



Article

More Knot Worms: Four New *Polygordius* (Annelida) Species from the Pacific and Caribbean

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Abstract: *Polygordius* is a clade of marine annelids mainly seen in coarse-grained habitats. They are notable for their smooth bodies, lacking in chaetae or obvious segments, and they resemble Nematoda or Nemertea. Though *Polygordius* taxa are found in all oceans of the world, identifying species based only on morphological characters can be challenging due to their relatively uniform external appearances. Diversity within the clade has likely been markedly underestimated. Where morphological characters are inconspicuous or even unreliable, molecular methods can provide clarity in delimiting species. In this study, morphological methods (examination under light and scanning electron microscopy) were integrated with molecular analyses (sequencing of Cytochrome c oxidase subunit I, 16S rRNA and Histone H3 gene fragments) to establish the systematic placement of *Polygordius* specimens collected from Australia, Belize, French Polynesia, Indonesia, Japan, and the U.S. west coast. These analyses revealed three new species of *Polygordius* from the Pacific Ocean (*P. erikae* n. sp., *P. kurthcarolae* n. sp., and *P. kurthsusanae* n. sp.) as well as one new species from the Caribbean Sea (*P. jenniferae* n. sp.). These new species are formally described, and a previously known Japanese species, *P. pacificus* Uchida, 1936, is redescribed. This study establishes the first molecular data set for *Polygordius* species from the Pacific region, as well as the first formal description of a Caribbean species of *Polygordius*. Phylogenetic relationships within *Polygordius* are summarized and discussed.

Keywords: integrative taxonomy; meiofauna; interstitial; diversity; distribution; new records

1. Introduction

Polygordius Schneider, 1868 [1], the only genus in Polygordiidae Czerniavsky, 1881 [2], is a clade whose members are all smooth-bodied marine annelids found living in the interstitial spaces of relatively coarse sediments ranging from the intertidal zone to continental slope environments worldwide [3,4]. Their tendency to tie themselves in knots when collected, and the etymological link to the legendary Gordian Knot, led us to coin the common name “knot worms” for this paper. Although 17 species and two subspecies have been described from the Atlantic, Pacific, Indian, and Southern Oceans, only 14 species are considered valid [3] (Table 1), and most of these have been described from the north Atlantic Ocean and Mediterranean Sea [5].

Table 1. Grouping of valid *Polygordius* species according to pygidial morphology based on findings of the present study (updated from [6]). Pygidial appendages/cirri refer to an extension/structure located either subterminally in the region between the glandular belt and anus or terminally from an anal lobe, thereby creating an elongate/enlarged lobe. Terminal pygidial appendages/cirri have also been previously referred to as anal cirri, pygidial papillae, anal papillae, and caudal appendages. Undetermined = description is incomplete or cannot be interpreted with confidence for this character.

Pygidium	Pygidial Appendages/ Cirri Absent	Pygidial Appendages/ Cirri Subterminal	Pygidial Appendages/ Cirri Terminal	Pygidial Appendages Undetermined
Pygidial glands present	<i>P. antarcticus</i> <i>P. lacteus</i> <i>P. neapolitanus</i>	<i>P. appendiculatus</i> <i>P. kiarama</i> <i>P. leo</i> <i>P. jenniferae</i> n. sp. <i>P. kurthcarolae</i> n.sp. <i>P. erikae</i> n.sp.	<i>P. eschaturus</i> <i>P. eschaturus brevipapillosus</i> <i>P. madrasensis</i> <i>P. pacificus</i>	<i>P. pacificus</i> <i>P. floreanensis</i> <i>P. ijimai</i>
Pygidial glands absent	<i>P. arafura</i> <i>P. jouinae</i> <i>P. triestinus</i> <i>P. kurthsusanae</i> n. sp.			
Pygidial glands undetermined	<i>P. uroviridis</i>			

Distinguishing features of *Polygordius* include a rounded or conical prostomium that tapers anteriorly into paired palps of varying length and proximity to one another, which are innervated and function as sensory receptors [7,8]. The prostomium also bears paired dorsolaterally-positioned nuchal organs used for sensory perception [6]. Completing the head is the peristomium, and its division from the prostomium is marked by a deep or shallow “head fold” situated in front of the ventral mouth [7,9]. The trunk shows no signs of external segmentation and is reminiscent of nematodes or ribbon worms (Nemertea), though the septa of the segments can be seen internally with the appropriate lighting. The trunk, which can have 200 or more segments, terminates in a pygidium that may be either inflated or cylindrical, and in many species of *Polygordius* it is encircled by pygidial glands that serve an adhesive function in the sediments [10]. These glands vary in size, shape, and number between different species [7]. Pygidial appendages/cirri may also be present either terminally or subterminally, with the tip of the pygidium forming distinctive anal lobes [4].

Identifying *Polygordius* species based on morphological characters alone can be challenging. Their long, cylindrical bodies appear relatively similar to one another under visual inspection, and the distinguishing features useful for morphology-based discrimination of species are small, requiring examination under scanning electron microscopy [3]. Even annelid species with larger and more conspicuous external features may be difficult to describe based on morphological methods alone [11], and species richness and diversity within Annelida in general and *Polygordius* specifically are likely much greater than is presently documented due to such challenges [10,12]. Molecular data can reveal both synonymous and cryptic species [5] and, combined with morphology-based techniques, provides a powerful tool for delineating species with greater accuracy.

After the first adult *Polygordius* was described, *P. lacteus* Schneider, 1868, the genus spent much of its history misinterpreted as a primitive group owing to a lack of annelid features such as chaetae and parapodia [4]. *Polygordius* was the first clade to be placed in the taxon Archiannelida by Hatschek [13]. He established the group for what he presumed to be the earliest annelid lineages, but Archiannelida was subsequently revealed as an invalid, polyphyletic taxon [3,4,14]. *Polygordius* is now viewed as a derived group that has adapted well to life in the interstitial environment [11,15]. Recent molecular systematic studies relate them most closely to Protodrilida [15], or Phyllodocida [11] as part of Errantia.

Ramey-Balci et al. [5] published the first phylogenetic hypotheses within *Polygordius*, focusing on European and Atlantic species. Their work confirmed six valid species, while synonymizing two, thus clarifying the diversity present in the region of study and providing a foundation for the use of molecular methods to understand evolutionary relationships within *Polygordius*. However, molecular data for *Polygordius* from other regions are lacking, and they have not been established for species outside of the Atlantic Ocean. In this study, we have generated molecular and morphological data for

three new species of *Polygordius* from the Pacific Ocean as well as the first known species from the Caribbean Sea. We provide data for several other specimens that we do not describe here, owing to a lack of complete specimens. We also redescribe *Polygordius pacificus* Uchida, 1935 [16] from Japan. We summarize the evolutionary relationships of these new species in relation to each other and the established species from the Atlantic Ocean.

2. Materials and Methods

2.1. Collection of Specimens

Specimens for all new species described in this study were collected from coarse sediments in locations across the Pacific Ocean and Caribbean Sea including French Polynesia, the east coast of Australia, Belize, and Japan. Specimens were collected over a depth range of 1–18 m and across a time span from 1996 to 2015 (Figure 1).

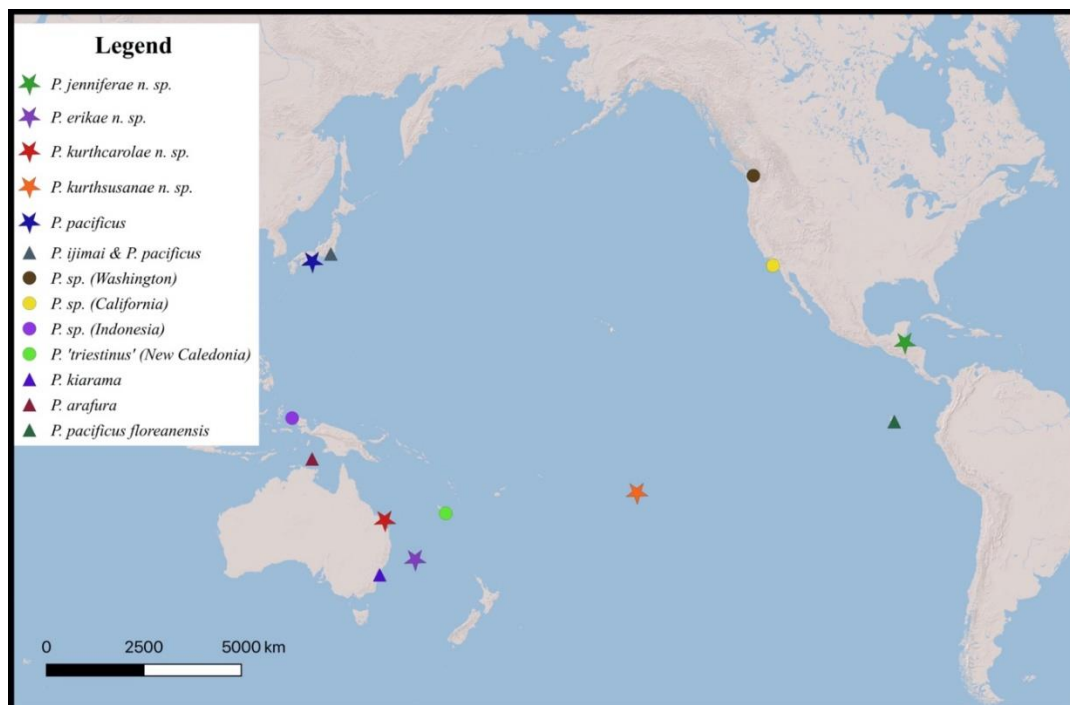


Figure 1. Geographic distribution (based on known collection records) for *Polygordius* species in the Pacific Ocean, Indian Ocean, and Caribbean Sea. Green star, *P. jenniferae* n. sp. (type locality). Purple star, *P. erikae* n. sp. (type locality). Red star, *P. kurthcarolae* n. sp. (type locality). Orange star, *P. kurthsusanae* n. sp. (type locality). Blue star, *P. pacificus* (collection record). Grey triangle, *P. ijimai* and *P. pacificus* (type locality). Brown circle, *Polygordius* sp. (Washington). Yellow circle, *Polygordius* sp. (California). Purple circle, *Polygordius* sp. (Indonesia). Green circle, *P. "triestinus" sensu* Jouin (New Caledonia)—this is an unconfirmed identification given limited information. Purple triangle, *P. kiarama* (type locality). Red triangle, *P. arafura* (type locality). Green triangle, *P. pacificus floreanensis* (type locality).

2.2. List of Museum Abbreviations

AM, Australian Museum, Sydney, Australia; MNHN, Muséum National d'Histoire Naturelle, Paris, France; MV, Museum Victoria, Melbourne, Australia; NTM, Museum and Art Gallery of the Northern Territory, Darwin, Australia; SMF, Senckenberg Museum Frankfurt, Frankfurt, Germany; SIO-BIC, Scripps Institution of Oceanography Benthic Invertebrates Collection, La Jolla, CA, USA; UMUTZ, University Museum, the University of Tokyo, Tokyo, Japan; USNM, National Museum

of Natural History, Smithsonian Institution, Washington DC, USA; YPM, Yale Peabody Museum of Natural History, Yale University, New Haven, Connecticut, USA; ZMB, Zoological Museum of Berlin, Berlin, Germany; ZMH, Zoological Museum Hamburg, Germany; ZUEC, Museu de Zoologia da Universidade Estadual de Campinas “Adão José Cardoso”, Campinas, Brazil.

2.3. Comparative Material Examined

2.3.1. Type Material

Polygordius arafura Avery, Ramey, Wilson 2009 [10]: holotype (NTM W22253), Arafura Sea, Gulf of Carpentaria, Australia, Station SS05/2005 007GR011 (39°50.1' S, 135°17.8' W), 83 m, calcareous poorly-sorted muddy fine sand, collected 4 May 2005; *Polygordius kiarama* Avery, Ramey, Wilson 2009 [10]: holotype (MV F165641), New South Wales, Coniston, S. of Port Kembla, Australia (34°28' S, 150°53' E), collected 3 August 2006; *Polygordius jouinae* Ramey, Fiege and Leander, 2006 [12]: holotype (YPM 38050), north-western Atlantic Ocean, United States, New Jersey, Tuckerton, Beach Haven Ridge, LEO-15 Station 9 (39°27.7' N, 74°15.8' W), ~12 m, coarse sand, collector P.A. Ramey, 19 May 2005; *Polygordius eschaturus brevipapillosus* Jouin and Rao, 1987 [17]: holotype (MNHN 1392) and paratype (MNHN 1164), Indian Ocean, North Andaman Island, south island beach (12°58.10' N, 92°59.28' E), 5 cm below surface, near low water mark, sandy sediment (400–700 mm), collector G.C. Rao, 28 April 1974; *Polygordius pacificus floreanensis* Schmidt and Westheide, 1977 [18]: holotype (ZMH P-13668), South Pacific Ocean, Galapagos Islands, Floreana, black beach, eulittoral, coarse black lava sand, leg. June 1972.

2.3.2. Nontype Material

Polygordius appendiculatus Fraipont, 1887 [19]: Mediterranean Sea, Island of Ischia (Italy), (40°45.1' N, 13°56.0' E), 7.5 m, coarse sand, grab, collector P.A. Ramey-Balci, 3 May 2011. Five specimens and 3 pygidia: SMF 22970, fixed in 70% ethanol, 1 complete specimen, I48; SMF 22248, SEM stub no. 1204b, head-pygidium-2 middle sections, I34; SMF 22244, SEM stub no. 1213, head-pygidium, I17; SMF 22971, SEM stub no. 1214, head-pygidium-middle section, I11; SMF 22972 SEM stub no. 1215, head-pygidium-2 middle sections, I12; SMF 22973, SEM stub no. 1216, 2 pygidia, I54 and I55; SMF 22974, SEM stub no. 1217, 1 pygidium, I22; *Polygordius triestinus* Hempelmann, 1906 [9]: Mediterranean Sea, northern Adriatic Sea, Rovinj (Croatia), Valdibora Harbour (RLV), collector B. Mikac, 20 September 2010. 3 specimens and 1 pygidium: SMF 22978, fixed in 70% ethanol, 1 complete specimen, RLV/2 (45°05.2' N, 13°38.2' E); SMF 22979, fixed in 70% ethanol, 2 complete specimens, SEM stub no. 1196, 1 complete specimen and 1 pygidium RLV/1 (45°05.2' N 13°38.3' E); *Polygordius eschaturus* Du-Bois Reymond Marcus 1948 [20]: South Atlantic Ocean, Praia de Estaleirinho (Estaleirinho beach), Santa Catarina, Brazil (27°3.14' S, 48°35.24' W), collector M. Di Domenico, 10 October 2010. 2 specimens, 1 head, and 2 pygidia: ZUEC 13537, SEM stub no. 1168, head; 13535, SEM stub no. 1186, head-pygidium; 13536, SEM stub no. 1188, pygidium; 13541/13542, SEM stub no. 1172/1173, head-pygidium; 13538, SEM stub no. 1169, pygidium.

2.4. Light and Scanning Electron Microscopy

Specimens of all new species described in this study were examined using light microscopy (Leica S8 Apo stereomicroscope and Canon EOS Rebel T6s camera, using Adobe Photoshop software, version 21.1.0) to measure more prominent morphological features (e.g., body length and width) and scanning electron microscopy (SEM) to examine minute features such as the number, size, and shape of the pygidial glands and anal lobes which are necessary for morphology-based discernment of species. Measurements were taken as follows: total length, from the base of the palps to the tip of the pygidium where it terminates in the anal lobes; prostomium length, which is the distance from the base of the palps to the top of the head fold (ventral transverse groove separating the prostomium and peristomium); body and pygidial width, measured at the widest section of the trunk and pygidium (at

pygidial inflation) respectively; “glandular belt width”, is the width of the ring of glands encircling the pygidium; gland width, measured in the middle region of several well-fixed glands; and the length of “elongate/enlarged anal lobes”, which is the distance from the base of the anal lobe to the tip of the elongate/enlarged extension (= lobe + pygidial cirrus). In order to determine whether the prostomium is “conical” or “rounded” the fixed specimen must be lying relatively flat, ventral-side-up or laterally. If the prostomium length is > its width, it is considered “conical”, whereas, if the prostomium width \geq its length, it is “rounded”. Specimens were prepared for SEM examination using a procedure adapted from the air-drying technique outlined by Murtey and Ramasamy [21]. Specimens were first moved from their respective preservatives (either ethanol or formalin) to water, then postfixed in osmium tetroxide for 80 min. Specimens were washed in water 4–5 times at 60-minute intervals followed by overnight immersion. After washing, specimens were dehydrated through a graded series of ethanol and hexamethyldisilazane (HMDS), beginning with immersion in 70% ethanol for 20 min, followed by two changes of 95% ethanol at 20-minute intervals, and two changes of 100% ethanol at 20-minute intervals. After immersion in pure ethanol, 50% HDMS was added to each specimen for a 20-minute interval, then 75% HDMS for a 20-minute interval, and finally three changes of 100% HDMS at 20-minute intervals. Specimens were allowed to dry overnight before being mounted on aluminum stubs, sputter-coated with gold-palladium (Au-Pd), and examined under a Zeiss EVO 10 scanning electron microscope.

2.5. DNA Extraction, Amplification, and Sequencing

DNA was extracted from samples of each specimen’s trunk tissue using the Zymo Research DNA-Tissue Miniprep Kit. All successful extractions were completed using the protocol outlined by the manufacturer. Two mitochondrial genes were then amplified and sequenced for the majority of specimens in this study: the 16S rRNA gene (16S) and cytochrome c oxidase subunit I (COI). The nuclear gene Histone H3 (H3) was also amplified and sequenced in order to compare specimens with more fragmented DNA (Table 2). The polymerase chain reaction (PCR) was used to amplify a section of each target gene in a 25 μ L solution of H₂O (8.5 μ L), taq DNA polymerase (12.5 μ L), primer sets (1 μ L each), and template DNA (2 μ L). (In some cases, volumes were adjusted based on the concentration of DNA in the extraction.) The following primer sets were used:

16S:

Ar1 5'-CGCCTGTTTATCAAAAACAT-3';
brH 5'- CCGGTCTGAACTCAGATCACGT-3' [22];

COI:

LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3';
HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' [23];
polyLCO 5'-GAYTATWTTCAACAAATCATAAAGATATTGG-3';
polyHCO 5'-TAMACTTCWGGGTGACCAARAATCA-3' [24];
Polyg347F 5'-CTGTGCCCACTCCTTTTTCT-3';
Polyg546R 5'- TACCGCTCATGCATTCCTAA-3' (this study);

H3:

H3F 5'-ATGGCTCGTACCAAGCAGACVGC-3';
H3R 5'-AT ATCCTTRGGCATRATRGTGAC-3' [25].

Table 2. Species names, localities, voucher information, and GenBank accession numbers for specimens used in this study. Museum abbreviations: see materials and methods. Names of new species described in this study appear in bold italics. Accession numbers of new sequences submitted to GenBank during this study appear in bold. COI = Cytochrome Oxidase Subunit I. Blank = no sequence.

Taxa	Location	Voucher	COI	16S rRNA	Histone H3
<i>Protodrilus pythionius</i>	Brazil	(Di Domenico et al.) [26]	KC763840	KJ451298	
<i>Polygordius appendiculatus</i>	Italy	(Ramey et al. 2018) [5]	MG603453	MG603479	
<i>Polygordius eschaturus</i>	Brazil	(Ramey et al. 2018) [5]	MG603404	MG603559	
<i>Polygordius jouinae</i>	USA, New Jersey	(Ramey et al. 2018) [5]	MG603410	MG603509	
<i>Polygordius lacteus</i>	Germany	(Ramey et al. 2018) [5], (Rousset et al. 2007) [27]	KT883951	MG603540	DQ779757
<i>Polygordius neapolitanus</i>	Italy	(Ramey et al. 2018) [5]	MG603433	MG603521	
<i>Polygordius triestinus</i>	Croatia	(Ramey et al. 2018) [5]		MG603537	
<i>Polygordius erikae</i> n. sp.	Australia	AM W.53108, A12259	MT263739-740	MT260195-196	
<i>Polygordius jenniferae</i> n. sp.	Belize	SIO-BIC A12260	MT263733	MT260193	
<i>Polygordius kurthcarolae</i> n. sp.	Australia	AM W.53109, SIO-BIC A12261, USNM1434789	MT263735-6,8	MT260191-192	MT263747
<i>Polygordius kurthsusanae</i> n. sp.	French Polynesia	USNM1432890, SIO-BIC A12287, A12289	MT263742-744	MT260187-189	
<i>Polygordius pacificus</i>	Japan	SIO-BIC A12402, A12265-6, A12284-6	MT263741	MT260197-201	MT263748-749
<i>Polygordius</i> sp.	Indonesia	SIO-BIC A4058	MT263745	MT260202	
<i>Polygordius</i> sp.	USA, California	SIO-BIC A12290	MT263734	MT260194	
<i>Polygordius</i> sp.	USA, Washington	SIO-BIC A11847	MT263746	KF511815	KF511879

The following cycling profiles were used for 16S amplification: (1) initial denaturation at 95 °C (3 min), followed by 40 cycles of denaturation at 95 °C (40 s), annealing at 50 °C (40 s), elongation at 72 °C (50 s), and final extension at 72 °C (5 min); (2) initial denaturation at 95 °C (3 minutes), followed by 35 cycles of denaturation at 95 °C (40 s), annealing at 55 °C (40 s), elongation at 68 °C (50 s), and final extension at 72 °C (5 min). For COI amplification, cycling profiles used were as follows: (1) initial denaturation at 95 °C (3 min), followed by 40 cycles of denaturation at 95 °C (40 s), annealing at 42 °C (45 s), elongation at 72 °C (50 s), and final extension at 72 °C (5 min); (2) initial denaturation at 94 °C (3 min), followed by 5 cycles of denaturation at 94 °C (30 s), annealing at 47 °C (45 s), elongation at 72 °C (60 s), then 30 cycles of denaturation at 94 °C (30 s), annealing at 52 °C (45 s), elongation at 72 °C (60 s), and final extension at 72 °C (5 min); (3) initial denaturation at 95 °C (3 min), followed by 40 cycles of denaturation at 95 °C (40 s), annealing at 49 °C (40 s), elongation at 72 °C (50 s), and final extension at 72 °C (5 min). Finally, for H3 amplification, the following profile was used: initial denaturation at 95 °C (3 min), followed by 40 cycles of denaturation at 95 °C (30 s), annealing at 53 °C (45 s), elongation at 72 °C (45 s), and final extension at 72 °C (5 min). Successful PCR products were purified using the ExoSAP-IT protocol (USB, Affymetrix), sequencing was conducted by Eurofins Genomics (Louisville, KY), and sequences were then assembled and edited using Geneious software (version 11.1.5).

2.6. Molecular Methods and Analysis

Sequences from previously published *Polygordius* species were obtained from GenBank (Table 2). Where sequences were available from multiple regions, then ones from nearest the type locality were chosen. Sequences were aligned using MAFFT [28] and then concatenated using SequenceMatrix v.1.8 [29]. A maximum likelihood (ML) analysis was performed on this data set using RAxML v.8.1.22 [30], partitioned by gene (and codon for COI), under the model GTR+G. Node support was assessed via the thorough bootstrapping (with 1000 pseudoreplicates). The protodrilid *Protodrilus pythionius* Di Domenico, Martínez, Lana and Worsaae, 2013 [26] was used as an outgroup, based on recent phylogenetic results [11,15]. Haplotype networks were generated for the COI data obtained for three of the new species using the TCS algorithm [31] in PopArt [32]. Uncorrected pairwise distances for COI were calculated using PAUP* [33].

3. Results and Discussion

Species Delimitation and Phylogeny

Table 3 shows uncorrected pairwise distances for COI sequences for available data on GenBank for five known *Polygordius* species (from type localities) and the new samples for this study. Most

distances among species are minimally 20% or more. The minimum distance found for any previously known species was between *P. lacteus* and *P. neapolitanus* Fraipont, 1887 [19] (15%). With respect to the new data, the undescribed *Polygordius* sp. from southern California was found to be 13% divergent from *P. jenniferae* n. sp. from Belize. *Polygordius* sp. from Indonesia was 17% divergent from *P. jouinae* (NE United States). *Polygordius* sp. from Washington was closest in distance and phylogeny (Figure 2) to a clade of three species from Germany and Italy, with *P. lacteus* being closest in distance (19%). *Polygordius eschaturus* from Brazil was 19% divergent from *P. pacificus* (Japan). The Pacific clade of three new *Polygordius* species from Australia and French Polynesia (*P. erikae* n. sp., *P. kurthcarolae* n. sp. and *P. kurthsusanae* n. sp.) were all at least 20% divergent from each other. These distances support the recognition of eight new species level taxa in *Polygordius*. One of these is recognized here as the known species *P. pacificus* and redescribed below. Sequences for *Polygordius* sp. (California), *Polygordius* sp. (Indonesia), and *Polygordius* sp. (Washington) were each obtained from incomplete individuals that were lacking pygidia, so these are not formally described here. The other species did have at least one complete specimen and so these are formally described below.

For the new species with multiple individuals, *P. erikae* n. sp., *P. kurthcarolae* n. sp., and *P. kurthsusanae* n. sp., haplotype networks for COI were made and these are shown in Figure 2. The two sequences for *P. erikae* n. sp. were both from the same locality (Lord Howe Island, NSW, Australia) but were 2% divergent from each other. We do not consider this to be enough to separate them, since this distance, while marked for specimens from the same locality, is within what is “normal” annelid intraspecific variation in annelids [34]. The maximum distance among the four COI sequences for *P. kurthsusanae* n. sp., all in one sand sample from Moorea, French Polynesia was also relatively high, for intraspecific distance, at 2.5%. *Polygordius kurthcarolae* n. sp. has a type locality on the Great Barrier Reef (Australia) and the two COI sequences from the type locality were identical. A *Polygordius* from Tetiaroa Atoll, French Polynesia, was 2% divergent from these Australian sequences, so to be consistent, we regard this specimen to be *P. kurthcarolae* n. sp., even over 6000 km away.

The maximum likelihood phylogeny based on the three gene fragments (mostly missing H3) is shown in Figure 2. The outgroup *Protodrilus pythonius* is not shown to save space, but the root position is based on its inclusion. A well-supported clade comprising *P. triestinus* (Croatia), *P. jouinae* (NE United States), and *Polygordius* sp. (Indonesia) was recovered as the sister group to the remaining *Polygordius*, but support was low. Within this major clade, *P. eschaturus* (Brazil) was the well supported sister group to *P. pacificus* (Japan) and this clade was then the sister group to the remaining *Polygordius*, which had two main subclades. One comprised the remaining European species from Germany and Italy, *P. appendiculatus*, *P. lacteus*, and *P. neapolitanus*, which formed a clade that was sister group to *Polygordius* sp. (Washington). The remaining clade of *Polygordius* was well-supported and comprised mainly Pacific terminals, including the three new Pacific species (*P. erikae* n. sp., *P. kurthcarolae* n. sp., and *P. kurthsusanae* n. sp.). These formed a poorly supported clade, though there was strong support for *P. kurthcarolae* n. sp. and *P. kurthsusanae* n. sp. as sister taxa. The new species from Belize (*P. jenniferae* n. sp.) was the well-supported sister group to *Polygordius* sp. (California). Overall, the tree had only a few poorly supported nodes, so the lack of any clear biogeographic patterns is somewhat surprising. The Atlantic/European/Mediterranean terminals were placed in three regions of the tree and none were close to the Caribbean *P. jenniferae* n. sp., which showed fairly low COI divergence from *Polygordius* sp. (California). The Australian/French Polynesian species did form a discrete clade. The phylogeny lacks sequence data for many of the known *Polygordius* species and further sequence data may make for more comprehensible biogeographical patterns. This paper includes the first data for Pacific and Caribbean *Polygordius* after the initial molecular study that focused on European/Atlantic region [5]. Obtaining sequences for *Polygordius* known from regions such as Antarctica and the Indian Ocean will be valuable for continuing phylogenetic studies.

Table 3. Uncorrected interspecific distances for *Polygordius* taxa used in this study based on COI data. Sequences for holotypes are used for the new species and specimens from type localities are used for previously published data (see Table 2). Distances in bold are discussed in the text.

Taxa	<i>lacteus</i>	<i>appendiculatus</i>	<i>neapolitanus</i>	<i>jouinae</i>	<i>eschaturus</i>	Washington	California	<i>pacificus</i>	Indonesia	<i>erikae</i>	<i>jenniferae</i>	<i>kurthcarolae</i>
<i>P. lacteus</i>	-											
<i>P. appendiculatus</i>	0.18	-										
<i>P. neapolitanus</i>	0.15	0.20	-									
<i>P. jouinae</i>	0.22	0.21	0.25	-								
<i>P. eschaturus</i>	0.23	0.21	0.22	0.22	-							
<i>P. Washington</i>	0.19	0.23	0.23	0.24	0.21	-						
<i>P. California</i>	0.23	0.20	0.22	0.24	0.25	0.24	-					
<i>P. pacificus</i>	0.22	0.20	0.20	0.23	0.19	0.23	0.21	-				
<i>P. Indonesia</i>	0.24	0.21	0.23	0.17	0.22	0.22	0.23	0.23	-			
<i>P. erikae</i> n. sp.	0.24	0.22	0.23	0.23	0.21	0.23	0.19	0.21	0.24	-		
<i>P. jenniferae</i> n. sp.	0.21	0.20	0.22	0.22	0.23	0.24	0.13	0.22	0.21	0.20	-	
<i>P. kurthcarolae</i> n. sp.	0.24	0.23	0.24	0.25	0.25	0.24	0.20	0.22	0.24	0.22	0.20	-
<i>P. kurthsusanae</i> n. sp.	0.24	0.25	0.26	0.24	0.23	0.26	0.23	0.26	0.24	0.22	0.21	0.23

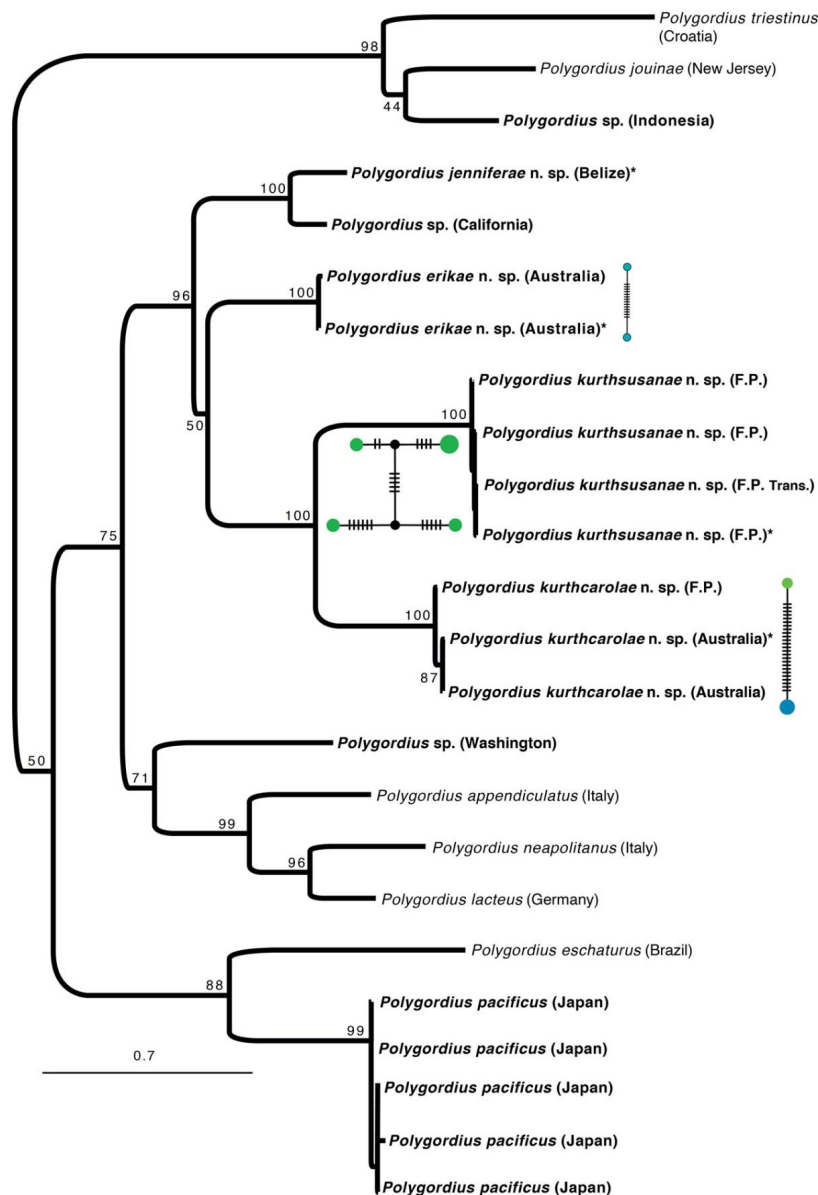


Figure 2. Maximum likelihood phylogeny obtained with the molecular combined data (COI, 16S, and H3) and with the protodrilid *Protodrilus pythonius* as outgroup (not shown). Values at nodes are shown as ML bootstrap values. Haplotype networks for COI data for three of the new species are shown next to *P. erikae* n. sp., *P. kurthcarolae* n. sp., and *P. kurthsusanae* n. sp. respectively. F.P. = French Polynesia and * = holotype.

4. Taxonomy

POLYGORDIIDAE Czerniavsky, 1881 [2]

Polygordius, Schneider, 1868 [1]

4.1. *Polygordius jenniferae* n. sp.

4.1.1. Material

Holotype (SIO-BIC A12260): complete adult (head/pygidium sections): on SEM stub (previously fixed in ethanol), molecular COI and 16S sequences are available at GenBank (MT263733, MT260193), North Atlantic Ocean, Caribbean Sea, off Carrie Bow Cay, Belize (16°48'6.08" N; 88°4'47.00" W), 18 m water depth in coarse sandy sediments, collector G. Rouse & N. Tuttle, 7 September 2015.

4.1.2. Etymology

Polygordius jenniferae n. sp. is named after the lead author's sister.

4.1.3. Description

Body measuring 48.4 mm in length and 0.47 mm wide (Figure 3A). Number of segments ~145. Prostomium conical with blunt tip (length 0.18 mm) and paired palps (length 0.18 mm), (Figure 4A). Length ratio of palp to prostomium 1:1 (Figures 3B and 4A). Pigment spots/"eyespot" absent. Head fold deep (Figure 4B). Pair of oval, nuchal organs positioned laterally at posterior margin of prostomium (Figure 4A). Ventrally positioned mouth with dorso-median fold that is subtriangular with deep cleft (Figure 4B). Pygidium inflated (width 0.28 mm), (Figures 3C and 4C,D). Pygidial glands present, ~40–54 elongate glands positioned close together longitudinally along body creating a thick glandular belt (width 30 μ m), (Figure 4D). Ratio of gland length to width ~3:1 (length 30 μ m; width 10–15 μ m) (Figure 4D). Pygidial appendages present. Three pygidial cirri attached subterminally between the glandular belt and anus including one dorsal median and two ventro-lateral (Figure 4C,D). Anus terminal with 6–12 anal lobes (Figure 4C). Dimensions of holotype are given in Table 4.

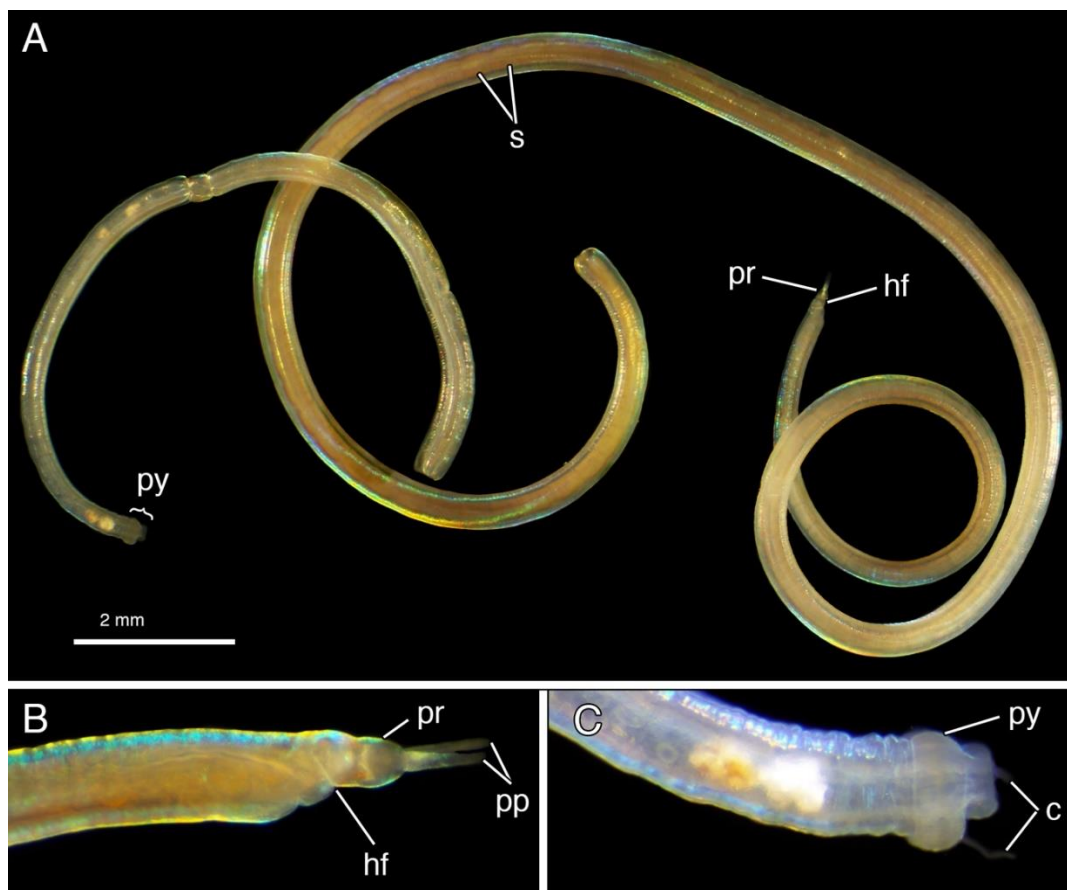


Figure 3. Light microscopy (LM) images of *Polygordius jenniferae* n. sp. holotype (SIO-BIC A12260), collected from Belize: (A) Live, complete adult specimen in two pieces. (B) Live, lateral view of head. (C) View of pygidium, photographed after fixation in ethanol. Abbreviations: c, cirri; hf, head fold; pp, paired palps; pr, prostomium; py, pygidium; s, segments.

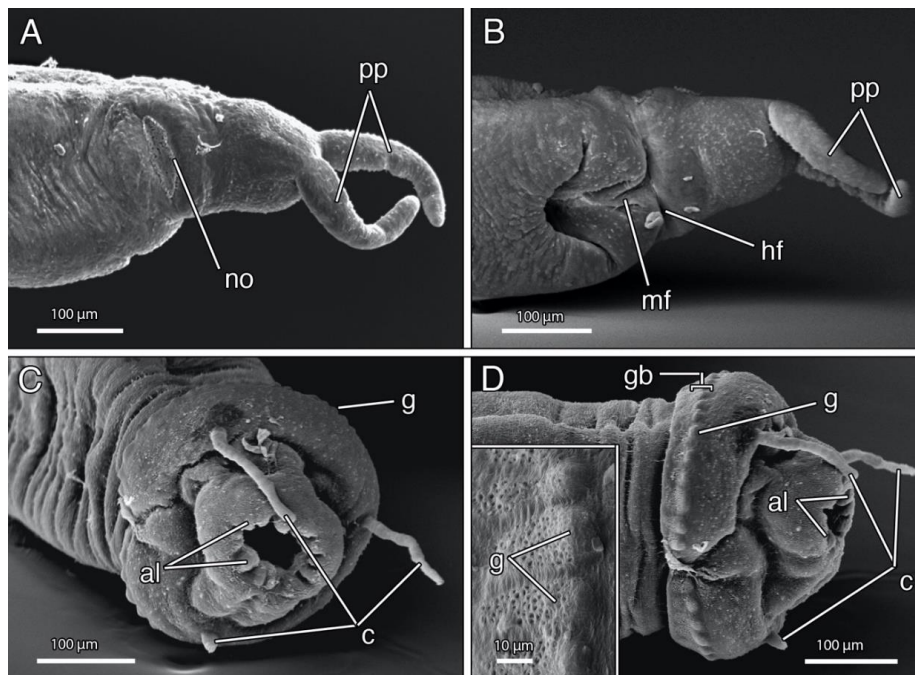


Figure 4. Scanning electron microscopy (SEM) images of *Polygordius jenniferae* n. sp. holotype (SIO-BIC A12260), collected from Belize, showing details of morphological characters: (A) Lateral view of head. (B) Ventral view of head. (C) Posterior view, tip of pygidium. (D) Lateral view of pygidium. (D-inset) Magnified view of pygidial glands. Abbreviations: al, anal lobes; c, cirri; g, gland; gb, glandular belt; hf, head fold; mf, median fold; no, nuchal organ, pp, paired palps.

4.1.4. Remarks

Polygordius jenniferae n. sp. (Caribbean Sea) is distinguished from most other *Polygordius* by having pygidial glands and subterminal pygidial appendages (Table 1). *Polygordius jenniferae* n. sp. is most closely related to *Polygordius* sp. (California), which is 13% divergent (Table 3), making these two species well-supported sister groups. Unfortunately, the *Polygordius* sp. (California) lacked a posterior end so could not be compared with *P. jenniferae* n. sp. in this sense. Morphologically, the most similar species to *P. jenniferae* n. sp. include *P. kiarama* from Australia and *P. appendiculatus* from the Mediterranean Sea (Tables 3 and 4). Although it cannot be determined unequivocally that the pygidial appendages of *P. ijimai* (Misaki, Japan) are subterminal (vs. terminal), they are described as being “filiform”, which is consistent with the appearance of subterminal pygidial cirri, and thus we also consider *P. ijimai* as similar to the new species (Table 4) [7,10]. *Polygordius jenniferae* n. sp. can be distinguished from these three species in having ~40–54 elongate pygidial glands, 3 pygidial cirri including two inserted ventro-laterally and one dorsal-median, 6–12 anal lobes, and a conical/blunt (not pointed) prostomium (Table 4). *Polygordius kiarama* has ~20 oval pygidial glands, 2 ventro-lateral pygidial cirri, and 7–8 anal lobes. *Polygordius appendiculatus* has ~30 round glands, 2 pygidial cirri positioned laterally, 5 anal lobes, a rounded prostomium with a palp: prostomium length ratio of 2:1 (vs. 1:1 for *P. jenniferae* n. sp.), and pigment/“eyespot” which are absent in the new species. *Polygordius ijimai* has 8 anal lobes, longer palps (1 mm vs. 0.18 mm), and is a relatively larger species (70–77 mm × 0.60–0.80 mm vs. 48 mm × 0.47 mm), (Table 4). Of the new species described herein, also having pygidial glands and subterminal pygidial appendages, *P. erikae* n. sp. (southeastern Australia) can be distinguished from *P. jenniferae* n. sp. in having ~40–52 oval glands, 2 ventro-lateral cirri, 5 anal lobes, and a rounded prostomium, and *P. kurthcarolae* n. sp. (northeastern Australia) has ~54–60 round glands, 2 ventro-lateral cirri, and 7–8 anal lobes. The genetic distance matrix indicated that the divergence value between *P. jenniferae* n. sp. and either of these new species is 20% (Table 3).

Table 4. Characters of *P. jenniferae* n. sp., *P. kurthcarolae* n. sp., and *P. erikae* n. sp. with morphologically most similar species of the genus. Morphological information for *P. appendiculatus*, *P. kiarama*, and *P. ijimai* was taken from the original species descriptions unless otherwise indicated. Brackets {}= paratype material.

Character/Species	<i>P. jenniferae</i> n. sp	<i>P. kurthcarolae</i> n. sp	<i>P. erikae</i> n. sp	<i>P. appendiculatus</i> Fraipont, 1887 [19]	<i>P. kiarama</i> Avery, Ramey, Wilson, 2009 [10]	<i>P. ijimai</i> Izuka, 1903 [35]
Prostomium shape	conical (blunt)	rounded	rounded	rounded (F, RC)	conical (blunt)	n.d.
Palp length (mm)	0.18	0.14 {0.25}	0.16 {0.11}	0.24–0.41(RC)	0.10–0.22	1.0
Prostomium length (mm)	0.18	0.15 {0.21}	0.13 {0.11}	n.d.	0.08–0.14	n.d.
Ratio (palp:prostomium)	1:1	~1:1	1:1	2:1 (F), >1:1 (RC)	1.2–2.0:1	n.d.
Pigment spots (eyespot)	absent	absent	absent	2; 1or 2 (RC)	absent	absent
Head fold	deep	deep	deep		deep	n.d.
Median fold shape	subtriangular with deep-cleft	subtriangular with deep-cleft	n.d.	n.d.	n.d.	
Pygidium shape/width (mm)	inflated/0.28	inflated/n.d. {0.35}	inflated/0.17	inflated/n.d.	inflated/0.12–0.40	inflated/n.d.
Pygidial glands (no)	40–54	n.d. {54–60}	40–52 {n.d.}	30 (RC)	20	n.d.
Pygidial gland shape/orientation	elongate/longitudinal	n.d. {round/n.a}	oval/longitudinal	round (F, RC)/ n.a.	oval/longitudinal	elongate/n.d.
Glandular belt (width) (µm)	30	n.d. {10–13}	17	n.d.	n.d.	n.d.
Gland (width) (µm)	10–15	n.d. {10–13}	10	n.d.		n.d.
Gland ratio length:width	3:1	n.d. {1:1}	1.7:1	1:1	2:1	n.d.
Subterminal pygidial cirri (no)	3	n.d. {2}	2	2	2	3
Subterminal pygidial cirri insertion point	2 ventro-lateral, 1 dorsal median	ventro-lateral	ventro-lateral	lateral	ventro-lateral	2 ventro-lateral, 1 dorsal median
Anal lobes (no.)	6–12	n.d. {7–8}	5	5;5 (RC)	7–8	8
Body length (mm)	48.4	n.d. {42.3}	21.4 {15.9}	max 20;20–45 (RC)	3.8–11.6	70–77
Body width (mm)	0.47	n.d. {0.50}	0.21 {0.18}	0.25;0.12–0.31 (RC)	0.3–0.28	0.6–0.8
Segment (no.)	145	n.d. {158}	56 {n.d.}	95–125	30–74	n.d.
Type locality	off Carrie Bow Cay, Caribbean Sea, Belize	One Tree Island Reef, Coral Sea, Northeastern Australia	Lord Howe, Island, Tasman Sea, Southwestern Australia	Mediterranean Sea, Gulf of Naples, North Atlantic	S of Port Kembla, Southeastern Australia	Misaki Mar. Biol. Lab., Japan
Region	North Atlantic	South Pacific	South Pacific	North Atlantic	South Pacific	North Pacific
Latitude	16°48'6.08" N	23°29'54.50" S	31°31'0.47" S		35°28'0" S	
Longitude	88°4'47.00" W	152°3'53.82" E	159°3'49.67" E		150°53'0" E	n.d.
Sediment type	coarse sand	coral rubble, gravel	n.d.	coarse sand	sandy, well sorted	sand, pebbles, shell-hash
Depth range (m)	18	5	3–5	n.d.	21–1650	lower intertidal

(RC) = for data taken from Rota and Carchini (1999) [7]; (F) = observed character based on Figure only, no = number; max = maximum, n.d. = no data.

4.2. *Polygordius erikae* n. sp.

4.2.1. Material

Holotype (AM W.53108): complete adult (head/pygidium sections): on SEM stub (previously fixed in ethanol), with molecular COI and 16S sequences available at GenBank (MT263740, MT260195), Southwest Pacific Ocean, Little Island off of Ned’s Beach, Lord Howe Island, New South Wales, Australia (31°31′0.48″ S, 159°3′49.68″ E), 3–5 m water depth, collector G. Rouse & N. Wilson, 8 April 2011. **Paratype** (SIO-BIC A12259): complete adult (head/pygidium sections): ethanol fixed, with molecular COI and 16S sequences available at GenBank (MT263739, MT260196), collected in the same location as holotype, collector G. Rouse & N. Wilson, 3 April 2011.

4.2.2. Etymology

Polygordius erikae n. sp. is named after the lead author’s sister.

4.2.3. Description

Body 15.9–21.4 mm in length and 0.18–0.21 mm wide (Figure 5A). Number of segments ~56. Prostomium rounded (length 0.11–0.13 mm) with paired palps 0.11–0.16 mm in length, (Figure 6A). Length ratio of palp to prostomium 1:1 (Figures 5B and 6A). Pigment spots/“eyespot” absent. Head fold deep. Pair of oval, nuchal organs positioned laterally at posterior margin of prostomium (Figure 6A). Ventrally positioned mouth. Pygidium inflated (width 0.17 mm), (Figure 5A,C and Figure 6B,C). Pygidial glands present, ~40–52 oval glands positioned close together encircling the pygidium (width 17 µm), (Figure 6B–D). Ratio of gland length to width ~1.7:1 (length 17 µm; width 10 µm) (Figure 6D). Pygidial appendages present. Two pygidial cirri attached subterminally between the glandular belt and anus positioned ventro-laterally (Figures 5C and 6B,C). Anus terminal with 5 anal lobes (Figure 6B). Dimensions of holotype given in Table 4.

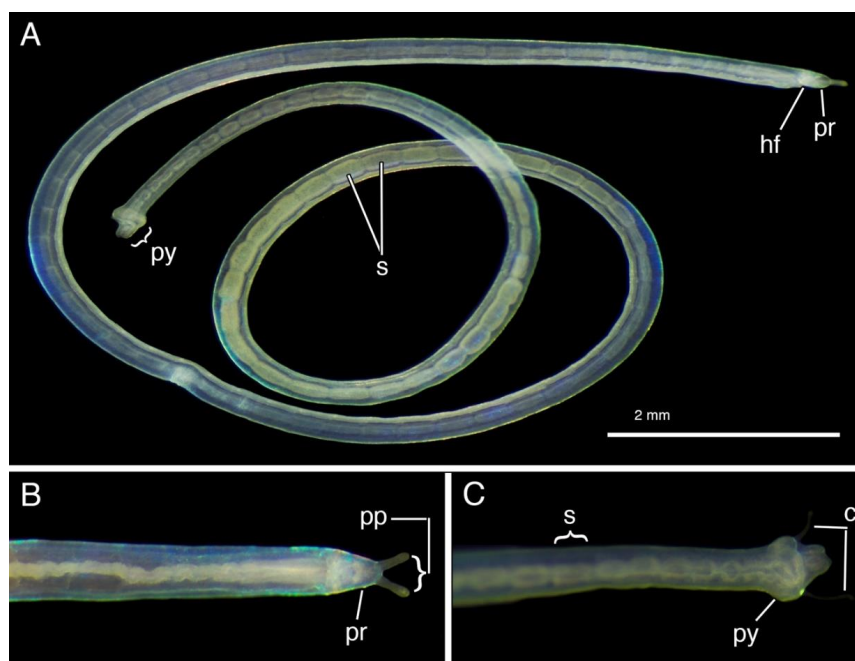


Figure 5. Light microscopy (LM) images of *Polygordius erikae* n. sp. holotype (AM W.53108), collected from Lord Howe Island, Australia, photographed alive before fixation in ethanol: (A) Complete adult specimen including head and pygidium. (B) Dorsal view of head. (C) View of pygidium. Abbreviations: c, cirri; hf, head fold; pp, paired palps; pr, prostomium; py, pygidium; s, segments.

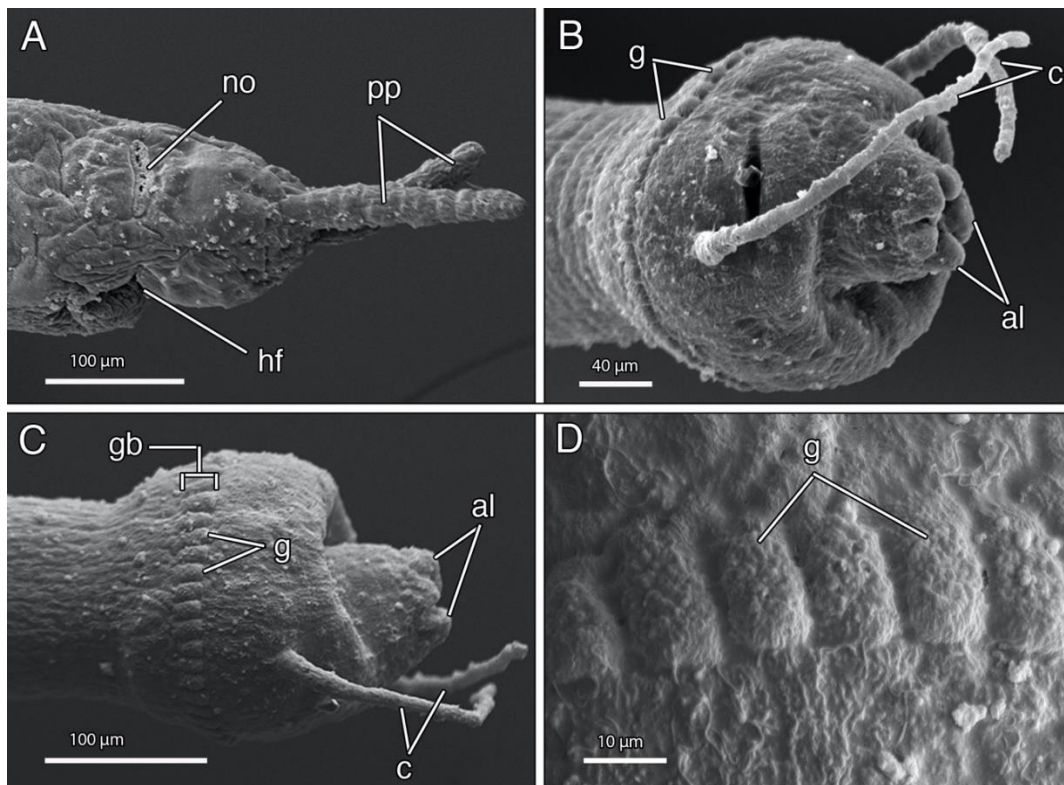


Figure 6. Scanning electron microscopy (SEM) images of *Polygordius erikae* n. sp. holotype (AM W.53108), collected from Lord Howe Island, Australia, showing details of morphological characters: (A) Lateral view of head. (B) Partial posterior view of pygidium showing anal lobes. (C) Lateral view of pygidium. (D) Magnified view of pygidial glands. Abbreviations: al, anal lobes; c, cirri; g, gland; gb, glandular belt; hf, head fold; no, nuchal organ, pp, paired palps.

4.2.4. Remarks

The new species from Australia and French Polynesia (*P. erikae* n. sp., *P. kurthcarolae* n. sp. and *P. kurthsusanae* n. sp.) formed a distinct clade (Figure 2), within which each species was distinguished by at least 20% divergence from the others (Table 3). *Polygordius erikae* n. sp. is not markedly similar to either *P. kurthcarolae* n. sp. or *P. kurthsusanae* n. sp. but its pygidial glands and subterminal pygidial cirri resembles *P. appendiculatus*, *P. ijimai* (see remarks of *P. jenniferae* n. sp., discussing interpretation of morphological characters for this species), and *P. kiarama* (Tables 3 and 4). It can be clearly distinguished from these species by the following characteristics including ~40–52 oval glands, 2 ventro-lateral cirri, 5 anal lobes, and a rounded prostomium (Table 4). In comparison, *P. appendiculatus* is easily distinguished from the new species in that it has ~30 round glands, a palp: prostomium length ratio of 2:1, and pigment/“eyespot” (Table 4). *Polygordius ijimai* has three pygidial cirri including two inserted ventro-laterally and one dorsal-median, whereas, *P. erikae* n. sp. has two ventral-lateral cirri (Table 4) *Polygordius erikae* n. sp. is most similar to *P. kiarama*, for which there is unfortunately no DNA data available. Although both species have oval pygidial glands, there are fewer in number for *P. kiarama* (20 vs. 40–52), which also has 7–8 anal lobes compared to 5 for *P. erikae* n. sp. The prostomium of *P. kiarama* is conical and blunt (not pointed), whereas it is rounded in *P. erikae*. (Table 4).

4.3. *Polygordius kurthcarolae* n. sp.

4.3.1. Material

Holotype (AM W.53109): head section: on SEM stub (previously fixed in ethanol), with molecular CO1 and H3 sequences available at GenBank (MT263736, MT263747), Pacific Ocean, off One Tree

Island, at entrance to 2nd Lagoon, Great Barrier Reef, Australia (23°29′54.50″ S, 152°3′53.82″ E), 5 m water depth in coral rubble and gravel, G. Rouse, 18 November 1996. **Paratypes:** complete adult (SIO-BIC A12263), head/pygidium sections on SEM stub (previously fixed in formalin), collected in the same sample/location/date as holotype; piece of body near pygidium (SIO-BIC A12261), on SEM stub (previously fixed in ethanol), with molecular CO1 and 16S sequences available at GenBank (MT263735, MT260191), collected in the same sample/location/date as holotype. **Non-type:** Anterior end (USNM 1434789): fixed in ethanol, DNA extraction located at USNM, with molecular CO1 and 16S sequences available at GenBank (MT263738, MT260192), South Pacific Ocean, Tetiaroa Atoll, Society Islands, north of Vaiare Pass, French Polynesia (17°30′15.84″S, 149°45′28.8″ W, 7 m water depth in coral rubble, 6 February 2012.

4.3.2. Etymology

Polygordius kurthcarolae n. sp. is named to commemorate Carol Marie Kurth in honor of her wedding. We celebrate her tying the knot with a new knot worm.

4.3.3. Description

Body 42.3 mm in length and 0.50 mm wide (Figures 7A and 8A). Number of segments ~158. Prostomium rounded (length 0.15–0.21 mm) with paired palps 0.14–0.25 mm in length, (Figure 8A,B and Figure 9A–C). Length ratio of palp to prostomium 1:1. Pigment spots/“eyespot” absent. Head fold deep (Figure 9A–C). Pair of oval, nuchal organs positioned laterally at posterior margin of prostomium (Figure 9A,B). Ventrally positioned mouth with dorso-median fold that is subtriangular with deep cleft (Figure 9C). Pygidium inflated (width 0.35 mm), (Figure 8A,C and Figure 9D,E). Pygidial glands present, ~54–60 round glands encircling the pygidium (width 10–13µm), (Figure 9D–F). Pygidial appendages present. Two pygidial cirri attached subterminally between the glandular belt and anus positioned ventro-laterally (Figures 8A and 9D–E). Anus terminal with 7–8 anal lobes (Figure 9D). Dimensions of holotype given in Table 4.

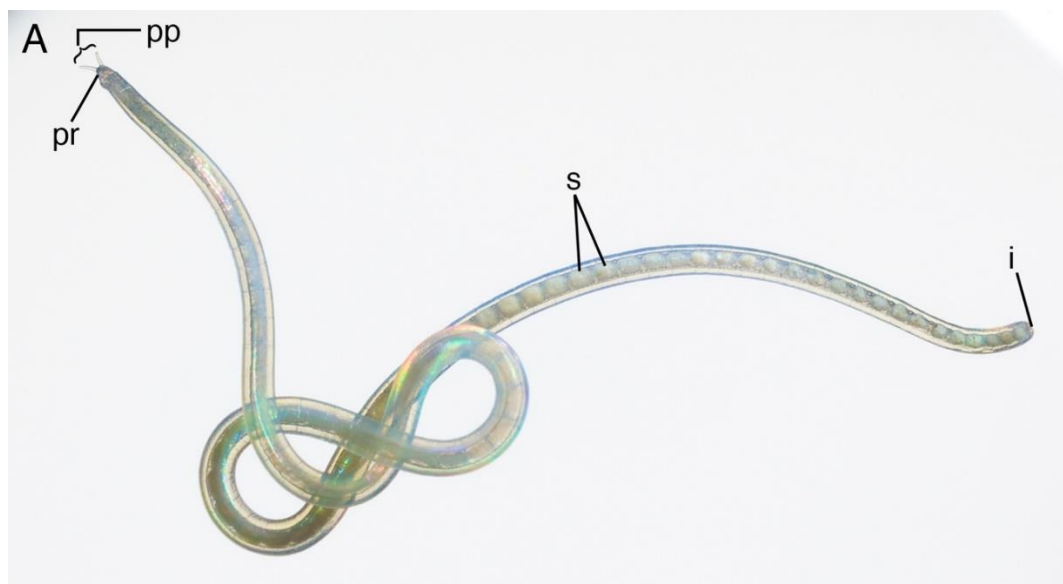


Figure 7. Light microscopy (LM) image of *Polygordius kurthcarolae* n. sp. (USNM 1434789), collected from Tetiaroa Atoll, French Polynesia, photographed by David Liittschwager, photographed alive before fixation in ethanol: (A) Incomplete adult specimen including head and trunk but missing pygidium. Abbreviations: i, incomplete; pp, paired palps; pr, prostomium; s, segments.

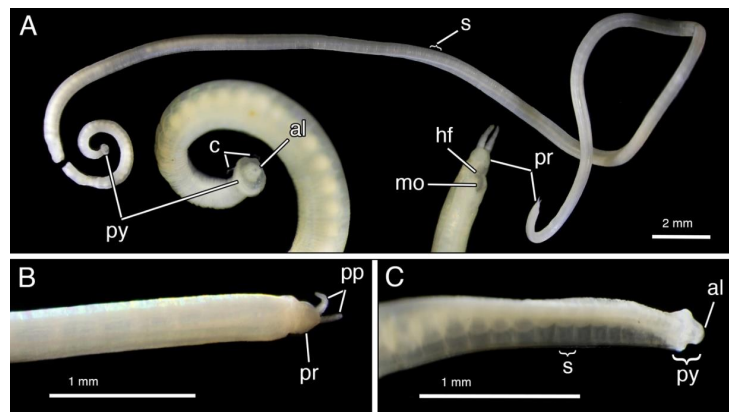


Figure 8. Light microscopy (LM) images of *Polygordius kurthcarolae* n. sp. specimens, collected from One Tree Island, Australia, photographed after fixation in formalin (A12263) and after fixation in ethanol (W.53109, A12261): (A) SIO-BIC A12263, complete adult specimen including head and pygidium (in two pieces). (B) AM W.53109 (holotype), lateral view of head. (C) SIO-BIC A12261, view of pygidium. Abbreviations: al, anal lobes; c, cirri; hf, head fold; mo, mouth; pp, paired palps; pr, prostomium; py, pygidium; s, segments.

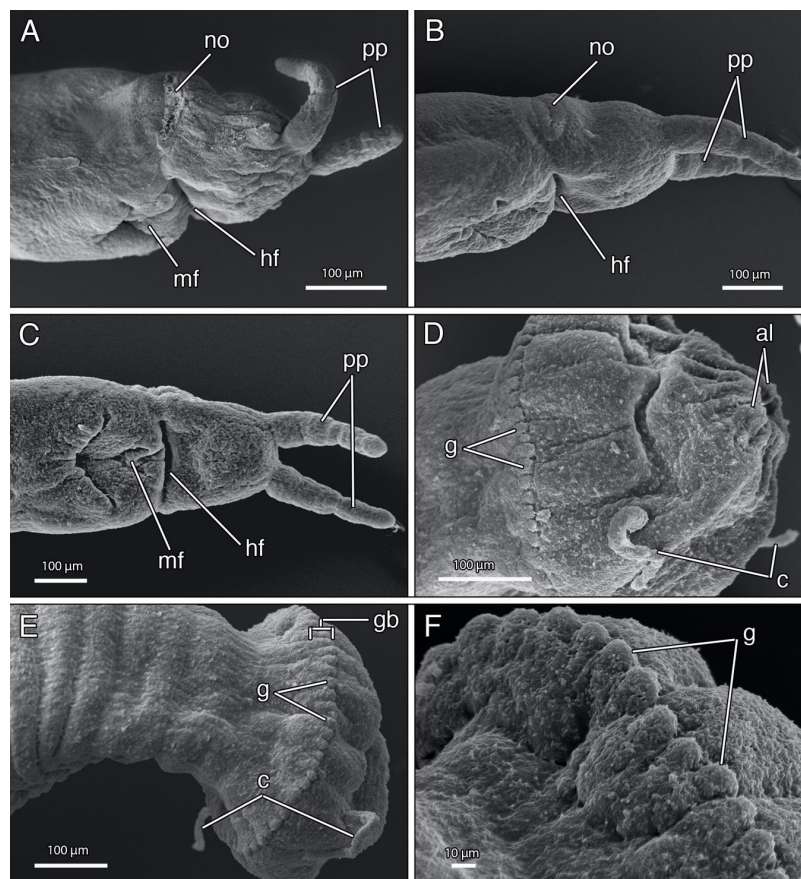


Figure 9. Scanning electron microscopy (SEM) images of *Polygordius kurthcarolae* n. sp. specimens, collected from One Tree Island, Australia, showing details of morphological characters: (A) AM W.53109 (holotype), ventral lateral view of head. (B) SIO-BIC A12263, lateral view of head. (C) SIO-BIC A12263, lateral view of head. (D) SIO-BIC A12263, lateral view of pygidium. (E) SIO-BIC A12263, partial posterior view of pygidium showing anal lobes. (F) SIO-BIC A12263, magnified view of pygidial glands. Abbreviations: al, anal lobes; c, cirri; g, gland; gb, glandular belt; hf, head fold; mf, median fold; no, nuchal organ, pp, paired palps.

4.3.4. Remarks

Polygordius kurthcarolae n. sp. is the well supported sister taxon to *P. kurthsusanae* n. sp. (Figure 2) though they are morphologically very distinct. *Polygordius kurthcarolae* n. sp. (Great Barrier Reef, Australia) is distinguished from most other *Polygordius* species by having pygidial glands and subterminal pygidial appendages (Table 1). Morphologically similar species include *P. appendiculatus*, *P. kiarama*, and *P. ijimai* (see remarks of *P. jenniferae* n. sp., discussing interpretation of morphological characters for this latter species), which also have pygidial glands and subterminal pygidial appendages (Tables 3,4). *Polygordius kurthcarolae* n. sp. can be distinguished from these three species by the following characteristics including ~54–60 round glands, 2 ventro–lateral cirri, and 7–8 anal lobes (Table 4). Although *P. appendiculatus* also has round glands, there are fewer of them in *P. appendiculatus* (30 vs. 54–60). Several other morphological characteristics of *P. appendiculatus* including 5 anal lobes, a palp: prostomium length ratio of 2:1, and the presence of pigment/“eyespot” differentiate it from *P. kurthcarolae* n. sp. (Table 4). *Polygordius ijimai* differs from the new species in that it has elongate pygidial glands and three pygidial cirri including two inserted ventro–laterally and one dorsal–median as compared to 2 ventro–lateral cirri in *P. kurthcarolae* n. sp. (Table 4). The prostomium of *Polygordius kiarama* is conical and blunt (not pointed), and the pygidium has ~20 oval pygidial glands as opposed to the new species which has a rounded prostomium and ~54–60 round glands. Moreover, *P. kiarama* is much smaller (3.8–11.6 mm × 0.28–0.30), compared to *P. kurthcarolae* n. sp. (42 mm × 0.50 mm), (Table 4). *Polygordius erikae* n. sp. differs from *P. kurthcarolae* n. sp., in that it has ~40–52 oval glands, 2 ventro–lateral cirri, and 5 anal lobes (Table 4). The genetic distance matrix indicated that the divergence value between these two new species is 22% (Table 3). All three new Pacific species (*P. erikae* n. sp., *P. kurthcarolae* n. sp., and *P. kurthsusanae* n. sp.) formed a distinct, though weakly supported, clade (Figure 2).

4.4. *Polygordius kurthsusanae* n. sp.

Polygordius n. sp. Andrade et al. (2015)

4.4.1. Material

Holotype (SIO-BIC A12287): complete adult (head/pygidium sections): on SEM stub (previously fixed in ethanol), with molecular CO1 and 16S sequences available at GenBank (MT263744, MT260188), South Pacific Ocean, off of Moorea, at station #BIZ-509, off of the channel MPA marker, French Polynesia (17°28′33.96″ S, 149°49′51.6″ W), 10–11 m water depth in coarse coral sand, collector G. Rouse and N. Wilson, 7 November 2010. **Paratypes**): complete adult (SIO-BIC A12288), head/pygidium sections on SEM stub (previously fixed in formalin), collected in the same sample/ location/date as holotype; head section (SIO-BIC A12289): on SEM stub (previously fixed in ethanol), with molecular COI and 16S sequences available at GenBank (MT263743, MT260189), collected in the same sample/location/date as holotype; Several specimens (SIO-BIC A2757) and DNA extraction (USNM1432890), COI and 16S sequences available at GenBank (MT263742, MT260187).

4.4.2. Etymology

Polygordius kurthsusanae n. sp. is named in honor of Susan Anne Kurth, who along with her sister Carol, “tied the knot” in 2019.

4.4.3. Description

Body 58.5 mm in length and 0.40–0.46 mm wide (Figure 10A). Number of segments ~146. Prostomium conical with blunt tip (length 0.20–0.25 mm), and paired palps 0.30 mm in length, (Figures 10B and 11A,B,E,F). Length ratio of palp to prostomium 1.5:1. Pigment spots/“eyespot” absent. Head fold deep. Pair of oval, nuchal organs positioned laterally at posterior margin of prostomium (Figure 11A,E). Ventrally positioned mouth with subtriangular dorso–median fold

(Figure 11F). Pygidium not inflated to minimally inflated (width 0.20 mm), (Figure 10A,C and Figure 11C,D,G,H). Pygidial glands and appendages absent (Figure 11C,D,G,H). Anus terminal with 4–5 anal lobes (Figure 11C,G). Dimensions of holotype are given in Table 3.

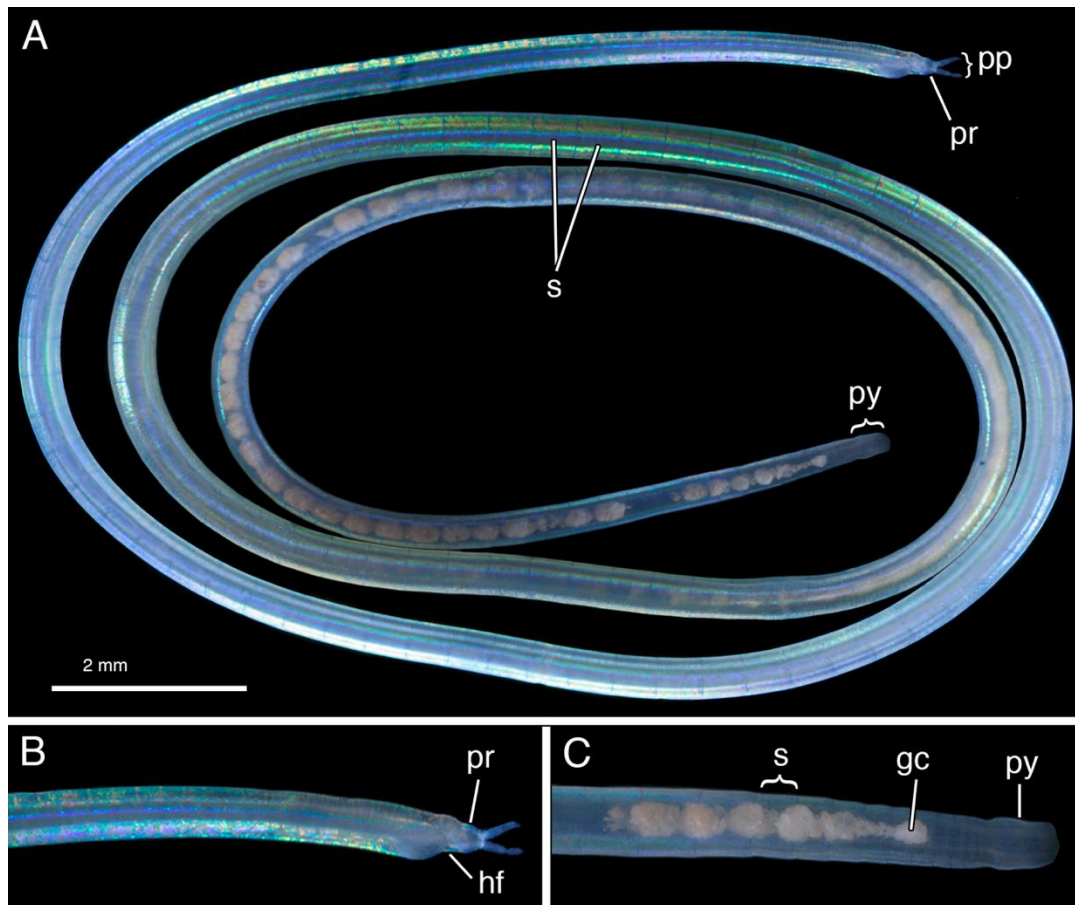


Figure 10. Light microscopy (LM) images of *Polygordius kurthsusanae* n. sp. specimen (SIO-BIC A12288), collected from Moorea, French Polynesia, photographed alive before fixation in formalin: (A) Complete adult specimen including head and pygidium. (B) Lateral view of head. (C) View of pygidium. Abbreviations: gc, gut content; hf, head fold; pp, paired palps; pr, prostomium; py, pygidium; s, segments.

4.4.4. Remarks

Polygordius kurthsusanae n. sp. (French Polynesia) is the well-supported sister taxon to *P. kurthcarolae* n. sp. (Figure 2), but can be distinguished from it and most other *Polygordius* species in that it lacks pygidial glands and appendages (Table 1). Morphologically, the most similar species also lacking pygidial glands and appendages include *P. arafura* from Australia, *P. jouinae* from New Jersey, USA, and *P. triestinus* from the Adriatic Sea (Table 1). *Polygordius kurthsusanae* n. sp. can, however, be clearly distinguished from these three species (Table 5). *Polygordius arafura*, for which there is unfortunately no DNA available, has shorter palps (0.04–0.06 mm) with a palp to prostomium length ratio of ~0.5:1 compared to 0.25 mm and ~1.5:1 respectively, for *P. kurthsusanae* n. sp. (Table 5). The median fold in *P. arafura* is subtriangular with a deep-cleft and is subtriangular without a deep-cleft in the new species. *Polygordius arafura* also has 7–8 anal lobes, whereas *P. kurthsusanae* n. sp. has 4–5. All three of these previously described species have a conical prostomium that is pointed at the tip, whereas, the “tip” is blunt in *P. kurthsusanae* n. sp. (Table 5). Moreover, *P. kurthsusanae* n. sp. is a relatively large species (58.5 mm × 0.40–0.46 mm), compared to *P. arafura* (2.4–11.6 mm × 0.08–0.18 mm), *P. jouinae* (13–43 mm

× 0.23–0.38 mm), and *P. triestinus* (30 mm) (Table 5). *Polygordius kurthsusanae* n. sp. was included in the phylogenomic analysis of annelid relationships by Andrade et al. 2015 [11] as *Polygordius* n. sp. The transcriptome of this species is available on GenBank (SRR2005365).

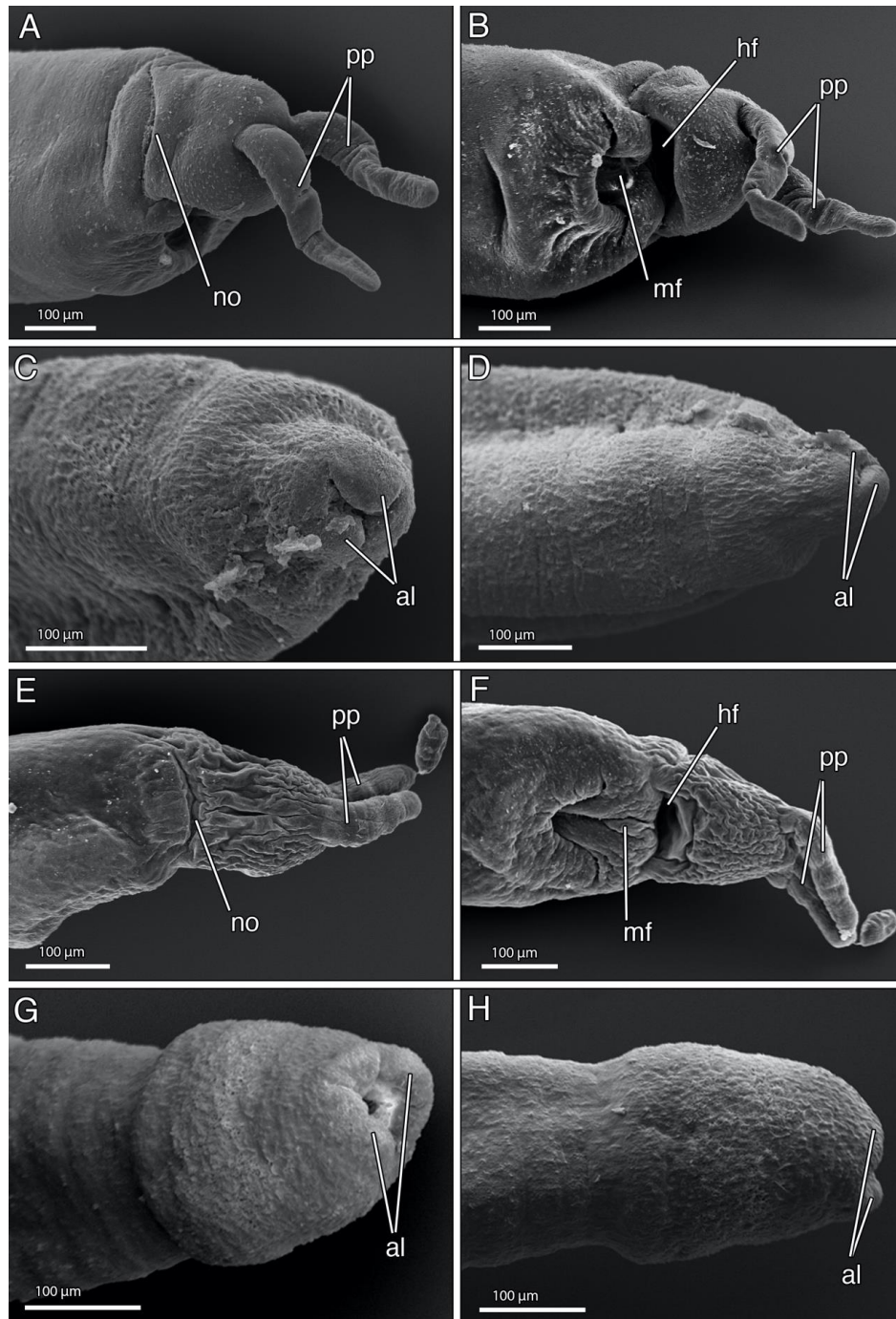


Figure 11. Scanning electron microscopy (SEM) images of *Polygordius kurthsusanae* n. sp. specimens, collected from Moorea, French Polynesia, showing details of morphological characters: (A) SIO-BIC A12287 (holotype), lateral view of head. (B) SIO-BIC A12287 (holotype), ventral view of head. (C) SIO-BIC A12287 (holotype), posterior view, tip of pygidium. (D) SIO-BIC A12287 (holotype), lateral view of pygidium. (E) SIO-BIC A12288, lateral view of head. (F) SIO-BIC A12288, ventral view of head. (G) SIO-BIC A12288, posterior view, tip of pygidium. (H) SIO-BIC A12288, lateral view of pygidium. Abbreviations: al, anal lobes; hf, head fold; mf, median fold; no, nuchal organ, pp, paired palps.

Table 5. Characters of *P. kurthSusanae* n. sp with morphologically most similar species of the genus. Morphological information for *P. triestinus*, *P. arafura*, and *P. jouinae* was taken from the original species descriptions. Brackets {} = paratype material.

Character/Species	<i>P. kurthSusanae</i> n. sp	<i>P. triestinus</i> Woltereck in Hempelmann, 1906 [9]	<i>P. arafura</i> Avery, Ramey, Wilson, 2009 [10]	<i>P. jouinae</i> Ramey, Fiege & Leander, 2006 [12]
Prostomium shape	conical [blunt]	conical (pointed)	conical (pointed)	conical (pointed)
Palp length (mm)	0.30 [n.d.*]	n.d.	0.04–0.06	0.11–0.15
Prostomium length (mm)	0.20 {0.25}	n.d.	0.06–0.12	0.11–0.15
Ratio (palp:prostomium)	1.5:1	n.d.	0.5–0.86	1:1
Pigment spots (eyespot)	absent	absent	absent	absent
Head fold	deep	shallow	deep	shallow
Median fold shape	subtriangular	n.d.	subtriangular deep cleft (F)	n.d.
Pygidium shape/width (mm)	not inflated to minimally inflated /0.20 {0.20}	not inflated	not inflated to minimally inflated	not inflated to minimally inflated
Pygidial glands (no)	absent	absent	absent	absent
Pygidial appendages/cirri pygidial cirri	absent	absent	absent	absent
Anal lobes (no.)	4–5	n.d.	7–8	7
Body length (mm)	n.d. {58.5}	max 30	2.4–11.6	13–43
Body width (mm)	0.46 {0.40}	n.d.	0.08–0.18	0.23–0.38
Segment (no.)	n.d. {146}	n.d.	27–74	82–93
Type locality	Moorea Island, French Polynesia	Northern Adriatic Sea, Italy	Australia, Gulf of Carpentaria	Beach Haven Ridge, Tuckerton, New Jersey
Region	South Pacific	North Atlantic	South Pacific	North Atlantic
Latitude	17°28'34.0" S	n.d.	9°50'6" S	39°27'42" N
Longitude	149°49'51.6" W	n.d.	135°17'48" E	74°15'48" W
Sediment type	coarse coral sand	mud	muddy-sand	sand
Depth range (m)	10–11	n.d.	69–92	5–152

(F) = observed character based on Figure only; no = number; n.d. = no data; * = palp broken.

4.5. *Polygordius pacificus* Uchida, 1935

4.5.1. Material

(SIO-BIC A12284): complete adult (head/pygidium sections): on SEM stub (previously fixed in ethanol), with molecular 16S sequence available at GenBank (MT260199), Northwest Pacific Ocean, off of Shirahama, Japan, by Fredrik Pleijel in front of the Seto Marine Laboratory (33°41'29.04" N, 135°20'11.04" E), collected from beach pebbles in the intertidal zone (1 m), 5 November 2008. Complete adult (head/pygidium sections) (SIO-BIC A12286): on SEM stub (previously fixed in ethanol), with molecular 16S sequence available at GenBank (MT260200), collected in the same sample/location/date as SIO-BIC A12284; head section (SIO-BIC A12285): previously fixed in ethanol, with molecular 16S sequence available at GenBank (MT260198), collected in the same sample/location/date as SIO-BIC A12284; complete adult (head/pygidium sections) (SIO-BIC A12266): on SEM stub (previously fixed in ethanol), with molecular 16S and H3 sequences available at GenBank (MT260201, MT263748), collected in the same location as SIO-BIC A12284, by Greg Rouse and Fredrik Pleijel, 26 May 1998; pygidium section (SIO-BIC A12265): on SEM stub (previously fixed in ethanol), with molecular H3 sequence available at GenBank (MT263749), collected in the same location as SIO-BIC A12284, 26 May 1998. Other material (SIO-BIC A12402): three complete individuals fixed in formalin, one head section fixed in ethanol, one individual sequenced, with molecular COI and 16S sequences available at GenBank (MT263741, MT260197), collected in the same location as SIO-BIC A12284, by Greg Rouse and Fredrik Pleijel, 26 May 1998.

4.5.2. Redescription

Body measuring 18.15–20.75 mm in length and 0.25–0.46 mm wide. Number of segments ~90–108. Prostomium rounded (length 0.09–0.11 mm) with paired palps (length 0.18–0.23 mm), (Figure 12A,E–F). Length ratio of palp to prostomium 2:1. Pigment spots/"eyespot" absent. Head fold deep (Figure 12E,F). Pair of oval, nuchal organs positioned laterally at posterior margin of prostomium (Figure 12E). Ventrally positioned mouth with elongate dorso-median fold on upper lip (Figure 12F). Pygidium inflated (width 0.30–0.38 mm), (Figure 12B–D,H). Pygidial glands present, ~44–75 elongate glands positioned close together longitudinally along the body creating a thick

glandular belt (width 100 μm), (Figure 12B–D,H). Ratio of gland length to width $\sim 7\text{--}10:1$ (length 100 μm ; width 10–15 μm). Terminal pygidial appendages present. Anus terminal with 6 anal lobes of which 2–3 are elongate/enlarged (length 20 μm), (Figure 12C,G,H).

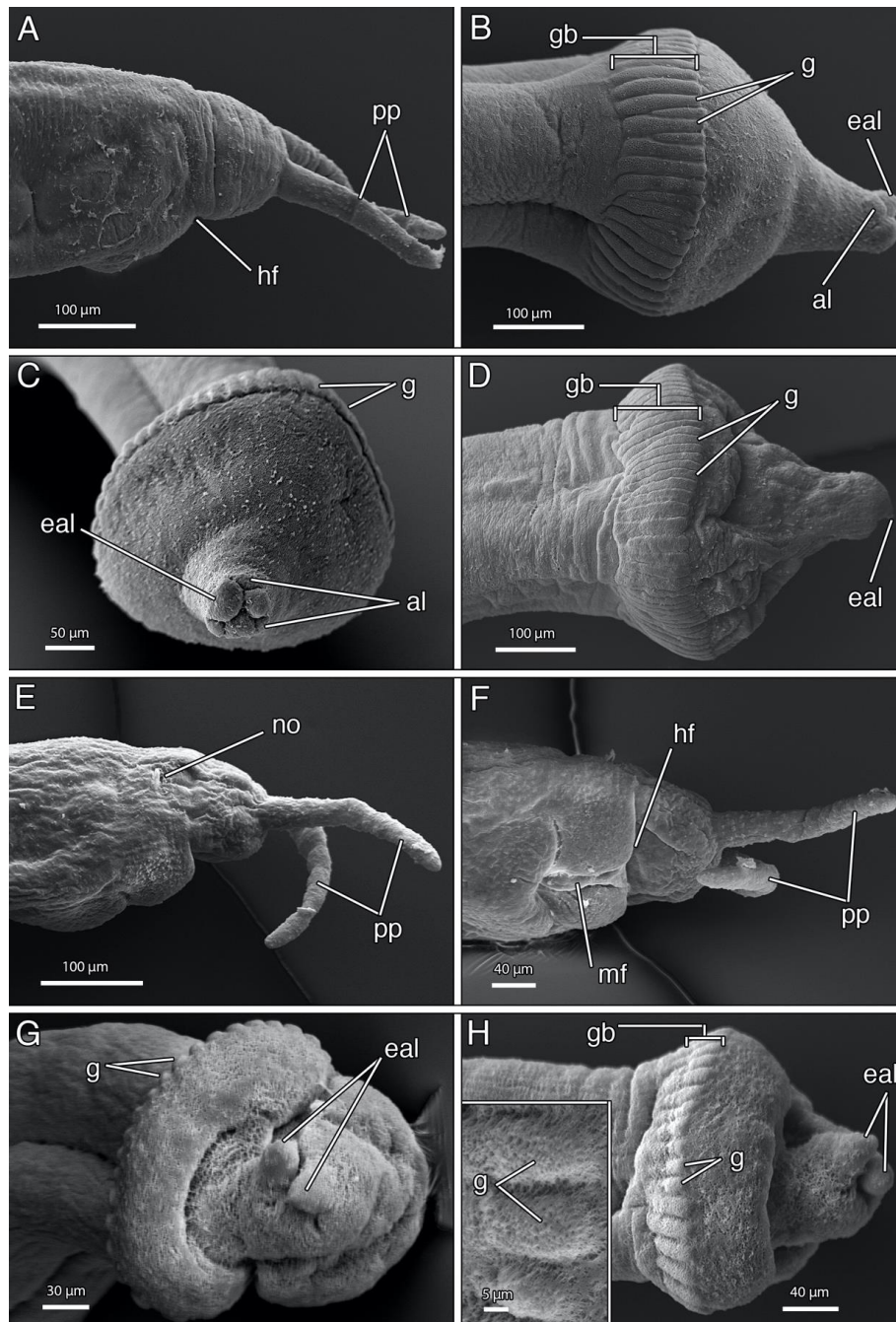


Figure 12. Scanning electron microscopy (SEM) images of *Polygordius pacificus* specimens, collected from Shirahama, Japan, showing details of morphological characters: (A) SIO-BIC A12284, lateral view of head (nuchal organ obscured). (B) SIO-BIC A12284, lateral view of pygidium. (C) SIO-BIC A12284, posterior view, tip of pygidium. (D) SIO-BIC A12286, lateral view of pygidium. (E) SIO-BIC A12266, lateral view of head. (F) SIO-BIC A12266, ventral lateral view of head. (G) SIO-BIC A12266, posterior view, tip of pygidium. (H) SIO-BIC A12266, lateral view of pygidium. (H-inset) Magnified view of pygidial glands. Abbreviations: al, anal lobes; eal, elongate/enlarged anal lobes; g, gland; gb, glandular belt; hf, head fold; mf, median fold; no, nuchal organ; pc, pygidial cirri; pp, paired palps.

4.5.3. Remarks

The type locality for *Polygordius pacificus* is near the Misaki Marine Biological Station, Honshu Japan. This is the same type locality as *P. ijimai* [35]. In the original description it was also noted that *P. pacificus* also sampled at the coast of the Japanese Sea [16]. The type material has apparently been lost (Eijiroh Nishi pers. comm.). The samples of *Polygordius pacificus* used in this study were also from Honshu, though 450 km south of the type locality. Given the distance from the type locality we do not nominate any of our specimens as a neotype.

Based on findings in the present study, *P. pacificus* is distinguished from most other *Polygordius* species by having pygidial glands and terminal pygidial appendages (Table 1). Morphologically similar species/subspecies include *P. pacificus floreanensis*, *Polygordius eschaturus*, *P. eschaturus brevipapillosus*, and *P. madrasensis* Aiyar and Alikunhi, 1944 [36], which also have pygidial glands and terminal pygidial appendages (Table 1). Of these, *P. pacificus* is most similar to *P. pacificus floreanensis*, *P. eschaturus*, and *P. eschaturus brevipapillosus* since they all have elongate pygidial glands (Table 6), whereas, *P. madrasensis* has oval glands (Aiyar and Alikunhi 1944 [36]). *Polygordius pacificus* can be easily distinguished from *P. pacificus floreanensis* by its rounded prostomium and ~2:1 palp to prostomium length ratio, whereas the prostomium of *P. pacificus floreanensis* is conical with a palp to prostomium length ratio of ~1:1 (Table 6). Unfortunately, examination of the single type specimen available for *P. pacificus floreanensis* could not provide details regarding the number of pygidial glands and anal lobes useful for species distinction, since SEM is needed to observe these and was not possible. *Polygordius pacificus* has 2–3 elongate/enlarged anal lobes similar to both *Polygordius eschaturus* and *P. eschaturus brevipapillosus* which have 2 and 2–3 elongate lobes respectively. The elongate lobes of these two species, however, are much longer (length 50–70 and 80 μm , respectively) than those of *P. pacificus* (length 40 μm), (Table 6). It is important to note that terminal pygidial cirri were absent in the original species description of *P. pacificus* (Table 6). This is not surprising, given that we only observed these with SEM. Furthermore, their morphology varied depending on fixation/condition of the specimen(s). In a relatively well-fixed individual, three of the six lobes appear enlarged (Figure 12B,C, 3 enlarged) rather than elongate (Figure 12G,H, 2 elongate). In the case of enlarged lobes, the tip of the lobe/pygidial cirrus is situated such that it is directed/turned “inward and downward” covering the anal opening (Figure 12B,C). The genetic distance matrix indicated that the divergence value between *P. pacificus* and *P. eschaturus* is 19%, making *P. eschaturus* a sister group to *P. pacificus* within their clade, but clearly denoting separate species.

Table 6. Characters of *P. pacificus* Uchida, 1935 [16] and specimens designated in the present study as compared to morphologically most similar species/subspecies including *P. pacificus floreanensis*, *P. eschaturus*, and *P. eschaturus brevipapillosus*. Morphological information was taken from the original species descriptions unless otherwise indicated.

Character/Species	<i>P. pacificus</i>	<i>P. pacificus</i> Uchida, 1935 [16]	<i>P. pacificus floreanensis</i> Schmidt & Westheide 1977 [18]	<i>P. eschaturus</i> Marcus, 1948 [20]	<i>P. eschaturus brevipapillosus</i> Jouin & Rao, 1987 [17]
Prostomium shape	rounded	n.d.	conical (F, P)	rounded (F)	rounded (F)
Palp length (mm)	0.18–0.23	n.d.	0.10–0.225	0.15–0.22	0.20–0.25
Prostomium length (mm)	0.09–0.11	n.d.	0.10 (P)	n.d.	n.d.
Ratio (palp:prostomium)	~2:1	n.d.	~1:1	n.d.	n.d.
Pigment spots (eyespot)	absent	absent	absent	absent	absent
Head fold	deep	n.d.	n.d.	deep (F)	deep
Median fold shape	elongate	n.d.	n.d.	n.d.	n.d.
Pygidium shape/width (mm)	inflated/0.35–0.38	inflated/n.d.	inflated/0.16	inflated/max 0.30	inflated/0.20–0.53
Pygidial glands (no)	44–75	n.d.	n.d.	n.d.	n.d.
Pygidial gland shape/orientation	elongate/ longitudinal	elongate/ ongitudinal	elongate/longitudinal	elongate/longitudinal	elongate/longitudinal
Glandular belt (width) (µm)	100	150 (F)	30 (F)	n.d.	108
Gland (width) (µm)	10–15	n.d.	n.d.	n.d.	n.d.
Gland ratio length:width	7–10:1	5:1 (F)	5:1 (F)	n.d.	n.d.
Terminal pygidial appendages/cirri	present	absent	n.d.	present	present
Anal lobes (total no.)	6	6–8	n.d.	n.d.	6–8
Elongate & enlarged anal lobes no./length (µm)	2-3/40	n.d.	n.d.	2/50–70	2–3/80
Body length (mm)	18.15–20.75	30–35	4.9–23	max 40	18–50
Body width (mm)	0.25–0.46	0.40–0.50	0.11	0.21	0.15–0.50
Segment (no.)	90–108	n.d.	35–75	120–180	110–160
Type locality	Seto Mar. Biol. Lab., Japan*	Misaki Mar. Biol. Lab., Japan	Floreana Isl., Galapagos	São Sebastião Isl., Brazil	Andaman Islands, India
Region	NW Pacific	NW Pacific	SE Pacific	SW Atlantic	Indian Ocean
Latitude	33°41'29.04" N	n.d.	n.d.	n.d.	12°58'06" N
Longitude	135°20'11.04" W	n.d.	n.d.	n.d.	92°59'17" E
Sediment type	pebbles	fine sand	coarse sand	coarse sand	coarse sand & shell-hash
Depth range (m)	intertidal	lower intertidal	lower intertidal	intertidal	lower intertidal

(F) = observed character based on Figure only, (P) = personal observation in type; no = number; n.d. = no data. *Not type locality; a new collection locality.

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