Underreported and taxonomically problematic: characterisation of sanguinicolid larvae from freshwater limpets (Burnupiidae), with comments on the phylogeny and intermediate hosts of sanguinicolids

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Abstract

Blood flukes of freshwater fish are understudied worldwide. Consequently, genetic information and data on their intramolluscan stages are scarce. In the current study, freshwater limpets of the genus *Burnupia* (Burnupiidae) from South Africa were examined for digeneans. Of 1645 specimens, 3.10% were infected by Sanguinicolidae larvae. Four sanguinicolids were distinguished by body size, number of penetration glands, tegumental spines’ patterns and relative sizes of the finfolds on the body and furcae. Analyses of 28S, 18S and ITS rDNA sequences showed that the morphotypes were distinct from each other and from sanguinicolids whose genetic data are available. The present study is the first genetic characterisation of sanguinicolids from Africa. Phylogenetic reconstruction revealed that the present species clustered with a sanguinicolid from Poland and were sister to *Sanguinicola* and *Pseudosanguinicola* from Russia and USA, respectively. The results indicate that the current species represent an unknown genus. What is more, blood fluke sequences from East Africa (presumed to be sanguinicolids), were distant from Sanguinicolidae and showed a closer relationship with acipensericolids from USA. Freshwater fish blood flukes seem to be more diverse than previously recorded and use species of at least thirteen gastropod families as intermediate hosts.

**Key words:** Blood flukes, brevifurcate cercaria, lophocercous-apharyngeate, rDNA sequences, scanning electron microscopy, *Burnupia*, biodiversity, Vaal River, Crocodile River
Introduction

Fish blood flukes have two-host life cycles in which cercariae from the intermediate hosts (gastropods, bivalves and polychaetes) penetrate the definitive hosts directly (Frandsen and Christensen, 1984; Warren and Bullard, 2019). The adult flukes inhabit the host's blood vessels, heart, liver and other highly vascularised organs (Paperna, 1996; Bullard and Overstreet, 2008). The flukes are associated with losses in aquaculture due to mortalities of the infected fish (Paperna, 1996; Bullard and Overstreet, 2008). The taxonomy of these important flukes has been problematic due to the paucity of genetic data and inadequate descriptions of putative species. Until recently, all blood flukes of fish were lumped in the family Aporocotylidae Odhner, 1912. However, 28S rDNA phylogenetic analyses of all the available sequences of marine and freshwater fish blood flukes revealed five distinct lineages (Cutmore et al. 2023). Consequently, based on morphological characteristics and genetic data, Warren and Bullard (2023) revised fish blood flukes into five families: Aporocotylidae Odhner, 1912; Sanguinicolidae Poche, 1926; Chimaerohemecidae Yamaguti, 1971; Acipensericolidae Warren and Bullard, 2023 and Elopicolidae Warren and Bullard, 2023.

Compared with their marine counterparts, blood flukes of freshwater fish are understudied (Zhokhov et al. 2021). Species of Sanguinicolidae and Acipensericolidae predominantly infect freshwater fishes (Warren and Bullard, 2023). Unlike acipensericolids that have been reported only from USA, sanguinicolids occur in Africa, Asia, Australia, Europe, North and South America (Orélis-Ribeiro and Bullard 2015; Zhokhov et al. 2021; Warren et al. 2023; Warren and Bullard, 2023). Despite being widespread, data on the taxonomy and ecology of sanguinicolids is scanty. According to Zhokhov et al. (2021), the deficiency of data might be due to their small size (1-2 mm), transparent nature and because they are easily lost through the breakage of blood vessels during parasitological examination of fish. What is more, there is little data on larval stages of fish blood flukes, and the intermediate hosts are largely
unknown. This can be attributed to difficulties in identifying their larvae which often have a very low prevalence in the intermediate hosts (Bullard and Overstreet, 2008). In addition to methodological challenges that hinder examination of sanguinicolids, there are very few parasitologists who study fish blood flukes (Warren et al. 2023).

Genetic data of adult sanguinicolids are available only for seven species. They are: *Sanguinicola volgensis* (Rašín, 1929) and *Sanguinicola plehnae* Warren and Bullard, 2023 from Russia; *Nomasanguinicola canthoensis* Truong and Bullard, 2013 and two unidentified Sanguinicolidae spp. from Vietnam; *Pseudosanguinicola occidentalis* Warren and Bullard, 2023 and unidentified species from USA (Warren et al. 2023; Warren and Bullard, 2023). In addition, DNA data are available for cercariae of 12 sanguinicolids from freshwater gastropods from Australia, East Africa, Poland and USA (Brant et al. 2006; Cutmore et al. 2023; Olson et al. 2003; Zemmer et al. 2020; Preston et al. 2021). However, the identities of some of the cercariae whose sequences are available, remain uncertain. For instance, Orélis-Ribeiro et al. (2014) raised concerns about the identity of *Sanguinicola cf. inermis* from Poland (Olson et al. 2003) due to the absence of morphological data. Also, there were significant morphological variations among the cercariae from East Africa, that were described by Brant et al. (2006), indicating that they belong to separate and distant taxa. Herein, detailed descriptions of sanguinicolid cercariae from south African freshwater limpets are presented. The taxonomic study of the specimens was based on the integration of morphometric descriptions using light and scanning electron microscopy (SEM) and genetic characterisation. Moreover, phylogenetic reconstruction has been used to explain the relationship between the present isolates and the other blood flukes of freshwater fish, for which DNA sequences are available.

Most investigations on gastropod-trematode associations in Africa have focussed on selected snails belonging to the families Bulinidae, Planorbidae, Lymnaeidae and Thiaridae (Brown, 1994; Outa et al. 2020). Other groups of snails, particularly limpets of the family...
Burnupiidae are understudied. Burnupiids are small sized limpets whose shells rarely reach 10 mm in length (Brown, 1994). The family has one genus (*Burnupia* Walker, 1912), from which 22 species have been described (MolluscaBase eds., 2021). Except for *Burnupia ingae* Lanzer, 1991 from Brazil, the distribution of *Burnupia* spp. is restricted to eastern, central and southern Africa (Bouchet *et al.*, 2017). Since they occur in submerged habitats and due to their small size, burnups often go unnoticed in field surveys (De Kock and Wolmarans, 2009; Outa and Avenant-Oldewage, 2023). To our knowledge, there are only two studies on the digeneans that are transmitted by burnupiids. Faust (1926) described a xiphidiocercaria and a brevifurcate, monostome, apharyngeate-lophocercous cercaria from *Burnupia trapezoidea* (Boettger, 1910) and *Burnupia capensis* (Walker, 1912) in South Africa. Porter (1938) reported nine cercarial types in *Burnupia verreauxii* (Bourguignat, 1853), *Burnupia gordonensis* (Melvill & Ponsonby, 1903), *Burn. trapezoidea* and *Bur. capensis* from selected water bodies in South Africa. The digeneans reported by Faust (1926) and Porter (1938) were classified in the placeholder genus ‘*Cercaria*’ based on cercarial morphology. Consequently, the species identity, genera and families of those digeneans, remain unresolved. Unfortunately, nearly nine decades since the original studies (Faust, 1926; Porter, 1938), digeneans of African limpets have not been revisited. The current study is a report of four new lineages of Sanguinicolidae from *Burnupia transvaalensis* (Craven, 1880), *Burnupia mooiensis* (Walker, 1912) and *Burn. trapezoidea*, which are endemic in South Africa.

**Materials and methods**

**Sampling and morphological analyses**

The study was conducted in two river systems in south Africa: Vaal River in the Orange River System and Crocodile River, which is part of the Limpopo River system. Snails were collected from four sites, two each from the Vaal and Crocodile River (Fig. 1). Site 1 is situated below
the Vaal Dam wall (26.872364 °S, 28.117173 °E); Site 2 is located below the Vaal River Barrage Reservoir (26.734854 °S, 27.634372 °E); Site 3 is at Lake Heritage (an impoundment on the Crocodile River) (25.959696 °S, 27.855555 °E) and Site 4 is in the river, below Lake Heritage (25.957086 °S, 27.858308 °E). Snails were collected from the sampling sites in summer and autumn (February-May) of 2022 and 2023. Sampling followed the procedures outlined by Outa and Avenant-Oldewage (2023). Accordingly, snails were handpicked from submerged reed stems, pebbles and boulders. Specimens were placed in 10L plastic buckets (50 individuals per container) half-filled with water from the sampling sites. Snails were protected from direct sunlight and kept aerated by partially covering the buckets. All specimens were transferred to an onsite field laboratory, identified based on shell morphology (Craven, 1880; Walker, 1912; Connolly, 1939; Brown, 1994) and examined for digeneans within 24 h of sampling.

Screening of snails for digeneans followed the methods described by Frandsen and Christensen (1984). Digenean larvae were isolated and studied alive in temporary mounts; stained with Nile blue or unstained. Illustrations of the specimens were made with the aid of a drawing tube and digitised on Corel DRAW ® Graphics Suite X6 software (Corel Corporation, Ottawa, Canada). Ten sporocysts and 30 cercariae, of each morphotype were preserved in 70 % ethanol for further morphological examination. Five to 10 specimens of each morphotype (from each snail), that had been examined in unstained mounts were transferred into 96 % ethanol for molecular analyses. Soft tissues of the snails (that harboured parasites) were preserved in 96 % ethanol for genetic identification. For morphometric analyses, sporocysts and 20 cercariae of each morphotype were processed following the procedures of Helmer et al. (2023). Representative morphotypes were studied using a Zeiss Axioplan 2 epifluorescence microscope and measurements were obtained using AxioVision 4.3 imaging software (Göttingen, Germany).
Ten cercariae of each morphotype were prepared for SEM based on the methods described by Nation (1983). Representative specimens were dehydrated through 70%, 80%, 90%, 96% and 100% ethanol. Subsequently, the specimens were transferred into a succession of 40%, 70% and 100% hexamethyldisilazane (Merck, Darmstadt, Germany), for five-seven minutes in each concentration. Specimens were mounted on adhesive conductive carbon tapes, fixed on copper stubs. Stubs with mounted specimens were placed in a Sanpla dry keeper desiccator cabinet (Kita-ku, Osaka, Japan) for 24 h. Mounted specimens were then sputter coated with gold, using an Emscope SC500 (Quorum Technologies, Newhaven, UK). Surface features of the specimens were studied using a Vega 3 LMH, Tescan (Brno, Czech Republic) scanning electron microscope at 6 kV.

**Genetic characterisation and phylogenetic analyses**

For genetic analyses, specimens (preserved in 96% ethanol) were dried, and DNA was extracted using E.Z.N.A.® Tissue DNA Kit (Omega, Bio-teck, Inc, Georgia, USA), following the manufacturer’s instructions. Genetic identification of the snails was based on analyses of mitochondrial CO1 gene. Partial fragments of CO1 gene were amplified using primers LCO1490 (5’-GGTCAACAAATCATAAGGATTTCGG-3’) and HCO2198 (5’-TAAACTTCAGGGTGACCAAAAAATCA-3’) (Folmer et al. 1994), following the PCR profile set by Albrecht et al. (2004). Digeneans were characterised using 28S, 18S and ITS rDNA. Partial sequences of 28S rDNA were amplified using primers dig12 (5’-AAGCATATCAAGGCGG-3’) and 1500R (5’-GCTATCCTGAGGAAAAACTTCG-3’) (Tkach et al. 2003). PCR profile (Tkach et al. 2003) was modified by adjusting the initial denaturation time (5 min), annealing (52 °C for 1 min) and the final extension (10 min). For 18S rDNA, primers 18S-E (5’-CCGAATTCGTCGACAACCTGGTGATCCTGCCAGT-3’) and WormB (5’-CTTGTTACGACTTTACTTCC-3’) (Littlewood and Olson, 2001) were
used, following the PCR conditions set by Králová-Hromadová et al. (2008). For ITS rDNA, fragments comprising of ITS1-5.8S-ITS2 were amplified using 81-f (5’-GTAACAAGGTTTCCGTTAGGTGAA-3’) (Gustinelli et al. 2010) and ITS2.S (5’-CCTGTTAGTTTTTCTCCG-3’) (Cribb et al. 1998). PCR profile set by Gustinelli et al. (2010) were modified by increasing the time for initial denaturation (3 min) and annealing (45 s).

Gel electrophoresis was performed using a SmartDoc™ 2.0 ultra-violet transilluminator (Benchmark Scientific, NJ, USA). Amplification of the fragments was verified visually in 1 % agarose gel loaded with SafeView™ FireRed (abm) stain. DNA sequencing was done using PCR forward and reverse primers, and the generated sequences were inspected, edited and aligned using Geneious Prime 2020.2.2, according to the procedures outlined by Kearse et al. (2012). Sequences were run through BLAST on GenBank, to find the nucleotide sequences with the closest similarity to the sequences generated in the present study. Sequences from the present study and those from GenBank were aligned and trimmed using MEGA7 software. Distances and differences in number of base pairs, between the aligned sequences were compared following Tamura et al. (2013).

Phylogenetic analyses were performed using 28S rDNA sequences since there are more fish blood fluke representative DNA sequences, compared with 18S and ITS rDNA. Maximum likelihood (ML) and Bayesian inference (BI) methods were performed, using final alignments of 1078-1221 bp. In addition to the sequences generated from the present study, the available GenBank sequences (25) of freshwater fish blood flukes were used. Species of the family Clinostomidae: Clinostomum complanatum (accession no. LC483164 and MH491531) and Euclinostomum spp. (accession no. MW604803-06), were used as outgroup. The alignment was run through a model estimation tool on MEGA7 to select appropriate nucleotide substitution model. ML reconstruction was done in MEGA7 using general time reversal (GTR).
model for nucleotide substitution, with five categories of discrete gamma (G) distribution. Phylogenetic BI reconstruction was done in BEAST v2.5.0 (Bouckaert et al. 2014) using GTR model and by applying 10 million Markov chain Monte Carlo (MCMC) analysis. In both ML and BI analyses, the estimations of nodal support values were based on 1000 bootstrap replicates.

Results

Prevalence and morphological descriptions

A total of 1645 specimens of Burnupia spp. were examined, of which 3.10% harboured furcocercariae. The cercarial characteristics: oral sucker modified into a cephalic penetration organ, aphyaryngeate, presence of a dorsal finfold on the body, furcae shorter than the tail stem (brevifurcate) and symmetrical, presence of dorsoventral finfolds on furcae and absence of a ventral sucker, were consistent with members of the family Sanguinicolidae (Erickson and Wallace, 1959; Schell, 1974; Simon-Martin et al. 1987; Kirk and Lewis, 1993; Sendersky and Dobrovolsky, 2004; Faltýnková et al. 2007). Detailed morphological and molecular studies of the specimens revealed that they represented four species. Co-occurrence of different sanguinicolid species in individual snails was not observed. Table 1 shows the prevalence of each sanguinicolid in the hosts from the sampling sites. The digeneans have been designated Sanguinicolidae gen. sp. 1 ZA, Sanguinicolidae gen. sp. 2 ZA, Sanguinicolidae gen. sp. 3 ZA and Sanguinicolidae gen. sp. 4 ZA. Descriptions of the parthenitae and cercariae are provided below. Morphometric measurements of the cercariae for each species are presented in Table 2. All measurements are given in micrometres; presented as mean values, and the ranges are in parentheses.
Sanguinicolidae gen. sp. 1 ZA

Localities: Vaal River, below Vaal Dam and below Vaal Barrage, (Orange River system) South Africa.

Host: *Burnupia transvaalensis* (Craven, 1880) and *Burnupia mooiensis* (Walker, 1912); (Gastropoda: Burnupiidae).

Genetic material: The representative 28S, 18S and ITS rDNA sequences; accession number ZZZ, respectively.

Descriptions: Sporocysts found embedded in digestive gland. Sporocysts ovoid, 275 (251-301) x 194 (178-214), contain three to four developed cercariae (Fig. 2A). Cercarial body curved ventrad, bearing dorsal finfold on convex side (Fig. 2C and 3A). Finfold pleated and aspinous, visible in live stained and unstained specimens, indistinct in some fixed specimens. Finfold originates in second quarter of body, extends over 68 (60-74) % of dorsal body surface; height, 28 (25-31) % of body width. Anterior part of body modified into ovate cephalic penetration organ, bearing protrusible tip. Transverse constriction of tegument separates cephalic organ from main body. Numerous cystogenous glands obscure internal body structures. Mouth opening discernible using SEM, subapical, located on ventral side of cephalic organ (Fig. 3C). Digestive tube indistinct; pharynx and ventral sucker absent. A pair of round structures resembling non-pigmented eyespots present posterior to apical organ on either side of body in some specimens. Six to eight (usually seven) pairs of penetration glands visible in stained and unstained specimens (Fig. 2C). Ducts from penetration glands extend laterally to cephalic organ apex. Excretory bladder present at posterior end of body. Excretory ducts undiscernible on tail stem, visible in each furca, open at furcal tip (Fig. 2D and 3G). Body tegument characterised by prominent dorsal and lateral longitudinal ridges. Ventral transverse folds present near posterior end of body (Fig. 3A). Tegument spinous; spines arise from furrows between longitudinal ridges. Spines pointed posteriorly, arranged in 32-35 concentric rows.
from anterior end to about 70% of body length. Cephalic organ bears seven circlets of spines: five on anterior part, two on posterior part and mid region devoid of spines (Fig. 3C). Anterior circlets of cephalic organ closely spaced, bear robust, sharp spines; posterior circlets widely spaced (three-four times wider than anterior circlets), bear slender spines. Tail stem straight, 1.8 (1.7-1.9) times longer than body. Dorsal longitudinal furrow extends posteriorly from tail base to about 70% of stem length. Tail stem bears posteriorly pointed slender spines and sensilla that arise from dorso-ventrally located papillae (Fig. 3D and E). Furcae, 2.6 (2.4-3.0) times shorter than tail stem; bear dorso-ventral finfolds. Finfolds indistinct in some fixed specimens when observed with light microscope. Each furca has 12-13 transverse rows of spines on lateral sides, from base to about 80% of furcal length (Fig. 3F). Furcal tip tapered, 18 (16-20) long, with two transverse constrictions (Fig. 2D); latero-dorsal sensillum present near tip (Fig. 3G).

Remarks

The cercaria differs from the other cercarial types from the current study (See Sanguinicolidae gen. sp. 2 ZA, Sanguinicolidae gen. sp. 3 ZA and Sanguinicolidae gen. sp. 4 ZA, described below) based on longer body finfold, relative to the body; longer furcae; more penetration glands; fewer spine-bearing circlets on the cephalic penetration organ and absence of spines in middle part of the cephalic organ. Compared with previous studies from Africa, the present cercaria resembles *Cercaria capensis* which was described by Porter (1938) from *Bur. capensis* in Oudtshoorn, South Africa. However, *Cercaria capensis* is produced in oblong and large sporocysts (350-500 long, 50-90 wide); different to the present species whose sporocysts are ovoid and smaller (251-301 long, 178-214 wide). What is more, the finfold of *Ce. capensis* extends from the cephalic organ to near the posterior end of the body (Porter, 1938, Plate LXXVII, Fig 3). In the present cercaria, the finfold originates posterior to the cephalic
penetration organ and covers approximately three quarters of the body length. Based on the features of the dorsal finfold and the numbers of large gland cells in the body, the present cercaria resembles four other digeneans from North America and Europe. These are: *Sanguinicola idahoensis* Schell, 1974 from *Fluminicola virens* (Lea, 1838) (Lithoglyphidae), *Sanguinicola lophophora* Erickson & Wallace 1959 and *Sanguinicola cristafer* Erickson & Wallace, 1959 from *Valvata* spp. (Valvatidae) in USA; *Sanguinicola armatus* Plehn, 1905 from *Lymnaea stagnalis* (Linnaeus, 1758) and *Sanguinicola* sp. from *Valvata macrostoma* Mörch, 1864 in Finland (Faltýnková et al., 2007). Indeed, the numbers of large gland cells in the body are nearly identical between the species: six to nine pairs in *S. lophophora* and *S. cristafer* (Erickson and Wallace, 1959), at least six pairs in *S. idahoensis* (Schell, 1974), eight pairs in *S. armatus* (Sendersky and Dobrovolsky, 2004), eight to 11 pairs in *Sanguinicola* sp. (Faltýnková et al. 2007), and six to eight pairs in the current specimens. However, unlike in the current specimens where the excretory bladder is nearly round, the excretory bladder is bilobed in *S. idahoensis* (Schell, 1974), V-shaped in *Sanguinicola armatus* (Sendersky and Dobrovolsky, 2004) and Y-shaped in *Sanguinicola* sp. (Faltýnková et al. 2007). *Sanguinicola lophophora* and *S. cristafer* are distinguished by the presence of long dorso-ventral caudal hairs (observed in living cercariae); a feature that was not observed on the cercaria from the present study. Finally, the present cercaria is distinguished from the other digeneans based on body length, relative to the dimensions of the tail stem and furcae (Table 2).

*Sanguinicolidae* gen. sp. 2 ZA

Localities: Vaal River, below Vaal Dam and below Vaal Barrage, (Orange River system) South Africa.

Host: *Burnupia transvaalensis* (Craven 1880) and *Burnupia mooiensis* (Walker, 1912); (Gastropoda: Burnupiidae).
Genetic material: The representative 28S, 18S and ITS rDNA sequences; accession number ZZZ, respectively.

Descriptions: Sporocysts found buried in digestive gland; difficult to separate from host’s tissues. Sporocysts ovoid, 418 (394-438) x 315 (302-334); bearing two to three germ balls and two developed cercariae (Fig. 4A). Cercarial body curved ventrally, bearing pleated, aspinous dorsal finfold (Fig. 4C). Finfold originates in second third of body, extends over 55 (50-61) % of dorsal body length; height, 47 (41-53) % of body width. Finfold observable in live specimens, nearly invisible in some preserved specimens when examined optically. Anterior part of body modified into pear-shaped cephalic penetration organ bearing protrusible tip. Cephalic organ differentiated from main body by transverse constriction of tegument. Mouth opening, visible with SEM, subapical, located on ventral side of cephalic organ (Fig. 5B). Intestine like tube extends from posterior part of cephalic organ to about half of body length. Pharynx and ventral sucker absent. Body bears a pair of poorly visible glands posterior to cephalic organ and five prominent pairs of penetration glands, arranged along body length, covering nearly same distance as body finfold (Fig. 4C). Genital primordium comprises of discoid mass of cells between posterior pair of penetration glands and excretory bladder (Fig. 4C). Excretory duct extends from excretory bladder, bifurcates at posterior end of tail stem, into two canals that terminate at lateral pores on furcal tips (Fig. 4C, D and 5G). Tegument bears well-defined longitudinal ridges, papillae and spines. Apex of cephalic organ lacks spines: bears circlets of ciliated and sheathed unciliated papillae (Fig. 5B). Tegumental spines pointed posteriorly, arranged in 34-35 concentric rows from anterior to about two thirds of body. Cephalic organ bears 11 circlets of spines: anterior five circlets densely spaced, consist of robust spines; posterior six circlets widely spaced, about two times wider apart than anterior circlets, bear slender spines, like spines on main body (Fig. 5B and C). Tail stem straight, 1.6 (1.3-1.7) times longer than body; bears posteriorly pointed slender spines and sensilla that arise
from dorso-ventral papillae (Fig. 5D and E). Furcae 3.1 (2.9-3.4) times shorter than tail stem, surrounded by dorso-ventral finfolds. Furcal finfolds visible in live specimens, indistinct in some fixed specimens when observed using light microscope. Lateral sides of furcae bear slender, sharp spines, densely arranged in 11-12 transverse rows, from base to about three quarters of furcal length (Fig. 5F). Single latero-dorsal sensillum present near tip of each furca (Fig. 5G); furcal tip attenuated, 12 (10-14) long.

Remarks
Cercaria of Sanguinicolidae gen. sp. 2 ZA differs from the other species in the present study based on its longer body (Table 2). Also, it bears 5 pairs of penetration glands, which is fewer than in the above species and more in number than in the two species described below. Finally, it bears 11 rows of spines on the cephalic organ, which is higher in number than in Sanguinicolidae gen. sp. 1 and Sanguinicolidae gen. sp. 4, but less than in Sanguinicolidae gen. sp. 3. Based on the presence of five prominent pairs of penetration glands, the present specimens resemble *Cercaria indicanonoides* reported by Porter (1938), from the bulinid, *Bulinus africanaum* (Krauss, 1848) in Sydenham, near Durban, South Africa and *Sanguinicola rutili* from a planorbid, *Ancylus fluviatilis* Müller, 1774 in Spain (Simon-Martin *et al.* 1987). The present species is characterised by large sporocysts (394-438 long), which are about double the size of mature sporocysts of *C. indicanonoides* (102-220 long) (Porter, 1938) and *S. rutili* (190-320 long) (Simon-Martin *et al.* 1987). Secondly, *Cercaria indicanonoides* is ocellate, a feature that distinguishes it from the present cercaria. Also, Sanguinicolidae gen. sp. 2 SA has a shorter finfold that originates in the second third of the body and terminates before the posterior end. In *C. indicanonoides*, the finfold originates at the constriction that demarcates the cephalic organ and extends to the posterior end of the body (Porter, 1938, Plate LXXX, Fig. 3). *Sanguinicola rutili* is distinguished by numerous long setae on the caudal stem, which
are absent in the present specimens. Finally, *S. rutili* has a smaller body and shorter tail stem compared with the present cercaria (Table 2).

**Sanguinicolidae gen. sp. 3 ZA**

Localities: Lake Heritage and below Lake Heritage, Crocodile River (Limpopo River system), South Africa.

Host: *Burnupia trapezoidea* (Boettger, 1910), (Gastropoda: Burnupiidae).

Genetic material: The representative 28S, 18S and ITS rDNA sequences; accession number ZZZ, respectively.

Descriptions: Sporocysts nearly spherical, 118 (109-132) x 110 (98-121), contain one developed cercaria and two germ balls (Fig. 6A). Cercarial body curved ventrad; convex side bears pleated, aspinous finfold (Figs. 6C and 7A). Finfold originates in second third of body, extends over 48 (41-52) % of dorsal body length; height, 47 (41-53) % of body breadth. Body finfold indiscernible in some preserved specimens when viewed using light microscope. Anterior part of body modified into ovoid cephalic organ bearing protrusible tip. Cephalic organ demarcated from main body by transverse constriction of tegument. Pharynx and ventral sucker absent; digestive tube not observed. Body bears four pairs of penetration glands with ducts that open at anterior end of cephalic organ (Fig. 6C). Genital primordium consists of aggregation of cells located between last pair of penetration glands and excretory bladder. Excretory bladder opens into posterior duct that extends along tail stem, bifurcates into canals that terminate at furcal tips (Fig. 6C and D). Body surface characterised by network of fine transverse and prominent longitudinal ridges. Tegumental spines pointed posteriorly, arranged in 29-31 concentric rows, from anterior to approximately two thirds of body length. Cephalic organ bears 12 transverse rows of spines: first three circllets have papillae interspersed between spines (Fig. 7C). Spines on anterior six rows of cephalic organ, robust, blunt and densely
arranged; posterior rows widely spaced (2-3 times wider than on anterior part), bear slender spines (Figs. 7B and C). Tail stem straight, 1.7 (1.6-1.9) times longer than body. Tail stem spinous, with papillae bearing sensilla, arranged in dorsoventral longitudinal rows (Fig. 7D). Furcae laterally flattened, 3.1 (2.8-3.1) times shorter than tail stem; surrounded by narrow dorso-ventral finfolds (Fig. 6D and 7E). Lateral surfaces of the furcae bear 12-13 transverse rows of spines, covering approximately three quarters of furcal length (Fig. 7E). The furcal tip tapered, 9.6 (8.6-11) long.

Remarks

Sanguinicolidae gen. sp. 3 ZA is distinguished from the other species from the current study by small sized sporocysts that are nearly spherical. It’s cercaria has four pairs of penetration glands, which is fewer than in the two species described above, and more than in Sanguinicolidae gen. sp. 4 ZA. Finally, it is characterised by an ovoid cephalic organ that bears 12 circlets of spines, which is more than in the other three species. Based on the number of penetration glands, the present cercaria resembles Cercaria britspennata Porter, 1938 that was found in thiarid Melanoides tuberculata (Müller, 1774) from the Crocodile River, South Africa (Porter, 1938), and S. inermis reported by Kirk and Lewis (1993) from lymnaeid Peregriana peregra (Müller, 1774) in England. Sanguinicolidae gen. sp. 3 ZA has a much shorter dorsal finfold that covers 0.41-0.52 of the body length, compared with C. britspennata whose finfold is about 0.80 of the body length (Porter, 1938, Plate LXXVIII, Fig 3). According to Kirk and Lewis (1993), SEM images of S. inermis revealed five rows of thick spines on the middle region of the cephalic organ. In contrast, the cephalic organ of the current cercaria bears 12 circlets of spines; the anterior six rows have thick spines, followed by seven posterior rows of thin spines. Finally, both C. britspennata and S. inermis are smaller in size, compared to the present cercaria (Table 2).
Sanguinicolidae gen. sp. 4 ZA

Localities: Lake Heritage, Crocodile River (Limpopo River system), South Africa.

Host: *Burnupia trapezoidea* (Boettger, 1910), (Gastropoda: Burnupiidae).

Genetic material: The representative 28S, 18S and ITS rDNA sequences; accession number ZZZ, respectively.

Descriptions: Cercariae found from one snail specimen; sporocysts not observed. Cercarial body curved ventrally, dorsal side bears pleated, aspinous finfold (Fig. 8B and 9A). Finfold originates from second third of body, extends over 51 (48-53) % of dorsal body surface; height, 30 (27-32) % of body width. Finfold indistinct in most preserved specimens when examined optically. Anterior end bears ovate cephalic organ, demarcated from main body by transverse constriction of tegument. Pharynx, digestive tube and ventral sucker not observed. Three pairs of prominent gland cells: an equatorial pair and two pairs located in posterior half of body (Fig 8B). Excretory canal runs through tail stem and furcae, open externally through latero-dorsal pores near furcal tips (Fig. 8C and 9F). Tegumental spines pointed posteriorly, occur in 25 concentric rows around body, covering about 40 % of body length from anterior end. Rim of the cephalic organ bears sheathed papillae that have sensilla (Fig. 9C). Cephalic organ has 10 circlets of spines (Fig. 9B). First four circlets on cephalic organ are closely arranged and bear robust spines; posterior six circlets widely spaced (2.5-3.0 times further apart than anterior rows) and consist of slender spines that resemble those on main the body. Tail stem 1.6 (1.4-1.7) times longer than body; spinous and bears papillae with sensilla, arranged in longitudinal dorsoventral rows (Fig. 9D). Furcae 3.6 (3.1-3.9) times shorter than tail stem. Each furca bears narrow finfold that extend dorsally along furcal length, over the tip and cover only distal half on ventral side (Fig. 8C and 9E); finfold poorly visible in preserved specimens when examined using light microscope. Furcal spines arranged in 10-12 transverse rows on lateral sides of each
furca, from base to about three quarters of furcal length. Furcal tip tapered, 10.1 (8.94-11.6) long.

Remarks

This species is distinguished from the other species in the current study by fewer rows of spines (25) that cover less than half of the body length. In contrast, spines cover at least two thirds of the body length in the other three species. Secondly, Sanguinicolidae gen. sp. 4 ZA is characterised by shorter furcal finfolds than in the other species. What is more, it has a unique arrangement of penetration glands, consisting of an equatorial pair and the rest occurring towards the posterior end of the body. The gross morphology of the current cercaria closely resembles Cercaria sewelli Faust 1926. Cercaria sewelli was reported in Bur. capensis from Kwazulu Natal, Bur. trapezoidea from Free State and Bulinus tropicus (Krauss, 1848) from Gauteng, South Africa (Faust 1926; Porter, 1938). Like C. sewelli, the present cercaria has a tail stem that is only slightly longer than the body and possesses short furcae that are covered with very thin finfolds (Faust 1926; Porter 1938). In addition, the body bears three pairs of prominent glands cells: an equatorial pair and two pairs located in the distal half (Faust, 1926, Plate VI, Fig 1). However, Faust (1926) considered the anterior pair of glands to be large eyespots. What is more, while the furcae of the present cercaria are nearly similar in length to C. sewelli, the body and tail stem of the latter are shorter (Table 2). Since SEM images and genetic characteristics of C. sewelli are unavailable, we cannot confirm if it is identical to Sanguinicolidae gen. sp. 4 ZA.

Genetic data and phylogenetic results

New partial sequences for 28S and 18S rDNA genes were successfully generated from at least five representative samples for the four sanguinicolids described above. For ITS rDNA, usable
sequences were obtained for all the morphotypes, except for Sanguinicolidae gen. sp. 4 SA. The newly generated 28S rDNA (1117-1161 bp) showed intraspecific variation of between one to three base pair differences. Blast results from GenBank showed that the present specimens had the highest genetic similarity with an isolate presumed to be Sanguinicola cf. inermis (accession no. AY222180.1; AY222098.1). Alignment of the 28S rDNA sequences obtained in the current study with ‘Sanguinicola cf. inermis’, showed that p-distances varied between 2.87-7.24%, corresponding to 32-92 nucleotide substitutions (Supplementary file 2). ‘Sanguinicolid’ spp. from East Africa (Brant et al. 2006) differed from the present specimens by a p-distance range of 21.5-25.0%. In general, the present specimens and ‘S. cf. inermis’ showed a high genetic divergence (11.9-26.9%) from other freshwater fish blood flukes for which 28S rDNA sequences are available (Supplementary file 2). Analyses of the 18S rDNA sequences from the current study showed that intraspecific variation did not exceed one nucleotide substitution. For 18S rDNA, alignment of the present sequences (1745-1766 bp) with ‘S. cf. inermis’ revealed that genetic divergence between the species ranged from 2.2-4.0%, corresponding to 21-67 base pair differences (Table 3). ‘Sanguinicolid’ spp. from East Africa (Brant et al. 2006) differed from the present specimens by 11.8-14.1% (Table 3). Based on ITS rDNA (1045-1245 bp) analyses, p-distances ranged from 5.60-10.4%, between the present species (Table 4). There are no ITS rDNA sequences for sanguinicolids, hence, the sequences generated from the current study provide data for future genetic comparisons.

The general topology of the phylogenetic trees was similar with that of fish blood flukes from previous studies (Cutmore et al. 2023; Warren and Bullard 2023). In both the BI and ML phylograms, sanguinicolids were monophyletic and formed four distinct clades (A-D). However, the BI reconstruction had higher values for nodal support than in ML (Fig. 10). In addition, there was a difference within clade A between the two phylograms. In the ML reconstruction, Pseudosanguinicola was basal to Sanguinicola and the subclade containing the
present species. In BI, *Pseudosanguinicola* and *Sanguinicola* formed a strongly supported subclade, and were sister to the subclade comprising of the species from the current study. In the two reconstructions, the species from the present study clustered with cercariae of ‘*S. cf. inermis*’ from Poland (Olson *et al.* 2003) in clade A. The blood fluke sequences from East Africa (‘Sanguinicolid’ sp. W1134 and W1284) formed a distinct and distant clade from well described Sanguinicolidae spp. Indeed, the 28S rDNA divergence between the East African isolates and sanguinicolids was large (17.2-25.0%). The accession numbers of the sequences and localities of the isolates that were used for phylogenetic analyses are provided in supplementary file 2.

Partial sequences for CO1 were obtained from seven isolates of each of the three snail species. The representative sequences (652-685 bp) have been submitted to GenBank; accession number zzz, for *Bur. transvaalensis*, *Bur. mooiensis* and *Bur. trapezoidea*, respectively. The sequences generated from the current study represent the first published genetic data for the three species. Descriptions of the snails are provided (Supplementary file 1). A comprehensive taxonomic and phylogenetic study of the snails will be the subject of a separate study.

**Discussion**

**Taxonomy**

Considering the paucity of data on genetic characterisation of sanguinicolids, the present study is a valuable contribution to the taxonomic study of the group. Data on genetic divergence and the results from phylogenetic analyses, showed that the present isolates and ‘*S. cf. inermis*’ belong to a genus that is monophyletic with *Sanguinicola* and *Pseudosanguinicola*. Cercaria of *Trematoda sp.* alias ‘*Sanguinicola*’ sp. from USA (Zemmer *et al.* 2020) formed a strongly supported subclade with *P. occidentalis*, indicating that it is a species of *Pseudosanguinicola*.  

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Sanguinicolidae sp. from Bass, *Micropterus* sp. from USA (Warren and Bullard, 2023) did not cluster with other isolates from North America, suggesting that it is representative of a distinct genus. The isolates from Vietnam formed a clade with *Nomasanguinicola canthoensis*, suggesting that they belong to *Nomasanguinicola*. Freshwater blood flukes from Australia formed two separate clades. The first group consists of cercariae of ‘sanguinicolid’ sp. W5003 (Brant *et al.* 2006) and ‘Aporocotylidae’ sp. B, C, D, F and G (Cutmore *et al.* 2023), which seem to represent a single genus within Sanguinicolidae. The second clade consisted of ‘Sanguinicolid’ sp. W5004, which was representative of a taxon that appear to be the most recent ancestor of sanguinicolids. It seems that ‘Sanguinicolid’ sp. W5004 belongs to an unidentified family. Its cercarial features deviates considerably from sanguinicolids. The cercaria was characterised by the presence of prominent eyespots, absence of finfolds on the body and furcae, and a large membrane on either side of the tail stem (Brant *et al.* 2006). These features are absent in cercariae of Sanguinicolidae spp. (Erickson and Wallace, 1959; Schell, 1974; Kirk and Lewis, 1993; Simon-Martin *et al.* 1987; Sendersky and Dobrovolsky, 2004; Faltýnková *et al.* 2007). ‘Sanguinicolid’ sp. W1134 and W1284 from East Africa (Brant *et al.* 2006) formed a distinct clade from all sanguinicolids and appears to be representative of an unknown taxon within an old lineage of freshwater fish blood flukes, that is closest to *Acipensericolidae*. Regarding their morphology, Brant *et al.* (2006) noted the absence of finfolds on the body and furcae of ‘sanguinicolid’ sp. W1284. In contrast, the presence of finfolds on the body and furcae is a common sanguinicolid feature.

The present study is the first to provide genetic data for Sanguinicolidae in Africa. Overall, there are only three reports of adult sanguinicolids from Africa, all of which lack genetic characterisation. *Sanguinicola chalmersi* Odhner, 1924 was reported in *Auchenoglanis occidentalis* (Valenciennes, 1840) and *Synodontis schall* (Bloch & Schneider, 1801) from Egypt and Sudan (Imam *et al.* 1984; Odhner, 1924; Paperna, 1996). In a recent revision of the
genus *Sanguinicola*, Warren *et al.* (2023) noted that *S. chalmersi* required more study. Imam *et al.* (1984) described *Sanguinicola clarias* from *Clarias gariepinus* (Burchell, 1822) in Egypt. However, Truong and Bullard (2013) noted that the morphological features of *S. clarias* were identical with those of the genus *Nomasanguinicola*. Therefore, its placement in the genus *Sanguinicola* is suspect. Ogambo-Ongoma (1975) reported on the occurrence of an unidentified *Sanguinicola* sp. in tilapiine cichlids from Lake Victoria, East Africa. The morphological descriptions of the sanguinicolid were not provided. Therefore, it appears that at least for now, *S. chalmersi* is the only valid representative of the genus *Sanguinicola* in Africa. Based on the present findings, it seems that there are three sanguinicolid genera in Africa: *Sanguinicola*, *Nomasanguinicola* and the genus comprising of the species in the present study. Future studies are likely to reveal more genera of blood flukes from African fishes.

Morphological characterisation of intramolluscan stages of Digenea often relies on the examination of parthenitae and cercariae using light microscopy (Frandsen and Christensen, 1984; Faltynkova *et al.* 2007). In the present study, morphological distinctions of the cercariae were made optically, based on overall body size, relative size of the finfolds on the body and furcae, and number of penetration glands in the body. The patterns of papillae and tegumental spines which were clearly discernible only after using SEM, also proved to be distinctive features for species delimitation. Prior to the present study, SEM data were available for cercariae of an unidentified *Sanguinicola* sp. (Simon-Martin and Gomez-Bautista, 1986) and *Sanguinicola inermis* (Kirk and Lewis, 1993). The present study provides additional tegumental features that were not mentioned in previous studies of sanguinicolids. Firstly, the occurrence of two groups of transverse circlets on the cephalic organ that differ not only in the size of their spines, but also in number and spacing. Second, the presence of sensilla near the furcal tips and the position of the excretory pore on the furcae.
Intermediate hosts of sanguinicolids

A vast majority of studies on sanguinicolid larval stages are from Europe, USA and Australia, with minimal reports from Asia, South America and Africa. The intermediate hosts of sanguinicolids from Europe have been presented in detail by Zhokhov et al. (2021). The data shows that snails from seven different families (Bithyniidae, Lithoglyphidae, Lymnaeidae, Melanopsidae, Neritidae, Planorbidae, Valvatidae) are intermediate hosts for sanguinicolids (Zhokhov et al. 2021). In North America, sanguinicolids have been reported from four gastropod families. According to Erickson and Wallace (1959) both S. cristafer and S. lophophorus use snails of the genus Valvata (Valvatidae) as the intermediate hosts. For S. idahoensis, the lithoglyphid F. virens was established to be the intermediate host (Schell, 1974). Preston et al. (2021) reported the occurrence of unidentified sanguinicolid cercaria in Juga plicifera (Lea, 1838) (Semisulcospiridae) from Oregon, USA. According to Zemmer et al. (2020), Elimia proxima (Say, 1825) (Pleuroceridae) is the intermediate host for an unidentified sanguinicolid species in Virginia, USA. Studies from Australia show that at least six sanguinicolid species use Posticobia brazieri (Smith, 1882) (Tateidae) and thiarid Plotiopsis balonnensis (Conrad, 1850) as intermediate hosts (Brant et al. 2006; Cutmore et al. 2023). Reports of intramolluscan stages of freshwater fish blood flukes from South America are very scarce. According to Alda and Martorelli (2014), larvae of an unidentified fluke that was suspected to parasitise freshwater fish, were found from Heleobia australis (d'Orbigny, 1835) (Cochliopidae) in Argentina. From Asia, larvae of S. lungensis have been reported only from the lymnaeid Radix plicatula (Benson, 1842) (Tang and Ling, 1975).

In Africa, reports on sanguinicolids are only from the Northeastern and southern parts of the continent. From East Africa, Ogambo-Ongoma (1975) reported Sanguinicola larvae in Radix natalensis (Krauss, 1848). However, since descriptions of those larvae have not been provided, their species identities are unknown. Morphological data shows that Cercaria sewelli...
from *Bur. capensis* and *Bur. trapezoidea*, *Cercaria britspennata* from *M. tuberculata* and *Cercaria capensis* from *Bur. capensis* which were reported from South Africa (Faust, 1926; Porter, 1938), might be species of Sanguinicolidae. Since sanguinicolids have scarcely been reported in other snail taxa from southern Africa, it seems that *Burnupia* spp. are the main transmitters of sanguinicolids in the region. Although the actual diversity of blood flukes of fish remains unknown in Africa, the group appears to be richer than initially thought. The definitive hosts of the African sanguinicolids are largely unknown. Indeed, the adults of only two species have been described from clariid, claroteid and silurid fishes, and only in Egypt and Sudan (Imam *et al*. 1984; Paperna, 1996). Considering the presence of clariid and claroteid fishes in South Africa (Skelton, 2001), we suspect that at least a species of those taxa to be the definitive hosts for the sanguinicolids recorded in the present study.

In summary, sanguinicolids use diverse species belonging to at least 13 different families of pulmonate and caenogastropod snails, as intermediate hosts. To our knowledge, there are no records of sanguinicolids from other invertebrate taxa. Also, it seems that the European sanguinicolids occur in a higher diversity of snail taxa, compared with the other regions of the world. However, this is probably a reflection of the paucity of studies on the intramolluscan stages of freshwater fish blood flukes in the other regions of the world.

**Supplementary material.** The supplementary material for this article can be found at [DOI].

**Data availability.** DNA sequences generated in the present study have been submitted to GenBank: accession numbers ZZZ.

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We thank Beric Gilbert, Lutfiyya Latief, Kenneth Matea, Mpho Maduenyane and Quinton Dos Santos for their assistance in the field and laboratory.

**Authors’ contributions.** JO and AA-O conceptualized the study. JO conducted field sampling and laboratory analyses. JO wrote the original draft of the article. JOO and AA-O reviewed and edited subsequent drafts.

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**Competing interests.** The authors declare there are no conflicts of interest.

**Ethical standards.** This research was granted approval by the University of Johannesburg Ethics Committee (Reference Number: 2022-08-05/Outa_Oldewage), and the study complied with the relevant institutional and national research standards.
References


Craven, AE (1880) On a collection of land and freshwater shells from Transvaal & Orange


First description of the adult stage of Clinostomum cutaneum Paperna, 1964 (Digenea: Clinostomidae) from grey herons Ardea cinerea L. and a redescription of the metacercaria from the Nile tilapia Oreochromis niloticus niloticus (L.) in Kenya. Systematic Parasitology 76, 39-51.


13.


Porter, A (1938) The larval Trematoda found in certain South African Mollusca with special reference to schistosomiasis (Bilharziasis). South African Institute for Medical Research 42 (8), 1-492.


Zemmer, SA, Detwiler, JT, Sokol ER, Da Silva Neto JG, Wyderko J and Potts, K, et al. (2020) Spatial scale and structure of complex lifecycle trematode parasite communities in

Figure 1. Map of southern Africa (A) and the study areas (B and C). Site 1: below the Vaal Dam (26.872364 °S, 28.117173 °E); Site 2: below the Vaal River Barrage Reservoir (26.734854 °S, 27.634372 °E); Site 3: Lake Heritage (25.959696 °S, 27.855555 °E) and Site 4: below Lake Heritage (25.957086 °S, 27.858308 °E).
Figure 2. Schematic drawings of Sanguinicolaidea gen. sp. 1 ZA. A, sporocyst; B, whole cercaria; C, cercarial body and D, furcae. Abbreviations: bo, body; co, cephalic organ; eb, excretory bladder; ed, excretory duct; ep, excretory pore; f, furca; ff, finfold; pg, penetration gland and ts, tail stem.
**Figure 3.** Scanning electron micrographs of Sanguinicolidae gen. sp. 1 ZA cercaria. A, cercarial body; B, enface view of anterior end showing circlet of papillae (inside the broken line circle); C, ventral view of anterior end; D, latero-dorsal view of mid region of tail stem; E, subventral view of the mid region of tail stem; F, lateral view of furcae and G, posterior end of furcae. Triangle arrow heads show rows of spines and winged arrows show papillae with sensilla. Abbreviations: co, cephalic organ; ep, excretory pore; f, furca; ff, finfold; m, mouth; pgd, tip of penetration gland ducts; tf, transverse folds and ts, tail stem.
Figure 4. Schematic drawings of Sanguinicolidae gen. sp. 2 ZA. A, sporocyst; B, whole cercaria; C, cercarial body and D, posterior end of tail. Abbreviations: bo, body; co, cephalic organ; eb, excretory bladder; ed, excretory duct; ep, excretory pore; f, furca; ff, finfold; gp, genital primordium; pg, penetration gland and ts, tail stem.
Figure 5. Scanning electron micrographs of Sanguinicolidae gen. sp. 2 ZA cercaria. A, cercarial body; B, lateral view of anterior end showing circle of papillae (inside the broken line circle); C, dorsal view of anterior end; D, latero view of mid region of tail stem; E, upclose view of the ventral side (mid region) of tail stem; F, lateral view of furcae and G, furcal posterior end. Triangle arrow heads show rows of spines and winged arrows show papillae with sensilla. Abbreviations: co, cephalic organ; ep, excretory pore; f, furca; ff, finfold; m, mouth; pgd, tip of penetration gland ducts; tf, transverse folds and ts, tail stem.
Figure 6. Schematic drawings of Sanguinicolidae gen. sp. 3 ZA. A, sporocyst; B, whole cercaria; C, cercarial body and D, posterior end of tail. Abbreviations: bo, body; co, cephalic organ; eb, excretory bladder; ed, excretory duct; ep, excretory pore; f, furca; ff, finfold; gp, genital primordium; pg, penetration gland and ts, tail stem.
Figure 7. Scanning electron micrographs of Sanguinicolidae gen. sp. 3 ZA cercaria. A, cercarial body; B, lateral view of the cephalic penetration organ; C, apical view of anterior end; D, latero view of the anterior half of tail stem; E, lateral view of furcae. Triangle arrow heads show rows of spines and winged arrows show papillae with sensilla. Abbreviations: co, cephalic organ; f, furca; ff, finfold; tip of penetration gland ducts and ts, tail stem.
Figure 8. Schematic drawings of Sanguinicolidae gen. sp. 4 ZA. A, whole cercaria; B, cercarial body and C, posterior end of tail. Abbreviations: bo, body; co, cephalic organ; ed, excretory duct; ep, excretory pore; f, furca; ff, finfold; pg, penetration gland and ts, tail stem.
Figure 9. Scanning electron micrographs of Sanguinicolidae gen. sp. 4 ZA cercaria. A, cercarial body; B, lateral view of anterior end; C, enface view of anterior end showing circlet of papillae (inside the broken line circle); D, lateral view of anterior region of tail stem; E, lateral view of furcae and F, furcal posterior end. Triangle arrow heads show rows of spines and winged arrows show papillae with sensilla. Abbreviations: co, cephalic organ; ep, excretory pore; f, furca; ff, finfold; pgd, tip of penetration gland ducts; tf, transverse folds and ts, tail stem.
Figure 10. Maximum likelihood (ML) and Bayesian inference (BI) reconstructions of the phylogenetic relationships between the present species (in bold) and other blood flukes of freshwater fish, based on 28S rDNA data. The branch length scale indicates the number of substitutions per site. Nodal support values that are below 80/0.8 have been excluded.
### Table 1. Prevalence (%) of sanguinicolid larvae in the examined snails from the study sites.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Digenea</th>
<th>Host</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaal River, below Vaal Dam (S1)</td>
<td>Sanguinicolidae gen. sp. 1 ZA</td>
<td><em>Burnupia transvaalensis</em></td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td>Sanguinicolidae gen. sp. 2 ZA</td>
<td><em>B. transvaalensis</em></td>
<td>1.19</td>
</tr>
<tr>
<td>Vaal Rive, below Vaal Barrage (S2)</td>
<td>Sanguinicolidae gen. sp. 1 ZA</td>
<td><em>B. transvaalensis</em></td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>Sanguinicolidae gen. sp. 2 ZA</td>
<td><em>Burnupia mooiensis</em></td>
<td>1.01</td>
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<tr>
<td></td>
<td></td>
<td><em>B. transvaalensis</em></td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. mooiensis</em></td>
<td>1.01</td>
</tr>
<tr>
<td>Lake Heritage (S3)</td>
<td>Sanguinicolidae gen. sp. 3 ZA</td>
<td><em>Burnupia trapezoidea</em></td>
<td>7.81</td>
</tr>
<tr>
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<td>Sanguinicolidae gen. sp. 4 ZA</td>
<td><em>B. trapezoidea</em></td>
<td>0.78</td>
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<tr>
<td>Crocodile River, below L. Heritage (S4)</td>
<td>Sanguinicolidae gen. sp. 3 ZA</td>
<td><em>B. trapezoidea</em></td>
<td>2.02</td>
</tr>
</tbody>
</table>

*B. transvaalensis*: S1 (n = 590), S2 (n = 132); *B. mooiensis*: S2 (n = 398); *B. trapezoidea*: S3 (n = 128), S4 (n = 397)
Table 2. Cercarial measurements (in μm) of the current species (in bold) and the previously described species of the family Sanguinicolidae.

<table>
<thead>
<tr>
<th>Species</th>
<th>Body length</th>
<th>Body width</th>
<th>Cephalic organ length</th>
<th>Cephalic organ width</th>
<th>Tail stem length</th>
<th>Tail stem width</th>
<th>Furcal length</th>
<th>Furcal base width</th>
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<tr>
<td><strong>Sanguinicolidae gen. sp. 1</strong></td>
<td>142 (135-147)</td>
<td>44 (41-51)</td>
<td>24 (23-25)</td>
<td>18 (16-20)</td>
<td>250 (238-270)</td>
<td>29 (25-34)</td>
<td>94 (89-98)</td>
<td>12 (10-13)</td>
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<td><strong>ZA</strong></td>
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<tr>
<td><strong>Sanguinicolidae gen. sp. 2</strong></td>
<td>160 (144-181)</td>
<td>43 (41-48)</td>
<td>25 (22-29)</td>
<td>20 (18-23)</td>
<td>251 (241-261)</td>
<td>31 (27-36)</td>
<td>82 (75-87)</td>
<td>12 (10-14)</td>
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<tr>
<td><strong>Sanguinicolidae gen. sp. 3</strong></td>
<td>132 (122-147)</td>
<td>35 (33-40)</td>
<td>25 (23-26)</td>
<td>18 (16-20)</td>
<td>225 (212-234)</td>
<td>30 (29-33)</td>
<td>73 (70-76)</td>
<td>13 (12-15)</td>
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<td><strong>Sanguinicolidae gen. sp. 4</strong></td>
<td>141 (123-169)</td>
<td>35 (31-43)</td>
<td>23 (20-26)</td>
<td>17 (16-18)</td>
<td>227 (222-232)</td>
<td>30 (28-30)</td>
<td>67 (58-75)</td>
<td>10 (9.4-11)</td>
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<td><strong>aCercaria sewelli</strong></td>
<td>150</td>
<td>48</td>
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<tr>
<td><strong>bCercaria britspennata</strong></td>
<td>67-131</td>
<td>20-28</td>
<td>13-20</td>
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<td>87-180</td>
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<td>35-46</td>
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<td>16-22</td>
<td>185-256</td>
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<td><strong>cSanguinicola cristata</strong></td>
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* Faust (1926); †Porter (1938); ‡Erickson and Wallace (1959); ‡Schell (1974); §Tang and Ling (1975) ‰Simon-Martinet et al. (1987); ††Kirk and Lewis (1993); †Sendersky and Dobrovolsky (2004); ‡‡Faltýnková et al. (2007).
Table 3. The number of base pair differences (below the diagonal) and sequence divergence (%) (above the diagonal), of the present specimens (in bold) and other freshwater blood flukes, based on 18S rDNA analyses.

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Table 4. The number of base pair differences (below the diagonal) and sequence divergence (%) (above the diagonal), of the present sanguinicolids, based on ITS rDNA analyses.

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