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Morphological variability and molecular characterization of *Pomphorhynchus zhoushanensis* sp. nov. (Acanthocephala: Pomphorhynchidae), with comments on the systematic status of *Pomphorhynchus* Monticelli, 1905

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ABSTRACT

Species of *Pomphorhynchus* Monticelli, 1905 commonly parasitize the digestive tract of freshwater fishes, and rarely occur in marine fishes and amphibians. In the present study, *Pomphorhynchus zhoushanensis* sp. nov., collected from the barred knifejaw *Oplegnathus fasciatus* (Temminck & Schlegel) (Perciformes: Oplegnathidae) in the East China Sea, was described using integrated approaches, including light and scanning electron microscopy, and the sequencing and analysing of ribosomal [small ribosomal DNA (18S) and internal transcribed spacer (ITS)] and mitochondrial [cytochrome c oxidase subunit 1 (*cox*1)] target regions. The results of the molecular analyses showed that morphological differences in the shape of the neck bulb (symmetrical or asymmetrical) among individuals of *P. zhoushanensis* sp. nov. are actually intraspecific variations. Moreover, phylogenetic analyses based on the 18S, ITS and *cox*1 sequences were constructed to evaluate the phylogenetic relationships between the new species and other pomphorhynchid species. The results of the phylogenetic analyses suggested that *Pomphorhynchus* is not a monophyletic group. Based on the results of the molecular and phylogenetic analyses, the taxonomic importance of the symmetry of the neck bulb for species identification in the genus *Pomphorhynchus* is questioned.

1. Introduction

The genus *Pomphorhynchus* Monticelli, 1905 currently comprises 29 nominal species, commonly parasitic in the digestive tract of freshwater fishes, and occasionally marine fishes and amphibians [1,2]. Only three species have been reported from the Chinese freshwater fishes, including *Pomphorhynchus cylindricus* Wang & Guo, 1983 (emend.) from *Tor yunnanensis* (Wang, Zhuang & Gao) (Cypriniformes: Cyprinidae), *P. yunnanensis* Wang, 1981 from *Poropuntius exiguus* (Wu & Lin) (Cypriniformes: Cyprinidae), and *P. perforator* (von Linstow, 1908) from *Schizothorax yunnanensis* Norman (Cypriniformes: Cyprinidae) [3,4].

Previous taxonomic studies of the Pomphorhynchidae were mainly based on classical morphological methods [1,5–9]. Recently, molecular approaches, utilising the ribosomal [small ribosomal DNA (18S) and internal transcribed spacer (ITS)] and/or mitochondrial [cytochrome c oxidase subunit 1 (*cox*1)] target sequences as genetic markers, have been used for distinguishing and identifying members of the Pomphorhynchidae [10–13]. Complementing conventional taxonomic work, molecular methods can help separate sibling species, reveal cryptic diversity, unambiguously identify eggs, larvae, females and fragments of parasites to the species level, and test the morphological variability of parasites in terms of intraspecific or interspecific variation.

During a helminthological survey of Chinese marine fishes, several pomphorhynchid acanthocephalans were collected from the barred knifejaw *Oplegnathus fasciatus* (Temminck & Schlegel) (Perciformes: Oplegnathidae) in the East China Sea. Their examination, using both light and scanning electron microscopy, revealed that these acanthocephalans represented an undescribed species of *Pomphorhynchus*. In addition, we also observed the presence of two different morphotypes among these parasites (i.e. some individuals had an asymmetrical neck bulb, whereas in others the neck bulb was symmetrical). According to conventional taxonomical criteria, the morphology of the neck bulb (for example, reduced or well developed, symmetrical or asymmetrical) is

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considered to be crucial for discriminating species of *Pomphorhynchus* [1,4,8,9,14]. In order to elucidate whether the two different morphotypes represent different species or a single species and to evaluate the taxonomic importance of the morphology of the neck bulb (symmetrical or asymmetrical), the specimens of the two different morphotypes were characterized using molecular methods. This was achieved by sequencing and analysing three different genes differing in their rate of evolution, including the ribosomal 18S and ITS rDNA and mitochondrial *cox*1. Moreover, the phylogenetic analyses based on these three different genetic markers were examined to determine the genetic relationships between the new taxon and the other pomphorhynchid species.

2. Material and methods

2.1. Light and scanning electron microscopy

A total of 16 specimens of *Oplegnathus fasciatus* (Temminck & Schlegel) (Perciformes: Oplegnathidae) were examined for parasites, which were caught by commercial trawlers in the East China Sea, off Zhoushan Islands (29°30′–31°00′N, 121°30′–125°00′E), Zhejiang Province, China. Live acanthocephalans collected from the guts of host fish were kept in tap water for a few hours until the proboscis was everted, and then fixed and stored in 80% ethanol until studied. For light microscopical studies, acanthocephalans were cleared in lactophenol. Drawings were made with the aid of a Nikon microscope drawing attachment. For scanning electron microscopy (SEM), specimens were fixed in 4% formaldehyde, post-fixed in 1% OsO4, dehydrated via an ethanol series and acetone, and then critical point dried. The specimens were coated with gold at 20 nm and examined using a Hitachi S-4800 scanning electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 20 kV. In order to observe the structure of the neck bulb, specimens are carefully dissected under a compound microscope (Nikon-SMZ18). Measurements (the range, followed by the mean in parentheses) are given in micrometres unless otherwise stated. Width measurements are of maximum width. For two-dimensional measurements, length is given before width. Type specimens are deposited in College of Life Sciences, Hebei Normal University, Hebei Province, P. R. China (accession numbers HBNU–F-A-2017001L–2017003L).

2.2. Molecular procedures

Three selected specimens were subjected to molecular analysis (Table 1). Genomic DNA from individual worms was extracted using a Column Genomic DNA Isolation Kit (Shanghai Sangon, China) according to the manufacturer's instructions. DNA was eluted in elution buffer and kept at − 20 °C until use. The partial 18S region was amplified by polymerase chain reaction (PCR) using the forward primer (5′-AGATTAAGCCATGCATGCGT-3′) and the reverse primer (5′-GCAGGTTCACCTACGGAAA-3′) [15]. The partial *cox*1 region was amplified by PCR using the forward primer (5′-GGTCAACAAATCATAAAGATATTGG-3′) and the reverse primer (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) [16]. The partial ITS region was amplified by PCR using the forward primer (5′-GTCGTAACAAGGTTTCCGTA-3′) and the reverse primer (5′-TATGCTTAAATTCAGCGGGT-3′) [10]. The cycling conditions were as described previously [13]. PCR products were checked on GoldView-stained 1.5% agarose gels and purified with Column PCR Product Purification Kit (Shanghai Sangon, China). Sequencing was carried out using a DyeDeoxyTerminator Cycle Sequencing Kit (v.2, Applied Biosystems, California, USA) and an automated sequencer (ABI-PRISM 377). Sequencing for each sample was carried out for both strands. Sequences were aligned using ClustalW2 and adjusted manually. The newly-generated sequences were compared (using the algorithm BLASTn) with those available in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

2.3. Phylogenetic analyses

Phylogenetic trees were constructed using maximum likelihood (ML) and maximum parsimony (MP) analyses based on partial 18S, ITS and *cox*1 sequences. *Acanthocephalus nanus* Van Cleave, 1925 was chosen as the outgroup. Sequences of 18S, ITS and *cox*1 were individually aligned using the MUSCLE algorithm in MEGA 7 with the default alignment parameters and then refined manually. The Kimura 2-parameter mode for 18S and ITS and the Hasegawa-Kishino-Yano model for *cox*1 were identified as optimal for the maximum likelihood analyses. The Tree-Bisection-Reconnection model for the 18S, ITS and *cox*1 was identified as optimal for the maximum parsimony analyses. Reliabilities for both ML and MP trees were tested using 1000 bootstrap replicates and bootstrap values exceeding 70 were considered well supported.

3. Results

Family Pomphorhynchidae Yamaguti, 1939.
Genus *Pomphorhynchus* Monticelli, 1905.

3.1. Pomphorhynchus zhoushanensis sp. nov.

3.1.1. Morphological diagnosis (Figs. 1, 2)

General. Palaeacanthocephala, Pomphorhynchidae, with characters of genus *Pomphorhynchus*. Worms small, yellowish-brown when alive. Trunk cylindrical, slightly enlarged anteriorly. Neck very long, conspicuously expanded in middle, forming distinct, asymmetrical or nearly symmetrical bulb (Figs. 1A, B, 2A, B). Proboscis short, club-shaped, distinctly expanded anteriorly, with 14–16 spiral, longitudinal rows of 7–11 hooks each (Figs. 1A–C, 2A–C). Proboscis armature almost identical in both sexes; usually, anteriormost hooks slightly shorter, but somewhat stouter. All hooks with simple roots directed posteriorly (Figs. 1D, 2G, H). Proboscis receptacle long, double-walled, extending into body-cavity for short distance. Outer muscular wall of receptacle complete posteriorly, with cerebral ganglion near posterior end (Fig. 1A, B). Lemnisci subequal, small, digitiform (Fig. 1A, B). Gonopore terminal in both sexes.

3.1.2. Morphotype I (with asymmetrical neck bulb)

Male [Based on 3 mature specimens]. Trunk 10.2–22.5 (15.3) mm long, 952–1500 (1251) wide. Neck 3.78–5.08 (4.58) mm long by 150–325 (242) wide, representing 22.6–37.1 (29.9)% of trunk length. Bulb 2.58–3.43 (3.00) × 1.75–2.38 (2.06) mm. Proboscis 309–644

Table 1
Specimens of *Pomphorhynchus zhoushanensis* sp. nov. collected from the Barred knifejaw *Oplegnathus fasciatus* (Temminck & Schlegel) (Perciformes: Oplegnathidae) in the East China Sea selected for molecular analysis.

Samples	GenBank nos. of 18S	GenBank nos. of ITS	GenBank nos. of *cox*1	Characteristics
1 Female	KY490051	KY472823	KY490047	Morphotype I, with asymmetrical bulb of neck
1 Male	KY490050	KY472822	KY490046	Morphotype I, with asymmetrical bulb of neck
1 Female	KY490049	KY472821	KY490045	Morphotype II, with symmetrical bulb of neck

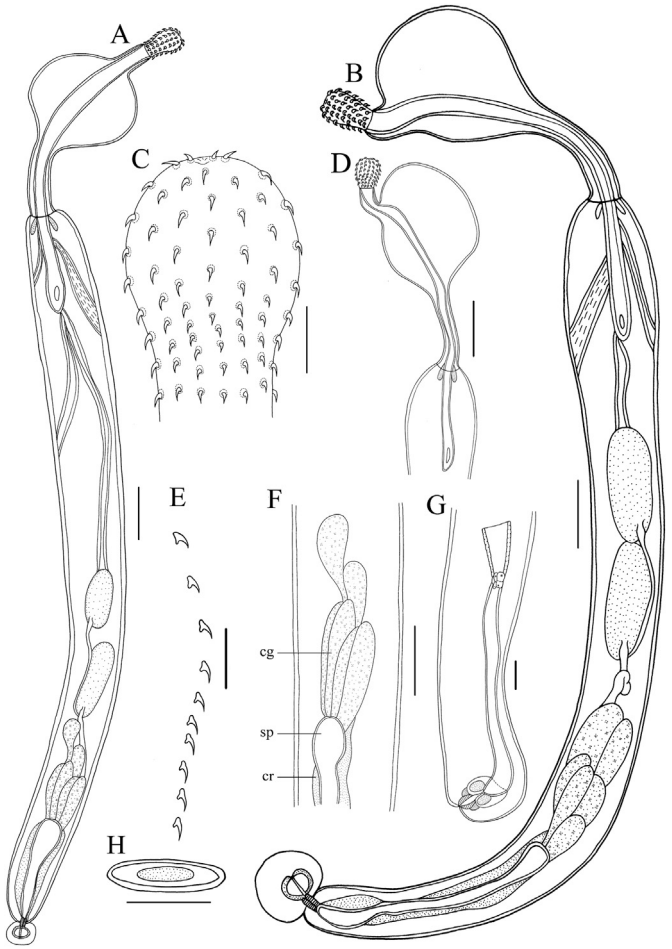

Fig. 1. *Pomphorhynchus zhoushanensis* sp. nov. from *Oplegnathus fasciatus* (Temminck & Schlegel) in the East China Sea. A, mature male with almost symmetric neck bulb; B, mature male with asymmetrical neck bulb; C, proboscis; D, anterior part of female; E, hooks in one row; F, cement glands; G, posterior part of female; H, mature egg. *Abbreviations*: cg–cement glands; sp–saeftigen's pouch; cr–cement reservoir. *Scale bars*: A, B, D, F = 1000 μm; C, G = 200 μm; E = 100 μm; H = 50 μm.

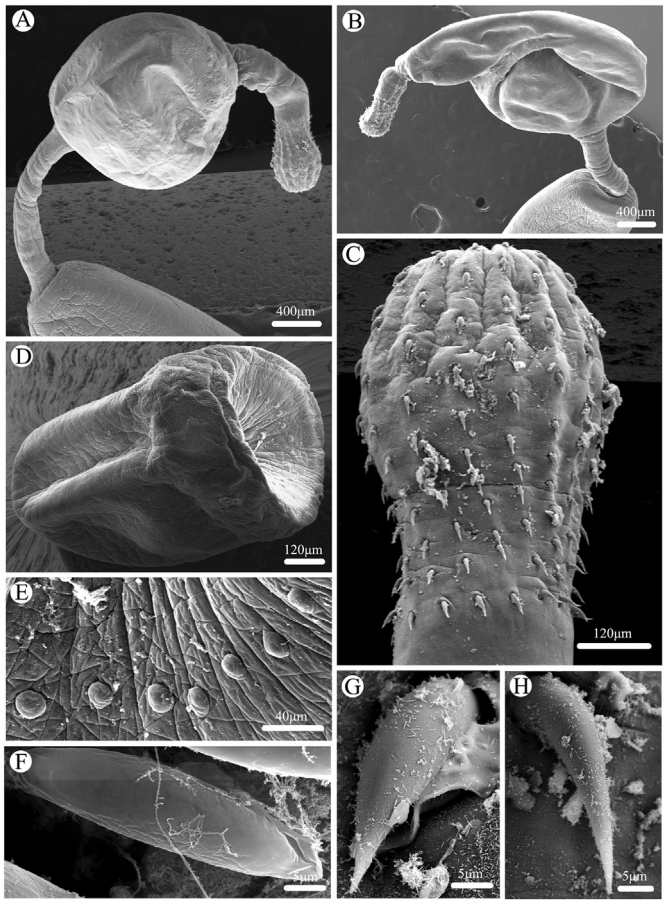

Fig. 2. Scanning electron micrographs of *Pomphorhynchus zhoushanensis* sp. nov. from *Oplegnathus fasciatus* (Temminck & Schlegel) in China. A, mature male with almost symmetrical neck bulb, lateral view; B, mature male with asymmetrical neck bulb (bulb collapsed), lateral view; C, proboscis of male, lateral view; D, copulatory bursa, lateral view; E, magnified image of sensory papillae of copulatory bursa; F, egg; G, magnified image of anteriormost hook of proboscis; H, Magnified image of posterior hook of proboscis.

(522) × 327–459 (401). Shortest (anteriormost) proboscis hooks 20–28 (25) × 13–18 (15); middle hooks 25–30 (28) × 12–15 (13); longest (posteriormost) hooks 29–38 (33) × 10–13 (12). Proboscis receptacle 5.78–6.30 (6.18) mm × 125–375 (233). Lemnisci almost equal; left lemniscus 252–317 (293) × 99–175 (128); right lemniscus 207–323 (271) × 50–109 (75). Testes oval, equatorial or slightly more anterior, usually contiguous (Fig. 1B). Anterior testis 1.07–1.91 (1.52) mm × 476–905 (644) wide, posterior testis 1.19–1.65 (1.42) mm × 476–650 (568) wide. Six cement-glands subequal, elongate-pyriform, closely arranged laterally or partly overlapping, short distance posterior to posterior testis, 475–1485 (951) × 143–374 (296) wide (Fig. 1B). Saefftigen's pouch just posterior to cement glands, 440–1120 (790) × 400–450 (420) anteriorly (Fig. 1E). Copulatory bursa 714–929 (845) × 762–1191 (974) wide, with about 30 sensory papillae arranged in single circle (Figs. 1B, 2D, E).

Female [Based on 3 gravid specimens]. Trunk 7.90–12.3 (10.1) mm × 850–1262 (1056). Neck 3.03–3.75 (3.39) mm × 250–425 (337), representing 30.5–38.4 (33.6)% of trunk length. Bulb 1.88–2.00 (1.94) × 1.15–1.30 (1.23) mm. Proboscis 359–680 (520) × 388–515 (452). Shortest (anteriormost) proboscis hooks 25–30 (28) × 12–16 (13); middle hooks 28–35 (32) × 10–13 (11); longest (posteriormost) hooks 30–42 (35) × 8–12 (10). Proboscis receptacle 4.68–5.28 (4.98) mm × 250–400 (325). Lemnisci almost equal; left lemniscus 337–396 (367) × 79–149 (114); right lemniscus 347–495 (421) × 78–99 (85) wide. Uterine bell funnel-shaped, 248–421 (334) × 194–286 (240). Uterus 1.63–1.89 (1.76) mm long; vagina 221–385 (343) × 149–243 (196) (Fig. 1F). Reproductive system 2.10–2.70 (2.43) mm long, about 22.0–26.6 (24.1)% of trunk length. Eggs fusiform, elongate, with concentric membranes and bluntly pointed polar ends; outer shell 63–67 (65) × 14–17 (16) (Figs. 1G, 2F).

3.1.3. Morphotype II (with almost symmetrical neck bulb)

Male [Based on 1 mature specimen]. Trunk 16.2 × 1.55 mm. Neck 4.98 mm × 500, representing 30.7% of trunk length. Bulb 2.28 × 2.30 mm. Proboscis 735 × 425. Shortest (anteriormost) proboscis hooks 24–32 (28) × 15–18 (16); middle hooks 26–38 (33) × 13–15 (14); longest (posteriormost) hooks 30–48 (42) × 10–13 (12). Proboscis receptacle 7.24 mm × 272. Lemnisci almost equal; left lemniscus 359 × 99; right lemniscus 396 × 90. Testes oval, equatorial or slightly more posterior, usually separate (Fig. 1A). Anterior testis 1.37 mm × 590; posterior testis 1.77 mm × 500. Six cement-glands subequal, elongate-pyriform, closely arranged laterally, short distance posterior to posterior testis, 833–1619 × 357–405 (Fig. 1A). Saefftigen's pouch just posterior to cement glands, 745 × 410 anteriorly. Copulatory bursa 495 × 852.

Female [Based on 1 gravid specimen]. Trunk 13.2 mm × 1444. Neck 5.11 mm × 410, representing 38.7% of trunk length. Bulb 1.92 × 2.05 mm. Proboscis 748 × 447. Size of proboscis hooks in female almost identical to that in male. Proboscis receptacle 7.51 × 296. Lemnisci almost equal; left lemniscus 398 × 111; right lemniscus 398 × 111. Uterine bell funnel-shaped, 444 × 220. Uterus 2.00 mm

long; vagina 323 × 190. Reproductive system 2.76 mm long, occupying 20.9% of trunk length. Eggs fusiform, elongate, with concentric membranes and bluntly pointed ends, outer shell 69–72 (71) × 15–17 (16).

Type-host and type-locality: Barred knifejaw *Oplegnathus fasciatus* (Temminck & Schlegel) (Perciformes: Oplegnathidae); East China Sea (off Zhoushan Islands) (29°30′–31°00′N, 121°30′–125°00′E), P.R. China.

Site in host: Intestine.

Prevalence and intensity of infection: 1 of 16 *O. fasciatus* were infected with 8 specimens.

Type specimens: Holotype: male (HBNU–F-A-17002L, with asymmetrical neck bulb), allotype: female (HBNU–F-A-17003L, with asymmetrical neck bulb), paratypes: 2 males, 2 females (HBNU–F-A-17004L, with asymmetrical neck bulb), paratypes: 1 male, 1 female (HBNU–F-A-17005L, with symmetrical neck bulb).

Etymology: The specific epithet refers to the type-locality, off the Zhoushan Islands.

3.1.4. Molecular characterization

3.1.4.1. 18S region. Two 18S sequences of the morphotype I and one 18S sequence of morphotype II of *P. zhoushanensis* sp. nov. were all 1660 bp in length; no nucleotide differences were detected between the three 18S sequences. There are three other *Pomphorhynchus* species with 18S sequences registered in GenBank, and pairwise comparison between *P. zhoushanensis* and these species produced 2.06% (*P. laevis*, GenBank no. AY423346) to 16.3% (*P. bulbocoli*, GenBank no. AF001841) nucleotide differences. The 18S sequences of *P. zhoushanensis* (KY490049–KY490051) are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

3.1.4.2. ITS region. Two ITS sequences of the morphotype I and one ITS sequence of morphotype II of *P. zhoushanensis* sp. nov. were all 633 bp in length, and there are no nucleotide differences detected between the three ITS sequences. There are three other *Pomphorhynchus* species with ITS sequences registered in GenBank, and pairwise comparison between *P. zhoushanensis* and these species showed 31.9% (*P. tereticollis*, GenBank nos. JF706705, AY424670) to 39.5% (*P. lucyi*, GenBank no. AY135418) nucleotide differences. The ITS sequences of *P. zhoushanensis* (KY472821–KY472823) are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

3.1.4.3. Cox1 region. Two *cox*1 sequences of the morphotype I and one *cox*1 sequence of morphotype II of *P. zhoushanensis* sp. nov. obtained herein were all 667 bp in length; no nucleotide differences were detected between the three *cox*1 sequences. There are three other *Pomphorhynchus* species with *cox*1 sequences registered in GenBank, and pairwise comparison between *P. zhoushanensis* and these species showed 26.6% (*P. tereticollis*, GenBank no. AY423353) to 39.5% (*P. bulbocolli*, GenBank no. DQ089709) nucleotide differences. The *cox*1 sequences of *P. zhoushanensis* (KY490045–KY490047) are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

3.1.4.4. Phylogenetic analyses. The ML and MP trees obtained based on the ITS, 18S and *cox*1 sequences are similar in topology (Fig. 3); both showed that the new species is sister to *Longicollum pagrosomi* Yamaguti, 1935 with high support values. The phylogenetic analyses based on ITS and 18S sequences indicated that *P. tereticollis* (Rudolphi, 1809) and *P. laevis* (Zoega in Müller, 1776) form a sister assemblage, which displays a close relationships to species of *Tenuiproboscis* Yamaguti, 1935 (Fig. 3A–D). However, in the phylogenetic trees based on the *cox*1 sequence, *P. tereticollis*, *P. laevis* and *P. bulbocolli* Linkins in Van Cleave, 1919 are clustered with *Tenuiproboscis* sp. NKS-2011, forming a paraphyletic group with low support values (Fig. 3E, F). The present results of phylogenetic analyses based on the three different genes all rejected the monophyly of the current concept of *Pomphorhynchus* [1,2].

4. Remarks

The genus *Pomphorhynchus* was established mainly based on the neck relatively long, forming bulb anteriorly, the proboscis with one type of hooks and six cement glands [1,2]. The gross morphology of the present specimens collected from the barred knifejaw *Oplegnathus fasciatus* (Temminck & Schlegel) (Perciformes: Oplegnathidae) in the East China Sea, especially the long neck possessing conspicuous symmetrical or asymmetrical bulb, clearly indicated they should belong to *Pomphorhynchus*. *Pomphorhynchus zhoushanensis* sp. nov. is the first species of *Pomphorhynchus* reported from Chinese marine fishes. The morphology of the new species is distinctly different from all the *Pomphorhynchus* species recorded from the China. For instance, *P. yunnanensis* has a special spindle-shaped trunk. As far as we are aware, in this genus only *P. spindletruncatus* Amin, Abdullah & Mhaisen, 2003 has a similar body shape [2]. The neck of *P. cylindricus* is only 1.28–1.76 mm long, which is distinctly shorter than that of *P. zhoushanensis* (3.0–5.1 mm). Furthermore, *P. cylindricus* has 12 longitudinal rows of proboscis hooks, slightly less than *P. zhoushanensis* (14–16 longitudinal rows). *Pomphorhynchus perforator* can be readily differentiated from the new species by having a much longer proboscis (about 1.48 mm) and shorter neck (not > 2.0 mm).

Among the other *Pomphorhynchus* species reported from the non-Chinese regions, The new species is similar to the following three species in having a very long neck forming a distinct bulb (neck > 3.0 mm), a relatively short proboscis (not > 1.0 mm) with 12–16 longitudinal rows of fewer than 18 hooks each and a cylindrical trunk with a length > 6.0 mm. These three species are *P. sebastichthydis* Yamaguti, 1939, *P. patagonicus* Ortubay, Ubeda, Semenas & Kennedy, 1991 and *P. rocci* Cordonnier & Ward [6,17,18]. *Pomphorhynchus zhoushanensis* sp. nov. differs from *P. sebastichthydis* in possessing more hook rows, fewer hooks per row and a normal sized basal hook (14–16 longitudinal rows of 7–11 hooks each vs 11–12 longitudinal rows of 10–12 hooks each and the basal hook distinctly longer than the others in *P. sebastichthydis*). In addition, the trunk and neck of the new species are much longer than those of *P. sebastichthydis* (trunk 7.9–22.5 mm, neck 3.0–5.1 mm in *P. zhoushanensis* vs trunk 3.2–10.0 mm, neck 2.2–3.9 mm in *P. sebastichthydis*). The protuberance of the neck bulb and the proboscis armed with many more hooks per row in *P. patagonicus* differs from *P. zhoushanensis* (12–16 hooks per row in the former vs 7–11 hooks per row in the latter). *Pomphorhynchus rocci* has its proboscis hooks in 12 longitudinal rows with 15–18 hooks in each row and much longer lemnisci than those of the new species (lemnisci small, 0.21–0.40 mm long).

To our knowledge, *Filisoma oplegnathi* Wang & Wang, 1988 (Echinorhynchida: Fessisentidae) and *Longicollum pagrosomi* Yamaguti, 1935 (Echinorhynchida: Pomphorhynchidae) have previously been also reported from *O. fasciatus* [13,19]. However, the morphology of the proboscis, neck and lemnisci, and the number of proboscis hooks and cement-glands in *F. oplegnathi* completely differ from the new species. Although the neck of *L. pagrosomi* is also very long and conspicuously expanded, *L. pagrosomi* has no true neck bulb; thus it is also different from *P. zhoushanensis*.

5. Discussion

The present work aims to test whether the morphological differences in the shape of neck bulb (symmetrical or asymmetrical) among individuals of *P. zhoushanensis* sp. nov. can be considered as intraspecific or interspecific variation. This does not negate the assumption that other species of *Pomphorhynchus* or of species of other pomphorhynchid genera may each have only one morphotype. The keys to the genera and species of the Pomphorhynchidae [1], emphasized the importance of the neck and bulb, but, in view of the present results, qualifying associated diagnoses with molecular data may be required.

The results of the molecular analysis of the two different

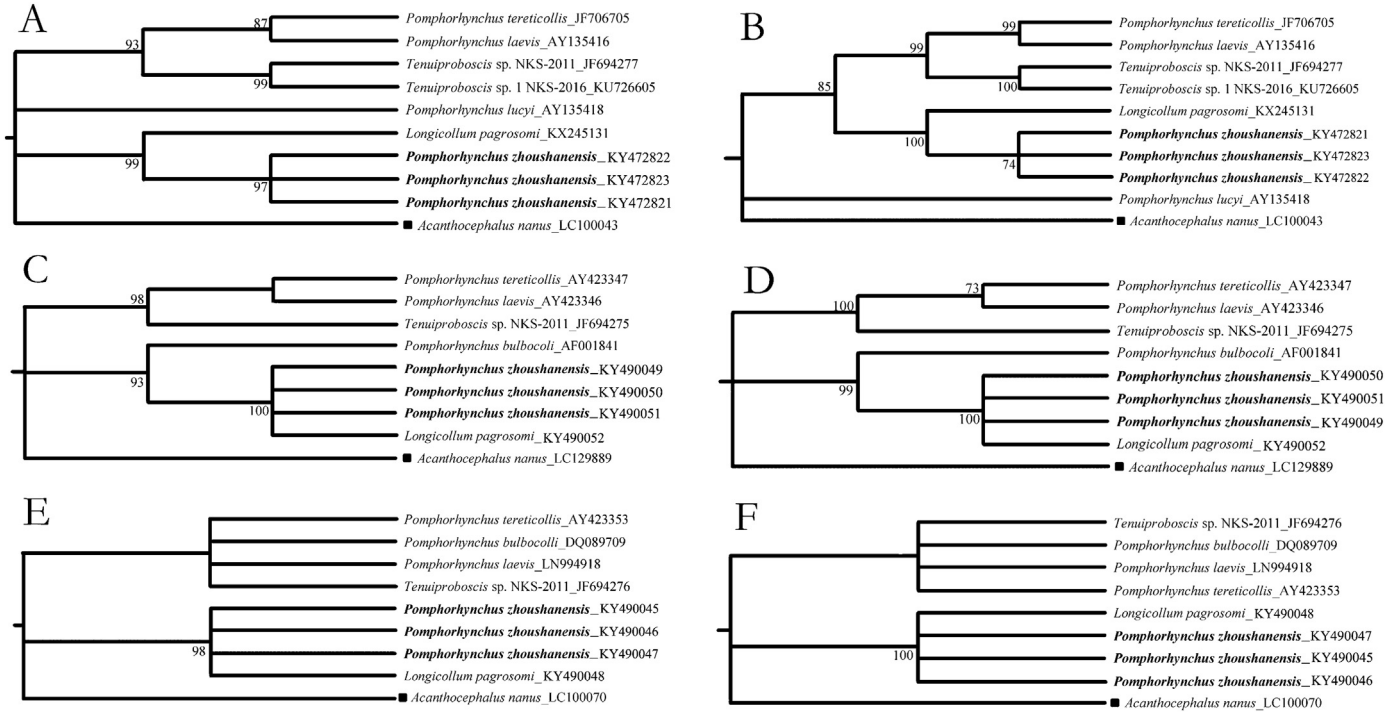

Fig. 3. Phylogenetic relationships between *Pomphorhynchus zhoushanensis* sp. nov. isolated in the present study (shown in bold) and other pomphorhynchid species registered in GenBank based on partial 18S, ITS and *cox*1 sequences. *Acanthocephalus nanus* Van Cleave, 1925 was chosen as the outgroup. Bootstrap values exceeding 70 in ML and MP trees were displayed. A, ML tree showing the genetic relationships between pomphorhynchid species based on partial ITS sequences; B, MP tree showing the genetic relationships between pomphorhynchid species based on partial ITS sequences; C, ML tree showing the genetic relationships between pomphorhynchid species based on partial 18S sequences; D, MP tree showing the genetic relationships between pomphorhynchid species based on partial 18S sequences; E, ML tree showing the genetic relationships between pomphorhynchid species based on partial *cox*1 sequences; F, MP tree showing the genetic relationships between pomphorhynchid species based on partial *cox*1 sequences.

morphotypes of the new species revealed that there are no nucleotide variations in the 18S, ITS and *cox*1 target regions, which indicates that the morphological difference in the shape of neck bulb (symmetrical or asymmetrical) between individuals of *P. zhoushanensis* should be interpreted as intraspecific variations. Such variations may be prompted by developmental or as yet unidentified factors. There is, consequently, a need to re-evaluate the taxonomic significance of this feature for the identification of species of *Pomphorhynchus*. However, because of our limited samples, this problem is still open to question. The issue may be solved once a comprehensive revision of *Pomphorhynchus*, integrating morphological and molecular approaches, is undertaken. In addition, the level of interspecific nucleotide variation in different DNA markers between *P. zhoushanensis* and other species of *Pomphorhynchus* registered in GenBank (2.06–16.3% in partial 18S region, 31.9–39.5% in partial ITS region, 26.6–39.5% in partial cox1 region) is distinctly greater than that of intraspecific nucleotide variation [*P. laevis*: 0–0.15% in partial 18S sequences (KF559309, AY423346, JX014223, AY218124), 0–0.83% in partial ITS sequences (KJ756498, KJ756500, KF559307, AY135415), 0–4.50% in partial *cox*1 sequences (AY423351–AY423353, EF051062–EF051071, KJ819957–KJ820005); *P. tereticollis*: 0–0.16% in partial ITS sequences (JF706705, AY424670), 0–3.30% in partial *cox*1 sequences (JN695504–JN695508, JF706706, AY423351, AY423352, LN994951–LN995000), no data in partial 18S sequence]. This result strongly supports the proposal that it is both fitting and practical to use the ribosomal 18S and ITS and mtDNA *cox*1 sequences as genetic markers for the accurate identification of *Pomphorhynchus* species.

The present phylogenetic analyses based on the three different genetic markers challenges the traditional classification of the Pomphorhynchidae and extends the taxonomic implications of the present findings beyond the genus *Pomphorhynchus*. According to our results, *Pomphorhynchus* is a polyphyletic taxon, because representatives of this genus were mixed with members of the genera *Longicollum* and *Tenuiproboscis*. If we want to eliminate the polyphyly of *Pomphorhynchus*, we need to determine the relationships between *Pomphorhynchus*, *Tenuiproboscis* and *Longicollum*. *Tenuiproboscis* is a poorly known pomphorhynchid genus, currently including seven species, most of which are reported from Indian marine fishes [2,20]. It was established mainly based on the following morphological characters: neck very long, uniformly cylindrical (without expansions); proboscis nearly filiform to claviform; lemnisci slender, digitiform or claviform; and cement glands 4–6 (usually 6), spherical to oval [21]. In fact, the most important characters differentiating *Tenuiproboscis* from *Pomphorhynchus* are the neck of *Tenuiproboscis* is uniformly cylindrical (not expanded in places) (vs neck not uniformly cylindrical, with anterior bulb in *Pomphorhynchus*) and the proboscis is nearly filiform to claviform. However, we do not consider these two features to be suitable generic criteria, because some *Pomphorhynchus* species (i.e. *P. dubious* Kaw, 1941, *P. orientali* Fotedar & Dhar, 1977, *P. lucyi* [14] and *P. omarsegundoi* Arredondo & Pertierra, 2010) also have an almost uniformly cylindrical neck (neck bulb very inconspicuous). Moreover, in our opinion, the shape of proboscis is only of taxonomic significance at the species level. In fact, this character is distinctly variable in different species of *Pomphorhynchus*, for example, *P. laevis* (Zoega in Müller, 1776), *P. tereticollis* (Rudolphi, 1809) and *P. kashmirensis* Kaw, 1941 have a cylindrical or claviform proboscis, *P. moyanoi* Olmos & Habit, 2007 and *P. zhoushanensis* sp. nov. have an almost club-shaped proboscis (distinctly enlarged anteriorly), and *P. rocci* Cordonnier & Ward, 1967 and *P. lucyi* have an almost vase-shaped proboscis (distinctly enlarged in middle). In our phylogenetic analyses, the species of *Tenuiproboscis* always nested within the core of *Pomphorhynchus*. Consequently, we consider that the current systematic position of *Tenuiproboscis* remains questionable and it is considered to be a genus *inquirendum*.

The genus *Longicollum* Yamaguti, 1935 was erected mainly based on the following morphological characters [21]: neck very long, more or

less spiral, conspicuously expanded on convex side but not forming true bulb; proboscis short, cylindrical; lemnisci short, saccular; and six cement glands, spherical to oval. The morphology of the neck (conspicuously expanded but not forming a true bulb) is considered as the most important diagnostic character differentiating between *Longicollum* and *Pomphorhynchus*. However, the use of this character as a diagnostic generic criterion is also dubious, because the morphology of the neck in species of *Pomphorhynchus* is very variable. For example, some species have a well-developed, symmetrical or asymmetrical neck bulb, but others have a reduced bulb (not a real bulb). The unique morphology of the neck in species of *Longicollum* may just represent different morphological forms of the neck. In addition, we have no knowledge of how the bulb forms and what its function is. We have observed, however, that the proboscis and the bulb of these parasites both penetrate the gut wall [13]. Some authors [22], among others, have also observed a similar situation in *L. pagrosomi* Yamaguti, 1935. We suggest that the neck bulb represents a structure that helps maintain the worm's attachment to the gut wall of its host. In his morphometric study of the development of *P. bulbocolli* Linkins in Van Cleave, 1919, one of the authors [23] found that recently ingested immature worms with a cylindrical neck ultimately grow and develop a prominent neck bulb to secure them in their final attachment sites in more posterior locations of their host's intestine. We found the bulb to comprise a cuticular inflation of the neck and consider that its morphology can vary. Consequently, we are uncertain as to the taxonomic importance of this traditional diagnostic feature (neck bulb reduced or well developed; symmetrical or asymmetrical) as either a generic or a specific criterion.

6. Conclusion

Our phylogenetic analyses have shown that *Pomphorhynchus*, as currently recognised, appears not to be a monophyletic group. Features of the neck and proboscis (i.e. neck uniformly cylindrical, fusiform or possessing anterior bulb; proboscis filiform, enlarged anteriorly or pyriform) used as generic criteria within the Pomphorhynchidae may be unreliable, and the systematic status of *Tenuiproboscis* and *Longicollum* is uncertain. Consequently, a more rigorous study of a wider range of pomphorhynchid taxa of is required to elucidate the phylogenetic relationship between *Pomphorhynchus* and *Longicollum* and *Tenuiproboscis*.

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