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Gyrodactylus sprostonae Ling, 1962 infects an indigenous cyprinid in southern Africa: An expanded description

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Research Paper

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Abstract

Gyrodactylus sprostonae Ling, 1962 is a highly invasive parasite reported across freshwater environments of the northern hemisphere. The taxon was originally described from *Carassius auratus* (Linnaeus, 1758) and *Cyprinus carpio* Linnaeus, 1758 in China. This parasite has never been reported in Africa or the southern hemisphere. Recently, this taxon was collected from an indigenous yellowfish, *Labeobarbus aeneus* (Burchell, 1822), in the Vaal River, South Africa. The present study includes the conclusive identification of the gyrodactylid parasites collected from *L. aeneus*, including additional taxonomic data, using microscopy and molecular techniques. Microscopy included light microscopy (LM) of whole worms and scanning electron microscopy (SEM) of isolated haptoral sclerites. Additionally, morphometric data were obtained from SEM and compared to that generated using LM. For molecular analysis, the internal transcribed spacer (ITS) region of rDNA was amplified and phylogenetic topologies constructed. The specimens were morphometrically and genetically highly similar to other data for *G. sprostonae*. Additional point-to-point measurements and ITS rDNA sequences were generated for the taxon, contributing to the morphometric and molecular data for *G. sprostonae*. The study also includes the first study of the isolated haptoral sclerites of the taxon using SEM, with similar morphometric results to LM. This is the first record of *G. sprostonae* in the southern hemisphere and from a new, indigenous African host, *L. aeneus*, indicating host switching to smallmouth yellowfish. Furthermore, these results expand on the knowledge of the distribution of invasive parasites in South Africa, as well as *Gyrodactylus* species diversity in Africa.

Introduction

Gyrodactylus von Nordmann, 1832 species have been reported as parasites of both marine and freshwater fishes globally (Harris *et al.* 2004). Forty-one species have been described from Africa (Christison *et al.* 2021; Dos Santos *et al.* 2019b; Van As & Basson 1984; Řehulková *et al.* 2018; Truter *et al.* 2022), with nine descriptions or records from South African freshwater and marine fishes (Table 1). Only three of these species parasitize cyprinids, *Gyrodactylus kherulensis* Ergens, 1974, *Gyrodactylus kobayashii* Hukuda, 1940 and *Gyrodactylus paludinosus* (Truter *et al.* 2022). The last taxon described was from South Africa, whereas *G. kherulensis* and *G. kobayashii* are suspected to be co-introduced with their ornamental and aquaculture fish host species, *Cyprinus carpio* Linnaeus, 1758 and *Carassius auratus* (Linnaeus, 1758) respectively (Smit *et al.* 2017).

Gyrodactylus sprostonae Ling, 1962, was first described from the gills of *C. auratus* and *C. carpio* in the middle and lower reaches of the Liaohe River, China (Ling 1962). Since then, it has been reported in other parts of Asia (e.g., Russia and China (Bykhovskaya-Pavlovskaya *et al.* 1964)), Japan (Ogawa & Egusa 1978), Iraq (Abdullah 2013; Mhaisen & Abdul-Ameer 2013), Iran (Daghigh Roohi *et al.* 2019)), North America (e.g., Mendoza-Garfias *et al.* 2017); Mexico (García-Vásquez *et al.* 2021), and Europe (e.g., Germany (Mattheis & Glaser 1970)), Croatia (Kiskaroly 1977), Poland (Rokicka *et al.* 2007; Ziętara & Lumme 2004), and Serbia (Djikanovik *et al.* 2012)). In England, the National Fisheries Services reported *G. sprostonae* as a cause of mass mortality of cultured carp, flagging it as an emerging pathogen and threat to fisheries (National Fisheries Services, www.gov.uk/environment-agency). Eight studies have provided morphometric data for *G. sprostonae* (Abdullah 2013; Barzegar *et al.* 2018; Bykhovskaya-Pavlovskaya *et al.* 1964; Daghigh Roohi *et al.* 2019; Ling 1962; Mattheis & Glaser 1970; Ogawa & Egusa 1978; Pugachev *et al.* 2009).

The haptor of gyrodactylids comprizes four different sclerites; the hamulus, ventral bar, dorsal bar, and marginal hooks. The haptoral sclerites and the male copulatory organ (MCO), which lies below the pharynx, are used for *Gyrodactylus* species identification (Malmberg 1970). There are seven sequences available for the internal transcribed spacer region of ribosomal DNA (ITS rDNA) of *G. sprostonae*. Of these sequences, only three (KP295469, AY278044, KT346368) span the entire ITS region of rDNA (Ziętara & Lumme 2004). Moreover, of all this data, only the

Table 1. List of *Gyrodactylus* species described or reported from South African marine and freshwater fishes. ⁺Description, ⁺⁺Distribution record, *Freshwater, **Marine.

Gyrodactylus sp.	Host	Province	Reference(s)
+***Gyrodactylus eyipayipi* Vaughan, Christison, Hansen & Shinn, 2010	*Syngnathus acus* Linnaeus, 1758	False Bay, Western Cape	Vaughan *et al.* (2010)
****Gyrodactylus kherulensis* Ergens, 1974	*Cyprinus carpio* Linnaeus, 1758	Vaal River, Gauteng Kuils River, Western Cape	Crafford *et al.* (2014) Maseng *et al.* (2009) Maseng (2010) unpublished
****Gyrodactylus kobayashii* Hukuda, 1940	*Carassius auratus* (Linnaeus, 1758)	Kuils River, Western Cape	Maseng *et al.* (2009) Maseng (2010) unpublished
+***Gyrodactylus molweni* Christison, Vaugham, Shinn & Hansen, 2021	*Chelon richardsoni* (Smith, 1846)	Table Bay, Western Cape	Christison *et al.* (2021)
+**Gyrodactylus paludinosus* Truter, Smit, Malherbe & Přikrylová, 2022	*Enteromius paludinosus* (Peters, 1852)	Barberspan wetland, North West Province	Truter *et al.* (2022)
****Gyrodactylus sturmbaueri* Vanhove, Snoeks, Volckaert & Huyse, 2011	*Pseudocrenilabrus philander* (Weber, 1897)	Nwanedi River, Limpopo	Zahradníčková *et al.* (2016)
****Gyrodactylus thlapi* Christison, Shinn & Van As, 2005	*Pseudocrenilabrus philander* (Weber, 1897)	Barberspan wetland, NorthWest Province	Truter *et al.* (2016)
+***Gyrodactylus transvaalensis* Prudhoe & Hussey, 1977	*Clarias gariepinus* (Burchell, 1822)	Marble hall, Limpopo	Prudhoe & Hussey (1977)
+***Gyrodactylus ulinganisus* García-Vásquez, Hansen, Christison, Bron & Shinn, 2011	*Oreochromis mossambicus* (Peters, 1852)	Stellenbosch, Western Cape	García-Vásquez *et al.* (2011)

sequence by Zietara & Lumme (2004) relates to a peer-reviewed publication. The latter study supported the position of *G. sprostonae* in the subgenera *Gyrodactylus* (*Limnonephrotus*) Malmberg, 1964 using ribosomal DNA fragments. There is currently no record of *G. sprostonae* from Africa or the rest of the southern hemisphere. This study, therefore, aimed to incorporate traditional and modern techniques to study the gyrodactylids collected from *L. aeneus* in the Vaal River, South Africa, and additional morphometric and molecular data for the taxon.

Materials and methods

Sample collection

A total of 40 smallmouth yellowfish, *L. aeneus* (0.12–1.59 kg), were collected in March 2022 using gill nets at two sites (20 fish per site) along the Vaal River in Gauteng, South Africa in accordance with the conditions of permit CPE2 0118 and ethical clearance from the University of Johannesburg (03 May 2016). Site 1 was below the Vaal Dam (26°52′12.38″S; 28° 7′13.99″E), and Site 2 was below the Vaal River Barrage (26°44′6.26″S; 27°38′4.73″E) (Figure 1). The skin of fish was checked for monogenean parasites by scraping the skin with a glass microscope slide and examined for parasites using a Zeiss Stemi 350 stereomicroscope (Carl Zeiss, Germany). Thereafter, fish were euthanized by severing the spinal cord according to the South African National Standard: Care and Use of Animals for Scientific Purposes (2008). The gills and fins were dissected and examined for monogenean parasites using a Zeiss Stemi 350 stereomicroscope. Parasites were collected with a micropipette and either stored in 96% ethanol (Sigma-Aldrich, Germany) for scanning electron microscopy (SEM) and molecular analysis or mounted fresh with glycerine ammonium picrate (GAP) (Malmberg 1957) to study with light microscopy (LM) as described below.

Infection statistics

The prevalence and 95% confidence interval (CI) of collected specimens from respective sites were calculated following Bush *et al.* (1997) using Microsoft Excel (Microsoft Corporation, USA).

Light microscopy

Twenty whole worms were individually placed on microscope slides in a small drop of water, and a coverslip was placed over the specimen with pressure to expose the sclerotized structures. The corners of the coverslip were affixed onto the slide with nail varnish, then excess water was removed from the sides by capillary action using filter paper. A drop of GAP was placed on the edge of the coverslip and left to diffuse slowly into the specimen. Lastly, nail varnish was used to seal the sides of the coverslip. The preparation process was observed using a Zeiss Stemi 350 stereomicroscope (Carl Zeiss, Germany). A Zeiss Axioplan 2 imaging light microscope with Axiovision 4.7.2 software was used to study the specimens and obtain light micrographs of the different haptoral sclerites as well as the MCO. Point-to-point measurements of the sclerites were made following Shinn *et al.* (2004). Additionally, body length and width were measured. All measurements (mean ± standard deviation (minimum–maximum)) were compared to those of *G. sprostonae* presented by Abdullah (2013), Barzegar *et al.* (2018), Bykhovskaya-Pavlovskaya *et al.* (1964), Daghigh Roohi *et al.* (2019), Ling (1962), Mattheis & Glaser (1970), Ogawa & Egusa (1978), and Pugachev *et al.* (2009). Line drawings of haptoral sclerites and the MCO were constructed using CorelDRAW

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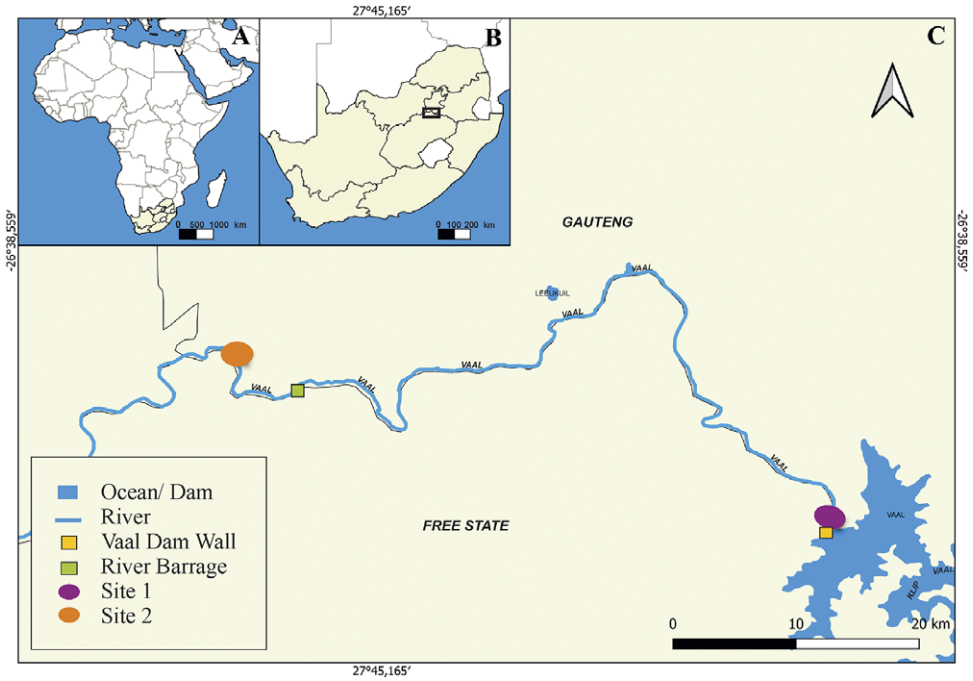

Figure 1. Collection sites along the Vaal River where *Labeobarbus aeneus* (Burchell, 1822) specimens were collected. **A** - African continent with South Africa shaded; **B** - map of South Africa indicating the area of collection sites; **C** - section of Vaal River system showing two collection sites, orange dot Site 1, purple dot Site 2.

(Taylor & Karney 1990) and compared to those presented in the aforementioned studies, all of which were redrawn for comparison.

Scanning electron microscopy

Thirteen whole worms (six from the first site and seven from the second site) stored in 96% ethanol were transferred to Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.96) overnight, then the buffer was changed three times over one-hour intervals. Thereafter, individual worms were placed on concavity slides with 0.5µl of digestion buffer from the E.Z.N.A.® Tissue DNA kit (Omega Bio-Tek Inc., Georgia, USA). The digestion protocol of Nation (1983) and Dos Santos & Avenant-Oldewage (2015) was followed but modified as in Maduenyane *et al.* (2022) to release and isolate haptoral sclerites. Following isolation, the sclerites were dried in a desiccator, and digested soft tissue was stored in a refrigerator for molecular analysis. Once dried, the sclerites were coated with gold using an Emscope SC500 sputter coater (Quorum Technologies, Lewes, UK) and micrographs taken at 6 kV acceleration voltage with a TESCAN Vega 3 LMH SEM (Brno, Czech Republic). Axiovision 4.7.2 software was used to generate point-to-point measurements of obtained micrographs for comparison with LM data. IBM SPSS version 28 was used for statistical analysis of haptoral sclerites and body measurements. Levene's test and histograms confirmed that the data were not normally distributed; therefore, the Kruskal-Wallis non-parametric test was conducted to test for significant differences between specimens and also between LM and SEM data.

Molecular analysis

Genomic DNA of the 13 specimens for which the sclerites were isolated was extracted using the E.Z.N.A.® Tissue DNA kit. The ITS rDNA was amplified with primer set; ITS1A (5'-GTA ACA AGG TTT CCG TAG GTG-3') (Matejusová *et al.* 2001) and ITS2R (5'-TCC TCC GCT TAG TGA TA-3') (Cunningham 1997). The following PCR conditions were used; 5 min @ 95°C, 35 cycles for 1 min @ 95°C, 1 min @ 55°C, 2 min @ 72°C then 5 min @ 72°C. Successful amplicons were verified using 1% agarose gel infused with SafeView™ Classic (Applied Biological Materials Inc., Richmond, Canada) and a SmartDoc™ 2.0 gel visualization and smartphone imaging system (Accuris instruments, Edison, NJ, USA). Standard BigDye chemistry was used to sequence the amplicons with an ABI 3137 Automated Sequencer (Applied Biosystems, Foster City, CA, USA). Obtained sequences were checked, aligned, assembled, and edited if needed with Geneious Prime version 2019.1.1 (http://www.genious.com), then compared to other *Gyrodactylus* species on GenBank. The Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990) was used to select sequences of *Gyrodactylus* species that were closest to data generated in the current study (online supplementary Table S1). Sequences from GenBank that did not cover 80% of the alignment of the ITS rDNA were excluded. MEGA 7 (Tamura *et al.* 2013) was utilized to compute genetic distances based on both uncorrected *p*-distances and number of base pair (bp) differences. Maximum likelihood (ML) and Bayesian inference (BI) approaches were used to construct phylogenetic topologies. The model selection tool in MEGA 7 was used to select the General Time Reversible model with Gamma distribution (5 categories (+G, parameter = 0. 3807)). A total of 1000 bootstrap replicates were used to assess the robustness of this topology. BEAST v2.5.0 (Bouckaert *et al.* 2014) with 10 million Markov chain Monte Carlo (MCMC) generations was used for BI analysis. Sequences generated from the present study were submitted to GenBank.

Results

Gyrodactylus sprostonae Ling, 1962

Type host: *Carassius auratus* (Linnaeus, 1758) and *Cyprinus carpio* Linnaeus, 1758

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Type locality: Liaohe River, China

New locality: Vaal River, South Africa (26°44′6.26″S; 27°38′4.73″E) and (26°52′12.38″S; 28° 7′13.99″E)

New host: *Labeobarbus aeneus* (Burchell, 1822)

Infection site: Gills

ITS rDNA reference sequences: Thirteen sequences submitted to GenBank (Accession numbers: OQ685901–13)

Infection statistics

Gyrodactylid parasites were not found on the skin and fins of fish but were present on the gills. Only one fish of 20 was infected with *G. sprostonae* from Site 1, and the prevalence was 5% (CI = –4.55–14.55%). From Site 2, nine hosts of 20 were infected; thus, the prevalence was 45% (CI = 23.2–66.8%). Aggregation was observed; most infected fish had only one gyrodactylid, while two fish (one from each site) had close to or more than 200 worms.

Morphometry (Figures 2–4)

All specimens with elongated bodies, total length 301 ± 80 (202–435) and width 66 ± 20 (40–114). Anterior bilobed and posterior comprised of haptor armed with pair of hamuli (Figures 2a & b) 49.4 ± 3.3 (41.3–54.3) long, root 18.6 ± 2.1 (15.1–21.6), and shaft 39.7 ± 2.4 (34.2–42.5) long. Hamuli connected by thin dorsal bar (Figure 2a) 17 ± 1.5 (14.7–20.8) long and 1.4 ± 0.3 (0.9–1.9) wide. Ventral bar (Figures 2d & e) 19.4 ± 2.2 (13.8–25.5) long and 19.6 ± 1.1 (18–22.3) wide, lies between hamuli, beneath dorsal bar (Figure 2a). Ventral bar comprised of thick, sclerotized horizontal bar, 19.6 ± 1.1 (18–22.3) wide, with sunken mid-point 3.5 ± 0.6 (2.8–5.1) long. Ventral bar with short, V-shaped, central process (Figure 2e) on dorsal side, and 14.6 ± 1.3 (10.4–16.2) long semi-sclerotized membrane. Haptor with 16 marginal hooks (Figure 2c), 24.9 ± 1.4 (21–26.7) long, with 19.7 ± 1.5 (16–22) long shaft, 5.2 ± 0.5 (3.7—6) long sickle, and 4.6 ± 0.4 (3.5–5.7) long sickle aperture. Marginal hook sickle has 3.5 ± 0.3 (2.9–4.2) proximal width and 3.2 ± 0.3 (2.6–3.9) distal width. Marginal hooks lack instep. Marginal hook sickle toe 1.6 ± 1.9 (1.1–1.9) long.

The MCO (Figure 4) is bulbous, muscular, and armed with one large spine and six small spines. Four spines near the middle are smaller than the two lateral spines. There was no statistically significant difference between point-to-point haptoral measurements (Table 2) of specimens from the two study sites (Kruskal-Wallis $p > 0.05$) based on LM observations. There was a statistically significant difference between the following LM and SEM point-to-point measurements: the hamulus total length (Kruskal-Wallis $p = 0.006$), point curve angle (Kruskal-Wallis $p < 0.001$), proximal shaft width (Kruskal-Wallis $p < 0.001$), inner aperture angle (Kruskal-Wallis $p < 0.001$), dorsal bar total length (Kruskal-Wallis $p = 0.008$), marginal hook sickle length (Kruskal-Wallis $p = 0.009$), sickle proximal width (Kruskal-Wallis $p < 0.001$), and toe length (Kruskal-Wallis $p < 0.001$). For the remaining 16 point-to-point measurements, there was no significant difference (Kruskal-Wallis $p > 0.05$).

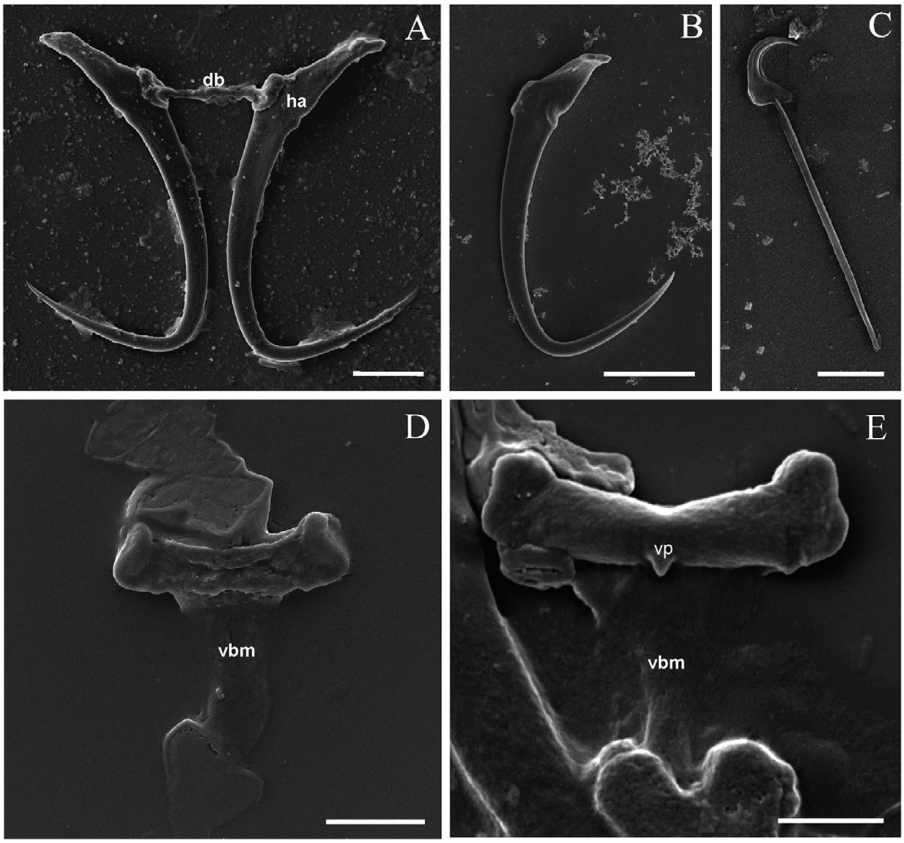

Figure 2. Scanning electron micrographs of isolated haptoral sclerites of *Gyrodactylus sprostonae* Ling, 1962 from the current study. **A** - ventral side of hamuli and dorsal bar (20 μm); **B** - dorsal side of hamulus (10 μm); **C** - marginal hook (5 μm); **D** - dorsal side of ventral bar (10 μm); **E** - ventral side of ventral bar (5 μm). hamuli (ha), dorsal bar (db), ventral bar membrane (vbm) v-shaped spike (vp).

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Table 2. Measurements (μm) of *Gyrodactylus sprostonae* Ling, 1962 from the present study and all other available studies based on light and scanning electron microscopy.

	Current study (LM) (South Africa) n = 20	Current study (SEM) (South Africa) n = 7	Ling 1962 (China)	Bykhovskaya-Pavlovskaya *et al.* 1964 (Russia & China)	Mattheis & Glaser (1970) (Germany)	Ogawa & Egusa (1978) (Japan)	Pugachev *et al.* 2009 (Russia & China)	Abdullah 2013 (Iraq)	Barzegar *et al.* 2018 (Iran)	Daghigh Roohi *et al.* 2019 (Iran)
BL	301 ± 80 (202–434)	–	192.7–426.4	Up to 420	300–420	296–543	200–400	420–500	270 ± 18 (25–300)	277 ± 73 (174–346)
BW	66 ± 20 (40–114)	–	57.4–106.6	Up to 100	70–100	81–267	–	10– –	81 ± 11 (67–92)	85 ± 9 (78–97)
Hamulus										
TL	49.4 ± 3.3 (41.3–54.3)	46.1 (42.7–48.3)	40.8–51.3	40–51	47–51	53 (51–57)	41–62	40–50	48.7 ± 3.7 (45–55)	51 ± 2.1 (48.5–54.2)
RL	18.6 ± 2.1 (15.1–21.6)	16.5 (12.7–18.1)	13.3–20.9	–	–	20 (17–22)	13–24	–	–	16.6 ± 1.9 (15.5–19.3)
SL	39.7 ± 2.4 (34.2–42.5)	39 (24.1–41.3)	36.1–39.9	–	–	40 (37–41)	–	–	38.8 ± 1.8 (36–41)	39.8 ± 1.7 (37.3–41.8)
PSW	4.4 ± 0.5 (3.8–5.4)	5.8 (5.2–6.3)	–	–	–	–	–	–	–	–
DSW	2.7 ± 0.5 (1.7–3.5)	2.6 (2.2–3.2)	–	–	–	–	–	–	–	–
PL	21.7 ± 2.3 (15.6–25)	22.6 (21.2–23.9)	17.1–22	–	22–23	22 (21–24)	17–25	–	19.5 ± 1.5 (18–22)	22.8 ± 0.9 (21.7–24.1)
AL	29.6 ± 3.5 (23.8–39.6)	28.2 (27.1–29)	–	–	–	–	–	–	–	–
AA	56.7 ± 6.3 (36.8–69.8)	54.5 (52.1–57)	–	–	–	–	–	–	70 ± 3.5 (65–75)	–
IAA	64 ± 3.5 (57.8–70.6)	60.3 (57.3–68.8)	–	–	–	–	–	–	–	–
ICL	1.6 ± 0.2 (1.4–1.9)	1.6 (1.3–2)	–	–	–	–	–	–	–	–
PCL	14.8 ± 2.2 (11.3–19.8)	17.2 (14.3–21.5)	–	–	–	–	–	–	–	–
Ventral bar										
TW	19.6 ± 1.1 (18–22.3)	28.2 (17.8–38.7)	14.2–20.9	–	17–20	18 (17–20)	–	–	19.7 ± 1.4 (18–22)	19.7 ± 1.3 (18–21.3)
TL	19.4 ± 2.2 (13.8–25.5)	28.9 (17.3–40.6)	–	13–20	–	–	13–26	–	–	–
ML	3.5 ± 0.6 (2.8–5.1)	5.2 (3–7.3)	2.8–3.8	3–4	–	2.5–4	4–7	–	2.9 ± 0.2 (2.5–3.2)	3.4 ± 0.4 (3–3.8)
PML	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	1–1.5	–	–	–	–
MML	14.6 ± 1.3 (10.4–16.2)	19.4 (11.4–27.4)	–	–	15–18	14–17	15–18	–	–	13.3 ± 1.1 (12.3–14.8)
Dorsal bar										
TL	19.8 ± 0.7 (19.2–20.6)	17 (14.7–20.8)	–	13–19	20– –	18–21	19–20	–	1.5 ± 0.2 (17–21)	–
TW	1.7 ± 0.3 (1.4–2.1)	1.4 (0.9–1.8)	–	1– –	–	1–2	1–2	–	9.7 ± 0.9 (8.5–11)	1 ± 0.1 (0.8–1.2)
Marginal hooks										
TL n=18	24.8 ± 1.4 (21–26.7)	23.9 (23.2–24.9)	19–24.7	24– –	22.–25	23–26	19–28	20–25	19.2 ± 1.5 (17.2– –)	–
SL n=18	19.7 ± 1.5 (16–22)	19.1 (17.9–19.8)	11.4–19.4	–	18–20	17–19	–	–	–	19.4 ± 0.9 (18.1–20.7)
SKL	5.2 ± 0.5 (3.7–6)	4.8 (4.4–5.1)	3.8–5.7	–	5– –	4.5–5	4–6	–	3.9 ± 0.3 (3.4–4.2)	4.5 ± 0.3 (4.1–4.9)
A	4.6 ± 0.4 (3.5–5.7)	4.5 (4.3–4.6)	–	–	–	–	–	–	–	–

(*Continued*)

Table 2. (*Continued*)

	Current study (LM) (South Africa) n = 20	Current study (SEM) (South Africa) n = 7	Ling 1962 (China)	Bykhovskaya-Pavlovskaya *et al.* 1964 (Russia & China)	Mattheis & Glaser (1970) (Germany)	Ogawa & Egusa (1978) (Japan)	Pugachev *et al.* 2009 (Russia & China)	Abdullah 2013 (Iraq)	Barzegar *et al.* 2018 (Iran)	Daghigh Roohi *et al.* 2019 (Iran)
SPW	3.5 ± 0.3 (2.9–4.2)	2.8 (2.7–3)	–	–	–	3–3.5	–	–	–	3.2 ± 0.3 (2.9–3.4)
SDW	3.2 ± 0.3 (2.6–3.9)	3.4 (2.4–4.1)	–	–	–	3–4	–	–	–	3.2 ± 0.5 (2.5–3.8)
STL	1.6 ± 1.9 (1.1–1.9)	1.3 (1.1–1.4)	–	–	–	–	–	–	–	–
IH	–	–	–	–	–	–	–	–	–	–
NS	6 small spines and 1 large spine	–	8 zigzag short spines and 1 curvy	–	–	6 small spines and 1 large spine	6 small spines and 1 large spine	–	–	–

BL - Body length; **BW** - body width; **TL** - total length; **RL** - root length; **SL** - shaft length; **PSW** - proximal shaft width; **DSW** - distal shaft width; **PL** - point length; **AL** - aperture length; **AA** - aperture angle; **IAA** - inner aperture angle; **ICL** - inner curve length; **PCA** - point curve angle; **TW** - total width; **ML** - median length; **PML** - process to mid-length; **PML** - process length; **MML** - Membrane length; **SKL** - sickle length; **A** - aperture; **SPW** - sickle proximal width; **SDW** - sickle distal width; **STL** - sickle toe length; **IH** - instep height; **NS** - number of spines
Key: (–) no measurement
(n) number of measured specimens

Remarks

Using the species identification key by Bykhovskaya-Pavlovskaya *et al.* (1964) and Pugachev *et al.* (2009), the specimens from the current study represent *G. sprostonae.* Moreover, the morphology of the specimens from the present study was highly similar to the original description of *G. sprostonae* by Ling (1962). Morphology of the haptoral sclerites from the current study (Figure 3a) were similar to those of *G. sprostonae* from previous studies (Abdullah 2013; Bykhovskaya-Pavlovskaya *et al.* 1964; Daghigh Roohi *et al.* 2019; Ling 1962; Mattheis & Glaser 1970; Ogawa & Egusa 1978; Pugachev *et al.* 2009) except for those by Barzegar *et al.* (2018). The ventral bar membrane was either absent or not detailed in most studies. The marginal hook sickle was most similar to the illustration of Bykhovskaya-Pavlovskaya *et al.* (1964) (Figure 3l), Mattheis & Glaser (1970) (Figure 3m), Ogawa & Egusa (1978) (Figure 3n), Pugachev *et al.* (2009) (Figure 3o), and Daghigh Roohi *et al.* (2019) (Figure 3r). Comparing sclerite and available MCO morphology of *Gyrodactylus* species reported from South African freshwater fishes to specimens from this study, there were no matches.

Genetic characterization

Identical sequences of ITS rDNA (1067–1163 bp) were obtained from all thirteen specimens (from both sites) in this study; thus only a single representative haplotype sequence was included for analyses. The alignment with selected *Gyrodactylus* data from GenBank was 1390 bp with 762 bp conserved, 624 bp variable, and 571 bp parsimony informative sites. The ITS rDNA phylogeny using both ML and BI showed that the sequences generated in the current study and sequences for *G. sprostonae* form a well-supported monophyletic group (Figure 5). A sister clade to the latter grouping comprized *G. kobayashii* and *Gyrodactylus gurleyi* Price, 1937. The intraspecific range for all included taxa for which more than one sequence is available was 0–0.07% (0–84 bp), while the interspecific range was 0–0.27% (6–327 bp) (excluding *Gyrodactylus pomeraniae x lavareti* hybrids) (online supplementary Table S2). Three sequences of *G. sprostonae* were included in the analysis and two sequences (KP295469 & AY278044) were identical to sequences generated in the present study (0%; 1–4 bp). The third sequence (KT346368) was 0.01% (7 bp) different from the data obtained here and other *G. sprostonae* data.

Discussion

This study presents the first record of *G. sprostonae* in the southern hemisphere and from a new, indigenous host, *L. aeneus.* The parasite was previously reported from indigenous and invasive hosts across the northern hemisphere (e.g., Abdullah 2013; Barzega *et al.* 2018; Bykhovskaya-Pavlovskaya *et al.* 1964; Daghigh Roohi *et al.* 2019; Ling 1962; Mattheis & Glaser 1970; Mhaisen & Abdul-Ameer 2013; Ogawa & Egusa 1978; Pugachev *et al.* 2009), mostly from its type hosts, *C. carpio* and *C. auratus* (e.g., Daghigh Roohi *et al.* 2019; Ling, 1962; Mendoza-Garfias *et al.* 2017; Ogawa & Egusa 1978). *Cyprinus carpio* has been reported as a host of several invasive monogenean parasites in the Vaal River system such as *G. kherulensis, Dactylogyrus extensus* Mueller & Cleave, 1932 and *Dactylogyrus minutus* Kulweic, 1927 (Crafford *et al.* 2014b). In a published conference abstract, Maseng *et al.* (2009) reported two invasive *Gyrodactylus* species, *G. kobayashii* and *G. kherulensis,* from *C. auratus* and *C. carpio* respectively. However, the locality of

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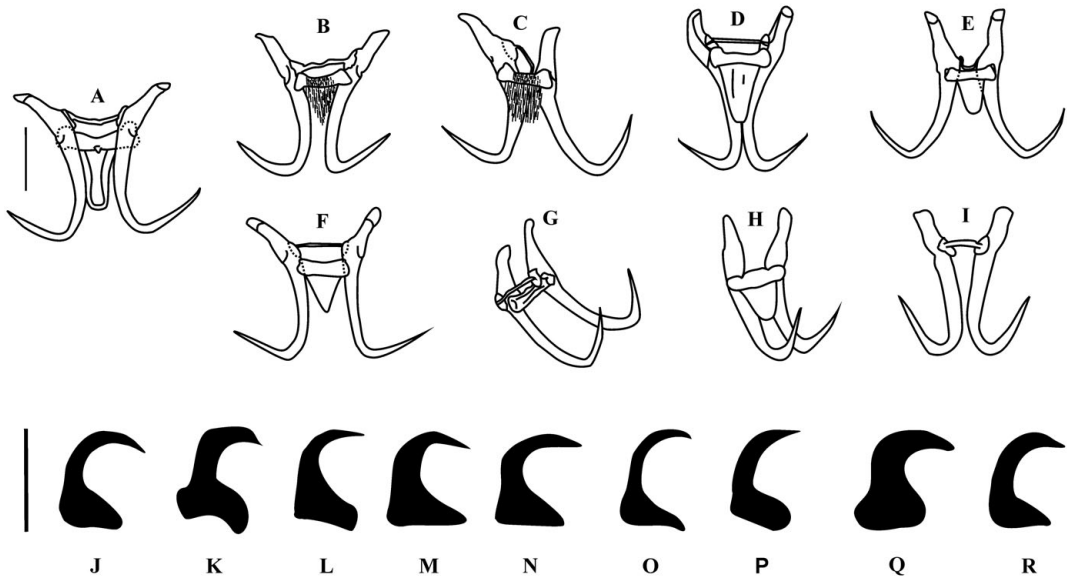

Figure 3. Illustrations of haptoral sclerites of *Gyrodactylus sprostonae* Ling, 1962 from **A**, **J** - present study; **B**, **K** - Ling (1962); **C**, **L** - Bykhovskaya-Pavlovskaya *et al.* (1964); **D**, **M -** Mattheis & Glaser (1970); **E**, **N** - Ogawa & Egusa (1978); **F**, **O -** Pugachev *et al.* (2009); **G**, **P** - Abdullah (2013); **H**, **Q** - Barzegar *et al.* (2018) and **I**, **R -** Daghigh Roohi *et al.* (2019). **A**-**I** - hamuli, dorsal and ventral bar (20 μm); **J**-**R** - marginal hook sickle (5 μm).

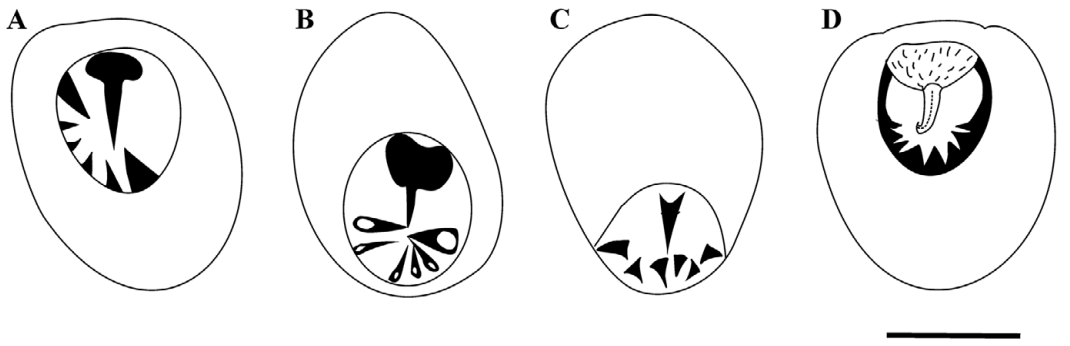

Figure 4. Illustrations of the male copulatory organ of *Gyrodactylus sprostonae* Ling, 1962 (5 μm). **A** - MCO from the present study; compared to MCO from **B** - Pugachev *et al.* (2009); **C** - Ogawa & Egusa (1978) and **D** - Ling (10962).

the fish hosts was not mentioned in the abstract, but it is mentioned in an unpublished Master of Science study by Maseng (2010). The latter stated that the fish were purchased from importers (imported from Asian and European countries), retailers and local breeders in Kuils River, South Africa. These records continue to remain uncertain as they have not been published in a peer-reviewed journal article. The presence of *G. sprostonae* on *L. aeneus* in the Vaal River system is suspected to be a recent introduction as it was not reported in previous monogenean surveys (Crafford *et al.* 2012; 2014a & b) conducted in this system. Furthermore, *L. aeneus* has been reported to host other invasive parasites, namely *Schyzocotyle acheilognathi* (Yamaguti, 1934) (Bertasso & Avenant-Oldewage 2005; Stadtlander *et al.* 2011), *Atractolytocestus huronesis* Anthony, 1958 (Dos Santos & Avenant-Oldewage 2022) and *Argulus japonicus* Thiele, 1900 (Kruger *et al.* 1983; Tam & Avenant-Oldewage 2006; Van As & Basson 1984;). The infection of *L. aeneus* with *A. huronensis* has also only recently been recorded, supporting Dos Santos & Avenant-Oldewage (2022) who speculated that a recent introduction of carp to the Vaal River system may have taken place. It is likely that *G. sprostonae* infected *L. aeneus* through host-switching from non-native host species that are present in the Vaal River such as *C. auratus*, *C. carpio* and *C. idella*.

Comparing morphometry of *G. sprostonae* presented here to that from previous studies (Table 2), differences were observed. The body length and width of specimens from the present study and all previous studies overlapped, excluding Abdullah (2013). The total and shaft length of the hamuli correlated with those in other studies, while the root length overlapped with that of Ogawa & Egusa (1978) but was longer than in all the other studies. Only Barzegar *et al.* (2018) measured the hamulus aperture angle, which was larger than in the current study. The hamulus point length was shorter in Ling (1962) and Pugachev *et al.* (2009) and overlapped with measurements presented by Mattheis & Glaser (1970), Ogawa & Egusa (1978), Barzegar *et al.* (2018), and Daghigh Roohi *et al.* (2019). The hamulus proximal and distal shaft width, inner curve and aperture length, inner aperture angle, and point curve angle are presented for the first time for *G. sprostonae* in the present study. The total length of the ventral bar matched that of Ogawa & Egusa (1978), Barzegar *et al.* (2018) and Daghigh Roohi *et al.* (2019). The ventral bar of specimens in the present study corresponded with all previous

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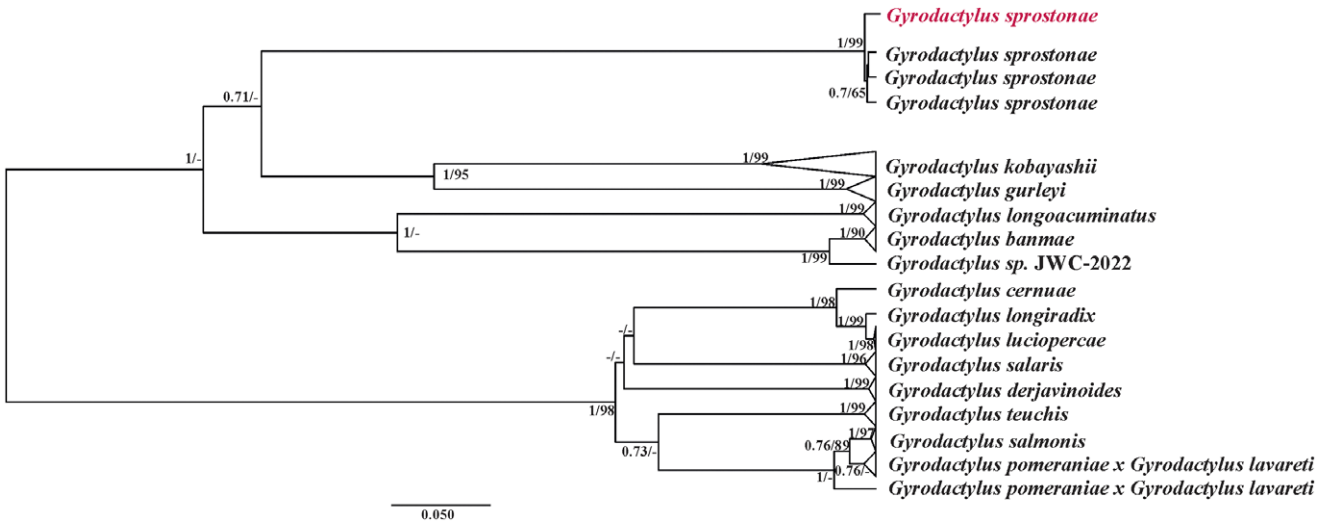

Figure 5. Combined maximum likelihood (ML) and Bayesian inference (BI) phylogenetic topology based on ITS rDNA analysis of *Gyrodactylus sprostonae* Ling, 1962 and selected *Gyrodactylus* species based on BI. Statistical support for respective methods is indicated at branch nodes (BI/ML); nodes with less than 50% support are indicated with dashes. Data generated in the present study are in pink.

studies but was wider than measurements in Ling (1962). Its median length was in range of other studies except that of Pugachev *et al.* (2009). Only four previous studies measured the ventral bar membrane length (Egusa 1978; ; Daghigh Roohi *et al.* 2019; Mattheis & Glaser 1970; Ogawa & Pugachev *et al.* 2009). The membrane length agreed with that by Ogawa & Egusa (1978) and Daghigh Roohi *et al.* (2019) but was shorter than those of Mattheis & Glaser (1970) and Pugachev *et al.* (2009). All specimens from the current and previous studies lack a ventral bar process, except for those studied by Ogawa & Egusa (1978). The dorsal bar length corroborated measurements from previous studies, except for those of Bykhovskaya-Pavlovskaya *et al.* (1964). The dorsal bar width overlapped with all previous studies, except for those studied by Barzegar *et al.* (2018). Four previous studies presented measurements for the shaft of the marginal hook, which was shorter in Ling (1962) and overlapped with all previous studies. The aperture and sickle toe length of the marginal hooks are measured for the first time in this study.

Illustrations of the hamuli and dorsal bar were similar across all compared studies (Figure 3). However, there was variation in the morphology of the ventral bar. This sclerite was either excluded (Daghigh Roohi *et al.* 2019), not drawn in detail (Abdullah 2013; Bykhovskaya-Pavlovskaya *et al.* 1964; Ling 1962,), or shown to have a V-shaped (Pugachev *et al.* 2009) or U-shaped membrane (Barzegar *et al.* 2018; Mattheis & Glaser 1970; Ogawa & Egusa 1978). The ventral bar membrane from the present study is U-shaped with a narrow depression at the centre. This study and all previous studies, except for Ogawa & Egusa (1978), agree that the horizontal rod of the ventral bar lack processes. The current study is the first to present a detailed illustration of the ventral bar, exposing a central V-shaped spike on the ventral side of this sclerite, following study with SEM. There was great variation in the comparison of the marginal hook sickle illustrations, as only five studies had a line drawing similar to that in the present study. This variation in illustrations is likely due to the sclerite morphology being altered by coverslip pressure or not flattened enough during LM preparation. It could also result from user error or incorrect identifications.

Using SEM for point-to-point haptoral sclerite measurements proved effective, as eight out of 24 measurements had statistically significant differences with LM measurements. This may be due to the technique allowing for the study of isolated sclerites, preventing alterations that could result from coverslip pressure in LM preparation as proposed by Mo & Appleby (1990) and Shinn *et al.* (1993). This isolation of haptoral sclerites and examination using SEM has successfully been applied in several monogenean studies (e.g., Dos Santos *et al.* 2019a & b; Dos Santos & Avenant-Oldewage 2015; Paladini *et al.* 2011; Shinn *et al.* 1993; Tu *et al.* 2015). It is likely that this technique is more accurate than LM, but it is not always comparable to previous studies that used only LM. As all previous studies used LM for morphological analysis of *G. sprostonae* (Abdullah 2013; Barzega *et al.* 2018; Bykhovskaya-Pavlovskaya *et al.* 1964; Daghigh Roohi *et al.* 2019; Ling 1962; Mattheis & Glaser 1970; Ogawa & Egusa 1978; Pugachev *et al.* 2009), the morphology of the ventral bar was either not detailed or excluded.

The elusive MCO was only presented in three studies (Ling 1962; Ogawa & Egusa 1978; Pugachev *et al.* 2009). The number of spines on the MCO corroborated the reports of Ogawa & Egusa (1978) and Pugachev *et al.* (2009), who counted six small and one large spine, but it is in contrast with the description by Ling (1962), who counted eight small, zigzagged spines and one large spine. It is suspected that the variation in the MCO may be due to the studied specimens being at different developmental stages, or that some of the soft tissue was misinterpreted for additional spines, as the number of small spines was consistent in all other studies. Even different preparation or microscopy techniques could produce different results. Several attempts to study the isolated MCO using SEM were unsuccessful here, as it could not be located after digestion.

Molecular characterization showed that specimens from the current study were identical or showed low intraspecific variability to available *G. sprostonae* ITS rDNA data, supporting the morphological analysis. The three *G. sprostonae* sequences that were compared to the haplotype from the current study are from different hosts and geographic regions in the northern hemisphere. Two of these sequences (KP295469 and AY278044) are identical to the haplotype from the Vaal River. They were collected from *C. auratus* in central China (unpublished study) and Poland (Ziętara & Lumme 2004), respectively. The third sequence (KT346368) was

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slightly different from the rest (0.01%; 7 bp), but the specimen for this sequence was collected from a different host, *Hypophthalmichthys nobilis* (Richardson, 1845), in western China (unpublished study). The genetic similarity between the sequences from *C. auratus* and those from the present study could support that *G. sprostonae* was co-introduced to the Vaal River alongside *C. auratus.* Phylogenetic topologies showed low support for the split node separating *G. sprostonae* sequences and the *G. kobayashii* and *G. gurleyi* cluster. These species parasitise similar hosts and have closely comparable morphological traits, thus supporting their phylogenetic proximity.

Labeobarbus aeneus is listed as a species of "least concern" by the International Union for Conservation of Nature (IUCN). It is endemic and widely distributed across the Orange-Vaal River system in South Africa but has been locally translocated through inter-basin water transfer schemes and for angling purposes (Skelton 2001). As a result, it is now present in the Limpopo and Olifants Rivers and several rivers of the Cape, such as Kei, Great Fish, Gourits, Sundays, and Kariega (Skelton 2001). Considering the loose host specificity of *G. sprostonae*, it is possible that this parasite may soon infect other indigenous hosts in the Orange-Vaal River system such as *Labeobarbus kimberleyensis* (Gilchrist & Thompson, 1913). The latter species is listed as near threatened under criteria B2b (ii.iii.v) by the IUCN. The prevalence of *G. sprostonae,* from the current and previous studies (Abdullah 2013; Daghigh Roohi *et al.* 2019; Ling 1962), is mostly below 35%, with the exception of one instance reported by Ling (1962) where the prevalence was 54.4%. Irrespective of the latter, this parasite has been reported to cause mass mortality of cultured carp in well-managed fisheries (National Fisheries Services, www.gov.uk/environment-agency), and thus may pose a threat to indigenous hosts.

To conclude, this study not only presents additional taxonomic data, a new locality, and host switching to a new host for *G. sprostonae*, but also the first SEM of isolated sclerites of this parasite. Generated morphometric and molecular data contribute to the existing literature about this emerging invasive pathogen. Moreover, generated taxonomic information for this parasite may be utilized in further studies to track the source and spread of its invasive hosts in South African freshwater systems and globally.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X23000202.

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Author contribution. Conceptualization: A.A.O, Q.M.D.S; Investigation: M. M:A.A.O., Q.M.D.S. Methodology: M.M, Q.M.D.S; Supervision: A.A.O, Q.M.D. S; Writing – review & editing: M.M, Q.M.D.S, A.A.O; Writing – original draft: M.M, Q.M.D.S, A.A.O; Formal analysis: M.M, Q.M.D.S, A.A.O.

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Competing interest. All authors declare that they have no conflict of interest.

Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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