Vestnik Zoologii, **52**(1): 37–46, 2018
DOI 10.2478/vzoo-2018-0005

DE GRUYTER OPEN

UDC 595.12

REDESCRIPTION AND NEW HOST RECORD OF *DIPLOSTAMENIDES SCIAENAE* (MONOGENEA, MICROCOTYLIDAE) AND ITS PHYLOGENETIC STATUS USING MOLECULAR MARKERS

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Redescription and New Host Record of *Diplostamenides sciaenae* (Monogenea, Microcotylidae) and its Phylogenetic Status Using Molecular Markers. Verma, A. K., Verma, J., Agrawal, N. — A new host and new locality is recorded for *Diplostamenides sciaenae* (Goto, 1894) Lebedev, Parukhin et Roitman, 1970 from *Johnius belangerii* at Versova dock landing centre, Mumbai, India. The morphometric comparison of *D. sciaenae* with previously published data, provided redescription complements results of molecular analysis. The partial 28S and 18S rRNA gene sequences of *D. sciaenae* were amplified, sequenced through PCR and deposited to GenBank database. The BLASTn searches revealed the significant closeness of *D. sciaenae* to other microcotylid parasites in large and small ribosomal subunits. The phylogenetic tree analyses with neighbor joining and minimum evolution methods also expressed belonging of *D. sciaenae* to Microcotylidae.
Key words: *Diplostamenides*, *D. sciaenae*, Microcotylidae, phylogeny, large and small ribosomal subunits.

Introduction

Unnithan (1971) established the genus *Diplostamenides* with the type species *D. umbrinae* Unnithan, 1971, infecting *Umbrina russelli* Cuvier, 1829 [(now known as *Dendrophysa russelii* (Cuvier, 1829), commonly known as 'goatee croaker' (Froese and Pauly, 2017)] from Trivandrum, India. Mamaev (1977) considered *Microcotyle hemiatriospinalis* Lebedev et al., 1970; *M. madrasi* Tripathi, 1957; *M. sciaenae* Goto, 1894 as synonym of *D. umbrinae* Unnithan, 1971. In 1986, Mamaev mentioned type species *D. sciaenae* comb. n. [syn. *M. sciaenae* Goto, 1894; *Atriostella sciaenae* (Goto, 1894) Unnithan, 1971; *D. umbrinae* Unnithan, 1971;

M. hemiatriospinalis Lebedev et al., 1970; *M. madrasi* Tripathi, 1957]. According to Mamaev (1977 & 1986) *D. umbrinae* Unnithan, 1971 is junior synonym of *D. sciaenae* (Goto, 1894) Lebedev, Parukhin et Roitman, 1970. Later Zhang et al. (2001 and 2003) also reported *D. sciaenae* from *Argyrosomus pawak* and *Sciaena russelli* from China.

D. sciaenae is redescribed here with new host and new locality record, because the descriptions by Goto (1894), Yamaguti (1958), Tripathi (1957), Lebedev et al. (1970), Unnithan (1971) and Zhang et al. (2001) were based upon limited material.

In addition to the redescription, the comparative metric account of *D. sciaenae* by several authors is also given. To improvise our understanding of relationships within Microcotylidae, newly sequenced partial 28S and 18S rRNA genes of *D. sciaenae* were analyzed with other polyopisthocotylean sequences available in GenBank.

Material and methods

Total 126 specimens of *Johnius belangerii* (Cuvier, 1830) were procured at Versova dock landing centre (19°7′60 N 72°47′60 E), Mumbai (India) of Arabian Sea Region, during 2014–2015, from local fishermen with the help of bottom trawls and boat seines. The fishes were identified on the basis of fish database by Froese and Pauly (2017) and identification sheets by Fischer and Bianchi (1984). Gills were e×cised and placed at 6 °C in refrigerator overnight for the separation of worms (Mizelle, 1936). Parasites were sorted using dissecting (Motic, ST-30 series) and compound (Motic, B1-220A) microscopes. A total of 85 parasites were collected from gills. For molecular analysis, parasites were stored in microfuge tubes containing absolute alcohol at — 20 °C. Temporary mounts were prepared in glycerine and for permanent mounts helminthes were stained with Gomori´s trichome stain (Gomori, 1950) and mounted in Canada balsam or DP× resin.

All measurements were taken in micrometers using the phase contrast microscope Olympus B×51; the clamp nomenclature provided according to Hollis, 1981. Measurements were represented as the range followed by mean in parentheses. Images were captured by digital camera (cool snap HQ, Olympus) and Image Pro-express 6.0 software, using ×4 to ×100 objective lenses. Drawings were made with the aid of drawing tube. Six voucher specimens were deposited to the parasite collection of the Parasites and Vectors Section, The Natural History Museum (NHM), London with accession number NHMUK 2015.12.15.1–6. Voucher specimens of *D. sciaenae* from other authors were not available for this study.

The genomic DNA was extracted from the alcohol preserved specimens using Qiagen DNeasy tissue kit (Qiagen, Hilden, Germany) according to manufacturer´s protocol. For amplification of 28S and 18S rDNA regions, primers were commercially synthesized. For 30 µl reaction volume, 1 ×PCR [(2 mM Tris-HCl (pH 8.4), 50 mM KCl)] buffer (Invitrogen, California, USA), 1.5 mM $MgCl_2$ (Invitrogen), 200 µM of dNTP mix (Promega, Wisconsin, USA), 0.4 µM of each forward and reverse primer, 1U/µl Taq DNA polymerase (Invitrogen), 8 µl of DNA and 14.86 µl of Milli Q water were added in a microfuge tube and processed through PCR machine. After amplification, PCR products were checked on 0.5–1 % agarose gel electrophoresis. Primers and PCR conditions were selected according to Plaisance et al. (2005). The 28S rDNA gene was amplified using the forward primer Ancy 55 (5′ GAGATTAGCCCATCACCGAAG 3′) and reverse primer LSU1200R (5′ GCATAGTTCACCATCTTTCGG 3′). The 18S rDNA gene was amplified using the forward primer Worm A (5′ ACGAATGGCTCATTAAATCAG 3′) and reverse primer Worm B (5′ CTTGTTACGACTTTTACTTCC 3′). Primers for ITS1 + 5.8S rRNA region (Cable et al., 2005) — forward P3b (5′ TAGGTGAACCTGCAGAAGGATCA 3′) and reverse F3 (5′ TTGCTGCACTCTTCATC 3′) were used. The PCR conditions for 28S and 18S rRNA genes were initially denatured at 94 °C for 3 min (35 cycles of 30 sec at 94 °C, 30 sec at 52 °C, 2 min at 72 °C), after which they were e×posed to the final extension at 72 °C for 10 min and followed by cooling at 4 °C. The PCR conditions for ITS1 + 5.8S rRNA region were initially denatured at 94 °C for 2 min, 35 cycles (15 sec at 94 °C, 30 sec at 60 °C, 2 min at 72 °C), e×posed to the final extension at 72 °C for 6 min and followed by cooling at 4 °C. PCR products were examined on 1 % agarose gel stained with ethidium bromide and visualized on gel documentation system. PCR products were purified and sequenced commercially by ×celris Labs Limited, Ahmadabad, India using Big Dye Terminater version 3.1 Cycle sequencing Kit (Applied Biosystems, California, USA). Two new sequences of *D. sciaenae* were submitted to GeneBank database and sequences of partial 28S and 18 rDNA of different microcotylids were retrieved from NCBI (table 2). Sequence for ITS1 + 5.8S rRNA region of *D. sciaenae* was also deposited to GenBank (accession no. KU872047), but not utilized in the present study.

BLASTn searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed for partial sequences of 28S and 18S rDNA to reveal the degree of resemblance between species. Phylogenetic analysis was conducted using MEGA version 6.06 software (Tamura et al., 2013). Each data set was analyzed through neighbor joining (NJ) and minimum evolution (ME), using ma×imum likelihood composite method and substitution including transitions, transversions, gaps and missing data were decimated. In the analysis, the codon positions (1st, 2nd and 3rd) were also included. Bootstrap values were calculated on the basis of 1000 replicates for 28S and 18S rDNA molecular data sets. The evolutionary trees were constructed with the help of neighbor joining (NJ) and minimum evolution (ME) methods for both 28S and 18S ribosomal subunits.

Table 1. Comparative measurements of *Diplostamenides sciaenae* specimen in the present study and published data (measurements in micrometers, range followed by mean in parentheses, — indicates not available, L = length, W = width)

Source	Goto (1894)	Yamaguti (1958)	Tripathi (1957)	Lebedev (1970)	Unnithan (1971)	Zhang (2001)	Present study
Locality	Mogi, Japan	Sagami Bay,Japan	Bay of Bengal, Madras	–	Trivandrum, India	Haikou, China	Versova, India
Host	*Sciaena sina*	*Nibea schlegeli*	*Pseudosciaena diacanthus*	*Seriola sp.*	*Umbrina russelli*	*Argyrosomus pawak, Sciaena russelli*	*Johnius belangeri*
Number of specimens	–	18	–	–	2	17	15
Body (L)	~4000	3100–4400	1980–2260	3330–4250	3670–4200	4536–5043	3112–3676 (3338)
Body (W)	–	440–800	188–210	410–450	750–850	572–594	514–789 (664)
Haptor (L)	–	–	725–1058	–	–	1793–1890	1532–1721 (1631)
Haptor (W)	48–911	–	240–370	–	–	–	650–923 (777)
Total clamps	–	114–157	–	–	50–63 both side	108–115	82–98
Clamps on long row	75	58–86	60	51–52	–	–	40–52
Clamps on short row	60	56–75	60	48–49	–	–	42–46
Clamp (L×W)	–	–	34–57×26–34	–	–	49–70×31–44	–
Long row clamp (L×W)	–	–	–	–	42×50–42×63	–	51–73 (60)×28–48 (39)
Short row clamp (L×W)	–	–	–	–	21×42–29×63	–	45–60 (53)×30–37 (33)
Mouth diameter	–	–	–	–	–	–	76–83 (80)
Oral sucker (L×W)	–	62–80×33–52	–	–	–	53–67×57–73	–
Oral sucker diameter	–	–	26	–	–	–	44–56 (50)
Pharyn× (L×W)	–	41–56×31–44	26–19	–	–	44–49×49–65	–
Pharyn× diameter	–	–	–	–	36–40	–	35–52 (44)
Oesophagus (L×W)	–	–	–	–	–	–	94–124 (111)×25–31 (28)
Left intestinal crus (L)		–	–	–	–	–	2160–2332 (2247)
Right intestinal crus (L)	–	–	–	–	–	–	1912–2132 (2065)
Intestinal crus (W)	–	–	–	–	–	–	86–173 (128)
Ovary (L×W)	–	220–500×180–300	–	–	–	238–356×65–97	240–296 (269)×60–112 (86)
Number of testes	~27	13–30	13–15	~20	18	15–19	17–20
Testes (L×W)	–	–	–	–	–	162–270×108–151	–
Testes diameter	–	–	38–45	–	60–75	–	42–65 (54)
Genital atrium (L×W)	–	56–86×46–65	–	–	70–45	73–93×39–57	58–93 (75)×49–69 (60)
Genital atrium diameter	–	–	133–152	45–64	–	–	–
Total no. of atrial spine	–	12–15	–	37–41	41	23–35	62–72
Atrial spine of outer arch (L)	110	20	38	17–19	4–12	–	14–18 (16)
Atrial spine of middle arch (L)	–	65	–	–	42	–	22–31(25)
Atrial spine of inner arch (L)	20	18	11	25–29	21	–	5–8 (6)
Atrial spine arrangement	2 curves	–	2 arches	2 arches	3 arches	–	3 arches
Vas deferens (L×W)	–	–	–	–	–	–	598–633 (618)×9–13 (11)
Vaginal pore diameter	–	–	–	–	–	–	–
Egg	–	–	–	–	–	–	–

Table 2. DNA sequences of polyopsithocotylean monogeneans retrieved and analyzed in this study (– = not available, * = sequence deposited to GenBank)

Species	Family	Host	Accession no.
28S Region			
Diclidophora minor	Diclidophoridae	*Micromesistius poutassou*	AF382048
Pedocotyle bravoi	Diclidophoridae	*Stellifer minor*	KJ397729
Urocotyle nibae	Diclidophoridae	–	FJ432588
Atrispinum acarne	Microcotylidae	*Pagellus acarne*	AF311702
Bivagina pagrosomi	Microcotylidae	–	AJ243678
Cynoscionicola branquialis	Microcotylidae	*Umbrina xanti*	AF382050
Diplostamenides sciaenae	Microcotylidae	*Johnius belangerii*	KU204208*
Kahawaia truttae	Microcotylidae	*Arripis truttacea*	GU263831
Metamicrocotyla cephalus	Microcotylidae	*Mugil cephalus*	AF131720
Microcotyle arripis	Microcotylidae	*Arripis georgianus*	GU263830
Microcotyle erythrinii	Microcotylidae	*Pagellus erythrinus*	AM157221
Microcotyle mugilis	Microcotylidae	*Mugil cephalus*	AF131722
Microcotyle sebastis	Microcotylidae	*Sebastes* sp.	AF382051
Microcotyle sp. AKV-2016	Microcotylidae	*Nemipterus japonicus*	KU926692
Omanicotyle heterospina	Microcotylidae	*Argyrops spinifer*	JN602095
Pagellicotyle mormyri	Microcotylidae	*Lithognathus mormyrus*	AF311713
Polylabris heterodus	Microcotylidae	*Diplodus annularis*	AF131716
Polylabris sillaginae	Microcotylidae	*Sillaginodes punctatus*	GU289509
Sparicotyle chrysophryii	Microcotylidae	*Sparus aurata*	AF311719
18S Region			
Heterobothrium okamotoi	Diclidophoridae	*Takifugu rubripes*	AB162155
Paraeurysorchis sarmientoi	Diclidophoridae	*Seriolella violacea*	KJ397724
Bivagina pagrosomi	Microcotylidae	*Chrysophrys aurata*	AJ228775
Cynoscionicola branquialis	Microcotylidae	–	AJ287495
Diplostamenides sciaenae	Microcotylidae	*Johnius belangerii*	KT185025*
Microcotyle sp. n. SU-2015	Microcotylidae	–	KT267180
Microcotyle sebastis	Microcotylidae	*Sebastesi* sp.	AJ287540
Polylabris bengalensis	Microcotylidae	–	KT267176
Polylabris sp. JYW-2010	Microcotylidae	*Siganus fuscescens*	HM545905

Results

Class Monogenea Carus, 1863
Subclass Polyopisthocotylea Odhner, 1912
Superfamily Microcotyloidea Unnithan, 1957
Family Microcotylidae Taschenberg, 1879
Subfamily Microcotylinae Monticelli, 1892
Genus *Diplostamenides* Unnithan, 1971
Diplostamenides sciaenae (Goto, 1894) Lebedev, Parukhin et Roitman, 1970
Type host: *Johnius dussumieri* (Cuvier, 1830), unaccepted name *Sciaena sina* (Cuvier, 1830).
Type locality: Mogi, Near Nagasaki, Japan.
Additional host: *Johnius belangerii* (Cuvier, 1830) (Sciaenidae), common name 'Belanger´s croaker´.
Additional locality: Versova dock landing centre, Mumbai, Arabian Sea region, India.
Site of infection: Gills.
Infection details: Worms collected from 15 infected fishes, infection prevalence — 6 %, mean intensity — 5 (Lebedev et al., 1970); a total of 85 worms collected from 19 infected fishes, examined fishes — 126, infection prevalence — 15.07 %, mean intensity — 4.47, relative density — 0.67 (present study).

Description (figs 1–2)

The description is based on 15 specimens. Body elongated, dorso-ventrally flat, tapered at both ends. Total body length including haptor (H) 3112–3676 (3338), width 514–789 (664) ma×imum at the level of haptor. Haptor distinct from body, relatively broad and pointed at posterior end, 1532–1721 (1631) long, ma×imum width at anterior margin 650–923 (777). Haptor asymmetrical with one long and one short rows of clamps (C), 40–52 and 42–46 clamps on long and short row, respectively. Clamps in long row bigger than clamps in short row. Long row clamp length 51–73 (60), width 28–48 (39), short row clamp length 45–60 (53), width 30–37 (33). Both long and short rows of clamps containing scleritum marginal dorsale (SMD), scleritum marginal ventrale (SMV), scleritum obliqum basale (SOB), scleritum median (SM), e×treme terminal dorsale (ETD) and e×treme terminal ventrale (ETV). SM with long ventral and short dorsal arms. Bident structure present both in long and short rows of clamps.

Mouth subterminal, sub-circular, with diameter of 76–83 (80). Two rounded, aseptate oral suckers (OS) 44–56 (50). Pharyn× (P) pyriform, laying behind oral suckers, its diameter 35–52 (44). Oesophagus (OE) tubular, narrow 94–124 (111) long, 25–31 (28) wide, branched above level of genital atrium (GA). Intestinal crura (IC) beginning at level of genital atrium and reaching haptoral region; posteriorly not confluent, left branch slightly longer than right, terminating at level of anterior haptor; long branch length 2160–2332 (2247), short branch length 1912–2132 (2065), width of both branches 86–173 (128). Genital atrium muscular, 58–93 (75) long, 49–69 (60) wide; three prominent rows of atrial spines (AS), first outer row of broad and thick spines 10–11, length 14–18 (16), second inner row of

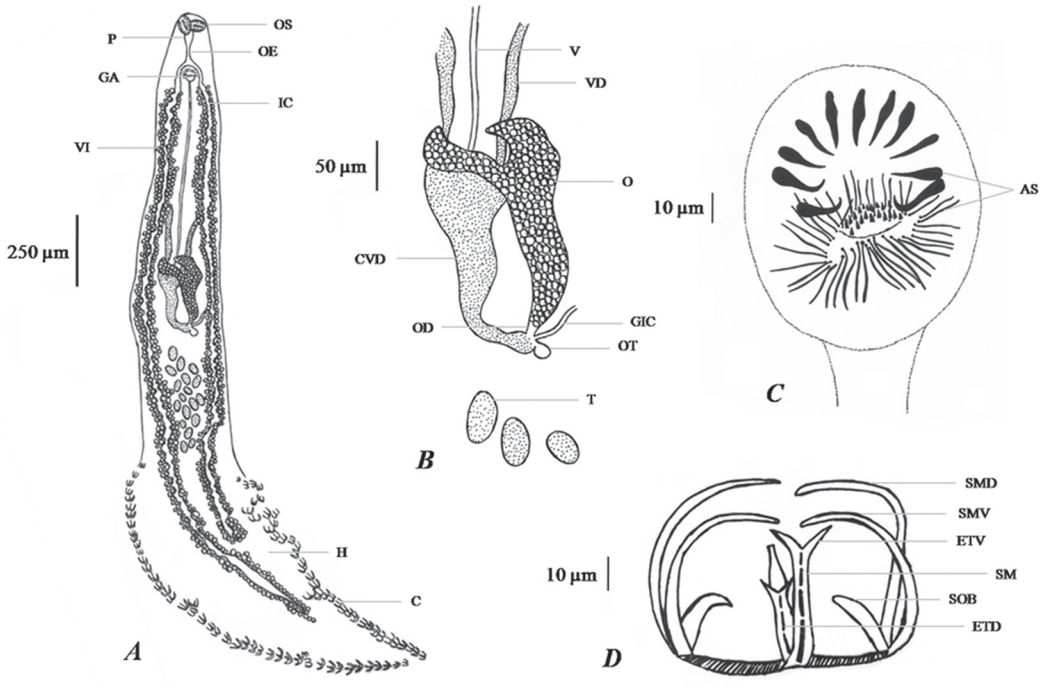

Fig. 1. *Diplostamenides sciaenae*: *A* — whole mount (ventral view): OS, oral sucker; P, pharyn×; OE, oesophagus; GA, genital atrium; IC, intestinal caecum; VI, vitellarium; H, haptor; C, clamp *B* — reproductive system: V, vas deferens; VD, vitelline duct; O, ovary; CVD, common vitelline duct; GIC, genitointestinal canal; OD, oviduct; OT, ootype; T, testes *C* — genital atrium and spines: AS, atrial spines; *D* — clamp and associated sclerites: SMD, scleritum marginal dorsale; SMV, scleritum marginal ventrale; SM, Scleritum median; SOB, scleritum obliqum basale; ETD, extreme terminal dorsale; ETV, extreme terminal ventrale.

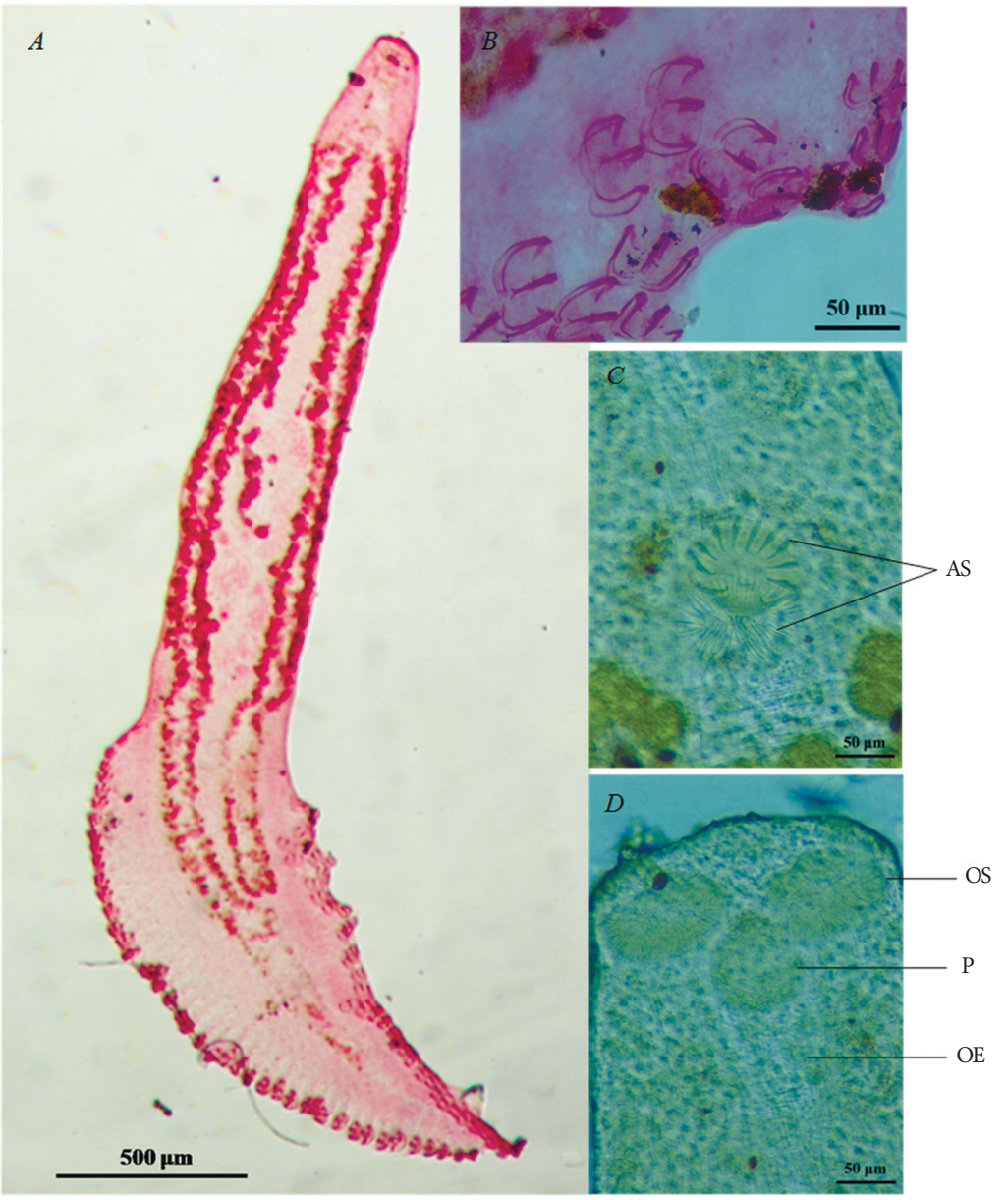

Fig. 2. *Diplostamenides sciaenae* digital phototmicrographs (present study): *A* — whole mount; *B* — clamp and associated sclerites; *C* — gential atrium and spines; *D* — anterior region with oral suckers, pharyn× and oesophagus. Abbreviations are provided in figure 1.

spines 40–45, length 22–31 (25), third innermost row of shortest spines 12–16 length 5–8 (6). Broader and thick spines of atrium comprising crown-like armature of cirrus.

Ovary (O) elongated, tube-like, situated in middle region of body anterior to testes (T), length 240–296 (269), width 60–112 (86). Oviduct (OD) short tube, running posteriorly from ovary, joining common vitelline duct (CVD) and genitointestinal canal (GIC), leading forward to ootype (OT). Common vitelline duct 225–264 (244) long, just behind ovary bifurcating into two vitelline ducts (VD), each 103–134 (119) long, reaching towards vitellaria (VI). Uterus and vaginal pore not observed. Testes (T) post-ovarian, intercaecal, limited to posterior region of body, roughly circular, 17–20 in number, diameter 42–65

(54). Vas deferens (V) as straight tube, originating from middle of body and opening into genital atrium, length 598–633 (618), width 9–13 (11). Vitellaria of several minute follicles, co-extensive with intestinal crura reaching up to haptor region. Eggs not observed.

Remarks

The comparative measurements of *D. sciaenae* in the present study along with other published records (Goto, 1894; Yamaguti, 1958; Tripathi, 1957; Lebedev et al., 1970; Unnithan, 1971; Zhang et al., 2001) are presented in table 1.

Molecular analysis

The nucleotide BLAST searches for *D. sciaenae* (India) showed the ma×imum similarity of 99 % with *D. sciaenae* (China, accession no. FJ432589), 93 % with *Cynoscionicola branquialis* and 88 % with *Omanicotyle heterospina* in case of 28S rDNA while 95 % with *Polylabris* sp. JYW-2010, 94 % with *Microcotyle sebastis* and 93% with *Bivagina pagrosomi* in case of 18S rDNA. This high magnitude of sequence identity is adequate to place this genus into Microcotylidae. The phylogenetic analyses with NJ and ME methods displayed similar tree topology with different bootstrap values for large and small ribosomal subunits. The tree analyses of both 28S and 18S partial rRNA sequences of *D. sciaenae* and other microcotylids clustered together with respect to outgroup (figs 3 and 4).

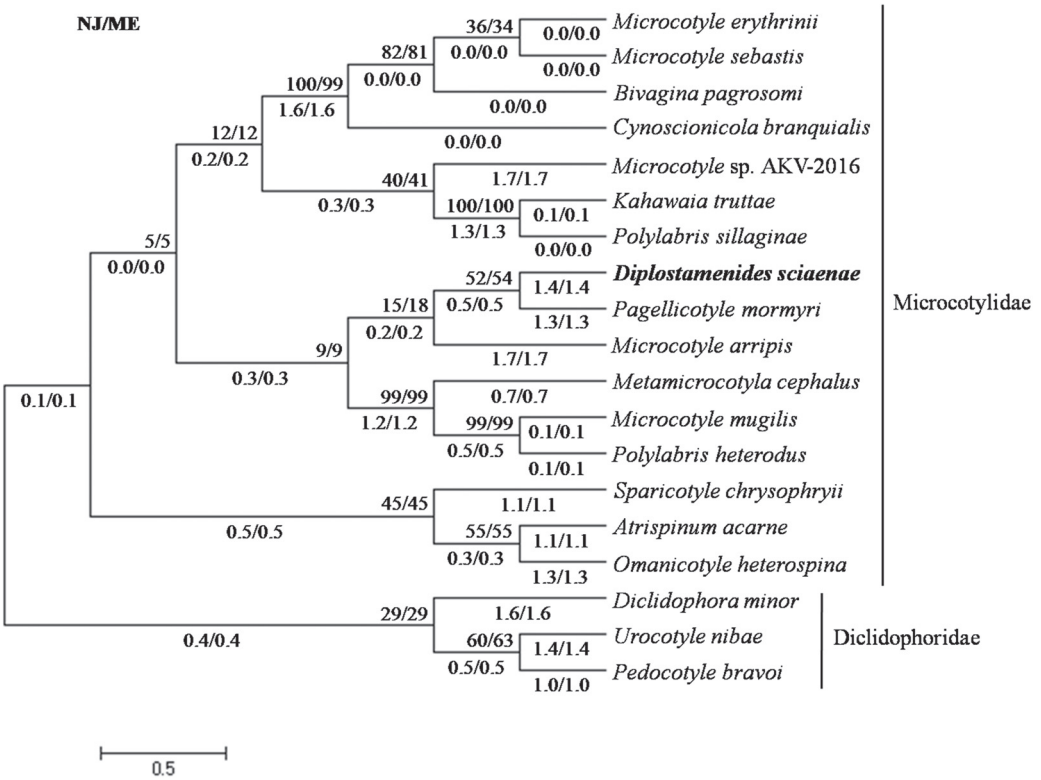

Fig. 3. Phylogenetic tree topology of parital 28S rRNA nucleotide sequence data for the members of microcotytlidae and outgroup of members of diclidophoridae through NJ and ME methods. The bootstrap values for 1000 replicates are shown as in the phylogram and branch length is genetic distance between taxa.

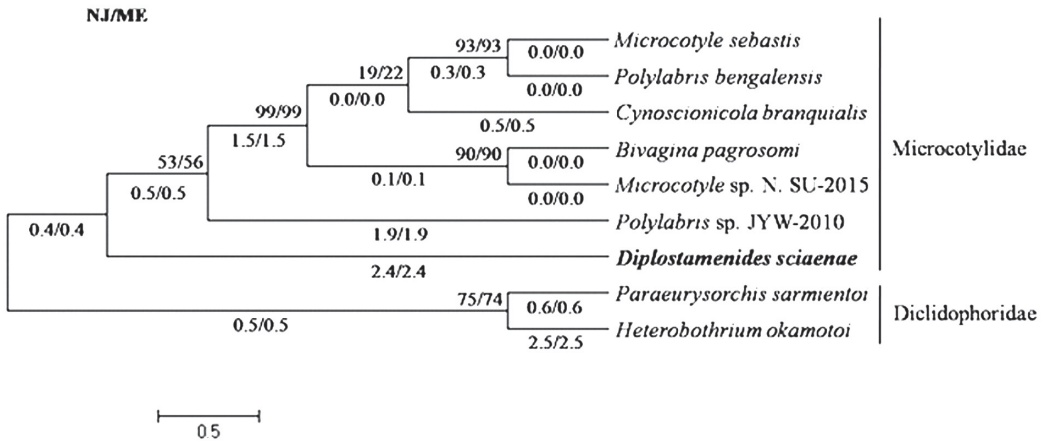

Fig. 4. Phylogenetic tree topology of partial 18S rRNA nucleotide sequence data for different microcotylids and outgroup as diclidophorids through NJ and ME methods. The bootstrap values for 1000 replicates are shown as in the phylogram and branch length is genetic distance between taxa.

Discussion

Goto (1894) originally described *Microcotyle sciaenae* from Japan. Later, Yamaguti (1958) redescribed *M. sciaenae*, agreeing with original description of Goto, 1894 on major aspects like genital atrium and its spine, except for few insignificant points [(58–86 clamps (75 after Goto) in long row and 56–75 clamps (60 after Goto) in short row and total number of clamps 114–157; number of testes 13–30 (around 27 after Goto)]. The present redescription of *D. sciaenae* shows minor differences with earlier descriptions (table 1) as follows: (1) total number of clamps are lowest in present specimen; (2) total number of atrial spines in male copulatory organ is higher in specimens studied by us; (3) uterus, vagina and vaginal pore were not detected in present study. Eggs were not observed by any investigators including the present study. In spite of these morphometric variations, the detailed anatomy of specimens studied by us is in consent with previous accounts by Goto (1894), Yamaguti (1958), Tripathi (1957), Lebedev et al. (1970), Unnithan (1971) and Zhang et al. (2001).

The host specificity of *D. sciaenae* in general (Rhode, 1979; Whittington et al., 2000) is appreciable as it is affecting only the members of Sciaenidae in published data and present study except the member of Carangidae mentioned by Lebedev et al. (1970).

Two different genetic markers (28S and 18S ribosomal subunits) were assigned to re-confirm the phylogenetic position and validity of *D. sciaenae* from India. The large and small ribosomal subunit gene sequences are proven milestone to facilitate the differentiation among homologous and heterologous sites and provide important signals to infer the phylogeny at generic and specific levels (Hillis and Dixon, 1991; Dixon and Hillis, 1993; Littlewood and Olson, 2000). Both LSU (large subunit) and SSU (small subunit) of rDNA support the morphological and molecular data to unriddle the phylogeny problems of monogeneans (Littlewood et al., 2001; Olson and Littlewood, 2002; Jovelin and Justine, 2001).

Conclusions

Diplostamenides sciaenae (Goto, 1894) Lebedev, Parukhin et Roitman, 1970 was recorded from *Johnius belangerii* at Versova dock landing centre, Mumbai, India. Morphological and molecular data analyses clearly concluded that the genus *Diplostamenides*

is valid. The global alignment searches for 28S and 18S rDNA of *D. sciaenae* revealed its significant homology with other microcotylids. The phylogenetic tree analyses clearly supported the BLAST analysis and firmly advocate the validity and position of *D. sciaenae* in Microcotylidae: Microcotylinae.

The authors are thankful to Central Institute of Fisheries Education (CIFE), Mumbai especially Dr. Gayatri Tripathi, Dr. S. K. Chakraborty, Dr. A. K. Jaiswar and Dr. K. Paniprasad for providing lab facilities and other arrangements during sample collection. Cooperation of Mr. Mukesh (CMFRI) during survey for collection of hosts is considerable. For morphological and molecular work, facilities from UGC-SAP (DRS- I & II), DST-PURSE program under the thrust area "Helminth Ta×onomy" of the Department of Zoology, University of Lucknow, Lucknow, utilized for the present study. We also acknowledge UGC for the award of a BSR- JRF (F25-1/2013-14 (BSR)/7-109/2007 (BSR) to Jyoti Verma. We would like to thank Dr. David I. Gibson of the Natural History Museum, London for the provided literature.

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Received 6 March 2017
Accepted 24 October 2017