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Molecular evidence of a putative new *Atractolytocestus* Anthony, 1958 (Cestoda: Caryophyllidea) species parasitic on common carp (*Cyprinus carpio*) in the People's Republic of China

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Research Paper

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Abstract

Three tapeworm species belonging to the genus *Atractolytocestus* Anthony, 1958 (Cestoda: Caryophyllidea) were reported from the common carp, *Cyprinus carpio* L., 1758 in the People's Republic of China so far: *Atractolytocestus sagittatus*; *Atractolytocestus tenuicollis*; and *Atractolytocestus huronensis*. In this study, we identified a putatively new tapeworm species in common carp from the Danjiangkou Reservoir in Central China (Hubei Province). The species is morphologically similar to *A. sagittatus*, but it differed conspicuously in sequences of three molecular markers. Ribosomal internal transcribed spacers 2 sequence identity of the new species was 94.7–95.5%, 91.8–92.7% and 80.0–83.9% with *A. huronensis*, *A. tenuicollis* and *A. sagittatus*, respectively. cytochrome *c* oxidase and NADH dehydrogenase subunit 3 sequence identity with these three species was lower than 92%. We conclude that this is a new tapeworm species, and we named it after the locality: *Atractolytocestus danjiangkouensis* n. sp.

Introduction

Currently there are three valid species in the tapeworm genus *Atractolytocestus* Anthony, 1958 (Caryophyllidea: Caryophyllaeidae): *Atractolytocestushuronensis* Anthony, 1958; *Atractolytocestus sagittatus* (Kulakovskaya & Akhmerov, 1965); and *Atractolytocestus tenuicollis* (Li, 1964). All three parasitize exclusively in the intestine of common carp (*Cyprinus carpio* L.) (Králová-Hromadová *et al.*, 2013). The cosmopolitan *A. huronensis* was originally described in the River Huron (Michigan, USA) (Anthony, 1958), and then it was recorded in Europe (Oros *et al.*, 2004; Kappe *et al.*, 2006; Bazsalovicsová *et al.*, 2011), South Africa (Scholz *et al.*, 2015) and Asia (Li *et al.*, 2017; Bazsalovicsová *et al.*, 2018). *Atractolytocestus sagittatus* was originally described as *Markevitschia sagittata* from the common carp in Russia (Kulakovskaya & Akhmerov, 1965), and then found in Japan and the People's Republic of China (PRC) (Scholz *et al.*, 2001; Xi *et al.*, 2009). In contrast, *A. tenuicollis* was only recorded in the PRC (Li, 1964; Králová-Hromadová *et al.*, 2013). *Atractolytocestus* species were recently surveyed in common carp from Central China. During this new investigation, *A. huronensis* has been found in Taibai Lake (Li *et al.*, 2017), and *A. tenuicollis* in Donghu and Niushan lakes (unpublished data). *Atractolytocestus sagittatus* was not found in this survey, but tapeworms whose internal transcribed spacers (ITS) sequences differed markedly from all three species of the genus were found in the Danjiangkou Reservoir. In the present paper, these tapeworms were characterized morphologically and genetically. We conclude that they belong to a new *Atractolytocestus* species.

Materials and methods

Tapeworm collection and morphological identification

Common carp specimens were collected in April 2018 from the Danjiangkou Reservoir (32°36′–33°48′N, 110°59′–111°49′E), Hubei Province, the PRC. Tapeworms, obtained from the intestine of the fish, were gently rinsed in 0.65% sodium chloride solution, and then fixed with hot 70% ethanol. Tapeworm specimens ($n = 3$) were cut into two parts from the neck. Each scolex segment was preserved in absolute ethanol and stored at 4°C for extraction of the genomic DNA. The remaining part and other complete tapeworms were preserved in 70% ethanol for morphological identification. Specimens for whole mounts ($n = 13$, including

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Table 1. Polymorphisms in repetitive microsatellite motifs within different ribosomal internal transcribed spacers 2 (ITS2) rDNA variants of *Atractolytocestus danjiangkouensis* from *Cyprinus carpio* in the People's Republic of China.

ITS2 variant	Microsatellite repeat 1	Microsatellite repeat 2	Microsatellite repeat 3	Size of ITS2 variant (bp)
1	(GTGC)4(GTTC)4	$(TGC)_3TGT(TGC)_2$	$(TTGGT)_3$	661
2	(GTGC)3(GTTC)6	$(TGC)_3TGT(TGC)_2$	$(TTGGT)_3$	665
3	$(GTGC)_4(GTTC)_5$	$(TGC)_3TGT(TGC)_2$	$(TTGGT)_3$	665

Table 2. Mitochondrial cytochrome *c* oxidase sequences of *Atractolytocestus* spp. used for the current phylogenetic analysis.

Mitochondrial haplotype code	Species	Country	Genbank accession numbers
Ha1	*Atractolytocestus huronensis*	Slovakia (SK) Hungary (HU) Croatia (CR) Romania (RO)	HM480475 HM480476 HM480477 HM480478
Ha2	*A. huronensis*	United Kingdom (UK) United States (USA) People's Republic of China (PRC)–Danjiangkou (CH–D) PRC–Poyang (CH–P)	HM480479 HM480480 MF959101 MF959102
Ha3	*Atractolytocestus sagittatus*	Japan (JP)	JF424669
Ha4	*Atractolytocestus tenuicollis*	PRC (CH)	KC834609–13
Ha5	*A. huronensis*	South Africa (SA)	KP635010
Ha6	*A. huronensis*	PRC–Danjiangkou (CH–D)	MF959103
Ha7	*A. huronensis*	PRC–Poyang (CH–P)	MF959104
Ha8			MF959105
Ha9			MF959106
Ha10	*Atractolytocestus danjiangkouensis*	PRC–Danjiangkou (CH–D)	MW533426
Ha11			MW533427
Ha12			MW533428
	Khawia sinensis	PRC	JN004232
	Breviscolex orientalis	Japan	JQ034055

the remaining part of the above three specimens) were stained with iron hydrochloric carmin, dehydrated in a graded ethanol series, cleared in xylene and mounted in Canada balsam as permanent preservation (Fu *et al.*, 2019). Seven individuals were used to perform histological sections using the standard protocols as follows: embedding the samples in paraplast; sectioning by microtome; staining with haematoxylin and eosin; and mounting in Canada balsam (Xi *et al.*, 2016).

DNA isolation, ribosomal internal transcribed spacers 2 (ITS2), cytochrome c oxidase (cox1) and NADH dehydrogenase subunit 3 (nad3) gene amplification and sequencing

Genomic DNA of the three tapeworm scolex parts was extracted using the Tissue Cell Genome Kit (Beijing, PRC) according to the manufacturer's instructions. Complete ITS2 sequence was amplified using primers 5.8S–2 (5′–GTC GAT GAA GAG CGC AGC–3′) and ITS2 (5′–AGG AGG CGA ATC ACT AT–3′) (Králová-Hromadová *et al.*, 2010). Polymerase chain reaction (PCR) amplification of ITS2 was conducted using LA Taq polymerase as follows: 5 min at 94°C as the initial step; then 35 cycles of 30 s at 94°C, 30 s at 50 °C, 1 min at 72°C. The final step was 7 min at 72°C. A fragment of the *cox1* gene was amplified with primers CFCYT2 (5′–ACT AAG TGT TTT CAA AA–3′) and CRCYT2 (5′–CCA AAA AAC CAA AAC AT–3′) (Bazsalovicsová *et al.*, 2012). Conditions of *cox1* gene PCR amplification were the same as that of ITS2, but the annealing temperature was 42°C. The primers NAD3F (5′–AAC GTA GCT AGT TAA GTG CTG AAT TCT–3′) and NAD3R (5′–CTT TTA ATT ATT AGC AGT AAC CGA TCT C–3′), designed with software Primer 6, were used to amplify the complete sequence of the *nad3* gene. Except for the 51°C annealing temperature, the PCR amplification of *nad3* was also the same as that of ITS2. All PCR products were loaded on a 1.5% agarose gel. After purification, PCR products were sequenced with the PCR primers described above, and the sequences were assembled manually with the software ContigExpress (except for ITS2 sequences). The purified PCR products of ITS2, amplified from three individuals, were cloned into the pGEM®–T Easy vector (Promega) following the manufacturer's protocol. Five recombinant clones were selected from each individual (DJ1/1–5, DJ2/1–5, DJ3/1–5) for sequencing. The boundaries of ITS2 were determined according to the sequences of *A. huronensis*.

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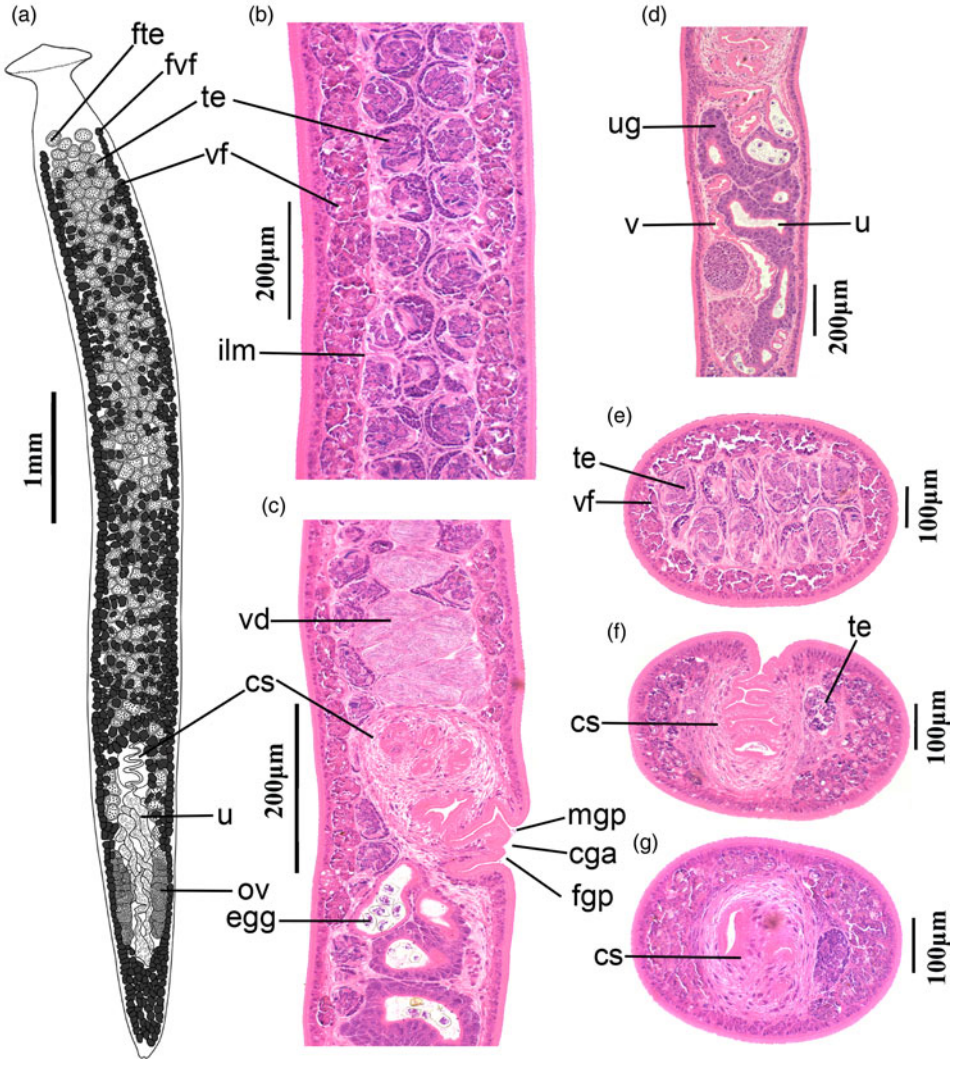

Fig. 1. Morphological characteristics of *Atractolytocestus danjiangkouensis* from *Cyprinus carpio* in the People's Republic of China. (a) total view; (b) sagittal section at the level anterior to cirrus- sac; (c) sagittal section at the level of cirrus-sac; (d) sagittal section at the level posterior to cirrus-sac; (e) cross-section at the level anterior to cirrus-sac; and (f, g) cross-section at the level of cirrus-sac. Abbreviations: cga, common genital atrium; cs, cirrus-sac; fg, female genital pore; ilm, inner longitudinal musculature; mg, male genital pore; ov, ovary; te, testes; ug, uterine glands; us, uterus; v, vagina; vd, vas deferens; vf, vitelline follicles.

The ITS2 sequences were compared with ITS2 variants of *A. huronensis*, *A. tenuicollis* and *A. sagittatus* (table 1). Newly obtained *cox1* sequences were compared with previously published *cox1* haplotypes of *A. huronensis*, *A. tenuicollis* and *A. sagittatus*, including haplotype 1 (Ha1) from continental Europe (EU), in particular Slovakia (SK), Hungary (HU), Croatia (CR) and Romania (RO), Ha2 from the United Kingdom (UK), the United States of America (USA), the Danjiangkou Reservoir and Poyang Lake, the PRC (CH–D, CH–P), Ha3 from Japan (JP), Ha4 from the PRC (CH), Ha5 from South Africa (SA), Ha6 from the Danjiangkou Reservoir (CH–D), and Ha7/8/9 from Poyang Lake (CH–P) (see table 2 and Bazsalovicsová *et al.*, 2018).

Phylogenetic analysis and genealogy reconstruction

The maximum likelihood (ML) method was used to construct phylogenetic trees using *cox1* sequences (table 2) in PhyML3.0 (Guindon *et al.*, 2010). Based on the Akaike information criterion, GTR + G was chosen as the optimal nucleotide substitution model for *cox1* (Lefort *et al.*, 2017). ML trees were obtained with 1000 bootstraps of the data. *Khawia sinensis* Hsü, 1935 (Accession no. JN004232) and *Breviscolex orientalis* Kulakovskaya, 1962 (Accession no. JQ034055) were used as outgroups in the *cox1* analysis. To visualize the level of intra-population and inter-population genetic variability of the sampled individuals and three valid species of genus *Atractolytocestus*, parsimony network was prepared for the *cox1* datasets using the program TCS (Clement *et al.*, 2002) implemented in PopART (Leigh & Bryant, 2015).

Results

Morphological identification

Based on 13 mounted specimens and seven histological sections, measurements are in micrometres (μm) unless otherwise stated (fig. 1).

Family Caryophyllaeidae Leuckart, 1878

Genus *Atractolytocestus* Anthony, 1958

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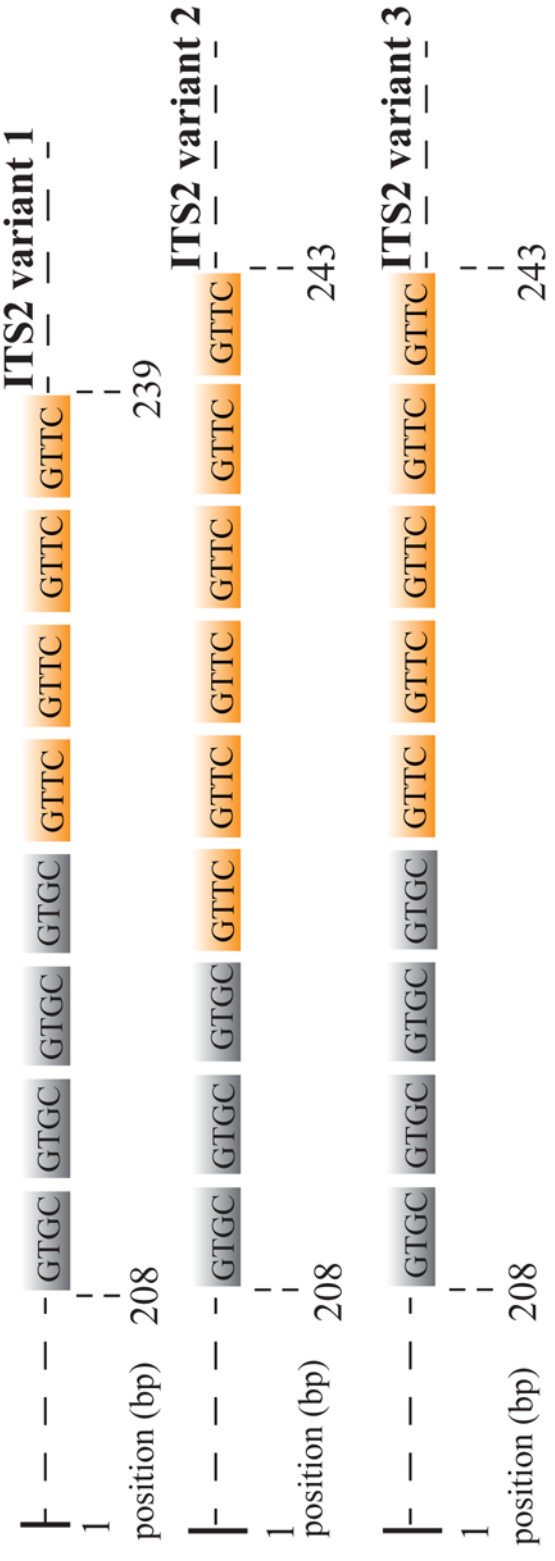

Fig. 2. Schematic presentation of ribosomal internal transcribed spacers 2 variants of the *Atractolytocestus danjiangkouensis* from *Cyprinus carpio* in the People's Republic of China.

Table 3. Comparison of sequence similarity of tapeworms of *Atractolytocestus danjiangkouensis* from *Cyprinus carpio* in the People's Republic of China with *Atractolytocestus huronensis*, *Atractolytocestus tenuicollis* and *Atractolytocestus sagittatus* in ribosomal internal transcribed spacers 2 (ITS2), cytochrome *c* oxidase (*cox1*) and NADH dehydrogenase subunit 3 (*nad3*), respectively.

	A. danjiangkouensis (%)		
Species	ITS2	*cox1*	*nad3*
A. huronensis	94.7–95.5	90.6–91.8	91.4
A. tenuicollis	91.8–92.7	88.8–89.3	–
A. sagittatus	80.0–83.9	75.9–76.0	77.1

***Atractolytocestus danjiangkouensis* n. sp.**

Host: *Cyprinus carpio* L. (Cypriniformes: Cyprinidae)

Locality: Danjiangkou Reservoir (Hubei Province) (110°7′–111°53′E, 32°15′N–33°22′N)

Type specimens: Voucher specimens (CN–ihb–PCA401–20) are deposited in the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan (Accession no. IHB20200910-1).

Representative DNA sequences: The newly generated sequences were submitted to the GenBank database under the accession numbers MW534425–39, MW533426–28 and MW506228–30.

Description (fig. 1)

Tapeworm body 7.4–9.6 mm long, with a maximum width of 0.59–0.67 mm. Scolex bulboacuminate or conical (0.62–0.85 mm wide), much wider than neck (0.29–0.43 mm wide). (fig. 1a). Testes medullary, spherical to oval, 96–130 long, 79–104 wide, and the number of testes numerous (approximately 200) (fig. 1b, c, e). Testes arranged in dorso-ventral rows, 10–12 on cross-section (fig. 1e). First testis anterior to the first vitelline follicle, testes anterior to the ovary (fig. 1a). Vitelline follicles cortical, extensive, oval, 53–79 long, 45–66 wide, uninterrupted alongside cirrus sac, uterine coils and ovarian arms (fig. 1a). Vas deferens forms several loops, anterior to cirrus-sac (fig. 1c). Cirrus-sac thick-walled, almost spherical, 263–273 × 255–265 (fig. 1a, c, f, g).

Ovary H–shaped, 560–728 long, 379–485 wide, with long ovarian lateral arms (fig. 1a). Vagina tubular, slightly sinuous, extending forward between ovarian isthmus and uterine loops (fig. 1d). Male genital pore anterior to female pore, opening into the common genital atrium (fig. 1c).

Uterus strongly coiled, forming loops between post-ovarian vitelline follicles and posterior margin of cirrus-sac (fig. 1a, c). Uterine glands present and well developed (fig. 1c, d). Eggs not examined.

Molecular characteristics and phylogenetic analysis

Divergent intragenomic copies were detected in the ITS2 ribosomal spacer of tapeworms from the Danjiangkou Reservoir. A total of 15 recombinant clones (Accession no. MW534425–39) obtained from three tapeworm individuals yielded eight different sequence types caused mainly by single nucleotide polymorphisms (SNPs) and varying numbers of the short repetitive region (GTGC)n(TTGGT)n. This allowed sorting of the ITS2 sequence types into three ITS2 variants (661, 665 and 665 base pairs (bp)) (table 1 and fig. 2). The ITS2 variant three was the most frequent and observed in a majority (nine out of 15) of the recombinant clones. ITS2 variant two was present only in two clones. The

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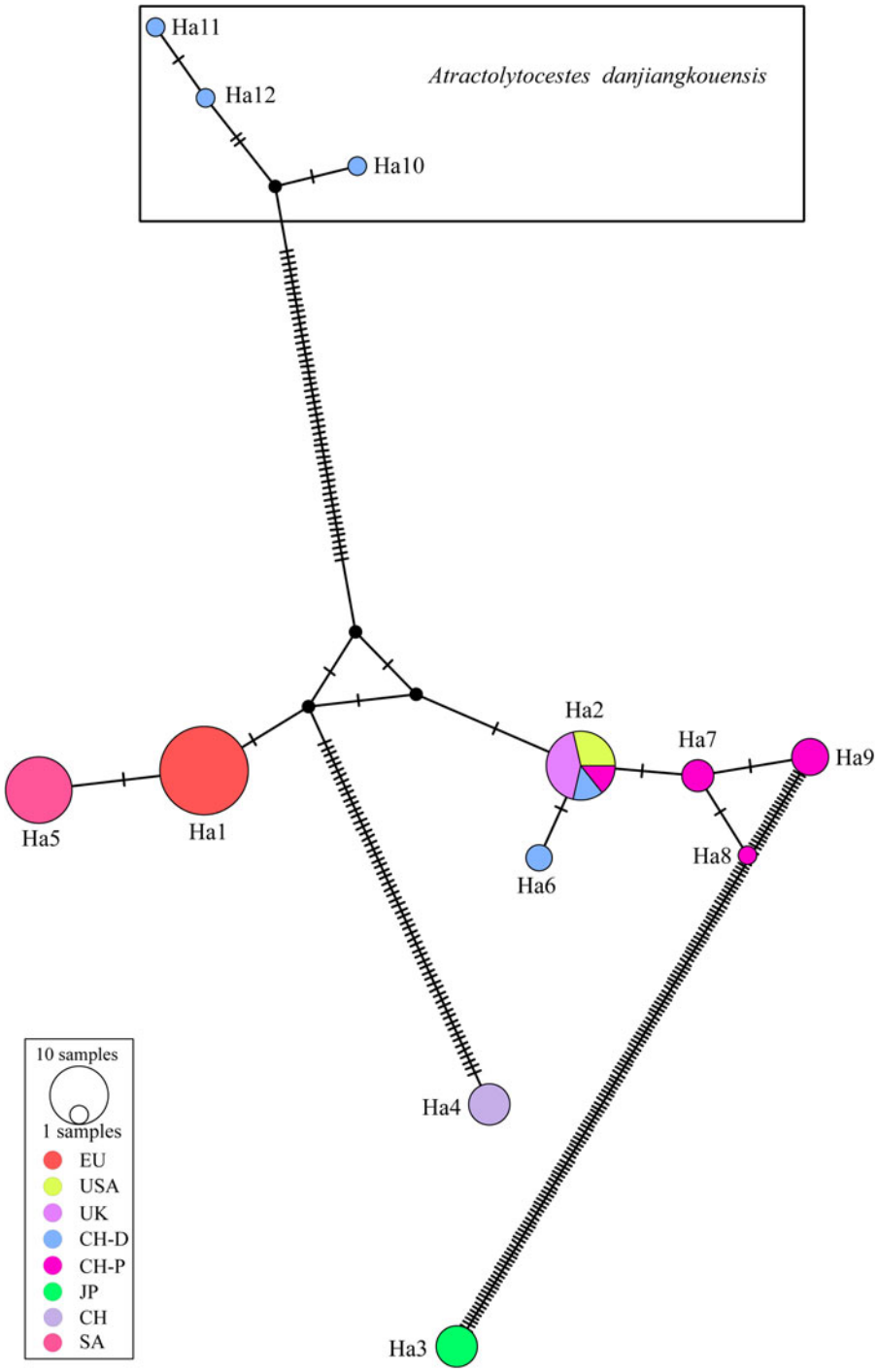

Fig. 3. Haplotype network based on mitochondrial *cox1* data on *Atractolytocestus* species. Country codes are as in table 2. EU stands for samples from continental Europe (SK, HU, CR and RO).

pairwise sequence identity among sequence types ranged between 99.1% and 100%. Compared with *A. huronensis*, *A. tenuicollis* and *A. sagittatus*, the ITS2 sequence identity of the newly collected tapeworms was 94.7–95.5%, 91.8–92.7% and 80.0–83.9%, respectively (table 3).

The total length of the amplified *cox1* sequences (Accession no. MW533426–28) was 672 bp, encoding for 224 amino acids of the protein. The mitochondrial *cox1* sequences revealed the presence of three new haplotypes, which differs from all so far determined *Atractolytocestus* haplotypes (we designated the new haplotypes as Ha10 to Ha12; haplotypes 1–9 have already been recognized in the three valid species of genus *Atractolytocestus*; table 3 and fig. 3). The pairwise sequence identity among haplotypes ranged between 99.4% and 99.9% (SNPs). However, all these mutations were silent and did not result in a change in the amino acid sequence. Interspecific pairwise identity of the haplotypes Ha10–12 with *A. huronensis*, *A. tenuicollis* and *A. sagittatus* was 90.6–91.8%, 88.8–89.3% and 75.9–76.0%, respectively (table 3).

The total length of the amplified *nad3* sequences (Accession no. MW506228–30) was 348 bp, encoding for 115 amino acids. The identity of *nad3* was 91.4% and 77.1% with *A. huronensis* and *A. sagittatus*, respectively (table 3).

Phylogenetic tree based on *cox1* indicated that tapeworms from the Danjiangkou Reservoir (Hubei, the PRC) were most

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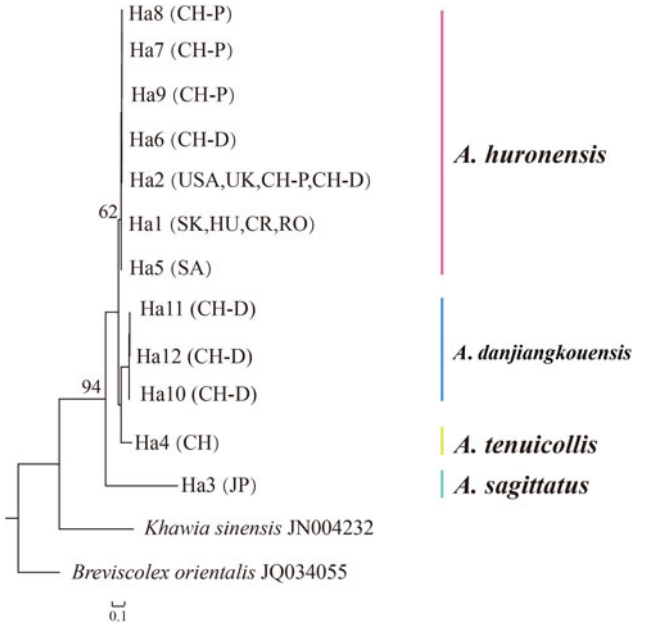

Fig. 4. Phylogenetic relationships of *Atractolytocestus danjiangkouensis* from *Cyprinus carpio* in the People's Republic of China inferred using maximum likelihood (ML) method and *cox1* sequences. Numbers at the branches represent bootstraps' values.

closely related to *A. tenuicollis*, followed by *A. huronensis*, and finally *A. sagittatus* (fig. 4).

Discussion

Morphological differentiation of the three valid species, *A. huronensis*, *A. sagittatus* and *A. tenuicollis*, is mainly based on the number of testes and the relative position of the first testes (fte) and the first vitelline follicles (fvf) (Králová-Hromadová *et al.*, 2013). Based on morphological characters, the newly collected tapeworm found in common carp from the Danjiangkou Reservoir differs from *A. huronensis* and *A. tenuicollis* (table 4). In comparison with *A. huronensis*, the new species had more testes (200 vs. 0–58). The fte were anterior to the fvf, but posterior in *A. huronensis*. Testes extended posteriorly to the anterior ovary in the new species, but only posterior to the cirrus-sac in *A. huronensis*. In addition, the ovarian arms of the new species were longer than those in *A. huronensis* (Oros *et al.*, 2004). Compared with *A. tenuicollis*, the fte were anterior to the fvf in the new species (Králová–Hromadová *et al.*, 2013). The morphological characteristics of the newly collected tapeworms were similar to those of *A. sagittatus* as characterized by Scholz *et al.* (2001), which exhibited a larger body size and shorter lateral arms.

However, significant molecular differences between tapeworms from the Danjiangkou Reservoir and *A. sagittatus* were found. The sequence identity between the nuclear ITS2 and mitochondrial *cox1* and *nad3* genes was not higher than 84% between the two species (80.0–83.9%, 75.9–76.0% and 77.1%, respectively). This suggests that the Danjiagkou population represents a different *Atractolytocestus* species, genetically distinct from from *A. sagittatus*. Although *A. sagittatus* was reported in Taihu and Niushan lakes, the PRC (Xi *et al.*, 2009), some morphological characteristics were different from the original description of *A. sagittatus* (Kulakovskaya & Akhmerov, 1965). The fte were posterior to the fvf (Xi *et al.*, 2009), which is opposite from *A. sagittatus*, but

Table 4. Morphological comparison of the *Atractolytocestus danjiangkouensis* from *Cyprinus carpio* in the People's Republic of China (PRC) with *Atractolytocestus huronensis*, *Atractolytocestus tenuicollis* and *Atractolytocestus sagittatus*.

Species	*A. danjiangkouensis*	*A. huronensis*[a]					*A. tenuicollis*[b]	*A. sagittatus*[c]
Location	PRC	USA	Hungary	Czech Republic	Slovakia	Germany	South Africa	PRC	Japan
body length (mm)	7.4–9.6	5.0–18.0	3.0–9.0	4.0–9.0	5.0–8.0	5.1–7.9	6.6–10.4	5.27–7.55	10.6–13.3
body width (mm)	0.6–0.7	0.9–2.0	0.5–1.2	0.5–0.9	0.5–0.8	0.9–1.3	1.04–1.38	0.83–1.25	1.34–2.23
number of testes	≈ 200	6–18	0–5	9–14	16–20	2–14	42–58	>200	100–200 (more >200)
size of testes (μm)	91–125 × 81–101	120–150	–	–	–	–	–	–	118–298 × 77–179
CS length (μm)	263–273	256–457	350–450	350–555	506–617	325–575	–	–	528–690
CS width (μm)	255–265	–	–	214–347	299–374	275–375	–	–	480–810
relative position of fte and fvf	anterior	posterior						posterior	anterior
O arm length (μm)	560–728	670–920	800–1100	365–691	446–703	–	680–1130	600–780	690–1048
O width (μm)	379–485	–	600–900	297–482	421–574	500–850	690–840	580–850	960–1580

[a]*A. huronensis* – data from Anthony (1958), Oros *et al.* (2004), Kappe *et al.* (2006) and Scholz *et al.* (2015).
[b]*A. tenuicollis* – data from Li (1964) and Králová-Hromadová *et al.* (2013).
[c]*A. sagittatus* – data from Scholz *et al.* (2001). '–': not available; CS: cirrus-sac; fte: first testes; fvf: first vitelline follicles; O: ovary.

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identical to *A. tenuicollis*. Subsequently, an *Atractolytocestus* species found in the same locality, Niushan Lake, was identified as *A. tenuicollis* based on molecular data (Králová-Hromadová *et al.*, 2013). Concerning the geographical distribution, *A. sagittatus* was firstly described as *Markevitschia sagittata* in the intestine of *C. carpio* from the Amur River basin in Russia (Kulakovskaya & Akhmerov, 1965), and subsequent studies mostly reported it from Japan (Scholz *et al.*, 2001; Bazsalovicsová *et al.*, 2012) Therefore, the identification of *A. sagittatus* recorded in the PRC by Xi *et al.* (2009) was most likely incorrect, and the species morphologically most probably corresponded to *A. tenuicollis*. Regarding the new putative *Atractolytocestus* species, it morphologically corresponded to *A. sagittatus*, but it differed in the molecular aspect.

Obvious differences in morphology were found between the putatively new tapeworm species and *A. huronensis* and *A. tenuicolli*, but ITS sequence identity was high among these species, with values ranging between 94.7–95.5% and 91.8–92.7%, respectively. Similar sequence identity levels, ranging between 91.7 and 95.2%, were also reported between *A. huronensis* and *A. tenuicolli* (Králová-Hromadová *et al.*, 2013). Divergent intra-genomic ITS2 sequences were also observed among different specimens of the new species, but the identity was higher than 99.4%. The same phenomenon had been observed previously in the congeners of *A. sagittatus* (Bazsalovicsová *et al.*, 2012) and *A. tenuicollis* (Králová-Hromadová *et al.*, 2013). The molecular differences in ITS2 sequence between the new species and *A. huronensis* and *A. tenuicolli* were higher than the within-species variation. The morphological and molecular analyses indicate that this tapeworm was a cryptic species most closely related to *A. sagittatus*. We named it *A. danjiangkouensis* n. sp.

In addition, *A. huronensis* was also recorded in the Danjiangkou Reservoir by Bazsalovicsová *et al.* (2018). It would be interesting to study the existence of these two congeners in sympatry, as it may help assess whether the origin of parthenogenetic *A. huronensis* may involve interspecific hybridization.

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Conflicts of interest. None.

Ethical standards. All applicable institutional, national and international guidelines for the care and use of animals were followed.

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