Smallmouth yellowfish, *Labeobarbus aeneus* (Teleostei: Cyprinidae), as a potential new definitive host of the invasive parasite *Atractolytocestus huronensis* (Cestoda: Caryophyllidea) from common carp: example of recent spillover in South Africa?

Quinton Marco Dos Santos and Annemariè Avenant-Oldewage*

Department of Zoology, University of Johannesburg, Auckland Park, P.O. Box 524, Johannesburg 2006, South Africa

Author e-mails: qmdossantos@live.co.za (QMDS), aoldewage@uj.ac.za (AAO)

*Corresponding author

**Abstract**

*Atractolytocestus huronensis* Anthony, 1958 has been co-introduced with its cyprinid host *Cyprinus carpio* Linnaeus, 1758, common carp, to several continents. This cestode was only recently (2012) detected in South Africa and occurs in two major river systems. In Africa, *A. huronensis* has only been reported from *C. carpio*. During routine parasitological surveys in the Vaal River system in central South Africa, unidentified cestodes were recorded from common carp at several localities. Using light and scanning electron microscopy, alongside genetic characterisation, they were identified as *A. huronensis*, greatly expanding the distribution of this parasite in the upper reaches of the Vaal River system and indicating rapid spread in the system. Thereafter, in November 2020, more caryophyllidean cestodes were detected infecting native smallmouth yellowfish, *Labeobarbus aeneus* (Burchell, 1822) just below the Vaal Dam wall. They were also morphologically and genetically identified as *A. huronensis*, indicating a possible new definitive host and spillover from carp. However, only juvenile worms (up to late stage 4) were detected in *L. aeneus*, suggesting a paradefinitive or accidental infection. Their pathological effect on the intestine of *L. aeneus* mimicked that described in acute infections in *common carp*, with damage limited to the intestinal epithelium and no prominent ulcerations. This apparent mild infection of an indigenous host needs to be monitored. The spillover to *L. aeneus* appears to be recent as no caryophyllidean cestodes were collected from this host species at the same locality and season the previous year, nor at any of the other well studied sites in the Vaal River system. Chronic infection of *L. aeneus* may still develop and indicate that the near threatened largemouth yellowfish, *Labeobarbus kimberleyensis* (Gilchrist & Thompson, 1913), may be at risk as well.

**Key words:** biological invasions, helminth parasites, *Cyprinus carpio*, pathology, DNA barcoding, SEM

**Introduction**

*Atractolytocestus huronensis* Anthony, 1958 was first described from common carp, *Cyprinus carpio* Linnaeus, 1758, in North America (Anthony 1958), but it has been speculated that it is of Asian origin (Oros et al. 2004; Scholz et al. 2015). These cestodes were exclusively recorded in
North America (Amin and Minckley 1996) until the first record in England in 1993 (Chubb et al. 1996; Kirk et al. 2003). Since then, they have been recorded from several other countries on four continents (Scholz et al. 2015; Bazsalovicsová et al. 2018), mostly from their type host. Oros et al. (2011) consider them strictly specific (oioxenous) to common carp, however, *A. huronensis* also been recorded from *Abramis brama* (Linnaeus, 1758) and *Rutilus rutilus* (Linnaeus, 1758) in Europe (Hanzelová et al. 2009; Oros and Hanzelová 2009). Whether these additional hosts were infected by gravid individuals or if the infections were chronic or acute in nature is not discernible from available literature, but it has been speculated that these represent auxiliary definitive hosts (Oros and Hanzelová 2009) or that they were instances of accidental hosts infections (Hanzelová et al. 2009; Oros et al. 2011).

Within Africa, the presence of *A. huronensis* has only very recently been recorded in South Africa (Scholz et al. 2015, 2018; Smit et al. 2017; Erasmus et al. 2020). Scholz et al. (2015) recorded it from common carp in several localities withing the Limpopo River Basin between 2012 and 2014, while Smit et al. (2017) reported that the cestode occurred in the same host in an undisclosed locality somewhere in the middle reaches of the Vaal River and in one of its tributaries, the Riet River, between 2013 and 2016. Erasmus et al. (2020) thereafter recorded *A. huronensis* from the upper reaches of the Limpopo River Basin in the Hex River in 2017 to 2018, again from *C. carpio*. No infection with *A. huronensis* in any local fish species has been recorded in Africa, with all records from common carp. As such, the cestodes are considered co-introduced into South Africa alongside *C. carpio* (Scholz et al. 2015; Smit et al. 2017). Common carp has been present in the freshwater systems of southern Africa for several decades, with the first record of its introduction in 1859 (De Moor and Bruton 1988). Thus, the very recent presence of *A. huronensis* has been attributed to a new and recent introduction (Scholz et al. 2015). Previous research on intestinal helminths of *C. carpio* in southern Africa did not report any trace of this cestode taxon (Boomker et al. 1980; Brandt et al. 1981; Van As and Basson 1984), supporting the view that this is a new introduction. This is not the first introduction of a cestode into the aquatic environment of southern Africa along with carp. *Schyzocotyle acheilognathi* (Yamaguti, 1934) is suspected to have been introduced into South Africa in 1975 with grass and/or silver carp (Boomker et al. 1980; Brandt et al. 1981; De Moor and Bruton 1988) and was subsequently reported from common carp (Boomker et al. 1980; Brandt et al. 1981; Van As et al. 1981; Van As and Basson 1984). This introduction has had a large impact on the local fauna, as *S. acheilognathi* has been able to colonise several native cyprinid species (Brandt et al. 1981; Van As et al. 1981; Mashego 1982; Bertasso and Avenant-Oldewage 2005; Retief et al. 2006, 2007, 2009; Degger et al. 2009; Stadtlander et al. 2011; Smit et al. 2017; Gilbert et al. 2020).
During standard parasitological surveys in the upper reaches of the Vaal River system in 2018, just below the Grootdraai Dam wall, cestodes preliminarily identified as \textit{A. huronensis} were recorded from common carp. To determine the extent of the spread of this invasive cestode in the system, carp were assessed for the presence of the cestodes during subsequent surveys at three additional sites (Figure 1). However, during the most recent survey (November 2020), caryophyllidean cestodes were also recorded from \textit{L. aeneus} just below the Vaal Dam wall. These unidentified cestodes resembled \textit{A. huronensis}, but were not gravid and no cestode other than \textit{S. acheilognathi} had been recorded from \textit{L. aeneus} previously (Scholz et al. 2018). The cestodes collected from both carp and yellowfish in the Vaal River system were studied using light and scanning electron microscopy, while also genetically characterised, to confirm their identity. The stages of development of the cestodes from yellowfish were assessed to determine if the infection was accidental or auxiliary, while also studying the histopathology caused by the worms to determine whether the infection was chronic or acute.

**Materials and methods**

**Sample collection**

During standard parasitological surveys from 2018 to 2020 in the Vaal River system, both common carp, \textit{C. carpio}, and smallmouth yellowfish, \textit{L. aeneus}, were collected using gill nets and electronarcosis. Samples were collected from four sites (Figure 1): Site 1 – Below the Grootdraai Dam...
Atractolytocestus huronensis infecting Labeobarbus aeneus in the Vaal River system

Dos Santos and Avenant-Oldewage (2022), Aquatic Invasions 17(2): 259–276, https://doi.org/10.3391/ai.2022.17.2.08

Site 2 – Vaal Dam (26.870528°S; 28.166916°E); Site 3 – Below the Vaal Dam wall (26.871111°S; 28.118611°E); Site 4 – Below Vaal River Barrage (26.732196°S; 27.632755°E). Fish were euthanized by severing the spinal cord posterior to the skull, the intestinal tract removed, and the intestine assessed for the presence of enteric helminths. Intestines were opened carefully in saline with fine forceps using a dissection microscope, and cestodes removed using a 000 Camel’s hair paintbrush. For morphological assessment, cestodes were fixed in either warm 10% neutral buffered formalin (NBF); warm triethanol-amine formaldehyde (TAF) (Courtney et al. 1955); or ambient temperature 70% ethanol. Additionally, some specimens were fixed in 96% ethanol for molecular study, while sections of infected intestinal tissue from L. aeneus were fixed in 10% NBF for pathological study. Infection statistics were calculated according to Bush et al. (1997). All institutional and national guidelines for the collection and study of fish were observed.

Morphology

For light microscopy (LM), whole mount preparations were stained with Semichon’s acetocarmine, differentiated in 1% acid alcohol, dehydrated in a graded ethanol series, cleared in xylene, and mounted in Entellan® (Merck KGaA, Darmstadt, Germany). Specimens were studied and micrographs obtained using a Zeiss AxioPlan 2 microscope operated with AxioVision 4.3 software (Carl Zeiss, Jena, Switzerland). Specimens were preliminary identified using the keys of Scholz et al. (2018), and then further identified using Oros et al. (2010). The developmental stages of specimens from L. aeneus were determined using the designation for the developmental stages of another caryophyllidean, Wenyonia virilis Woodland, 1923, by Ibraheem and Mackiewicz (2006).

For scanning electron microscopy (SEM), whole specimens fixed in 70% ethanol were prepared by dehydrating through a graded ethanol series, followed by a graded series of hexamethyldisilazane (HMDS) (Nation 1983; Dos Santos et al. 2015). Specimens were dried in a Sanpla dry keeper desiccator cabinet (Kita-Ku, Osaka, Japan) and gold coated using an Emscope SC500 sputter coater (Quorum Technologies, Newhaven, U.K.). They were then studied using a Vega 3 LMH scanning electron microscope (Tescan, Brno, Czech Republic) at 5–6 kV acceleration voltages.

Pathology

Sections of L. aeneus intestine with attached cestodes fixed in 10% NBF were dehydrated, washed in running tap water overnight, and then dehydrated to 70% ethanol. Samples were further dehydrated using a graded series of acetone, infiltrated with TAAB Transmit resin (TAAB Ltd, Aldermaston, UK), and cured. Semi-thin sections (5–7 µm) were made
using a rotary microtome with a glass blade, floated on a glass slide with albumin solution, and allowed to dry and affix. Resin was removed with absolute ethanol saturated with sodium hydroxide, after which sections were stained using haematoxylin and eosin, coverslips mounted using Entellan®, and slides studied as above. Observations were compared to those of Molnár et al. (2003) and Gjurčević et al. (2012) for the infection of *C. carpio* with *A. huronensis*.

**Genetic study**

Tissue fragments were excised from the posterior of eight cestodes collected from *C. carpio* (two from each site) and three from *L. aeneus* (Site 3), and genomic DNA extracted using a NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) following the manufacturer’s instructions. Thereafter, 18S rDNA was amplified using primers WormA (5’–GGG AAT GGC TTA AAT CAG –3’) and WormB (5’–CTT GTT ACG ACT TTT ACT TCC-3’) (Littlewood and Olson 2001), while 28S rDNA was amplified using primers C1 (5’-ACC CGC TGA ATT TAA GCA T-3’) and D2 (5’-TCC GTG TTT CAA GAC GG-3’) (Hassouna et al. 1984). PCR conditions from Scholz et al. (2013) and Jovelin and Justine (2001) were used for the 18S and 28S rDNA primer pairs respectively. Successful amplification was verified using a 1% agarose gel, impregnated with GelRed™ (Biotium Inc., Fremont City, California), and visualized with a UV transilluminator. Sequencing was done in both directions using PCR primers following Avenant-Oldewage et al. (2014). Generated sequence data were aligned, inspected, edited if necessary, and reads merged using Geneious Prime 2020.2.2 (https://www.geneious.com). Obtained sequence data was deposited to GenBank. To determine the identity of the cestodes, obtained sequences were analysed using BLAST (Altschul et al. 1990) and aligned to available 18S and 28S rDNA sequence data for *Atractolytocestus* spp. downloaded from GenBank (details in Table 1) using MEGA7 (Kumar et al. 2016) and MAFFT (Katoh et al. 2002; Katoh and Standley 2013). *Atractolytocestus tenuicollis* (Li, 1964) was not included as no data for the selected markers are currently available for this species. *Khawia sinensis* Hsü, 1935 and *Breviscolex orientalis* Kulakovskaya, 1962 were included as outgroups. Due to published sequence data for both markers being produced from the same specimens, 18S and 28S rDNA data was combined and analysed together. Pairwise distances were estimated by uncorrected *p*-distance with 1000 bootstrap replicate variance estimation using MEGA7. Evolutionary history was investigated employing both maximum likelihood (ML) and Bayesian inference (BI) methods. For ML analyses, the Tamura-Nei (TN93) model (Tamura and Nei 1993) with discrete Gamma distribution (5 categories (+G, parameter = 0.05)) was selected as the best nucleotide substitution model using MEGA7. This was supported by 1000 bootstrap replicates. Bayesian inference (BI) analysis was performed with BEAST v2.5.0 (Bouckaert
Table 1. List of the species, host, collection sites and accession numbers for the 18S and 28S rDNA data for *Atractolytocestus* spp. used in the present study. *Breviscolex orientalis* and *Khawia sinensis* included as outgroups.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host species</th>
<th>Locality</th>
<th>Isolate ID</th>
<th>18S rDNA</th>
<th>28S rDNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atractolytocestus</em></td>
<td><em>Labeobarbus</em></td>
<td>Below Vaal Dam wall, Vaal River, South Africa</td>
<td>AhLa1–3; LH1</td>
<td>OM972659</td>
<td>OM927748</td>
<td>Present study</td>
</tr>
<tr>
<td><em>huronensis</em></td>
<td><em>aeneus</em></td>
<td>Below Groothdraai Dam, Vaal River, South Africa; Vaal Dam, South Africa; Below Vaal Dam wall, Vaal River, South Africa; Below Vaal River Barrage, Vaal River, South Africa</td>
<td>AH1–5; 7–8 (CH1; 28S rDNA)</td>
<td>OM972658</td>
<td>OM927746</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Cypinus carpio</em></td>
<td></td>
<td>Below Vaal River Barrage, Vaal River, South Africa</td>
<td>AH6 (CH2; 28S rDNA)</td>
<td>OM972658</td>
<td>OM927747</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Velké Trakany, Tisa River, Slovakia</em></td>
<td></td>
<td>Hungary</td>
<td>PBI-44/TS-02/58 (SLO 3d)</td>
<td>MW027424</td>
<td>MW027484</td>
<td>Scholz et al. (2021)</td>
</tr>
<tr>
<td><em>Breviscolex orientalis</em></td>
<td><em>Hemibarbus</em></td>
<td>Bracknell, England, United Kingdom</td>
<td>BMNH.2005.10.5.13</td>
<td>JQ034132</td>
<td>JQ034115</td>
<td>Brabec et al. (2012)</td>
</tr>
<tr>
<td><em>Tina River, East Slovakia</em></td>
<td></td>
<td></td>
<td>EU343748</td>
<td>EU343738</td>
<td></td>
<td>Olson et al. (2008)</td>
</tr>
<tr>
<td><em>Lake Biwa, Honshu, Japan</em></td>
<td></td>
<td></td>
<td>C-340 (JP8b)</td>
<td>AF286978</td>
<td>AF286910</td>
<td>Olson et al. (2001)</td>
</tr>
<tr>
<td><em>Cyprinus sagittatus</em></td>
<td><em>Cyprinus</em></td>
<td>Lake Biwa, Honshu, Japan</td>
<td>BMNH.2001.1.30.1-4</td>
<td>AF286978</td>
<td>AF286910</td>
<td>Olson et al. (2001)</td>
</tr>
<tr>
<td><em>carpio</em></td>
<td><em>labeo</em></td>
<td>Hiroi River, at Kotobuki, Iyama City, Nagano Prefecture, Honshu, Japan</td>
<td>–</td>
<td>JQ034134</td>
<td>JQ034117</td>
<td>Brabec et al. (2012)</td>
</tr>
<tr>
<td><em>Khawia sinensis</em></td>
<td><em>Cyprinus</em></td>
<td>Vlhavy pond, South Bohemia, Czech Republic</td>
<td>PBI-57/TS-09/310</td>
<td>MW027430</td>
<td>MW027490</td>
<td>Scholz et al. (2021)</td>
</tr>
<tr>
<td></td>
<td><em>carpio</em></td>
<td>Latorica River, Slovakia</td>
<td>sk32</td>
<td>JN004250</td>
<td>JN004261</td>
<td>Scholz et al. (2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lake Biwa, Japan</td>
<td>sk104</td>
<td>JN004253</td>
<td>JN004264</td>
<td>Scholz et al. (2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lake Dong-Hu, Wuhan, China</td>
<td>09_104</td>
<td>JN004254</td>
<td>JN004265</td>
<td>Scholz et al. (2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Engleand, United Kingdom</td>
<td>BMNH.2005.10.5.14</td>
<td>EU343746</td>
<td>EU343740</td>
<td>Olson et al. (2008)</td>
</tr>
</tbody>
</table>

et al. 2014) using 10 million Markov chain Monte Carlo (MCMC) generations and the TN93 model. Due to the similarity between BI and ML analyses, a single topology based on BI analyses is given with both ML and BI support indicated at relative nodes. Only nodes with support above 65% are indicated.

**Results**

*Morphology, development and infection statistics*

Macroscopically similar caryophyllidean cestodes were collected from both *C. carpio* (Figure 2) and *L. aeneus* (Figure 3). All cestodes were identified as members of *Atractolytocestus* Anthony, 1958 based on their body lacking a tail-like posterior; afossate, bulbocuminate scolex; genital pores located near posterior; postovarian vitelline follicles; and lateral ovarian lobes. Cestodes collected from *C. carpio* were mostly gravid (Figure 2A), while those collected from *L. aeneus* were at various stages of sexual maturity, but not gravid (Figure 3A). Underdeveloped structures in immature specimens collected from *L. aeneus* hindered morphological identification, but all cestodes were identified as *A. huronensis* due to the number of testes and the scolex morphology, which was supported by molecular characterisation. Scolexes were highly variable in shape, with the most prevalent form the characteristic arrow-head shape (Figures 2A, B, 3A, B), but some with...
Atractolytocestus huronensis infecting Labeobarbus aeneus in the Vaal River system

Dos Santos and Avenant-Oldewage (2022), Aquatic Invasions 17(2): 259–276, https://doi.org/10.3391/ai.2022.17.2.08

Figure 2. Light and scanning electron micrographs of adult Atractolytocestus huronensis collected from Cyprinus carpio in the Vaal River system. A: Light micrograph of gravid cestode stained with Semichon’s acetocarmine. Scanning electron micrographs of: B: Whole adult cestode; C: Protruding cirrus of A. huronensis; D: Distal end of cirrus; E: Female genital pore with egg. Photomicrographs by Quinton M. Dos Santos.

A sharpened, tapering scolex (Figure 3C) and others a broad, mushroom-like scolex (Figure 3D). The protruding cirrus sac of A. huronensis was also observed for the first time using SEM (Figure 2C, D). This was only observed in samples collected from C. carpio. The protruding cirrus sac was finger-like and unarmed, with a folded, rosette-like distal tip. An egg was also observed in the female genital pore (Figure 2E).

Using the designation of juveniles of W. virilis by Ibraheem and Mackiewicz (2006), the cestodes collected from L. aeneus were confirmed to be juveniles from stages 2 to 4. A late stage 4 cestode can be seen in Figure 3A, B. Late final stage juveniles (stage 4) were identified by the presence of follicular ovaries, fully differentiated vitelline follicles and testes, numerous wide uterine folds, and fully formed male and female genital pores, that are open to the exterior (Figure 3E, F). No cercomers were observed.

Regarding cestode infections in cyprinids collected in the Vaal River system (Supplementary material Table S1), A. huronensis had a prevalence of 60–100% in C. carpio and 20% in L. aeneus. Intensities in C. carpio ranged from 1 to 64 individuals, with the highest number of individuals recorded from L. aeneus (1–94). A mixed infection with a single specimen of S. acheilognathi was recorded from one L. aeneus individual at site 3 in November 2020.
Atractolytocestus huronensis infecting Labeobarbus aeneus in the Vaal River system

Figure 3. Light and scanning electron microscopy images of immature Atractolytocestus huronensis collected from Labeobarbus aeneus in the Vaal River system. A: Light micrograph of stage 4 cestode stained with Semichon’s acetocarmine. Scanning electron micrographs of: B: Whole stage 4 cestode; C: Sharpened, tapering shape of scolex; D: Broad, mushroom-like shape of scolex; E: Fully formed genital pores of late stage 4 cestode. F: Epi-fluorescent micrograph of open genital pores of late stage 4 cestode stained with Semichon’s acetocarmine. F – Female genital pore, M – Male genital pore. Photomicrographs by Quinton M. Dos Santos.

Genetic characterisation

Obtained 18S rDNA data for all specimens analysed were identical, irrespective of the host or locality from which they were collected, representing a single haplotype (2071 bp) containing three polymorphic sites. For 28S rDNA (1127 bp), two haplotypes were observed, with most specimens from both hosts and all localities being identical, and a single specimen displaying a second haplotype. The only difference between these two haplotypes was the number of polymorphic sites, the first haplotype from C. carpio (CH1) and L. aeneus (LH1) with six polymorphic sites and the second haplotype from C. carpio (CH2) with a single polymorphic site. Representative haplotype data was deposited to GenBank (18S – OM972658–OM972659; 28S – OM927746–OM927748). Using combined 18S and 28S
rDNA from the present study, BLAST analyses supported the similarity of the specimens to *Atractolytocestus*. Using representative haplotype data and published data, an alignment of 3231 bp with 3042 conserved, 185 variable, and 146 parsimony informative sites was produced. Sequence data from the present study was 0–0.07% (0–2 bp) distant to data for *A. huronensis* (Table S2). This limited variation is identical to the calculated intraspecific variability for *A. huronensis*, and lower than the intrageneric variation for *Atractolytocestus* (between *A. huronensis* and *A. sagittatus* (Kulakovskaya and Akhmerov, 1965)) of 1.72–1.92% (54–58 bp), supporting the morphological identification of all specimens as *A. huronensis*. The distance of the produced sequence data to *A. huronensis* was also similar to the intraspecific distance calculated for *B. orientalis* (0.03%; 1 bp), and lower than the intraspecific distance of *K. sinensis* (0–0.42%; 0–13 bp).

Phylogenetic reconstruction (Figure 4) placed the current data in a strongly monophyletic clade with all available 18S and 28S rDNA data for *A. huronensis*. This clade is sister to data for *A. sagittatus*, indicating a clear separation of the two taxa, confirming that the specimens from the present study belong to *A. huronensis*.

**Pathology**

In both carp and yellowfish, caryophyllidean cestodes occurred in the anterior region of the intestine, close but posterior to the opening of the bile duct.
Atractolytocestus huronensis infecting Labeobarbus aeneus in the Vaal River system

Cestodes were attached to the intestinal wall with their scolexes wedged in between the intestinal folds, penetrating into the intestinal crypts. In histological sections of infected intestines of L. aeneus, cestodes were not observed to penetrate past the epithelial layer of the mucosa (Figure 5A–D). The columnar epithelium of the intestinal folds and crypts were mostly discernible at attachment sites, remaining mainly intact. However, thinning and degeneration of the epithelium (Figure 5B), alongside rupture...
of the brush border (Figure 5C) were seen. The epithelial layer was occasionally absent (Figure 5D), presumably removed by mechanical abrasion by the scolex, leaving the cestode in direct contact with the lamina propria. No cestodes were observed inside the lamina propria or approximating the lamina muscularis of the mucosa. The space between the tegument of the worms and the epithelium of the intestine was often filled with loose cells and cell debris including cytoplasm, nuclei and occasionally monocytes (Figure 5E, F). Similar cells and artifacts were observed surrounding the cestodes’ tegument in the lumen of the intestine (Figure 5G).

Discussion

All caryophyllidean cestode specimens collected from both common carp and smallmouth yellowfish in the Vaal River system were identified as *Atractolytocestus huronensis* using both morphometry and genetic characterisation. The prevalence of 100% seen in carp during some collections is based on single hosts collected, thus this is not an accurate reflection of the infection. Thus, the only reliable sample size was collected at the Vaal Dam in March 2019 (prevalence 60%) as 15 host were collected. It appears that the spillover of *A. huronensis* to *L. aeneus* occurs at increased infectivity (mean intensity 35; abundance 11.8) compared to carp (mean intensity 19.7; abundance 7), which is concerning. Furthermore, previous infections in host species other than carp had very low prevalence. Hanzelová et al. (2009) and Oros and Hanzelová (2009) noted prevalence of 1.5% (intensity 1) and 3.95% (intensity 1) in *R. rutilus* and *A. brama* respectively, compared to that in *L. aeneus* (prevalence 20%; intensity 1–94). As such, the infection in *L. aeneus* does not appear to be incidental but rather represent a potential new definitive host for *A. huronensis*. However, as no gravid caryophyllidean cestodes were observed from *L. aeneus*, the infections may be paradefinitive or even accidental (similar to the findings of Hanzelová et al. 2009; Oros and Hanzelová 2009; and Oros et al. 2011). The co-occurrence of *A. huronensis* and *S. acheilognathi* in *L. aeneus* is also noteworthy as the latter cestode was also introduced into southern Africa with carp. The spillover of *S. acheilognathi* to yellowfish was so successful, that in most surveys by the present authors’ research team in the Vaal River System, this cestode is virtually absent from all carp species, but is instead present predominantly in the near threatened *Labeobarbus kimberleyensis* (Gilchrist & Thompson, 1913). It is thus possible that *A. huronensis* may follow the same progression, arriving with carp, then infecting *L. aeneus* and possibly other native cyprinids, and finally establishing in *L. kimberleyensis*.

As noted, none of the cestodes collected from *L. aeneus* were gravid, but late stage 4 juveniles were observed and thus they may have been on the verge of developing eggs. Unfortunately, the development of juvenile *A. huronensis* in the definitive host is not well studied, and the present
extrapolated interpretation from the work of Ibraheem and Mackiewicz (2006) may be premature. Scholz (1993) studied the development of another caryophyllidean, Khawia baltica Szidat, 1942, and noted that several factors determine the speed of development in the definitive host. Thus, it is not possible to determine if the cestodes collected from *L. aeneus* recently inoculated the host, or whether they had been present for some time, with development either delayed or halted due to infection of a sub-optimal host.

The effect of *A. huronensis* on common carp has been studied on several occasions (Mackiewicz et al. 1972; Molnár et al. 2003; Gjurčević et al. 2012), indicating severe pathology on the host due to mechanical displacement and epithelial loss. No study of the effect of this cestode on other host species has been recorded. Therefore, the pathology observed due to the infection of *L. aeneus* with *A. huronensis* was compared to that for common carp. Similar to what was observed in carp, the scolex of *A. huronensis* attached to the intestinal wall of *L. aeneus*, presumably with a tapered scolex (Figure 3E) that penetrate via the intestinal crypts, widening to an arrow-head (Figures 2A, B, 3A, B) or mushroom-like (Figure 3F) shape to embed (Molnár et al. 2003). In *L. aeneus*, the intestinal epithelium is damaged, but cestodes were not seen to penetrate beyond the epithelium, or enter the lamina propria, as in carp. Other pathological changes, similar to that observed in carp, included compressed epithelium; free nuclei and cell debris in the space between the cestode and host tissue; absence of severe inflammatory responses; and the presence of cellular bodies surrounding the parasite body in the intestinal lumen. The shallow ulcerations observed when cestodes penetrated into the submucosa of carp (Gjurčević et al. 2012) were not seen in *L. aeneus*. This indicates that the infections of *A. huronensis* encountered in *L. aeneus* have either not advanced to a chronic state, which is supported by the cestodes not penetrating into the lamina propria, or that they do not achieve a chronic state in this host. The first hypothesis is supported by the fact that no gravid cestodes were recorded from *L. aeneus* and thus the cestodes were still maturing and establishing, but the latter cannot be disregarded as the infection may die off in yellowfish before the cestodes fully mature and penetrate the mucosa, and thus not advance to a chronic stage. Additional sampling, and possibly experimental observations, will be necessary to determine if the cestode can become gravid in yellowfish hosts and establish a chronic infection. However, previous research concluded that infections in carp are usually mild and do not pose a serious risk, with no host deaths associated with infection by *A. huronensis* (Molnár et al. 2003; Kappe et al. 2006; Gjurčević et al. 2012). This does not mean that these cestodes do not pose a threat to native yellowfish populations, some of which are endangered or with populations on the decline, and as such further investigation is needed.
The age of hosts does not seem to impact on the infection of carp (Molnár et al. 2003; Gjurčević et al. 2012), as fry of a few weeks old are equally infected compared to older fish. The age of the fish in the present study were not determined, but they were all of similar sizes and the possible effect of age could therefore not be determined.

Although limited information is available for the intermediate host of these cestodes, aquatic annelids are the usual suspects (Oros et al. 2011). In *A. sagittatus*, *Tubifex* Lamarck, 1816 or *Limnodrilus* Claparède, 1862 have been confirmed to act as intermediate host (Oros et al. 2011). *Tubifex*, along with many other aquatic oligochaetes (about 50 species), are present in the freshwater systems of southern Africa (Day 2015). Thus, the presence of *A. huronensis* in southern Africa is not surprising as both the definitive and possible viable intermediate hosts are present in the region. Additionally, the spillover to *L. aeneus* is probable as these fish feed on benthic invertebrates (Skelton 2001). It is also likely that they will be susceptible to infection from an early age, similar to what has been seen in carp (Molnár et al. 2003; Gjurčević et al. 2012), due to juvenile yellowfish feeding on microscopic organisms, which could include smaller oligochaetes (Skelton 2001). It is interesting that both Scholz et al. (2015) and Smit et al. (2017) recorded *A. huronensis* at similar times in two very distant geographical locations within South Africa. Additionally, the present authors routinely survey both *L. aeneus* and *C. carpio* in the Vaal River system and had not come across caryophyllidean cestodes in cyprinids until 2018. This may indicate that infected carp may have been introduced into both the Limpopo and Vaal Rivers systems at similar times.

The similarity between the 18S and 28S rDNA for specimens collected from *L. aeneus* and *C. carpio* confirms that they are infected by the same species of caryophyllidean cestode. The presence of an alternate haplotype from a single specimens collected from *C. carpio* needs further investigation, but as the only difference between this haplotype (CH2) and those for all other specimens from both hosts (CH1 and LH1) is the absence of polymorphic sites, this variation may be due to sequencing or the amplification of only one allele. The polymorphic sites encountered in CH1 (and LH1) also correspond to those seen in published sequence data, indicating that these polymorphisms are present in other populations of *A. huronensis*. Similarly, CH2 is similar to other data for *A. huronensis* with resolved polymorphic sites. Thus, with the exception of one site, all variation observed for 18S and 28S rDNA data is likely due to the alternate resolution of sites where polymorphisms occur. This reiterates that the regions analysed are suitable for the identification of species and that the present identification is reliable. Unfortunately, no 18S or 28S rDNA data has been generated for previous collections of *A. huronensis* in Africa and thus genetic comparison is not possible. However, data for the second internal transcribed spacer
(ITS2) rDNA and the cytochrome c oxidase subunit 1 (cox1) mtDNA have been generated for samples collected in South Africa (Bazsalovicsová et al. 2018). It was revealed that the samples collected in South Africa were genetically similar to those from Slovakia, Hungary, Croatia and Romania, suggesting that they were introduced from continental Europe. This is supported by the notion that common carp was originally introduced to southern Africa from Germany (De Moor and Bruton 1988). However, this introduction was in 1859 and can thus be excluded as the cause of the introduction of A. huronensis which is likely closer to 2013. Additional markers will be useful to determine whether the specimens collected in the Vaal River system belong to the same cohort as those from the Limpopo River system, but the present authors have not been able to generate comparative data for these markers. Further genetic analyses may also assist in determining whether the inoculation of the Vaal River system occurred in the upper reaches near the Limpopo River Basin, spreading downstream, or whether it occurred further downstream and spread upstream during spawning migration or translocation of fish.

The present study is the first to describe the protruding cirrus of A. huronensis using SEM. This was only observed in specimens collected from carp. Anthony (1958) note that the cirrus sac is slightly oval when internal, becoming more pyriform when extruded. Majoros et al. (2003) also make note of the protruding cirrus, but only mention that it is long. The protruding cirri observed in the present study were more finger-like than pyriform, with a rosetted structure at the distal tip. Grey and Mackiewicz (1980) observed a “conspicuous fingerlike projection or branch extending from the side of the body just anterior to the cirrus” (sic.). These authors do not refer to the structure as the cirrus, but rather liken them to duplications of the reproductive system noted by Mackiewicz (1978) in triploid caryophyllideans.

**Conclusions**

The present study not only confirms the presence of A. huronensis in common carp in the upper reaches of the Vaal River system using morphology and genetic characterisation for the first time, expanding on previously recorded distribution of this cestode in the system and southern Africa, but also spillover to native smallmouth yellowfish. Pathological changes in L. aeneus were acute and lacked ulcerations. This spillover to L. aeneus appears very recent and needs further monitoring in these as well as other native cyprinids. Further investigation into the source of the introduction of these cestodes into the system and the drivers behind apparent spillover to a native host is needed, especially as other native host fishes could be at risk.
Atractolytocestus huronensis infecting Labeobarbus aeneus in the Vaal River system

Acknowledgements
The authors would like to thank the members of the Parasitology Laboratory at the University of Johannesburg for their assistance during field collections, specifically Dr. Beric Gilbert. The Spectrum Analytical Facility at the University of Johannesburg is also thanked for providing infrastructure for acquiring scanning electron micrographs. The authors would also like to express their sincere appreciation to the owners of the property below the Vaal Dam wall were collections for the current study took place, Bryan and Julia Webster. Finally, the reviewers and editorial board are thanked for their input and assistance during the preparation of the manuscript.

Funding declaration
The authors would like to thank the University of Johannesburg (UJ) Science Faculty and Central Research Committee for funding to AAO, and the UJ Global Excellence and Stature post-doctoral fellowship for funding to QMDS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author’s contribution
AAO – research conceptualization, sample design and methodology, data analysis and interpretation, ethics approval, funding provision, review and editing. QMDS – research conceptualization, sample design and methodology, investigation and data collection, data analysis and interpretation, first draft, review and editing.

Ethics and permits
Fish were collected and euthanized in accordance with permit CPE2-0118 from Nature Conservation of Gauteng Province Government, South Africa; permit MPB. 5601 from the Mpumalanga Tourism and Parks Agency; and ethics reference 2016-5-03 from the University of Johannesburg.

References


**Supplementary material**

The following supplementary material is available for this article:

**Table S1.** Dates, collection sites, coordinates and collection data for cestodes from *Cyprinus carpio* and *Labeobarbus aeneus* in the Vaal River system.

**Table S2.** Genetic distances (below diagonal) and number of base pair differences (above diagonal) of combined 18S and 28S rDNA data for *Atractolytocestus huronensis* collected from *Cyprinus carpio* and *Labeobarbus aeneus* in the Vaal River system to available data for *Atractolytocestus* spp., *Khavia sinensis* and *Breviscolex orientalis*.

This material is available as part of online article from: http://www.reabic.net/aquaticinvasions/2022/Supplements/AI_2022_DosSantos_Oldewage_SupplementaryMaterial.xlsx