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FISH PARASITOLOGY - ORIGINAL PAPER

Molecular and morphological characterisation of four diplostomid metacercariae infecting *Tilapia sparrmanii* (Perciformes: Cichlidae) in the North West Province, South Africa

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Abstract
Despite their pathogenic effects on fish, the diversity of trematodes from the family Diplostomidae remains vastly unexplored in Africa and specifically South Africa. To date, only six species of diplostomids have been reported from freshwater fishes in this country, with only two species being molecularly characterised. In this study, combined morphological and molecular analyses were used to identify and describe metacercariae of the Diplostomidae (Digenea) parasitising banded tilapia *Tilapia sparrmanii* (Perciformes: Cichlidae) collected within the North West Province, South Africa. Metacercariae found on the body surface and muscles of the fish were separated into four groups based on the infection site, the colour of the cysts and the morphology of excysted specimens. Isolates from each group were further identified through molecular analyses. Comparative analyses of the newly generated 28S rDNA, ITS1-5.8S-ITS2 and *cox*1 sequences revealed the presence of four species of which three were identified as *Bolbophorus* sp. 3 (28S rDNA, ITS1-5.8S-ITS2 and *cox*1), *Posthodiplostomum* sp. 9 (28S rDNA and ITS1-5.8S-ITS2) and *Uvulifer* sp. 4 (28S rDNA, ITS1-5.8S-ITS2 and *cox*1), respectively, and the fourth species belonging to the Diplostomidae gen. sp. (28S rDNA, ITS1-5.8S-ITS2 and *cox*1). Morphology of metacercariae of *Posthodiplostomum* sp. was compared with metacercariae of this genus previously reported in fishes in Africa. This study presents the first molecular data for species of *Bolbophorus* Dubois, 1935, *Posthodiplostomum* Dubois, 1936 and *Uvulifer* Yamaguti, 1934 from Africa, and it highlights the need for future research on the diversity of diplostomid parasites in South Africa and in Africa as whole.

Keywords Digenea · Metacercariae · *Tilapia sparrmanii* · DNA · South Africa

Introduction

The Diplostomidae Poirier, 1886 (Digenea) is a large and diverse family of widely distributed digeneans that infect numerous mammals and bird species. The life cycles of diplostomids typically include freshwater snails, fish and amphibians as intermediate hosts and mammals and fish-eating birds as definitive hosts (Niewiadomska 2002). The metacercarial stages of some species of the genera *Bolbophorus* Dubois, 1935, *Crassiphiala* Van Haitsma, 1925, *Diplostomum* von Nordmann, 1832, *Posthodiplostomum* Dubois, 1936, *Tylodelphys* Diesing, 1850 and *Uvulifer* Yamaguti, 1934 are known to be pathogenic to their fish intermediate hosts and can cause severe morbidity and occasionally mortalities in cases of high infection intensities (Terhune et al. 2003; López-Jiménez et al. 2017; Blasco-Costa and Locke 2017 and references therein). Although this group, particularly its type genus *Diplostomum*, has become in recent years a focus of intensive studies in Europe and North America (Blasco-Costa and Locke 2017), our knowledge of these parasites in Africa remains incomplete due to the absence of dedicated studies and presence of numerous ambiguous reports that lack of morphological and molecular evidence (Van As and Basson 1984; Khalil and Polling 1997; Florio et al. 2009; Akoll et al.

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2012; Jansen van Rensburg et al. 2013; Grobbelaar et al. 2014; Scholz et al. 2016; Kudlai et al. 2018).

To date, 21 species of the Diplostomidae have been reported from freshwater fishes in Africa (Table 1). Only six species of these, *Diplostomum* sp. (Chibwana et al. 2013, Moema et al. 2013), *Tylodelphys mashonensis* (Chibwana et al. 2013, Moema et al. 2013), *Tylodelphys* sp. 1 and *Tylodelphys* sp. 2 (Chibwana and Nkwengulila 2010; Chibwana et al. 2013), *Tylodelphys* sp. 2 (Otachi et al. 2015) and "Diplostomid metacercaria B" (Moema et al. 2013), were characterised molecularly.

Little attention has been paid to diplostomid parasites from the freshwater fishes in South Africa with six species, *Diplostomum* sp. type I and *Diplostomum* sp. type II (Prudhoe and Hussey 1977), *Neodiplostomum* sp. (Prudhoe and Hussey 1977; Van As and Basson 1984), *Ornithodiplostomum* sp. (Barson and Avenant-Oldewage 2006), *T. mashonensis* (Mashego and Saayman 1989; Moema et al. 2013) and "Diplostomid metacercaria B" (Moema et al. 2013), and a few reported metacercariae assigned to the different morphotype groups within Diplostomidae currently known (Prudhoe and Hussey 1977; Van As and Basson 1984; Khalil and Polling 1997; Barson and Avenant-Oldewage 2006; Grobbelaar et al. 2014, 2015). Although there are over 180 species of fish that occur in the freshwater systems in the country (Froese and Pauly 2017), only three species, namely, *Clarias gariepinus* (Burchell, 1822) (Siluriformes: Clariidae), *Tilapia sparrmanii* Smith, 1840 (Perciformes: Cichlidae) and *Pseudocrenilabrus philander* (Weber, 1897) (Perciformes: Cichlidae), were reported as the intermediate hosts for six diplostomid species (Mashego and Saayman 1989; Barson and Avenant-Oldewage 2006; Chibwana and Nkwengulila 2010; Moema et al. 2013; Chibwana et al. 2013 and references therein). Of

Table 1 Summary data on diplostomid trematodes from freshwater fishes in Africa

Species	Country	Source
Diplostomum garrae Zhokhov, 2014	Ethiopia	Zhokhov (2014)
Diplostomum heterobranchi Wedl, 1861	Egypt	Khalil and Polling (1997)
Diplostomum longicollis Zhokhov, 2014	Ethiopia	Zhokhov (2014)
Diplostomum magnicaudum El-Naffar, 1979	Egypt	Khalil and Polling (1997)
Diplostomum montanum Zhokhov, 2014	Ethiopia	Zhokhov (2014)
Diplostomum tilapiae Zhokhov, 2014	Ethiopia	Zhokhov (2014)
Diplostomum sp.	Nigeria	Chibwana et al. (2013)
Diplostomum sp. type I	South Africa	Prudhoe and Hussey (1977)
Diplostomum sp. type II	South Africa	Prudhoe and Hussey (1977)
Dolichorchis tregenna Nazmi Gohar, 1932	Ethiopia, Sudan	Zhokhov et al. (2010)
Neodiplostomum sp.	South Africa	Prudhoe and Hussey (1977)
Neodiplostomum type I	Botswana	Jansen Van Rensburg et al. (2013)
Ornithodiplostomum sp.	South Africa	Barson and Avenant-Oldewage (2006)
Posthodiplostomoides leonensis Wiliams, 1969	Sierra Leone	Khalil and Polling (1997)
Posthodiplostomum biellipticum Dubois, 1958 (syn. *Posthodiplostomum nanum* Dubois, 1937)	Sierra Leone, Ghana	Williams (1967), Khalil and Polling (1997)
Tylodelphys grandis Zhokhov, Morozova and Tessema, 2010	Ethiopia	Zhokhov et al. (2010)
Tylodelphys mashonensis Beverley-Burton, 1963	South Africa, Tanzania, Zambia, Zimbabwe	Mashego and Saayman (1989), Khalil and Polling (1997), Chibwana et al. (2013), Moema et al. (2013)
Tylodelphys sp. 1	Tanzania	Chibwana and Nkwengulila (2010), Chibwana et al. (2013)
Tylodelphys sp. 2	Tanzania	Chibwana and Nkwengulila (2010), Chibwana et al. (2013)
Tylodelphys sp. 2	Kenya	Otachi et al. (2015)
"Diplostomid metacercaria B"	South Africa	Moema et al. (2013)
Bolbophorus sp. 3	South Africa	Present study
Posthodiplostomum sp. 9	South Africa	Present study
Uvulifer sp. 4	South Africa	Present study
Diplostomidae gen. sp.	South Africa	Present study

these, five species were found in *C. gariepinus*, whereas *T. sparrmanii* and *P. philander* were reported as the hosts for an unidentified diplostomid "Diplostomid metacercaria B" (Moema et al. 2013).

All of the above clearly indicates that the knowledge on the diversity of diplostomid parasites in South African and in African freshwater fishes is incomplete. Thus, the aim of our study was to examine *T. sparrmanii*, a widespread cichlid fish species in Southern and Central Africa (Skelton 2001), for the presence of diplostomid trematodes in the North West Province, South Africa, and characterise any diplostomids found with the application of morphological and molecular techniques.

Materials and methods

Sample collection

A total of 47 *T. sparrmanii* individuals were collected at three sites in the North West Province, South Africa: Vaal River (26° 52′ 07.5″ S, 27° 17′ 30.6″ E), Mooi River (26° 41′ 02.9″ S, 27° 05′ 58.5″ E) and Boskop Dam (26° 32′ 35.2″ S, 27° 07′ 08.1″ E) (Fig. 1), using electrofishing, fyke and seine nets in March and April of 2017. The Mooi River is a tributary of the Vaal River, and Boskop Dam is an impoundment in the Mooi River. Fish were collected under the permit HO 09/03/17-125NW. Fish were euthanized by cranial pithing and spinal severance and examined for the presence of diplostomid parasites on the body surface, fins, muscles, eyes and brain. Encysted metacercariae were excysted under a dissecting microscope using fine needles. When possible, metacercariae were studied live, whereafter specimens were preserved in molecular grade ethanol. All metacercariae were counted and separated into four groups based on their infection site in the fish, the colour of the cysts and morphological characters. Representative metacercariae from each group were selected for DNA isolation, sequencing and morphological analyses. The voucher material preserved in ethanol was deposited in the Parasite Collection of the National Museum, Bloemfontein, South Africa (NMB).

The map with species sampling locality information was compiled in ArcGIS 10.6 (available from https://support.esri.com/en/downloads).

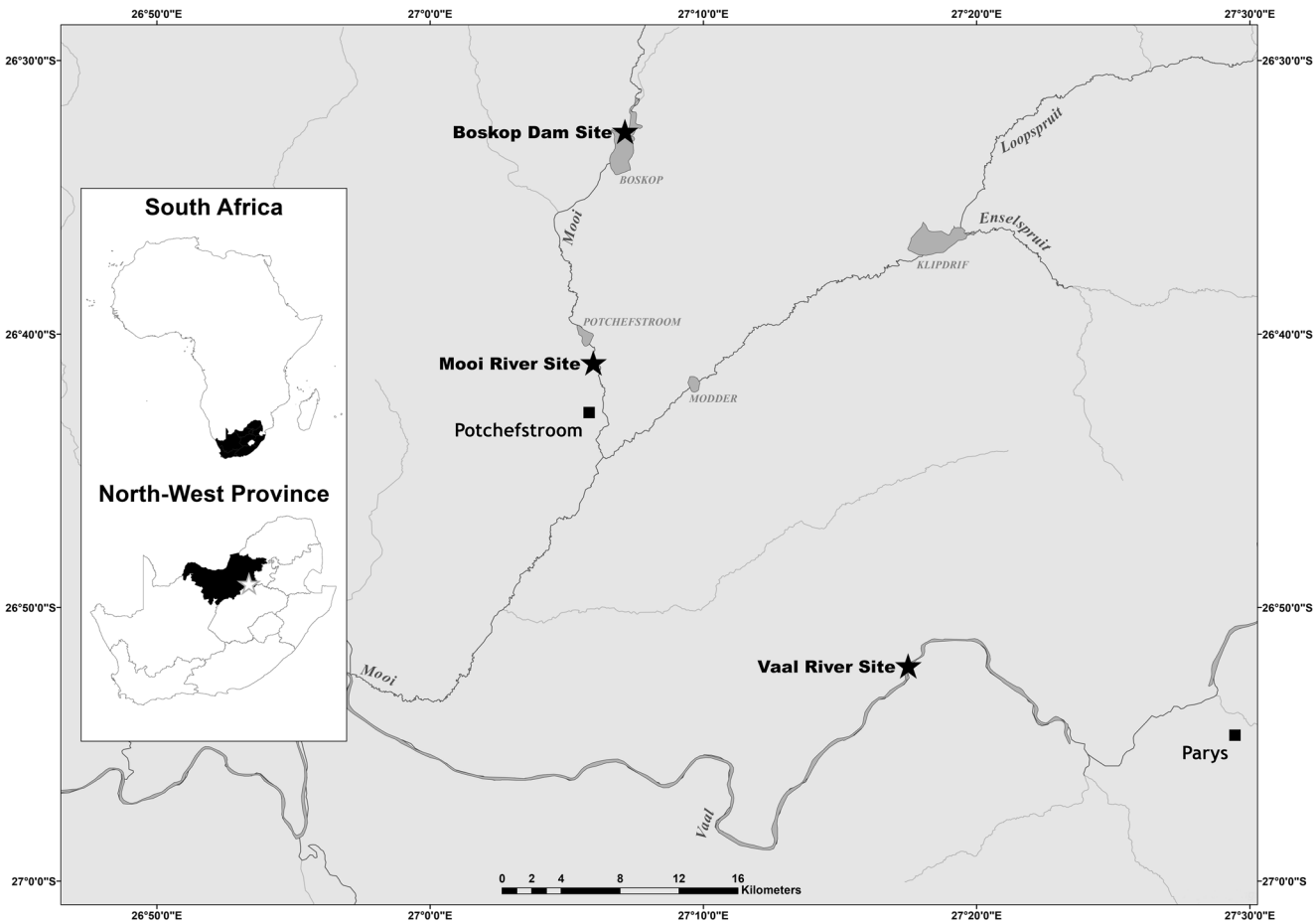


Fig. 1 Map of sampling sites in the North West Province, South Africa (sampling sites marked with stars)

Generation of molecular data

Genomic DNA from selected metacercariae was extracted using KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) following the manufacturer's protocol. DNA amplifications were performed following different polymerase chain reaction (PCR) protocols (Snyder and Tkach 2001; Galazzo et al. 2002; Tkach et al. 2003; Suzuki et al. 2006; Moszczynska et al. 2009; Van Steenkiste et al. 2015; Wee et al. 2017) using forward and reverse primers (Table 2) to amplify partial fragments of the cytochrome *c* oxidase subunit 1 (*cox*1), 18S rRNA and 28S rRNA genes and the entire ITS1-5.8S-ITS2 gene cluster. The partial fragment of the 18S rRNA gene was amplified for *Bolbophorus* sp. found in the present study in order to compare with *Bolbophorus levantinus* Dubois, 1970 and *Bolbophorus confusus* (Krause, 1914) Dubois, 1936 reported from Israel following the protocol described by Suzuki et al. (2006) using primers listed in Table 2. PCR amplicons were visualised by 1% agarose gel electrophoresis and sent to Inqaba Biotechnical Industries (Pty) Ltd. (commercial sequencing company) in Pretoria, South Africa, for purification and sequencing. Geneious v. 11 was used to assemble and edit sequences (Biomatters, Auckland, New Zealand). Sequences were deposited in GenBank under the accession numbers MK604814 (18S), MK604820–MK604826 (28S), MK604879–MK604886 (ITS1-5.8S-ITS2) and MK605688–MK605692 (*cox*1).

Phylogenetic analyses

Sequences obtained in the present study were used for basic local alignment search tool (BLAST) analyses against the sequences deposited in GenBank (NCBI, http://www.ncbi.nlm.nlh.gov) to compare and detect closely related sequences. Three alignments were constructed according to the gene/region fragment amplified using MUSCLE (Edgar 2004) implemented in Geneious v. 11 under default parameter values. The 28S alignment (973 nucleotides (nt)) included five novel sequences obtained during the study and 21 sequences retrieved from GenBank (Table S1 in the supplementary material). The ITS1-5.8S-ITS2 alignment (733 nt) comprised eight novel sequences along with 42 sequences from GenBank (Table S1 in supplementary materials). *Australapatemon niewiadomski* Blasco-Costa, Poulin and Presswell, 2016 was the selected outgroup for 28S and ITS1-5.8S-ITS2 analyses based on the results of the phylogenetic analyses by Blasco-Costa and Locke (2017). The *cox*1 alignment (316 nt) included five novel sequences and 47 sequences retrieved from GenBank (Table S1 in supplementary materials), with *Australapatemon burti* Miller, 1923 as the selected outgroup for the analyses based on the results of the phylogenetic analyses by Blasco-Costa and Locke (2017). Bayesian inference and maximum likelihood analyses were performed to assess the taxonomic position of species collected from *T. sparrmanii*. The best-fitting model was estimated prior to analyses using the Akaike information criterion (AIC) implemented in jModelTest 2.1.2 (Guindon and Gascuel 2003; Darriba et al. 2012). The best-fitting model was the general time-reversible model incorporating invariant sites and gamma distributed among-site rate variations (GTR + I + G) for the 28S and ITS1-5.8S-ITS2 datasets. The best-fitting model for the *cox*1 dataset was the HKY + I + G model. Bayesian inference analysis was performed with the aid of MrBayes v. 3.2.6 (Ronquist et al. 2012) using Markov chain Monte Carlo (MCMC) chains running 10,000,000

Table 2 Primers used for amplification and sequencing

Locus	Primer	Sequence	Source
18S	18SU467F	5′-ATCCAAGGAAGGCAGCAGGC-3′	Suzuki et al. (2006)
	18SL1310R	5′-CTCCACCAACTAAGAACGGC-3′	Suzuki et al. (2006)
28S	Digl2	5′-AAGCATATCACTAAGCGG-3′	Tkach et al. (2003)
	1500R	5′-GCTATCCTGAGGGAAACTTCG-3′	Snyder and Tkach (2001)
ITS1-5.8S-ITS2	D1	5′-AGGAATTCCTGGTAAGTGCAAG-3′	Galazzo et al. (2002)
	D2	5′-CGTTACTGAGGGAATCCTGGT-3′	Galazzo et al. (2002)
*cox*1	Plat-diploCOX1F	5′-CGTTTRAATTATACGGATCC-3′	Moszczynska et al. (2009)
	Plat-diploCOX1R	5′-AGCATAGTAATMGCAGCAGC-3′	Moszczynska et al. (2009)
	DICE1F	5′-ATTAACCCTCACTAAATTWCNTTRGATCATAAG-3′	Moszczynska et al. (2009)
	DICE11R	5′-TAATACGACTCACTATAGCWGWACHAAATTTHCGATC-3′	Van Steenkiste et al. (2015)
	DICE14R	5′-TAATACGACTCACTATACCHACMRTAAACATATGATG-3′	Van Steenkiste et al. (2015)
	Dig_cox1Fa	5′-ATGATWTTYTTYTTYYTDATGCC-3′	Wee et al. (2017)
	Dig_cox1R	5′-TCNGGRTGHCCRAARAAYCAAAA-3′	Wee et al. (2017)

generations and sampling trees every 1000 generations. Bayesian inference analyses were performed using MrBayes on CIPRES Science Gateway v. 3.3 (Miller et al. 2010). The log-likelihood scores were plotted, and only the final 75% of trees were used to produce the consensus trees by setting the "burn-in" parameter at 2500 (disregarding the first 25% of trees sampled). The results were visualised in Tracer ver. 1.6 (Rambaut et al. 2014) to assess convergence and proper sampling and to identify the "burn-in" period. Maximum likelihood analyses were carried out using PhyML v. 3.0 (Guindon et al. 2010) and run on the ATGC bioinformatics platform (available from http://www.atgc-montpellier.fr/). Nonparametric bootstrap validation based on 100 pseudoreplicates was applied. FigTree v. 1.4 software (Rambaut 2012) was used to visualise phylogenetic trees. The *p*-distance and number of differences between sequences were calculated in MEGA v. 7 (Kumar et al. 2016).

Morphological analysis

The morphology of the metacercariae used to generate molecular sequences was examined in live and fixed specimens. A series of photomicrographs were taken with a dedicated digital microscope camera attached to a Nikon Eclipse N*i* microscope using NIS-Elements BR Camera analysis software. The measurements for each isolate were taken from the digital images with the aid of ImageJ (available from https://imagej.nih.gov/ij/download.html). The morphometrical data are presented as range, followed by the mean of the measurements taken in parentheses. All measurements are given in micrometres.

Results

General observations

A total of 47 specimens of *T. sparrmanii* from the Mooi River (7 specimens), Vaal River (20) and Boskop Dam (20) were examined. Infection with metacercariae of the Diplostomidae was found in 28 specimens of fish (prevalence = 60%) with two infected specimens from the Mooi River, 14 specimens from the Vaal River and 12 specimens from the Boskop Dam. Metacercariae detected on the body surface of one fish were enclosed within transparent cysts, whereas metacercariae recovered from the muscles of 27 fish were enclosed within transparent, green and black cysts. As mentioned above, all found metacercariae were initially assigned to four groups based on the site in the fish and colour of the cysts and referred to as "transparent cysts on body surface" (TCB), "transparent cysts in muscles" (TCM), "black cysts in muscles" (BCM) and "green cysts in muscles" (GCM).

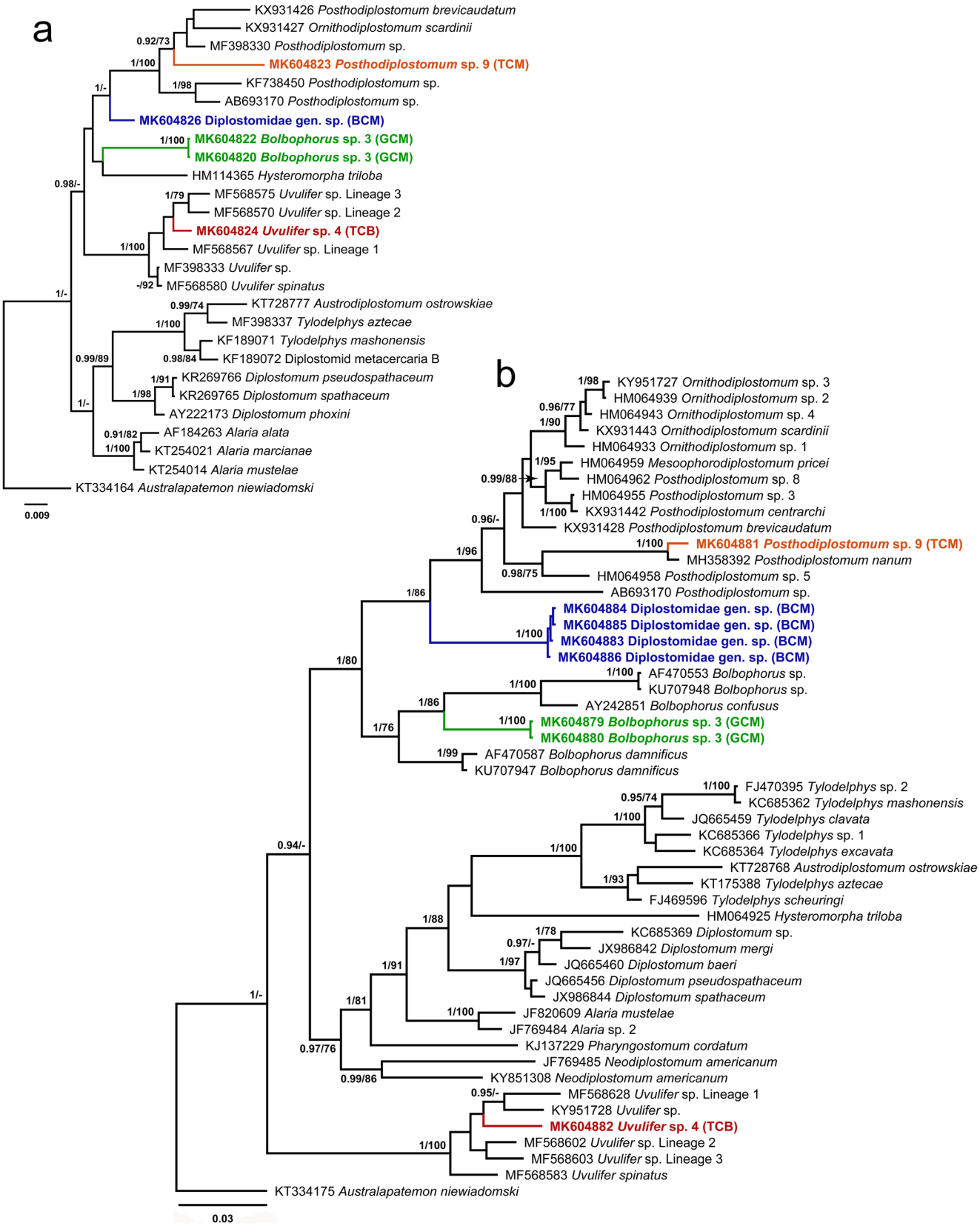
Molecular identification of metacercariae

Twenty-one novel sequences were generated during this study for metacercaria isolates selected from each group: one sequence for the partial 18S rRNA gene, eight sequences for the ITS1-5.8S-ITS2 region, seven sequences for the partial 28S rRNA gene and five sequences for the partial *cox*1 gene.

Bayesian inference (BI) and maximum likelihood (ML) analyses of the 28S rDNA dataset yielded similar phylogenetic topologies (Fig. 2a). The newly generated sequences fell into four distinct clades. Sequences generated for metacercariae from TCM fell with a strong support within the clade comprising four *Posthodiplostomum* spp. and *Ornithodiplostomum scardinii* (Schulman, 1952). The sequence for metacercariae from BCM formed a separate branch at the basal position to the "*Posthodiplostomum* + *Ornithodiplostomum*" clade. Two sequences for metacercariae from GCM clustered with the sequence for *Hysteromorpha triloba* (Rudolphi, 1819) (HM114365) with low support in both BI and ML analyses. The sequence for metacercariae from TCB clustered within the clade for *Uvulifer* spp. with a strong nodal support.

Both BI and ML phylogenetic analyses based on the ITS1-5.8S-ITS2 alignment resulted in consensus trees with similar topologies (Fig. 2b). Sequences for the isolate from TCM clustered within the strongly supported "*Posthodiplostomum* + *Ornithodiplostomum* + *Mesoophorodiplostomum*" clade demonstrating close relationships to the species *Posthodiplostomum nanum* Dubois, 1937 (MH358392) reported from Brazil. The interspecific divergence between these species was 1% (6 nt) based on 733 nt alignment positions. Four identical sequences for the isolates from BCM formed a strongly supported clade basal to the "*Posthodiplostomum* + *Ornithodiplostomum* +*Mesoophorodiplostomum*" clade. Sequences for two metacercariae from GCM were identical and fell within a strongly supported clade containing three species of *Bolbophorus* reported from the USA and Israel. The interspecific divergence observed between the present species and *B. confusus* (AY242851) within the ITS1-5.8S-ITS2 alignment was 5.5% (34 nt) and between the present species and *Bolbophorus damnificus* Overstreet and Curran, 2002 (AF470587 and KU707947) and *Bolbophorus* sp. (AF470553 and KU707948) was 5.6% (35 nt). The isolate for the metacercariae from TCB clustered within the clade comprising five species of *Uvulifer* reported from North America with the present isolate exhibiting the lowest interspecific divergence (2.9%, 18 nt) with *Uvulifer spinatus* López-Jiménez, Pérez-Ponce de León and García-Varela, 2018 and *Uvulifer* Lineage 4 sensu López-Jiménez et al. (2017).

The trees from both BI and ML analyses of the *cox*1 dataset yielded similar phylogenetic hypotheses (Fig. 3). The three sequences for the isolates from GCM were identical and clustered within the clade together with four species of *Bolbophorus*



(KM538081, KT831373, KU707937 and KU707938) from North America. The interspecific divergence within this clade ranged between 14.8–18.4% (46–57 nt), with novel sequences of *Bolbophorus* sp. and *B. damnificus* (KU707937) exhibiting

◀ **Fig. 2** Bayesian inference (BI) and maximum likelihood (ML) trees for Diplostomidae spp. based on the **a** partial 28S rDNA sequences and **b** ITS1-5.8S-ITS2 sequences. Nodal support from BI and ML analyses is indicated as BI/ML; values < 0.90 (BI) and < 70 (ML) are not shown. The scale bar indicates the expected number of substitution per site. The newly generated sequences are highlighted in bold. Abbreviations: BCM, black cysts in muscles; GCM, green cysts in muscles; TCB, transparent cysts on body surface; TCM, transparent cysts in muscles

the lowest interspecific divergence (14.8%, 46 nt), whereas the divergence between our isolate and *Bolbophorus* sp. (KU707938) from USA was the highest (18.4%, 57 nt). The position of the novel sequence generated for metacercaria isolates from BCM on the phylogenetic tree was not resolved. It tentatively clustered in the large clade comprising the representatives of the genera *Alaria* Schrank, 1788, *Austrodiplostomum* Szidat and Nani, 1951, *Bolbophorus*, *Diplostomum*, *Hysteromorpha* Lutz, 1931, *Neodiplostomum* Railliet, 1919 and *Tylodelphys* at a basal position. Within this clade, the isolate from BCM demonstrated the lowest percent of sequence divergence from the isolate for *Tylodelphys excavata* (KC685344) (16.1%, 50 nt) and the highest percent of sequence divergence from the isolate for *Posthodiplostomum* sp. 5 (FJ477219) (23.2%, 72 nt). The sequence for the metacercariae from TCB clustered with a strong support with an unidentified species of *Uvulifer* (MF124281) from North America. The interspecific divergence between these two isolates was 12% (37 nt).

Additionally, the partial 18S rDNA sequence was obtained for metacercariae from GCM in order to compare with the sequences of *B. levantinus* (AF490576) and *B. confusus* (AY24285) reported from Israel and for which only the 18S sequences are available. The genetic divergence between our sequence and *B. levantinus* was 1.2% (11 nt) and between our sequence and *B. confusus* was 1.3% (12 nt) which is within interspecific variability.

Based on the results of molecular analyses, we identified metacercariae from TCB as a species of the genus *Uvulifer*, metacercariae from TCM as a species of *Posthodiplostomum* and metacercariae from GCM as a species of *Bolbophorus*. Metacercariae from BCM were identified only to the family level, as Diplostomidae gen. sp.

Following the previously published studies that provided numbers for unidentified species of *Bolbophorus*, *Posthodiplostomum* and *Uvulifer* (Levy et al. 2002; Locke et al. 2010; Stoyanov et al. 2017; López-Jiménez et al. 2017), we give the subsequent numbers for the species found in our study, i.e., *Bolbophorus* sp. 3, *Posthodiplostomum* sp. 9 and *Uvulifer* sp. 4.

Morphological description of metacercariae

Genus *Bolbophorus* Dubois, 1935

Bolbophorus sp. 3

Second intermediate host: *Tilapia sparrmanii* Smith, 1840 (Perciformes: Cichlidae).

Site in host: Muscles.

Localities: Vaal River (26° 52′ 07.5″ S, 27° 17′ 30.6″ E) and Boskop Dam (26° 32′ 35.2″ S, 27° 07′ 08.1″ E).

Prevalence: 55% (Vaal River); 55% (Boskop Dam).

Intensity of infection: 1–7 (Vaal River); 1–17 (Boskop Dam).

Voucher material: 10 voucher specimens deposited in NMB P 494.

Representative DNA sequences: 18S, 1 sequence (MK604814); 28S, 3 sequences (MK604820–MK604822); ITS1-5.8S-ITS2, 2 sequences (MK604879, MK604880); *cox*1, 3 sequences (MK605688–MK605690).

Description

[Based on 15 excysted fixed metacercariae; Fig. 4a–d]. Cysts oval, green, transparent with thin walls, diameter, 756–976 (861). Body large, indistinctly bipartite, elongate-oval, 1139–1766 × 393–534 (1374 × 483), with maximum width at level of ventral sucker. Forebody long, 966–1503 × 393–563 (1181 × 486), covered with numerous tiny spines. Forebody/hindbody length ratio 1:0.14–0.18 (1:0.16). Hindbody short, aspinose, 136–263 × 202–321 (192 × 255). Forebody /hindbody width ratio 1:0.51–0.57 (1:0.52). Oral sucker terminal, elongate-oval, 54–79 × 36–57 (66 × 48). Pseudosuckers elongate-oval, 58–95 × 36–69 (74 × 48). Ventral sucker medium-sized, transversely oval, in forebody, 64–80 × 71–84 (73 × 77), larger than oral sucker [sucker width ratio 1:1.32–2.08 (1:1.63)]. Prepharynx short, 6–18 (12). Distance from ventral sucker to anterior extremity of body 485–633 (545) and to posterior extremity 676–806 (732). Pharynx muscular, elongate-oval, 35–54 × 19–33 (45 × 25). Oesophagus short 16–27 (22), bifurcates posterior to pharynx. Intestinal bifurcation in anterior quarter of forebody. Holdfast organ elongate-oval, opens via longitudinal slit, 109–196 × 97–192 (150 × 135) located in the posterior part of forebody. Distance from holdfast organ to ventral sucker 279–433 (346). Excretory vesicle, V-shaped, two branches extending from excretory vesicle into forebody. The paranephridial system, divided into 5 longitudinal canals filled with small excretory granules in forebody. One wide median channel, two narrow lateral channel pairs connected with transverse commissures in anterior half of body. Excretory pore terminal.

Genus *Posthodiplostomum* Dubois, 1936

Posthodiplostomum sp. 9

Second intermediate host: *T. sparrmanii*.

Site in host: Muscle.

Localities: Boskop Dam (26° 32′ 35.2″ S, 27° 07′ 08.1″ E).

Prevalence: 5% (1 of 20).

Intensity of infection: 1.

Representative DNA sequences: 28S, 1 sequence (MK604823); ITS1-5.8S-ITS2, 1 sequence (MK604881).

Description

[Based on 1 fixed metacercaria; Fig. 5a]. Cyst oval, transparent, with thin wall. Body distinctly bipartite, 677 × 435.

Forebody subspherical, 410 × 435, larger than hindbody. Forebody/hindbody length ratio 1:1.02. Hindbody subspherical, 417 × 390, narrower than forebody. Forebody/hindbody width ratio 1:0.90. Oral sucker, subterminal, elongate-oval, 45 × 33. Pseudosuckers absent. Ventral sucker, elongate-oval, in forebody, 55 × 45, larger than oral sucker (sucker width ratio 1:1.36). Distance from ventral sucker to anterior extremity of body, 238. Distance from ventral sucker to posterior extremity of body, 125. Prepharynx absent or very short. Pharynx elongate-oval, muscular, 48 × 32. Oesophagus short. Intestinal bifurcation in anterior quarter of forebody. Large, dark granular content spread across forebody. A few

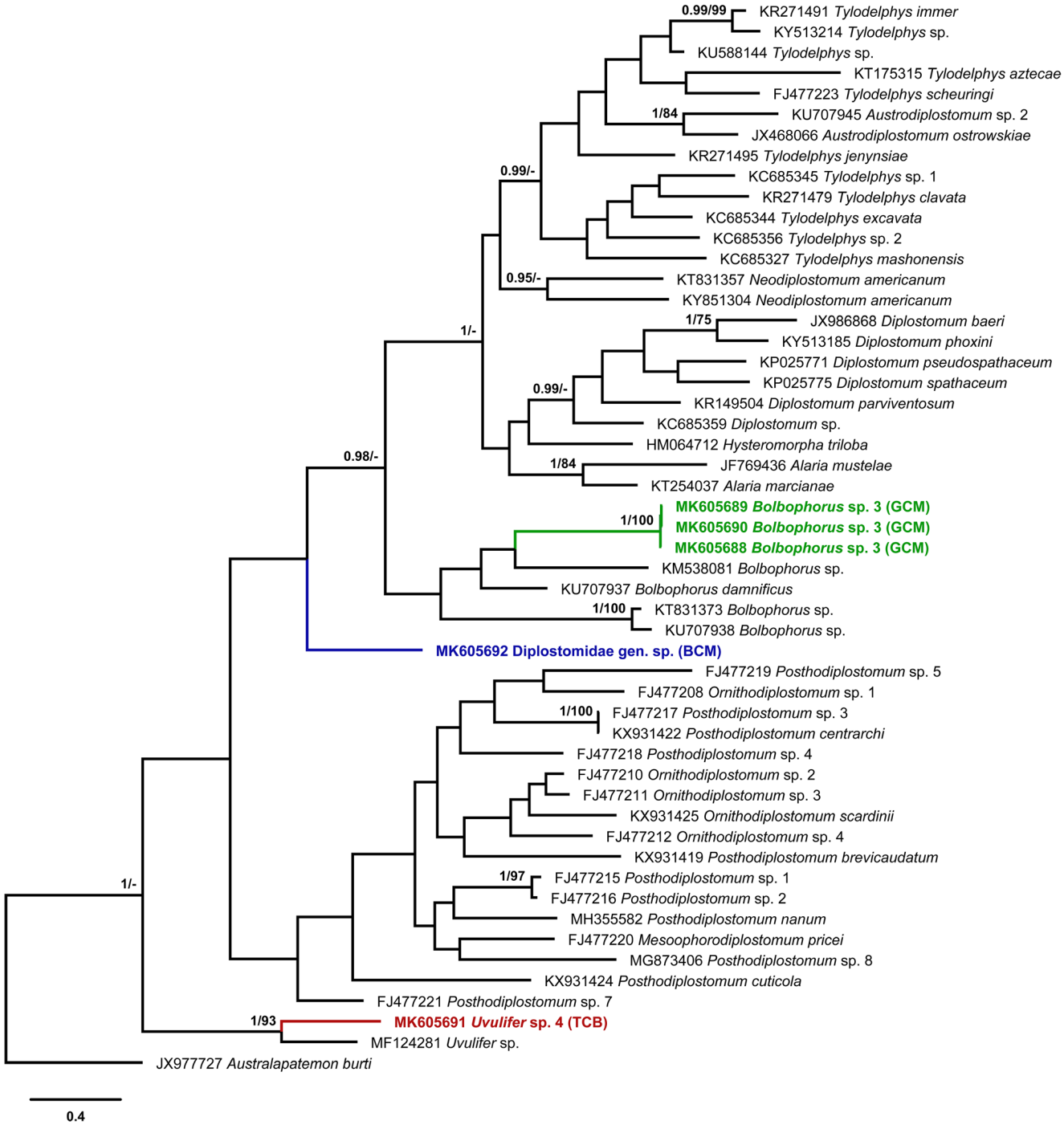


Fig. 3 Bayesian inference (BI) and maximum likelihood (ML) trees for Diplostomidae spp. based on the *cox*1 sequences. Nodal support from BI and ML analyses is indicated as BI/ML; values < 0.90 (BI) and < 70 (ML) are not shown. The scale bar indicates the expected number of substitution per site. The newly generated sequences are highlighted in bold. Abbreviations: BCM, black cysts in muscles; GCM, green cysts in muscles; TCB, transparent cysts on body surface

Fig. 4 Microphotographs of metacercariae of *Bolbophorus* sp. 3 ex *Tilapia sparrmanii*. **a** Fixed excysted, ventral view. **b** Fixed encysted. **c** Pseudosuckers, ventral view. **d** Posterior part of the body, ventral view: 1, holdfast organ; 2, excretory vesicle

fine, small granules in hindbody. Holdfast organ transversely oval, 103 × 194. Distance from ventral sucker to holdfast organ, 13. Excretory system of "neascus" type, composed of one median and two lateral canals forming net in forebody. Excretory pore subterminal.

Genus *Uvulifer* Yamaguti, 1934

***Uvulifer* sp. 4**

Second intermediate host: *T. sparrmanii*.

Site in host: Body surface.

Localities: Mooi River (26° 41′ 02.9″ S, 27° 05′ 58.5″ E).

Prevalence: 14% (1 of 7) (Mooi River).

Intensity of infection: 2 (Mooi River).

Representative DNA sequences: 28S,2 sequences (MK604824, MK604825); ITS1-5.8S-ITS2, 1 sequence (MK604882); *cox*1, 1 sequence (MK605691).

Description

[Based on 1 live metacercaria; Fig. 5b]. Cyst elongate-oval, 1015 × 850. Body distinctly bipartite, 1286 × 262. Forebody elongate-oval, 678 × 262. Hindbody claviform 688 × 149. Forebody/hindbody length ratio 1:1.01. Forebody /hindbody width ratio 1:0.57. Oral sucker terminal, oval, 71 × 65. Pseudosuckers absent. Ventral sucker anterior to holdfast organ. Holdfast organ elongate-oval, 297 × 259.

Diplostomidae gen. sp.

Second intermediate host: *T. sparrmanii*.

Site in host: Muscle.

Localities: Mooi River (26° 41′ 02.9″ S, 27° 05′ 58.5″ E) and Vaal River (26° 52′ 07.5″ S, 27° 17′ 30.6″ E).

Prevalence: 33% (Mooi River); 30% (Vaal River).

Intensity of infection: 1 (Mooi River); 1–2 (Vaal River).

Voucher material: 1 voucher specimens deposited in NMB P 495 .

Representative DNA sequences: 28S, 1 sequence (MK604826); ITS1-5.8S-ITS2, 4 sequences (MK604883–MK604886); *cox*1, 1 sequence (MK605692).

Description

[Based on 1 live encysted metacercaria; Fig. 5c]. Cyst elongate-oval, 1195 × 776, enclosed in black capsule. Body

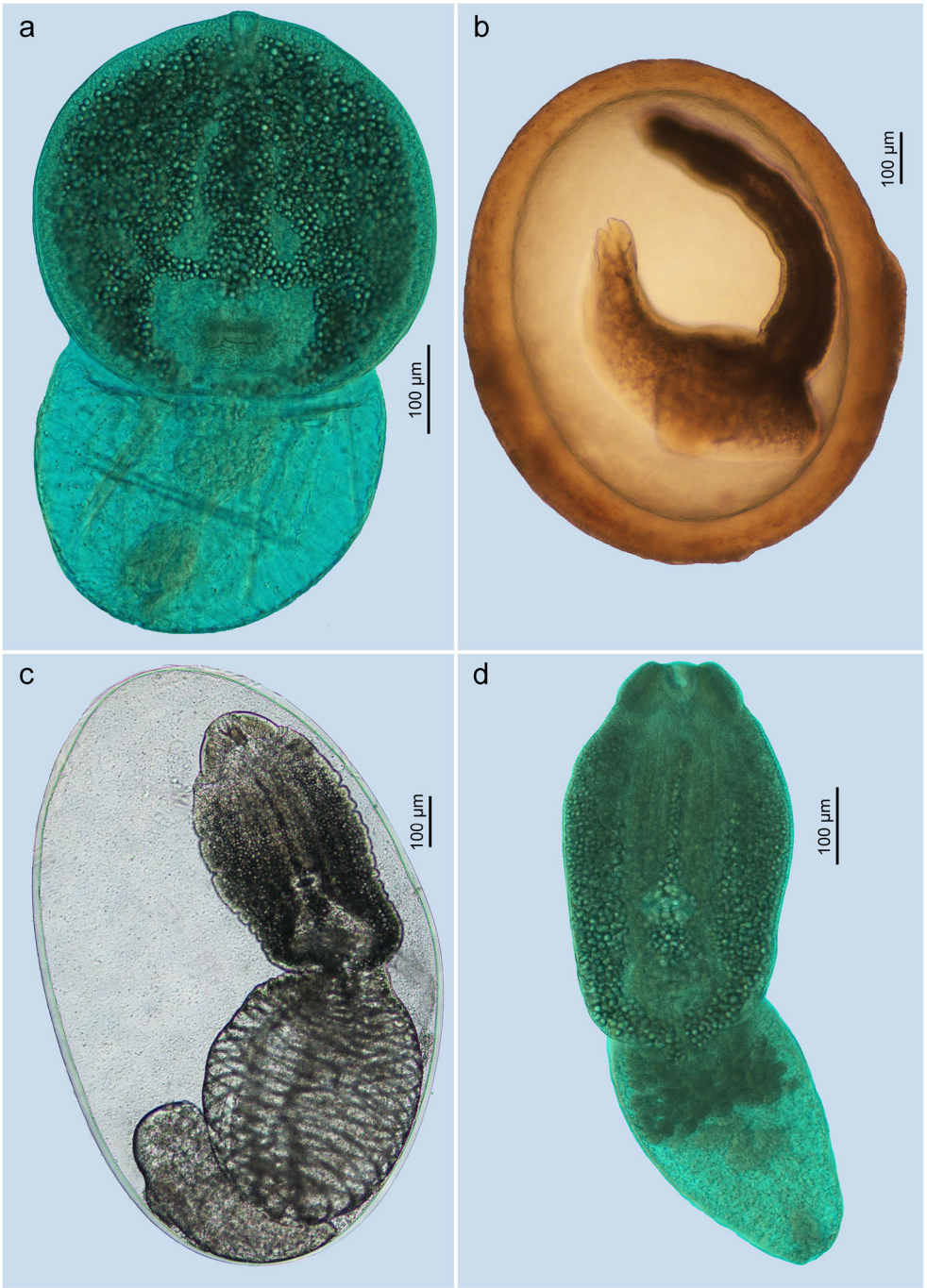


Fig. 5 Microphotographs of metacercariae of the Diplostomidae ex *Tilapia sparrmanii*. **a** *Posthodiplostomum* sp. 9, fixed excysted, ventral view. **b** *Uvulifer* sp. 4, live encysted, lateral view. **c** Diplostomidae gen. sp., live encysted, ventral view. **d** Diplostomidae gen. sp., fixed excysted, ventral view

distinctly bipartite, 1082 × 424. Forebody elongate-oval, 555 × 313. Hindbody larger than forebody, bulb-shaped, 526 × 424. Forebody width/hindbody width ratio 1:1.35. Oral sucker subterminal, 223 × 212. Pseudosuckers elongate-oval, 93 × 52. Ventral sucker small, elongate-oval, 36 × 62. Distance from ventral sucker to anterior extremity of body 348; distance from ventral sucker to posterior margin of forebody, 164. Medium- to large-sized granules spread across forebody. Smaller granules spread across in hindbody, in a web-like manner. Holdfast organ transversely oval, 71 × 96.

[Based on 7 fixed excysted metacercariae; Fig. 5d]. Body distinctly bipartite, 716–1148 × 256–429 (956 × 325). Forebody elongate-oval, 461–649 × 269–345 (560 × 289), larger than hindbody. Forebody/hindbody length ratio 1:0.67–0.87 (1:0.73). Hindbody elongate-oval, leaf ($n = 2$) or bulb-shaped ($n = 5$), 309–565 × 205–429 (407 × 306), wider than forebody. Forebody/hindbody width ratio 1:0.76–1.24 (1:1.06). Oral sucker subterminal, elongate-oval, 52–66 × 39–64 (57 × 52). Pseudosuckers, large, elongate-oval, 66–96 × 35–46 (80 × 41). Ventral sucker transversely oval, 46–

68 × 69–80 (59 × 75), larger than oral sucker [sucker width ratio 1:1.25–1.73 (1:1.41)], in posterior half of forebody. Prepharynx absent. Pharynx elongate-oval, muscular, 35–40 × 17–35 (38 × 24). Oesophagus short, narrow, 18–20 (19). Intestinal bifurcation in anterior quarter of forebody. Numerous medium-sized granules spread across entire forebody. Large transversely oval concretions in anterior half of hindbody. Holdfast organ elongate-oval, 64–75 × 53–88 (70 × 68). Distance from ventral sucker to anterior body extremity 265–415 (337). Distance from ventral sucker to posterior extremity of forebody, 130–179 (153). Distance from holdfast organ to ventral sucker 38–43 (41). Excretory system of "neascus" type, composed of ramified median and two lateral canals forming net in forebody. Excretory pore terminal.

Discussion

The present study advances our knowledge on the diversity of digeneans from the family Diplostomidae in Africa by providing novel morphological and genetic characterisations for metacercariae found in *T. sparrmanii* in the North West Province, South Africa. The initial delineation of found metacercariae into four species based on the differences in their site of infection in the fish, the colour of the cysts and the morphology of excysted specimens was supported by phylogenetic analyses of *cox*1, ITS1-5.8S-ITS2 and 28S data.

Metacercariae found enclosed within transparent cysts on the body surface of *T. sparrmanii* belonged to the genus *Uvulifer* and identified as *Uvulifer* sp. 4. Morphologically, these metacercariae possess features that are fully consistent with metacercariae of "neascus" type, in particular, the foliaceous forebody and elongate well-developed hindbody, absence of pseudosuckers, a reserve bladder consisting of ramified median and two lateral canals that form a net in the forebody. Metacercariae of *Uvulifer* sp. 4 differ from metacercariae of *Uvulifer* sp. reported in *Poeciliopsis occidentalis* Baird and Girard in Mexico (López-Jiménez et al. 2017) based on the smaller total body length (1286 vs 592–677 (636)), a lower range of ratios for forebody/hindbody length (1:1.01 vs ≈ 1:0.46) and forebody/hindbody width (1:0.57 vs ≈ 1:0.75) and the shape of the forebody (elongate-oval vs spatulated) and hindbody (claviform vs bulb- to oval-shaped). Our study is the first to report metacercariae of this genus in the freshwater fishes in Africa where three species of *Uvulifer* have previously been reported from birds: *Uvulifer cerylou* Dollfus, 1950 from *Ceryle rudis* Linnaeus, 1758 in the Democratic Republic of the Congo (Dollfus 1950) and Zimbabwe (Dubois and Beverley-Burton 1971), *Uvulifer murinum* Baer, 1971 from small rodents in the Republic of Côte d'Ivoire (Baer 1971) and *Uvulifer pseudoprosocotyle* Dubois and Beverly-Burton, 1971 from *C. rudis* in Zimbabwe (Dubois and Beverley-Burton 1971). It is possible that the metacercariae from *T. sparrmanii* may represent a larval stage of one of the species listed above.

Metacercariae of the three remaining species discovered in this study were extracted from the muscle tissue of *T. sparrmanii*. Specimens found in transparent cysts were identified as *Posthodiplostomum* sp. 9 based on analyses of molecular and morphological data. The metacercariae possess characteristics of "neascus" type: the oval forebody and well-developed hindbody, absence of the pseudosuckers, reserve bladder with ramified median and two lateral canals that form a net in the forebody. Two species of the genus *Posthodiplostomum* have been reported in Africa. *Posthodiplostomum biellipticum* Dubois, 1958 was described from a striated heron *Butorides striata atricapilla* (Afzelius, 1804) in the Republic of the Congo (Dubois 1958). Metacercariae of *Posthodiplostomum nanum* Dubois, 1937, a species described from a green heron *Butorides virescens* (Linnaeus, 1758) (Ardeidae) collected in Brazil (Dubois 1937), were reported from fishes, *Epiplatys spilargyreius* Duméril, 1861, *Epiplatys sexfasciatus* Gill, 1862, *Coptodon zillii* (Gervais, 1848), *Hemichromis fasciatus* Peters, 1857 and *Heterobranchus longifilis* Valenciennes, 1840, in Ghana and Sierra Leone (Williams 1967; Fischthal and Thomas 1968). The identification of the metacercariae of *P. nanum* was performed based on adults experimentally obtained from infected chickens and cattle egrets *Bubulcus ibis* Linnaeus, 1758 by Williams (1967). Later, *P. nanum* of Williams (1967) and Fischthal and Thomas (1968) was synonymised with *P. biellipticum* Dubois, 1958 by Dubois (1970). Although metacercariae found in our study appeared to be phylogenetically close to *P. nanum*, they are morphologically and genetically distinct. Morphologically, metacercariae of *Posthodiplostomum* sp. 9 differs from metacercariae of *P. nanum* recently reported from experimentally infected guppies *Poecilia reticulata* Peters, 1859 in Brazil (López-Hernández et al. 2018) by being smaller in total length (677 vs 686–932 (820)) and having a higher range for the ratios: forebody/hindbody length (1: 1.02 vs ≈ 1:0.50, respectively) and forebody/hindbody width (1:0.90 vs ≈ 1:0.60) and by the shape of the forebody (subspherical vs spatulated) and hindbody (subspherical vs conical). The genetic divergence between the two species within the ITS1-5.8S-ITS2 alignment was 1% (6 nt). Our specimen differs from metacercariae of *P. biellipticum* (identified as *P. nanum* by Williams (1967)) in possessing a shorter forebody (410 vs 530–1010 (650)) and longer and wider hindbody (417 vs 180–390 (310) and 390 vs 170–330 (250), respectively), a higher range for the ratios: forebody/hindbody length (1:1.02 vs ≈ 1: 0.48, respectively) and forebody/hindbody width (1:0.90 vs ≈ 1:0.59), a larger pharynx length (48 vs 20–30 (35)) and width of the holdfast organ (194 vs 90–140 (110)). Thus, we assume that *Posthodiplostomum* sp. 9 may represent a species not previously described or reported in Africa.

General morphology of the metacercariae extracted from the green cysts found in the muscles of *T. sparrmanii* corresponded well to the previous descriptions for metacercariae of *Bolbophorus* spp. reported by Fox (1965), Overstreet et al. (2002), Yost (2008) and Rosser et al. (2016), in particular, the foliaceous elongate forebody and short hindbody, presence of the pseudosuckers, reserve bladder with ramified median and two lateral canals forming a net in the forebody.

Bolbophorus spp. are typically encysted in an ovoid, transparent, light green or yellow cysts located in the muscles of the fish host but may also occur in the dermis of the trunk and caudal regions (Rosser et al. 2016). Phylogenetic analyses confirmed the taxonomic position of the found species within the genus *Bolbophorus*. Akoll et al. (2012) reported metacercariae of *Bolbophorus* sp. from *Oreochromis niloticus* (Linnaeus, 1758) in Uganda, Africa. However, the report was not supplemented with any morphological or molecular evidence. Metacercariae of *Bolbophorus* sp. 3 found in *T. sparrmanii* are genetically distinct from all currently sequenced species from North America, Europe and Asia and may represent a new species from the African continent. *Bolbophorus* sp. 3 was the most abundant species found in the present study with the highest prevalence and intensity recorded in *T. sparrmanii*.

Metacercariae found in the black cysts in the fish muscles were identified only to the family level as Diplostomidae gen. sp. The specimens possess characteristics of the "neascus" morphotype according to Niewiadomska (2002). Based on 28S rDNA and ITS1-5.8S-ITS2 phylogenetic analyses, sequences of this species formed a clade associated with a strong support with sequences for the species of the genera *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum* and can be considered to be a member of the Crassiphialinae.

Further identification of *Bolbophorus* sp. 3, *Posthodiplostomum* sp. 9, *Uvulifer* sp. 4 and Diplostomidae gen. sp. to the species level requires the adult worms from the definitive hosts. According to Niewiadomska (2002), the definitive hosts for species from these genera are typically piscivorous birds from the order Pelecaniformes for *Bolbophorus* spp., from the order Ciconiiformes for *Posthodiplostomum* spp. and from the family Alcedinidae for *Uvulifer* spp. Eight species of ciconiiform, 28 species of pelecaniform (including suliform) and nine species of alcedinid birds are present in the North West Province, South Africa (Lepage and Warnier 2014) and may be potential hosts for the diplostomid species found in our study. Eleven species of molluscs that occur in the water bodies where the fish for this study were collected may act as the first intermediate hosts for diplostomid parasites; these are *Bulinus africanus* Krauss, 1848, *Bulinus depressus* Haas, 1936, *Bulinus tropicus* Krauss, 1848, *Ceratophallus natalensis* Krauss, 1848, *Gyraulus connollyi* Brown and Van Eeden, 1969, *Gyraulus costulatus* Krauss, 1848 (Planorbidae), *Burnupia mooiensis* (Walker, 1912) (Burnupiidae), *Radix natalensis* (Krauss, 1848), *Lymnaea* (*Galba*) *truncatula* (Müller, 1874), *Pseudosuccinea columella* Say, 1817 (Lymnaeidae) and *Physella acuta* (Draparnaud, 1805) (Physidae) (Pretorius 2017).

Another major result from our phylogenetic analyses based on the partial 28S rRNA gene is that the unidentified species of "Diplostomid metacercaria B" found in *T. sparrmanii* by Moema et al. (2013) in the Limpopo Province, South Africa, clustered within a strongly supported clade of *Tylodelphys* spp. This result together with the general morphology of metacercariae supports the position of this species within the genus *Tylodelphys*.

The discovery of four species from the Diplostomidae in one species of hosts in the relatively small geographical area of a single river system demonstrates that the diversity of this group of parasites in South Africa is potentially much higher. These results, combined with previous knowledge, showed that at least five species of the Diplostomidae, *Bolbophorus* sp. 3, *Posthodiplostomum* sp. 9, *Uvulifer* sp. 4, *Tylodelphys* sp. and Diplostomidae gen. sp., utilise *T. sparrmanii* as the second intermediate host. We believe both morphological descriptions and molecular sequence data provided in the present study will enable the future research to improve our understanding on life cycles, distribution and taxonomy of diplostomid parasites in Africa.

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Compliance with ethical standards All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

Disclaimer Opinions expressed and conclusions arrived at are those of the authors and are not necessarily those of the NRF.

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