

## COI barcoding of *Hydroides*: a road from impossible to difficult

Y. Sun<sup>A</sup>, E. K. Kupriyanova<sup>B</sup> and J. W. Qiu<sup>A,C</sup>

<sup>A</sup>Hong Kong Baptist University, Department of Biology, 224 Waterloo Road, Kowloon, Hong Kong, China.

<sup>B</sup>The Australian Museum, Marine Invertebrates Section, 6 College Street, Sydney, Australia.

<sup>C</sup>Corresponding author. Email: [qiujuw@hkbu.edu.hk](mailto:qiujuw@hkbu.edu.hk)

**Abstract.** A fragment of the cytochrome *c* oxidase subunit I (COI) gene has been used increasingly for species identification and discovery in eukaryotes. However, amplifying COI has proven difficult, or even impossible, in some taxa due to non-homology between the universal primers and the target DNA region. Among the most problematic animal groups is Serpulidae (Annelida). These sedentary marine animals live in self-secreted calcareous tubes and many of them, especially of the genus *Hydroides*, are economically important reef-builders, foulers, and biological invaders. We developed novel taxon-specific primers for amplifying COI from *Hydroides*, and for the first time generated 460-bp COI sequences from 11 of 14 species attempted. Average Kimura-2-parameter interspecific sequence distance (26.2%) was >60 times greater than the average intraspecific distance (0.43%), indicating that the COI gene is effective for species delimitation in *Hydroides*. Although applicability of the new primers for a wide range of serpulids needs to be tested, barcoding of *Hydroides* is now on its way from impossible to difficult. We anticipate that COI barcoding will provide a modern species identification tool and, combined with other molecular markers, yield important insights in phylogeny and evolutionary ecology of this large and important genus.

**Additional keywords:** DNA, polychaeta, serpulidae.

Received 11 April 2012, accepted 13 September 2012, published online 19 December 2012

### Introduction

DNA barcoding is a tool of species identification and discovery using a short and standardised gene region (Hebert *et al.* 2003). The best barcode candidate for animal groups, a 648-base-pair locus of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, has several desirable properties: it is present in all animal taxa, short enough to be sequenced easily and long enough to provide sufficient interspecific variations, and sequenceable using ‘universal’ PCR primers. This DNA barcode has been proven effective in diverse groups of animals at the species level, with a success rate of >98% (Park *et al.* 2011) for as many as 116 369 animal species registered with a barcode according to BOLD3 (<http://www.barcodinglife.com>, accessed on 14 August 2012). DNA barcoding has many applications, such as determination of species status and description of new species (Halt *et al.* 2009), revealing cryptic species (Hebert *et al.* 2004b; Smith *et al.* 2006), linking adult with juvenile (Thomas *et al.* 2005) or male with female of the same species (Willassen 2005).

One important prerequisite for applying DNA barcoding is successful PCR amplification of the gene fragment of interest. Previous research has revealed that the standard primer set LCO1490/HCO2198 (Folmer *et al.* 1994) cannot be used for amplification of the COI gene from some sponges (Erpenbeck *et al.* 2006), insects (Kondo *et al.* 2008), annelids (Halanych and Janosik 2006; Halt *et al.* 2009; Carr *et al.* 2011) and fish (Sevilla *et al.* 2007). This problem has led some researchers to propose

alternative genes for barcoding animals, including some evolving at a lower rate than COI (Sevilla *et al.* 2007; Smith *et al.* 2007). However, this deviates from the original idea of barcoding using one standardised gene region for species-level identification in all animals, making it more difficult to catalogue and compare the data. Therefore, efforts have been directed to develop new primers in order to recover the COI barcoding region for those taxa that are not responsive to the standard primer set (Carr *et al.* 2011; Park *et al.* 2011).

Polychaeta, the largest class of Annelida, with 81 families and over 12 000 described species, is often the dominant group of macrobenthos in marine ecosystems in terms of both species numbers and abundance (Hutchings 1998). Although COI has been demonstrated to be a good barcode to distinguish species and reveal cryptic species of polychaetes, it is difficult to amplify in some members of Cirratulidae, Nephtyidae, Spionidae, Sabellidae, and especially Serpulidae (Pleijel *et al.* 2009; Barroso *et al.* 2010; Nygren and Pleijel 2011; Carr *et al.* 2011). Serpulidae is a monophyletic clade of polychaetes (Kupriyanova *et al.* 2006; Lehrke *et al.* 2007) living in self-secreted calcareous tubes and using a beautiful branchial crown for both feeding and respiration. From a barcoding point of view, Serpulidae is by far the most problematic polychaete family, appropriately termed a ‘nightmare group’ by Dirk Steinke (pers. comm.). Most attempts to amplify and sequence COI in serpulids using universal primers (e.g. *Hydroides*: Toril Loennechen Moen,

pers. comm.; *Marifugia*: Valerija Zakšek, pers. comm.; *Spirobranchus*: Craig Starger and Paulo Paiva, pers. comm.) or to develop taxon-specific primers (*Galeolaria*: Halt *et al.* 2009) known to us have not been successful. The only successful amplifications were reported by Carr *et al.* (2011), who obtained sequences using primers designed specifically for polychaetes.

Because of their hard calcareous tubes and long-living planktotrophic larvae, some serpulids, especially in the genera *Hydroides*, *Ficopomatus*, *Serpula* and *Spirobranchus*, are economically important reef-builders, foulers, and bio-invasers (Schwindt *et al.* 2001; Nishi and Kato 2004). *Hydroides* Gunnerus, 1768, the largest genus of Serpulidae with ~100 species (ten Hove and Kupriyanova 2009), includes several species of bio-invasers and foulers (Qiu and Qian 1997; Lewis 2006; Otani and Yamanishi 2010; Tovar-Hernández *et al.* 2009). These worms can form dense aggregates on underwater structures such as aquaculture nets, seawater intake pipes and ship hulls and buoys (Qiu and Qian 1997), and therefore are important nuisances to marine aquaculture, navigation, shipping industries and power plants. Recently, *Hydroides elegans* was reported to be the major fouler of submarine seawater piping systems on boats based in Sydney, and was also problematic for the new submarines built in Adelaide (John Lewis, pers. comm.). Millions of dollars are spent each year to prevent the fouling of marine organisms, especially *Hydroides*, on man-made structures (Dürr and Watson 2010). Foulers can modify ecosystem dynamics and species assemblages through competition for space and food. For example, outbreaks of introduced *Hydroides elegans* caused serious damage to cultured oyster crops in Japan (Arakawa 1971). Of the 18 polychaete species on the list of 100 'worst invasives' in the Mediterranean (Streftaris and Zenetos 2006), 12 species were serpulids, including eight species of *Hydroides*.

Identification of the species in question is the first and foremost step towards an understanding of the risk of potential bioinvasion. Revision based on morphological characters (Bastida-Zavala and ten Hove 2002, 2003) has shown that many species of this genus can be distinguished easily by the structures of the operculum, a modified radiole of the tentacle crown serving as a plug to close the opening of the tube. Some species with identical opercula, such as *H. elegans* and *H. norvegicus*, can still be distinguished by the morphology of the chaetae (ten Hove 1974). However, in many other species, such as *H. albiceps* and *H. trivesiculosus*, *H. bispinosus* and *H. crucigera*, *H. elegans* and *H. longispinosus*, the opercula are very similar and their intraspecific variations in morphological characters largely overlap with interspecific variations (ten Hove and Jansen-Jacobs 1984; Fiege and Sun 1999; Bastida-Zavala and ten Hove 2002). Clearly, an efficient molecular approach for species identification such as barcoding is urgently needed for serpulids, especially *Hydroides*.

The goal of this study was to develop taxon-specific primers that can be used to reliably amplify and sequence the barcoding fragment of COI in *Hydroides*. We reassessed the potential application of the universal COI primers (Folmer *et al.* 1994) and the polychaete-specific primers (Carr *et al.* 2011) for use in *Hydroides*, designed novel *Hydroides*-specific primers, and tested the effectiveness of the new primers for barcoding *Hydroides* and a non-*Hydroides* serpulid available to us.

## Materials and methods

### Taxon sampling and identification

A total of 21 serpulid specimens belonging to 14 species of *Hydroides* and one species of *Serpula* were used in this study. The details of taxon names, collection localities, voucher numbers, and GenBank accession numbers are given in Table 1. Freshly

**Table 1. Species included in the present study**  
Collection locations, voucher numbers and accession numbers are shown (for those successfully amplified by PCR)

Species	Collection location	Voucher no.	Accession no.
<i>Hydroides albiceps</i> (Grube, 1870) (1)	Hong Kong, China	AM W.40534	–
<i>Hydroides albiceps</i> (Grube, 1870) (2)	Chiba, Japan	AM W.40535	–
<i>Hydroides brachyacanthus</i> Rioja, 1941	Mazatlan, Mexico	AM W.40536	JQ885941
<i>Hydroides</i> cf. <i>brachyacanthus</i> Rioja, 1941	Mazatlan, Mexico	AM W.40537	JQ885942
<i>Hydroides crucigera</i> (Mörch, 1863)	Mazatlan, Mexico	AM W.40538	JQ885947
<i>Hydroides diramphus</i> Mörch, 1863	Hong Kong, China	AM W.40539	JQ885946
<i>Hydroides elegans</i> (Haswell, 1883) (1)	Hong Kong, China	AM W.40540	JQ885938
<i>Hydroides elegans</i> (Haswell, 1883) (2)	Sydney, Australia	AM W.40541	JQ885939
<i>Hydroides exaltatus</i> (Marenzeller, 1885)	Hong Kong, China	AM W.40542	–
<i>Hydroides ezoensis</i> Okuda, 1934 (1)	Qingdao, China	AM W.40543	–
<i>Hydroides ezoensis</i> Okuda, 1934 (2)	Vladivostok, Russia	AM W.40544	JQ885951
<i>Hydroides fuscicola</i> Mörch, 1863	Manazuru, Japan	AM W.40545	JQ885950
<i>Hydroides longistylaris</i> Wu & Chen, 1980	Hong Kong, China	AM W.40546	–
<i>Hydroides recurvispina</i> Rioja, 1941	Mazatlan, Mexico	AM W.40547	JQ885945
<i>Hydroides sanctaerucis</i> Krøyer [in] Mörch, 1863 (1)	Hong Kong, China	AM W.40548	JQ885944
<i>Hydroides sanctaerucis</i> Krøyer [in] Mörch, 1863 (2)	Mazatlan, Mexico	AM W.40549	JQ885943
<i>Hydroides operculatus</i> (Treadwell, 1929)	Hong Kong, China	AM W.40550	JQ885949
<i>Hydroides</i> cf. <i>operculatus</i> (Treadwell, 1929)	Hong Kong, China	AM W.40551	JQ885948
<i>Hydroides tambalagamensis</i> Pillai, 1961	Hong Kong, China	AM W.40552	–
<i>Hydroides trivesiculosus</i> Straughan, 1967	Queensland, Australia	AM W.40553	JQ885940
<i>Serpula</i> cf. <i>granulose</i> Marenzeller, 1885	Hong Kong, China	AM W.40554	JQ885952

collected individuals were fixed in 90% ethanol and preserved in 95% ethanol. Specimens were initially identified to species on the basis of descriptions in Straughan (1967), Imajima (1976), Wu and Chen (1980), Fiege and Sun (1999), Bastida-Zavala and ten Hove (2002, 2003), and Pillai (2009). Vouchers are deposited in the Australian Museum (AM) in Sydney.

#### DNA amplification and sequencing

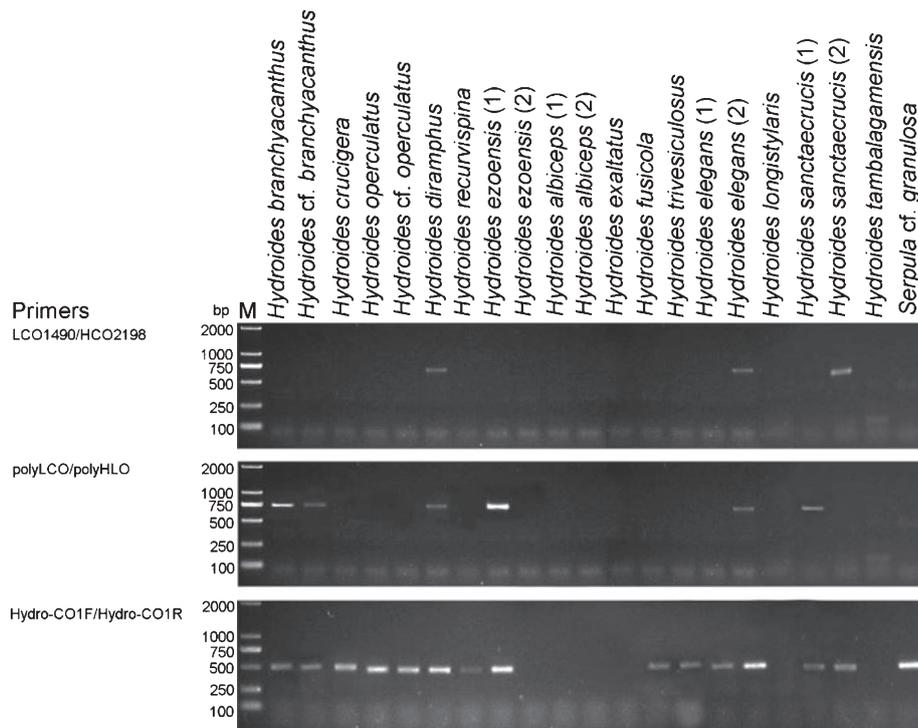
For each individual, the tentacle crown was separated from the body for DNA extraction to reduce the chance of contamination by exogenous DNA due to the presence of algae or bacteria in the gut. The crown was immersed in 0.5 mL TE buffer for 3 h with a change in buffer every 1 h to remove ethanol in the tissue. Qiagen DNeasy Blood and Tissue Kit was used to extract the genomic DNA according to the manufacturer's protocol. Initial attempts to amplify COI were performed with the universal primer set (LCO1490/HCO2198: Folmer *et al.* 1994), and the primer set for polychaetes (polyLCO/polyHCO: Carr *et al.* 2011). PCR reaction mixture (total volume: 20  $\mu$ L) contained 10 $\times$  buffer (2  $\mu$ L), 50 mM MgCl<sub>2</sub> (0.6  $\mu$ L), 10 mM dNTPs (0.4  $\mu$ L), 10  $\mu$ M primer mix (1  $\mu$ L), ddH<sub>2</sub>O (15.42  $\mu$ L), Invitrogen Platinum Taq Polymerase (0.08  $\mu$ L) and template (0.5  $\mu$ L). All amplifications were carried out in an Eppendorf Mastercycler Gradient thermocycler using the following PCR protocol: 94°C for 5 min, 5 cycles at 94°C for 30 s, 47°C for 30 s and 72°C for 40 s, 30 cycles at 94°C for 30 s, 51°C for 30 s and 72°C for 40 s, final extension at 72°C for 7 min. PCR products were separated by electrophoresis using 1.0% agarose gel. The gel was stained with

ethidium bromide and visualised using an UV transilluminator equipped with a digital camera.

Out of the 21 samples, amplifications were successful in three samples by using LCO1490/HCO2198 and six by using polyLCO/polyHCO (Fig. 1). PCR products were purified with QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol. Because the PCR products from a single amplification with the universal primers LCO1490/HCO2198 were insufficient for sequencing, they were diluted 1 : 10, and a 0.5- $\mu$ L aliquot was used as template for a secondary amplification under identical PCR protocol. Nevertheless, the second PCR failed in all reactions. The products from the first PCR were then cloned directly into the pMD™ 18-T Vector (Takara) to sequence these amplicons. Sequencing reactions were performed using an AB SOLiD™ 4.0 automatic sequencer with the universal primer M13F for vector sequencing. PCR products amplified with polyLCO/polyHCO were sequenced directly without cloning.

#### Sequence alignment and primer design

Sequences obtained with universal and polychaete-specific primers were compared with those in GenBank ([www.ncbi.nlm.nih.gov/Genbank/index.html](http://www.ncbi.nlm.nih.gov/Genbank/index.html)) by BLAST search to check whether the correct gene fragments had been amplified. All sequences were not obvious pseudogenes (i.e. no frameshift). However, the six sequences obtained by using polyLCO/polyHCO showed >90% similarity with the COI sequences of bacteria such as *Vesicomya* spp. and thus were likely



**Fig. 1.** Gel images of PCR amplicons for 20 *Hydroides* specimens and one *Serpula* specimen using LCO1490/HCO2198, polyLCO/polyHCO and Hydro-CO1F/Hydro-CO1R, respectively. 'M' denotes molecular size ladder in base pairs. The specimen labels are also shown in Table 1.

contaminants of prokaryotes. These sequences were not used for further analysis. The three sequences obtained using LCO1490/HCO2198 were aligned with ClustalX using default settings (15-gap opening penalty and 6.66-gap extension penalty), and subsequently checked by eye using BioEdit to identify the homogenous regions for design of new primers. The new primers were tested against the same 21 individuals under the same PCR protocol as for the other two sets of primers. The PCR amplicons after gel purification provided sufficient products for direct sequencing with new primers.

#### DNA analysis

A matrix of sequence data obtained by using the newly developed primers was generated using ClustalX under default settings. Genetic distances among sequences were calculated using MEGA4 (Tamura *et al.* 2007). The Kimura-2-parameter (K2P) model was chosen as it is appropriate for comparison of taxa with low genetic distances (Nei and Kumar 2000) and has previously been used in studies of mtDNA barcoding (e.g. Hebert *et al.* 2004a).

To infer relationships between *Hydroides* and other polychaetes, the COI sequences of all polychaete species available in GenBank were downloaded and combined with the sequences of *Hydroides* and *Serpula* obtained in this study. The Neighbour-joining (NJ) analysis was applied as it is suitable for analysis of large assemblages of taxa (Kumar and Gadagkar 2000). The construction of NJ tree was performed with the K2P model using MEGA4 to provide a graphical representation of the result. Bootstrap analyses were performed with 1000 replicates using MEGA4.

#### Results

Amplifications with universal primers LCO1490/HCO2198 resulted in amplicons from three samples of three species: *Hydroides diramphus*, *H. sanctaecrucis* and *H. elegans* (Fig. 1). The read length of these sequences, after removing primers and vector sequences, was 658 bp. Alignment of the sequences revealed two distinct regions with high homogeneity (Fig. 2), which were used to develop a set of primers for *Hydroides*:

Hydro-COIF: 5'-TCWRTWRTKACDGTKCATGCTA-3' and

Hydro-COIR: 5'-CMRYAGGWTSAAARAACCTAGTA-3',

where degenerate positions are represented by the following ambiguity codes: D = A|G|T; K = G|T; R = A|G; S = C|G; W = A|T; Y = C|T (Fig. 2).

We then tested the new primers against the 20 individuals of 14 *Hydroides* species and one individual of *Serpula* cf. *granulosa*. Thirteen amplicons belonging to 10 species were successfully obtained from the 20 *Hydroides* samples (79% success based on the number of species used; 52% success based on the number of samples used); one amplicon from *Serpula* cf. *granulosa* was also obtained (Fig. 1). The PCR reaction provided sufficient products for direct sequencing. The read length was 460 bp in all reactions. No stop codons were present in any of the sequences.

Interspecific sequence divergence ranged from 10.4 to 36.9%, with a mean of 26.2%. Intraspecific sequence divergence was much smaller, ranging from 0 to 0.9% (mean: 0.43%). There was no intraspecific genetic divergence between *Hydroides elegans* collected from Hong Kong and that from Australia. The intraspecific genetic divergence between *H. sanctaecrucis* collected from Hong Kong and Mexico was only 0.41%. The genetic divergence between *H. operculatus* and *H. cf. operculatus*, and between *H. branchyacanthus* and *H. cf. branchyacanthus* was 0.4% and 0.9%, respectively.

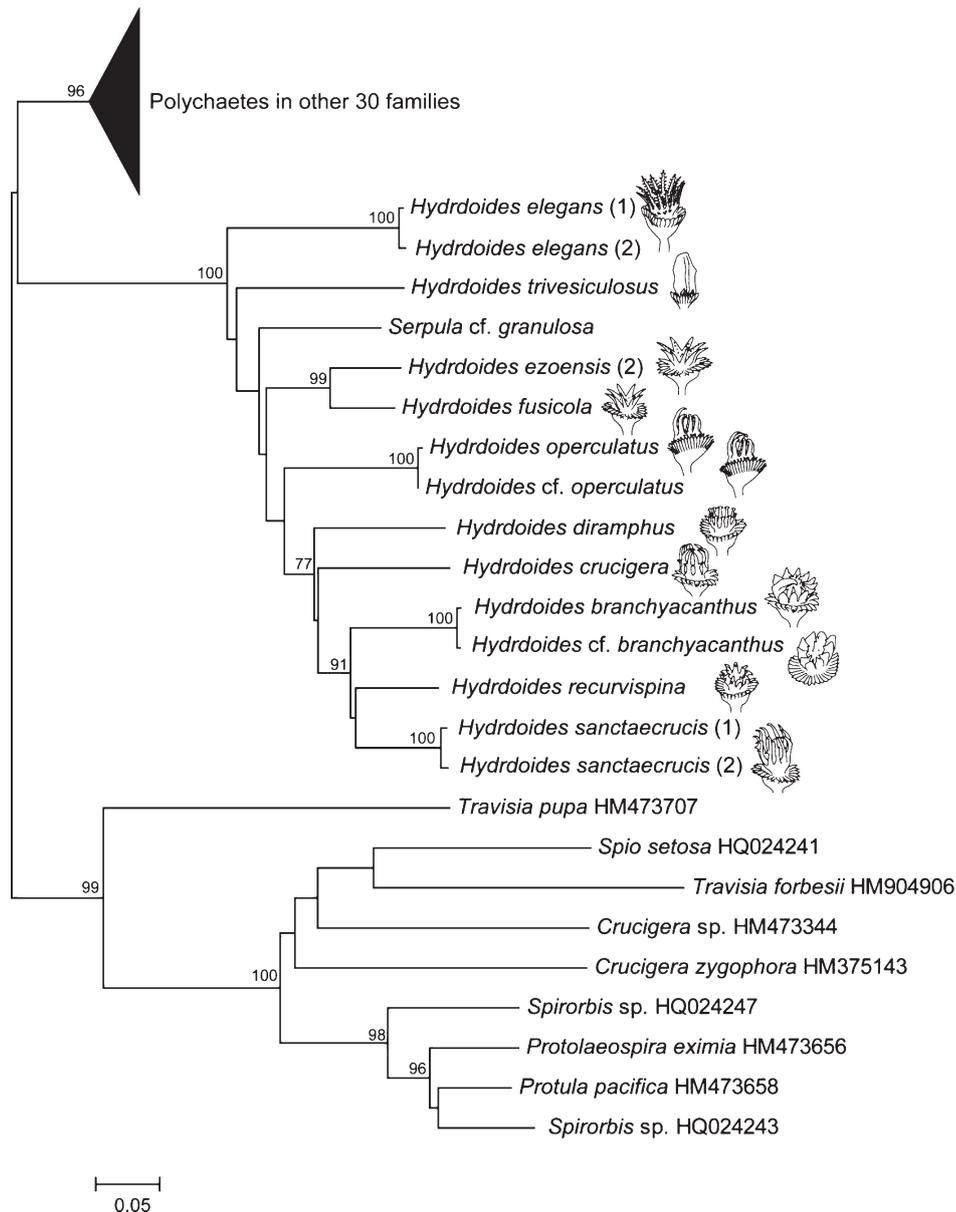
In every species of *Hydroides* for which more than one individual was used, the species was monophyletic in the NJ tree (Fig. 3). The clade of *Hydroides* was not clustered with the clade of other serpulids sequenced in an earlier study (Carr *et al.* 2011). The NJ tree indicated a base position of the *Hydroides* clade in relation to other polychaetes except for certain genera of Opheliidae (*Travisia*), Spionidae (*Spio*) and Serpulidae (*Crucigera*, *Protula*, *Protolaespira* and *Spirorbis*) (Fig. 3). Compared with the clade of serpulids comprising *Crucigera*, *Protula*, *Protolaespira* and *Spirorbis*, *Hydroides* had a closer relationship with other groups of polychaetes.

#### Discussion

This study represented the first successful attempt to amplify the COI sequences from *Hydroides*, the most speciose genus in Serpulidae, with ~100 species distributed worldwide (ten Hove and Kupriyanova 2009; Pillai 2009). The failure to amplify the



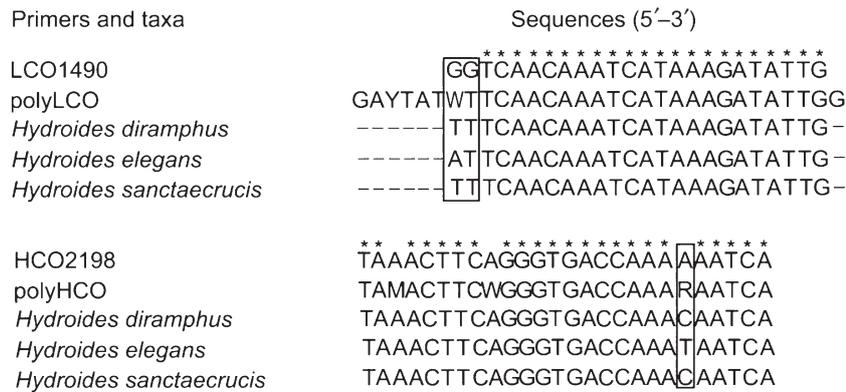
**Fig. 2.** Alignment of the three COI sequences showing the conserved regions of the COI locus used to design degenerate oligonucleotide primers Hydro-COIF/Hydro-COIR. Asterisks indicate conserved positions.



**Fig. 3.** Neighbour-joining tree for polychaete species. The number next to each node indicates a bootstrap value (>75%) as a percentage of 1000 replicates. Only clades with serpulids are shown. The species-level classification is shown in Supplementary Material 1. The labels of samples are also shown in Table 1. To the right of the *Hydrdoides* species names are drawings of the opercula. Sequences cited from Carr *et al.* (2011) are downloaded from NCBI with the accession number after each species name. BLAST search results indicate that sequences of *Travia pupa*, *Spio setosa*, *Travia forbesii*, *Crucigera* sp., *Crucigera zygophora*, *Spirorbis* sp., *Protolaeospira eximia*, *Protula pacifica*, *Spirorbis* sp. are not of polychaete origin.

COI barcoding region with the universal primer set in previous studies of polychaetes is thought to be due to the non-homology between the primers and the target DNA region (Halanych and Janosik 2006). Efforts to overcome the problem included designing primer sets/cocktail according to the published sequences of other species that are phylogenetically close to the target species (Halt *et al.* 2009; Carr *et al.* 2011), or searching for primer sites on the tRNA gene, which usually lies within 200 bp upstream of the COI gene (Park *et al.*

2011). When the sequences amplified using LCO1490/HCO2198 were compared with the primer sequences, two substitutions were identified in the 5' end of the target barcoding region (G → T/A, G → T) and one in the 3' end (A → C/T) (Fig. 4). These nucleotide substitutions could have led to the failure of primer annealing, which eventually resulted in the failure of the PCR reaction. PolyLCO/polyHCO contains modifications to LCO1490/HCO2198 in order to recover the three substitutions on the



**Fig. 4.** Alignment of the three COI sequences obtained by using LCO1490/HCO2198, showing three substitutions on the primer regions of *Hydroides* COI sequences, compared with the sequences of LCO1490/HCO2198. Asterisks indicate conserved positions.

primer regions. Specifically, W (a position with the possibility of being A or T) and T were used to recover the double Gs in the 5' end; and R (a position with the possibility of being C or T), was used to recover A in the 3' end, respectively. However, using this modified primer set for *Hydroides* samples, we only generated amplicons of prokaryotic genes. The fact that COI primers originally designed for invertebrates might effectively amplify the COI locus of certain bacteria has been reported in studies of shrimp and mollusks, which is due to the high homogeneity between the primers and the corresponding region of the bacterial COI gene (Siddall *et al.* 2009). Our new primers were designed within the COI region to avoid using the region that is homogenous to the prokaryotic gene. Using the newly designed primer set, we successfully amplified 79% of the tested *Hydroides* species and one *Serpula* species. This result indicates that barcoding *Hydroides* using a fragment of the COI gene is now not an impossible goal, although it might still not be easy.

The NJ tree indicated a deep divergence between the *Hydroides* clade obtained in this study and the other serpulids (*Crucigera*, *Protula*, *Protolaeospira* and *Spirorbis*) sequenced by Carr *et al.* (2011). This result is suspicious for several reasons. All previous phylogenetic studies based on morphological and molecular data indicated that Serpulidae is a monophyletic group (e.g. Kupriyanova *et al.* 2006, 2008; Lehrke *et al.* 2007; Kupriyanova and Rouse 2008) and *Crucigera* is most closely related to *Serpula* (Kupriyanova and Rouse 2008; Kupriyanova *et al.* 2008). Moreover, a BLAST search showed a high similarity between sequences from Carr *et al.* (2011) and sequences from prokaryotes such as *Pseudoalteromonas* sp. (98%), *Colwellia psychrerythraea* (94%), *Galeomma turtoni* (83%), *Vesicomys* sp. (80%) and *Methyloversatilis universalis* (76%). In contrast, the positive hits of our *Hydroides* sequences were all invertebrates, with 60–70% sequence similarity with polychaetes (*Eulalia levicornuta*, *Eumida merope*, *Eumida sanguinea*, *Nereiphylla castanea*, *Pseudomystides limbata*), arthropods (*Holopedium glacialis*, *Triops longicaudatus*), mollusks (*Gulella usambarica*, *Schizobranchium polycotylum*, *Odostomia plicata*) and jellyfishes (*Crambionella orsini*, *Aurelia aurita*). Besides, serpulid sequences from Carr *et al.* (2011) have 2–3 indels in the reading frame and the location of the indels is inconsistent, so these sequences are not currently ruled out as being from bacteria.

Although polyLCO/polyHCO is useful for several families of polychaetes, as demonstrated by Carr *et al.* (2011), our results suggest that this primer set is not suitable for barcoding serpulids.

Levels of interspecific sequence divergence reported in the present study of *Hydroides* are high (10.4–36.9%), but are consistent with data from other studies of polychaetes, which also reported high values, i.e. 16% in *Harmothoe* (Hardy *et al.* 2011); mean of 16.5% for 333 species (Carr *et al.* 2011). In the polychaete *Pectinaria koreni*, the intraspecific sequence divergence could reach 16.4% (Jolly *et al.* 2005), but this might indicate the presence of cryptic species and the data might actually reflect interspecific sequence divergence. The mean COI K2P sequence divergence between the *Hydroides* species was 60 times higher than within the species. Application of the average within-species variation threshold (i.e. 10× variation: see Hebert *et al.* 2004a; Witt *et al.* 2006) indicated the presence of 10 *Hydroides* species among our samples, which was consistent with the number of identified species. Entries for the same species were tightly clustered and distinct from the other species (Fig. 5). The large gap between the intraspecific and interspecific COI divergence indicated that the COI fragment amplified by our primer set is a good candidate for distinguishing species of *Hydroides* and can be an effective barcoding tool for this genus. It is especially useful for species identification when expertise in morphology-based identification is not available or morphology-based methods do not work. For example, *H. branchyacanthus* and *H. cf. branchyacanthus* were identified on the basis of the presence of geniculate spines with a well developed knob protruding above a subapical incurving tip in both species, nevertheless they differ in the spine size: in *H. branchyacanthus* the dorsal spine is larger than the other spines, whereas in *H. cf. branchyacanthus* all spines are similar in size and shape. Since the two taxa were placed in one cluster with low divergence (0.9%), we consider both as *H. branchyacanthus*. Similarly, *H. operculatus* and *H. cf. operculatus*, identical with respect to several morphological characters (i.e. 9 verticil spines, spines of identical shape, and the presence of a basal internal spinule) but differing in the number of large spines (one in *H. operculatus* versus three in *H. cf. operculatus*), showed only 0.4% sequence divergence from each other but at least

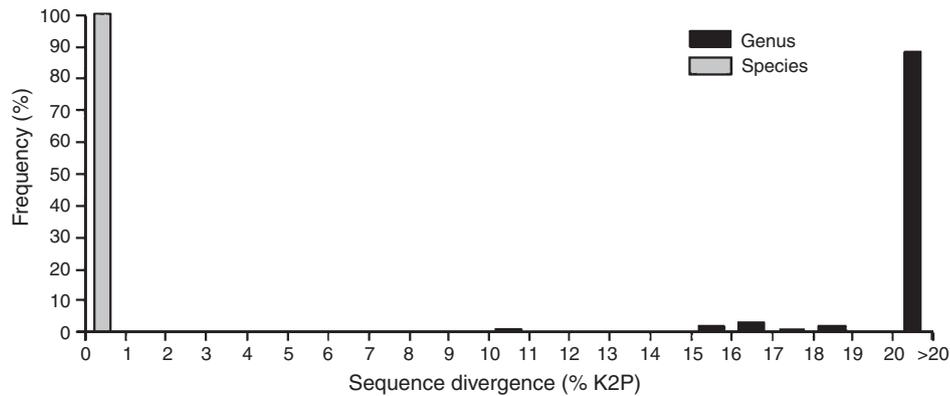


Fig. 5. COI (K2P) distances for the barcode region within species and genera.

22.5% divergence from other clades; therefore both should be considered as *H. operculatus*.

In the present study, individuals of the same species collected from widely different geographical locations show low genetic divergence. This was the case for *Hydroides elegans* collected from China and Australia, *Hydroides sanctaerucis* collected from Mexico and China, and *Hydroides ezoensis* from China and Russia. The great distance between collection sites of the same species but lack of genetic divergence is consistent with the observation that these species are common fouling organisms that are easily transported by ship (ten Hove and Kupriyanova 2009) and their wide distribution could be the result of recent introduction. In fact, biofouling was proposed as a major mode of dispersal for *Hydroides elegans* as inferred from microsatellite loci (Pettengill *et al.* 2007). Therefore, the COI fragment amplified by our newly designed primer set can be useful for confirmation of the specific status of invasive *Hydroides*.

In summary, our newly designed primer set has opened the door for barcoding of *Hydroides*, the most speciose and economically important genus of Serpulidae. In addition, it can amplify the COI fragment from *Serpula cf. granulosa* and thus, may be useful for other non-*Hydroides* serpulids. As already noted, the new primer set failed to amplify the barcode region of three tested *Hydroides* species. But the successfully amplified *Hydroides* sequences can serve as reference sequences for the design of novel primers and confirmation of the feasibility of creating a barcode library for *Hydroides* when Hydro-COIF/Hydro-COIR does not work. With the increasing application of COI barcoding and expansion of the DNA reference library, we anticipate a clearer distinction between *Hydroides* species, especially for those species whose identity cannot be resolved solely on the basis of morphological characters. When data from the COI barcode and other mitochondrial gene sequences become available for most species of *Hydroides*, analyses can then be conducted to better understand the speciation and evolutionary history of *Hydroides*.

### Acknowledgements

We thank Emma Johnston, Ana-Maria Tovar, and Vasily Radashevsky for collecting specimens from Australia, Mexico and Russia, respectively, and Paulo Paiva, Craig Starger, Dirk Steinke, Toril Loennechen Moen, and Valerija Zakšek for sharing their experience in barcoding serpulids, and

John Lewis for providing information on fouling caused by *Hydroides*. This project was supported by a grant from Environment and Conservation Fund, Hong Kong (ECF 7/2007).

### References

- Arakawa, K. Y. (1971). Notes on a serious damage to cultured oyster crops in Hiroshima caused by unique and unprecedented outbreak of a serpulid worm, *Hydroides norvegica* (Gunnerus) in 1969. *Venus (Fukuyama-Shi, Japan)* **30**, 75–82.
- Barroso, R., Klautau, M., Solé-Cava, A. M., and Paiva, P. C. (2010). *Eurythoe complanata* (Polychaeta: Amphinomidae), the ‘cosmopolitan’ fireworm, consists of at least three cryptic species. *Marine Biology* **157**, 69–80. doi:10.1007/s00227-009-1296-9
- Bastida-Zavala, J. R., and ten Hove, H. A. (2002). Revision of *Hydroides* Gunnerus, 1768 (Polychaeta: Serpulidae) from the western Atlantic region. *Beaufortia* **53**, 103–178.
- Bastida-Zavala, J. R., and ten Hove, H. A. (2003). Revision of *Hydroides* Gunnerus, 1768 (Polychaeta: Serpulidae) from the eastern Pacific Region and Hawaii. *Beaufortia* **53**, 67–110.
- Carr, C. M., Hardy, S. M., Brown, T. M., Macdonald, T. A., and Hebert, P. H. N. (2011). A tri-oceanic perspective: DNA barcoding reveals geographic structure and cryptic diversity in Canadian polychaetes. *PLoS ONE* **6**, e22232. doi:10.1371/journal.pone.0022232
- Dürr, S., and Watson, D. I. (2010). Biofouling and antifouling in Aquaculture. In ‘Biofouling’. (Eds S. Dürr and J. C. Thomason.) pp. 267–287. (Wiley-Blackwell: Oxford.)
- Erpenbeck, D., Hooper, J. N. A., and Worheide, G. (2006). COI phylogenies in diploblasts and the ‘Barcoding of Life’ – are we sequencing a suboptimal partition? *Molecular Ecology Notes* **6**, 550–553. doi:10.1111/j.1471-8286.2005.01259.x
- Fiege, D., and Sun, R. (1999). Polychaeta of Hainan Island, South China Sea. Part I. Serpulidae (Annelida: Polychaeta: Serpulidae). *Senckenbergiana Biologica* **79**, 109–141.
- Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**, 294–299.
- Halanych, K. M., and Janosik, A. M. (2006). The state of annelid phylogenetics. *Integrative and Comparative Biology* **46**, 533–543. doi:10.1093/icb/iccj052
- Halt, M. N., Kupriyanova, E. K., Cooper, S. B., and Rouse, G. W. (2009). Naming species with no morphological indicators: species status of *Galeolaria caespitosa* (Annelida: Serpulidae) inferred from nuclear and mitochondrial gene sequences and morphology. *Invertebrate Systematics* **23**, 205–222. doi:10.1071/IS09003

- Hardy, S. M., Carr, C. M., Hardman, M., Steinke, D., Corstorphine, E., and Mah, C. (2011). Biodiversity and phylogeography of Arctic marine fauna: insights from molecular tools. *Marine Biodiversity* **41**, 195–210. doi:10.1007/s12526-010-0056-x
- Hebert, P. D. N., Cywinska, A., Ball, S. L., and deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings Biological Sciences* **270**, 313–321. doi:10.1098/rspb.2002.2218
- Hebert, P. D. N., Stoeckle, M. Y., Zemplak, T. S., and Francis, C. M. (2004a). Identification of birds through DNA barcodes. *PLoS Biology* **2**, e312. doi:10.1371/journal.pbio.0020312
- Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H., and Hallwachs, W. (2004b). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 14 812–14 817. doi:10.1073/pnas.0406166101
- Hutchings, P. (1998). Biodiversity and functioning of polychaetes in benthic sediments. *Biodiversity and Conservation* **7**, 1133–1145. doi:10.1023/A:1008871430178
- Imajima, M. (1976). Serpulinae (Annelida, Polychaeta) from Japan. I. The genus *Hydroides*. *Bulletin of the National Science Museum (A, Zoology)* **2**, 229–248.
- Jolly, M. T., Jollivet, D., Gentil, F., Thiebaut, E., and Viard, F. (2005). Sharp genetic break between Atlantic and English Channel populations of the polychaete *Pectinaria koreni*, along the north coast of France. *Heredity* **94**, 23–32. doi:10.1038/sj.hdy.6800543
- Kondo, T., Gullan, P. J., and Williams, D. J. (2008). Coccidology. The study of scale insects (Hemiptera: Sternorrhyncha: Coccoidea). *Revista Corpoica – Ciencia y Tecnología Agropecuaria* **9**, 55–61.
- Kumar, S., and Gadagkar, S. R. (2000). Efficiency of the neighbor-joining method in reconstructing deep and shallow evolutionary relationships in large phylogenies. *Journal of Molecular Evolution* **51**, 544–553.
- Kupriyanova, E. K., and Rouse, G. W. (2008). Yet another example of paraphyly in Annelida: molecular evidence that Sabellidae contains Serpulidae. *Molecular Phylogenetics and Evolution* **46**, 1174–1181. doi:10.1016/j.ympev.2007.10.025
- Kupriyanova, E. K., Macdonald, T. A., and Rouse, G. W. (2006). Phylogenetic relationships within Serpulidae (Annelida: Polychaeta) inferred from molecular and morphological data. *Zoologica Scripta* **35**, 421–439. doi:10.1111/j.1463-6409.2006.00244.x
- Kupriyanova, E. K., Bastida-Zavala, R., Halt, M. N., Lee, M. S. Y., and Rouse, G. W. (2008). Phylogeny of the *Serpula-Crucigera-Hydroides* clade (Serpulidae: Annelida) using molecular and morphological data: implications for operculum evolution. *Invertebrate Systematics* **22**, 425–437. doi:10.1071/IS08011
- Lehrke, J., ten Hove, H. A., Macdonald, T. A., Bartolomeus, T., and Bleidom, C. (2007). Phylogenetic relationships of Serpulidae (Annelida: Polychaeta) based on 18S rDNA sequence data, and implications for opercular evolution. *Organisms, Diversity & Evolution* **7**, 195–206. doi:10.1016/j.ode.2006.06.004
- Lewis, J. A. (2006). Establishment of the Caribbean serpulid tubeworm *Hydroides sanctaerucis* Krøyer [in] Mörch, 1863, in northern Australia. *Biological Invasions* **8**, 665–671. doi:10.1007/s10530-005-2062-7
- Nei, M., and Kumar, S. (2000). 'Molecular Evolution and Phylogenetics.' (Oxford University Press: New York.)
- Nishi, E., and Kato, T. (2004). Introduced and globally invaded polychaetous annelids. *Japanese Journal of Benthology* **59**, 83–95.
- Nygren, A., and Pleijel, F. (2011). From one to ten in a single stroke – resolving the European *Eumida sanguinea* (Phyllococidae, Annelida) species complex. *Molecular Phylogenetics and Evolution* **58**, 132–141. doi:10.1016/j.ympev.2010.10.010
- Otani, M., and Yamanishi, R. (2010). Distribution of the alien species *Hydroides dianthus* (Verrill, 1873) (Polychaeta: Serpulidae) in Osaka Bay, Japan, with comments on the factors limiting its invasion. *Plankton and Benthos Research* **5**, 62–68. doi:10.3800/pbr.5.62
- Park, D.-S., Suh, S.-J., Hebert, P. D. N., Oh, H.-W., and Hong, K.-J. (2011). DNA barcodes for two scale insect families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae). *Bulletin of Entomological Research* **101**, 429–434. doi:10.1017/S0007485310000714
- Pettengill, J. B., Wendt, D. E., Schug, M. D., and Hadfield, M. G. (2007). Biofouling likely serves as a major mode of dispersal for the polychaete tubeworm *Hydroides elegans* as inferred from microsatellite loci. *Biofouling* **23**, 161–169. doi:10.1080/08927010701218952
- Pillai, T. G. (2009). Descriptions of new serpulid polychaetes from the Kimberleys of Australia and discussion of Australian and Indo-West Pacific species of *Spirobranchus* and superficially similar taxa. *Records of the Australian Museum* **61**, 93–199. doi:10.3853/j.0067-1975.61.2009.1489
- Pleijel, F., Rouse, G., and Nygren, A. (2009). Five colour morphs and three new species of *Gyptis* (Hesionidae, Annelida) under a jetty in Edithburgh, South Australia. *Zoologica Scripta* **38**, 89–99. doi:10.1111/j.1463-6409.2008.00356.x
- Qiu, J. W., and Qian, P. Y. (1997). Combined effects of salinity, temperature and food on early development of the polychaete *Hydroides elegans*. *Marine Ecology Progress Series* **152**, 79–88. doi:10.3354/meps152079
- Schwindt, E., Bortolus, A., and Iribarne, O. O. (2001). Invasion of a reef-builder polychaete: direct and indirect impacts on the native benthic community structure. *Biological Invasions* **3**, 137–149. doi:10.1023/A:1014571916818
- Sevilla, R. G., Diez, A., Noren, M., Mouchel, O., Jerome, M., Verrez-Bagnis, V., van Pelt, H., Favre-Krey, L., and Bautista, J. M. (2007). Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome b and nuclear rhodopsin genes. *Molecular Ecology Notes* **7**, 730–734. doi:10.1111/j.1471-8286.2007.01863.x
- Siddall, M. E., Fontanella, F. M., Watson, S. C., Kvist, S., and Erseus, C. (2009). Barcoding bamboozled by bacteria: convergence to metazoan mitochondrial primer targets by marine microbes. *Systematic Biology* **58**, 445–451. doi:10.1093/sysbio/syp033
- Smith, M. A., Woodley, N. E., Janzen, D. H., Hallwachs, W., and Hebert, P. D. N. (2006). DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proceedings of the National Academy of Sciences of the United States of America* **103**, 3657–3662. doi:10.1073/pnas.0511318103
- Smith, M. A., Wood, D. M., Janzen, D. H., Hallwachs, W., and Hebert, P. D. N. (2007). DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 4967–4972. doi:10.1073/pnas.0700050104
- Straughan, D. (1967). Some Serpulidae (Annelida: Polychaeta) from Heron Island, Queensland. *University of Queensland Papers* **1**, 27–45.
- Streftaris, N., and Zenetos, A. (2006). Alien marine species in the Mediterranean – the 100 'worst invasives' and their impact. *Mediterranean Marine Science* **7**, 87–118.
- Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). Mega4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599. doi:10.1093/molbev/msm092
- ten Hove, H. A. (1974). Notes on *Hydroides elegans* (Haswell, 1883) and *Mercierella enigmatica* Fauvel, 1923, alien serpulid polychaetes introduced into the Netherlands. *Bulletin Zoologisch Museum Universiteit van Amsterdam* **4**, 45–51.
- ten Hove, H. A., and Jansen-Jacobs, M. J. (1984). A revision of the genus *Crucigera* (Polychaeta; Serpulidae): a proposed methodical approach to serpulids, with special reference to variation in *Serpula* and *Hydroides*. In 'Proceedings of the First International Polychaete Conference'. (Ed. P. A. Hutchings.) pp. 143–180. (Linnean Society of New South Wales: Sydney.)

- ten Hove, H. A., and Kupriyanova, E. K. (2009). Taxonomy of Serpulidae (Annelida, Polychaeta): the state of affairs. *Zootaxa* **2036**, 1–126.
- Thomas, M., Raharivololoniaina, L., Glaw, F., Vences, M., and Vieites, D. R. (2005). Montane tadpoles in Madagascar: molecular identification and description of the larval stages of *Mantidactylus elegans*, *Mantidactylus madecassus*, and *Boophis laurenti* from the Andringitra Massif. *Copeia* **2005**, 174–183. doi:10.1643/CH-03-293R2
- Tovar-Hernández, M. A., Méndez, N., and Villalobos-Guerrero, T. F. (2009). Fouling polychaete worms from the Southern Gulf of California: Sabellidae and Serpulidae. *Systematics and Biodiversity* **7**, 319–336. doi:10.1017/S1477200009990041
- Willassen, E. (2005). New species of *Diamesa* (Diptera: Chironomidae) from Tibet: conspecific males and females associated with mitochondrial DNA. *Zootaxa* **1049**, 19–32.
- Witt, J. D. S., Threlloff, D. L., and Hebert, P. D. N. (2006). DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. *Molecular Ecology* **15**, 3073–3082. doi:10.1111/j.1365-294X.2006.02999.x
- Wu, B. L., and Chen, M. (1980). Two new species of the family Serpulidae from South China Sea. *Acta Zootaxonomica Sinica* **6**, 247–249.