Molecular phylogeny, morphology, and distribution of *Polygordius* (Class: Polychaeta;
Family Polygordiidae) in the Atlantic and Mediterranean

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Abstract

Low morphological diversity among interstitial taxa makes it difficult to delimit species and their geographic boundaries based solely on morphology and molecular data often reveal cryptic species. *Polygordius* (Annelida, Polygordiidae) have low morphological diversity, but are unusual among interstitial species in their comparatively large size due to their elongated form, high fecundity, and potential for long-distance dispersal via a planktotrophic larval stage. *Polygordius* species collected from 14 localities in the Northwest Atlantic, Mediterranean Sea, and Southwest Atlantic including several of the respective type localities were analysed. This study presents the first phylogeny of the genus *Polygordius* and combines molecular data, sequences of COI, 16S and ITS1/2 genes, and morphological data for a systematic re-evaluation focusing on Atlantic species, with an emphasis on populations from European waters. Phylogenetic analyses recovered six valid species (*P. appendiculatus*, *P. lacteus*, *P. neapolitanus*, *P. triestinus*, *P. jouinae*, and *P. eschaturus*) and their distinctness is confirmed by haplotype network analyses. Thus, molecular data supported the validity of the previously recognized morpho-species and no new species were present. *P. erythrophthalmus* and *P. villoti* are invalid species being synonymous with *P. lacteus*. Subtle differences in head and pygidial morphology and larval type (endolarva vs. exolarva), were useful characters for discrimination. Yet seemingly significant variation in characters among individuals in some species was not diagnostic (e.g., number of pygidial cirri). Highly similar species based on adult morphology were shown to be sister taxa occurring in allopatry. Present day distribution patterns of species are summarized in light of this study.

1. Introduction

The marine interstitium or the space between the particles of sediments that cover much of the ocean floor and beaches, is one of the earth's largest and oldest ecosystems (Snelgrove, 1999). It was once considered to be void of animals, but during the last century it has been shown to

contain a rich variety of life with representatives from most animal taxa (Giere, 2009; Higgins and Thiel, 1988; Noodt, 1974), forming a major component of marine faunal diversity (Snelgrove, 1999). The interstitial environment is generally characterized by an extreme and strict requirement for small size due to the narrow but highly structured three-dimensional space for organisms to inhabit (Struck, 2006; Westheide, 1977, 1987). Thus, interstitial species are small with only very thin individuals being longer than 1 mm. Bodies are long and slender, worm-like (cylindrical), or broad and flat, and in comparison to their larger in- or epifaunal relatives, interstitial species often have a high degree of morphological “simplicity” and “uniformity” (Giere, 2009; Higgins and Thiel, 1988; Swedmark, 1964). Given their small size and therefore limited energetic resources, they produce only a few eggs and offspring. Consequently fertilization of the eggs is usually direct as is development (Swedmark, 1964; Westheide, 1984). Most interstitial species lack planktonic larval and/or juvenile stages and have low mobility as adults (Gerlach, 1977; Giere, 2009; Sterrer, 1973). Thus, their dispersal abilities are limited. Yet, many interstitial species appear to be widespread, having amphiatlantic or cosmopolitan distributions, a phenomenon known as the Meiofauna Paradox (Gerlach, 1977; Giere, 2009; Sterrer, 1973; Westheide, 1977).

There are some interstitial species, however, that do have the potential for wide dispersal (Higgins and Thiel, 1988). One example are species in the annelid taxon Polygordiidae (Fig. 1). *Polygordius* species have a strong affinity for highly energetic habitats with coarse sandy sediments in intertidal to subtidal and continental shelf/slope environments worldwide (Ramey, 2008; Ramey-Balcı et al., 2013). The relatively larger interstitial spaces afforded by coarser sediment grains allow for larger body size. Polygordiid species are usually several centimeters long, but very slender (i.e., width: 0.08–1.50 mm; length: 10–100 mm; Fig. 1A; Supplementary material 1: video) (Ramey-Balcı et al., 2013). One consequence of their greater size is that more energetic resources are available for reproduction, and indeed

compared to other interstitial species, polygordiids can produce relatively large numbers of gametes (e.g., 1,000s of eggs with max diameter 62 μm ; Ramey 2008; also see Rota and Carchini, 1999). Fertilization is indirect as sperm structure is consistent with external fertilization via broadcast spawning (Franzén, 1977; Ramey, 2008). Development is also indirect with a planktotrophic larval phase that has two distinct, species-specific forms (i.e., exolarvae vs. endolarvae) which differ in structure and development (e.g., Fig. 1D–E) (Agassiz, 1867; Cowles, 1903; Hatschek, 1878; Herrmann, 1986; Lovén, 1843; Ramey-Balcı and Ambler, 2014; Woltereck, 1902). Although few studies have examined the population dynamics of adult/larval stages of polygordiids (Nordheim, 1984; Ramey 2008, Ramey-Balcı and Ambler, 2014), studies on *Polygordius jouinae* Ramey et al. 2006, indicate their potential for long distance dispersal. Large numbers of exolarvae were found to made-up > 90% of the meroplankton abundance (highest density 4,013 ind m^{-3} ; station 1; July 2006) off Chincoteague Virginia spending at least two weeks to a month in the plankton (Ramey et al., 2006; Ramey-Balcı and Ambler, 2014; see also Shanks, 2009).

To date only 17 species and two subspecies of polygordiids have been described from the Atlantic including the Mediterranean Sea (9 species), Indian (2 species; 1 subspecies), Pacific (5 species; 1 subspecies), and Southern Oceans (1 species). Polygordiids are generally not morphologically diverse. The elongate, thin, cylindrical body is smooth and lacks external signs of segmentation as well as typical polychaete features such as parapodia and chaetae. Morphological features for species distinction are limited to their overall shape and minute, subtle differences in structures on the head/prostomium and posterior end/pygidium (Ramey et al., 2006; Ramey-Balcı et al., 2013) (Fig. 1A–C; Supplementary material 2: video). Although most species are distinguished based on adult morphology, larval morphology has also been used (Fig. 1D–E).

The validity of some species in this group has been repeatedly questioned and remains unresolved. For example, *Polygordius neapolitanus* Fraipont, 1887 is very similar morphologically to *Polygordius lacteus* Schneider, 1868, so similar it was once considered a synonym (Hempelmann, 1906 p. 528) and then separated again (Hempelmann, 1931 p. 157). In 1905, Woltereck attempted inter-breeding experiments with these two species and although fertilization was achieved (despite different reproductive seasons), development did not continue beyond the earliest trochophora stage (Woltereck, 1904; 1905). Thus results were inconclusive and have been used to both lend support and refute the validity of *P. neapolitanus* (e.g., Marcus, 1948; Hempelmann, 1906). Giard (1880) considered the presence of red “eyes/eyespot” as sufficient to describe, *Polygordius erythrophthalmus* Giard, 1880, that was otherwise similar in all other respects to *P. lacteus*. The red pigment spots on the prostomium of *P. erythrophthalmus*, have recently been shown to be pigmented coelomic cells rather than sensory structures and a limited molecular analysis (including 3 species and 34 COI sequences) indicated that *P. erythrophthalmus* should be considered a junior synonym of *P. lacteus* (see Lehmacher et al., 2016). Likewise Perrier (1875) described *Polygordius villoti* Perrier, 1875 distinguishing it from *P. lacteus*, the only other species of *Polygordius* known at the time, by details of the circulatory apparatus, presence of segmental organs for evacuation of sexual products, and by an erroneous difference in body length (body length: *P. villoti* >10cm vs. *P. lacteus* 10 mm in Perrier 1875; *P. lacteus* 10 cm in Hempelmann, 1906; 40-50 mm in Schneider 1868). Details of the circulatory apparatus and mode of spawning of *P. villoti* described by Perrier (1875) have been questioned by Fraipont (1887) in his monographic work on *Polygordius*, and to our knowledge *P. villoti* has never been reported again (also see Cabioch et al. 1968). It is likely that this species is also a junior synonym of *P. lacteus*. Moreover, *Polygordius triestinus* Hempelmann 1906, and *P. jouinae* are distinguished by only a few, very slight morphological differences on the prostomium, and by

P. triestinus being hermaphroditic (Ramey et al., 2006). The reproductive mode and validity of *P. triestinus* have been questioned (e.g., Westheide, 2008). Moreover *P. triestinus*, has not been collected in the Gulf of Trieste despite frequent benthic studies conducted there from 1966 to 2003 (V. Solis-Weiss, personal communication) (also see Ramey et al. 2006). With the exception of *P. jouinae*, type material for these species has either been lost or was never deposited (Ramey et al. 2006).

Low morphological diversity among interstitial species makes it especially difficult to delimit species and their geographic boundaries (Jörger et al., 2012) without using additional techniques. For many interstitial taxa, detailed examinations of widespread species using molecular studies have shown that gene flow has not occurred over long distances and genetically distinct units could be recognized, which are usually regarded as cryptic species (e.g., Jörger and Schrödl, 2013; Kieneke et al., 2012; Rocha-Olivares et al., 2001; Schmidt and Westheide, 2000; Suatoni et al., 2006; Todaro et al., 1996; Von Soosten et al., 1998; Westheide and Hass, 2001). To date no comprehensive molecular studies have been conducted on Polygordiidae.

This study examines eight of the nine species of *Polygordius* described from the Atlantic and Mediterranean Sea (*P. lacteus*; *P. neapolitanus*; *P. erythrophthalmus*; *P. villoti*; *P. jouinae*; *P. appendiculatus* Fraipont, 1887; *Polygordius eschaturus* Marcus, 1948; and *P. triestinus*; unfortunately specimens of *P. leo* Marcus, 1955 were not available to us), and presents a molecular phylogeny based on mitochondrial COI and 16S rRNA, as well as nuclear ITS1 and ITS2 sequences, and combine molecular and morphological data for a systematic re-evaluation within the genus. Specifically we: 1. test the validity of six recognized morpho-species, 2. determine whether cryptic species are present within recognized morpho-species and new species need to be described, 3. assess the usefulness of currently applied morphological characters for species distinction, 4. evaluate the

phylogenetic relationships of morphologically highly similar species and 5. provide an update of the current distribution of *Polygordius* species in the Atlantic and Mediterranean.

2. Material and Methods

2.1. Collection, fixation and identification of Polygordiidae

Polygordiidae were collected between 2008 and 2011 from either subtidal or intertidal sandy sediments using a grab or dredge. A total of 104 specimens belonging to six species were collected from fourteen localities including type localities in the Northeast Atlantic (Helgoland, Germany; Beg Meil, France; Roscoff, France: localities Primel and Drezen), Mediterranean Sea (Banyuls-sur-Mer, France; Ischia, Italy; Rovinj, Croatia: localities Punta Croce, near Figarola Island, Valdibora harbor and Veštar), Northwest Atlantic (Tuckerton, New Jersey and Chincoteague Virginia, United States), and South Atlantic (Santa Catarina and Rio de Janeiro, Brazil) (Table 1; Fig. 2). Samples were gently elutriated with running seawater over a sieve (mesh size 40 or 500 μm) to facilitate live sorting of specimens from the sediment under a stereomicroscope. Entire (unbroken) specimens were sectioned into three parts including the head, middle, and pygidium. The head and pygidium with their important morphological characters were either fixed in 4% buffered formaldehyde seawater solution or in Steedman's Fixative (Griffiths et al., 1976) which was later replaced with Steedman's preservative (Steedman, 1976), whereas the middle section was fixed in 95% non-denatured ethanol for molecular work. Morphological species identifications were conducted using light microscopy and/or scanning electron microscopy (SEM) on specimens fixed in formalin or Steedman's Fixative. Specimens (head and pygidium) for SEM analysis were dehydrated using a graded ethanol series (10 min each in 70, 90 and 2 x 100% ethanol), critical point dried using CO_2 , mounted on aluminium stubs, and sputter coated with gold-palladium. Specimens were examined using a CamScan CS 24 SEM and detailed imaging of external

morphology was conducted using Orion software 6.63. Voucher specimens and DNA extractions have been deposited in collections at Senckenberg Museum Frankfurt (SMF), and Museu de Zoologia da Universidade Estadual de Campinas “Adão José Cardoso” (ZUEC) (Supplementary material 3).

2.2. Determination of COI, 16S and ITS1 and ITS2 sequences

Genomic DNA was extracted using DNeasy Tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. We amplified and sequenced two mitochondrial genes, the barcoding gene cytochrome c oxidase subunit I (COI) and the 16S ribosomal gene (16S), as well as part of the nuclear rDNA cluster, the internal transcribed spacers ITS1 and 2 (ITS1/2). Primers used in this study are listed in Supplementary material 4 along with the polymerase chain reaction (PCR) cycling conditions for each gene.

Fragment length, approximate yield of DNA from each reaction, and detection of potential contamination in negative controls were verified by agarose gel electrophoresis. Successful PCR products were purified using a Gel Extraction Kit (PeQLab) according to the manufacturer’s instructions. Sequencing was conducted on an ABI 3730xl capillary sequencer following the manufacturer’s instructions at the Senckenberg Biodiversity and Climate Research Centre (SBIK-F) Laboratory Centre, Frankfurt/Main. A consensus of the forward and reverse sequences for each individual was assembled using SeqMan with a minimum match percentage of 95% (DNA STAR, Lasergene 7.1.0). A blast search was performed in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) to ensure that sequence(s) were not a result of contamination by non-annelid material.

2.3. Analyses of molecular datasets

192 We obtained COI and 16S sequence data from the complete mitochondrial genomes of
193 *Nephtys* sp., *Orbinia latreillii*, *Platynereis dumerilii*, *Riftia pachyptila* and *Terebellides*
194 *stroemi* as well as ITS1/2 sequence data of *Perinereis aibuhitensis* and *Harmothoe imbricata*
195 as outgroup taxa (Supplementary material 3). The sequences of each gene were first aligned in
196 MUSCLE (Edgar, 2004a, b) implemented in MEGA 5.1 using default settings (Molecular
197 Evolutionary Genetics software package; Tamura et al., 2011), and subsequently corrected
198 manually (COI and 16S only) in GeneDoc 2.6.002 (Nicholas et al., 1997). The alignments
199 were subsequently trimmed from both ends until the first and last position of the alignment
200 had at least 90% coverage (MEGA 5.1). For the 16S alignment, additional ambiguously
201 aligned positions were masked using AliScore (Kück et al., 2010; Misof and Misof, 2009)
202 with *n* option, which treats gaps as ambiguous characters, since it is most consistent with the
203 settings in the ML analysis using RAxML (Stamatakis, 2006). The COI gene sequence
204 alignment was also masked, but it produced the same dataset as before masking, so all
205 subsequent analyses were conducted on the original unmasked alignment. Additionally, we
206 concatenated the two mitochondrial genes (COI and 16S masked) into one dataset for all
207 individuals for which both genes were present (COI/16S). For ITS1/2, given that the lengths
208 of the ITS1/2 of the *Polygordius* species were substantially longer than those of the outgroup
209 species we generated three different datasets. The first dataset comprised all *Polygordius*
210 ITS1/2 sequences as well as those of the outgroup taxa. However, the alignment possessed
211 many areas with gaps as the small outgroup sequences had to be fitted to the much longer
212 ingroup sequence. Therefore, for the second dataset the outgroup taxa were excluded and then
213 the ITS1/2 aligned anew. In the third dataset, ambiguously aligned positions of the second
214 dataset (i.e., without the outgroup taxa) were masked using AliScore (Kück et al., 2010; Misof
215 and Misof, 2009). Maximum Likelihood (ML) analyses of each of the seven datasets
216 described above were conducted with RAxML 7.3.1 (Stamatakis, 2006). As RAxML allows

only the GTR substitution model we applied GTR+I+ Γ 4 for each gene. For each concatenated dataset (i.e., the COI/16S and the three ITS1/2 datasets) we applied a partitioned approach, where each gene was assigned its own substitution model and branch lengths. Moreover, for the COI/16S dataset we further tested if the protein-coding genes COI should be partitioned further based on codon positions using PartitionFinder2 (Guindon et al., 2010; Lanfear et al., 2012; Lanfear et al. 2016), and performed final analyses under independent GTR+I+ Γ 4 substitution models and branch lengths for each codon position. Confidence values for the internal branches of the ML tree were computed using the automatic bootstrapping option (-#autoMRE) in RAxML to a maximum of 1,000 bootstrap (BP) replicates (Felsenstein, 1985).

Additionally, we conducted phylogeographic analyses for the COI, 16S and COI/16S datasets without the outgroup taxa (ITS1/2 dataset was not used since the number of specimens sequenced was too few compared to those obtained for COI and 16S, see results). For all three datasets, haplotype networks were reconstructed using TCS (Clement et al., 2000) with the lowest possible connection threshold of 90%. F_{ST} values of the COI and 16S data were calculated using Arlequin (Excoffier and Lischer, 2010) to compare individuals and populations grouped by species assignments based on morphology and the ML analyses.

3. Results

3.1. Species identification—morphology and distribution

Morphological identification of specimens collected indicated six species of Polygordiidae including *P. lacteus*, *P. appendiculatus*, *P. neapolitanus*, *P. triestinus*, *P. jouinae* and *P. eschaturus* (Table 1; Fig. 2; Supplementary material 3). Initial identifications of specimens based on original species descriptions (also summarized by Rota and Carchini, 1999) were not straightforward, especially for the first three species listed; so some explanation is warranted. Specimens from Helgoland were identified as *P. lacteus* (type locality Helgoland) and

P. appendiculatus (Table 1; Supplementary material 3). These two species co-occurred in sediment with medium-coarse shell-hash and sand grains (locally referred to as Amphioxus sand where *Amphioxus* (= *Branchiostoma*) is abundant). Sexually mature individuals (males and females) of both species were present in early July (Table 1). Distinction between the two species was mainly based on the presence of pygidial cirri in *P. appendiculatus* (Fig. 1A, C, F–G) (which are absent in *P. lacteus*) and the relatively larger size of *P. lacteus* (see videos in Supplementary material 1 and 2, *P. lacteus* and *P. appendiculatus* respectively). Examination of individuals of *P. appendiculatus* from Helgoland showed considerable morphological variability in the number and structure of pygidial cirri (> 2 pygidial cirri in 17% of specimens examined; cirri forked or unforked) (Fig. 1F–G; also see individuals with “**” in Supplementary material 3). Since variability was less common for *P. appendiculatus* from Ischia in the Bay of Naples (the type locality of this species) with respect to the Helgoland population, we considered the hypothesis of a new undescribed species occurring in Helgoland.

Several individuals collected from sediments containing coarse shell-hash mixed with small pieces of coralline algae at Beg Meil had dark-red dots on the prostomium resembling “eyes/eyespots”. These individuals were initially identified as *P. erythrophthalmus* (type locality Beg Meil) (see individuals with “*” in Supplementary material 1; Fig. 1a’ in Lehmacher et al. 2016).

Specimens from Ischia were identified as *P. neapolitanus* and *P. appendiculatus*, which were both originally described from the Gulf of Naples. Sexually mature individuals of both species co-occurred in coarse sandy sediments in early May (Table 1). Distinction between these two species was again based on the presence of pygidial cirri in *P. appendiculatus* (Fig. 1A, C, F–G) (which are absent in *P. neapolitanus*), and the larger size of *P. neapolitanus*. On the other hand, *P. neapolitanus* could not be morphologically

distinguished from *P. lacteus* from Helgoland. Thus, identification of specimens from Ischia as *P. neapolitanus* was primarily based on the fact that this species had been originally described in the Gulf of Naples along with previous studies indicating that larval type differed for these two species with *P. neapolitanus* having an exolarva and *P. lacteus* exhibiting an endolarva (Fraipont, 1887; Woltereck, 1925; 1926). Specimens superficially resembling *P. lacteus*, *P. neapolitanus* and *P. villoti* (type locality Roscoff) collected from Roscoff (including specimens collected from Primel) and from Beg Meil (specimens without red “eyes/eyespot”), as well as from Rovinj (collected from Punta Croce, and Veštar) were initially identified as *Polygordius* spp. since they could not be definitively determined as belonging to any of these species. Although *P. appendiculatus* was also found in Roscoff, it did not co-occur with *P. lacteus*, as it did in Helgoland, and was instead found in relatively finer sediments primarily made-up of fine shell-hash and sea anemone spines (Table 1).

Polygordius jouinae and *P. triestinus* were easily distinguished from each other in part due to geographic distribution but also longer palps and larger size of *P. jouinae* (see Table 1; Fig. 2; Supplementary material 3) and from the other species in lacking pygidial appendages and glands. *Polygordius eschaturus* was easily distinguished from the other species with pygidial appendages and/or glands by having terminally positioned appendages and numerous elongate glands, compared to subterminally positioned and relatively fewer round or oval shaped glands. *Polygordius eschaturus* was found in the intertidal zone, whereas all other species were found subtidally.

3.2 Molecular datasets

The finalized datasets for CO1, 16S and ITS1/2 genes included 96 sequences (average length 648 bp $\pm$ 30), 99 sequences (average length 918 bp $\pm$ 139), and 39 sequences (average length 811 bp $\pm$ 41), respectively. A total of 93 sequences formed the combined CO1/16S dataset.

Further details of the COI, 16S and ITS datasets including total number of individuals, the average sequence length with standard deviation and range are given for each population (species/location) (Supplementary material 5). The length of the 16S alignment was 972 bp (25% of the positions were removed after masking, resulting in 729 bp), and the length of the second ITS1/2 dataset (i.e., without outgroup taxa) was 983 bp (28.1% of the positions removed after masking, resulting in 707 bp). The COI dataset had a total of 68 different haplotypes and on average each haplotype was represented by 1.29 specimens (range = 1 to 8 specimens per haplotype). Similarly, the 16S datasets resulted in 75 haplotypes and on average each haplotype was represented by 1.63 specimens per haplotype (range = 1–7 specimens per haplotype).

3.3. Phylogenetic analyses

The phylogenetic reconstruction based on the analysis of the combined mitochondrial markers (COI/16S) using ML found clades corresponding to five described *Polygordius* species based on morphology and distribution (Fig. 3). *Polygordius lacteus* is present in the Northeast Atlantic (Roscoff, Beg Meil, Helgoland) and *P. neapolitanus* in the Mediterranean (Rovinj, Ischia). *Polygordius appendiculatus* is present in both the Northeast Atlantic (Roscoff, Helgoland) and the Mediterranean (Rovinj, Ischia, Banyuls) (Fig. 3). Population structure was not detectable despite its broad range. *Polygordius eschaturus* was present in the Southwest Atlantic (Santa Catarina, Rio de Janeiro), and *P. jouniae* in the Northwest Atlantic (Tuckerton, Chincoteague). The monophyly of each species was significantly supported with bootstrap values of 100 (Fig. 3). The morphologically nearly identical species *P. lacteus* and *P. neapolitanus* are sister taxa based on COI/16S data (bootstrap support of 98). The other *Polygordius* species present in the Northeast Atlantic and Mediterranean Sea, *P. appendiculatus*, is sister to this clade (bootstrap support of 100). Interestingly, the sister to this clade is not *P. jouniae*, which also has a Northern Atlantic distribution, but *P. eschaturus*

from the Southern Atlantic (bootstrap support of 98). The distribution of the *Polygordius* species examined in the present study is summarized in Fig. 2.

Analyses of the individual COI and 16S datasets were congruent with the ML tree of the combined COI/16S dataset (Supplementary material 6 and 7, respectively). We also obtained 16S data for 5 individuals of a sixth *Polygordius* species, *P. triestinus*, whose monophyly was significantly supported with bootstrap values of 100 (Supplementary material 7). Interestingly, *P. triestinus* is not closely related to another *Polygordius* species with a Northeastern or Mediterranean distribution, but to *P. jouinae* with a Northwest Atlantic distribution (bootstrap support of 100).

We also obtained nuclear ITS1/2 data for fewer individuals of all six species. Analyses of the three different ITS-datasets were congruent with each other, keeping in mind that two of three analyses were conducted without the outgroup taxa (Fig. 4). In these two cases the basal relationships of the *Polygordius* species to each other could not be resolved, as these trees could not be rooted (indicated by na in Fig. 4). Overall these analyses were generally in agreement with the phylogenetic patterns revealed for the mitochondrial genes in recovering the monophyly of each of the five species and the same phylogenetic relationships with the exception of *P. eschaturus*. In the nuclear analyses the clade containing *P. triestinus* and *P. jouinae* was sister to the clade with *P. lacteus*, *P. neapolitanus* and *P. appendiculatus* rather than *P. eschaturus*.

3.4. Network analyses

Results of the haplotype network analyses conducted on combined COI/16S, COI and 16S datasets were generally similar (Fig. 5: COI/16S; Supplementary material 8–9: COI and 16S, respectively). Thus we primarily focus on presenting the combined COI/16S network (Fig. 5). All species recognized by the phylogenetic analyses and morphology were also clearly

separated by the network analyses. The network of *P. lacteus* was characterized by one common haplotype present in six specimens and several unique haplotypes. The maximum number of substitutions between any two haplotypes was 10. Similarly, the network of *P. appendiculatus* had one common haplotype (containing five specimens) along with two other haplotypes (containing 3 and 2 specimens respectively) and several unique haplotypes. The maximum difference between any two haplotypes was 25 substitutions (Fig. 5). Interestingly the individuals of *P. appendiculatus* from the Northern Atlantic populations (Helgoland and Roscoff) were generally more similar to each other compared to individuals from the Mediterranean populations which were usually located on the outskirts of the networks and exhibited stronger differences. In contrast, to these two species, the networks of *P. jouinae*, *P. neapolitanus*, and *P. eschaturus* only contained unique haplotypes (with the exception of the 16S network of *P. jouinae*, which possessed a common haplotype with seven specimens, see Supplementary material 9). The maximum difference between any two haplotypes was 13 substitutions in *P. jouinae*, and as much as 33 and 21 substitutions in *P. neapolitanus*, and *P. eschaturus*, respectively. In particular one haplotype in *P. eschaturus* found in a specimen from Santa Catarina, was more than 23 substitutions, the 90% connection threshold value, different from any of the other haplotypes (including the hypothetical ones) and, hence, was not connected to the network of the other haplotypes of *P. eschaturus*.

3.5. F_{ST} data

Analyses of F_{ST} values calculated for COI and 16S genes showed that each species had a high degree of genetic differentiation from each other with high and significant F_{ST} values of 0.80 or greater (Fig. 6A & B). The F_{ST} values of COI between populations of the same species, which included *P. appendiculatus* with five populations, *P. lacteus* with three and *P. neapolitanus* with two, were much lower ranging from 0 to 0.55 (Fig. 6A). Among these, relatively higher F_{ST} values (0.31-0.55) were only found for populations of *P. appendiculatus*

that were farthest apart including Rovinj vs. Helgoland, Rovinj vs. Roscoff, and Rovinj vs. Banyuls-sur-Mer (Fig. 5A).

4. Discussion

A combination of morphological, molecular and distributional information was used to distinguish species of polygordiids from the Atlantic and Mediterranean Sea. The phylogenetic and network reconstructions of both the mitochondrial and nuclear data unequivocally support six valid morphologically defined species as monophyletic groups: *P. appendiculatus*, *P. lacteus*, *P. neapolitanus*, *P. triestinus*, *P. jouinae*, and *P. eschaturus*. Moreover, the F_{ST} values indicate that the significant differences between each species pair are fixed. Among these species we did not detect independent clades or strong population structure that would have supported the presence of previously unknown species. However, the phylogenetic and network analyses of mitochondrial genes revealed that within *P. eschaturus* one individual from Santa Catarina differed from all others. Nuclear data and more individuals of *P. eschaturus* from additional locations along the South American coastline are needed to resolve the phylogeography and taxonomy of *P. eschaturus* in more detail. Additionally, there is one species described from the Southwestern Atlantic (*P. leo*) that was not included in our study, as it was not collected. It was described from the same type locality as *P. eschaturus* (Island of São Sebastião, Brazil), but is easily distinguished from *P. eschaturus* in having numerous (8-15) subterminal pygidial cirri. Based on molecular analyses, *Polygordius* spp. from Roscoff were identified as *P. lacteus* (see Table 1; Fig. 2; Supplementary material 3), and both *P. erythrophthalmus* and *P. villoti* are junior synonyms of *P. lacteus*. Thus, to date, there are seven well-defined *Polygordius* species distributed in the Atlantic Ocean (Fig. 2).

Molecular analyses thus answered the long-standing question of the validity of *P. neapolitanus* which is morphologically similar to *P. lacteus* based on adult morphology. Consistent with this, a comprehensive preliminary examination of morphological characters using detailed light and SEM for these two species indicates considerable overlap in variability of characters important in species distinction (e.g., palp length, length ratio palp:prostomium, and number/shape of pygidial glands; Ramey-Balcı, unpublished data). These two species are only discernible based on differences in larval type having an exolarva and endolarva, respectively. Other highly similar species in this group could only be distinguished based on a few, subtle differences in morphology. For example, the distinction between *P. jouinae* and *P. triestinus* as adults is mainly based on *P. jouinae* having longer palps and larger size. Both species have an exolarva. These two species are thought to differ in their reproductive biology; however, the hermaphroditism of *P. triestinus* has been questioned and is unlikely (Schroeder and Hermans 1975; Westheide 2008); all other species of polygordiids, for which reproduction is known, are gonochoristic.

Slight morphological differences such as those observed in the present study, have often been considered sufficient to delineate species in taxonomy. This is especially the case for some interstitial species as slight differences in morphology and/or ecological preferences have aided in their delineation (e.g., Casu and Curini-Galletti, 2006; Derycke et al., 2008; Leasi and Todaro, 2009; Rocha-Olivares et al., 2001; Struck et al., 2017; Westheide and Rieger, 1987). For example, in the *Cletocamptus deitersi* species complex (Harpacticoidea, Crustacea) the difference is an additional inner chaeta on the exopod of the third leg, or in the gastrotrich species complex *Xenotrichula intermedia* the pharynx varies in length (Leasi and Todaro, 2009; Rocha-Olivares et al., 2001).

Following the perspective put forth by Struck et al. (2018), *P. neapolitanus* and *P. lacteus* as well as *P. jouinae* and *P. triestinus* could potentially be considered cryptic

species based solely on biological properties (i.e., degree of morphological disparity and divergence time). However, to verify that significantly lower degrees of morphological disparity are exhibited given the level of genetic divergence, statistically significant studies including detailed analyses of the morphology as well as the genetic divergence would have to be conducted for all *Polygordius* species.

In the present study, species with only very subtle morphological differences were also shown to be sister taxa, but occurred in allopatry. For example, *P. triestinus* is found in the Mediterranean Sea, whereas *P. jouinae* is found in the Northwest Atlantic. The same is true for *P. lacteus* being present in the Atlantic, and *P. neapolitanus* in the Mediterranean Sea.

Our study also found marked morphological differences that were not useful in delineating some species. For example, the number and structure (i.e., forked vs. unforked) of pygidial cirri especially in the Helgoland population of *P. appendiculatus* were variable and this variability was not related to sex or size differences (Ramey-Balcı, unpublished observations). Hempelmann (1906) also found (Fig. 17, p. 587) a single individual of *P. appendiculatus* with three pygidial cirri (collection locality not provided). Likewise, Giard (1880) considered the presence of red “eyes/eyespot” as sufficient to distinguish, *P. erythrophthalmus* from *P. lacteus*. In our analyses, the individuals with similar numbers of appendages or having red “eyes/eyespot” did not cluster together. Molecular analyses verified that *P. erythrophthalmus* is an invalid species being synonymous with *P. lacteus* (also see Lehmacher et al., 2016). Similarly, in the *Gyptis* species complex surface color patterns of these polychaetes (Hesionidae), were diagnostic with two out of three clades, showing one unique color pattern without strong variation. However, the third clade exhibited color variation with three different patterns (Pleijel et al., 2009). Thus the recognition of subtle morphological differences is important for species delimitation; however, it is not straightforward, as significant morphological variation can also confound the delineation

process. Detailed examination of the variation in morphological characters in adults/reproductive stages (on live and fixed material) and distribution patterns, combined with molecular analyses becomes necessary to ultimately distinguish species. This is especially essential in taxa that have a high degree of morphological “simplicity” and “uniformity” such as those living in the interstitial environment.

Some species of *Polygordius* appear to have relatively widespread distributions such as *P. appendiculatus*. This species was originally described from the western Mediterranean (type locality: Gulf of Naples). The current study not only “re-confirmed” the presence of *P. appendiculatus* in western Mediterranean and the NE Atlantic but also extended its range into the eastern Mediterranean and Adriatic Sea (Fig. 2). *Polygordius lacteus*, however, is restricted to the Northeast Atlantic (type locality: Helgoland), and has not been found in the Mediterranean Sea. In contrast to the other species, both *P. appendiculatus* and *P. lacteus* exhibit a dominant haplotype in the NE Atlantic. This could be indicative that populations in this area were affected by a relatively recent decrease in population size. Interestingly, of the areas sampled in our study, this area was most strongly affected by the last glaciations (Behre, 2007; Lambeck, 1995; 1997) which could be hypothesized to have caused a decline in population sizes in this area. *Polygordius neapolitanus* was described from the western Mediterranean (type locality: Gulf of Naples) and with this study its range is extended to the eastern Mediterranean and Adriatic Sea. The distribution of *P. triestinus* (type locality: Gulf of Trieste) is thus far restricted to the Adriatic Sea and this is the first record of this species in more than eight decades (Fig. 2). *Polygordius jouinae* (Fig. 2; type locality: Beach Haven Ridge LEO-15) is found in the Northwest Atlantic in the Mid-Atlantic Bight, North of Cape Hatteras (Ramey and Ambler 2014), whereas, *P. eschaturus* is found in the Southwestern Atlantic off the coast of Brazil (Fig. 2; type locality: Island of São Sebastião). *Polygordius leo* is to date only known from its type locality, the Island of São Sebastião in the Southwestern

Atlantic off the coast of Brazil (Fig. 2). Although *Polygordius* spp. having a planktotrophic larval stage may allow for such widespread distributions compared to other interstitial species, distribution patterns among species in the present study were not related to larval type (exolarvae vs. endolarvae). For example, although *P. appendiculatus* has a more widespread distribution than *P. lacteus*, both of these species exhibit an endolarvae, whereas all other species for which larval type is known have an exolarvae (*i.e.*, *P. jouinae*, *P. triestinus*, *P. neapolitanus*).

Moreover, because the species occurring in the study area had not been thoroughly revised to date, and due to difficulties to distinguish among *Polygordius* species based on morphology alone, identification of *Polygordius* species is often restrained to genus level as *Polygordius* sp., *P. sp. A*, etc., (see Ramey et al., 2006). On the other hand, identification of *Polygordius* might for practical reasons be restricted to the use of distinct characters like presence/absence of pygidial cirri, which might lead to an underestimation of the diversity of *Polygordius* species in a particular location. Thus for a more comprehensive determination of the distributional range of *Polygordius* species occurring in the Atlantic and Mediterranean, beyond that of the present study, previous records need to be identified based on the results presented here. In summary, the resolved phylogeny and taxonomic revision presented here provide the basis to further explore the complex evolutionary history of the genus *Polygordius*. Future work with more rapidly evolving recombining marker systems such as microsatellites or RADseq data may shed light on the phylogeography of species and existing gene-flow among and between their populations.

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509 **Table 1.** List of species of Polygordiidae, locality, latitude/longitude (degrees/decimal minutes), collection date (day/month/year), depth (m),
 510 habitat description based on sediment type, and the number of specimens collected during the present study. Localities in bold represent
 511 species/specimens collected at or near type localities. Sediment type: sh = shell-hash including some larger shell material mixed with fine-coarse
 512 sand; fsh = fine shell-hash and sea anemone spines; csh1 = coarse shell-hash (Punta Croce, Croatia: median Φ = 0; Veštar, Croatia: median Φ = 1);
 513 csh2 = coarse shell-hash mixed with small pieces of coralline algae; mcsh = medium-coarse shell-hash and sand grains (Helgoland: amphioxus sand,
 514 *Amphioxus* (= *Branchiostoma*) is abundant), mdys = muddy sand (Rovinj, Croatia RLV1: median Φ = 4.85; Rovinj; Croatia RLV3: median Φ =
 515 3.94); fcs = fine-coarse sand (glycerids and *Amphioxus* abundant); cs = coarse sand (Santa Catarina, Brazil: median Φ = 1.2-1.5; Tuckerton, United
 516 States: mean 1 Φ ; Figarola Island, Croatia: median Φ = 0.84), and cvs = very coarse sand mixed with some shell-hash, I = intertidal, wc = water
 517 column.

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Species	Locality	Latitude/longitude	Collection date	Depth (m)	Sediment type	Number of specimens	Total
<i>Polygordius lacteus</i>	Helgoland, Germany	54°15.1'N, 7°56.9'E	08.07.10	20	mcs	4	21
	Roscoff, Primel, France	48°43.4'N, 3°51.0'W	17.03.11	25	csh1	8	
	Beg Meil, France	47°50.9'N, 3°58.2'W	19.03.11	10	csh2	9	
<i>Polygordius appendiculatus</i>	Helgoland, Germany	54°15.1'N, 7°56.9'E	23.09.10/08.07.10	20	mcs	9	35
	Helgoland, Germany	54°11.6'N, 7°52.7'E	13.07.10	4	csh1	1	
	Roscoff, Drezen, France	48°45.5'N, 4°05.6'W	16.03.11/25.03.11	50/54	fsh	3/5	
	Roscoff, Drezen, France	48°44.5'N, 4°05.7'W	16.03.11	70	sh	1	
	Ischia, Italy	40°45.1'N, 13°56.0'E	03.05.11	7.5	cs	10	
	Rovinj, Punta Croce, Croatia	45°6.8'N, 13°36.5'E	23.07.08	7	csh1	3	
	Rovinj, Figarola Is., Croatia	45°5.7'N, 13°37.2'E	29.09.11	13	cs	1	
	Banyuls-sur-Mer, "Le Troc" France	42°28.8'N, 3°8.6'E	21.10.10	2-3	fcs	2	
<i>Polygordius neapolitanus</i>	Ischia, Italy	40°45.1'N, 13°56.0'E	03.05.11	7.5	cs	8	19
	Rovinj, Veštar, Croatia	45°2.9'N, 13°41.1'E	23.07.08	6	csh1	1	
	Rovinj, Punta Croce, Croatia	45°6.8'N, 13°36.5'E	23.07.08/17.05.11	7/4	csh1	10	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	27°3.1'S, 48°35.3'W	10.10.10	i	cs	12	13
	Rio de Janeiro, Brazil	22°56.7'S, 43°9.3'W	10.10.10	i	cs	1	
<i>Polygordius jouinae</i>	Tuckerton, New Jersey, USA	39°27.7'N, 74°15.8'W/	13.05.08/	12	cs	6/2	11
		39°27.9'N, 74°15.1'W	20.07.10				
	Chincoteague, Virginia, USA	37°35.9'N, 74°45.9'W	23.06.10	wc	n/a	3	
<i>Polygordius triestinus</i>	Rovinj, Valdibora harbor, Croatia	45°05.1'N, 13°38.0'E/ 45°05.2'N, 13°38.3'E	30.09.11	19/12	mdys	4/1	5
Total specimens collected							104

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Figure legends

Figure 1. Light microscope images and scanning electron micrographs of polygordiids showing morphology: A. adult *P. appendiculatus* from North Sea (Helgoland, Germany; 2.5 cm total length); B. head, C. pygidium of adult *P. appendiculatus* Mediterranean Sea (Ischia, Italy); D. exolarvae, and E. endolarvae of *P. jouinae* and *Polygordius* sp. respectively from western Atlantic (off Chincoteague, Virginia), and F-G. *P. appendiculatus* from Helgoland with 3-4 cirri and forked cirri respectively. *Scale bars* A 480 μm ; B 60 μm ; C 25 μm ; D 125 μm ; E 140 μm ; F 100 μm ; G 50 μm . *al* anal lobes, *ao* apical organ, *c* cirri, *ep* episphere, *g* gland, *h* hyposphere, *hf* head fold, *m* metatroch, *mo* mouth, *no* nuchal organs, *p* prototroch, *pp* paired palps, *pr* prostomium, *py* pygidium, *t* worm trunk

Figure 2. Summary of the geographic distribution of: A. six species of *Polygordius* considered to be valid (to date) based on collections/material examined in the present study. Colored circles in (A) indicate regions sampled in the present study (legend in lower left corner). Note: different locations/populations within regions not indicated. Numbers indicate species, with white numbers indicating species collected at or near a type locality. 1 = *P. lacteus* Schneider, 1868; 2 = *P. neapolitanus* Fraipont, 1887; 3 = *P. appendiculatus* Fraipont, 1887; 4 = *P. triestinus* Hempelmann 1906; 5 = *P. eschaturus* Marcus, 1948; 6 = *P. jouinae* Ramey, Fiege, Leander, 2006; 7 = *P. leo* Marcus, 1955; NS = North Sea; MS = Mediterranean Sea. Raw map image from OBIS <http://www.iobis.org/>

Figure 3. Phylogram of the ML analysis of mitochondrial dataset (i.e., combined COI/16S). Only bootstrap values ≥ 70 shown above the branches. For branches for which the bootstrap value could not be placed, the corresponding branch is indicated by a line. Species in bold/italics and number of individuals in each population provided in brackets. Individual specimens labeled by the collection number (see Supplementary material 3) and color-coded by population (legend in the upper right corner).

Figure 4. Phylogram of the ML analysis of nuclear ITS dataset with outgroup. The analyses without outgroup were congruent with unrooted tree of this dataset. Therefore, bootstrap values are shown at the corresponding branches. Only bootstrap values ≥ 70 are shown above the branches. The upper value is from the analysis with the outgroup, middle with the outgroup excluded, and lower with outgroup excluded and aligned as well as masked anew. For branches for which the bootstrap value could not be placed, the corresponding branch is indicated either by a line or a bracket. Species in bold/italics and number of individuals in each population provided in brackets. Individual specimens labeled by the collection number (see Supplementary material 3) and color-coded by population (legend in the upper right corner). n.a. = not applicable.

Figure 5. Haplotype networks of the COI/16S dataset. Species in italics. Size of the circles correspond to number of specimens with this haplotype (see legend lower right corner). For each haplotype with more than one specimen the number of specimens from each population is provided. Each population is color-coded (legend in the lower right corner). Black dots represent reconstructed haplotypes at edges of the network, which were not found. Numbers above the connecting branches indicate the number of substitutions between two haplotypes. If no number is given the value is 1.

Figure 6. Pairwise comparisons of FST values for the CO1 (A) and the 16S (B) datasets differentiating between the clades (i.e., species) reconstructed in Fig. 3. Species were compared at the population level. The scale bar to right of each plot indicates the color scale for the FST values. BAF = Banyuls-sur-Mer (France), BMF = Beg Meil (France), HG = Helgoland (Germany), II = Ischia (Italy), P.a. = *P. appendiculatus*, P.e. = *P. eschaturus*, P.j. = *P. jouinae*, P.l. = *P. lacteus*, P.n. = *P. neapolitanus*, P.t. = *P. triestinus*, RC = Rovinj (Croatia), RF = Roscoff (France), SB = Santa Catarina/Rio de Janeiro (Brazil), TU = Tuckerton/Chincoteague (USA).

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 6

Supplementary material 1. Video of *P. lacteus* burrowing into coarse sandy sediments (0.63x magnification). Collected at Helgoland Germany.

Supplementary material 2. Video of *P. appendiculatus* (female with eggs) showing the pygidial cirri and how the glands surrounding the pygidium can be used to anchor it to the substrate (in this case the petridish) (0.63x magnification). Collected at Helgoland Germany.

Supplementary material 3

List of species, locality, and corresponding sequences generated in the present study and submitted to GenBank. For each sequence the corresponding catalog number, field collection number and tube number of the DNA extract and/or voucher material used for morphological analysis is provided (SMF = Senckenberg Museum Frankfurt; and ZUEC = Museu de Zoologia da Universidade Estadual de Campinas “Adão José Cardoso”). Accession numbers of previously published sequences of Polygordiidae ($n = 13$) and outgroup species ($n = 7$) used in the study appear in bold. Specimens collected at or near type localities indicated in bold. Blank = no sequence. COI = Cytochrome Oxidase Subunit 1; 16S = 16S ribosomal RNA; ITS1/2 = Internal transcribed spacer 1 and 2; * = prostomial red “eyesspots” observed thus initially identified as *Polygordius erythrophthalmus* (see Lehmacher et al. 2016); ** = more than 2 cirri and may be forked.

Species	Locality	Catalogue number (SMF)	Collection number	Tube number	Accession number		
					COI	16S	ITS1/2
<i>Polygordius lacteus</i>	Helgoland, Germany	22006	H1 24	25	KT883953	MG603539	
<i>Polygordius lacteus</i>	Helgoland, Germany	22007	H1 25	14	KT883952	MG603538	MH128715
<i>Polygordius lacteus</i>	Helgoland, Germany	22008	H1 29	15	KT883951	MG603540	
<i>Polygordius lacteus</i>	Helgoland, Germany	22009	H1 31	21	KT883950	MG603541	MH128716
<i>Polygordius lacteus</i>	Roscoff, Primel, France	21700	P 1	227	MG603415	MG603547	
<i>Polygordius lacteus</i>	Roscoff, Primel, France	21701	P 2	228	MG603416	MG603548	
<i>Polygordius lacteus</i>	Roscoff, Primel, France	21702	P 3	229	MG603417	MG603549	
<i>Polygordius lacteus</i>	Roscoff, Primel, France	21703	P 4	230			MH128706
<i>Polygordius lacteus</i>	Roscoff, Primel, France	21704	P 5	231	MG603418	MG603553	MH128707
<i>Polygordius lacteus</i>	Roscoff, Primel, France	21705	P 6	232	MG603419	MG603551	
<i>Polygordius lacteus</i>	Roscoff, Primel, France	21706	P 7	233	MG603420	MG603552	MH128708
<i>Polygordius lacteus</i>	Roscoff, Primel, France	21707	P 8	234	MG603421	MG603550	
<i>Polygordius lacteus</i>	Beg Meil, France	22213	BM 7	52	KT883948	MG603542	
<i>Polygordius lacteus</i>	Beg Meil, France	22214	BM 8	53	KT883949	MG603543	
<i>Polygordius lacteus</i>	Beg Meil, France	21715	BM 10	55	MG603412	MG603544	MH128709
<i>Polygordius lacteus</i>	Beg Meil, France	21716	BM 11	56	MG603413	MG603545	MH128710

<i>Polygordius lacteus</i>	Beg Meil, France	21717	BM 12	57	MG603414	MG603546	MH128711
* <i>Polygordius lacteus</i>	Beg Meil, France	22207	BM 1	47	KT883937	MG603554	MH128712
* <i>Polygordius lacteus</i>	Beg Meil, France	22208	BM 2	48	KT883938	MG603555	MH128713
* <i>Polygordius lacteus</i>	Beg Meil, France	22215	BM 9	54	MG603411	MG603556	MH128714
* <i>Polygordius lacteus</i>	Beg Meil, France	22218	BM 26	58	KT883939	MG603557	
<i>Polygordius appendiculatus</i>	Helgoland, Germany	22033	H2 9	6	MG603445	MG603481	MH128702
<i>Polygordius appendiculatus</i>	Helgoland, Germany	22017	H2 11	181	MG603441	MG603487	
<i>Polygordius appendiculatus</i>	Helgoland, Germany	22035	H2 16	5	MG603446	MG603483	
<i>Polygordius appendiculatus</i>	Helgoland, Germany	22018	H2 12	182	MG603442	MG603488	
** <i>Polygordius appendiculatus</i>	Helgoland, Germany	22019	H1 13	183	MG603440	MG603482	
<i>Polygordius appendiculatus</i>	Helgoland, Germany	22004	H1 22	3	MG603443	MG603484	
** <i>Polygordius appendiculatus</i>	Helgoland, Germany	22011	H1 33	4	MG603444	MG603485	
** <i>Polygordius appendiculatus</i>	Helgoland, Germany	22012	H1 34	146	MG603459	MG603499	MH128700
<i>Polygordius appendiculatus</i>	Helgoland, Germany	22013	H1 35	147	MG603457	MG603486	MH128701
<i>Polygordius appendiculatus</i>	Helgoland, Germany	22015	H1 52	149	MG603466	MG603502	
<i>Polygordius appendiculatus</i>	Roscoff, Drezen, France	22219	D 2	59	MG603455	MG603495	
<i>Polygordius appendiculatus</i>	Roscoff, Drezen, France	21728	D 5	61	MG603456	MG603496	
<i>Polygordius appendiculatus</i>	Roscoff, Drezen, France	21741	D 6	165	MG603461	MG603490	
<i>Polygordius appendiculatus</i>	Roscoff, Drezen, France	21742	D 10	166	MG603465	MG603491	MH128697
<i>Polygordius appendiculatus</i>	Roscoff, Drezen, France	21743	D 11	167	MG603458	MG603501	
<i>Polygordius appendiculatus</i>	Roscoff, Drezen, France	21744	D 16	168	MG603464	MG603492	MH128698
<i>Polygordius appendiculatus</i>	Roscoff, Drezen, France	21745	D 23	169	MG603467		
<i>Polygordius appendiculatus</i>	Roscoff, Drezen, France	21746	D 25	170	MG603462	MG603493	
<i>Polygordius appendiculatus</i>	Roscoff, Drezen, France	21747	D 27	171	MG603463	MG603494	MH128699
<i>Polygordius appendiculatus</i>	Ischia, Italy	21786	I 3	113	KT883955	MG603500	
<i>Polygordius appendiculatus</i>	Ischia, Italy	21787	I 4	114	KT883954	MG603478	
<i>Polygordius appendiculatus</i>	Ischia, Italy	21788	I 8	115	MG603453	MG603479	
<i>Polygordius appendiculatus</i>	Ischia, Italy	22242	I 15	109	MG603450	MG603475	MH128694
<i>Polygordius appendiculatus</i>	Ischia, Italy	22229	I 16	110	MG603451	MG603476	MH128695
<i>Polygordius appendiculatus</i>	Ischia, Italy	22243	I 18	111	MG603452	MG603477	MH128696
<i>Polygordius appendiculatus</i>	Ischia, Italy	22231	I 23	73	MG603447	MG603472	
<i>Polygordius appendiculatus</i>	Ischia, Italy	22233	I 33	75	MG603448	MG603473	
<i>Polygordius appendiculatus</i>	Ischia, Italy	21800	I 40	205	MG603454	MG603480	
<i>Polygordius appendiculatus</i>	Ischia, Italy	22241	I 50	81	MG603449	MG603474	
<i>Polygordius appendiculatus</i>	Rovinj, Punta Croce, Croatia	22167	SCC 4	27	MG603468	MG603503	MH128704
<i>Polygordius appendiculatus</i>	Rovinj, Punta Croce, Croatia	22170	SCC 7	35	MG603469	MG603504	MH128705
<i>Polygordius appendiculatus</i>	Rovinj, Punta Croce, Croatia	22172	SCC 9	37	MG603470	MG603505	
<i>Polygordius appendiculatus</i>	Rovinj, Figarola Is., Croatia	22179	1RLK 1	212	MG603460	MG603497	MH128703
<i>Polygordius appendiculatus</i>	Banyuls-sur-Mer, France	22036	B 1	9	MG595331	MG603489	
<i>Polygordius appendiculatus</i>	Banyuls-sur-Mer, France	22036	B 2	10	MG595332	MG603498	

<i>Polygordius neapolitanus</i>	Ischia, Italy	22227	I 10	70	MG603434	MG603516	
<i>Polygordius neapolitanus</i>	Ischia, Italy	22230	I 21	72	MG603431	MG603517	MH128723
<i>Polygordius neapolitanus</i>	Ischia, Italy	22232	I 32	74			MH128724
<i>Polygordius neapolitanus</i>	Ischia, Italy	22234	I 35	76	MG603432	MG603531	MH128725
<i>Polygordius neapolitanus</i>	Ischia, Italy	22236	I 36	204	MG603436	MG603520	
<i>Polygordius neapolitanus</i>	Ischia, Italy	22238	I 38	78	MG603435	MG603518	
<i>Polygordius neapolitanus</i>	Ischia, Italy	22239	I 39	79	MG603437	MG603519	
<i>Polygordius neapolitanus</i>	Ischia, Italy	21801	I 41	206	MG603433	MG603521	
<i>Polygordius neapolitanus</i>	Rovinj, Veštar, Croatia	22169	SCV 6	29	MG603427	MG603530	MH128721
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22166	SCC 3	26	MG603426	MG603527	
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22173	SCC 10	38	MG603429	MG603529	MH128722
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22164	SC 1	7	MG603430	MG603526	MH128720
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22171	SCC 8	36	MG603428	MG603528	
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22182	RB 2	101	MG603422	MG603522	MH128717
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22185	RB 5	104	MG603423	MG603523	
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22186	RB 6	105	MG603438	MG603532	MH128718
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22187	RB 7	106	MG603424	MG603524	MH128719
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22188	RB 8	107	MG603439		
<i>Polygordius neapolitanus</i>	Rovinj, Punta Croce, Croatia	22189	RB 9	108	MG603425	MG603525	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	ZUEC13545 & 13535	BZ BC1	30	MG603404	MG603559	MH128690
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	ZUEC13546	BZ BC2	31	MG603392	MG603560	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	ZUEC13547 & 13536	BZ BC3	32	MG603393	MG603561	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	ZUEC13548	BZ BC4	39	MG603396	MG603562	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	ZUEC13449	BZ BC5	40	MG603397	MG603563	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	22348	BZ BC6	41	MG603398	MG603564	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	22349	BZ BC7	42	MG603399	MG603565	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	22350	BZ BC8	43	MG603400	MG603566	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	22351	BZ BC9	44	MG603401	MG603567	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	22352	BZ BC10	45	MG603402	MG603558	
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	22353	BZ BC11	46	MG603403	MG603568	
<i>Polygordius eschaturus</i>	Rio de Janeiro, Brazil	ZUEC13550	BZ PV1	33	MG603394	MG603569	MH128687
<i>Polygordius eschaturus</i>	Santa Catarina, Brazil	ZUEC13551	BZ PV2	34	MG603395	MG603570	MH128688
<i>Polygordius jouinae</i>	Tuckerton, New Jersey, USA	24362	11 A7	186	MG603405	MG603511	MH128691
<i>Polygordius jouinae</i>	Tuckerton, New Jersey, USA	24369	12 B7	187		MG603512	
<i>Polygordius jouinae</i>	Tuckerton, New Jersey, USA	24360	13 C7	188	MG603406	MG603513	MH128692
<i>Polygordius jouinae</i>	Tuckerton, New Jersey, USA	24363	14 D7	189	MG603471		MH128693
<i>Polygordius jouinae</i>	Tuckerton, New Jersey, USA	24361	15 E7	190	MG603407	MG603514	
<i>Polygordius jouinae</i>	Tuckerton, New Jersey, USA	24364	16 F7	191	MG603408	MG603515	

<i>Polygordius jouinae</i>	Tuckerton, New Jersey, USA	22357	LEO 1	18	MG603410	MG603509	
<i>Polygordius jouinae</i>	Tuckerton, New Jersey, USA	22358	LEO 2	19	MG603409	MG603510	
<i>Polygordius jouinae</i>	Chincoteague, Virginia, USA	22361	8 exo	96	JQ011388	MG603507	
<i>Polygordius jouinae</i>	Chincoteague, Virginia, USA	22362	12 exo	97	JQ011386	MG603508	
<i>Polygordius jouinae</i>	Chincoteague, Virginia, USA	22360	17 exo	95	JQ011384	MG603506	
<i>Polygordius triestinus</i>	Rovinj, Valdibora harbor, Croatia	22174	0RLV 1	207		MG603537	
<i>Polygordius triestinus</i>	Rovinj, Valdibora harbor, Croatia	22178	1RLV 3	211		MG603536	MH128689
<i>Polygordius triestinus</i>	Rovinj, Valdibora harbor, Croatia	22176	2RLV 3	209		MG603534	
<i>Polygordius triestinus</i>	Rovinj, Valdibora harbor, Croatia	22175	3RLV 3	208		MG603533	
<i>Polygordius triestinus</i>	Rovinj, Valdibora harbor, Croatia	22177	4RLV 3	210		MG603535	
<i>Riftia pachyptila</i>					AY741662		
<i>Orbinia latreillii</i>					AY961084		
<i>Terebellides stroemi</i>					EU236701		
<i>Nephtys sp.</i>					EU293739		
<i>Platynereis dumerilii</i>					AF178678		
<i>Perinereis aibuhitensis</i>					AF33215,		
					GQ478872		
<i>Harmothoe imbricata</i>					GQ478872		

Supplementary material 4. Primers used in amplification and sequencing along with polymerase chain reaction (PCR) cycling conditions for each gene provided below.

Name	Sequence 5' to 3'	Direction	Reference
COI			
LCO1490	GGT CAA CAA ATC ATA AAG ATA TTGG	forward	Folmer et al. 1994
HCO 2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	reverse	Folmer et al. 1994
LCO_external	TGA TTA TTC TCA ACC AAT CAT AAA GA	forward	This study
16S rDNA			
16SAN-F	TAC CTT TTR CAT CAT GG	forward	Zanol et al. 2010
16SAN-R	GCT TAC GCC GGT CTG AAC TCA G	reverse	Zanol et al. 2010
16SarL	CGC CTG TTT ATC AAA AAC AT	forward	Palumbi et al. 1991
16SbrH	CCG GTC TGA ACT CAG ATC ACG T	reverse	Palumbi et al. 1991
ITS1/2			
18Sf	GGA AGT AAA AGT CGT AAC AAG	forward	Böggemann 2009
28SR	AAT GCT TAA ATT CAG CGG GTA	reverse	Westheide and Schmidt 2003

Polymerase chain reaction (PCR) cycling conditions for COI included: initial denaturation at 94°C for 3 min; 50 cycles of denaturation at 94°C for 30 sec; annealing at 45°C for 30 sec; elongation at 65°C for 3 min; final extension at 65°C for 7 min. PCR reactions took place in a 25- μ l solution containing: 0.3 μ l Hot Master Taq DNA Polymerase (5 U μ l⁻¹) (Promega); ~1 μ l genomic DNA template; 17.5 μ l ddH₂O; 2.5 μ l 10x buffer with MgCl₂ (25 mM); 2.0 μ l dNTP (2.5 mM); and 1 μ l (10 pmol μ l⁻¹) of each primer. Protocol for PCR of 16S included: initial denaturation at 94°C for 4 min; 40 cycles of denaturation at 94°C for 30 sec (decreasing 0.2°C each cycle); annealing at 51°C for 30 sec; elongation at 65°C for 2 min; final extension at 65°C for 5 min. PCR reactions took place in a 25- μ l solution containing: 0.25 μ l Hot Master Taq DNA Polymerase (5 U μ l⁻¹) (Promega); ~1 μ l genomic DNA template, 13.25 μ l ddH₂O; 2.5 μ l 10x buffer with MgCl₂ (25 mM); 4.0 μ l dNTP (2.5 mM); and 2 μ l (10 pmol μ l⁻¹) of each primer. Protocol for ITS1/2 included initial denaturation at 94°C for 2 min; 50 cycles of denaturation at 94°C for 20 sec; annealing at 53°C for 20 sec; elongation at 65°C for 3 min; final extension at 65°C for 6 min. PCR reactions took place in a 25- μ l solution containing: 0.1 μ l Hot Master Taq DNA Polymerase (5 U μ l⁻¹) (Promega); ~1 μ l genomic DNA template; 16.4 μ l ddH₂O; 2.5 μ l 10x buffer with MgCl₂ (25 mM); 4.0 μ l dNTP (2.5 mM); and 0.5 μ l (10 pmol μ l⁻¹) of each primer.

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Supplementary material 5. Summary of sequence data used in analyses including the number of sequences (no. seq.), average length (no. bp) with standard deviation (st. dev.), minimum number of base pairs (min no. bp), and the maximum number of basepairs (max no. bp) for CO1, 16S and ITS1/2 genes for each species at each location sampled.

Gene	Species	Location	No. seq.	Average length (no. bp)	St. dev.	Min (no. bp)	Max (no. bp)
CO1	<i>P. lacteus</i>	Roscoff	7	685	8	676	697
		Helgoland	9	625	38	595	680
		Beg Meil	4	618	13	612	652
	<i>P. neapolitanus</i>	Rovinj	11	634	28	598	689
		Ischia	7	667	19	628	685
	<i>P. appendiculatus</i>	Roscoff	9	667	29	595	683
		Helgoland	10	664	18	630	683
		Rovinj	4	675	2	672	677
		Ischia	10	641	35	615	675
		Banyuls	2	649	0.7	648	649
	<i>P. jouinae</i>	Tuckerton/Chincoteague	10	645	30	603	678
	<i>P. triestinus</i>	Rovinj	0				
	<i>P. eschaturus</i>	Santa Catarina/Rio de Janerio	13	629	11	618	654
16S	<i>P. lacteus</i>	Roscoff	7	971	35	924	1010
		Helgoland	4	975	31	934	1006
		Beg Meil	9	961	7	951	971
	<i>P. neapolitanus</i>	Rovinj	10	966	15	948	991
		Ischia	7	965	17	930	978
	<i>P. appendiculatus</i>	Roscoff	8	953	22	922	995
		Helgoland	10	960	41	902	1014
		Rovinj	4	963	5	959	969
		Ischia	10	956	15	929	985
		Banyuls	2	977	21	962	991
	<i>P. jouinae</i>	Tuckerton/Chincoteague	10	570	227	447	1000
	<i>P. triestinus</i>	Rovinj	5	883	21	866	912
	<i>P. eschaturus</i>	Santa Catarina/Rio de Janerio	15	956	8	946	965
ITS1/2	<i>P. lacteus</i>	Roscoff	3	836	1.4	835	838
		Helgoland	2	773	0.7	772	773
		Beg Meil	6	782	9	771	793
	<i>P. neapolitanus</i>	Rovinj	6	796	24	778	842
		Ischia	3	794	3.4	789	797
	<i>P. appendiculatus</i>	Roscoff	3	825	25	795	856
		Helgoland	3	820	42	761	853
		Rovinj	3	777	19	759	803
		Ischia	3	776	0.9	775	777
		Banyuls	0				

	<i>P. jouinae</i>	Tuckerton/Chincoteague	3	879	3	876	882
	<i>P. triestinus</i>	Rovinj	1	909			
	<i>P. eschaturus</i>	Santa Catarina/Rio de Janeiro	3	864	8	855	871

Supplementary material 6.

Phylogram of the ML analysis of CO1 dataset. Only bootstrap values ≥ 70 shown above the branches. For branches for which the bootstrap value could not be placed, the corresponding branch is indicated either by a line or bracket. Species in bold/italics and number of individuals in

each population provided in brackets. Individual specimens labeled by collection number (see Supplementary material 3) and color coded by population (legend in the upper right corner).

Supplementary material 7.

Phylogram of the ML analysis of 16S dataset. Only bootstrap values ≥ 70 shown above the branches. For branches for which the bootstrap value could not be placed, the corresponding

branch is indicated either by a line or a bracket. Species in bold/italics and number of individuals in each population provided in brackets. Individual specimens labeled by the collection number (see Supplementary material 3) and color-coded by population (legend in the upper right corner).

Supplementary material 8.

P. lacteus

P. neapolitanus

P. eschaturus

P. appendiculatus

P. jouinae

Haplotype networks of the COI dataset. Species in italics. Size of the circles correspond to number of specimens with this haplotype (see legend lower right). For each haplotype with more than one specimen the number of specimens from each population is provided. Each population is color-coded (legend in the upper right corner). Black dots represent reconstructed haplotypes at

edges of the network, which were not found. Numbers above the connecting branches indicate the number of substitutions between two haplotypes. If no number is given the value is 1.

Supplementary material 9.

Haplotype networks of the 16S dataset. Species in italics. Size of the circles correspond to number of specimens with this haplotype (see legend lower right). For each haplotype with more than one

specimen the number of specimens from each population is provided. Each population is color-coded (legend in the upper right corner). Black dots represent reconstructed haplotypes at edges of the network, which were not found. Numbers above the connecting branches indicate the number of substitutions between two haplotypes. If no number is given the value is 1.