Resolution of the identity of three species of *Diplostomum* (Digenea: Diplostomidae) parasitising freshwater fishes in South Africa, combining molecular and morphological evidence

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ABSTRACT

Reliable data on the diversity of the genus *Diplostomum* (Digenea: Diplostomidae) parasitising freshwater fishes in South Africa, as well as in Africa, is almost non-existent. Most of the morphology-based identifications of species within this genus reported from Africa require critical revision. The aim of the present study was to determine the diversity of *Diplostomum* metacercariae in South African fishes applying molecular and traditional morphological techniques. To achieve this aim, a total of 216 fishes belonging to 21 species collected in the Rivers Phongolo, Riet, Usuthu and Mooi in three provinces of South Africa were examined. Metacercariae of *Diplostomum* were recovered from the eye lenses of 38 fishes belonging to five species of the families Anguillidae, Cichilidae and Mochokidae, with an overall low prevalence of infection (18%). Metacercariae were subjected to morphological study and molecular sequencing of the partial mithochondrial *cox*1 and ribosomal 28S rDNA genes as well as of ribosomal ITS1-5.8S-ITS2 region. Morphological and phylogenetic analyses revealed the presence of three species which matched those previously reported from Nigeria, Iraq and China, therefore those from *Tilapia sparrmanii* and *Synodontis zambezensis* were named *Diplostomum* sp.; those from *Anguilla labiata*, *Oreochromis mossambicus* and *S. zambezensis* were named *Diplostomum* sp. 14; and those from *Pseudocrenilabrus philander* were named *Diplostomum* sp. 16. Geographic distribution of several species of *Diplostomum* appeared to be wider than expected. Morphological description and novel sequence data generated during this study will contribute to the elucidation of the life cycles of *Diplostomum* sp., *Diplostomum* sp. 14 and *Diplostomum* sp. 16 and advance further research of diplostomids in Africa.

1. Introduction

Trematodes from the genus *Diplostomum* von Nordmann, 1832 (Digenea: Diplostomidae) are intestinal parasites of fish-eating birds, reported from all continents, but with the majority of species described from the Nearctic and Palaearctic (Shigin, 1986, 1993). For the successful completion of their life cycles, species of *Diplostomum* utilise freshwater snails and fish as intermediate hosts (Niewiadomska, 2002). Metacercarial stages are regarded as pathogenic for their fish hosts and therefore they remain the focus of numerous ecological, behavioural and evolutionary studies (Ballabeni and Ward, 1993; Owen et al., 1993; Kalbe and Kurtz, 2006; Seppälä et al., 2004, 2011; Benesh and Kalbe, 2016; Klemme et al., 2016). A significant amount of research effort has recently been invested in developing the molecular sequence library for species within this genus (Galazzo et al., 2002; Moszczynska et al., 2009; Locke et al., 2010a, 2010b; 2015; Behrmann-Godel, 2013; Georgieva et al., 2013; Pérez-del-Olmo et al., 2014; Blasco-Costa et al., 2014; Selbach et al., 2015; Kuhn et al., 2015; Soldánová et al., 2017; Kudlai et al., 2017; Enabulele et al., 2018). The complete mitochondrial genomes of two closely related species from the Palaearctic, *D. spathaceum* (Rudolphi, 1819) and *D. pseudospathaceum* Niewiadomska, 1984 were characterised (Brabec et al., 2015). Currently, molecular data for *Diplostomum* available in GenBank includes sequences for eight species and 38 unidentified species/species-level genetic lineages from Europe (4 species and 15 unidentified species/species-level genetic lineages), North America (4 and 19, respectively), Asia (1 and 3, respectively) and Africa (1 unidentified species) (see Chibwana et al., 2013; Locke et al., 2015; Kudlai et al., 2017; Soldánová et al., 2017; Gordy and Hanington, 2019). Nevertheless, the utility of available molecular data remains limited due to the heavy bias nature towards

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larval stages. Among the sequences of *Diplostomum* spp. currently available in GenBank, only 6% were generated from the adult isolates collected from bird definitive hosts, 19% from the cercarial isolates collected from snails and 75% from the metacercarial isolates collected from fishes. The majority of molecular sequences were derived from the metacercarial stages that, in contrast to adult stages, lack sufficient morphological characteristics used for accurate species identification. Therefore, numerous metacercarial isolates remain unidentified and require the sequences from their adult in bird definitive hosts. To date, sequences for nine species generated from the adult isolates have been published, with only six being identified to species level. Of these, two species are from Europe: *D. spathaceum* and *D. pseudospathaceum*; and seven species are from North America: four named species, *Diplostomum ardeae* Dubois 1969, *Diplostomum baeri* Dubois, 1937, *Diplostomum huronense* (La Rue, 1927), *Diplostomum indistinctum* (Guberlet, 1923); and three unidentified species, *Diplostomum* sp. 1, 3, 4 *sensu* Locke et al. (2010a, 2010b).

Studies of the global diversity of *Diplostomum* are restricted due to sampling insufficiencies. Although numerous research studies have been done in the Northern Hemisphere, there were no comprehensive studies published from the Southern Hemisphere. To date, ten species of *Diplostomum* were described and reported from Argentina [*D. minutum* Szidat, 1964], Antarctica [*D. antarcticum* Freiler, 1986, *D. dominicanum* Freiler, 1986 and *D. minutum* Szidat 1964], Australia [*D. amygdalum* Dubois and Pearson, 1965, *D. auriculosum* Dubois and Pearson, 1967, *D. murrayense* (Johnston and Cleland, 1938), *D. parvulum* Dubois and Angel, 1972 and *D. triangulare* (S. J. Johnston, 1904)], and Brazil [*D. compactum* (Lutz, 1928)]. However, no molecular evidence for the distribution of *Diplostomum* spp. in the Southern Hemisphere exists. In Africa, eight species have been described and reported from freshwater fishes (Kudlai et al., 2018; Hoogendoorn et al., 2019) based on morphological examination. Thus far, molecular data is only available for one unidentified species, *Diplostomum* sp. from Nigeria (Chibwana et al., 2013).

As part of an ongoing survey of diplostomid trematodes from freshwater fishes in South Africa, we studied the diversity of the genus *Diplostomum* based on combined morphological and multi-locus molecular analyses involving the partial nuclear 28S rRNA and mitochondrial *cox*1 genes, as well as the ribosomal internal transcribed spacer region ITS1-5.8S-ITS2. We provide detailed morphological descriptions for the metacercariae of the detected species of *Diplostomum*, explore their phylogenetic position and relationships within the genus and provide the first molecular evidence for species of *Diplostomum* distributed in the Southern Hemisphere.

2. Materials and methods

2.1. Sample collection

Samples were collected at five localities in three provinces of South Africa: **KwaZulu-Natal Province** [River Phongolo Site 1 (26°55′47″S, 32°19′30″E), River Phongolo Site 2 (26°52′58″S, 32°184′1″E), Lake Nyamithi of the River Phongolo system (26°53′35″S, 32°17′35″E), and Shokwe Pan of the River Usuthu system (26°51′50″S, 32°12′55″E) within the Ndumo Game Reserve in 2016, 2017 and 2018], **Northern Cape Province** [River Riet (28°59′60″S, 24°28′50″E) within the Mokala National Park in 2016], and **North West Province** [River Mooi at Boskop Dam (26°33′58″S, 27°07′16″E) within the Boskop Dam Nature Reserve in 2019] (Fig. 1). Sampling was carried out under the permits OP 1582/2018 (Ezemvelo KZN Wildlife) and NW 8065/03/2019 (Department of Rural, Environmental and Agricultural Development). Fishes were identified using Skelton (2001) and taxonomy followed FishBase (Froese and Pauly, 2019). The vitreous humour, retina, eye lenses and brain of each of the 216 fishes belonging to 21 species from 11 families (Table 1) were examined for the presence of metacercariae of *Diplostomum*. The metacercariae were examined, counted and

preserved directly in 96% molecular grade ethanol. Selected representatives of metacercariae were prepared for morphological analyses, DNA isolation and sequencing. Voucher material was deposited in the Parasite Collection of the National Museum (NMB), Bloemfontein, South Africa.

2.2. Morphological examination

The selected metacercariae were initially studied live under a Nikon dissecting microscope or Nikon Eclipse Ni compound microscope (when possible), thereafter the parasites were transferred to molecular grade ethanol and re-examined. Photomicrographs of live and fixed metacercariae were captured with a digital camera attached to a Nikon Eclipse Ni microscope using NIS-Elements BR Camera analysis software. All measurements for the representative isolates were taken from the digital images with the aid of ImageJ (Available from https://imagej. nih.gov/ij/download.html). Twenty morphometric variables were measured from the digital images: BL, body length; BW, body width; FL, forebody length; FW, forebody width; HL, hindbody length; HW, hindbody width; OSL, oral sucker length; OSW, oral sucker width; PPHL, prepharynx length; PHL, pharynx length; PHW, pharynx width; PSL, pseudosucker length; PSW, pseudosucker width; VSL, ventral sucker length; VSW, ventral sucker width; HOL, holdfast organ length; HOW, holdfast organ width; VSDAB, distance from ventral sucker to anterior end of body; VSDPFB, distance from ventral sucker to posterior end of forebody; VSDHO, distance between ventral sucker and holdfast organ.

2.3. Generation of sequence data

Total genomic DNA was isolated from a single specimen of metacercaria following manufacturer's protocol of the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa). DNA amplifications for the partial cytochrome *c* oxidase subunit 1 (*cox*1) and 28S rRNA genes, and the entire ITS1-5.8S-ITS2 gene cluster were performed using forward and reverse primers (Table 2) following polymerase chain reaction (PCR) protocols as described in Galazzo et al. (2002), Tkach et al. (2003), Moszczynska et al. (2009), and Van Steenkiste et al. (2015). PCR amplicons were purified and sequenced at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa (a commercial sequencing company). The original PCR primers as well as the internal primers (Table 2) were used for sequencing of the 28S rDNA amplicons. Contiguous sequences were assembled and edited using Geneious v. 11 (Biomatters, Auckland, New Zealand). Sequences were deposited in GenBank under the accession numbers MN808616-MN808629; MN813526-MN813549.

2.4. Phylogenetic analyses

Datasets for the phylogenetic analyses included 38 novel sequences obtained in the present study and 143 sequences for *Diplostomum* spp. available in GenBank (Supplementary Table 1). Analyses were performed using three alignments that were built according to the gene/region fragment amplified using MUSCLE (Edgar, 2004) implemented in Geneious v. 11 under default parameter values. Sequences of two species of the genus *Tylodelphys* Diesing, 1850, *Tylodelphys mashonensis* Beverly-Burton, 1963 (28S, KF189071) and *Tylodelphys clavata* (von Nordmann, 1832) (ITS, JQ665459; cox1, JX986909) were used as the outgroup based on the results of the phylogenetic analyses of *Diplostomum* published by Georgieva et al. (2013). The *cox*1 dataset was aligned with reference to the amino acid translation, using the trematode mitochondrial code (translation table 21; https://www.ncbi.nlm. nih.gov/Taxonomy/Utils/wprintgc.cgi#SG21) (Garey and Wolstenholme, 1989; Ohama et al., 1990). Phylogenetic trees were constructed through Bayesian inference (BI) and maximum likelihood (ML) analyses based on GTR + I + G model for ITS1-5.8S-ITS2 and
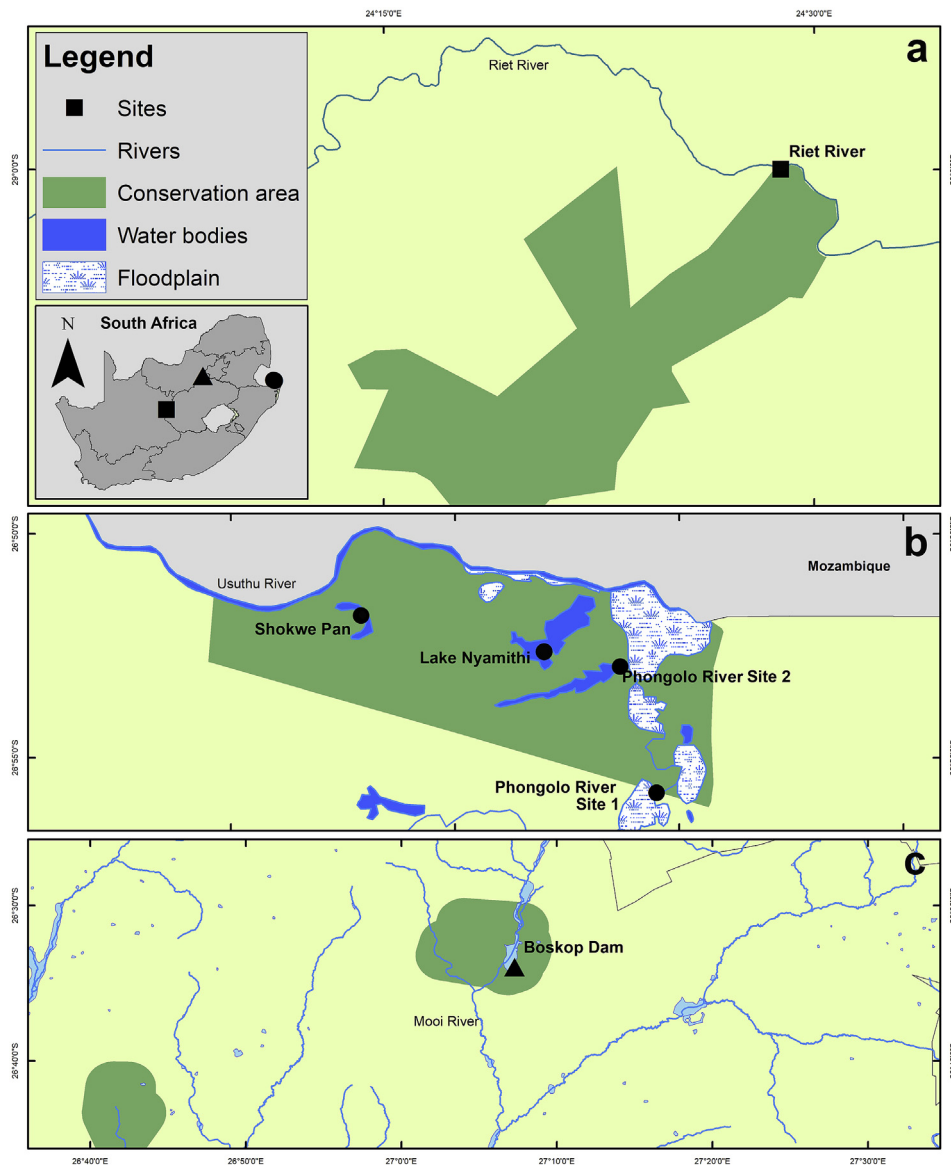
Fig. 1. Map illustrating the sampling localities on (a) River Riet in Mokala National Park (b) River Phongolo (Site 1, Site 2 and Nyamithi Lake) and the River Usuthu (Shokwe Pan) in Ndumo Game Reserve and (c) River Mooi (Boskop Dam) in Boskop Dam Nature Reserve, South Africa. The illustration was compiled in ArcGIS 10.6 (Available from https://support.esri.com/en/downloads).

*cox*1 datasets and HKY + I + G model for the 28S rDNA dataset. The jModelTest 2.1.2 (Guindon and Gascuel, 2003; Darriba et al., 2012) was used to estimate the best-fitting model of nucleotide substitution based on Akaike information criterion (AIC). Bayesian inference analyses were performed using MrBayes v. 3.2.6 software (Ronquist et al., 2012) run on CIPRES Science Gateway v. 3.3 (Miller et al., 2010). Markov chain Monte Carlo (MCMC) chains were run for 10, 000, 000 generations, 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 2,500. Maximum likelihood analyses were performed using PhyML v. 3.0 (Guindon et al., 2010) and run on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/). Nodal support was estimated using a bootstrap value of 100 pseudoreplicates. Phylogenetic trees were visualised using FigTree v. 1.4 software (Rambaut, 2012). MEGA v. 6 was used to calculate the pairwise genetic distances (p-distance) and number of nucleotide differences between sequences.

3. Results

3.1. General observations

Metacercariae of *Diplostomum* spp. were found in the eye lenses of 38 fishes belonging to five species: African mottled eel *Anguilla labiata* (Peters, 1852) (n = 3), Mozambique tilapia *Oreochromis mossambicus* (Peters, 1852) (n = 1), Southern mouthbrooder *Pseudocrenilabrus philander* (Weber, 1897) (n = 9), Plain squeaker *Synodontis zambezensis* Peters, 1852 (n = 24) and Banded tilapia *Tilapia sparrmanii* Smith, 1840 (n = 1). The overall prevalence of infection with metacercariae of *Diplostomum* was rather low (18%). The overall intensity of infection appeared to be relatively high in *P. philander* (3–21 metacercariae per fish) collected in the River Mooi and was low (1–12 metacercariae per fish) in other fish hosts from the Phongolo, Riet and Usuthu rivers.

3.2. Molecular identification of metacercariae

Thirty-eight novel sequences were generated for 16 isolates during

Table 1
Number of fish examined during the study.

Fish species	River Mooi	River Phongolo Site 1	River Phongolo Site 2	River Phongolo Lake Nyamithi	River Usuthu	River Riet	Total
Alestidae							
Brycinus imberi (Peters, 1852)	–	11	–	–	–	–	11
Hydrocynus vittatus Castelnau, 1861	–	1	–	–	–	–	1
Anguillidae							
Anguilla bengalensis labiata (Peters, 1852)	–	4	–	–	–	–	4
Centrarchidae							
Micropterus salmoides (Lacepède, 1802)	6	–	–	–	–	–	6
Cichilidae							
Coptodon rendalli (Boulenger, 1897)	–	7	–	–	–	–	7
Oreochromis mossambicus (Peters, 1852)	–	18	2	9	–	–	29
Pseudocrenilabrus philander (Weber, 1897)	10	1	–	–	–	4	15
Tilapia sparrmanii Smith, 1840	11	6	–	–	–	6	23
Clariidae							
Clarias gariepinus (Burchell, 1822)	–	6	3	–	5	5	19
Cyprinidae							
Cyprinus carpio Linnaeus, 1758	–	–	–	–	–	1	1
Labeo capensis (Smith, 1841)	–	–	–	–	–	3	3
Labeo congoro Peters, 1852	–	1	–	–	–	–	1
Labeo cylindricus Peters, 1852	–	2	–	–	–	–	2
Labeobarbus aeneus (Burchell, 1822)	–	–	–	–	–	7	7
Labeobarbus marequensis (Smith, 1841)	–	3	–	–	–	–	3
Gobiidae							
Glossogobius giuris (Hamilton, 1822)	–	7	–	–	–	–	7
Mochokidae							
Synodontis zambezensis Peters, 1852	–	46	–	–	–	–	46
Mormyridae							
Marcusenius macrolepidotus (Peters, 1852)	–	6	–	–	5	–	11
Petrocephalus wesseli Kramer and van der Bank, 2000	–	2	–	–	3	–	5
Schilbeidae							
Schilbe intermedius Rüppell, 1832	–	12	–	–	–	–	12
Sparidae							
Acanthopagrus berda (Forsskål, 1775)	–	3	–	–	–	–	3

this study: eight sequences for the partial 28S rRNA gene, 16 sequences for the ITS1-5.8S-ITS2 region and 14 sequences for the partial *cox*1 gene.

The 28S dataset (1,230 nt positions) consisted of eight sequences obtained in the present study and three sequences for *Diplostomum* spp. from GenBank (see Supplementary Table 1). Bayesian inference and ML analyses resulted in consensus trees with similar topologies (Fig. 2). The three newly-generated sequences for the isolates DAB1P, DSZ1P and DOM1P (Table 3) clustered with *D. spathaceum* (KR269765) reported from the Black-headed gull, *Larus ridibundus* (Linnaeus), from the Czech Republic (Brabec et al., 2015). All four isolates were identical. The sequences of two isolates (DPP3B and DPP4B) obtained from *P. philander* (Table 3) clustered with the sequence of *D. pseudospathaceum* (KR269766) also from *L. ridibundus* collected in the Czech Republic (Brabec et al., 2015) with low support. The genetic divergence between

two identical sequences of our isolates and sequence of *D. pseudospathaceum* was 0.3% (4 nt). Sequences of the three remaining isolates (DSZ6P, DSZ7P and DTS1R) collected from the eye lenses of *T. sparrmanii* and *S. zambezensis* clustered with *Diplostomum phoxini* (Faust, 1918) (AY222173) from the Eurasian minnow, *Phoxinus phoxinus* (L., 1758) collected in the United Kingdom (Olson et al., 2003). The sequences of our isolates from both hosts were identical, but differed from the sequence of *D. phoxini* by 1.5% (8 nt).

Sixteen novel sequences and 42 sequences downloaded from GenBank were used in the ITS1-5.8S-ITS2 alignment (963 nt positions). The phylogenetic tree resulted from BI and ML analyses demonstrated that the isolates sequenced in the present study clustered into three clades (Fig. 2b). Sequences of the metacercarial isolates collected from *S. zambezensis* (DSZ6P and DSZ7P) in the River Phongolo and *T. sparrmanii* (DTS1R) in the River Riet clustered with the sequence of

Table 2
Primers used for amplification and sequencing.

| Locus/primer	Sequence	Source
28S		
Digl2 (forward)	5′-AAGCATATCACTAAGCGG-3′	Snyder and Tkach (2001)
1500R (reverse)	5′-GCTATCCTGAGGGAAACTTCG-3′	Tkach et al. (2003)
300F[a] (forward)	5′-CAAGTACCGTGAGGGAAAGTTG-3′	Littlewood et al. (2000)
ECD2[a] (reverse)	5′-CCTTGGTCCGTGTTTCAAGACGGG-3′	Littlewood et al. (1997)
ITS1-5.8S-ITS2		
D1 (forward)	5′-AGGAATTCCTGGTAAGTGCAAG-3′	Galazzo et al. (2002)
D2 (reverse)	5′-CGTTACTGAGGGAATCCTGGT-3′	Galazzo et al. (2002)
***cox*1**		
Plat-diploCOX1F (forward)	5′-CGTTTRAATTATACGGATCC-3′	Moszczynska et al. (2009)
Plat-diploCOX1R (reverse)	5′-AGCATAGTAATMGCAGCAGC-3′	Moszczynska et al. (2009)
Dice1F (forward)	5′-ATTAACCCTCACTAAATTWCNTTRGATCATAAG-3′	Van Steenkiste et al. (2015)
Dice14R (reverse)	5′-TAATACGACTCACTATACCHACMRTAAACATATGATG-3′	Van Steenkiste et al. (2015)

[a] Internal primers.
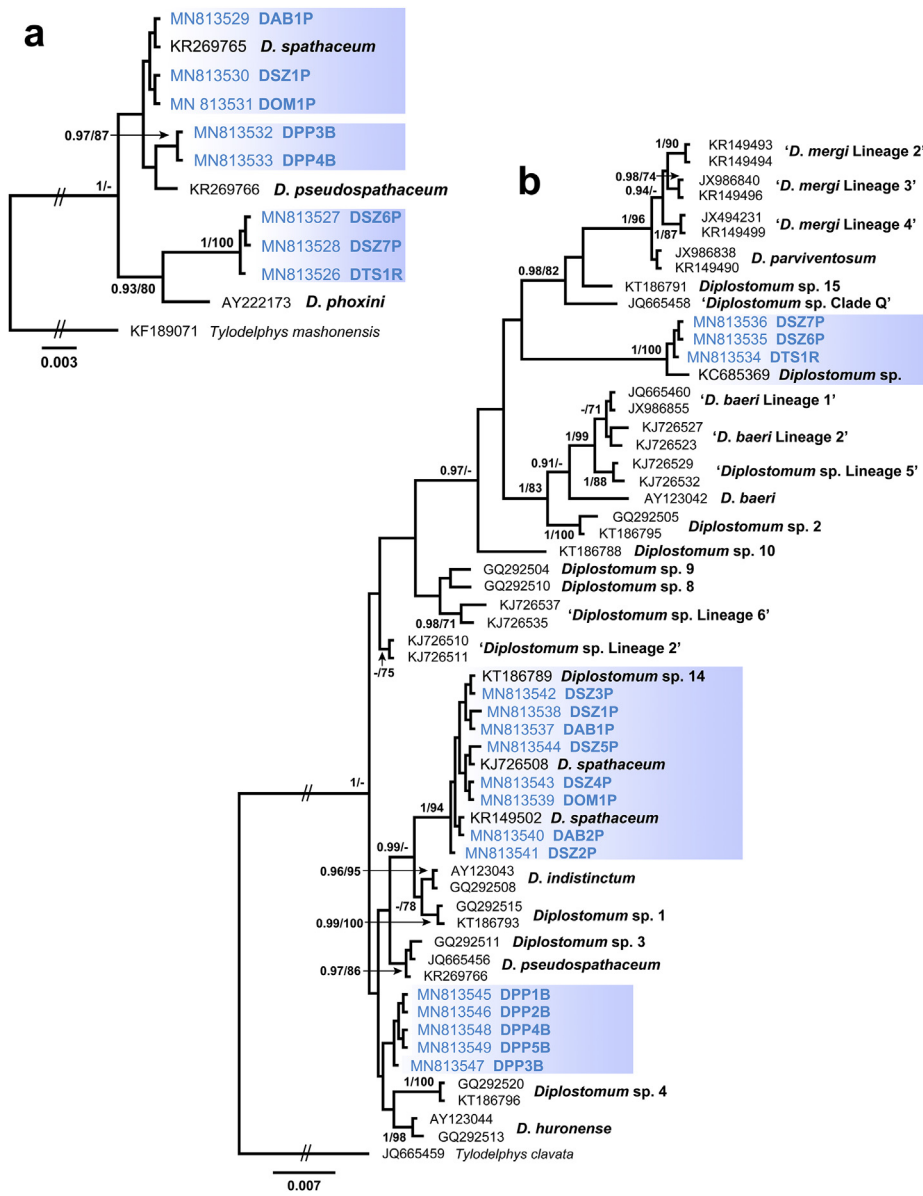
Fig. 2. Bayesian inference (BI) and maximum likelihood (ML) phylograms reconstructed using (a) partial 28S rDNA sequences (b) ITS1-5.8S-ITS2 sequences for species of *Diplostomum*. Nodal support from BI and ML analyses indicated as BI/ML; only values > 0.90 (BI) and > 70 (ML) are displayed. Scale-bar indicates the expected number of substitution per site. Sequences generated in this study are in bold and indicated by blue rectangles. Codes with isolate information for newly generated sequences are provided in Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Summary data for the sequences of *Diplostomum* spp. obtained during this study.

Species	Isolate	Host	Locality	GenBank accession numbers		
				28S	ITS1-5.8S-ITS2	*cox*1
Diplostomum sp.	DSZ6P	*Synodontis zambezensis*	River Phongolo Site 1	MN813527	MN813535	MN808617
Diplostomum sp.	DSZ7P	*Synodontis zambezensis*	River Phongolo Site 1	MN813528	MN813536	MN808618
Diplostomum sp.	DTS1R	*Tilapia sparrmanii*	River Riet	MN813526	MN813534	MN808616
Diplostomum sp. 14	DAB1P	*Anguilla bengalensis labiata*	River Phongolo Site 1	MN813529	MN813537	MN808619
Diplostomum sp. 14	DAB2P	*Anguilla bengalensis labiata*	River Phongolo Site 1	–	MN813540	–
Diplostomum sp. 14	DOM1P	*Oreochromis mossambicus*	River Phongolo Site 2	MN813531	MN813539	MN808621
Diplostomum sp. 14	DSZ1P	*Synodontis zambezensis*	River Phongolo Site 1	MN813530	MN813538	MN808620
Diplostomum sp. 14	DSZ2P	*Synodontis zambezensis*	River Phongolo Site 1	–	MN813541	–
Diplostomum sp. 14	DSZ3P	*Synodontis zambezensis*	River Phongolo Site 1	–	MN813542	MN808622
Diplostomum sp. 14	DSZ4P	*Synodontis zambezensis*	River Phongolo Site 1	–	MN813543	MN808623
Diplostomum sp. 14	DSZ5P	*Synodontis zambezensis*	River Phongolo Site 1	–	MN813544	MN808624
Diplostomum sp. 16	DPP1B	*Pseudocrenilabrus philander*	River Mooi	–	MN813545	MN808625
Diplostomum sp. 16	DPP2B	*Pseudocrenilabrus philander*	River Mooi	–	MN813546	MN808626
Diplostomum sp. 16	DPP3B	*Pseudocrenilabrus philander*	River Mooi	MN813532	MN813547	MN808627
Diplostomum sp. 16	DPP4B	*Pseudocrenilabrus philander*	River Mooi	MN813533	MN813548	MN808628
Diplostomum sp. 16	DPP5B	*Pseudocrenilabrus philander*	River Mooi	–	MN813549	MN808629

Diplostomum sp. recorded in the lenses of *Synodontis nigrita* Valenciennes, 1840 in Nigeria (Chibwana et al., 2013) in a strongly supported clade. The sequence divergence between our isolates and the isolate from *S. nigrita* was 0.3% (2 nt). Isolates collected from *A. labiata* (DAB1P and DAB2P), *O. mossambicus* (DOM1P) and *S. zambezensis* (DSZ1P–DSZ5P) in the River Phongolo, similarly to the results of 28S rDNA analyses, demonstrated close relationships with the isolates of *D. spathaceum* (KJ726508; KR149502). This clade also included a sequence of an unidentified species *Diplostomum* sp. 14 *sensu* Locke et al. (2015) (KT186789), recently reported by Locke et al. (2015) from the Tench, *Tinca tinca* (Linnaeus, 1758), in China (Supplementary Table 1). The sequence divergence within the clade was 0.1% (1 nt). The clade consisting of sequences of the isolates recovered from the lenses of *P. philander* collected in the River Mooi received no support from both BI and ML analyses and was recovered as a sister clade to two species from North America, *D. huronense* and *Diplostomum* sp. 4 (Fig. 2b). Sequences of all isolates from *P. philander* were identical and did not match any sequence of *Diplostomum* spp. currently available in GenBank.

For the *cox*1 dataset, three sequences per species or per species-level genetic lineages (the longest possible) reported from different countries were selected. The BI and ML analyses based on the *cox*1 alignment (347 nt positions, 114 sequences) yielded similar phylogenetic trees (Fig. 4), however different from the hypotheses based on nuclear markers, 28S rDNA and ITS1-5.8S-ITS2. Novel sequences of the isolates collected from *P. philander* in the River Mooi (DPP1B–DPP5B) formed a strongly supported clade with the sequences of an unidentified species of *Diplostomum*, *Diplostomum* sp. 16 recently reported from the Tigris bleak, *Alburnus caeruleus* Heckel, 1843 in Iraq (Locke et al., 2015). No sequence divergence was found between our isolates and the isolate of *Diplostomum* sp. 16. Sequences of the metacercarial isolates collected from the three fish species in the River Phongolo (DAB1P, DOM1P, DSZ1P and DSZ3P–DSZ5P) (Table 3) clustered together with the two isolates of *Diplostomum* sp. 14 collected from the Snakehead, *Channa argus* (Cantor, 1842), and *T. tinca* in China (Locke et al., 2015) and one isolate of *Diplostomum* sp. 14 collected from Tigris kingfish, *Cyprinion macrostomum* Heckel, 1843, in Iraq (Locke et al., 2015) in the strongly supported clade remoted from the clade of *D. spathaceum* (Fig. 3). The sequence divergence within this clade ranged between 0 and 3.3% (0–9 nt). Sequences of the three metacercarial isolates from *S. zambezensis* (DSZ6P and DSZ7P) and *T. sparrmanii* (DTS1R), similarly to the results of the ITS1-5.8S-ITS2 analyses, clustered with the isolates of *Diplostomum* sp. from *S. nigrita* in Nigeria (Chibwana et al., 2013). The sequence divergence ranged between 0 and 1.1% (0–3 nt) which is considered as intraspecific.

The species identification of the metacercarial isolates recovered from the lenses of the freshwater fishes in South Africa during this study was based on the results of the *cox*1 gene analyses. A total of three species were identified and they appeared to be conspecific to the three species previously reported from Nigeria (*Diplostomum* sp.), China (*Diplostomum* sp. 14) and Iraq (*Diplostomum* sp. 14 and *Diplostomum* sp. 16). Therefore, the three species are referred to as *Diplostomum* sp. *sensu* Chibwana et al. (2013), *Diplostomum* sp. 14 *sensu* Locke et al. (2015) and *Diplostomum* sp. 16 *sensu* Locke et al. (2015). The interspecific divergence between *Diplostomum* sp. and *Diplosotmum* sp. 14 was 13.2–14.7% (36–40 nt), *Diplostomum* sp. and *Diplostomum* sp. 16 was 11.8–12.1% (32–33 nt) and *Diplostomum* sp. 14 and *Diplostomum* sp. 16 was 11.8–12.5% (32–34 nt). Although the three species can be well distinguished using molecular sequence data, they also exhibit several prominent characteristics that can be used for the identification based on morphology. The morphological descriptions of the present metacercariae are provided below.

3.3. Morphological descriptions of metacercariae

3.3.1. *Diplostomum sp. sensu* Chibwana et al. (2013)
Description (Fig. 4a – c)

[Based on one live metacercaria]. Body large, oval, 738 × 532, with maximum width at level of ventral sucker. Tegument covered with numerous tiny spines. Forebody subspherical, 590 × 532, larger than hindbody. Hindbody conical, short, 168 × 223, rounded. Forebody/hindbody length ratio 1:0.28, forebody/hindbody width ratio 1:0.42. Pseudosuckers sunken, at level of pharynx (Fig. 4c). Oral sucker subterminal, oval, 63 × 56. Prepharynx long, 21; pharynx muscular, elongate-oval, 68 × 37; oesophagus short; caeca long, reach posterior to holdfast organ. Ventral sucker transversely oval, in forebody, 82 × 102, distinctly larger than oral sucker [sucker width ratio 1:1.82]. Distance from ventral sucker to anterior end of body, 364, and to posterior end of forebody, 237. Holdfast organ large, transversely oval, 103 × 170, in posterior part of forebody. Distance from holdfast organ to ventral sucker, 11. Excretory vesicle large, V-shaped; reserve excretory system of diplostomid type. Excretory granules medium-sized, numerous, scattered throughout forebody. Excretory pore subterminal, oriented ventrally.

[Based on three ethanol-fixed metacercariae. Measurements are provided in Table 4]. Body large, subspherical, with maximum width at level of ventral sucker. Tegument covered with numerous tiny spines. Forebody transversely oval, larger than hindbody. Hindbody short, bluntly rounded. Forebody/hindbody length ratio 1:0.13–1:0.16 (1:0.15), forebody/hindbody width ratio 1:0.56–1:0.63 (1:0.60). Pseudosuckers sunken, at level of pharynx. Oral sucker subterminal, subspherical. Prepharynx long; pharynx muscular, elongate-oval; oesophagus short; caeca long, reach posterior to holdfast organ. Ventral sucker transversely oval, distinctly larger than oral sucker [oral/ventral sucker width ratio 1:1.98–1:2.29 (1:2.12)]. Distance from ventral sucker to anterior end of body, 174–298 (256), and to posterior end of forebody, 91–208 (163). Holdfast organ large, transversely oval, in posterior part of forebody, contiguous with ventral sucker. Excretory vesicle large, V-shaped; reserve excretory system of diplostomid type. Excretory granules medium-sized, numerous, scattered throughout forebody. Excretory pore subterminal, oriented ventrally.

Second intermediate host: Plain squeaker *Synodontis zambezensis* Peters, 1852 (Siluriformes: Mochokidae); Banded tilapia *Tilapia sparrmanii* Smith, 1840 (Perciformes: Cichlidae).

Localities: River Phongolo Site 1 (26°55′47″S, 32°19′30″E) and River Riet (28°59′60″S, 24°28′50″E).

Prevalence: 5% (*S. zambezensis*, River Phongolo Site 1); 1 of 6 (*T. sparrmanii*, River Riet).

Intensity of infection: 1 metacercaria per fish.

Representative DNA sequences: 28S – 3 sequences (MN813526 – MN813528), ITS1-5.8S-ITS2 – 3 sequences (MN813534 – MN813536), *cox*1 – 3 sequences (MN808616 – MN808618).

3.3.2. *Diplostomum sp. 14 sensu* Locke et al. (2015)
Description (Fig. 4d – f)

[Based on 10 live metacercariae]. Body elongate-oval, 300–472 × 175–294 (354 × 229), with maximum width at level of ventral sucker or just anterior to ventral sucker. Tegument covered with numerous tiny spines. Forebody elongate-oval, 277–443 × 178–294 (336 × 235), longer than hindbody. Hindbody rounded, short, 53–84 × 57–100 (68 × 78). Forebody/hindbody length ratio, 1:0.16–1:0.26 (1:0.21). Forebody/hindbody width ratio 1:0.24–1:0.47 (1:0.34). Pseudosuckers elongate-oval, 35–57 × 20–25 (42 × 22). Oral sucker subterminal, subspherical (n = 8), 40–51 × 40–54 (45 × 46) or transversely oval (n = 2), 44–48 × 51–54 (46 × 53). Prepharynx very short, 3–10 (5) or absent; pharynx muscular, elongate-oval, 25–37 × 17–26 (31 × 20); oesophagus short; caeca thick, long, reach posterior to holdfast organ. Ventral sucker transversely oval, postequatorial, 34–46 × 42–53 (39 × 46), smaller or equal to oral sucker [oral/ventral sucker width ratio 1:0.82–1:1.08 (1:0.96)]. Distance from ventral sucker to anterior end of body, 146–224 (177) and to posterior end of forebody, 96–168 (126). Holdfast organ subspherical, 52–67 × 57–78 (61 × 68). Distance from holdfast organ to ventral
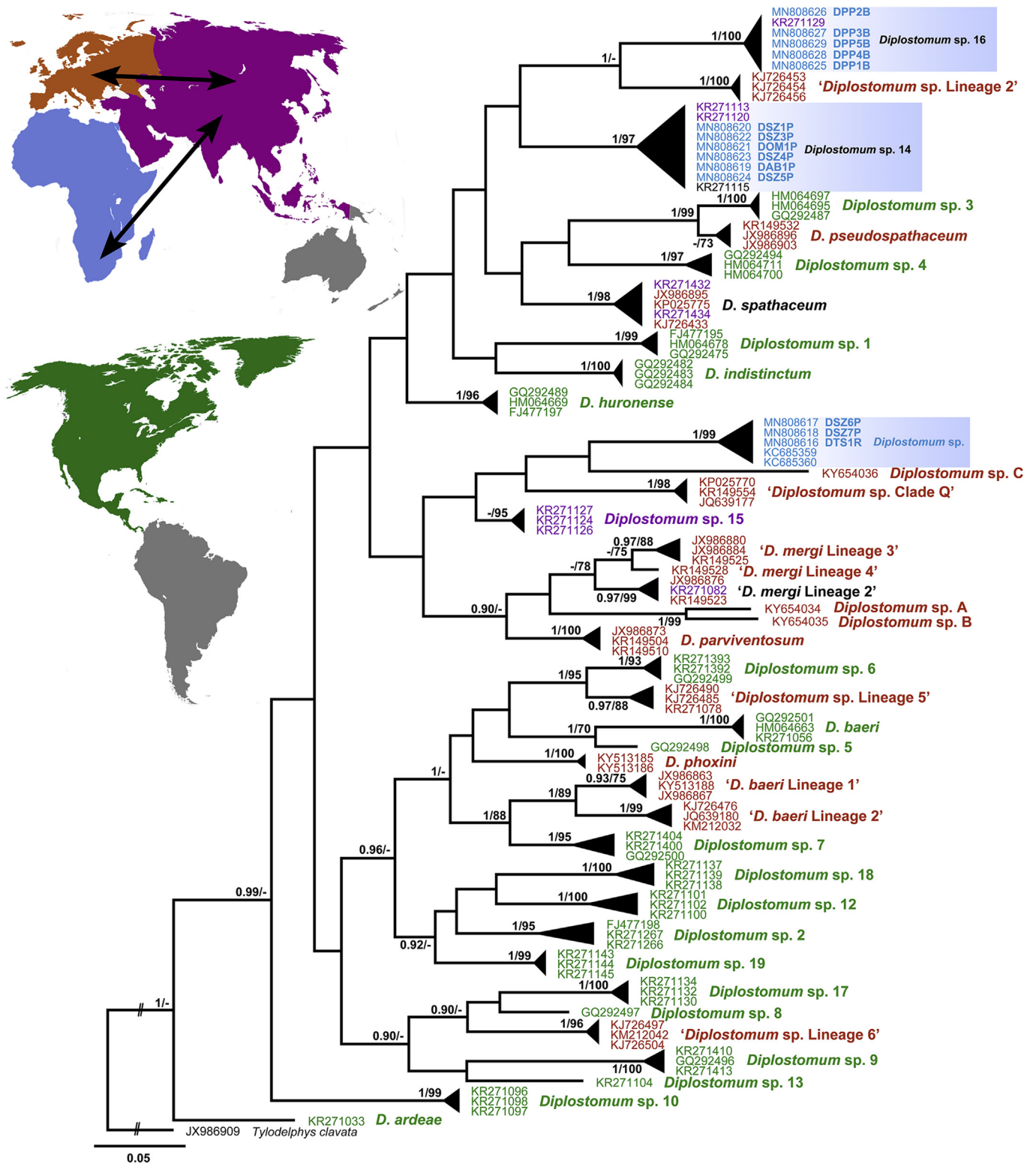
Fig. 3. Bayesian inference (BI) and maximum likelihood (ML) phylogram reconstructed using *cox*1 sequences for species of *Diplostomum*. Nodal support from BI and ML analyses indicated as BI/ML; only values > 0.90 (BI) and > 70 (ML) are displayed. Scale-bar indicates the expected number of substitution per site. Sequences generated in this study are in bold and indicated by blue rectangles. Codes with isolate information for newly generated sequences are provided in Table 3. Sequences derived from Africa are highlighted in blue, from Asia in purple, from Europe in orange, from North America in green (according to the map) and sequences reported from more than one continent are highlighted in black. Black arrows on the map demonstrate distribution of *Diplostomum spathaceum* and '*D. mergi* Lineage 2' in both, Asia and Europe, and *Diplostomum* sp. 14 and *Diplostomum* sp. 16 in both, Africa and Asia. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
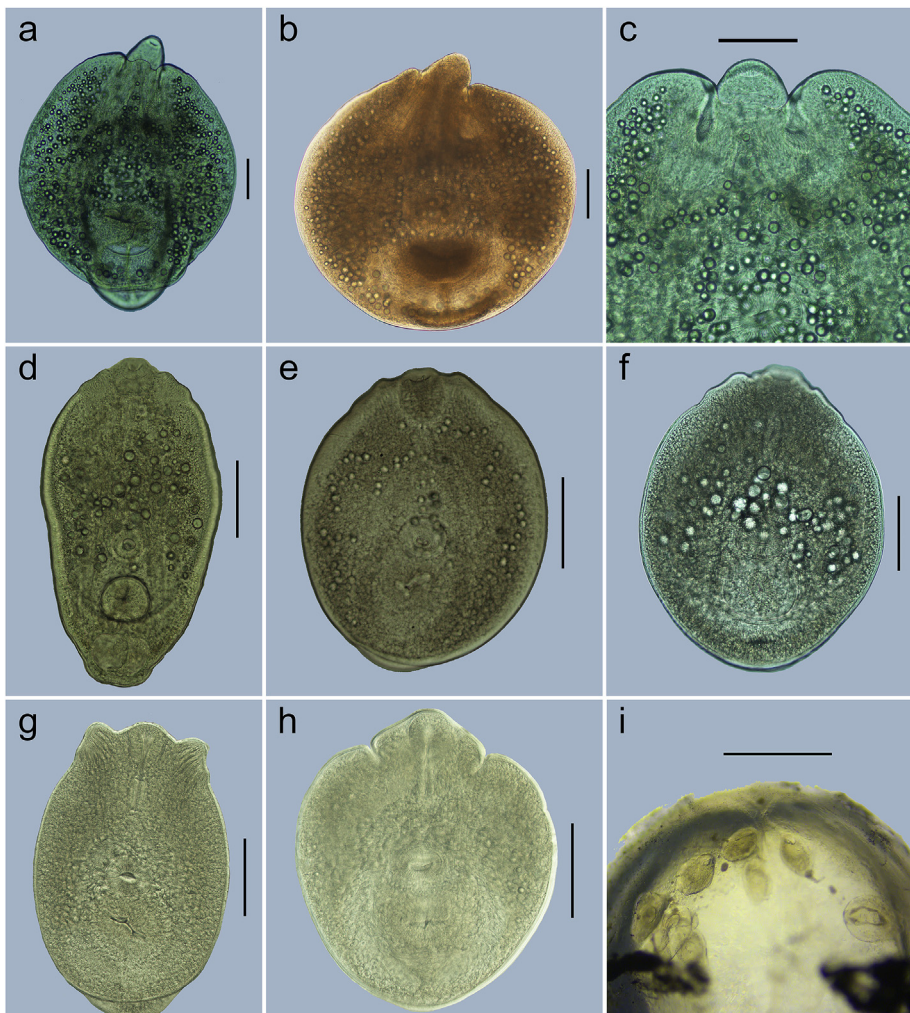
Fig. 4. Metacercariae of *Diplostomum* spp. from eye lenses of different fish hosts; (a) *Diplostomum* sp. from *Tilipia sparrmanii*, live, ventral view (b) *Diplostomum* sp. from *Tilipia sparrmanii*, fixed, ventral view (c) *Diplostomum* sp. from *Tilipia sparrmanii*, live, sunken pseudosuckers (arrowhead) (hologenophore, GenBank MN813526, MN813534 and MN808616) (d) *Diplostomum* sp. 14 *sensu* Locke et al. (2015) from *Synodontis zambezensis*, live, ventral view (hologenophore, GenBank MN813541) (e) *Diplostomum* sp. 14 *sensu* Locke et al. (2015) from *Oreochromis mossambicus*, fixed, ventral view, small excretory granules (arrowhead) (hologenophore, GenBank MN813531, MN813539 and MN808621) (f) *Diplostomum* sp. 14 *sensu* Locke et al. (2015) from *Synodontis zambezensis*, fixed, ventral view, large excretory granules (arrowhead) (hologenophore, GenBank MN813541) (g) *Diplostomum* sp. 16 *sensu* Locke et al. (2015) from *Pseudocrenilabrus philander*, fixed, ventral view, everted pseudosuckers (arrowhead) (hologenophore, GenBank MN813532, MN813547 and MN808627) (h) *Diplostomum* sp. 16 *sensu* Locke et al. (2015) from *Pseudocrenilabrus philander*, fixed, ventral view, inverted pseudosuckers (arrowhead) (hologenophore, GenBank MN813533, MN813548 and MN808628) (i) *Diplostomum* sp. 16 *sensu* Locke et al. (2015) from *Pseudocrenilabrus philander*, live metacercariae inside of fish lens. Scale bars: a–h = 100 μm; i = 700 μm.

Table 4
Comparative metrical data on *Diplostomum* spp. (fixed specimens).

Species	*Diplostomum* sp.		*Diplostomum longicollis* Zhokhov (2014)	
Host	*Synodontis zambezensis, Tilapia sparrmanii*		*Enteromius humilis, Garra dembecha*	
Country	South Africa		Ethiopia	
Source	Present study		Zhokhov (2014)	
Character	Range	Mean	Range	Mean
BL	379–615	497	612–1008	748
BW	456–525	491	378–576	490
FL	337–568	491	–	–
FW	456–563	515	–	–
HL	55–76	66	–	–
HW	288–296	292	–	–
OSL	38–55	48	66–72	63
OSW	48–54	50	66–72	65
PPHL	42–92	60	72–180	123
PHL	42–65	50	60–66	63
PHW	31–41	35	30–42	39
VSL	66–87	75	72–96	89
VSW	100–112	106	96–120	104
HOL	104–123	115	132–180	158
HOW	141–196	165	150–252	183
OSW:VSW	1:1.98–2.29	1:2.12	1:1.45–1.67[a]	1:1.6[a]

[a] Estimated from measurements provided in Zhokhov (2014).

sucker, 7–15 (11). Excretory granules, medium- or large sized, scattered in forebody, but generally grouped into two lateral extracaecal and one median field. Excretory vesicle V-shaped; reserve excretory system of diplostomid type. Excretory pore subterminal, oriented ventrally.

[Based on 14 ethanol-fixed metacercariae. Measurements are provided in Table 5]. Body oval, with maximum width just anterior to ventral sucker. Tegument covered with numerous tiny spines. Forebody oval, longer than hindbody. Hindbody rounded, short Forebody/hindbody length ratio 1:0.08–1:0.26 (1:0.18), forebody/hindbody width ratio, 1:0.28–1:0.60 (1:0.40). Pseudosuckers elongate-oval. Oral sucker subterminal, subspherical. Prepharynx very short or absent; pharynx muscular, elongate-oval; oesophagus short; caeca long, thick, reach posterior to holdfast organ. Ventral sucker subspherical, postequatorial, smaller to larger than oral sucker [oral/ventral sucker width ratio 1:0.84–1:1.28 (1:1.05)]. Distance from ventral sucker to anterior end of body, 85–187 (133) and to posterior end of forebody, 89–144 (105). Holdfast organ subspherical, in posterior part of forebody. Distance from holdfast organ to ventral sucker, 5–6 (5) or holdfast organ contiguous with ventral sucker. Excretory granules, medium- (Fig. 4e) or large (Fig. 4f) sized, scattered in forebody, but generally grouped into two lateral extracaecal and one median field. Excretory vesicle V-shaped; reserve excretory system of diplostomid type. Excretory pore subterminal, oriented ventrally.

Second intermediate host: African mottled eel *Anguilla labiata* (Peters, 1852) (Anguilliformes: Anguillidae), Plain squeaker *Synodontis zambezensis* Peters, 1852 (Siluriformes: Mochokidae); Mozambique tilapia *Oreochromis mossambicus* (Peters, 1852) (Perciformes: Cichlidae).

Table 5
Comparative metrical data on *Diplostomum* spp. (fixed specimens).

Species	*Diplostomum* sp. 14		*Diplostomum* sp. 16		*Diplostomum garrae* Zhokhov (2014)		*Diplostomum montanum* Zhokhov (2014)		*Diplostomum tilapiae* Zhokhov (2014)	
Host	*Anguilla bengalensis labiata, Oreochromis mossambicus, Synodontis zambezensis*		*Pseudocrenilabrus philander*		*Garra dembecha*		*Enteromius humilis, Garra dembecha, Labeobarbus gorgorensis, Varicorhinus beso*		*Oreochromis niloticus*	
Country	South Africa		South Africa		Ethiopia		Ethiopia		Ethiopia	
Source	Present study		Present study		Zhokhov (2014)		Zhokhov (2014)		Zhokhov (2014)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
BL	237–372	302	284–434	356	306–414	380	432–621	552	531–828	653
BW	206–271	240	212–306	254	252–306	283	240–372	289	198–234	212
FL	216–346	274	293–414	346	–	–	–	–	–	–
FW	206–271	237	231–277	250	–	–	–	–	–	–
HL	41–91	61	56–96	81	–	–	–	–	33–121	61
HW	66–102	81	72–130	104	–	–	–	–	–	–
OSL	36–55	45	51–62	55	54–72	65	60–78	63	53–66	58
OSW	35–53	43	38–59	49	54–66	59	48–72	67	53–57	55
PPHL	3–7	5	8–22	15	7–24	17	–	–	–	–
PHL	28–38	33	31–39	36	36–54	44	30–48	41	29–40	33
PHW	15–27	22	20–28	24	24–30	29	24–36	31	22–29	24
PSL	30–56	41	43–63	53	60–78	60	–	–	33–66	47
PSW	18–32	23	28–35	32	–	–	–	–	–	–
VSL	31–49	40	40–55	49	42–66	56	36–80	60	42–55	46
VSW	34–53	44	52–68	61	60–66	66	48–90	99	48–57	53
HOL	52–87	68	77–99	91	90–120	106	84–120	111	88–121	98
HOW	58–91	73	84–124	101	90–120	112	84–120	115	88–103	72
OSW:VSW	1:0.84–1.28	1.05	1:0.95–1:1.50	1:1.25	1:1–1.11[a]	1.12[a]	1:1–1.26[a]	1.48[a]	1:0.91–1[a]	0.96[a]

[a] Estimated from measurements provided in Zhokhov (2014).

Localities: River Phongolo Site 1 (26°55′47″S, 32°19′30″E) and River Phongolo Site 2 (26°52′58″S, 32°18′41″E).

Prevalence: 55% (*S. zambezensis*); 3 of 4 (*A. labiata*); 1 of 3 (*O. mossambicus*, River Phongolo Site 2).

Intensity of infection: 1–12 metacercariae per fish (*S. zambezensis*, River Phongolo Site 1); 2–6 metacercariae per fish (*A. labiata*, River Phongolo Site 1); 1 metacercaria per fish (*O. mossambicus*, River Phongolo Site 2).

Voucher material: 14 voucher specimens deposited in NMB P 526–530 [NMB P 526 (2 specimens), NMB P 527 (2 specimens), NMB P 528 (3 specimens), all from *S. zambezensis*, River Phongolo, KwaZulu-Natal, South Africa; NMB P 529 (3 specimens) and NMB P 530 (4 specimens), both from *A. labiata*, River Phongolo, KwaZulu-Natal, South Africa].

Representative DNA sequences: 28S – 3 sequences (MN813529 – MN813531), ITS1-5.8S-ITS2 – 8 sequences (MN813537 – MN813544), *cox*1 – 6 sequences (MN808619 – MN808624).

3.3.3. *Diplostomum sp. 16 sensu* Locke et al. (2015)

Description (Fig. 4g – i)

[Based on 15 ethanol-fixed metacercariae. Measurements are provided in Table 5]. Body elongate-oval, with maximum width at level of ventral sucker or just anterior to ventral sucker. Tegument covered with numerous tiny spines. Forebody elongate-oval, longer than hindbody. Hindbody rounded, short. Forebody/hindbody length ratio 1:0.19–1:0.27 (1:0.23), forebody/hindbody width ratio 1:0.31–1:0.49 (1:0.41). Pseudosuckers elongate-oval, everted (n = 14; Fig. 4g) or inverted (n = 1; Fig. 4h). Oral sucker subterminal, elongate-oval. Prepharynx short; pharynx muscular, elongate-oval; oesophagus short; caeca long, reach posterior to holdfast organ. Ventral sucker transversely oval, equatorial, equal or larger than oral sucker [oral/ventral sucker width ratio 1:0.95–1:1.50 (1:1.25)]. Distance from ventral sucker to anterior end of body, 128–207 (167) and to posterior end of forebody, 105–158 (133). Holdfast organ transversely oval, in posterior part of forebody, contiguous with ventral sucker. Excretory granules,

medium-sized, grouped into two lateral extracaecal and one median field. Excretory vesicle V-shaped; reserve excretory system of diplostomid type. Excretory pore subterminal, oriented ventrally.

Second intermediate host: Southern mouthbrooder *Pseudocrenilabrus philander* (Weber, 1897) (Perciformes: Cichlidae).

Localities: River Mooi (26°33′58″S, 27°07′16″E).

Prevalence: 9 of 10.

Intensity of infection: 3–21 metacercariae per fish.

Voucher material: 15 voucher specimens deposited in NMB P 531–533 [NMB P 531 (7 specimens), NMB P 532 (5 specimens), NMB P 533 (3 specimens), all from *P. philander*, River Mooi, North West Province, South Africa].

Representative DNA sequences: 28S – 2 sequences (MN813532; MN813533), ITS1-5.8S-ITS2 – 5 sequences (MN813545 – MN813549), *cox*1 – 5 sequences (MN808625 – MN808629).

3.4. Remarks

The three species of *Diplostomum* described above represent species that were previously reported from freshwater fishes in Nigeria (Chibwana et al., 2013), Iraq and China (Locke et al., 2015) based on the analyses of molecular data. The previous reports did not include morphological descriptions of the metacercarial isolates and, thus our study provides the first morphological characterisation of these three species of *Diplostomum* linked to molecular sequences. Morphologically, metacercariae of the present species are well-distinguishable from each other. The most characteristic feature differentiating metacercariae of *Diplostomum* sp. from the two other species in our study is the presence of pseudosuckers of the sunken type. In addition the metacercariae of *Diplostomum* sp. differs from both, *Diplostomum* sp. 14 and *Diplostomum* sp. 16 by the shape (subsperical body *vs* elongate-oval *vs* elongate-oval, respectively) and size of body [379–615 × 456–525 (497 × 491) *vs* 237–372 × 206–271 (302 × 240) *vs* 284–434 × 212–306 (356 × 254)], longer prepharynx [42–92 (60) *vs* 3–7 (5) *vs* 8–22 (15)], larger pharynx [42–65 × 31–41 (50 × 35) *vs* 28–38 × 15–27

(33 × 22) vs 31–39 × 20–28 (36 × 24)], ventral sucker [66–87 × 100–112 (75 × 106) vs 31–49 × 34–53 (40 × 44) vs 40–55 × 52–68 (49 × 61)], oral/ventral suckers ratio [1:1.98–1:2.29 (1:2.12) vs 1:0.84–1:1.28 (1:1.05) vs 1:0.95–1:1.50 (1:1.25)] and holdfast organ [104–123 × 141–196 (115 × 165) vs 52–87 × 58–91 (68 × 73) vs 77–99 × 84–124 (91 × 101)]. Furthermore, the size and distribution of the excretory granules in the metacercariae of Diplostomum sp., i.e. medium-sized and scattered throughout the forebody, differs from the state observed in the two other species where the excretory granules are of small to large size and grouped into two lateral extracaecal and one median field.

Although the body length and width of the metacercariae of Diplostomum sp. 14 and Diplostomum sp. 16 overlap in range [237–372 × 206–271 (302 × 240) vs 284–434 × 212–306 (356 × 254)], the measurements of the metacercariae of Diplostomum sp. 14 is on average smaller than those of Diplostomum sp. 16. Diplostomum sp. 14 further differ from Diplostomum sp. 16 in having a smaller oral sucker [36–55 × 35–53 (45 × 43) vs 51–62 × 38–59 (55 × 49)], shorter prepharynx [3–7 (5) vs 8–22 (15)], smaller ventral sucker [31–49 × 34–53 (40 × 44) vs 40–55 × 52–68 (49 × 61)] and smaller holdfast organ [52–87 × 58–91 (68 × 73) vs 77–99 × 84–124 (91 × 101)].

Morphologically, the metacercariae of Diplostomum sp. strongly resemble the metacercariae of Diplostomum longicollis Zhokhov (2014) reported by Zhokhov (2014) from Enteromius humilis (Boulenger, 1902) and Garra dembecha Getahun and Stiassny, 2007 in Ethiopia in the presence of pseudosuckers of the sunken type. However, morphometric data comparisons of the fixed metacercariae revealed that the specimens in our study exhibit shorter body [379–615 (497) vs 612–1,008 (748)], smaller oral sucker [36–55 × 48–54 (48 × 50) vs 66–72 × 66–72 (63 × 65)], shorter prepharynx (42–92 vs 72–180), lower low limits for pharynx length (42–65 vs 60–66) and ventral sucker length (66–87 vs 72–96), and smaller holdfast organ [104–123 × 141–196 (115 × 165) vs 132–180 × 150–252 (158 × 183)].

The metacercariae of Diplostomum sp. 14 are morphologically most similar to the metacercariae of D. montanum Zhokhov (2014) from the eye lenses of E. humilis, G. dembecha, L. gorgorensis, V. beso and D. tilapiae Zhokhov (2014) from the eye lenses of O. niloticus collected in Ethiopia (Zhokhov, 2014). These similarities include: the shape of the body and pseudosuckers, position and size of the holdfast organ in relation to the ventral sucker and position of the ventral sucker. However, almost all body dimensions of metacercariae in our material are smaller than those of metacercariae of D. montanum and D. tilapiae as described by Zhokhov (2014) (see Table 5 for details).

The metacercariae of Diplostomum sp. 16 possess features similar to metacercariae of Diplostomum garrae Zhokhov (2014) found in the eye lens of G. dembecha in Ethiopia (Zhokhov, 2014). These include shape of the body, pseudosuckers and holdfast organ and the position of the ventral sucker. Metacercariae of Diplostomum sp. 16 can further be distinguished from D. garrae in having a lower low limits for a number of features, including length and width of body, oral sucker, pharynx, ventral sucker, holdfast organ and the length of the prepharynx (see Table 5 for details).

It should be noted that metacercariae reported in the present study were not compared to the four species of Diplostomum, D. heterobranchi Wedl, 1861, D. magnicaudum El-Naffar (1979), Diplostomum sp. type I Prudhoe and Hussey (1977) and Diplostomum sp. type II Prudhoe and Hussey (1977) collected in the brain or encysted in the mesenteries of the North African catfish Clarias gariepinus (Burchell, 1822) in Egypt and South Africa (Prudhoe and Hussey, 1977; Khalil and Polling, 1997), because detailed examination of the descriptions and illustrations of the metacercariae of these species suggested that their placement within the genus Diplostomum was erroneous.

4. Discussion

Freshwater ecosystems in South Africa are characterised by a rich fish diversity with over 180 species currently recognised (Froese and Pauly, 2019). Even though the members of the Cichlidae and Cyprinidae dominate the ichtyofauna, much information concerning diplostomid trematodes originates from studies on C. gariepinus (Siluriformes: Clariidae) (Prudhoe and Hussey, 1977; Khalil and Polling, 1997; Barson and Avenant-Oldewage, 2006; Moema et al., 2013), a species widely used in commercial aquaculture in Africa. Whilst five species of the Diplostomidae known in South Africa have been found parasitising C. gariepinus (Khalil and Polling, 1997; Kudlai et al., 2018; Hoogendoorn et al., 2019), cichlid fishes (T. sparrmanii and P. philander) have been reported as hosts only for a single species (Moema et al., 2013). Recently, Hoogendoorn et al. (2019) examined T. sparrmanii in the North West Province, South Africa and reported four diplostomid species (Bolbophorus sp. 3, Posthodiplostomum sp. 9, Uvulifer sp. 4 and Diplostomidae gen. sp.) that were not previously detected neither in cichlids or cyprinids nor in C. gariepinus. Therefore, it is not surprising that a wider sampling effort for potential hosts, including 21 species from 11 families collected in three provinces of South Africa resulted in the discovery of at least three species of Diplostomum not previously reported from this region. Two out of the three species reported here, Diplostomum sp. and Diplostomum sp. 14 were found to infect more than one host species from different fish families (Anguillidae, Cichlidae and Mochokidae). The wide host range of Diplostomum sp. 14 in South Africa is not surprising as the host range in Iraq and China is even broader and includes members of the Channidae, Cyprinidae, Hemiramphidae, Odontobutidae, Bagridae, Gobiidae, Percichthyidae. However, the wide host range of Diplostomum sp. is interesting and unexpected since it has been previously reported from only a single host species (S. nigrita) in Nigeria (Chibwana et al., 2013). Our data of numerous fish hosts therefore extends its host range. In the present study Diplostomum sp. 16 was only found parasitising a cichlid species, P. philander; which is similar to its previous record where it was found infecting a single host species (A. caeruleus) of cyprinid fish in Iraq (Locke et al., 2015). The fact that metacerariae of all three species in the present study were found in cichlid fishes suggests that their transmission is mainly associated with this group. Further comprehensive assessment of freshwater fishes in other parts of southern Africa may reveal more information on the host ranges of Diplostomum.

One of the major impediments for accurate species delineation and identification of Diplostomum based on their metacercariae is the simple morphology of larval stages (Shigin, 1986; Georgieva et al., 2013; Kudlai et al., 2017). However, the initial delineation of metacercariae in our material into three species based on morphological and morphometric characteristics was further supported by sequence data analyses. It is worth mentioning that one of the most noticeable and rather rare characteristic in metacercariae of Diplostomum sp. that we found in T. sparmanii and S. zambesensis is the pseudosuckers of the sunken type. To the best of our knowledge, this type of pseudosuckers was previously documented in metacercariae for only four species, Diplostomum gobiorum Shigin, 1965, D. pungiti Shigin, 1965, D. volvens von Nordmann, 1832 and D. longicollis (Shigin, 1986; Zhokhov, 2014).

Following the methodological recommendations in previous studies that focused on Diplostomum (Georgieva et al., 2013; Brabec et al., 2015; Locke et al., 2015), molecular identification of our material was based on multiple molecular markers including the partial 28S rRNA and cox1 genes, and ITS1-5.8S-ITS2 gene cluster. The results of both analyses that employed ribosomal markers suggested that specimens collected from A. labiata, O. mosambicus and S. zambezensis in Ndumo Game Reserve was conspecific with D. spathaceum (Fig. 2). In contrast, analyses based on the mitochondrial cox1 gene, that is considered to be more effective for species identification, demonstrated remarkable differences between these species (Fig. 3) suggesting that the specimens isolated from above mentioned hosts is in fact conspecific with

Diplostomum sp. 14 reported from Iraq and China (Locke et al., 2015). This observation has once again proved the importance of the application of multiple genetic markers for accurate species delineation and identification with mitochondrial genes being a priority choice.

The results of our study not only improve the knowledge on the species diversity within *Diplostomum* in South Africa, but also uncover their geographical range and provide the first molecular evidence for the distribution of *Diplostomum* spp. in the Southern Hemisphere. The analysis of the data available for *Diplostomum* spp. from four continents (Africa, Asia, Europe and North America) (see Fig. 3), demonstrated a very broad distribution of several species. *Diplostomum* sp. recently reported from *S. nigrita* in Nigeria (Chibwana et al., 2013) appeared to also parasitise *S. zambezensis* and *T. sparrmanii* in South Africa, thus this species has a much wider geographical distribution within the Afrotropical region than previously recorded. To date, both *Diplostomum* sp. 14 and *Diplostomum* sp. 16 were only known from the Asian continent (Iraq and China) and the presence of these species in South Africa was rather unexpected, especially since most species of *Diplostomum* have a relatively restricted geographical distribution and have been reported from only one zoogeographical region. Prior to our study, based on molecular data, the only two species *D. spathaceum* and *D. mergi* Lineage 2 were known to be distributed across two continents, Europe and Asia within the Palaearctic region (Locke et al., 2015). Our results provide evidence for species within *Diplostomum* to have a much broader geographical distribution by being common in both Northern (within Palaearctic region) and Southern (within Afrotropical region) hemispheres. The transmission between the continents is primarily associated with the migratory patterns of their definitive hosts – piscivorous birds. Four out of six sampling localities of our survey were situated within the Ndumo Game Reserve, an area known for its high diversity of resident and migratory birds. It accommodates 430 bird species that is 19% of all species present on the African continent (Marnewick et al., 2015). Moreover, this area is located within the African-Eurasian flyways for the migratory birds that has previously been shown to be involved in the transmission of numerous bird parasites.

5. Conclusions

Based on an intensive review of the literature and data obtained in the present study, there are currently seven species of *Diplostomum* known to exploit freshwater fishes in Africa, namely *Diplostomum garrae* Zhokhov (2014), *Diplostomum longicollis* Zhokhov (2014), *Diplostomum montanum* Zhokhov (2014), *Diplostomum tilapiae* Zhokhov (2014), *Diplostomum* sp., *Diplostomum* sp. 14 and *Diplostomum* sp. 16. Of these, three species are distributed in South Africa. The discovery of these three species strongly suggests that the diversity of this genus is highly underestimated and understudied in the Southern Hemisphere. Moreover, our findings of these species from five fishes belonging to the families Anguillidae, Cichlidae and Mochokidae, significantly contributes to our knowledge on the host ranges and parasite-host associations in South Africa. In this study, we provide the first detailed morphological descriptions together with molecular data evidence for the three newly discovered species in South Africa, revealing a broader geographical distribution of *Diplostomum* spp. across the Palaearctic and Afrotropic regions. The expansion of the geographical range of *Diplostomum* between the Northern and Southern hemispheres based on molecular data evidence in particular, demonstrates the importance of the integration of morphological and molecular characterisation of these parasites. However, detailed descriptions of adults from piscivorous birds are still required for species identification and it is advised to follow a cautionary approach in the identification of new species. The development of a much needed baseline will be beneficial for future studies on the diversity, distribution and life histories of this genus, particularly in the Southern Hemisphere. A comprehensive study on the migratory patterns of piscivorous birds of the Palaerctic and Afrotropics

should be considered for the study on the transmission and distribution of *Diplostomum* between these zoogeographical regions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.12.003.

Policy and ethics

Animal handling was approved by the North-West University Animal Care, Research Ethics Committee (Ethics number: NWU-00160-18-S5).

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