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First report of a fish blood fluke from sub-Saharan Africa: *Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard, 2023 infecting African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) Teugles, 1982 in the Kavango River, Namibia, and a revised phylogeny for Sanguinicolidae Poche, 1926

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ABSTRACT

We herein provide a supplemental description of *Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard, 2023 (Digenea: Sanguinicolidae) and provide a revised *28S* phylogeny to test relationships among freshwater fish blood flukes. We examined the heart of three African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) Teugles, 1982 from the Kavango River (northeastern Namibia) that was infected with adults of *N. dentata*. This blood fluke differs from *N. canthoensis* by having a body 5.3–6.7 longer than wide (vs. 3.5–4.6), an anterior esophageal swelling 7–8% (vs. 14–24%) of total esophageal length, a posterior esophageal swelling 3–5% (vs. 8–10%) of total esophageal length, a pre-cecal (vs. wholly post-cecal) testis, and an ovary that does not extend laterally beyond the nerve cords. The *28S* sequence for *N. dentata* differed from that of *N. canthoensis* by 144 bp (9% difference). The phylogenetic analysis recovered these species as sister taxa and Sanguinicolidae as monophyletic. This is the first report of a fish blood fluke from sub-Saharan Africa, and the first report of a species of *Nomasanguinicola* from Africa in ~40 yrs.

1. Introduction

Relative to North America and Mexico, African freshwater fishes are under-sampled for parasites (Smit et al. [1]; Scholz and Choudhury [2]), and the taxonomic descriptions of fish blood flukes from Africa are incomplete. In fact, no parasite has been reported from >80% of the fish species in the four largest African rivers (Niger, Nile, Congo, and Zambezi) and the African Great Lakes (Lakes Victoria, Malawi, and Tanganyika) (Smit et al. [1]). The two fish blood flukes (*Sanguinicola chalmersi* Odhner, 1924 and *Nomasanguinicola clarias* [*Imam*, Marzouk, Hassan, and Itman, 1984] Warren and Bullard, 2023) that have been reported from African catfishes (Siluriformes), both have incomplete morphological descriptions and lack nucleotide data. Further, both were collected from northern Africa (*S. chalmersi* [Sudan] and *N. clarias* [Egypt]) (Woodland [3]; Odhner [4]; *Imam* et al. [5]; Warren et al. [6]).

Presently, three species are assigned to *Nomasanguinicola* Truong and Bullard, 2013 (*Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard, 2023, *N. clarias, Nomasanguinicola canthoensis* Truong and

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Bullard, 2013) (Paperna [7]; *Imam* et al. [5]; Truong and Bullard [8]; Warren and Bullard [9]; Warren et al. [6]). *Nomasanguinicola clarias* is morphologically similar to, infects the same host (African sharptooth catfish, *Clarias gariepinus* [Burchell, 1822] Teugles, 1982), and was collected in the same geographic location (Egypt vs. Israel) as *N. dentata* (Paperna [7], Truong and Bullard [8]). However, *N. dentata* also has an incomplete and seemingly erroneous morphological description and lacks a nucleotide sequence. Hence, supplemental descriptions of both *N. dentata* and *N. clarias* are needed to determine if they should be synonymized.

Herein, we provide a supplemental description for *N. dentata* and present a phylogenetic analysis using the large subunit ribosomal DNA (*28S*) to support the morphological description and test relationships and monophyly of the Sanguinicolidae Poche, 1926. We also comment on the monophyly of catfish blood flukes.

2. Materials and methods

2.1. Fish collection, parasite specimen collection, preparation, and deposition

On 7 Dec 2021, three African sharptooth catfish (*C. gariepinus*) from the Kavango River (northeastern Namibia) were captured by hook and line and identified using Skelton [10] by having a mottled dorsal surface, a white ventral surface, three pairs of barbels (one pair dorsal, one pair lateral, one pair vertical), palantine teeth fused and inverse V-shaped, first and second dorsal fin rays half the length of third ray, dorsal fin rays (71), anal fin rays (53), pelvic fin rays (4), and pectoral fin rays (9) counts. The heart was excised intact, sliced longitudinally, immersed and shaken in saline, and examined with the aid of a dissecting microscope and fiber optic light source. The heart was teased apart with forceps to reveal adult blood flukes, and sediment from the fixed heart was taken from a settling column and examined. Adult specimens intended for morphology were observed microscopically, heat-killed on glass slides using a butane hand lighter under no coverslip pressure and fixed in 10% neutral buffered formalin (n.b.f.). Flukes collected for DNA extraction were wet mounted on glass slides and examined to confirm their identity, preserved in 95% ethanol (EtOH), and stored at −20 °C. Upon returning to the laboratory morphological specimens were rinsed with distilled water, stained overnight in Van Cleave's hematoxylin with 3 additional drops of Ehrlich's hematoxylin, dehydrated using an ethanol series, cleared in clove oil, permanently mounted in Canada balsam, illustrated using an Olympus BX51 microscope (Olympus Corporation of the Americas, Center Valley, Pennsylvania, USA) equipped with differential interference contrast (DIC), measured using a Jenoptik Gryphax camera (Jenoptik AG, Jena, Germany), and illustrated using a drawing tube. Measurements are reported in micrometers (μm) as the range followed by the mean, standard deviation, and sample size in parentheses unless otherwise indicated. Scientific names including taxonomic authorities and dates for fishes follow Eschmeyer et al. [11]. Classification and anatomical terms for fish blood flukes follow Warren and Bullard [9] and Warren et al. [6]. Vouchers were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D. C.).

2.2. DNA extraction, amplification, sequencing, and phylogenetic analysis

Two EtOH-preserved and microscopically identified fish blood flukes were used for DNA extraction and sequencing. DNA extraction, primers used, PCR amplification, sequencing, sequence assembly and analysis follow that of Warren et al. [6,12,13,25]. The phylogenetic analyses included the new freshwater fish blood fluke sequence and selected sequences representing species of sanguinicolids that were available on GenBank (Warren et al. [6]; Warren and Bullard [9]). The out-group comprises sequences representing the chimaerohemecids from Warren and Bullard [9] for the analysis. Sequences were aligned with the multiple alignment tool using fast Fourier transform (MAFFT) (Katoh and Standley [14]) and trimmed to the length of the shortest sequence presented herein (1362 [*28S*] base pairs [bp]). JModelTest 2 version 2.1.10 was implemented to perform statistical selection of the best-fit models of nucleotide substitution based on Bayesian Information Criterion (BIC) (Darriba et al. [15]). Aligned sequences were reformatted (from .fasta to .nexus) using the web application ALTER (Glez-Peña et al. [16]) to run Bayesian inference (BI). BI was performed in MrBayes version 3.2.7a (Ronquist and Huelsenbeck [17]) using substitution model averaging ("nst-mixed") and a gamma distribution to model rate-heterogeneity. Defaults were used in all other parameters. Three independent runs with 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al. [18]) and the "sump" command in MrBayes: all runs appeared to reach convergence after discarding the first 25% of generations as burn-in. A majority rule consensus tree of the post burn-in posterior distribution was generated with the "sumt" command in MrBayes. The inferred phylogenetic tree was visualized using FigTree v1.4.4 (Rambaut et al. [18]) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

3. Results

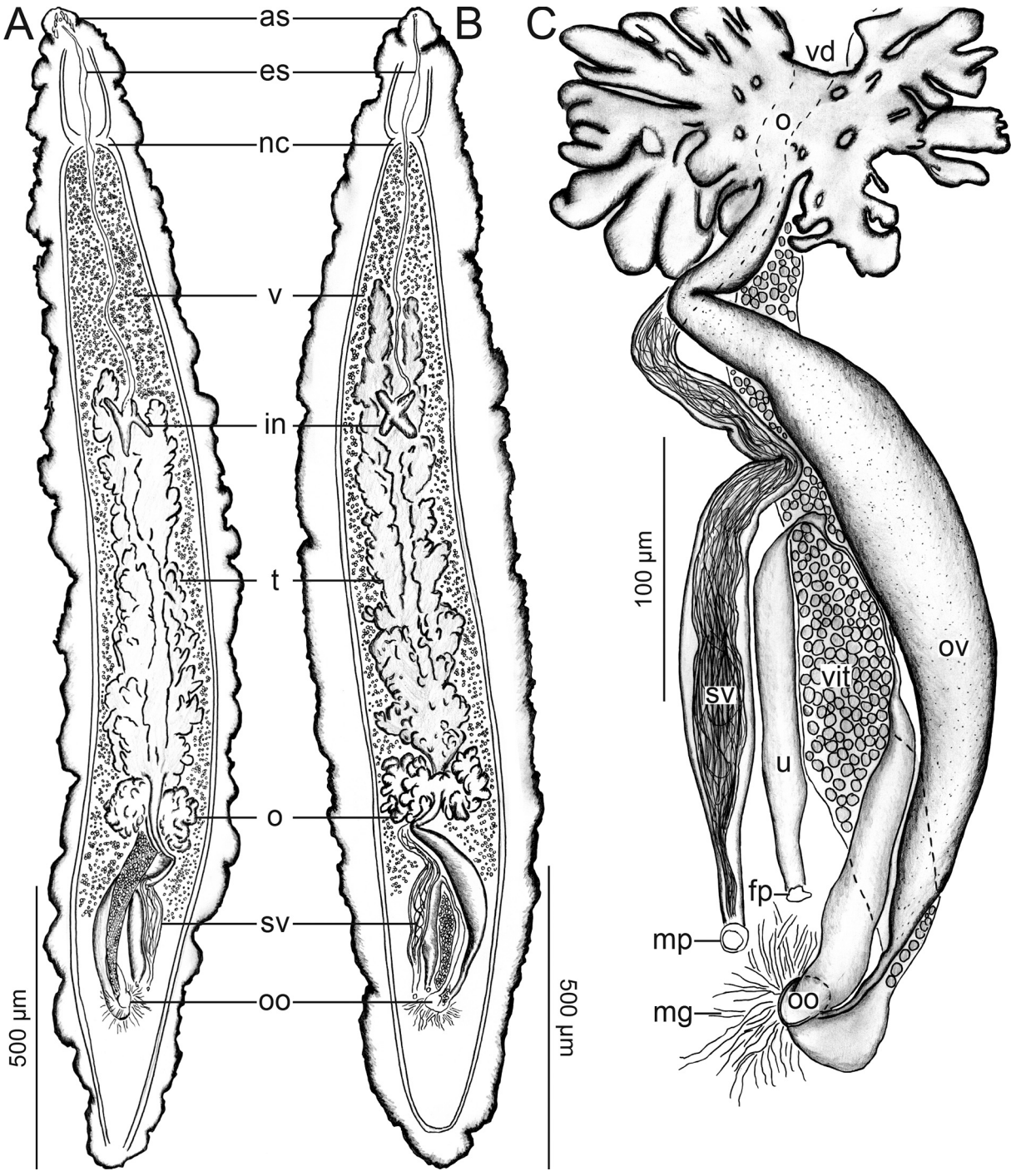
3.1. Nomasanguinicola dentata (Paperna, 1964) Warren and Bullard, 2023 (Figs. 1A–C, 2A–D)

3.1.1. Light microscopy of adult based on 4 whole-mounted adult specimens: USNM collection nos. 1606938–1606941)

Body elongate, ovoid, 1814–3249 (2229 ± 683, 4) long, 303–482 (364 ± 81, 4) in maximum width, 5.3–6.7 × longer than wide (Fig. 1A, B); lateral body margin having regularly spaced tegumental papillae, 2.3–8.6 (4.6 ± 3, 40) long, 2.1–16 (6.9 ± 6.4, 40) wide (Fig. 2D). Ventrolateral nerve cords 10–15 (12 ± 2, 4) wide near mid-body, 48–84 (59 ± 17, 4) or 14–17% of body width from body margin; secondary branches and dorsolateral nerve cords not evident; commissure of ventrolateral nerve cord 210–316 (243 ± 49, 4) or 10–12% of body length from anterior body end, 54–84 (67 ± 13, 4) across body width, 15–18 (16 ± 2, 4) in breadth, coursing dorsal to esophagus (Fig. 1A, B). Tegumental sensory papillae present, distributing across ventral and dorsal surface of body from level of anterior sucker to posterior end, approximately 4.2–6.4 (5.4 ± 0.9, 4) in diameter, cilia not observed.

Anterior sucker 38–57 (50 ± 8, 4) long, 55–72 (61 ± 8, 4) wide at base or 15–18% of body width, 1–1.9 × wider than long (Fig. 2A); terminal papillae on anterior margin of anterior sucker not observed. Denticles of anterior sucker 23–24 (23.6 ± 0.3, 4) in total length, 4–4.5 (4.2 ± 0.4, 4) in maximum width, extending 4 (7) from tegument; shaft 13–17 (15 ± 2, 4) long, 4.1–4.3 (4.2 ± 0.1, 4) in maximum width; aperture 8.8–10 (9.3 ± 0.7, 4); with each flanking column 28–41 (34 ± 6, 4) long or 56–79% of anterior sucker length (Figs. 2A–C). Mouth 3–4 (3.3 ± 1, 4) in diameter, medioventral, 13–16 (14 ± 1.5, 4) or 28–29% of anterior sucker length from anterior end, trilobate (Fig. 2A); pharynx not observed. Esophagus 600–960 (726 ± 160, 4) in total length or 30–36% of body length, beginning as narrow tube extending posteriad 94–143 (114 ± 22, 4) or 4–7% of body length before connecting with anterior esophageal swelling; anterior esophageal swelling 48–69 (56 ± 10, 4) long or 7–8% of esophagus total length, 10–14 (11 ± 2, 4) wide or 3–4% of maximum body width, at level midway between ventrolateral nerve commissure and anterior sucker; esophagus narrowing from anterior esophageal swelling and extending posteriad; posterior esophageal swelling immediately anterior to ceca 26–35 (30 ± 4, 4) long or 3–5% of esophageal total length, 20–33 (26 ± 6, 4) wide or 1–3% × maximum esophagus width (Figs. 1A, B). Esophageal gland indistinct.

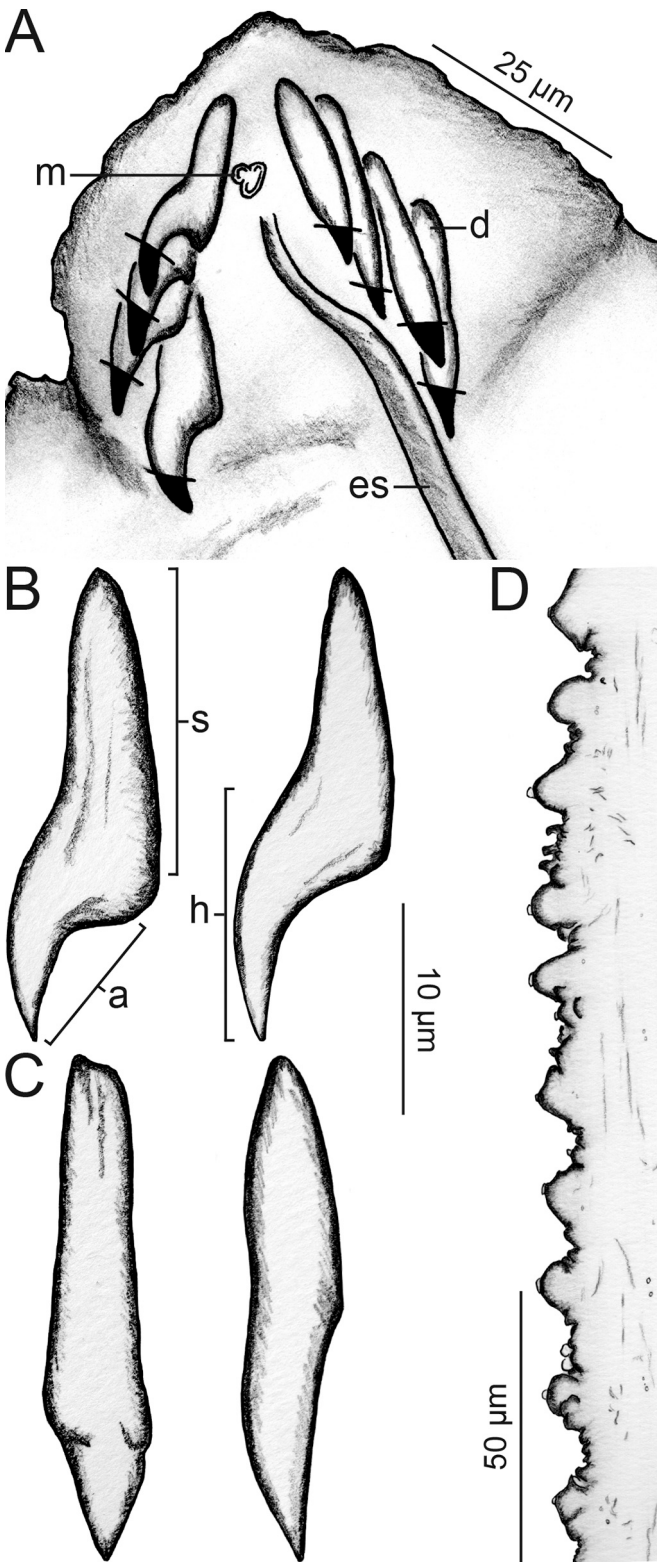
Intestine X-shaped, having 4 distinct radial ceca (Fig. 1A, B); cecal intersection 608–967 (732 ± 160, 4) or 30–36% of body length from



Figs. 1. *Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard, 2023 (Digenea: Sanguinicolidae Poche, 1926) infecting the heart of African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) Teugles, 1982 (Siluriformes: Clariidae) from the Kavango River (northeastern Namibia). Scale values aside bars. (**A**) Body of voucher (USNM No. 1606939) showing anterior sucker (as), esophagus (es), nerve commissure (nc), vitellarium (v), intestine (in), testis (t), ovary (o), seminal vesicle (sv), and oötype (oo). Ventral view. (**B**) Body of voucher (USNM No. 1606938). Dorsal View. (**C**) genitalia of voucher (USNM No. 1606938) showing vas deferens (vd), ovary (o), oviducal seminal vesicle (ov), vitelline duct (vit), seminal vesicle (sv), uterus (u), female genital pore (fp), male genital pore (mp), oötype (oo), and Mehlis' gland (mg). Dorsal View.

anterior body end; anterior ceca 33–54 (43 ± 11, 4) long or 2% of body length, 17–24 (19 ± 4, 4) wide, containing granular material within lumen of some individuals; posterior ceca 42–54 (47 ± 6, 4) long or 2–3% of body length, 20–24 (22 ± 2, 4) wide, ventral to testis; post-cecal space 1161–2253 (1465 ± 527, 4) long or 63–69% of body length.

Testis 697–1571 (931 ± 427, 4) long or 35–48% of body length, 107–165 (123 ± 28, 4) wide or 31–36% of body width, 6–10 × longer than wide, lobed, extending anterior to cecal bifurcation (Fig. 1A, B); post-testicular space 566–1039 (732 ± 210, 4) long or 29–36% of body length. Vasa efferentia comprising several dendritic ducts, 18–26 (21 ± 3, 4) wide. Vas deferens 122–205 (159 ± 35, 4) long, 9–17 (14 ± 3, 4) wide, emanating from postero-ventral portion of testis, curving sinistrally before sharply curving toward midline and becoming confluent with seminal vesicle. Cirrus-sac present, having wall approximately 5–9 (7 ± 1.7, 4) thick, including seminal vesicle, cirrus indistinct; seminal vesicle 158–503 (293 ± 148, 4) long, 27–51 (36 ± 11, 4) wide, 5–10 ×



Figs. 2. *Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard, 2023 (Digenea: Sanguinicolidae Poche, 1926) infecting the heart of African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) Teugles, 1982 (Siluriformes: Clariidae) from the Kavango River (northeastern Namibia). Scale values aside bars. (A) Anterior sucker of voucher (USNM No. 1606939) showing mouth (m), denticles (d), and esophagus (es). Ventral view. (B) Lateral view of denticles of voucher (USNM No. 1606939) showing shaft (s), crescent-shaped hook (h), and aperture (a). (C) Dorso-ventral view of denticles of voucher (USNM No. 1606939). (D) Lateral tegumental margin of voucher (USNM No. 1606941) showing lateral papillae.

longer than wide (Fig. 1A–C); male genital pore toward midline, 4–12 (8 ± 3, 4) in diameter, dorsal, post-ovarian, sinistral to and posterior to female genital pore, 285–377 (323 ± 40, 4) or 12–17% of body length from posterior body end.

Ovary medial, lobed, 116–299 (166 ± 89, 4) in maximum length or 6–9% of body length, 138–222 (168 ± 38, 4) wide or 42–49% of body width, immediately post-testicular; post-ovarian space 541–767 (605 ± 108, 4) long or 24–30% of body length (Fig. 1A–C). Oviduct arching in dextral half of body posterior to ovary and medial to cirrus sac, 405–616 (467 ± 100, 4) long or 19–23% of body length including oviducal seminal receptacle; oviducal seminal receptacle 207–290 (245 ± 41, 4) long or 47–62% of oviduct length, 38–79 (51 ± 19, 4) wide or 4–7 × longer than wide (Fig. 1C). Vitellarium appearing as loose follicles, occupying space dorsal and lateral to testis and ceca, extending from nerve commissure to seminal vesicle (Figs. 1A, B); collecting duct 799–1846 (1322 ± 740, 4) long, 27–55 (43 ± 13, 4) wide. Oötype 12–21 (17 ± 5, 4) long, 12–14 (13 ± 1, 4) wide; post-oötype space 258–321 (288 ± 32, 4) long or 10–14% of body length from posterior end (Fig. 1C). Mehlis' gland 65–94 (79 ± 14, 4) wide, surrounding oötype (Fig. 1C). Uterus inverse U-shaped, post-cecal, post-gonadal, occupying space between seminal vesicle and oviducal seminal receptacle; ascending uterus 168–373 (237 ± 93, 4) long or 8–12% of body length, 9–14 (12 ± 2, 4) in maximum width; descending uterus 152–347 (212 ± 91, 4) long or 84–93% of ascending uterus length, 16–56 (28 ± 19, 4) in maximum width (Fig. 1C). Female genital pore medial, dorsal, post-ovarian, dextral to and anterior to male genital pore. Excretory system indistinct.

3.1.2. Taxonomic summary

Type and only known host: African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) Teugles, 1982 (Siluriformes: Clariidae).

Type locality: Hule Nature Reserve, Israel.

Other localities: Kavango River, Namibia.

Prevalence and intensity of infection: One of 3 African sharptooth catfish was infected by 6 specimens of *N. dentata*.

Specimens deposited: Vouchers (USNM 1606938, 1606939, 1606940, 1606941).

Site of infection: Heart.

3.1.3. Taxonomic remarks

We identified the newly collected specimens herein as *N. dentata* by having an anterior sucker with two columns of denticles flanking the mouth, a lateral body margin having regularly spaced tegumental papillae, an esophagus with an anterior swelling, an intestine with 4 radial ceca, and a testis that extends anterior to the intestine. *Nomasanguinicola dentata* differs from *N. canthoensis* by having a body 5.3–6.7 longer than wide (vs. 3.5–4.6), an anterior esophageal swelling 7–8% (vs. 14–24%) of total esophageal length, a posterior esophageal swelling 3–5% (vs. 8–10%) of total esophageal length, a pre-cecal (vs. wholly post-cecal) testis, and an ovary that does not extend laterally beyond the nerve cords. The denticles of *N. dentata* should be viewed in lateral view to accurately illustrate the morphology of the denticle as having a distinct shaft and hook (which are both mostly embedded in the tegument). If the denticle is in frontal or dorso-ventral view, the denticle outline appears as elongate oval or pyriform (Figs. 2A, C) (Truong and Bullard [8]). Further, in the largest specimen of *N. dentata* examined (USNM 1606941), the denticles apparently lacked a pointed tip. Specimens of *N. canthoensis* likely have a similar denticle morphology but confirmation is needed as this character state is likely an important synapomorphy for the sanguinicolids.

The species identities of *N. dentata* and *N. clarias* are doubtful because no type specimen could be located for either species, the illustrations are superficial and incomplete, and both species are reported to infect *C. gariepinus* in Israel and Egypt (Paperna [7]; *Imam* et al. [5]; *Imam* and El-Askalany [19]). The species description of *N. clarias* includes the presence of lateral tegumental spines, but no spine was

illustrated in the original description. We cannot be certain of whether this description represents a valid species or if it is conspecific with *N. dentata*. Because of that, we consider *N. clarias* as a *species inquirenda* until a new specimen from Egypt is collected and studied (*Imam* et al.'s [5]). A neotype should be designated for *N. clarias* as well as *N. dentata*.

3.2. Phylogenetic analysis

The amplified *28S* fragments representing the two specimens of *N. dentata* were identical and comprised 1546 nucleotides. They differed from that of *N. canthoensis* by 144 bp (9% difference). The new sequences differed from species of *Sanguinicola* (*Sanguinicola volgensis* [Rašín, 1926] McIntosh, 1934, *S.* cf. *volgensis*, *Sanguinicola plehnae* Warren and Bullard, 2023) and *Pseudosanguinicola occidentalis* (Van Cleave and Mueller, 1932) Warren and Bullard, 2023 by >350 bp (22% difference). The phylogenetic analysis recovered *N. dentata* and *N. canthoensis* as sister taxa and sister to two innominate sanguinicolids that infect two other catfishes in the Mekong River, Vietnam (GenBank numbers: OQ709105, OQ709106) as well as a monophyletic Sanguinicolidae (see Warren and Bullard [9]; Fig. 3). *Nomasanguinicola dentata* differs from the innominate species infecting *Pangasius* cf. *macronema* (Siluriformes: Pangasiidae) (OQ709106) and *Mystus* cf. *mysticetus* (Siluriformes: Bagridae) (OQ709105) by >165 bp (> 10% difference) nucleotides. This monophyletic catfish-infecting clade is recovered sister to all other species assigned to Sanguinicolidae.

4. Discussion

Presently, seven families of catfishes are known hosts for sanguinicolids (Pimelodidae Swaison, 1838; Clariidae Bonaparte, 1846; Bagridae Bleeker, 1858; Pangasiidae Bleeker, 1858; Auchenipteridae Bleeker, 1862; Claroteidae Bleeker, 1862; Mochokidae Jordan, 1923). These records are from Africa, South America, and Asia (Woodland [3]; Odhner [4]; Szidat [7]; Wang [20]; *Imam* et al. [5]; Lunaschi [21]; Paperna [7]; Truong and Bullard [8]; Orélis-Ribeiro and Bullard [22,23]). No record of a catfish blood fluke exists from a marine catfish (Ariidae), and none has been reported from North America or Australia. The sequences generated for the present study and those by Warren and Bullard [9] ($n = 4$) are the only available catfish blood fluke sequences. Given the diversity of siluriforms (4159 species assigned to 501 genera of 40 families) (Fricke et al. [24]), there are likely several distinct freshwater fish blood fluke lineages awaiting discovery. For example, the innominate sanguinicolid that infects *M.* cf. *mysticetus* from Vietnam (OQ709105) has an anterior sucker with two rows of denticles flanking the mouth and lateral tegumental spines. However, the denticles associated with this innominate species are in rows of 5 (vs 4) (Truong and Bullard [8]; present study) and lateral tegumental spines are only described from *N. clarias*, *S. chalmersi*, and *S. sanliense* (*Imam* et al. [5]; Wang [20]; Warren et al. [6]). The other innominate sanguinicolid infecting *P.* cf. *macronema* from Vietnam (OQ709106) is further unique by lacking spines or denticles.

All known catfish blood flukes represented by nucleotides share a common ancestor (Fig. 3), objectively suggesting a high level of phylogenetic host specificity of this lineage of blood flukes to the catfishes. This level of phylogenetic host specificity will be tested as additional blood fluke species are discovered and described. Based on available evidence, there is no pattern of cophyly between freshwater fishes and their blood flukes (Warren and Bullard [9,25]; Betancur et al. [26]). In the future, freshwater sanguinicolids should be the focus from Africa and elsewhere as they will likely reveal several unknown lineages that can better the systematics of the group.

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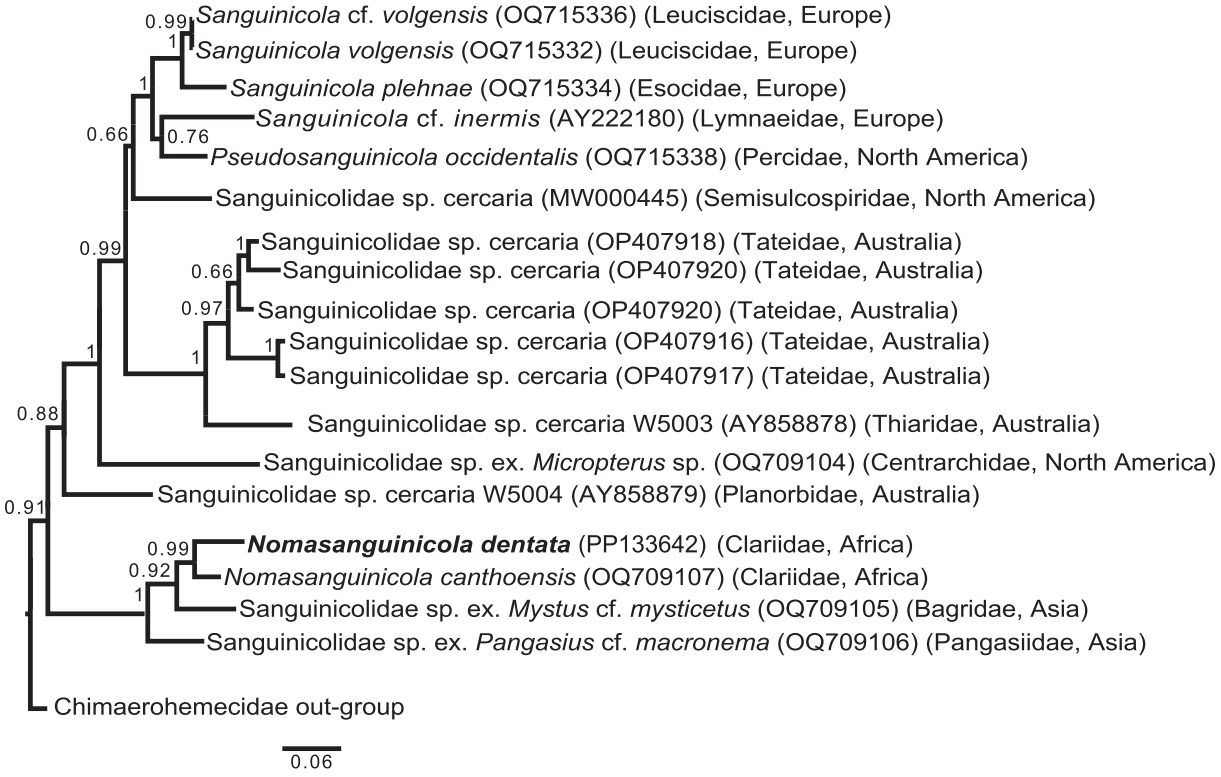

Fig. 3. Phylogenetic relationships of freshwater fish blood fluke species assigned to Sanguinicolidae Poche, 1926. Reconstructed using Bayesian inference analysis using the large subunit ribosomal DNA (*28S*) gene. New sequence is shown in bold and GenBank accession number, host family, and continent locality aside taxa.

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CRediT authorship contribution statement

Micah B. Warren: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Francois Jacobs:** Resources. **Haley R. Dutton:** Resources, Data curation. **Edward C. Netherlands:** Resources. **Louis H. DuPreez:** Resources, Project administration, Data curation. **Stephen A. Bullard:** Supervision, Resources, Project administration, Funding acquisition, Data curation.

Declaration of competing interest

No author has a competing interest.

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