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Morphological and molecular description of *Allocreadium apokryfi* sp. n. (Digenea: Allocreadiidae) from native *Labeobarbus aeneus* (Cyprinidae) in South Africa, including notes on its biology, evolutionary history and an updated key of African *Allocreadium*

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Abstract: Adult trematodes of *Allocreadium* Looss, 1900 (Digenea) infect the intestine of mostly freshwater fishes in Asia, Europe, Africa and the Americas. During routine parasitological surveys in the Vaal River system, adult trematodes were collected from the intestine of smallmouth yellowfish, *Labeobarbus aeneus* (Burchell). The trematodes were confirmed to represent a member of *Allocreadium* and did not match any existing taxon. Therefore, they are described as a new species, *Allocreadium apokryfi* sp. n. The morphology of the new species most closely resembles that of *Allocreadium aswanense* El-Naffar, Saoud et Hassan, 1984, but it differs from it by having a bipartite internal seminal vesicle, wider eggs, a shorter intertesticular distance, an intestinal bifurcation at the ventral sucker level, a ventral sucker that is larger than the oral sucker, and a genital pore near the intestinal bifurcation or the ventral sucker. The surface topology of the new species is notably different from that of other allocreadiids. Papillae were observed in the ventral sucker and surrounding both ventral and oral suckers, but the number and arrangement of the latter were not consistent among specimens. The protruding cirrus of *A. apokryfi* sp. n. was described using SEM and is the first such observation for the genus. Genetic characterisation showed that the new species was clearly distinct from other *Allocreadium* spp. using both 18S (nucleotide difference 1.3–9.1%) and 28S (4.7–6.5%) rDNA, forming a well-supported clade in *Allocreadium*. The presence of *A. apokryfi* sp. n. in a well-studied river is unexpected, and considering the diet of its host and the scarcity of *Allocreadium* in Africa, the possible biology of this species is discussed herein.

Key words: Smallmouth yellowfish, endoparasitic helminths, Trematoda, Africa, 28S rDNA, 18S rDNA, SEM, taxonomic key.

The trematode genus *Allocreadium* Looss, 1900 includes small to medium-sized parasites inhabiting the digestive tract of a remarkable range of mostly freshwater fish families (Caira and Bogéa 2005). Adults have an elongated, dorsoventrally flattened body, well-developed pharynx and suckers, testes in tandem, vitellarium extending from the forebody at various levels to the posterior end of the body, with vitelline follicles in two lateral fields anteriorly but confluent posteriorly (Caira and Bogéa 2005).

The genus *Allocreadium* has been speculated to have originated in southern Asia, spread into Eurasia, then North America, and to some extent into Africa, with African species having affinities to Indian taxa (Thomas 1957, Manter 1963). Recently, species have also been described from South America (Shimazu et al. 2000, Flores et al. 2004). All species of the genus are strictly from freshwater environments (Manter 1963, El-Naffar 1986), and no species have been recorded from more than one continent. This indicates that the evolution and spread of species of this genus may be useful in indicating intercontinental connections (Manter 1963). Delineation among species of the genus is based on adult morphology (i.e., sucker size, ovary position, vitellarium distribution, oesophagus length, pharynx size, genital pore position, extent of intertesticular space, and host range) (Thomas 1957, Saoud et al. 1974).

Systematics of the genus has been problematic due to taxonomy being based predominantly on morphometry and a lack of genetic studies (Vainutis 2020). It would appear that a high level of intraspecific variability exists among species of this genus, which has resulted in many species being relegated to synonymy (see Peters 1957, Kakaji 1969). Thomas (1957) accepted 30 species, whereas

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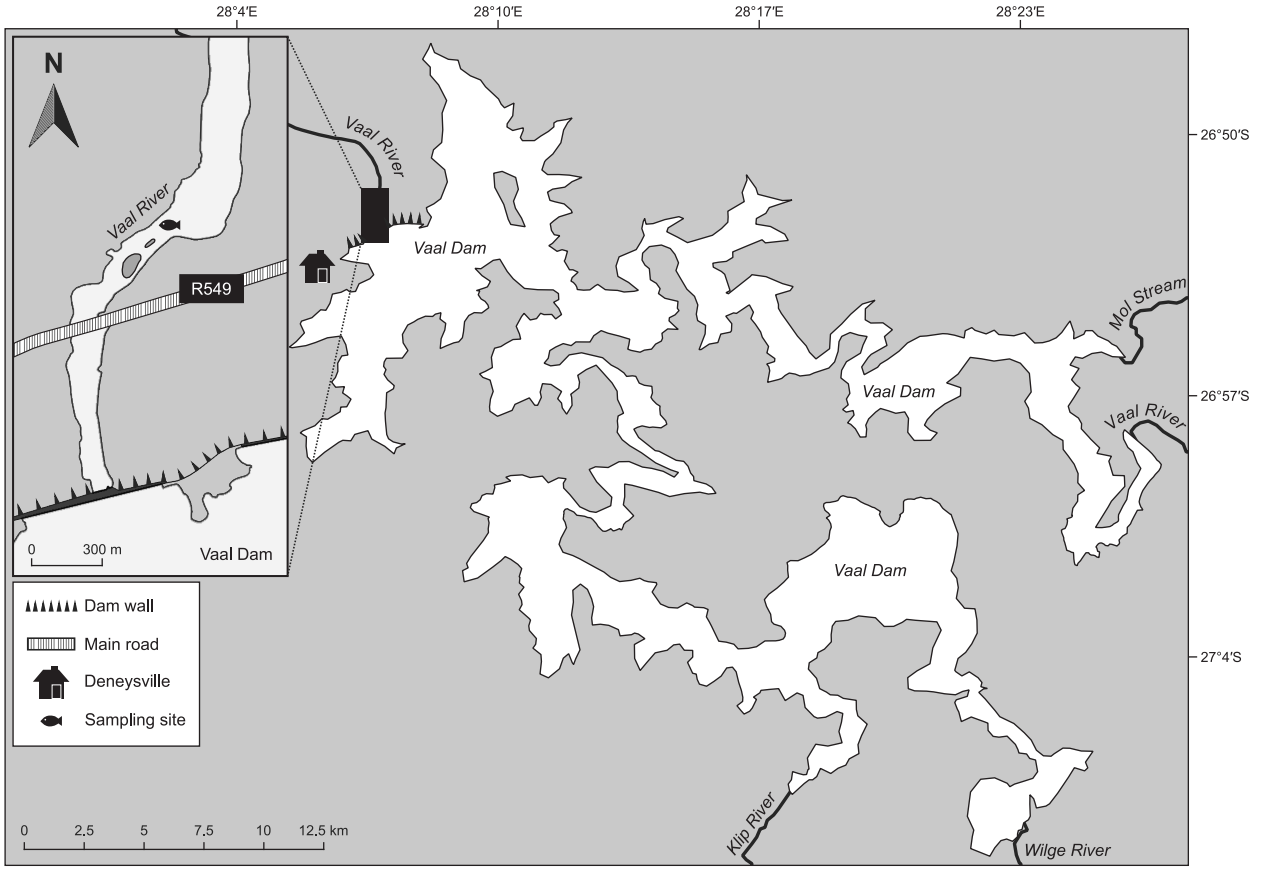

Fig. 1. Map of the sample collection site in the Vaal River below the Vaal Dam, South Africa. Insert shows the specific sampling site for *Labeobarbus aeneus* (Burchell) in the Vaal River.

Peters (1957) in a review based on morphological features of cercariae retained 16 of the 31 species described at the time. In contrast, Saoud et al. (1974) accepted 45 species. Currently, the literature on species of the genus *Allocreadium* reveals large species diversity (e.g. WoRMS 2020), mostly described from Asia, Europe, Africa and the Americas (Vainutis 2020).

Eight species were described from Africa: *Allocreadium voltanum* Thomas, 1957; *Allocreadium indistinctum* Baer, 1959; *Allocreadium mazoense* Beverley-Burton, 1962; *Allocreadium ghanense* Fischthal et Thomas, 1972; *Allocreadium engraulicypridis* Khalil et Thurston, 1973; *Allocreadium sudanense* Saoud, Abdel-Hamd et Ibrahim, 1974; *Allocreadium aswanense* El-Naffar, Saoud et Hassan, 1984; and *Allocreadium bynni* El-Naffar, 1986. These species have been described from hosts in the families Cyprinidae, Alestidae, Clariidae and Mochokidae (Thomas 1957, Baer 1959, Beverley-Burton 1962, Fischthal and Thomas 1972, Khalil and Thurston 1973, Saoud et al. 1974, El-Naffar et al. 1984, El-Naffar 1986). However, while Asian species of the genus are frequently encountered in many regions (Gao et al. 2008), they seem to be quite scarce in Africa (Mbahinzireki 1987). This is supported by the large gaps between some of the most recent records of *Allocreadium* in Africa in 1987 and 2010 (Mbahinzireki 1987, Mwita and Nkwengulila 2010), with the current record more than a decade later.

In the current study, digeneans belonging to *Allocreadium* were collected from the intestine of *Labeobarbus aeneus* (Burchell) in the Vaal River, South Africa. Trematodes of this group have not been recorded from this host nor locality previously, and were studied using an integrative taxonomic approach involving morphological data (light and scanning electron microscopy) in combination with DNA characterisation. The parasites showed morphometric and molecular traits that set them apart from congeneric taxa described in Africa, as well as in the rest of the world, and as such were designated as a new species. A key for African species of *Allocreadium* is provided, based on the revised and updated key by El-Naffar (1986).

MATERIALS AND METHODS

Sample collection

Smallmouth yellowfish, *Labeobarbus aeneus*, were collected in November 2019 using gill nets and electrofishing approximately 1.5 km downstream of the wall of the Vaal Dam (Fig. 1) in the Vaal River (26.871111°S, 28.118611°E). Fish were euthanised by severing the spinal cord posterior to the skull, the intestinal tract was removed and examined for the presence of parasites. Intestines were opened carefully in saline with fine forceps using a dissection microscope and trematodes removed using a 000 Camel's hair paintbrush. For morphological study, trematodes were fixed in warm 10% neutral buffered formalin or warm 70% etha-

nol using a temporary mount (coverslip suspended on dollops of petroleum jelly) to prevent specimens from folding. Additionally, some specimens were fixed in 70% ethanol for scanning electron microscopy (SEM) study or 96% ethanol for molecular study at room temperature. Infection parameters were calculated according to Bush et al. (1997). Fish were collected and sacrificed in accordance with permit CPE2-0118 from the Nature Conservation of Gauteng Province Government, South Africa and ethics reference 2016-5-03 from the University of Johannesburg. All institutional and national guidelines for the collection and study of fish were followed.

Light microscopy

For whole mount preparations, ovigerous adult specimens (n = 19) were stained with acetocarmine, differentiated in 0.5% acid alcohol, dehydrated in a graded ethanol series, cleared in beechwood creosote, and mounted in Canada balsam (Thatcher 2006). Photomicrographs and measurements were obtained with an Olympus BX53 compound microscope and Olympus Soft Imaging Solutions (Olympus, Münster, Germany), then used for illustrations with Corel DRAW® Graphics Suite X6 software (Corel Corporation, Ottawa, Canada). All measurements pertain to specimens fixed using a temporary mount and are given in micrometres unless otherwise stated, given as the range followed by the mean in parentheses. All measurements (including standard deviation) are given alongside those of all other African *Allocreadium* spp. as per their original descriptions in Table 1.

Scanning electron microscopy

Seven whole specimens fixed in 70% ethanol were prepared for SEM study by dehydration through a graded ethanol series, followed by a graded series of hexamethyldisilazane (Merck, Darmstadt, Germany) (Nation 1983, Dos Santos et al. 2015). Specimens were dried in a Sanpla dry keeper desiccator cabinet (Kita-Ku, Osaka, Japan) and coated with gold using an Emscope SC500 sputter coater (Quorum Technologies, Newhaven, UK). A Vega 3 LMH scanning electron microscope (Tescan, Brno, Czech Republic) was used to study the specimens at 5–6 kV. Microdissection of dried specimens was also used to study eggs *in utero*.

Genetic study

Genomic DNA was extracted from five whole trematodes using a NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The D1–D3 region of 28S rDNA was amplified with primers dig12 (5' – AAG CAT ATC ACT AAG CGG – 3') (Tkach et al. 2003) and 1500R (5' – GCT ATC CTG AGG GAA ACT TCG – 3') (Olson et al. 2003, Tkach et al. 2003), while 18S rDNA was amplified with JLR24 (5' – CGG AAT TCG CTA GAG GTG AAA TTC TTG G – 3') and JLR25 (5' – CCG AAT TCC GCA GGT TCA CCT ACG G – 3') (Campos et al. 1998). PCR conditions were adjusted from Tkach et al. (2003) for 28S rDNA (initial denaturation 5 minutes, final elongation 10 minute and annealing temperature 52 °C), and Mwita and Nkwengulila (2010) for 18S rDNA (annealing temperature 50 °C). Successful amplification was verified using a 1% agarose gel, impregnated with GelRed™ (Biotium Inc., Fremont City, California), and visualised with a UV transilluminator. Sequencing was done according to Avenant-Oldewage et al. (2014) using PCR primers in both directions.

Generated sequence data were aligned, inspected, edited if necessary, and reads merged using Geneious Prime 2020.2.2 (Kearse et al. 2012). To determine the distinctness of the trematodes, obtained sequences were analysed using BLAST (Altschul et al. 1990) and aligned to all previously published *Allocreadium* 18S and 28S rDNA sequences (and *Palaeorchis* [*Cercariaeum*] *crassus* [Wesenburg-Lund, 1934]) downloaded from GenBank (details in Table 2) using MEGA7 (Kumar et al. 2016) and MAFFT (Katoh et al. 2002, Katoh and Standley 2013). Pairwise distances were estimated by uncorrected *p*-distance with 1,000 bootstrap replicate variance estimation using MEGA7.

Evolutionary history was studied using 28S rDNA, employing both maximum likelihood (ML) and Bayesian inference (BI) methods. For ML analyses, the General Time Reversible model (GTR) (Nei and Kumar 2000) with discrete Gamma distribution (5 categories (+G, parameter = 0.4408)) was selected as the best nucleotide substitution model using MEGA7. This was supported by 1,000 bootstrap replicates. Bayesian inference analyses were performed with BEAST v2.5.0 (Bouckaert et al. 2014) using 10 million Markov chain Monte Carlo (MCMC) generations and the GTR model. Due to the similarity between BI and ML analyses, a single topology based on BI analyses is given with both ML and BI support indicated at respective nodes and rooted with both *Acrolichanus auriculatus* (Wedl, 1858) (MN750364) and *Crepidostomum oschmarini* Zhokhov et Pugacheva, 1998 (MH159994) as outgroup.

RESULTS

***Allocreadium apokryfi* sp. n.** Figs. 2–5

ZooBank number for species:
urn:lsid:zoobank.org:act: 97514610-93E3-473E-900F-BBB35068F5A8

Description (based on 19 whole mounts of gravid trematodes and 7 whole trematodes examined with SEM). Body elongate 1.75–3.33 mm (2.74 mm) long, 622–1.030 (846) wide, rounded anteriorly, tapering towards posterior, widest at level of ventral sucker (Figs. 2A, 3A). Both suckers well-developed. Oral sucker anterior, ventrally subterminal, subspherical, 212–338 (256) long, 146–355 (254) wide (Figs. 2A, 3B). Ventral sucker subspherical, wider than long, 247–349 (299) long, 254–350 (310) wide (Figs. 2A, 3C). Tegumental papillae present in both oral and ventral suckers. Papillae at oral sucker present inside oral opening, around rim of sucker (in two rows), and more distant from opening (Fig. 3B). The number and placement of papillae inconsistent among specimens. Sensory openings present on anterior rim of oral sucker (Fig. 3B and insert). Ventral sucker with 4 to 6 tongue-like papillae on inner lip, with additional papillae present around ventral sucker of some specimens (Fig. 3C). Tegument aspinous (Figs. 3, 4), transverse striations observed on some specimens, generally more striking in ventral part of forebody (Figs. 3A, 4A), dissipating posteriorly (Figs. 3A, 4B), absent on dorsal body surface (Fig. 4D). Cobblestone-like protrusions intermittently present at high magnification (Fig. 4C). Eye spot pigment not observed. Oral sucker unarmed; mouth opens directly to pharynx (Fig. 3B). Prepharynx absent.

Table 1. Measurements (in µm unless otherwise stated) of adult *Allocreadium apokryfi* sp. n. (values in bold) and all other species of *Allocreadium* Looss, 1900 described from Africa.

Species	*A. voltanum*	*A. indistinctum*	*A. mazoense*	*A. ghanense*	*A. engraulicypridis*	*A. sudanense*	*A. aswanense*	*A. bynni*	***Allocreadium* n. sp.**
Locality	West Africa	Congo	Zimbabwe	Ghana	Uganda	Sudan	Egypt	Egypt	**South Africa**
Reference	Thomas (1957)	Baer (1959)	Beverley-Burton (1962)	Fischthal and Thomas (1972)	Khalil and Thurston (1973)	Saoud et al. (1974)	El-Naffar et al. (1984)	El-Naffar (1986)	**Present study**
Body length (mm)	4.51–7.12	0.875–1.04	1.69–2.62	2.88–3.26	2.04 (2.32)[b]	2.89 –3.71	2.24–4.56	2.20–3.40	**1.75–3.33 (2.74±0.37)**
Body width	1,440–1,710	390–455	650–980	900–940	880 (1,020)[b]	920–1,330	880–1,330	990–1200	**622–1,030 (846±118)**
Forebody length	—	—	—	1,110–1,155	—	—	—	640–810	**382–995 (697±140)**
Hindbody length (mm)	—	—	—	1.35–1.68	—	—	—	1.15–1.76	**1.13–2.35 (1.74±0.28)**
Prepharynx length	—	—	—	26–50	—	—	—	—	**—**
Oesophagus length	—	—	270	95–135	—	—	330–560	66–92	**114–724 (488±148)**
Pharynx length	150–220	105	120–150	200–220	130 (140)[b]	160–210	110–200	95–125	**100–166 (130±21)**
Pharynx width	140–190	91	90–160	150	170 (170)[b]	140–160	140–170	98–129	**114–174 (143±18)**
Oral sucker length	300–380	229–250	190–280	400–420	300 (330)[b]	320–390	260–380	370–400	**212–338 (256±37)**
Oral sucker width	370–410	—	220–300	410–420	300 (330)[b]	360–380	330–390	400–470	**146–355 (254±43)**
Ventral sucker length	400–530	274–320	230–320	420–460	450	400–460	320–390	380–410	**247–349 (299±29)**
Ventral sucker width	450–600	69–90	260–360	480–520	410	360–470	290–390	440–480	**254–350 (310±31)**
Ovary length	200–250	114–123	210–250	120–150	180 (190)[b]	300–320	190–310	150–230	**152–250 (199±26)**
Ovary width	190–270	114–123	190–270	150–160	120 (140)[b]	—	150–250	130–210	**161–252 (201±26)**
Anterior testis length	400–500	183	220–300	300–330	230 (290)[b]	470–630	330–480	340–410	**228–438 (308±52)**
Anterior testis width	650–780	142-160	140–300	320	170 (210)[b]	480–550	330–420	330–440	**201–379 (296±48)**
Posterior testis length	500–570	183	270–330	350–450	220 (330)[b]	420–480	330–440	340–520	**241–592 (364±76)**
Posterior testis width	600–670	142–160	170–320	250–270	180 (260)[b]	470–490	360–440	340–480	**201–382 (302±55)**
Egg length	82.5–85	100–110	88–95	90–101	104	82–94	70–80	84–90	**83–92 (87±3)**
Egg width	60	55–64	56–60	55–64	61	53–59	30–40	55–62	**60–74 (66±4)**
Seminal receptacle length	330–360	—	—	230–330	112	270–290	160–280	230–280	**86–256 (173±51)**
Seminal receptacle width	110–180	—	—	120–210	85	180–190	80–120	90–100	**79–160 (113±25)**
Cirrus-sac length	220–310	274–320	260–300	360–450	154	510–540	440–670	570–740	**480–754 (602±72)**
Cirrus-sac width	180–270	69–91	160–190	120–130	112	290–330	110–240	270–330	**137–261 (187±28)**
Previtelline distance (mm)	—	—	—	—	—	—	—	—	**0.72–1.72 (1.19±0.25)**
Vitelline field length (mm)	—	—	—	—	—	—	—	—	**0.91–1.88 (1.56±0.23)**
Vitelline follicle length	—	—	—	—	—	130	—	—	**89–152 (121±19)**
Vitelline follicle width	—	—	—	—	—	130	—	—	**78–129 (95±15)**
Ventral sucker to ovary distance	—	—	—	0–27	—	—	—	—	**0–580 (114±141)**
Ovary to anterior testis distance	—	—	—	0–60	—	—	—	—	**10–402 (194±100)**
Intertesticular distance	0	0	0	0	0	0	30–170	0	**0–82 (26±26)**
Post-testicular distance	—	—	—	410–640	—	—	—	—	**481–922 (708±102)**
Postvitelline distance	—	—	—	—	—	—	—	—	**46–122 (87±25)**
Oral sucker length (%)[a]	—	—	—	—	—	—	—	—	**7–13 (9±2)**
Ventral sucker length (%)[a]	—	—	—	—	—	—	—	—	**8–15 (11±2)**
Oral to ventral sucker-length ratio	—	—	—	1 : 1.05–1.10	—	—	—	—	**0.70–1.12 (0.85±0.14)**
Oral to ventral sucker-width ratio	—	—	—	1 : 1.12–1.24	—	—	—	—	**0.44–1.1 (0.82±0.13)**
Forebody (%)[a]	—	—	—	—	—	—	—	—	**19–32 (25±3)**
Hindbody (%)[a]	—	—	—	—	—	—	—	—	**51–71 (63±4)**
Previtelline distance (%)[a]	—	—	—	—	—	—	—	—	**37–59 (43±6)**
Ventral sucker to ovary dist. (%)[a]	—	—	—	—	—	—	—	—	**0–17 (4±4)**
Cirrus-sac length (%)[a]	—	—	—	—	—	—	—	—	**16–26 (21±3)**
Intertesticular distance (%)[a]	—	—	—	—	—	—	—	—	**0–3 (0.9±0.9)**
Post-testicular distance (%)[a]	—	—	—	—	—	—	—	—	**22–30 (26±2)**
Postvitelline distance (%)[a]	—	—	—	—	—	—	—	—	**1.6–6.8 (3.3±1.3)**
Ovary length (%)[a]	—	—	—	—	—	—	—	—	**5.9–10 (7.4±1.0)**
Vitelline field length (%)[a]	—	—	—	—	—	—	—	—	**50–63 (57±4)**

[a] – percentage relative to total body length of specimen; [b] – measurements in parentheses for live specimens.

Pharynx muscular, subspherical, smaller than oral sucker, 100–166 (130) long, 114–174 (143) wide, leading to long slightly curved oesophagus, 114–724 (488) long. Intestinal bifurcation at level of ventral sucker; intestinal caeca almost uniform in diameter throughout entire length, running into hindbody, terminate close to posterior end of body.

Testes two, oval in dorsoventral view, smooth, tandem, intercaecal, post-equatorial, separated by intertesticular distance 0–82 (26); anterior testis 228–438 (296) long, 201–379 (364) wide; posterior testis larger, 241–592 (364) long, 201–382 (302) wide; post-testicular distance 481–922 (708) (Figs. 2A). Cirrus-sac 480–754 (602) long, 137–261 (187) wide, retort-shaped, in forebody, situated near intestinal bifurcation, distal part of cirrus-sac reaching anterior to mid-level of ventral sucker, mostly medial, occasionally slightly overlapping ventral sucker, intercaecal, enclosing bipartite internal seminal vesicle, anterior part of cylindrical shape, posterior part large, looped, connecting to vas deferens (Fig. 2A,B). Protruding cirrus unarmed, tip blunt and flattened, lace-like texture on surface of protruded cirrus (Fig. 5A). Pars prostatica elliptical; prostatic gland cells spherical, thin-walled vesicular cells with nuclei. Ejaculatory duct forms widened part prior to opening into genital atrium. Vas deferens bifurcates at level of ovary into two vasa efferentia that connect to respective testes (Fig. 2C).

Ovary approximately oval, smooth in outline, generally smaller than testes, 152–250 (199) long, 161–252 (201) wide. Oviduct runs from anterior edge of ovary, connecting

Table 2. List of allocreadiid trematodes included in the molecular analyses with *Allocreadium apokryfi* sp. n.

Species	Host	Accession no.	Reference	Locality
28S rDNA				
Allocreadium neotenicum Peters, 1957	*Hydroporus rufifrons* (beetle)	JX983204	Bray et al. (2012)	Lake District (South), Cumbria, United Kingdom
Allocreadium mazoense Beverly-Burton, 1962	*Clarias gariepinus* (fish)	DQ813450	Mwita and Nkwengulila (2010)	Lake Victoria (South), Tanzania
18S rDNA				
Allocreadium gotoi (Hasegawa et Ozaki, 1926)	*Misgurnus anguillicaudatus* (fish)	LC215274	Shimazu (2017)	Midori, Iiyama City, Nagano Prefecture, Japan
Allocreadium handiai Pande, 1937	*Mystus tengara* (fish)	KX344072*	Chaudhary et al. (2016)	Fish market, Hastinapur and Meerut, India
Allocreadium hemibarbi Roitman, 1963	*Hemibarbus labeo* (fish)	MK211220–3	Vainutis (2020)	Russia, Khankaisky district, Komissarovka River
Allocreadium isoporum (Looss, 1894)	Ba*rbatula bar*batula (fish)	MH143102	Petkevičiūtė et al. (2018)	River Il'd, upper Volga River basin, Khankaisky district, Russia
	Alburnus alburnus (fish)	GU462125–6	Petkevičiūtė et al. (2010)	Lake Oster, Karelia, Russia
Allocreadium khankaiensis Vainutis, 2020	*Rhynchocypris oxycephalus* (fish)	MK211211–2	Vainutis (2020)	Poperechny spring, Komissarovka River basin, Russia
		MK211213–9	Vainutis (2020)	Komissarovka River, Khankaisky district, Russia
Allocreadium lobatum Wallin, 1909	*Semotilus atromaculatus* (fish)	DQ029327	Platta and Choudhury (2006)	Rose Isle Creek, Manitoba, Canada
	Semotilus corporalis (fish)	EF032693	Curran et al. (2006)	Moosehead Lake, Maine, USA
Allocreadium neotenicum Peters, 1957	*Hydroporus rufifrons* (beetle)	JX977132	Bray et al. (2012)	Lake District (South), Cumbria, United Kingdom
	Oreodytes sanmarkii (beetle)	KY513132–3	Soldánová et al. (2017)	Lake Takvatn, Troms County, Norway
	Pisidium casertanum (bivalve)	MH143103	Petkevičiūtė et al. 2018)	River Burulcha, Crimea, Ukraine
		MH143104	Petkevičiūtė et al. (2018)	Lake Takvatn, Norway
	Pisidium sp. (bivalve)	MH143105	Petkevičiūtė et al. (2018)	Lake Nordersjoen, Norway
Allocreadium sp.	*Phoxinus phoxinus* (fish)	MK211209–10	Vainutis (2020)	River Nezhinka tributary, Razdolnaya River basin, Russia
Allocreadium sp.	*Carassius carassius* (fish)	MK258685–7	Unpublished	Russia
Allocreadium sp.	*Sphaerium corneum* (bivalve)	GU462121**	Petkevičiūtė et al. (2010)	River Belka, Dnieper River basin, Ukraine
Palaeorchis crassus (Wesenburg-Lund, 1934)	*Pisidium amnicum* (bivalve)	GU462117–20	Petkevičiūtė et al. (2012)	River Žeimena, Lithuania
		JF261141–3	Petkevičiūtė et al. (2012)	Siilaisenpuro River, Finland
		JF261144	Petkevičiūtė et al. (2012)	River Ūla, Lithuania

* Sequence does not fall into the *Allocreadium* ingroup. More closely related to *Haplorchoides* Chen, 1949; ** Noted as *Crepidostomum* sp. 'larva' in Petkevičiūtė et al. (2010).

to seminal receptacle. Vitelline duct joins posterior part of seminal receptacle, vitelline duct enlarged at posterior, running parallel to oviduct, connecting to vitellarium (Fig. 2C). Vitellarium occupies lateral fields, from level of ovary mostly extracaecal, crossing into intercaecal space from level of testes, then confluent in post-testicular region, 46–122 (87) from posterior extremity of body. Vitelline follicles densely clustered, irregular-shaped, 89–152 (121) long, 78–129 (94.6) wide. Seminal receptacle pear-shaped, in median line posterior to ovary, dilated posteriorly, curved anteriorly, 86–256 (173) long, 79–160 (113) wide (Fig. 2A,C). Laurer's canal not clearly visible. Mehlis' gland surrounding ootype, along oviduct, oviduct opens into uterus (Fig. 2C).

Uterus coiled extensively, filling inter- and extracaecal space from mid-anterior testis to ventral sucker, partially overlapping ovary, ascending coils run dextrally to ventral sucker, opening into genital atrium, metraterm on both sides of distal part of uterus approaching genital atrium (Fig. 2B). Genital pore median, anterior to ventral sucker and caecal bifurcation (Figs. 2A, 3A). Eggs oval, operculate (Fig. 5B), shell yellow, often collapsed, 83–92 (87) long, 60–74 (66) wide. Excretory vesicle tubular, short, not reaching posterior testis (Fig. 2A). Excretory pore terminal (Figs. 3A, 4E).

Type host: *Labeobarbus aeneus* (Burchell), smallmouth yellowfish (Cypriniformes: Cyprinidae).

Type locality: Vaal River, downstream (± 1.5 km) of the Vaal Dam wall (26.871111°S, 28.118611°E).

Site of infection: Anterior to middle intestine.

Infection parameters: prevalence 33% (7 of 21 fish infected); intensity 1–15 (mean 8.3); abundance 2.8.

Specimens deposited: Holotype – ovigerous adult specimen deposited at the Iziko South African Museum, Cape Town, South Africa (SAMC-A092079); paratypes: three specimens deposited at the Iziko South African Museum, Cape Town, South Africa (SAMC-A092080–2); three specimens deposited at the Natural History Museum, London, UK (NHMUK 2020.12.16.1–3); and three specimens deposited at the Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic (IPCAS D-831).

Representative DNA sequences: All genetic material produced deposited to GenBank, five sequences for 28S rDNA (MW907591–MW907595) and five sequences for 18S rDNA (MW907958–MW907962).

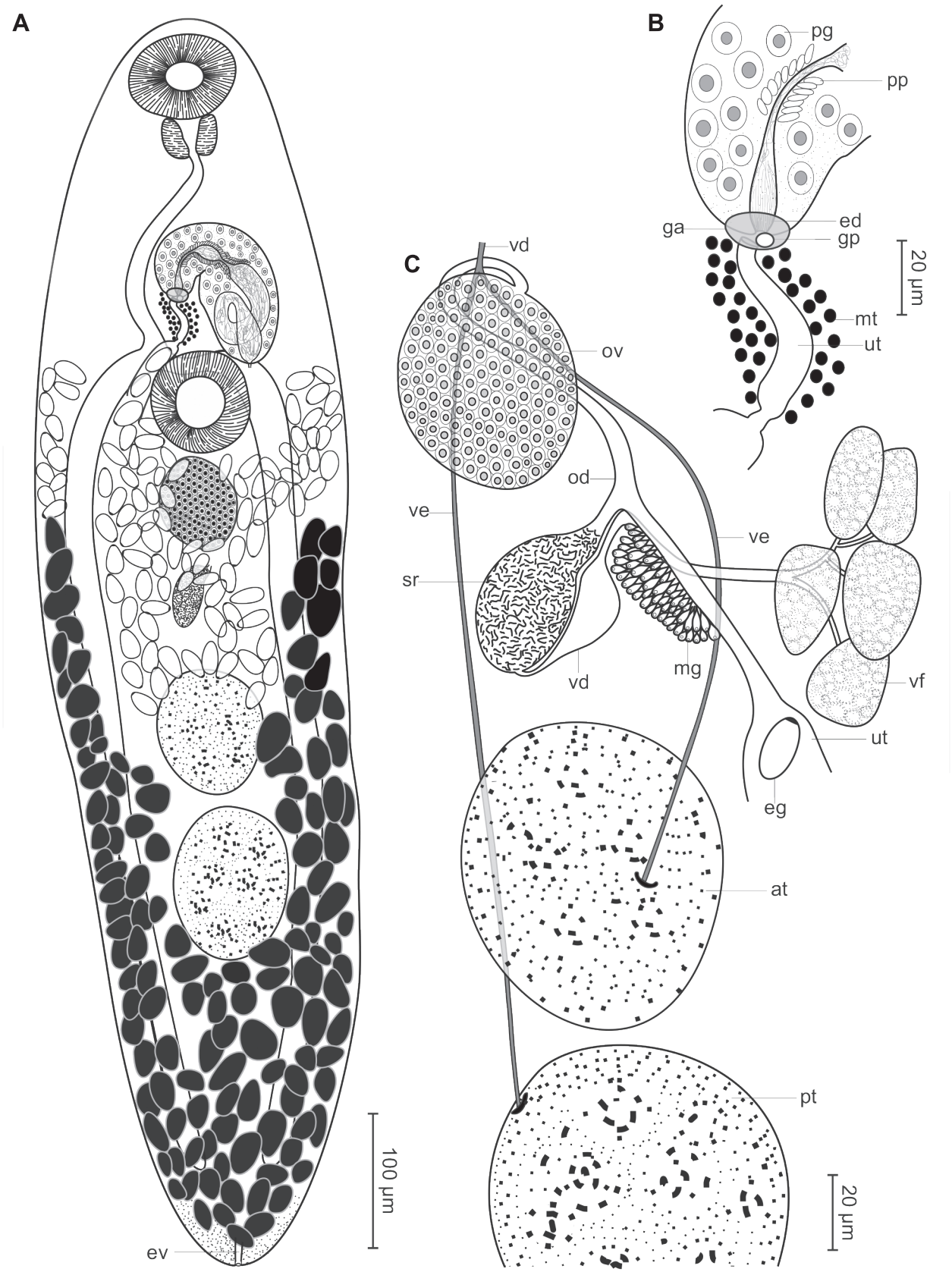

Fig. 2. *Allocreadium apokryfi* sp. n. from *Labeobarbus aeneus* (Burchell) in South Africa. **A** – ventral view of whole specimen; **B** – termination of genitalia at genital pore; **C –** reproductive organs. *Abbreviations*: at – anterior testis; ed – ejaculatory duct; eg – egg; ev – excretory vesicle; ga – genital atrium; gp – genital pore; mg – Mehlis gland; mt – metraterm; od – oviduct; ov – ovary; pg – prostatic gland cell; pp – pars prostatica; pt – posterior testis; sr – seminal receptacle; ut – uterus; vd – vasa deferens; vd – vitelline duct; ve – vas efferentia; vf – vitelline follicle.

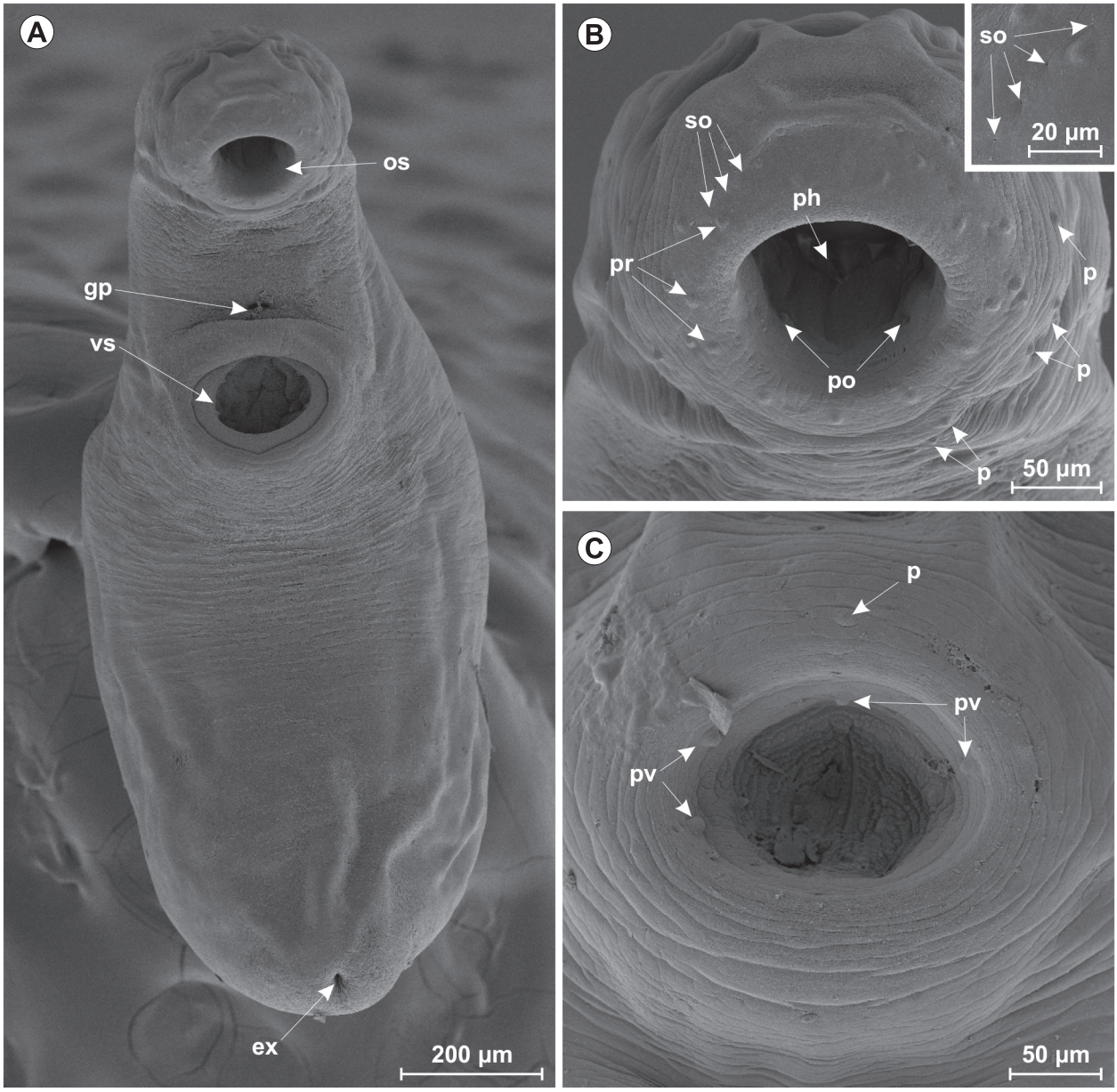

Fig. 3. Scanning electron micrographs of *Allocreadium apokryfi* sp. n. from *Labeobarbus aeneus* (Burchell) in South Africa. **A** – ventral view of whole specimen; **B** – oral sucker (insert showing possible sensory openings); **C** – ventral sucker. *Abbreviations*: ex – excretory pore; gp – genital pore; os – oral sucker; p – papillae; ph – pharynx; po – papillae in oral sucker; pr – papillae on rim of oral sucker; pv – papillae in ventral sucker; so – sensory openings; vs – ventral sucker.

Etymology: The species name is based on the Greek word απόκρυφος (apókryfos; ἀπό [apó, "from"] + κρύπτω [krúptō, "I hide"]), referring to the cryptic nature of the species and that it has remained hidden in a well-studied river for so long.

Remarks

In having an oral sucker without muscular papillae, caeca extending to near the posterior extremity of the body, uterus not extending posteriorly beyond testes, long oesophagus, vitellarium mostly restricted to hindbody, and the ovary not restricted to the anterior third of the body (Caira and Bogéa 2005), specimens of the present study are assigned to the genus *Allocreadium*. *Allocreadium* spp. previously described from Africa (n = 8) are all described based on adult specimens and are similar to *Allocreadium apokryfi* sp. n. in that the ventral sucker is larger than the oral sucker (see Table 1), with the exception of *Allocreadium indistinctum* and *A. aswanense* for which the suckers are nearly equal.

Among African species of *Allocreadium*, *A. apokryfi* sp. n. most closely resembles *A. aswanense*, with both species possessing a vitellarium that extends from the ovary level and the presence of an intertesticular space (El-Naffar et al. 1984). Nevertheless, *A. apokryfi* sp. n. differs from *A. aswanense* by having a bipartite internal seminal vesicle (vs. unipartite), a genital pore near the ventral sucker (vs. pharynx), an intestinal bifurcation at the ventral sucker level (vs. anterior to the ventral sucker), a larger ventral than the oral sucker (vs. suckers equal in size) and wider eggs.

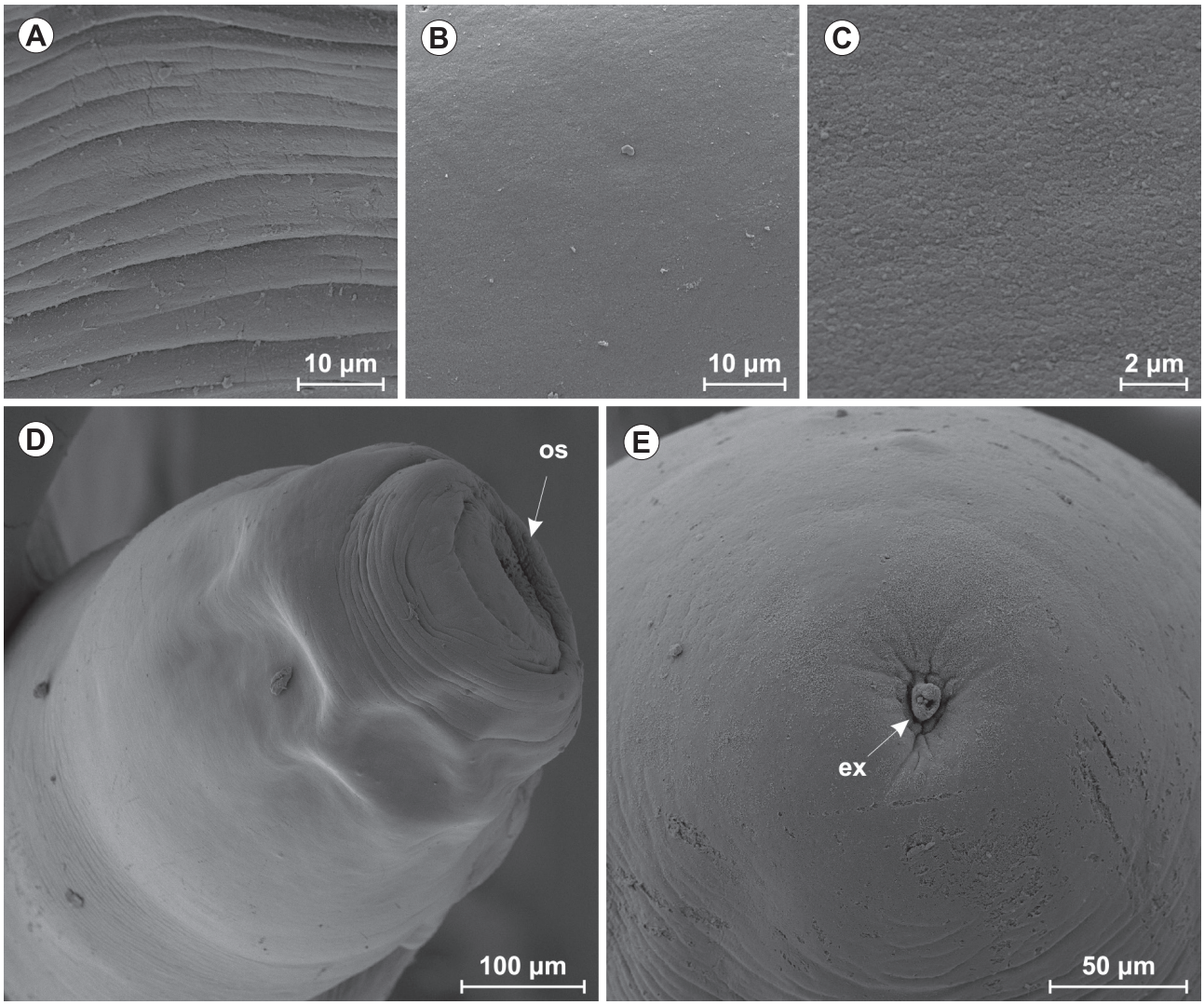

Fig. 4. Scanning electron micrographs of the tegument and posterior extremity of *Allocreadium apokryfi* sp. n. from *Labeobarbus aeneus* (Burchell) in South Africa. **A** – tegumental folds in the forebody; **B** – posterior ventral tegument; **C** – cobblestone-like structure of tegument; **D** – ventral view of the forebody; **E** – posterior extremity of the body. *Abbreviations*: ex – excretory pore; os – oral sucker.

The new species and *A. aswanense* further differ in the type host and locality with *A. apokryfi* sp. n. from *L. aeneus* in South Africa, whereas *A. aswanense* is from the Niger barb, *Labeobarbus bynni* (Forsskål), in Egypt. However, the congeneric nature of the hosts of *A. apokryfi* sp. n. and *A. aswanense* may explain their similarity.

Allocreadium apokryfi sp. n. can be distinguished from the remainder of African species of *Allocreadium* by a dorsally bifurcating oesophagus at the level of the ventral sucker instead of anterior to the ventral sucker (Thomas 1957, Fischthal and Thomas 1972, Khalil and Thurston 1973, Saoud et al. 1974, El-Naffar et al. 1984, El-Naffar 1986). Additionally, both *A. sudanense* and *A. indistinctum* possess overlapped testes (Baer 1959, Saoud et al. 1974), whereas *Allocreadium bynni* has oblique testes (El-Naffar 1986), rather than the presence of an intertesticular space in the new species. *Allocreadium voltanum* is the only African species having lobed testes (Thomas 1957), whereas they are rounded and smooth in the new species. In both *Allocreadium ghanense* and *Allocreadium engraulicypridis*, the vitellarium extends deeply into the forebody to the pharynx level, whereas it extends to the ovary level in *A. apokryfi* sp. n. Furthermore, *A. ghanense* possesses a prepharynx and eye spot pigment granules at the pharynx level (Fischthal and Thomas 1972) whereas they are absent in the new species. The genital pore of *A. engraulicypridis* opens posterior to the caecal bifurcation rather than more anteriorly as seen in the new species. The genital pore position of *Allocreadium mazoense* is similar to that of *A. apokryfi* sp. n. (Beverley-Burton 1962).

The new species differs from the majority of European, Asian and American species of *Allocreadium* in that the vitellarium extends to the ovary level and not deeply into the forebody towards the pharynx, near the caecal bifurcation or ventral sucker (Odhner 1901, Wallin 1909, Mueller and Van Cleave 1932, Rankin 1937, Peters 1957, Odening 1959, Akhmerov 1960, Rai 1962, Rees 1968, Kakaji 1969, Koval 1972, Fischthal and Nasir 1974, Shimazu et al. 2000, Flores et al. 2004, Shimazu 2016, Vainutis 2020). The new species also differs from other Asian and Ameri-

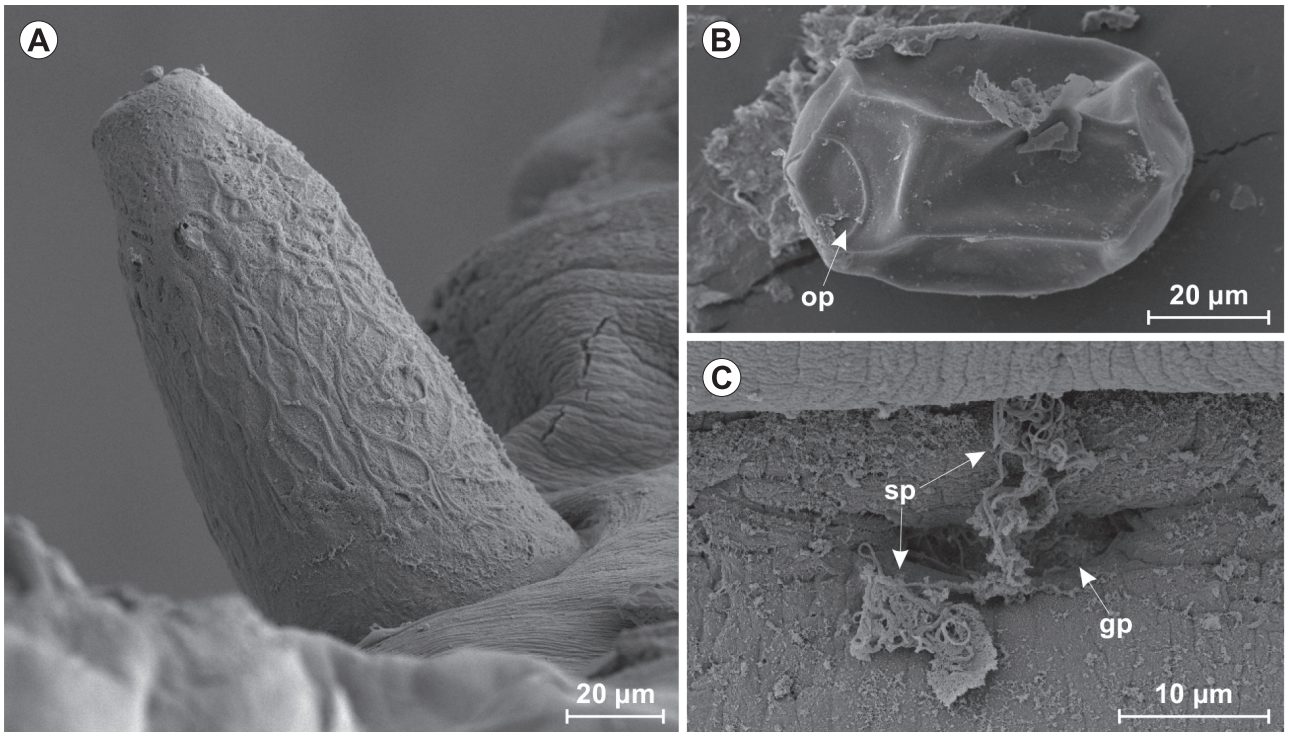

Fig. 5. Scanning electron micrographs of reproductive structures of *Allocreadium apokryfi* sp. n. from *Labeobarbus aeneus* (Burchell) in South Africa. **A** – protruding cirrus; **B** – egg; **C** – genital pore with possible sperm. *Abbreviations*: go – genital pore; op – operculum; sp – sperm.

can species of *Allocreadium* in that the oesophagus bifurcates at the level of the ventral sucker instead of anterior to the ventral sucker (Pande 1937, Gupta 1956, Rai 1962, Agrawal 1964), and that the testes are round and smooth, not lobed (Wallin 1909, Fischthal and Nasir 1974).

Molecular data analyses

Sequence data for both 18S and 28S rDNA fragments were successfully obtained from all specimens of *A. apokryfi* sp. n. analysed (n = 5). Only a single haplotype was observed for each marker from all individuals, indicating no intraspecific variation in these samples for the designated rDNA fragments. BLAST of the obtained 18S rDNA sequence (904 bp) confirmed the similarity of the specimens to species of *Allocreadium*. Generated 18S rDNA was aligned to two 18S sequences for species of *Allocreadium* from GenBank, producing an alignment of seven sequences consisting of 905 bp, with 849 conserved, 55 variable, and 1 parsimony informative sites. Based on *p*-distances of 18S rDNA, *A. apokryfi* sp. n. is 1.2% different from *Allocreadium neotenicum* Peters, 1957 (JX983204), while it was 9.1% from *A. mazoense* (DQ813450).

Using all other rDNA sequence data on species of *Allocreadium* available, 41 28S rDNA sequences for the genus were aligned to that of *A. apokryfi* sp. n. (haplotype of 1,255 bp). The produced alignment contained 46 sequences and consisted of 1,258 bp, with 1,102 conserved, 156 variable, and 149 parsimony informative sites. Evolutionary divergence between sequences based on *p*-distances is presented in Table 3. *Allocreadium apokryfi* sp. n. was most closely related to *Allocreadium hemibarbi* Roitman, 1963 (4.7%, MK211220–MK211223), whereas *Allocreadium lobatum* Wallin, 1909 was the most distant (6.5%, DQ029327). Using only sequence data for specimens identified to species level, intraspecific distances of 0–0.3% were calculated with interspecific distances of 0.3–6.5%.

Based on the BI topology (Fig. 6) based on analysis of the 28S rDNA alignment, sequences for *A. apokryfi* sp. n. form a distinct clade. This clade is well supported by both BI and ML methods, with branching within the clade being negligible. *Allocreadium apokryfi* sp. n. groups sister to most other species of *Allocreadium*, except for unidentified specimens from Russia (MK258685–7; unpublished), which are basal to the other species of the genus. All taxa included form well supported (> 90%) clades supporting their distinctness, except for *A. lobatum* and *A. neotenicum*, in which only BI support is strong enough for the formation of a clade of the first species and ML support is low (> 65%). However, intraspecific nodes for the genus are well supported only by BI analyses, with most ML nodes having low support. Based on the large distances observed between both the 18S and 28S rDNA haplotypes generated in the current study and published sequence data, and the formation of a distinct clade in Fig. 6, *A. apokryfi* sp. n. represents a species not genetically characterised before.

Updated key to the species of *Allocreadium* from African freshwater fishes

The key is based on the rearrangement of those by El-Naffar et al. (1984) and El-Naffar (1986), using the following additional features: pharynx length, level of the

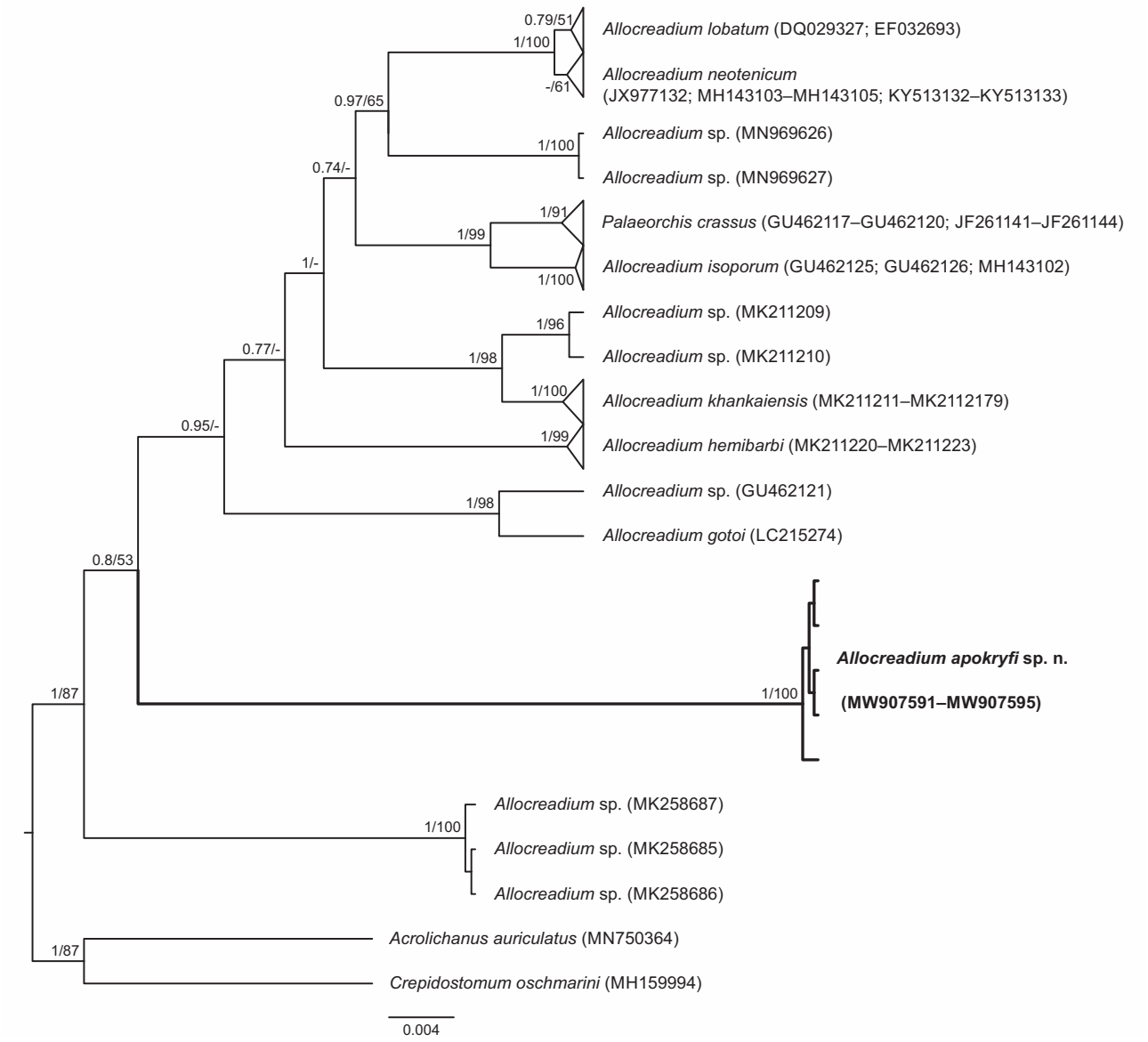

Fig. 6. Topology based on 28S rDNA using Bayesian Inference (BI) approaches indicating the evolutionary history of *Allocreadium apokryfi* sp. n. in relation to other species of *Allocreadium* and *Palaeorchis crassus* (Wesenburg-Lund, 1934), with *Acrolichanus auriculatus* (Wedl, 1858) and *Crepidostomum oschmarini* Zhokhov et Pugacheva, 1998 used as outgroup. Support for BI (10 million MCMC), and maximum likelihood (ML, 1000 bootstrap replicates) indicated at nodes (BI/ML), nodes with less than 50% support indicated with "-".

intestinal bifurcation, extent of tegumental spines, and position of the genital pore and cirrus-sac.

1a Testes lobed …… *A. voltanum*

1b Testes spherical or oval with smooth outline ……2

2a Vitellarium extending to the pharyngeal level ……3

2b Vitellarium not extending anteriorly beyond the ventral sucker……4

3a Cirrus-sac elongate enclosing a bipartite internal seminal vesicle …….... *A. ghanense*

3b Cirrus-sac roughly oval enclosing a unipartite internal seminal vesicle …… *A. engraulicypridis*

4a Tegumental spines present anteriorly …… *A. mazoense*

4b Tegumental spines absent …… 5

5a Vitellarium extending only to the anterior testis, the eggs longer than 95µm ……...…… *A. indistinctum*

5b Vitellarium extending to the ovary; eggs shorter than 95µm …..…….…… 6

6a Oesophagus short; intertesticular space absent …… 7

6b Oesophagus long; two testes separated by intertesticular space ……..…….....……. 8

7a Testes symmetrical; ovary immediately posterior to the anterior testis ……. *A. sudanense*

7b Testes oblique; ovary separated from the anterior testis by a space ….…….…….... *A. bynni*

8a Intestinal bifurcation anterior to the ventral sucker; genital pore near the pharynx …… *A. aswanense*

8b Intestinal bifurcation at level of the ventral sucker; genital pore near the ventral sucker .... ***Allocreadium apokryfi* sp. n.**

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Table 3. Genetic distance of *Allocreadium apokryfi* sp. n. (values in bold) from other species of *Allocreadium* Looss, 1900 based on 28S rDNA. Sequence divergence (%) based on average uncorrected *p*-distance below the diagonal, with the number of base differences per sequence above. Intraspecific distances indicated by shaded cells.

	Accession number	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	**MW907591–MW907595**	***Allocreadium apokryfi* sp. n.**	-	**59**	**61**	**61**	**61**	**61**	**60**	**63**	**53**	**63**	**62**	**66**	**66**	**66**	**66**	**66**	**72**	**73**	**73**	**73**	**74**	**72**	**72**	**72**	**76**
2	MK211220–MK211223	*Allocreadium hemibarbi*	**4.7**	-	32	32	32	32	32	34	30	22	27	27	27	27	27	27	43	26	27	32	33	37	37	37	42
3	JX977132	*Allocreadium neotenicum*	**4.9**	2.6	-	0	0	0	0	2	2	41	42	34	34	34	34	34	55	38	39	42	43	38	38	38	38
4	MH143104	*Allocreadium neotenicum*	**4.9**	2.6	0	-	0	0	0	2	2	41	42	34	34	34	34	34	55	38	39	42	43	38	38	38	38
5	MH143105	*Allocreadium neotenicum*	**4.9**	2.6	0	0	-	0	0	2	2	41	42	34	34	34	34	34	55	38	39	42	43	38	38	38	38
6	MH143103	*Allocreadium neotenicum*	**4.9**	2.6	0	0	0	-	0	2	2	41	42	34	34	34	34	34	55	38	39	42	43	38	38	38	38
7	KY513132–KY513133	*Allocreadium neotenicum*	**5.2**	2.8	0	0	0	0	-	2	2	41	42	34	34	34	34	34	54	37	38	41	42	38	38	38	35
8	EF032693	*Allocreadium lobatum*	**5.1**	2.8	0.2	0.2	0.2	0.2	0.2	-	2	43	44	34	34	34	34	34	57	39	40	43	44	38	38	38	39
9	DQ029327	*Allocreadium lobatum*	**6.5**	3.7	0.3	0.3	0.3	0.3	0.3	0.3	-	37	36	35	35	35	35	35	45	34	35	38	38	36	36	36	32
10	LC215274	*Allocreadium gotoi*	**5**	1.8	3.3	3.3	3.3	3.3	3.6	3.5	4.6	-	10	37	37	37	37	37	41	39	39	41	42	47	47	47	53
11	GU462121	*Allocreadium* sp.	**5.3**	2.3	3.6	3.6	3.6	3.6	3.8	3.8	4.4	0.9	-	37	37	37	37	36	42	36	36	38	39	47	47	47	53
12	GU462117–GU462120	*Palaeorchis crassus**	**5.4**	2.2	2.8	2.8	2.8	2.8	3	2.8	4.3	3	3.2	-	0	0	0	0	53	29	30	37	38	14	14	14	44
13	JF261143	*Palaeorchis crassus**	**5.4**	2.2	2.8	2.8	2.8	2.8	3	2.8	4.3	3	3.2	0	-	0	0	0	53	28	29	36	37	14	14	14	43
14	JF261142	*Palaeorchis crassus**	**5.4**	2.2	2.8	2.8	2.8	2.8	3	2.8	4.3	3	3.2	0	0	-	0	0	53	28	29	36	37	14	14	14	43
15	JF261141	*Palaeorchis crassus**	**5.5**	2.2	2.8	2.8	2.8	2.8	3	2.8	4.3	3.1	3.2	0	0	0	-	0	52	28	29	36	37	14	14	14	43
16	JF261144	*Palaeorchis crassus**	**5.6**	2.3	2.8	2.8	2.8	2.8	3	2.8	4.3	3.1	3.1	0	0	0	0	-	53	28	29	36	37	14	14	14	43
17	MK258685–MK258687	*Allocreadium* sp.	**5.7**	3.4	4.4	4.4	4.4	4.4	4.7	4.6	5.6	3.3	3.6	4.3	4.3	4.3	4.3	4.4	-	54	54	57	58	63	63	63	69
18	MK211209	*Allocreadium* sp.	**5.8**	2.1	3	3	3.1	3.1	3.2	3.2	4.2	3.1	3.1	2.4	2.3	2.3	2.3	2.3	4.3	-	1	10	11	39	38	38	43
19	MK211210	*Allocreadium* sp.	**5.8**	2.2	3.1	3.1	3.1	3.1	3.3	3.3	4.3	3.1	3.1	2.4	2.4	2.4	2.4	2.4	4.3	0.1	-	11	12	40	39	39	44
20	MK211211–MK211217, MK211219	*Allocreadium khankaiensis*	**5.8**	2.6	3.4	3.4	3.4	3.4	3.6	3.5	4.7	3.3	3.2	3	2.9	2.9	3	3	4.5	0.8	0.9	-	1	45	44	44	47
21	MK211218	*Allocreadium khankaiensis*	**5.9**	2.6	3.4	3.4	3.5	3.5	3.7	3.6	4.7	3.4	3.3	3.1	3	3	3.1	3.1	4.6	0.9	1	0.1	-	46	45	45	48
22	MH143102	*Allocreadium isoporum*	**5.8**	3	3.1	3.1	3.1	3.1	3.3	3.1	4.4	3.8	4	1.1	1.1	1.1	1.2	1.2	5.1	3.2	3.3	3.7	3.7	-	0	0	48
23	GU462125	*Allocreadium isoporum*	**5.9**	3	3.1	3.1	3.1	3.1	3.3	3.1	4.4	3.8	4	1.1	1.1	1.1	1.2	1.2	5.1	3.1	3.2	3.6	3.7	0	-	0	47
24	GU462126	*Allocreadium isoporum*	**5.9**	3	3.1	3.1	3.1	3.1	3.3	3.1	4.4	3.8	4	1.1	1.1	1.2	1.2	1.2	5.1	3.1	3.2	3.6	3.7	0	0	-	47
25	MN969626–MN969627	*Allocreadium* sp.	**6.1**	3.4	3	3	3.1	3.1	3	3.2	4	4.2	4.6	3.6	3.5	3.6	3.6	3.6	5.5	3.4	3.5	3.7	3.8	3.9	3.8	3.8	-

* Noted as *Cercariaeum crassum* Wesenberg-Lund, 1934

DISCUSSION

Taxonomy

The present study increases the number of species of *Allocreadium* recorded from Africa to nine. Globally, more than 100 species of this genus are accepted as biologically distinct (WoRMS 2020). The first key to African species of *Allocreadium* was presented by Saoud et al. (1974) and included five species distinguished by the testis shape, egg size and the structure and extent of the vitellarium. El-Naffar et al. (1984) and El-Naffar (1986) updated the key to include eight nominal species based on the shape and position of the testes, extent of the vitellarium and the oesophagus length. By reworking these keys and adding the information on *Allocreadium apokryfi* sp. n., a new key was produced based on the length of the pharynx, arrangement of tegumental spines and the position of the genital atrium and cirrus-sac. However, a revision of all known African species, including updated morphology and molecular characterisation, may alter the key.

Surface ultrastructure of the new species studied using SEM differed from what has previously been observed for species of *Allocreadium*. Currently, only one other species of the genus, *Allocreadium danjiangense* Gao, Wang, Xi, Yao et Nie, 2008 has been studied using SEM (Gao et al. 2008). Although the tegument of both *A. apokryfi* sp. n. and *A. danjiangense* is smooth, with tegumental striations present, the tegumental striations dissipate posteriorly in *A. apokryfi* sp. n., whereas they become denser and shallower in the posterior part of the body of *A. danjiangense*. Additionally, the protuberant rugae on the dorsal surface, dorsal tubercles and muscular grids around the oral and ventral sucker edges were not observed in *A. apokryfi* sp. n. Tongue-like tubercles were seen in the ventral sucker of the new species but did not occur in a groove. The tortoise shell-like tegumental structure between the oral sucker of *A. danjiangense* was similar to the cobblestone-like tegumental nature of *A. apokryfi* sp. n. Unfortunately, Gao et al. (2008) did not produce any molecular data and thus the differences in surface topology cannot be related to phylogenetic relationships of both taxa. However, the surface ultrastructure of these two allocreadiids is far more similar to one another than to other allocreadiid species studied using SEM, such as *Crepidostomum farionis* (Müller, 1780), *Crepidostomum metoecus* (Braun, 1900) and *Crepidostomum oschmarini* in that no tegumental bosses, ciliated papillae or lobes are present (Moravec 2002, Petkevičiūtė et al. 2018).

It would be an interesting future endeavour to determine if the papillae of species of *Allocreadium* can also be separated into different types as observed by Žďárská and Nebesářová (2004) in *C. metoecus* using transmission electron microscopy, although ciliated receptors were absent in *A. apokryfi* sp. n. Interestingly, structures similar to the minute sensory receptors (Moravec 2002) or non-papillate sensory endings (Petkevičiūtė et al. 2018) observed on the dorsal part of the oral sucker in *Crepidostomum* spp. appear to be present in *A. apokryfi* sp. n. and have been noted as possible sensory openings. However, their composition and function need further investigation, including TEM observation. The number and arrangement of the tegumental papillae on the ventral tegument around the oral and ventral suckers of *A. apokryfi* sp. n. will also need further investigation as this was not consistent among the specimens studied here.

The observation of the protruded cirrus of *A. apokryfi* sp. n. is the first of its kind for this genus. The cirrus appears to be far shorter and stouter than that of other allocreadiids like *C. oschmarini*. The elevation of the genital pore around the cirrus is similar to that seen by Petkevičiūtė et al. (2018) for *C. oschmarini*, but the cirrus surface in *A. apokryfi* sp. n. is not smooth; it is covered with lace- or sponge-like structures. Whether this feature is characteristic of all species of *Allocreadium* or only the new species will have to be confirmed. The observation of possible sperm at the genital pore opening is also noteworthy and may be the result of interrupted copulation. The further study of these structures may be interesting.

Both 18S and 28S rDNA fragments assessed for the new species support that this species is clearly distinct from all other taxa for which sequence data are available. The distance between the new species for both regions amplified (1.2–9.1% for 18S and 4.7–6.5% for 28S) is large and in the case of 28S rDNA, far higher than the observed intraspecific range of 0–0.3%. It is also possible that the overlap resulting from the high intraspecific distances observed between the two sequences for *A. lobatum* (0.3%) and the low interspecific distances between *A. lobatum* and *Allocreadium neotenicum* (0.2–0.3%) may indicate that these species are synonyms, as has been suggested in earlier work (Bray et al. 2012). This would mean that the intra- and interspecific ranges for 28S rDNA of *Allocreadium* are actually 0–0.3% and 0.8–6.5%, respectively. Additionally, *A. apokryfi* sp. n. forms a well-supported, distinct clade. However, sequence data are currently only available for seven identified species of *Allocreadium*, excluding another four possibly distinct taxa which are not identified, and *Palaeorchis crassus* which falls within the ingroup. Even if all the unidentified species are considered distinct and *P. crassus* is considered a congener, only 12 taxa have representative molecular data, which is strikingly less than the 107 suggested *Allocreadium* spp. on WoRMS (2020).

The low number of taxa for which genetic information is available, the high number of unidentified species for which data are available, the inclusion of *P. crassus* in the *Allocreadium* ingroup, and the low bootstrap support observed at deeper nodes of the produced topology, all indicate that there is still much to be elucidated regarding the phylogeny of this genus. However, the basal placement of the unpublished sequences for an unidentified *Allocreadium* sp. collected in Russia (MK258685–MK258687), with those of *A. apokryfi* sp. n. in the ingroup, may support the speculated origin of the group in the south of the Far East (Manter 1963, Vainutis 2020). Nevertheless, the need for additional molecular study of this group is exemplified by the exclusion of sequence KX344072 (Chaudhary et al. 2016) in the current work. It was designated as *Allocreadium handiai* Pande, 1937 but does not relate to oth-

er 28S rDNA for *Allocreadium* spp. and instead appears to be more closely related to *Haplorchoides* Chen, 1949 (Heterophyidae) when using BLAST. Similarly, sequences GU462111 and GU462116 are designated as *Allocreadium isoporum* (Looss, 1894) in their respective publication (Petkevičiūtė et al. 2010), but are identified as *Bunodera luciopercae* (Müller, 1776) in GenBank, which is supported by BLAST analyses. This accentuates the need for a more in-depth investigation into the molecular identity of this genus. Other markers (COI mtDNA and ITS rDNA) have also been used for the study of allocreadiids, but all taxa for which data are available have been included in either the 18S or 28S rDNA analyses.

Definitive hosts and specificity

The definitive hosts of these trematodes appear to be mostly species of Actinopterygii (ray-finned fishes), with various groups and families serving as hosts to adults of species of the genus. In Africa, definitive hosts for species of *Allocreadium* have been recorded from four orders: Characiformes (n = 1), Perciformes (n = 1), Siluriformes (n = 3), and Cypriniformes (n = 7) (Thomas 1957, Baer 1959, Beverley-Burton 1962, Fischthal and Thomas 1972, Khalil and Thurston 1973, Saoud et al. 1974, Moravec 1977, Jones 1982, Mashego 1982, El-Naffar et al. 1984, El-Naffar 1986, Mbahinzireki 1987, Mwita and Nkwengulila 2010, Mwita 2014). However, adult *A. neotenicum* has also been collected from beetles in Europe, *Hydroporus rufifrons* (Müller) by Bray et al. (2012) and *Oreodytes sanmarkii* (Sahlberg) by Soldánová et al. (2017), indicating other groups as possible hosts of progenetic stages.

Allocreadium mazoense is one of the few *Allocreadium* spp., and the only species from Africa, which has been recorded from more than one host species (six fish species from three families). Although *A. mazoense* was described from *Clarias gariepinus* (Burchell), it has been speculated that the Cyprinidae are the true hosts for this species as most recorded hosts are of this family (Mashego 1982). This is supported by the fact that sampling efforts in which the parasites were recorded from cyprinids did not reveal infections in *C. gariepinus* even when the fish was present (Mashego 1982). The spread and identity of *A. mazoense* in Africa may also need to be revised as some of the records may have been misidentified. For example, Jones (1982) examined *A. mazoense* from *Enteromius camptacanthus* (Bleeker) but did not observe anterior tegumental spines. These spines appear to be a key feature of the species, and thus the trematode studied by Jones (1982) may have represented a distinct species. Additionally, Jones (1982) analysed the type material of *A. mazoense* and could not detect the spines noted in the original description. This could indicate a need to use SEM and molecular approaches to solve this riddle.

In contrast to the loose specificity of *A. mazoense*, three species of *Allocreadium* from Africa, *A. bynni*, *A. sudanense* and *A. aswanense*, were described from the same host species (*Labeobarbus bynni*) within the same river system. The identity and host allocation of these three species also require revision. In the description of *A. sudanense* by Saoud et al. (1974), and in later papers (El-Naffar et al. 1984, El-Naffar 1986), the host is noted as *L. bynni*, but in El-Naffar (1986) it is also given as *Bagrus bajad* (Forsskål), a catfish referred to as a cyprinid. Additionally, the description of *A. bynni* is accredited to El-Naffar (1970) in El-Naffar et al. (1984) but is then described in El-Naffar (1986) as a new taxon. This may be why *A. bynni* is not mentioned again in other papers, with later publications and online databases not listing the taxon (Khalil and Polling 1997, Kudlai et al. 2018, WoRMS 2020). Again, this would be a good opportunity to use modern techniques to determine the distinctness or synonymy of these taxa. Other species of *Allocreadium* in Africa, like *Allocreadium voltanum*, appear to fit the more general host specificity for members of *Allocreadium*, displaying strict host specificity (Thomas 1957). Thus, the host specificity of *Allocreadium* in Africa may not be as highly variable as reported.

Intermediate hosts in Africa

The intermediate hosts of species of *Allocreadium* in Africa are nearly unknown, with only Mbahinzireki (1987) broaching the topic. The author collected *A. mazoense* from the cichlid *Haplochromis teegelaari* Greenwood et Barel in Lake Victoria, noting that the host is molluscivorous. Using the feeding and ecology of the host, he inferred the possible life cycle of the trematode. He hinted at a level of host specificity in the system as a second congeneric molluscivore, *Haplochromis ptistes* Greenwood et Barel was not infected. He attributes this to either the level of adaptation of the parasite to the host, or the depth at which the two cichlid species occur, the latter of which may relate to the depth at which infective stages would be encountered. The first of these hypotheses seems unlikely due to the loose host specificity of *A. mazoense* as discussed, meaning that the second scenario is more likely. Mbahinzireki (1987) noted that *Sphaerium* sp. has been recorded as intermediate host of *Allocreadium* spp. previously, and thus the Mwanza Gulf clam (*Sphaerium* sp.) may be the local vector for *A. mazoense*. Unfortunately, the bivalve mollusc diversity and distribution in the Vaal River system are not well known. According to Appleton and Miranda (2015), species of three genera of the Sphaeriidae occur in southern Africa, of which only species of *Pisidium* Pfeiffer appear to occur in the central region of South Africa where the Vaal River system is situated. However, the identification and distribution of the eight species of *Pisidium* and their possible presence in the Vaal River system have not been well studied. Only *Pisidium langleyanum* Melvill et Ponsonby is noted in the reaches of the Vaal River system (De Kock and Wolmarans 2008) and thus may be the prime suspect as an intermediate host for *A. apokryfi* sp. n. The definitive host, *Labeobarbus aeneus*, is not exclusively molluscivorous, but rather broadly omnivorous (Skelton 2001). However, Skelton (2001) does make specific mention of bivalve molluscs forming a large part of the diet of *L. aeneus*, further supporting that they may be the source of the infection.

Ecology

The parasite fauna of fish in the Vaal River system has been well studied for several decades. Especially the intestinal helminths of *L. aeneus* have been observed for several years from many sites in the system, with no record of trematodes having ever been detected (Bertasso and Avenant-Oldewage 2005). Therefore, the unexpected observation of a new trematode species in *L. aeneus* in the Vaal River is surprising. Three possibilities have been considered in this regard. The first is that the trematodes were introduced into the system with an invasive intermediate or definitive host, but based on our morphometric and molecular data, it would appear that the species does not match an existing taxon from Africa or the rest of the world. Additionally, no known allocreadiid has been recorded as invasive in southern Africa (Smit et al. 2017), nor from the fish or mollusc species which have been introduced to the area. The second scenario is that the range of a native, innominate species recently changed due to environmental or anthropogenic activities, but without accurate temporal and spatial data, along with the lack of knowledge on the distribution of molluscs in southern Africa, this is difficult to discuss at this time. The last scenario is that this species is native to the host and locality, but has not been detected before for some reason. Possible reasons may be a very short-lived period in the fish host and that the detection of species of *Allocreadium* is rare, which is supported by the findings of Mbahinzireki (1987), or that the range of the species has been limited to this single locality due to intermediate host populations, ecological factors, or even extinction in other regions. At present, we are unable to decide which of these scenarios are the most likely, but it could be a topic for future investigations to unravel the life cycle of the new species in order to elucidate its origin and transmission.

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