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Morphological and Molecular Characterization of *Polydora websteri* (Annelida: Spionidae), with Remarks on Relationship of Adult Worms and Larvae using Mitochondrial COI Gene as a Molecular Marker

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Authors' Contributions
LY and JW conceived the idea, designed the experiments and wrote the article. LY, CC and BT collected the specimens and conducted the experimental work. TY prepared the figures. RW helped in identification of polydorid species and analyses of molecular data.

Key words
***Polydora websteri*, Larvae, Morphological plasticity, Mitochondrial COI, Genetic distance, Adult worm.**

ABSTRACT

The shell-boring *Polydora websteri* is described in detail in the present study for future unambiguous identification using an integrative taxonomic approach that combines morphology and molecular analysis of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene. Adult *P. websteri* exhibit a high degree of morphological plasticity in the palp pigmentation pattern, the shape of the anterior edge of the prostomium, the shape of the major spines on chaetiger 5, and the shape of the pygidium. The COI gene sequence demonstrated that the intraspecific distance of *P. websteri* was 0.33%, whereas the interspecific distance of *P. websteri* ranged from 18.88% (with *P. brevipalpa*) to 24.79% (with *Boccardia proboscidea*). The intraspecific genetic distances of polydorids examined in the present study ranged from 0.33% to 1.67%, whereas the interspecific distances ranged from 18.88% to 24.79%. Such large barcoding gaps between intra- and inter-specific distances indicate that the COI is a suitable gene marker for molecular identification of polydorid species. Our results demonstrate that not only did all COI sequences from the larvae show greater than 99% sequence identity to those from adults, but some larvae share the same haplotypes as adults. These findings clearly indicate that the larvae collected from sea waters around an oyster farm belong to *P. websteri*, the same species as the adult worms collected from the oyster *Crassostrea hongkongensis* in that locality. Two polydorid-specific primers were successfully designed, for the first time, to amplify target fragments of the COI gene. This study is the first to molecularly validate unidentified larvae from the aquatic environment through the known COI sequences of adults.

INTRODUCTION

Polydorid polychaetes, some of which are shell-boring worms, can severely affect the growth and development of many economically important mollusc species (Skeel, 1979; Blake, 1996; Handley and Bergquist, 1997; Bilbao *et al*., 2011). Most of the shell-boring polydorids have two developmental stages in their life cycles: the larval stage and the adult stage. The larvae develop from eggs in the brood capsules of adults, release into the plankton, and remain pelagic for an extended period in the water column prior to settlement (Blake, 1996; Blake and Arnofsky, 1999). The post larvae then settle on the shells, build their burrows inside the shells, and gradually mature to the adult stage until they produce the next generation. The taxonomy and ecology of adult shell-boring polydorids has been investigated quite intensively, because of their significant damage to mollusc fisheries and aquaculture (Blake and Evans, 1973; Read, 1975; Sato-Okoshi, 1999; Radashevsky *et al*., 2006; Silina, 2006; Simon, 2011; Sato-Okoshi and Abe, 2012; Diez *et al*., 2013; Radashevsky and Pankova, 2013). In contrast, there is a comparative paucity of information on the taxonomy and biology of polydorid larvae which are free-living in the water environment (Hopkins, 1958; Blake, 1969; Day and Blake, 1979; Zajac, 1991; Radashevsky, 2005; Abe *et al*., 2011; David *et al*., 2014). As far as we know, there has been no research on the validation of corresponding relationships between

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free-living polydorid larvae in the water environment and shell-boring polydorid adult worms.

Traditional identification of polydorids has mainly relied on the phenotypic distinction of adult worms based on microscopic observations (Blake, 1996; Walker, 2011; Radashevsky, 2012). However, as in many other marine species, this method may lead to a greater number of synonyms of morphologically similar, but genetically distinct species, often resulting in the inflation of assumed geographic distributions (Pfenninger and Schwenk, 2007). In contrast, molecular markers have become increasing important in the taxonomy of polydorids as molecular data can provide comparatively definite species delimitations, independent of phenotypic, ontogenetic and ecological variations (Rice *et al*., 2008; Simon *et al*., 2009; Radashevsky and Pankova, 2013; David *et al*., 2014). For the identification of polydorid larvae, most researchers firstly identify the species of the maternal adults, then the egg capsules are cultured to each developmental stage in the laboratory, and finally the morphology of the larvae are observed (Blake and Woodwick, 1975; Day and Blake, 1979). However, it is difficult to quickly and accurately identify those larvae free-living in the water column as their morphology is completely different from that of the adults, their bodies are rather simple, and they lack discrete characteristics to distinguish between larvae of different species. The integration of molecular data from larvae and adults may significantly improve our knowledge of accurate species identification of the free-living larvae.

Polydora websteri was originally described and named *P. caeca* by Dr. Webster, and renamed *P. websteri* by Dr. Hartman due to misleading and erroneous original descriptions (Loosanoff and Engle, 1943). Since then, *P. websteri* has been reported worldwide and can be found in almost all coastal waters, such as the gulf coasts of North America, the coast of South America (Peru and Ecuador), Australia, Japan, Korea, Black Sea, and the United Kingdom (Blake, 1969, 1971, 1983, 1996; Handley and Bergquist, 1997; Surugiu, 2005, 2012; Radashevsky *et al*., 2006; Lisitskaya *et al*., 2010; Read, 2010; Walker, 2011; Sato-Okoshi *et al*., 2012; Sato-Okoshi and Abe, 2013). This species was also recorded to bore into the shell of a variety of mollusc species, such as oysters, scallops, and mussels (Blake and Evans, 1973). However, species identification in most of these studies was based on morphology, and to determine whether *P. websteri* has many host species and widespread distribution requires further reliable evidence, such as molecular techniques.

The mitochondrial cytochrome c oxidase subunit 1 (COI) gene is a universally accepted marker for the molecular identification of annelids as it exhibits a marked divergence between intra- and inter-specific genetic distance (Bely and Wray, 2004; Erseus and Kvist, 2007; Nygren and Pleijel, 2011; Siqueira *et al*., 2013; Pérez-Losada *et al*., 2015). However, only one report identified the polydorids using COI gene sequence data (Rice *et al*., 2008). In the present study, we successfully designed specific primers for amplifying the COI gene sequences from polydorids. In order to avoid ambiguous identification of *P. websteri*, we used an integrative taxonomic approach that combined morphology and molecular analysis with the COI gene for a detailed description of this species. In addition, in order to assess whether the adult worms boring into the shells of oysters and the larvae free-living around the oyster farm waters belonged to the same species, the COI gene sequence was obtained from a number of adult and larval samples. Comparisons of the genetic distance among polydorids were also performed to assess the delimitation of intraspecific and interspecific variation.

MATERIAL AND METHODS

Material and morphological observation

Approximately 200 cultured oysters (*Crassostrea hongkongensis*) were collected from an oyster farm (21°44′46.60″N, 111°45′23.14″E) in Yangxi County, Guangdong Province, China. The oyster shells were broken into fragments using a hammer and pliers and the polydorid species were extracted from the burrows, and transferred to Petri dishes (containing filtered seawater) to keep the worms alive until further examination. The prevalence and abundance were counted according to definition of Bush *et al*. (1997). The prevalence of polydorids was calculated as the percentage of oyster valves occupied. The abundance of adults on oysters was calculated as the numbers of specimens per valve. The abundance of polydorid larvae was counted according to the method of Abe *et al*. (2011). Plankton larvae were collected by trawling with a WP-2 net (mesh size 77 μm) equipped with a 1 L closed cod-end at slow speed through surface water around the oyster farm. The Petri dish containing plankton larvae were lit obliquely using a gooseneck lamp. Polydorid larvae can be separated and collected from other plankton because they had stronger phototropism. Several drops of 5% $MgCl_2$ solution were added and the adult worms and larvae were examined and photographed using a stereomicroscope (SZX7, Olympus, Japan) equipped with a digital camera. Some adult and larval specimens were fixed in 10% neutral formalin for further morphological identification, and other specimens were fixed in 80% alcohol for the molecular study. For scanning electron microscopy (SEM), the specimens were fixed with 2.5% glutaraldehyde solution, dehydrated by graded ethanol, critical-point dried in carbon dioxide, coated with gold palladium, and then

examined and photographed by SEM (Hitachi S-3400N, Japan).

DNA extraction, amplification and sequencing

Approximately one third of the length of each adult worm was cut for DNA extraction. To extract enough DNA from plankton larvae, more than 13-chaetiger larvae were selected. A total of 25 specimens were used for DNA extraction, including 13 adult worms and 12 large-sized plankton larvae. DNA was extracted using the Tissues/ Cells Genomic DNA Extraction Kit (BioTek, Beijing, China) following the manufacturer's protocol.

For further confirmation of the accuracy of the morphological identification of the polydorid species, gene fragment of the nuclear 18S rDNA was amplified and sequenced according to Ye *et al*. (2015). In order to amplify a fragment of the COI gene from polydorid species, several primer pairs (Table I) were designed using Primer Premier 5.0 software according to a comparison of the COI region of numerous annelid sequences. The primer pairs X1-FF2 and X1-R6 successfully amplified approximately 1000 bp of the COI sequence of the polydorid species. When these primer pairs were unsuccessful, approximately 850 bp of the COI fragment was amplified using the primer pairs X1-F2 and X1-R2. Each 50 µL reaction contained 25 µL of 2× PCR mixture (Dongsheng, China), 2 µL of template, 19 µL of distilled water, and 2 µL of each primer. A Takara PCR Thermal Cycler Dice was used with the following cycling profile: 95°C for 5 min, followed by 35 cycles of 50 s at 94°C, 50 s at 50°C and 90 s at 72°C, and then a final extension of 10 min at 72°C. The PCR results were confirmed on 1% agarose gel stained with SYBR green. PCR products were sequenced in both directions using the amplification primers and ABI Big Dye Terminator Chemistry on an ABI 3730XL automatic DNA sequencer (Applied Biosystems, USA). The forward and reverse complementary sequences were merged into a consensus using SeqMan 4.0 (DNAstar v7.0). The consensus sequences were submitted to NCBI and registered in Genbank (accession nos. KR337461-72).

Table I.- PCR and sequencing primers of mitochondrial COI gene in this study.

Primer name	Sequence 5'-3'	Position
X1-FF2	CCTWGTDATACCTRTCWTAATT	195-216
X1-R6	CCTGTAAATARAGGGAATCA	1196-1177
X1-F2	CCWGATATRGCATTCCC	259-265
X1-R2	GCKARYCADCTAAATACTTTAA	965-944

Note, Position numbers refer to the *Lumbricus terrestris* complete mitochondrial sequence (GenBank Accession No. U24570). The sequences in Italics indicate reverse primers.

Data analyses

The COI sequences obtained from 25 polydorid specimens were edited and aligned with the Clustal W alignment tool in Bioedit (Hall, 1999) using default parameters and further verified manually. In order to avoid nuclear pseudogene amplification, the COI sequences were translated into amino acid sequences using an invertebrate mitochondrial code. The identities of the sequences were confirmed by BLAST searches in GenBank.

Haplotype diversity, nucleotide diversity, and the average number of nucleotide differences were calculated using DnaSP5.0 (Librado and Rozas, 2009). Pairwise and overall distances among haplotype sequences were calculated using MEGA 6.06 software (Tamura *et al*., 2013) with default parameters. The variation between and within groups of *P. websteri* (adult and larvae) was determined using an AMOVA in Arlequin version 3.5. A haplotype network was constructed in Network 4.613 (http://www.fluxus-engineering.com) using the median joining network (MJN) approach with Maximum Parsimony (MP) Calculation (Polzin and Daneschmand, 2003).

In order to calculate the pairwise distances, seven different polydorids mitochondrial COI gene sequences were retrieved from Genbank: *P. brevipalpa* (KP231319-25; KR052121-4; KR052126-7; KR052130-5), *P. websteri* (KR337461-72), *P. aura* (KR052136-40), *B. androgyna* (JX276718-9), *B. syrtis* (JX276729-31), *B. acus* (JX276663-717), *B. proboscidea* (JX276721; JX276723-8). The data set was aligned using Clustal X 1.83 with default parameters, and resulting alignments were manually edited using the BioEdit program (Hall, 1999). Pairwise distances for intraspecific and interspecific polydorid species were calculated using MEGA 6.06 (Tamura *et al*., 2013) with the Kimura-2-parameter (K2P) model, and are presented in Table IV.

RESULTS

Adult worms and larvae

During the study of *P. websteri* on the oyster *C. hongkongensis*, we found that the prevalence and abundance of *P. websteri* on large hosts (more than 10 cm in diameter) were higher than those on small hosts (less than 10 cm in diameter). The worms often formed mud blisters in the inner layer of the shells. In most cases, sinuous tubes and several worms were often found in the same mud blister. On three occasions (Feb 4, 2015; Mar 10, 2015; Apr 3, 2015) the larval density of *P. websteri* was investigated in the same locality around the oyster farm waters. We found that the larval density reached 1000 ind m^{-3} on Feb 4, 2015, which was higher than that on Mar 10, 2015 and Apr 3, 2015. At these two time points, the larval density

Table II.- Morphological characteristics of *Polydora websteri* which showed 99.4% to 100% sequence identity to one another based on mitochondrial COI sequences.

Speciemen No.	Palp pigmentation	Prostomium (anterior edge)	Caruncle (maximal length)	Major spines on chaetiger 5	pygidium
YJ-3	groove-edge, continuously	weakly incised	end of chaetiger 3	lateral flange or sheath	disc-like
YJ-7	groove-edge, continuously	weakly incised	end of chaetiger 3	lateral flange or sheath	disc-like
YJ-8	bar-like, uncontinuously	weakly incised	end of chaetiger 3	lateral flange or sheath	disc-like
YJ-14	absent	incised	middle of chaetiger 4	lateral flange or sheath	cup-like
YJ-15	absent	weakly incised	end of chaetiger 3	flange absent	disc-like
YJ-16	groove-edge, continuously	incised	end of chaetiger 3	lateral flange or sheath	disc-like
YJ-17	bar-like, uncontinuously	weakly incised	middle of chaetiger 4	flange absent	cup-like
YJ-22	groove-edge, continuously	incised	end of chaetiger 3	lateral flange or sheath	disc-like
YJ-25	groove-edge, continuously	incised	middle of chaetiger 4	lateral flange or sheath	cup-like
YJ-26	groove-edge, continuously	weakly incised	end of chaetiger 3	flange absent	disc-like
YJ-40	absent	weakly incised	middle of chaetiger 4	lateral flange or sheath	disc-like
YJ-44	groove-edge, continuously	weakly incised	end of chaetiger 3	flange absent	cup-like

ranged from 5 to 10 ind m^{-3}. All stages of developing larvae were found on Feb 4, 2015. Three-chaetiger to seven-chaetiger larvae were dominant.

Morphological characterization

Systematics

Family Spionidae Grube, 1850

Genus *Polydora* Bosc, 1802

Polydora websteri Hartman *in* Loosanoff and Engle, 1943

Material examined

Yangxi county, Guangdong Province, China (21°44′46.60″N, 111°45′23.14″E), from the shells of oysters *Crassostrea hongkongensis*, coll. L. T. Ye, 4 Feb 2015, NSB20150010 (5 spec.), NSB20150011-15 (40+ spec.).

Description of adult worms

Adults worms measuring up to 15 mm long and 1.0 mm wide at chaetiger 5, with up to 100 chaetigers. Body pale or light tan in life (Fig. 1a, b). Palps with continuously groove-edge pigmentation, or non-continuous bar-like pigmentation line (Fig. 1a) along edges of food groove, or pigmentation absent (Table II). Rod-like papillae scattered inside the groove and along the margin of palps (Fig. 1d). Pygidium white in colour (Fig. 1b). Eyes absent or present. If present, four eyes trapezoidal in arrangement. Prostomium weakly bilobed or incised, caruncle extending back to the end of chaetiger 3 or middle of chaetiger 4 (Fig. 1c, Table II). Occipital antenna absent.

Chaetiger 1 with capillary neurochaetae, notochaetae absent. Special notopodial spines absent on posterior chaetigers. Neuropodial bidentate hooded hooks from chaetiger 7, up to 10 per series in middle chaetigers, decreasing to 3 in number on posterior chaetigers, with constriction on shaft. Branchiae from chaetiger 7, absent from the last several posterior chaetigers (Fig. 1f). Pygidium disc-like or cup-shaped, with a dorsal to narrow incision (Fig. 1b, f, Table II).

Chaetiger 5 greatly modified, almost twice as long as adjacent ones, with several winged capillary noto- and neurochaetae. 5-8 heavy spines arranged in a slightly oblique row, alternating with pennoned companion chaetae. Heavy spines falcate, with lateral flange or sheath on concave side, or flange absent (Fig. 1e, Table II).

Description of larvae

Three-chaetiger larvae measured on average 267.9 µm long and 142.88 µm wide at the head. Two pigmented eyespots were apparent on the dorsal surface of the head (Fig. 2a). Seven to ten long, serrated chaetae were present on each side of chaetiger 1 (Fig. 2a, d). Fewer serrated chaetae were present on chaetiger 2 and chaetiger 3. The prototroch with a band of fine cilia encircling the head, with the exception of the dorsal part (Fig. 2a, d). Five-chaetiger larvae measured on average 303.6 µm long and 145.88 µm wide at the head. Four pigmented eyespots were present, the anterior pair larger than the posterior pair (Fig. 2b). Two rows of pigment were scattered across the dorsal surface of each chaetiger (Fig. 2b). The vestibule with bundles of cilia, extended posteriorly to chaetiger 2 (Fig. 2g). The telotroch with a circle of cilia was observed near the posterior end (Fig. 2h). The nototroch began from chaetiger 3, and one row of cilia was seen across the dorsal side of each chaetiger (Fig. 2e). Thirteen-chaetiger larvae measured on average 750.1 µm long and 160.7 µm wide at the head. A pair of round palps was present on both sides of the head (Fig. 2c, f).

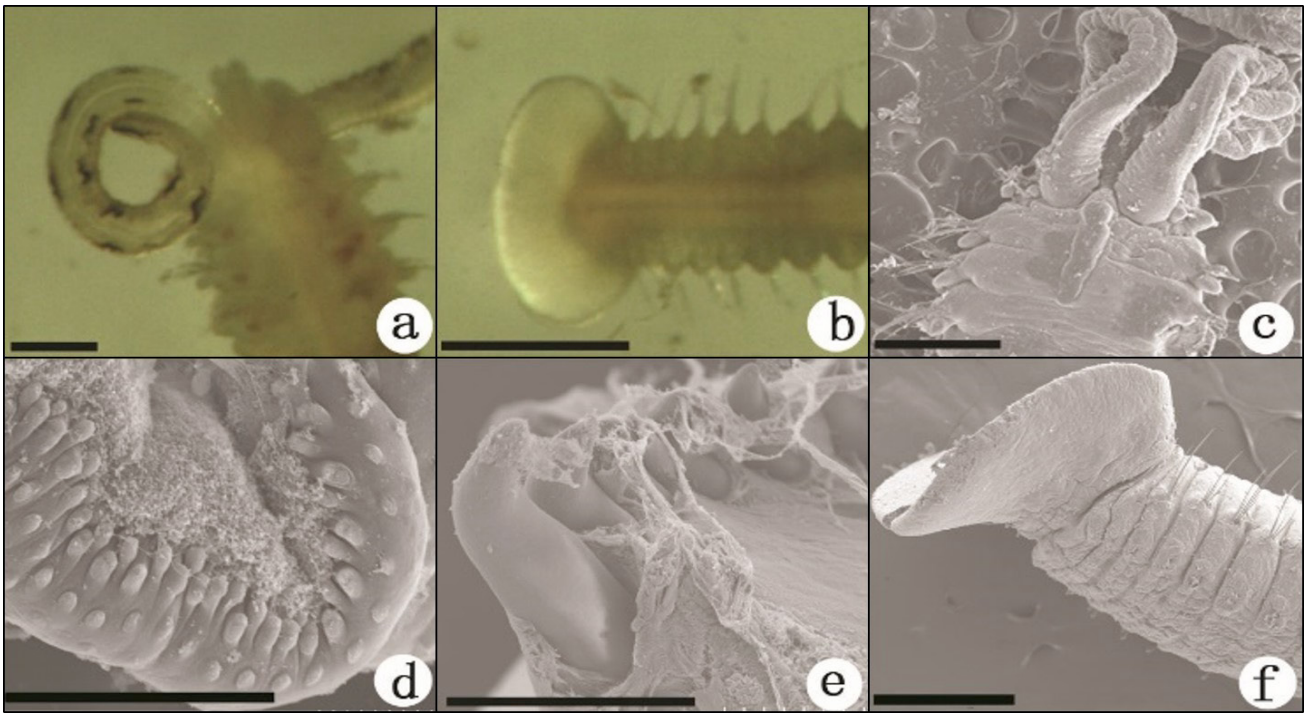

Fig. 1. Adult worms of *Polydora websteri*. **a–b,** Light photographs; **c–f,** SEM images: **a,** Anterior end, dorsal view, showing non-continuous bar-like pigmentation line along the groove edges of palps; **b,** Posterior end, ventral view, showing cup-shaped pygidium; **c,** Anterior chaetigers with the caruncle extending back to posterior end of chaetiger 3; **d,** Margin of palps on which rod-like papillae are scattered; **e,** Heavy spines of chaetiger 5; **f,** Posterior chaetigers with cup-shaped pygidium. Scale bar = 50 µm.

Table III.- Analysis of molecular variance (AMOVA) results for *Polydora websteri* between populations of adult worms and planktotrophic larvae based on mitochondrial COI sequences. A total of 25 individuals are used for analysis, including 13 adult worms and 12 planktotrophic larvae.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	F_{st}	P
Among population	1	0.264	0.07384 Va	-6.64		
Within population	23	27.256	1.18506 Vb	106.64		
Total	24	27.520	1.11123	100	-0.066	0.980

Molecular characterization

After aligning and editing the 25 COI sequences of *P. websteri*, a fragment of 853 bp was further analyzed. There were 11 polymorphic sites and no insertions or deletions. Among 11 polymorphic sites, six were parsimony informative sites, and five were singleton variable sites (Fig. 3). A total of 10 haplotypes were identified. Sequence divergence among the 10 haplotypes, according to the model of Tamura and Nei (1993), ranged from 0.12% to 0.71%, with an average of 0.27%. The hierarchical AMOVA test indicated that 106.64% of the genetic variation was attributed to within populations of adult worms and planktotrophic larvae, while negative variation (–6.64%) was attributed to variability between populations of adult worms and planktotrophic larvae (Table III). No significant genetic structure (Fst=–0.066, P=0.980) was detected among populations of adult worms and planktotrophic larvae (Table III). A parsimony network showed that 8 of 10 haplotypes were unique and represented by a single individual, including three adult individuals, and five larvae (Fig. 4). The most common haplotype (H3) accounted for 48% (12 of 25) of all individuals sampled, including seven adult individuals, and five larvae (Fig. 4). Haplotype H1 was shared by five individuals, three adult individuals and two larvae (Fig. 4). The value of haplotype diversity (h) and nucleotide diversity (π) was 0.75±0.08 and 0.00269±0.00040, respectively. This indicated that the COI sequences of *P. websteri* had a high level of haplotype diversity and low nucleotide diversity.

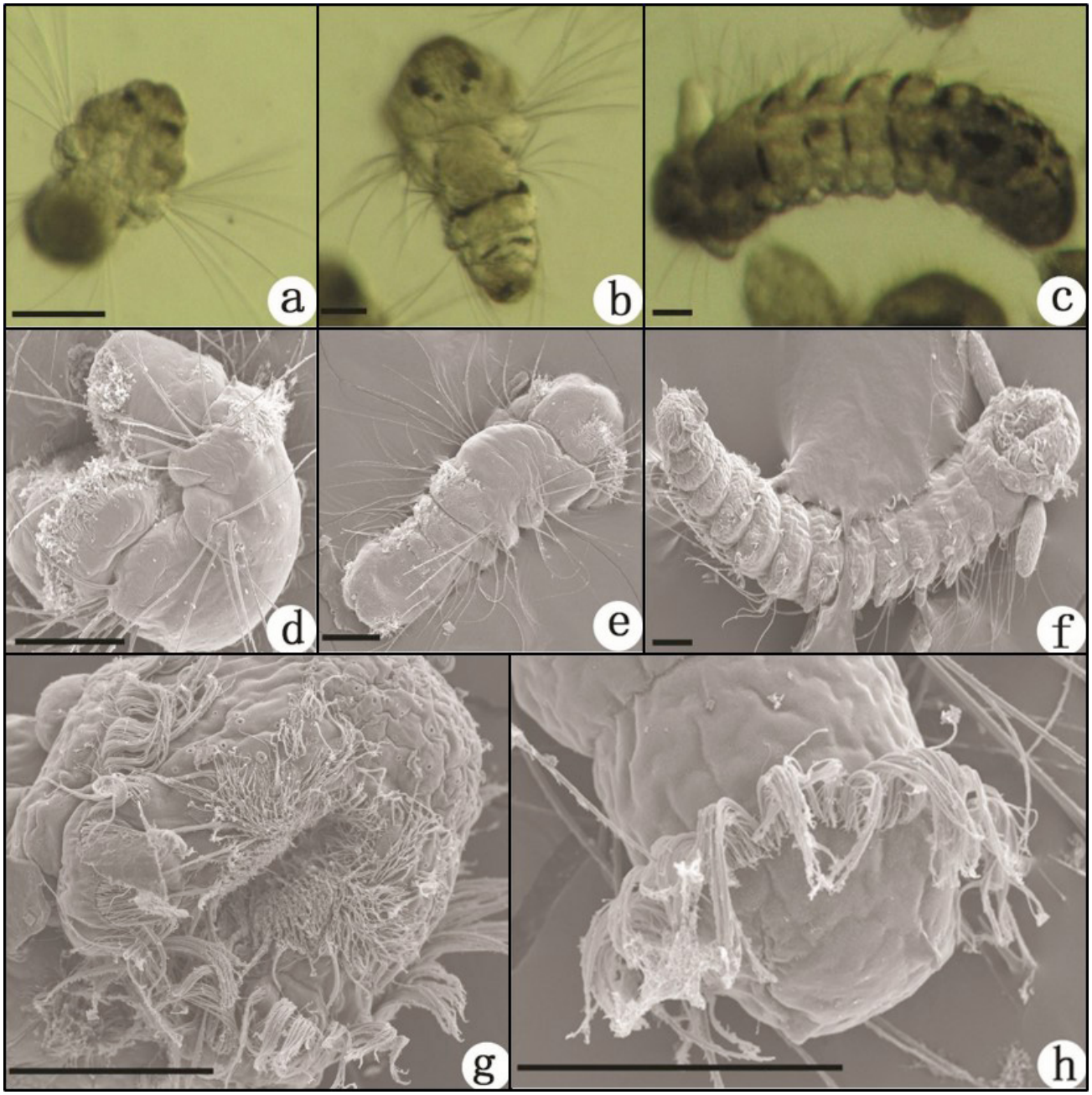

Fig. 2. Larvae of *Polydora websteri*. **a–c,** Light photographs; **d–h,** SEM images: **a,** Three-chaetiger larva, dorsal view, showing two pigmented eyespots on the dorsal surface of the head; **b,** Five-chaetiger larva, dorsal view, two rows of pigment scattered across the dorsal surface of each chaetiger; **c,** Thirteen-chaetiger larva, dorsal view; **d,** Three-chaetiger larva, lateral view, serrated chaetae present on each side of the chaetigers; **e,** Five-chaetiger larva, dorsal view, the prototroch with a band of fine cilia, encircling the head, with the exception of the dorsal part; **f,** Thirteen-chaetiger larva, ventral view, a pair of round palps present on both sides of the head; **g,** Ventral view of the head, showing the vestibule with bundles of cilia; **h,** Dorsal view of posterior end, the telotroch with a circle of cilia. Scale bars = 50 μm.

Genetic distance analyses showed that the intraspecific distance of the polydorids ranged from 0.33% to 6.42%, whereas the interspecific distance ranged from 12.87% to 24.79% (Table IV). The intraspecific distance of

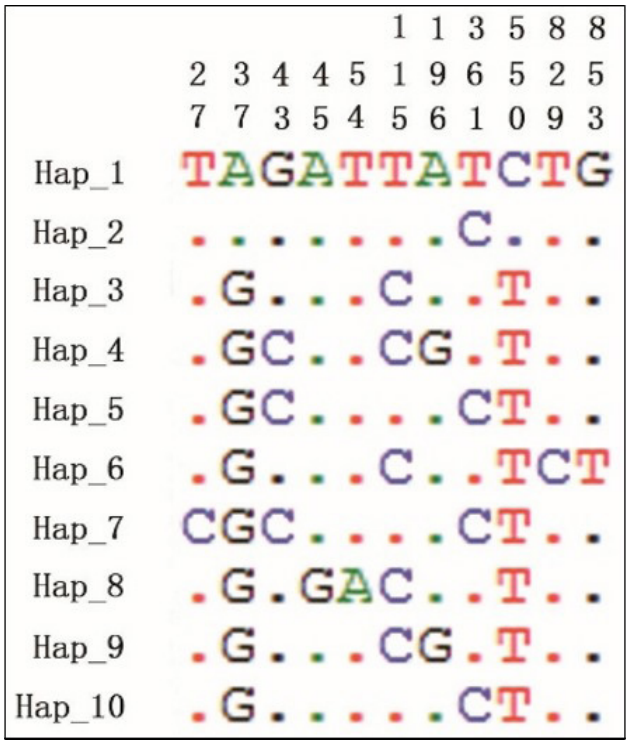

Fig. 3. Location of mutative nucleotide acids in 10 haplotypes of mitochondrial COI sequences from *Polydora websteri*. Hap_1—Hap_10 represent the names of 10 haplotypes. Numbers on the top represent locations of mutative nucleotide acids in the 10 haplotypes. Dots indicate the bases which are the same as Hap_1.

P. websteri was 0.33%, whereas *P. websteri* had the largest interspecific distance compared with *B. proboscidea* (24.79%), and the smallest distance compared with *P. brevipalpa* (18.88%). The COI gene sequences of *P. websteri* showed 99.4% to 100% sequence identity to one another, and 79.1% (vs. *B. proboscidea*) to 82.7% (vs. *P. brevipapa*) sequence identity to other polydorid species.

DISCUSSION

In the present study, morphological characteristics of the adult polydorid worms such as palps with groove-edge black pigmentation, chaetiger 5 bearing broad-flanged spines, and the body lacking dorsal black pigmentation, were in good agreement with the identification characteristics of *P. websteri* (Read, 2010; Sato-Okoshi and Abe, 2013).

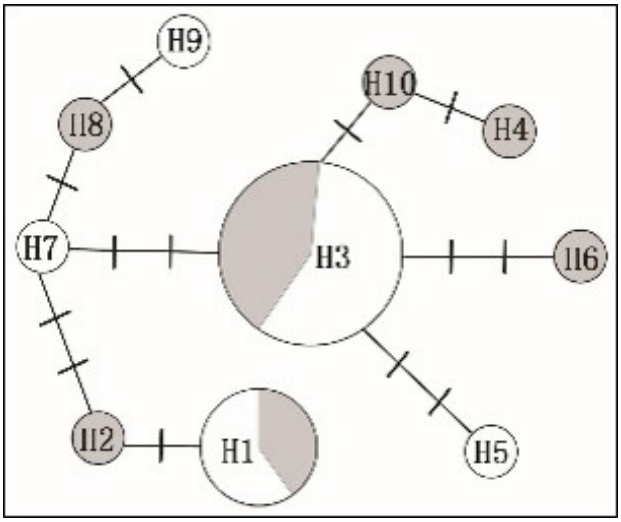

Fig. 4. Mitochondrial haplotype network of *Polydora websteri* based on COI sequences. Network represents 10 haplotypes; 8 unique, 1 shared by 5 individuals and 1 shared by 12 individuals. Each perpendicular line represents one mutation step. The areas in grey represent the larval individuals, and the areas in white represent adult individuals. The areas of the circles are proportional to the number of samples of each haplotype.

Table IV.- Intraspecific and interspecific Kimura-2-parameter (K2P) distances relating to the mitochondrial gene COI of the polydorins retrieved from Genbank. Taxon names are represented by names and numbers. P: *Polydora*; B: *Boccardia*.

Species	No. of specimen	Origin	Intraspecific distance (%)	Interspecific distance (%)					
				1	2	3	4	5	6
P. brevipalpa	16	China	0.33						
P. aura	5	China	1.34	23.66					
P. websteri	25	China	0.38	18.88	22.56				
B. androgyna	2	Canada	1.08	20.43	22.66	21.07			
B. syrtis	3	Canada	6.42	24.86	20.75	22.31	18.97		
B. acus	42	Canada	1.51	23.31	19.92	18.92	19.40	12.87	
B. proboscidea	6	Canada	1.67	23.89	24.17	24.79	23.36	22.47	21.04

In addition, the 18S rDNA sequences of the worms (Genbank no. KP231302) showed 100% sequence identity to those of *P. websteri* collected from Japan (Genbank No. AB705402) and Australia (Genbank No. AB705405) (Sato-Okoshi and Abe, 2013). Therefore, we can assume that the adult polydorid worms in the present study belonged to *P. websteri*. By comparing the morphological characteristics of *P. websteri,* which showed 99.4% to 100% sequence identity to one another based on mitochondrial COI sequences, we found that *P. websteri* showed variation in palp pigmentation pattern, the shape of the anterior edge of the prostomium, the shape of the major spines on chaetiger 5, and shape of the pygidium (Table III). The distinct pigmentation patterns on palps were regarded as one of the main characteristics in the morphological identification of *P. websteri* (Blake, 1996; Read, 2010; Sato-Okoshi and Abe, 2013). However, our results demonstrated that palp pigmentation of *P. websteri* was variable: some had continuous groove-edge pigmentation, some had non-continuous bar-like pigmentation, and some had no pigmentation (Table III). Such intraspecific variations in palp pigmentation is common, possibly caused by adaption to different environments and food sources (Sato-Okoshi and Abe, 2012). Furthermore, palp and body pigmentation quickly disappeared after fixation in formalin (Radashevsky and Pankova, 2006; Sato-Okoshi and Abe, 2013). Therefore, this characteristic was unsuitable to be a key for the identification of *P. websteri*. In view of the large intraspecific variations in morphological characteristics, it is hard to differentiate between *P. websteri* and other similar species. The evaluation of other characteristics such as sperm shape, differences in methyl green staining, and boring activity are necessary to avoid misidentification of the species (Radashevsky and Pankova, 2006; Read, 2010; Sato-Okoshi and Abe, 2013).

In contrast to the variations in phenotypic characteristics, molecular characteristics exhibit consistent and obvious variations between different species, especially sibling species. Several molecular gene markers have been used for species identification in polydorids including the 18S ribosomal RNA (18S rRNA) and mitochondrial genes (Rice *et al*., 2008; Sato-Okoshi and Abe, 2013; Radashevsky and Pankova, 2013; David *et al*., 2014). Sato-Okoshi and Abe (2012) first demonstrated that 18S rRNA gene sequences were effective for distinguishing sibling polydorid species in the Pacific and Asian waters. Radashevsky and Pankova (2013) and Ye *et al*. (2015) also found that integration with 18S rRNA gene sequences was helpful in the morphological identification of polydorid species. However, by comparing the 18S rRNA gene sequences of polydorids available from Genbank, it can be seen that the interspecific variations are commonly very small, some even almost overlapped with intraspecific variations. For example, the interspecific variation of *P. websteri* (Genbank No. AB705405) and *P. haswelli* (Genbank No. AB705402) was only 0.53% (9 nt /1706 nt), whereas the intraspecific variation of *P. aura* collected from Japan (Genbank No. AB705409) and China (Genbank No. KR052141) was 0.35% (6 nt /1716 nt). Such intra- and inter-specific variations are small enough to cause confusion in the molecular identification of morphologically similar species.

Due to the comparatively large barcoding gaps between intra- and inter-specific distances, the mitochondrial COI gene is regarded as a standard marker and is frequently used for the identification of metazoans (Erseus and Kvist, 2007). Our results demonstrated that, with the exception of *B. syrtis*, the intraspecific genetic distances of polydorids ranged from 0.33% to 1.67%, whereas interspecific distances ranged from 18.88% to 24.79% (Table IV). However, *B. syrtis* from Canadian waters showed 6.42% intraspecific variation and 12.87% interspecific variation compared with *B. acus* (Table IV). Hebert *et al*. (2003) showed that most metazoans exhibited lower than 2% variation in genetic distances between their congeners, and 10–25 % variation between species. Hebert *et al*. (2004) proposed that the interspecific variation should be more than 10 times the mean intraspecific variation for the group under study. With the exception of *B. syrtis*, our results are in good agreement with the variation ranges and standard threshold proposed by these authors. Further study on the COI sequences of *B. syrtis* is required to determine whether such a large interspecific variation existed or whether the sample contained some cryptic species.

Rice *et al*. (2008) first used the COI gene for the molecular identification of polydorid polychaetes. A large genetic divergence between the nominal *P. cornuta* suggested that populations of *P. cornuta* may comprise some cryptic species. However, with the exception of the *P. cornuta* complex of species, no other COI sequences of polydorid species were mentioned in their study. Hence, the study did not assess the delimitation of intraspecific and interspecific variation among polydorid species. Furthermore, the primer to amplify the target fragments of the COI gene was not provided in the study. Different from most other metazoans, our preliminary experiments demonstrated that the so-called “universal” PCR primers (Folmer primers, HCO2198-LCO1490) were not all able to amplify the target fragments of the COI gene among polydorid species. In the present study, two polydorid-specific primers were successfully designed to amplify

approximately 700–1000 bp fragments of the COI gene. This study represents the first attempt to utilize polydorid-specific COI primers to study the species diversity of polydorids. Our results demonstrated that the COI gene provided high resolution in the classification of polydorid species. However, of nine genera of polydorid species, only a few species in the genus *Polydora* and *Boccardia* were related in the present study. Furthermore, our sampling was limited to Chinese coastal waters, and the number of samples was small. Hence, in order to fully evaluate the usefulness of COI as a general barcode for polydorid species, extensive sampling in terms of individuals and geographical coverage is urgently required in future studies.

Among the limited number of morphological characteristics, chromatophore pigmentation pattern and arrangement is regarded as the most effective criterion for morphological identification of polydorid larvae (Blake and Woodwick, 1975; Day and Blake, 1979; Radashevsky, 2005). However, there are significant limitations based on the morphological identification of larvae. First, it is difficult to differentiate sibling larvae as the pigmentation pattern shows great variation in the different developmental stages of the larvae even between congeners. Second, morphological identification of larvae relies on viviperception of the specimens, as the fixation of larvae may lead to deformation, or the loss of morphological characteristics such as pigmentation pattern. Third, some polydorid species have two morphologically different larvae (adelphophagic and planktotrophic) in their growth stage, thus it is difficult to confirm whether they belong to the congeners based on morphological identification (David *et al*., 2014). Finally, the exact identification of larvae to their corresponding adults relies on laboratory-reared specimens, and most larvae are difficult to artificially rear in the laboratory, possibly due to the effect of the unique environmental factors (Blake, 1969). Our results demonstrate that all COI sequences from the larvae showed greater than 99% sequence identity to those from adults, and some larvae even shared the same haplotypes as adults (Fig. 4). This clearly indicates that the larvae collected from sea waters around the oyster farm belong to *P. websteri*, the same species as the adult worms collected from *C. hongkongensis* oysters in the same locality. This represents the first molecular identification of unidentified larvae from the aquatic environment through the known COI sequences of adult worms. Further studies should focus on monitoring the species diversity of polydorid larvae and their variation in abundance in the aquatic environment using the molecular approach.

In future studies, the COI sequences should be integrated with morphological descriptions for unambiguous identification of polydorid species. The COI gene marker can also play an important role in resolving synonym problems in polydorid species, validating their distribution range, and elucidating genealogical relationships among the polydorid taxa.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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