonic aspect.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Phylogenetic relationships of *C. masanorii* inferred from the maximum likelihood analysis of the four concatenated markers (18S rRNA, 28S rRNA, 16S rRNA and *COI*). Numbers in the branches indicate bootstrap support values.

**Movie S1.** Anterior end of one individual live *C. masanorii*, and posterior part of another one, showing the chaetae.

**Movie S2.** Live individuals of *C. masanorii* living on *Be. campana*.

**Movie S3.** Live regenerating/subadult forms of *C. masanorii* feeding and moving.

**Movie S4.** 3D reconstruction of the anterior end of *Ctenophoricola* sp. from the Gulf of California based on histological thin sections showing the exterior surface, the structure of the eyes, the nervous system, and the digestive tract (green). Eyes: transparent orange; lenses: yellow; receptor layer of the retina: white; nuclear layer of the retina (receptor cells): red; optic nerve: light blue; central brain and remaining nervous system: dark blue.

**Movie S5.** Compilation of thin sections (1 µm) of *Ctenophoricola* sp. from the Gulf of California stained with Richardson's blue. The specimen is damaged on the outer surface and appears to be pierced through several times by undetermined spines.