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Masenia nkomatiensis n. sp. (Digenea: Cephalogonimidae) from *Clarias gariepinus* (Burchell) (Clariidae) in Incomati Basin, Mozambique

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Abstract A new species of *Masenia* Chatterji, 1933 is described based on material from the intestine of *Clarias gariepinus* (Burchell) in the Incomati River, Mozambique. The combination of morphological characteristics and analysis of 18S and 28S rDNA sequences delineated the specimens found in the present study as a distinct species. The new form is distinguished from other *Masenia* spp. in having a large reniform seminal receptacle, a cirrus-sac ending anterior to the ventral sucker, intestinal caeca extending into the hindbody to the level of the posterior testis, and the vitelline fields extending anteriorly to the ventral sucker and posteriorly to the middle of the ovary. Notably, the new form is the only record of African species having a sac-shaped excretory vesicle. Analysis of 28S rDNA sequence data supported its placement in the Cephalogonimidae Looss, 1899. 18S analyses also supported its placement in this family but showed it was not closely related to *Masenia bangweulensis* (Beverley-Burton, 1962), the sole other African species for which genetic data is currently accessible. The total pairwise differences for 18S and 28S sequences showed the new form differing from other cephalogonimids. Thus, both morphological and genetic characteristics indicate that the present form represents a distinct species, here described as *Masenia nkomatiensis* n. sp. An updated key to African *Masenia* spp., now five, is provided.

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Introduction

Cephalogonimidae Looss, 1899 is a small family containing small, spinose digeneans parasitic in the gastro-intestinal tract of fishes, amphibians and reptiles (Jones & Bray, 2008). Adult cephalogonimids have a genital pore at or near the anterior extremity and a long cirrus-sac terminating at different levels in the forebody (Jones & Bray, 2008). It comprises five genera: *Cephalogonimus* Poirier, 1886; *Paracephalogonimus* Skrjabin, 1950; *Cephalogonimoides* Brooks & Buckner, 1976; *Emoleptalea* Looss, 1900; and *Masenia* Chatterji, 1933.

Species of *Masenia* (syns *Eumasenia* Srivastava, 1951 and *Pseudomasenia* Pan, 1984) inhabit the intestine of marine and freshwater fishes of the class Actinopterygii throughout Africa and Asia (Jones & Bray, 2008; Madhavi, 2011). Adults of species of *Masenia* can be distinguished from the other cephalogonimids by the possession of two rows of enlarged circumoral spines and vitelline follicles located mainly in the hindbody (Beverley-Burton, 1962; Jones & Bray, 2008). Species delineation within the genus is based on the body shape and size, arrangement of the internal organs, number of the circumoral spines, the shape of the excretory vesicle, the size of the eggs and the extension of vitelline follicles, the caeca and the cirrus-sac (Khalil & Thurston, 1973).

Currently, 22 *Masenia* spp. are considered valid with a remarkable breadth of fish families infected. They are clustered into two groups, those described from Africa and those from Asia (Table 1). The Asian group includes 18 species; of these 14 were described from freshwater hosts in the Channidae Fowler, Bagridae Bleeker and Heteropneustidae Hora, and four species were described from marine hosts in the Engraulidae Gill, Carangidae Rafinesque, Sciaenidae Cuvier and Mullidae Rafinesque (see Madhavi, 2011). The African group contains four species described from fishes of the freshwater families Mochokidae Regan and Clariidae Bonaparte (see Khalil & Thurston, 1973; Khalil & Polling, 1997).

In Africa, few recent contributions on the biology and taxonomy of *Masenia* are available and all descriptions and reports on *Masenia* spp. were based on morphological data; only one DNA sequence is currently available for *M. bangweulensis* (Beverley-Burton, 1962) from Tanzania (Mwita & Nkwengulila, 2010). An unidentified species of *Masenia* was collected from *C. gariepinus* in Mozambique; it is here described as a new species. As no *Masenia* spp. has been subject to electron microscopical examination, scanning electron microscopy (SEM) and histology were performed together with morphometric analysis of the internal morphology of the new species. This study increases the knowledge of African *Masenia* species diversity by describing a new species based on a combination of morphological and molecular characters of adult specimens collected in a freshwater system in Mozambique.

Materials and methods

Study area

The Incomati River is one of the major trans-national basins and traverses three countries, i.e. Republic of South Africa, Kingdom of eSwatini (previously Swaziland), and Republic of Mozambique; in the latter country it plays a major role in shaping the natural habitat (Vaz & Pereira, 2000; Da Silva & Rafael, 2014). For the present study, five collections were performed at two sites on the Incomati River: three collections at site 1 (Manhica, 25°44′16.6″S, 32°36′8.4″E); two collections at site 2 (Calanga, 25°25′44.04″S, 32°48′44.7″E).

Collection and examination of fish

Sharptooth catfish, *C. gariepinus* [n = 154, standard length 19–64 (37 ± 9) cm] were collected using gill nets of 2 mm and 4 mm mesh size. Fish were euthanised by severing the spinal cord with a single cut posterior to the skull, dissected and the intestine examined for digenean trematodes. Freshly recovered parasites were washed in saline solution and preserved either with 70% ethanol for light and scanning electron microscopy (SEM) study (Dos Santos et al., 2015; Jyrwa et al., 2016), 10% neutral buffered formalin for histological study, or 96% ethanol for genetic analyses (Blasco-Costa et al., 2016). Additionally, some of the parasites were slightly flattened in a drop of alcohol-formalin-acetic acid (AFA) between a glass slide and coverslip, and subsequently transferred to 70% ethanol (Jyrwa et al., 2016).

Preparation of parasites for light microscopy

For light microscopy (LM), whole ovigerous adult specimens (n = 25) were stained with acetocarmine, differentiated in acid alcohol, dehydrated in a graded ethanol series, cleared in beechwood creosote and mounted in Canada balsam under a coverslip on a glass slide (Bakke, 1986; Thatcher, 2006). Specimens for histology were washed overnight in running water, dehydrated in ethanol and embedded in TAAB Transmit LM Resin Kit (TAAB, UK) following the standard protocol suggested by the manufacturer. Sections 5 µm thick were cut, stained with hematoxylin-eosin and examined by LM for digestive and excretory systems' termination and the structure of the cirrus-sac. Drawings were made from whole mounts

Table 1 List of Asian and African species of *Masenia* with information regarding habitat, host and type-locality

Species	Host	Habitat	Locality	Reference
African group				
M. bangweulensis (Beverley-Burton, 1962)	*Clarias ngamensis* Castelnau	Freshwater	Zambia	Beverley-Burton (1962)
M. proteropora (Thomas, 1958)	*Clarias anguillaris* (Linnaeus)	Freshwater	Ghana	Thomas (1958)
M. synodontis (Khalil & Thurston, 1973)	*Synodontis victoriae* Boulenger	Freshwater	Uganda	Khalil & Thurston (1973)
M. ghanensis (Fischthal & Thomas, 1968)	*Heterobranchus longifilis* Valenciennes	Freshwater	Ghana	Fischthal & Thomas (1968)
Asian group				
M. quiloni (Gupta & Tandon, 1984)	*Thryssa mystax* (Bloch & Scheneider)	Marine	India	Gupta & Tandon (1984)
M. carangai (Gupta & Tandon, 1984)	*Carangoides armatus* (Rüppell)	Marine	India	Gupta & Tandon (1984)
M. orissai (Gupta & Tandon, 1984)	*Protonibea diacanthus* (Lacépède)	Marine	India	Gupta & Tandon (1984)
M. upeneusi Gupta & Puri, 1982	*Upeneus macronemus* (Bleeker)	Marine	India	Gupta & Puri (1982)
M. collata Chatterji, 1933 (type-species)	*Clarias batrachus* (Linnaeus)	Freshwater	Myanmar	Chatterjii (1933)
M. fossilisi Gupta, 1953	*Heteropneustes fossilis* (Bloch)	Freshwater	India	Gupta (1953)
M. moradabadensis (Srivastava, 1951)	*Heteropneustes fossilis*	Freshwater	India	Srivastava (1951)
M. gomtia Agrawal, 1963	*Mystus vittatus* (Bloch)	Freshwater	India	Agrawal (1963)
M. vittatusia Agrawal, 1963	*Mystus vittatus*	Freshwater	India	Agrawal (1963)
M. ritai Sircar & Sinha, 1970	*Rita rita* (Hamilton)	Freshwater	India	Sircar & Sinha (1970)
M. fukienensis (Tang & Lin, 1973)	*Clarias fuscus* (Lacépède)	Freshwater	China	Chung-Ti & Sui-Ming (1973)
M. gwaliorensis (Bhadauria & Dandotia, 1986)	*Clarias batrachus*	Freshwater	India	Bhadauria & Dandotia (1986)
M. kwangtungensis (Pan, 1984)	*Clarias* sp.	Freshwater	China	Pan (1984)
M. agarwali Hasnain & Sahay, 1994	*Heteropneustes fossilis*	Freshwater	India	Hasnain & Sahay (1994)
M. chauhani Maurya, Agarwal & Singh, 1989	*Rita rita*	Freshwater	India	Maurya et al. (1989)
M. jaunpurensis Maurya & Singh, 2004	*Channa gachua* (Hamilton)	Freshwater	India	Maurya & Singh (2004)
M. pushpanjalii Singh, Shankar, Singh & Gupta 2006	*C. gachua*	Freshwater	India	Singh et al. (2006)
M. dayali Gupta, 1953	*Clarias batrachus*	Freshwater	India	Gupta (1953)

using a drawing tube attachment and terminologies are in accordance to Manter (1970). Measurements and photomicrographs were obtained with a Zeiss Axioplan 2 imaging LM and AxioVision software Release 4.7.2 (Carl Zeiss, Jena, Switzerland). Measurements are given in micrometres unless otherwise stated and are shown as the range, followed by the mean in parentheses.

Preparation of parasites for scanning electron microscopy

Adult flukes (n = 60) fixed for SEM were dehydrated through an ascending series of ethanol to absolute ethanol and transferred to increased concentrations of hexamethyldisilazane (HMDS): absolute ethanol (Merck, Germany) for 15 min each (Dos Santos et al., 2015). Specimens were mounted on a strip of carbon conductive tape fixed to a microscope slide and then dried in a Sanpla dry keeper desiccator cabinet (Sanplatec Corporation, Japan) for at least 48 h, coated with gold in an Emscope SC500 sputter coater (Quorum Technologies, Newhaven, UK) and viewed with a VEGA 3LMH scanning electron microscope (Tescan, Brno, Czech Republic) at an acceleration voltage of 5 kV.

In the present study, due to the geographical region in which the specimens were collected, the key for African maseniids was used. For the amended key of Khalil & Thurston (1973) for *Masenia* from African fishes, characteristics such as the number of circumoral spines, the extension of intestinal caeca, and the shape of the body, oral sucker and excretory vesicle were considered.

Molecular analysis

Genomic DNA was extracted from seven ethanol-fixed specimens from different fish. Before extraction, each specimen was rehydrated through a descending ethanol series and washed in water overnight. DNA was extracted using the DNeasy® Blood and Tissue kit (Qiagen, Manchester, UK) following the standard protocol suggested by the manufacturer. Polymerase chain reaction (PCR) for the 18S rRNA gene (spanning the V4-V5 variable regions) was performed using the forward primer JLR24 (5′-CGG AAT TCG CTA GAG GTG AAA TTC TTG G-3′) and the reverse primer JLR25 (5′-CCG AAT TCC GCA GGT TCA CCT ACG G-3′) according to Campos et al. (1998). The 28S rRNA gene (D1-D3 variable regions) was amplified using the forward primer dig12 (5′-AAG CAT ATC ACT AAG CGG-3′) and the reverse primer 1500 (5′-GCT ATC CTG AGG GAA ACT TCG-3′) according to Tkach et al. (2003), using a MultiGene^{TM} OptiMax Thermal Cycler (Labnet International Inc, North America).

For each marker, the total volume for the PCR reaction was 30 µl, including 1U *Taq* polymerase, 1 µl of 100 µM dNTP, 3µl of 2.5 mM of $MgCl_2$, 3 µl 1× reaction buffer, 2 µl of each primer (10 µM) and 10 µl of DNA template. PCR conditions were: initial denaturation for 15 min at 95 °C, 40 cycles of denaturation for 30 s at 93 °C, annealing for 90 s at 52 °C, and an extension for 2 min at 72 °C, followed by a final extension step for 5 min at 72 °C. Successful amplification was confirmed using 1% agarose gel (1g of agarose in 100 ml TBE buffer), impregnated with GelRed® (Biotuim), in an ENDURO^{TM} Electrophoresis Systems (Labnet International Inc, North America). Amplicons were sequenced following Avenant-Oldewage et al. (2014) in both directions. Electropherograms were inspected and edited manually (if required) using MEGA v6.06 (Tamura et al., 2013) and then combined in the BioEdit sequence alignment editor (Hall, 1999). A hundred similar sequences obtained through BLAST were aligned to the obtained sequences using MUSCLE algorithm (Edgar, 2004) as implemented in MEGA v6.06. Eighteen 18S rDNA sequences and 15 28S rDNA sequences were included in the final analysis. Pairwise differences were estimated for each dataset using the following conditions according to Bray et al. (2018): "variance estimation method = none", "model/method = No. of differences" and "Substitutions to include = d: Transitions + Transversions" and "Gaps/Missing Data Treatment = complete deletion" in MEGA. Phylogenetic analysis through maximum likelihood (ML) method based on Tamura-Nei model (Tamura & Nei, 1993) and parsimony (MP) method using subtree-pruning-regrafting (SRP) algorithm (Nei & Kumar, 2000) were conducted to explore relationships among taxa with MEGA v6.06 (default settings). Nodal supports for produced topologies (rooted) were calculated using 1,000 bootstrap replicates (Felsenstein, 1985). Given the position of members of the family Haematoloechidae [*Skrjabinoeces similis* (Looss, 1899) (GenBank: AJ287575) and *Haematoloechus longiplexus* (Stafford, 1990) (GenBank: AJ287520)] in Olson et al. (2003), they were designated as functional outgroup taxa. Representative sequences for both 18S and 28S rDNA generated in this study were submitted to the GenBank database.

Family Cephalogonimidae Looss, 1899
Genus *Masenia* Chatterji, 1933

Masenia nkomatiensis n. sp.

Type-host: *Clarias gariepinus* (Burchell) (Siluriformes: Clariidae).

Type-locality: Incomati River (25°44′16.6″S, 32°36′8.4″E), Manhiça District, Mozambique (coll. March 2017).

Other locality: Calanga (25°25′44.04″S, 32°48′44.7″E), Manhiça District, Mozambique.

Type-material: Holotype: ovigerous adult specimen was deposited in the Iziko South African Museum, Cape Town, South Africa (accession no. SAMC-A091284). Paratypes: 8 specimens were deposited in the Iziko South African Museum, Cape Town, South Africa (accession nos SAMC-A091285-A091292); 8 specimens were deposited in collection of the Natural History Museum, London, UK (accession nos NHMUK 2019.1.17.1-8); and 8 specimens were

deposited in the Royal Museum for Central Africa in Tervuren, Belgium (accession nos RMCA_-VERMES_38600 -RMCA_VERMES_38607).
Site in host: Intestine (anterior section).
Prevalence and mean intensity: 18.83% and 26.11 worms per infected fish.
Representative DNA sequences: Gen Bank: MH142267 (18S rRNA gene); MH142268 (28S rRNA gene).
ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Masenia nkomatiensis* n. sp. is urn:lsid:-zoobank.org:act:5E6056CA-A151-44DC-BBBE-3BEB7B08545C
Etymology: The name of the species refers to the Nkomati River in which the host was collected.

Description (Figs. 1, 2)

[Based on 25 specimens; metrical data in Table 2]. Body ellipsoidal, 645–1,533 (1124) long, 276–549 (447) wide at level of ovary. Tegument spinous; spines dense on anterior, middle, 4 long; scattered in posterior region, 3 long. Forebody 222–492 (332) long, hindbody 430–982 (676) long. Oral sucker funnel-shaped, terminal, 103–171 × 94–198 (137 × 125) (Fig. 1A); 2 rows of alternating spines interrupted mid-dorsally at level of genital pore present (Fig. 2B); oral and aboral spines conical, acuminate, directed posteriorly; oral spines 8–16 (13) long, aboral spines 7–10 (9) long (Fig. 2B, C). Anterior region, of opening of oral sucker with 22 ciliate papillae with symmetrical distribution pattern, mostly aggregated on mid-dorsal interruption; single papillae present between circumoral spines (Fig. 2C, D). Ventral sucker spherical, 112–165 × 106–171 (140 × 137), with 2 rows of small tegumental papillae on outer lip and nine larger papillae on inner lip (Fig. 2E). Oral to ventral sucker length ratio 1:0.67–1.26 (1:0.99); width ratio 1:0.68–1.34 (1:0.93). Prepharynx 0–22 (4) long, not visible in some specimens. Pharynx four-lobed anteriorly, 33–57 × 33–64 (43 × 51) (Fig. 1A). Oesophagus 0–30 (7) long. Intestinal bifurcation in forebody. Caeca extend into hindbody to level of posterior testis.

Testes 2, smooth, oblique, post-equatorial, oval in dorso-ventral view; anterior testis dextral, 66–163 × 89–175 (111 × 128), connected to seminal vesicle through vas efferens; posterior testis 68–177 × 104–208 (114 × 143), vas efferens not observed (Fig. 1A); post-testicular distance 191–560 (353). Cirrus-sac 399–762 × 51–114 (587 × 75), contains bipartite seminal vesicle (Fig. 1A), both portions long and slender, anterior portion 68–123 (94) long, posterior portion 73–134 (100) long. Prostatic cells, without diverticulum, surround seminal vesicle. Pars prostatica spherical, thin-walled by vesicular cells without nuclei. Ejaculatory duct opens into genital atrium. Genital atrium circular, large, dorsal to oral sucker. Genital pore in anterior region, dorsa-medial, at level of oral sucker (Fig. 2D). Gland cells in antero-dorsal region of body (Fig. 1B).

Ovary smooth, equatorial, 69–167 × 78–154 (121 × 114). Seminal receptacle reniform, saccular, near posterior margin of ovary (Fig. 1A), 100–171 × 61–91 (112 × 73). Laurer's canal and Mehlis' gland not observed. Vitelline follicles in lateral fields from anterior margin of ventral sucker to middle of ovary, intra- and extracaecal, not confluent, 234–518 (341) from anterior extremity (Fig. 1A); follicles aciniform, 16–24 in number, 41–78 × 31–43 (58 × 37). Uterus extensively looped in hindbody, extending extracaecally; uterus ascends dextrally to genital atrium. Eggs elliptical, operculate, 20–26 × 10–16 (23 × 12).

Excretory pore ventrally subterminal (Fig. 2F). Excretory vesicle irregularly sac-shaped (Fig. 1A, C).

Key to the species of *Masenia* from freshwater fishes in Africa

1a	Oral sucker spherical or slightly oval …… 2
1b	Oral sucker funnel-shaped ……..…… 3
2a	Body oval; 50–56 circumoral spines …… …….. *M. proteropora*
2b	Body pyriform; 36–40 circumoral spines …… *M. synodontis*
3a	Body ellipsoidal, excretory vesicle sac-shaped …… *M. nkomatiensis* n. sp.
3b	Body elongate, excretory vesicle Y-shaped ……..… 4

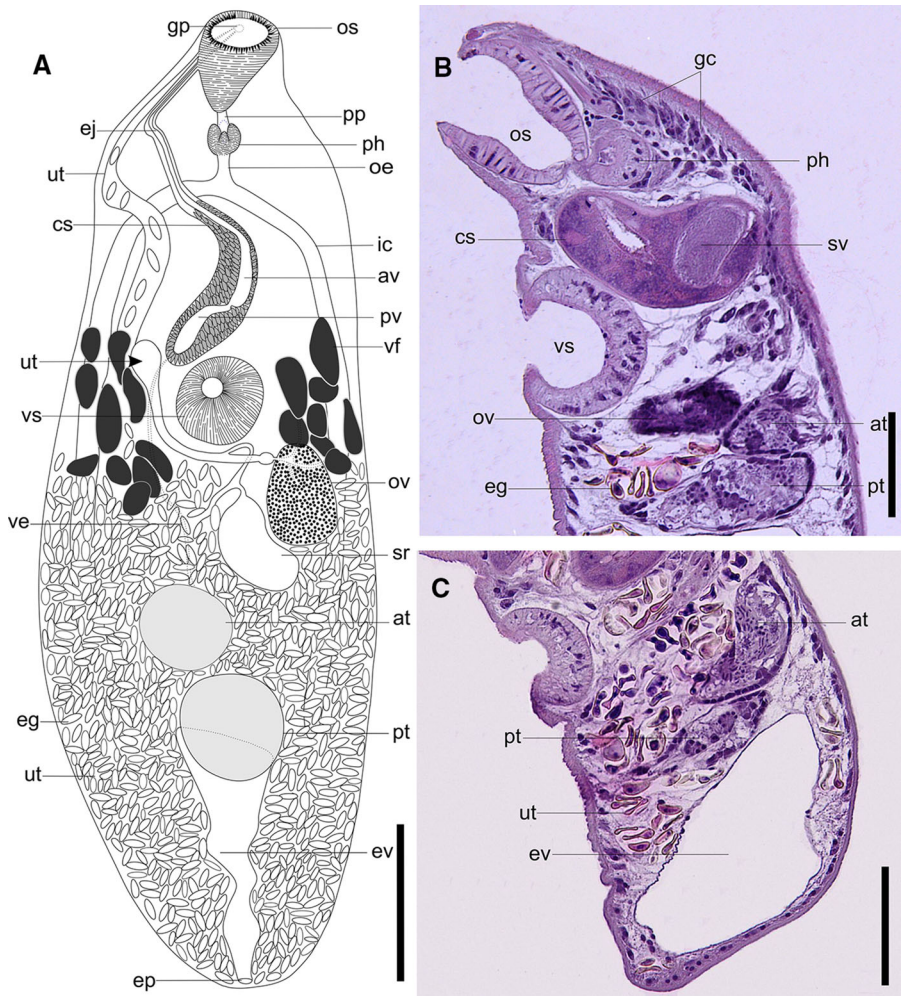

Fig. 1 *Masenia nkomatiensis* n. sp. collected from *Clarias gariepinus* in Mozambique. A, Ventral view of whole specimen. *Abbreviations*: at, anterior testis; av, anterior portion of seminal vesicle; cs, cirrus-sac; eg, egg; ej, ejaculatory duct; ep, excretory pore; ev, excretory vesicle; gp, genital pore; ic, intestinal caeca; oe, oesophagus; os, oral sucker; ov, ovary; ph, pharynx; pp, prepharynx; pt, posterior testis; pv, posterior portion of seminal vesicle; sr, seminal receptacle; ut, uterus; ve, vas efferens; vf, vitelline follicle; vs, ventral sucker. B, C, Photomicrographs of sagital section showing details of anterior and posterior regions. *Abbreviations*: gc, gland cells; sv, seminal vesicle. *Scale-bars*: A, 100 μm; B, C, 50 μm

4a Intestinal caeca extending to level of posterior margin of posterior testis; 56 circumoral spines ……..… *M. ghanensis*

4b Intestinal caeca extending to level of posterior margin of ventral sucker; 48 circumoral spines …….. *M. bangweulensis*

Molecular data

For this study we generated 18S and 28S sequences of ribosomal DNA for seven specimens, producing a single haplotype for each marker. The 18S sequence for *M. nkomatiensis* n. sp. was 940 bp long and the 28S sequence was 1,293 bp long. Only one species of *Masenia* has been sequenced previously, *M. bangweulensis* (GenBank: DQ813461), and only 18S sequence is available for it. Sequences from GenBank included in the analysis are closely related taxa to *M. nkomatiensis* n. sp. in the superfamily Plagiorchioidea (Tables 3, 4).

The interspecific pairwise differences between *M. nkomatiensis* with related 18S rDNA sequences ranged from 9–29 bp (Table 5). Intraspecific

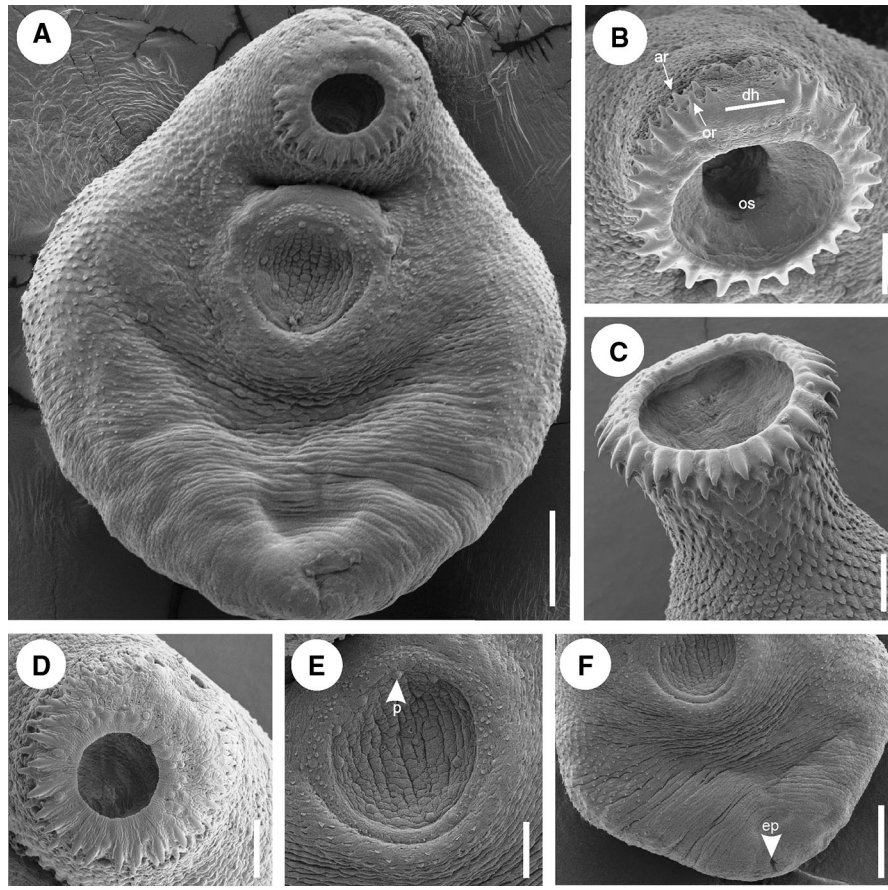

Fig. 2 Surface ultrastructure of the adult of *Masenia nkomatiensis* n. sp. from the intestine of *Clarias gariepinus*. A, Ventral view of whole mount; B–D, Anterior extremity showing rows of circumoral spines; E, Ventral sucker. F, Posterior region. *Abbreviations*: ar, aboral row of spines; ep, excretory pore; gp, genital pore; dh, dorsal hiatus; or, oral row of spines; os, oral sucker; p, papillae on inner lip. *Scale-bars*: A, F, 50 μm; B–E, 20 μm

variability was only assessed for *Choanocotyle hobbsi* Platt & Tkach, 2003, but none could be detected. Interestingly, there is a variability between the *Choanocotyle* sp. sequences included. The nodal support of the phylogenetic topology (Fig. 3A) were generally low, but *Cephalogonimus retusus* Walton, 1938 formed a clade with *M. nkomatiensis* n. sp. [72% ML and 70% MP support; lowest total pairwise differences (9 bp)] despite of considerable morphological differences. Interestingly, the new species and *M. bangweulensis* did not form a clade and differed by 29 bp. The three cephalogonimids (*M. nkomatiensis* n. sp., *M. bangweulensis* and *C. retusus*) differed by 9–29 bp.

For 28S rDNA, total pairwise differences ranging from 65–105 bp separated *M. nkomatiensis* n. sp. from other sequences (Table 6). Intraspecific variability was not assessed through the included sequences. *Masenia nkomatiensis* n. sp. formed a strongly-supported clade with species of *Cephalogonimus* (96% ML and 87% P nodal support) (Fig. 3B). The three cephalogonimids (*M. nkomatiensis* n. sp., *C. retusus* and *C. americanus*) each differed from one another by 42–73 bp. Thus, the molecular results strongly support the following relationships: (i) distinctness of the Cephalogonimidae in the Plagiorchioidea (Fig. 3A, B); (ii) molecular evidence of inclusion of *M. nkomatiensis* n. sp. in the

Table 2 Morphometric data for *M. nkomatiensis* n. sp. and four African and two Asian species of *Masenia* with mid-dorsal interruption in collar of circumoral spines

Species	*M. proteropora*	*M. bangweulensis*	*M. ghanensis*	*M. synodontis*	*M. moradabadensis*	*M. fukienensis*	*M. nkomatiensis* n. sp.
Locality	Ghana	Zambia	Ghana	Ghana	India	China	Mozambique
Source	Thomas (1958)	Beverley-Burton (1962)	Fischthal & Thomas (1968)	Khalil & Thurston (1973)	Srivastava (1951)	Chung-Ti & Sui-Ming (1973)	Present study
Body length	670–950	780–810	543–1,044	720–970	500–1,000	1,040–1,680	645–1,533 (1,124)
Body width	610–650	380–400	177–350	360–450	150–418	320–630	276–549 (447)
Forebody length	–	–	182–315	162–212	–	–	222–492 (332)
Hindbody length	–	–	288–605	414–483	–	–	430–982 (676)
Prepharynx length	45–65	7	12–31	–	12	22–64	0–22 (4)
Oesophagus length	–	–	5–37	–	30	86–111	0–30 (7)
Pharynx length	45–65	50–60	33–49	30–38	40	34–64	33–57 (43)
Pharynx width	50–65	50	34–54	42–50	36	43–73	33–64 (51)
Oral sucker length	125–155	150–170	94–140	96–104	145–143	141–173	103–171 (137)
Oral sucker width	–	120–130	77–123	116–135	145–143	135–189	94–198 (125)
Number of circumoral rows/ spines	2/25–28 each	2/24 each	2/28 each	2/36–40	2/26 each	2/25–32 each	2/25 each
Oral spine length	17	22	10–16	–	–	17–25	8–16 (13)
Aboral spine length	17	12	8–12	–	–	17–25	7–10 (9)
Ventral sucker length	150–180	130–140	73–134	112–123	132	141–169	112–165 (140)
Ventral sucker width	150–180	130–140	72–136	112–123	130	137–176	106–171(137)
Ovary length	130–135	80–100	74–104	65–96	110	76–179	69–167 (121)
Ovary width	125–120	90	59–102	71–92	138	83–150	78–154 (114)
Anterior testis length	100–120	50–70	52–111	38–77	100	78–134	66–163 (111)
Anterior testis width	105–145	60–90	64–122	85–112	138	124–129	89–175 (128)
Posterior testis length	100–120	80	57–131	53–62	130	60–141	68–177 (114)
Posterior testis width	105–145	90–100	65–140	116–123	90	120–236	104–208 (143)
Egg length	25–28	23–26	21–26	23–27	20–24	22–31	20–26 (23)
Egg width	16–20	12–14	13–18	15–16	11–15	15–19	10–16 (12)
Seminal receptacle length	–	40	–	–	74	64–159	100–171 (112)
Seminal receptacle width	–	20	–	–	45	42–107	61–91 (73)
Cirrus-sac length	–	700	262–480	406–605	460	677– 960	399–762 (587)
Cirrus-sac width	–	80	46–87	46–61	–	71–99	51–114 (75)

Table 2 continued

Species	*M. proteropora*	*M. bangweulensis*	*M. ghanensis*	*M. synodontis*	*M. moradabadensis*	*M. fukienensis*	*M. nkomatiensis* n. sp.
Locality	Ghana	Zambia	Ghana	Ghana	India	China	Mozambique
Source	Thomas (1958)	Beverley-Burton (1962)	Fischthal & Thomas (1968)	Khalil & Thurston (1973)	Srivastava (1951)	Chung-Ti & Sui-Ming (1973)	Present study
Anterior portion of seminal vesicle length	–	270	53–92/ 31–55	81–96	–	–	68–123 (94)
Posterior portion of seminal vesicle length	–	–	60–114/ 23–59	58–73	–	–	73–134 (100)
Previtelline distance	–	–	–	–	–	–	234–518 (341)
Vitelline field length	–	–	–	–	–	–	149–565 (256)
Ventral sucker to ovary distance	–	–	–	–	–	–	0–97 (21)
Ovary to anterior testis distance	–	–	–	–	–	–	0–48 (18)
Inter-testicular distance	–	–	–	–	–	–	0–20 (4)
Post-testicular distance	–	–	197–355	290–387	–	–	191–560 (354)
Post-vitelline distance	–	–	–	–	–	–	225–707 (512)
Oral sucker length (%)[a]	–	–	–	–	–	–	9.73–15.98 (12)
Ventral sucker length (%)[a]	–	–	–	–	–	–	8.9–16.11 (12)
Oral to ventral sucker-length ratio	–	–	1: 0.69–0.96	1: 1.12–1.23	–	–	1: 0.67–1.26 (0.99)
Oral to ventral sucker-width ratio	–	–	–	–	–	–	1: 0.68–1.34 (0.93)
Forebody (%)[a]	–	–	–	–	–	–	23–35 (29)
Hindbody (%)[a]	–	–	–	–	–	–	54–68 (59)
Pre-vitelline distance (%)[a]	–	–	–	–	–	–	21–39 (31)
Ventral sucker to ovary distance (%)[a]	–	–	–	–	–	–	1–5 (2)
Cirrus-sac length (%)[a]	–	–	–	–	–	–	40–72 (53)
Inter-testicular distance (%)[a]	–	–	–	–	–	–	0–2 (0.3)
Post-testicular distance (%)[a]	–	–	–	–	–	–	21–39 (31)
Post-vitelline distance (%)[a]	–	–	–	–	–	–	29–53 (45)
Ovary length (%)[a]	–	–	–	–	–	–	6–13 (10)
Vitelline field length (%)[a]	–	–	–	–	–	–	16–48 (23)

[a]Percent of body length

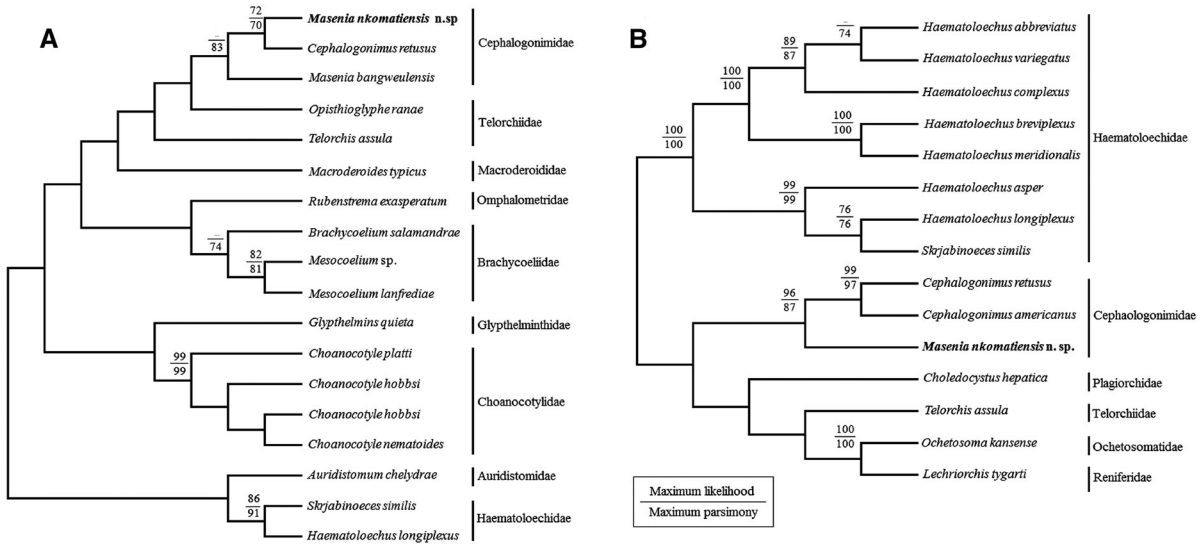

Fig. 3 Phylogenetic trees inferred by maximum likelihood and maximum parsimony methods based on 18S rDNA (A) and 28S rDNA (B) of *Masenia nkomatiensis* n. sp. and related sequences within the Plagiorchioidea. Bootstrap support for maximum likelihood/maximum parsimony are given at the respective nodes (only bootstrap support above 70% is shown)

Cephalogonimidae (Fig. 3A, B); and (iii) distinctness of *M. nkomatiensis* n. sp. and *M. bangweulensis* (Fig. 3A).

Discussion

Morphological delimitation

In having a genital pore close to the anterior extremity, two rows of circumoral spines and a Y- or sac-shaped excretory vesicle, the specimens of *M. nkomatiensis* n. sp. are consistent with the diagnosis of *Masenia* as presently proposed by Jones & Bray (2008). Other cephalogonimids such as *Cephalogonimus* spp., *Emoleptalea* spp. or *Paracephalogonimus* spp., have a genital pore at or close to the anterior extremity but lack a collar of circumoral spines, and *Cephalogonimoides* spp. have three to six rows of circumoral spines and a terminal genital pore (Jones & Bray, 2008).

Four species of *Masenia* are currently recognised in Africa and all are clearly distinct from the present species by the small body length and size of the ovary, seminal receptacle and testes. Specifically, *M. bangweulensis* possesses 44–48 circumoral spines around the oral sucker and a tubular excretory vesicle (Beverley-Burton, 1962), *Masenia proteropora* (Thomas, 1958) possesses 50–56 spines of the same length and Y-shaped excretory vesicle (Fischthal &Thomas, 1972), *Masenia ghanensis* (Fischthal & Thomas, 1968) has 56 circumoral spines, and an I- to Y-shaped excretory vesicle (Fischthal & Thomas, 1968), and *Masenia synodontis* (Khalil & Thurston, 1973) has 36–40 spines and a Y-shaped excretory vesicle (Khalil & Thurston, 1973); all distinct from 50 spines and sac-shaped excretory vesicle in the new species. This is the only record of African *Masenia* species possessing a sac-shaped excretory vesicle; in other species it is either tubular and I- to Y-shaped.

The present species resembles two Asian species, *Masenia dayali* Gupta, 1953 and *Masenia fukienensis* (Chung-Ti & Sui-Ming, 1973) by having a sac-shaped excretory vesicle but differs in being smaller in overall size. Furthermore, *M. dayali* has a conical seminal receptacle, a lobed ovary and small and non-operculated eggs (Gupta, 1953), rather than the reniform seminal receptacle, ovoid ovary, large and operculated eggs present in the new species. Non-operculated eggs and a lobed ovary are also present in *Masenia fossilis* Gupta, 1953, thus differing from the new species as well. *Masenia fukienensis* is characterised by large operculated eggs and small oval seminal receptacle (Chung-Ti & Sui-Ming, 1973), instead of the small eggs and large reniform seminal receptacle in the new species.

Table 3 List of species included in the molecular analysis of 18S rDNA dataset

Digenean species	Host species	Host class	Geographical origin	GenBank ID	Reference
Auridistomidae Srunkard, 1924					
Auridistomum chelydrae (Stafford, 1990)	*Chelydra serpentina* Schweigger	Reptilia	Mississipi, USA	AY222159	Olson et al. (2003)
Cephalogonimidae Looss, 1899					
Cephalogonimus retusus Walton, 1938	*Rana ridibunda* Pallas	Amphibia	Kolkaljane, Bulgaria	AJ287489	Olson et al. (2003)
Masenia bangweulensis (Beverley-Burton, 1962)	*Clarias liocephalus* Boulenger	Actinopterygii	Lake Victoria, Tanzania	DQ813461	Mwita & Nkwengulila (2010)
Masenia nkomatiensis n. sp.	*Clarias gariepinus*	Actinopterygii	South Region, Mozambique	MH142267	Present study
Haematoloechidae Freitas & Lent, 1939					
Haematoloechus longiplexus (Stafford, 1990)	*Rana catesbiana* Shaw	Amphibia	Keith County, Nebraska, USA	AJ287520	Olson et al. (2003)
Skrjabinoeces similis (Looss, 1899)	*Rana ridibunda*	Amphibia	Kolkaljane, Bulgaria	AJ287575	Olson et al. (2003)
Omphalometridae Looss, 1899					
Rubenstrema exasperatum (Rudolphi, 1819)	*Crocidura leucodon*	Mammalia	Kolkaljane, Bulgaria	AJ287572	Olson et al. (2003)
Telorchiidae Looss, 1899					
Telorchis assula (Dujardin, 1845) Dollfus, 1957	*Natrix natrix* (Linnaeus)	Reptilia	Kiev Region, Ukraine	AY222156	Olson et al. (2003)
Opisthioglyphe ranae (Frolich, 1791)	*Rana arvalis* Nilsson	Amphibia	Ivano-Frankivsk, Ukraine	AY222157	Olson et al. (2003)
Macroderoididae McMullen, 1937					
Macroderoides typicus (Winfield, 1929)	*Lepisosteus platostomus* Rafinesque	Actinopterygii	Tennessee, USA	AY222158	Olson et al. (2003)
Brachycoeliidae Looss, 1899					
Mesocoelium lanfrediae Gomes, Melo, Giese et al. 2013	*Rhinella marina* (Linnaeus)	Amphibia	Amazonia, Brazil	JQ886404	Olson et al. (2003)
Mesocoelium sp.	*Bufo marinus* (Linnaeus)	Amphibia	Queensland, Australia	AJ287536	Olson et al. (2003)
Brachycoelium salamandrae (Frölich, 1789)	*Salamandra salamandra* (Linnaeus)	Amphibia	Zakarpatska Region near Rakhiv, Ukraine	AY222160	Olson et al. (2003)
Glypthelminthidae Cheng, 1959					
Glypthelmis quieta (Stafford, 1900) Stafford, 1905	*Rana catesbiana*	Amphibia	Keith County, Nebraska, USA	AJ287517	Olson et al. (2003)
Choanocotylidae Jue Sue & Platt, 1998					
Choanocotyle hobbsi Platt & Tkach, 2003	*Chelodina oblonga* Gray	Reptilia	Western Australia, Australia	AY116868	Olson et al. (2003)
Choanocotyle platti Tkach & Snyder, 2007	*Chelodina rugosa* Ogilby	Reptilia	Northern Territory, Australia	EU196355	Tkach & Snyder (2007)
Choanocotyle hobbsi Platt & Tkach, 2003	*Chelodina oblonga*	Reptilia	Western Australia, Australia	EU196356	Tkach & Snyder (2007)
Choanocotyle nematoides Jue Sue & Platt, 1998	*Emydura kreftii* Gray	Reptilia	Queensland, Australia	EU196357	Tkach & Snyder (2007)

Table 4 List of species included in the molecular analysis of 28S rDNA dataset

Digenean species	Host species	Host class	Geographical origin	GenBank ID	Reference
Plagiorchiidae Lühe, 1901					
Choledocystus hepaticus (Lutz, 1928)	*Rhinella marina*	Amphibia	San Pedro las Playas, Guerrero, Mexico	AY875679	Razo-Mendivil et al. (2006)
Reniferidae Pratt, 1902					
Lechriorchis tygarti Talbot, 1933	*Lithobates sylvaticus* (LeConte)	Amphibia	North Dakota, USA	JF820603	Pulis et al. (2011)
Cephalogonimidae Looss, 1899					
Cephalogonimus retusus Walton, 1938	*Pelophylax ridibundus* (Pallas)	Amphibia	Kokaljane, Bulgaria	AY222276	Olson et al. (2003)
Cephalogonimus americanus Stafford, 1902	*Ambystoma velasci* (Dugès)	Amphibia	Quechulac, Puebla	HM137615	Razo-Mendivil & Perez-Ponce de Leon (2011)
Masenia nkomatiensis n. sp.	*Clarias gariepinus*	Actinopterygii	South Region, Mozambique	MH142268	Present study
Haematoloechidae Freitas & Lent, 1939					
Haematoloechus longiplexus (Stafford, 1902)	*Rana catesbiana* Shaw	Amphibia	Gage County, Nebraska, USA	AF387801	Snyder & Tkach (2001)
Haematoloechus asper Looss, 1899	*Rana arvalis* Nilsson	Amphibia	Ivano-Frankivsk, Region, Ukraine	AF151934	Tkach et al. (2000)
Haematoloechus breviplexus (Stafford, 1902)	*Rana catesbiana*	Amphibia	Cochise County, Arizona, USA	AF387800	Snyder & Tkach (2001)
Haematoloechus meridionalis León-Règagnon, Brooks & Zelmer, 2001	*Lithobates vaillanti* (Brocchi)	Amphibia	La Victoria, Catemaco, Veracruz, Mexico	HM137618	Razo-Mendivil & Perez-Ponce de Leon (2011)
Haematoloechus complexus (Seely, 1906)	*Rana blairi* (Mecham, Littlejohn, Oldham, Brown & Brown)	Amphibia	Gage County, Nebraska, USA	AF387797	Snyder & Tkach (2001)
Haematoloechus abbreviatus (Bychowsky, 1932)	*Bombina variegata* (Linnaeus)	Amphibia	Zakarpatska Region, Ukraine	AF184251	Tkach et al. (2000)
Haematoloechus variegates (Rudolphi, 1819)	*Rana ridibunda* Pallas	Amphibia	Ivano-Frankivsk,Region, Ukraine	AF151916	Tkach et al. (1999)
Skrjabinoeces similis (Looss, 1899)	*Rana ridibunda*	Amphibia	Bulgaria	AY222279	Olson et al. (2003)
Ochesomatidae Leão, 1945					
Ochetosoma kansense Looss, 1899	*Drymarchon corais* (Holbrook)	Reptilia	Florida, USA	AF433671	Tkach et al. (2001)
Telorchiidae Looss, 1899					
Telorchis assula (Dujardin, 1845)	*Natrix natrix* (Linnaeus)	Reptilia	Kiev Region, Ukraine	AF151915	Tkach et al. (1999)

Masenia upeneusi Gupta & Puri, 1982, *Masenia carangai* Gupta & Tandon, 1984 and *Masenia orissai* Gupta & Tandon, 1984 are marine species with very long cirrus-sac extending beyond the ventral sucker (Gupta & Puri, 1982; Gupta & Tandon, 1984), while that of the new species extends anteriorly to ventral sucker. *Masenia moradabadensis* Srivastava, 1951 has a shorter body length and smaller size of the eggs than

Table 5 Sequence divergence based on total pairwise differences of 18S rDNA dataset for *Masenia nkomatiensis* n. sp. and related sequences

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*Auridistomum chelydrae*	–																	
2	*Telorchis assula*	13	–																
3	*Rubenstrema exasperatum*	8	12	–															
4	*Choanocotyle hobbsi*	8	15	10	–														
5	*Choanocotyle platti*	8	15	10	0	–													
6	*Choanocotyle hobbsi*	8	15	10	0	0	–												
7	*Choanocotyle nematoides*	8	15	10	0	0	0	–											
8	*Skrjabinoeces similis*	10	18	13	13	13	13	13	–										
9	*Haematoloechus longiplexus*	10	17	12	10	10	10	10	7	–									
10	*Mesocoelium* sp.	10	9	6	12	12	12	12	13	14	–								
11	*Brachycoelium salamandrae*	9	11	5	11	11	11	11	10	11	3	–							
12	*Mesocoelium lanfrediae*	12	10	8	14	14	14	14	15	16	3	5	–						
13	*Opisthioglyphe ranae*	13	13	15	17	17	17	17	18	19	16	16	18	–					
14	*Macroderoides typicus*	9	12	11	13	13	13	13	14	13	11	10	13	14	–				
15	*Glypthelmins quieta*	15	17	15	15	15	15	15	18	17	15	14	16	24	18	–			
16	*Cephalogonimus retusus*	11	10	8	15	15	15	15	15	17	12	11	12	12	14	20	–		
17	*Masenia bangweulensis*	30	28	27	30	30	30	30	29	32	28	27	30	30	30	36	24	–	
18	*Masenia nkomatiensis* n. sp.	17	17	14	21	21	21	21	21	23	18	17	20	19	20	27	9	29	–

Table 6 Sequence divergence based on total pairwise differences of 28S rDNA fragments among similar species of the Plagiorchiodea

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Choledocystus hepatica*	–														
2	*Telorchis assula*	48	–													
3	*Lechriorchis tygarti*	51	63	–												
4	*Ochetosoma kansense*	48	59	6	–											
5	*Haematoloechus longiplexus*	72	82	80	77	–										
6	*Skrjabinoeces similis*	72	84	81	78	27	–									
7	*Haematoloechus asper*	74	86	82	79	38	42	–								
8	*Haematoloechus variegatus*	76	80	85	82	65	62	71	–							
9	*Haematoloechus abbreviatus*	75	80	80	77	62	58	70	18	–						
10	*Haematoloechus breviplexus*	79	92	88	85	66	66	77	45	34	–					
11	*Haematoloechus complexus*	94	101	100	99	80	81	93	49	38	57	–				
12	*Haematoloechus meridionalis*	81	94	90	87	70	68	81	46	36	17	61	–			
13	*Cephalogonimus americanus*	67	79	87	86	93	99	102	88	95	105	111	102	–		
14	*Cephalogonimus retusus*	64	75	87	84	90	95	96	93	95	102	113	103	42	–	
15	*Masenia nkomatiensis* n. sp.	65	76	84	83	87	93	93	94	94	99	105	95	70	73	–

the new species. Moreover, this species has a tubular excretory vesicle and prostatic cells with a lateral diverticulum, rather than the sac-shaped excretory vesicle, and prostatic cells lacking lateral diverticulum in the new species.

Masenia quiloni (Gupta & Tandon, 1984) possesses vitelline fields joining centrally, posterior to the

ventral sucker, while in the new species the vitelline follicles form two, non-confluent lateral fields. In *Masenia pushpanjalii* Singh, Shankar, Singh & Gupta, 2006, *M. chauhani* Agarwal & Singh, 1989 and *Masenia jaunpurensis* Maurya & Singh, 2004, the vitelline fields extend from the level of the oesophagus to the region between the anterior and posterior margins of the anterior testis, while in the new species the vitellarium forms shorter fields, between the anterior region of the ventral sucker and mid-level of the ovary. Additionally, the caeca in *M. jaunpurensis* extend to the level of the anterior testis (Maurya & Singh, 2004), while in the new species the caeca reach the posterior testis. *Masenia ritai* Sircar & Sinha, 1970 possesses large vitelline follicles, extending between the mid-level of ventral sucker to the posterior margin of the anterior testis and a tubular excretory vesicle (Sircar & Sinha, 1970), rather than the large extension of vitelline fields and a sac-shaped excretory vesicle observed the new species.

Masenia vittatusia Agrawal, 1963, *Masenia collata* Chatterji, 1933, *Masenia gomtia* Agrawal, 1963 and *Masenia agarwali* Hasnain & Sahay, 1994 have an oral sucker smaller than ventral sucker and partly spinose tegument, distinct from the new species which possesses an oral sucker larger than ventral sucker and a tegument completely armed with spines, which decrease in number and size posteriorly.

Host specificity

Species of *Masenia* have a striking specificity for their definitive host, which are generally fishes of the Siluroidei. The African *Masenia* species have been described from three clariid hosts (*Clarias melandi* Boulenger, *C. senegalensis* Valenciennes and *Heterobranchus longifilis* Valenciennes) and one mochokid host (*Synodontis victoriae* Boulenger). Only *M. bangweulensis* has been recorded in *C. gariepinus* by El-Naggar et al. (1992), Zhokhov et al. (2017), Mwita & Nkwengulila (2004, 2008, 2010). The new species was found exclusively parasitising *C. gariepinus*.

Molecular analysis

A limited genetic dataset exists for *Masenia*, as well as for the family Cephalogonimidae. Total pairwise differences ranging from 9–73 bp for the two markers confirmed the distinctness of *M. nkomatiensis* n. sp. from exiting genetic data for cephalogonimids. According to the 18S topology, the new species formed a clade with *C. retusus* rather than with *M. bangweulensis*. However, based on this result, it can be suggested that the sequence for *C. retusus* was based on misidentified material and is actually representing a species of *Masenia*, or the genus concept for *Masenia* is poor. Pairwise divergence between the two *Masenia* species was 29 bp. In the 28S analysis, the new species also formed a clade with *Cephalogonimus* spp. with strong nodal support.

Jones & Bray (2008) transferred the genus *Masenia* from Maseniidae Yamaguti, 1954 to Cephalogonimidae (syn. Maseniidae), based on morphological features. In the present study, the close genetic relationship of *Masenia* and *Cephalogonimus* (type-genus) serves as additional evidence for supporting the inclusion of *Masenia* in the Cephalogonimidae. Although *Masenia* spp. are genetically close to *C. americanus* and *C. retusus*, they differ in having a collar of circumoral spines around the oral sucker, while species of *Cephalogonimus* lack circumoral spines (Jones & Bray, 2008). Furthermore, considering the biogeography, species of *Masenia* are restricted to continental Africa and Asia, while *Cephalogonimus* spp. are distributed through South and North America and Europe (Tkach et al., 2003).

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Compliance with ethical standards

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

Ethical approval The study was approved by the Ethics Committee of the Faculty of Science, University of Johannesburg, nr 2016-11-28/Dumbo and all procedures for collection, transportation and examination of fish comply with the ethical standard guidelines for manipulation and use of laboratory animals in South Africa. The collection permit for this study was issued by the Ministry of Sea, Inland Waters and Fisheries of

Mozambique, number 1148/590/GM-MIMAIP/SIC/2016 of 16 June 2016.

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