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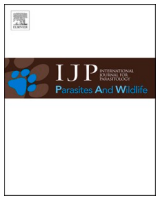

Hidden diversity of cestodes in wild African carnivores: I. Non-taeniid cyclophyllideans

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ABSTRACT

Our knowledge of parasites in wildlife remains limited, primarily due to restricted access to samples, especially of parasites from protected species. This present study contributes to the comprehension of the enigmatic world of helminths of African wild mammals and cestode biodiversity by combining both molecular and morphological analysis. Cestode samples were opportunistically collected from 77 individual definitive hosts in South Africa, Namibia and Ethiopia, encompassing 15 different species of wild African carnivores and additionally domestic cats. The analysis revealed 32 different cyclophyllidean species of which 21 (65.6 %) represent previously unknown genetic entities. They belong to the families Mesocestoididae, Hymenolepididae, Dipylidiidae and Taeniidae. Here we cover the non-taeniid cestodes, while the taeniids will be addressed in a separate publication. Three of the non-taeniid species uncovered in this study could be assigned to the genus *Mesocestoides* and were isolated from servals and domestic cats. The white-tailed mongoose was found to be a suitable host for a species belonging to the Hymenolepididae, which was identified as *Pseudandrya* cf. *mkuzii*. Both feline and canine genotypes of *Dipylidium caninum* were detected in domestic cats, the canine genotype also in an African wolf. In addition to these, a novel species of *Dipylidium* was discovered in an aardwolf. Lastly, four distinct species of *Joyeuxiella* were found in this study, revealing a cryptic species complex and emphasizing the need for a taxonomic reassessment of this genus. Despite the limited scope of our study in terms of geography and sample size, the results highlight that biodiversity of cestodes in African wild mammals is grossly under-researched and follow-up studies are urgently required, in particular linking morphology to gene sequences.

1. Introduction

With more than 3000 known species, the order Cyclophyllidea is the largest among mammal cestodes (Mariaux et al., 2017). Life cycles of cyclophyllideans include a mammalian definitive host and one or more intermediate hosts that may be vertebrates or invertebrates. While the cestode species of humans, livestock and companion animals are relatively well known, this is not so for most taxa which are adapted to wild mammals. On estimate, only about one-third of all global cestode species have been described to date (Caira et al., 2017). This is partly due to the restricted access to samples, in particular concerning parasites from legally protected wildlife. Also, most cestode species had been described in the past based on morphological features alone, which can be ambiguous and has led to misclassification (Schmidt, 1970; Khalil et al., 1994). Routine application of gene sequencing has become available relatively recently, leading to the discovery of novel (cryptic) species and uncovering phylogenetic relationships. Thanks to this molecular method, it has become apparent that the actual cestode diversity exceeds by far what can be observed at the morphological level (De León and Nadler, 2010; Mariaux et al., 2017; Cháves-González et al., 2022).

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Therefore, numerous taxa still await reassessment, especially cestodes from wild animals.

Sub-Saharan Africa still harbours an impressive number of wild mammal species, and their cestode fauna – as described so far – is equally diverse (Mettrick and Beverley-Burton, 1961; Round, 1968; Hunkeler, 1974). For numerous African wildlife cestodes, detailed morphological descriptions date back to the nineteenth and the beginning of the twentieth century, valuable achievements which still serve as principal references for morphological identification (Setti, 1897; Baer, 1926; Baer, 1933; Ortlepp, 1938). Thus, eight cyclophyllidean families are known from African terrestrial mammals as definitive hosts: Anoplocephalidae (mostly ungulates and Rodentia) (Round, 1968; Hunkeler, 1974; Haukisalmi, 2008, 2013), Davaineidae (Hyracoidea, Carnivora, Manoidea and Rodentia) (Yamaguti, 1959; Quentin, 1964; Round, 1968; Hunkeler, 1974), Dilepididae (Rodentia) (Quentin, 1964; Hunkeler, 1974); Dipylidiidae (Carnivora) (Round, 1968; Schuster, 2020), Taeniidae (mainly Carnivora, with other mammals as intermediate hosts) (Round, 1968; Verster, 1969; Loos-Frank, 2000), Hymenolepididae (Carnivora) (Round, 1968; Hunkeler, 1974; Greiman and Tkach, 2012), Mesocestoididae (Hyracoidea and Carnivora) (Round, 1968) and Catenotaeniidae (Rodentia) (Round, 1968; Haukisalmi et al., 2018).

In addition to their definitive hosts, most cyclophyllidean cestodes require one or more intermediate hosts to complete their life cycle. Intermediate hosts may be arthropods, amphibians, reptiles or, as is the case for Taeniidae, other mammals. Depending on the cestode species, the metacestode (larval stage) is nested in various tissues or lumina of the intermediate host. Generally, the definitive host acquires cestodes by preying on (or accidentally ingesting) the final intermediate host with the metacestode. The cestode will develop into its adult stage within the definitive host and reproduce. The parasite's eggs are released into the environment with the faeces of the definitive host and taken up by the (first) intermediate host. Any subsequent intermediate hosts, if present, usually acquire the cestode through a predator-prey relationship. However, often-times the life cycles of cestodes and their host ranges are only incompletely known. The vast majority of these parasites of wild mammals, of which descriptions were published more than fifty years ago, have not been re-evaluated nor have they been assessed by molecular means.

As systematic large-scale sampling of wild mammals is usually not possible in the present age of dwindling wildlife, new insights into wild mammal parasites depend on opportunistic sampling. This is often unsatisfactory in terms of sample sizes, geographical coverage and quality of the sampled material, but, as in the present study, can yet provide valuable insights into the hidden diversity of mammalian parasites. Here we report our effort to identify cestodes from opportunistically collected wild carnivores from northeastern and southern Africa, combining genetic and morphological data. The present publication focusses on non-taeniid tapeworms only.

2. Materials and methods

2.1. Collection of adult cestodes

The cestode samples originate from 77 mammals of 16 species collected in South Africa, Namibia, and Ethiopia (Table 1). The study focussed on collection of wild animals, but some free-roaming domestic cats (*Felis catus*) were included, as their prey spectrum and predatory behaviour closely resemble that of small wild felids. Furthermore, only carcasses of domestic cat from the same areas as the wild animals were included in the study.

All Ethiopian host animals were roadkills collected between the cities Addis Ababa and Dire Dawa. Parasites from South Africa were obtained from roadkills in the northern provinces Limpopo and Mpumalanga. The Namibian samples consisted of faecal material of individual animals from the Etosha National Park or parasites found during necropsy of legally hunted jackals (*Lupulella mesomelas*) and cheetahs (*Acinonyx jubatus*) on farmland that had been collected in the context of other surveys (Wassermann et al., 2015; Aschenborn et al., 2023).

Table 1
Host species and their numbers collected in Ethiopia, Namibia and South Africa. Cestodes collected from hosts were obtained either from faecal samples (F) or by necropsy (N). Numbers in square brackets [n] specify the number of hosts for which this collection method applied.

Origin	Host species	n	Collection method
Ethiopia	*Canis lupaster*	4	N
	Crocuta crocuta	4	N
	Felis catus familiaris	5	N
Namibia	*Acinonyx jubatus*	4	F [3], N [1]
	Caracal caracal	2	F
	Lupulella mesomelas	12	F [2], N [10]
	Lycaon pictus	1	F
	Panthera leo	9	F
South Africa	*Civettictis civetta*	1	N
	Crocuta crocuta	3	N
	Felis catus familiaris	6	N
	Felis lybica	1	N
	Genetta genetta	2	N
	Genetta maculata	5	N
	Ichneumia albicauda	1	N
	Leptailurus serval	5	N
	Lupulella mesomelas	4	N
	Lycaon pictus	1	N
	Panthera leo	4	N
	Panthera pardus	2	N
	Proteles cristata	1	N

To obtain adult cestodes, the host's intestines were opened, the content visually examined, and macroscopically visible parasites isolated, rinsed with water and preserved in 70% ethanol until further investigation. In case of faecal samples, zinc-chloride flotation was performed for egg isolation as described previously (Mathis et al., 1996; Wassermann et al., 2015).

For comparative purpose, adult *Mesocestoides litteratus* and *Joyeuxiella* sp. aff. *pasqualei* were included in the analyses. The specimen of *M. litteratus* was collected from a red fox (*Vulpes vulpes*) in Southern Germany and genetically confirmed by the analysis of the mitochondrial *nad1* gene. Material of *J.* sp. aff. *pasqualei* was collected from a domestic cat in the United Arab Emirates. The adult worm was morphologically evaluated by two independent researchers to match the descriptions of *J. pasqualei*.

2.2. Molecular identification and phylogenetic analysis

Small pieces of strobila (~0.1 cm^3) or single eggs were transferred into separate 0.2 ml tubes, containing in case of tissue 20 µl or for eggs 10 µl 0.02 M NaOH solution, and were lysed at 95 °C for 10 min (Nakao et al., 2003). The lysate was used directly as template for the following PCRs.

Primary target sequence was a fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene (approximately 400 bp, length variation depending on the species). Samples of interest were further investigated for the complete *cox1* and NADH dehydrogenase subunit 1 (*nad1*) genes, as well as partial sequences of cytochrome *b* (*cob*), the 12S and nuclear 18S ribosomal RNA (rRNA) genes. The complete *cox1* gene was amplified in two fragments with overlapping sequences.

Primers for *cox1*, *nad1* and *cob* were designed to amplify a broad spectrum of cyclophyllidean cestode species (Table 2). The *nad1* primer set 'nad1-Taen' was preferred for taeniid specimens, 'nad1-Alt' was used with non-taeniid samples. The PCR reaction mixtures consisted of 2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each forward and reverse primers, 1x AmpliTaq PCR Buffer II and AmpliTaq Polymerase (0.625 U for the first PCR, 1.25 U for nested PCR) (Thermo Fischer Scientific). For the first PCR, 1 µl of lysate was added to 24 µl reaction mixture. For the

Table 2
Primer sequences used for the amplification of target sequences of cyclophyllidean cestodes.

Gene	Region	Primer Pair	Step	Forward Primer	Reverse Primer	length (bp)	T_a
cox1	centre	Cyclo-ID	first	5′-TTTGATCGTAAATTTAGTTCTGC-3′	5′-GCAACAACAAATCAAGTATCATG-3′	~450	50 °C
			nested	5′-GTTCTGCTTTTTTTGATCC-3′	5′-GTATCATGTAGAACTTTATC-3′	~400	50 °C
	front	Cycox1w-F	first	5′-GTTACTGCTAATAATTTTTTGTC-3′	5′-GCATATAHAACCAAGTAAAHACC-3′	~950	50 °C
			nested	5′-GTCAATTDRTTTTGACTTTCTC-3′	5′-CCAAGTAAAHACCTTTATACC-3′	~940	55 °C
	back	Cycox1w-B	first	5′-WATAAAGGTTTRTTATTTGCTATG-3′	5′-ATCHAWTAAGCATGATGCAAAAGG-3′	~780	50 °C
			nested	5′-TATGTTTTCAATAGTBTGTTTAGG-3′	5′-CATGATGCAAAAGGCAAAWAAACC-3′	~760	55 °C
nad1	complete	nad1-Taen	first	5′-TGATGATTTGTCTAGTC-3′	5′-TTCTTGAAGTTAACAGC-3′	~900	45 °C
			nested	5′-GATTTGTCTAGTCATAGATG-3′	5′-CTTGAAGTTAACAGCATCACG-3′	~890	55 °C
		nad1-Alt	first	5′-TTAAGAATGTTGGGTTTGC-3′	5′-GACCAAAGGTCCCCAAAACC-3′	~900	45 °C
			nested	5′-AGAATGTTGGGTTTGCGTC-3′	5′-GGTCCCCAAAACCATCATTC-3′	~890	55 °C
cob	front	Cycob-F	first	5′-CATTTTGTTGATTATGTTTG-3′	5′-CTAAWATHAYAAAACCYAAAC-3′	~520	48 °C
			nested	5′-TAAACTGRTARATTGTGG-3′	5′-CCYAAACAAAYATGHACAG-3′	~510	50 °C
12S rRNA	centre	60.for/375.rev [1]	single	5′-TTA AGA TAT ATG TGG TAC AGG ATT AGA TAC CC-3′	5′-AAC CGA GGG TGA CGG GCG GTG TGT ACC-3′	~310	55 °C
18S rRNA	front	SSU18A/ SSU9R [2]	single	5′-AAA GAT TAA GCC ATG CAT G-3′	5′-AGC TGG AAT TAC CGC GGC TG-3′	~510	52 °C

Abbreviation: T_a = annealing temperature; bp = base pairs. *References:* 1. Von Nickisch-Rosenegk et al. (1999); 2. Blaxter et al. (1998). All other primers were designed for this study.

amplification of the *cox1*, *nad1* and *cob* genes, 1 µl of primary PCR products were used as templates for follow-up nested PCRs with a total reaction volume of 50 µl. PCRs were performed with initial denaturation (5 min, 95 °C) followed by 35 cycles of denaturation (30 s, 95 °C), annealing (30 s), extension (60 s, 72 °C) and a final extension step (5 min, 72 °C). The respective annealing temperatures (T_a) were set as shown in Table 2.

The *nad1* gene of the Namibian egg samples were amplified by nested PCR as described by Wassermann et al. (2015).

The amplicons were purified using the High Pure PCR Purification Kit following the manufacturers instruction (Roche, Mannheim, Germany) and sequenced by Microsynth Seqlab GmbH (Göttingen, Germany).

Sequences were analysed and trimmed with GENtle 1.9.4. (Manske, 2006). Alignments were performed with MEGA X using the ClustalW implementation (Kumar et al., 2018), mitochondrial sequences were translated respecting the echinoderm and flatworm code (translation table 9). Phylogenetic trees and genetic distances were also calculated with MEGA X. Nucleotide substitution models used to compute the maximum likelihood trees were chosen based on PhyML Smart Model Selection and the Akaike information criterion (Lefort et al., 2017). For a dataset of 18S rRNA sequences the HKY + G + I model was chosen (Hasegawa et al., 1985). All other trees were built using the GTR + G + I model (Nei and Kumar, 2000). Support values are based on 1000 bootstrap replications.

For the construction of phylogenetic trees, the new sequences were analysed with reference sequences from GenBank. In the different phylograms, the integrated reference species and the number of sequences vary depending on the availability and length of comparative sequences from specific cestode families in the NCBI database. Since many reference sequences are significantly shorter than the sequences obtained here, two phylogenetic trees were generated in all cases. The first was calculated using a data set where all sequences were trimmed to a common consensus length. The second is based on the entire length of the analysed gene(s), including the associated alignment gaps and missing data (supplementary figures). Sequences taken from GenBank are identified as such with their respective accession numbers.

Concatenated sequences were constructed using only sequences obtained from the same specimen.

All sequences obtained in this study were deposited in NCBI GenBank under the following accession numbers: PP028545-PP028549, PP028783-PP028805, PP035833-PP035843, PP054311, PP054312, PP057931-PP057938 (for a detailed list, see Supplementary Table S1).

2.3. Morphological examination

Proglottids and scoleces of adult specimens were stained in hematoxylin and 70% ethanol (1:2 v/v) for at least 30 min and then briefly washed in 70% ethanol with 5% hydrochloric acid to de-stain the cuticle. Stained specimens were dehydrated through an ethanol series (70–100%) and then cleared with a eugenol series (0.5–100%). The tapeworm fragments were subsequently mounted in Canada balsam.

Microscopic photographs were processed with ZEN imaging software (Carl Zeiss AG). Morphological features were counted and measured. Exemplary specimens served as templates for model sketches for morphological descriptions.

3. Results

The sequences obtained from the cestodes of the 77 examined mammals could be tentatively allocated to 32 different cyclophyllidean species. Of these, only 11 could be specifically identified to species level by comparison with deposited sequences. Most of these (9) belonged to the Taeniidae, which are not covered in this article. The only identifiable non-taeniids were two separate *Dipylidium caninum* genotypes ("feline genotype" in seven domestic cats and "canine genotype" in one domestic cat and one African wolf, *Canis lupaster*) which are herein treated as separate species.

The sequences of the remaining 21 putative species did not match any GenBank deposits (Table 3). A phylogenetic analysis based on fragments of the *cox1* and *nad1* genes showed that 12 of these sequences are firmly rooted in the family Taeniidae (data not shown). The others belonged to Mesocestoididae (three species), Hymenolepididae (one species), and Dipylidiidae (one species). The last four unknown species were identified as *Joyeuxiella* spp.

3.1. Molecular and morphological analyses of non-taeniid cestodes

A phylogenetic tree was constructed based on *cox1* sequences (with a consensus length of 355 bp) of 20 different species from eight cestode families plus the nine novel African non-taeniid sequences discovered in this study (Fig. 1), reflecting the phylogenetic relation between the families and the novel sequences. Supplementary Fig. S1 shows the same compilation based on complete *cox1* sequence length including missing data.

3.1.1. Hymenolepididae - Pseudandrya cf. mkuzii

One specimen isolated from a white-tailed mongoose (*Ichneumia albicauda*) from South Africa yielded a 942 bp long *cox1* and 230 bp 12S

Table 3
Cyclophyllidean cestode lineages collected in this study, from definitive hosts in Ethiopia [E], Namibia [N] and South Africa [S]. The number of hosts examined is indicated by n. Novel lineages identified in this study are *Taenia* spp. a-k, *Hydatigera* sp. and all species listed in bold. The superscript numbers indicate the number of animals in which the respective species was found.

Definitive host species		n	Cyclophyllidean cestodes found
Acinonyx jubatus	Cheetah	4 [N]	*Taenia* spp. (a [1],b [2])[N]
Canis lupaster	African Wolf	4 [E]	*Dipylidium caninum* canine genotype [1E], ***Joyeuxiella* sp. IV** [2E], *Taenia hydatigena* [1E]
Caracal caracal	Caracal	2 [N]	*Taenia* sp. (c) [2N]
Civettictis civetta	African Civet	1 [S]	***Joyeuxiella* sp. I** [1S]
Crocuta crocuta	Spotted Hyena	4 [E] +3 [S]	*Taenia crocutae* [2S], *Taenia* sp. I [4E,*], *Taenia* sp. III [2S,a], *Taenia* spp. (d [1E], e [1S])
Felis catus familiaris	Domestic Cat	6 [S] + 5 [E]	*Dipylidium caninum* canine genotype [1E], *Dipylidium caninum* feline genotype[2E,5S], *Hydatigera taeniaeformis*[3E,1S], ***Joyeuxiella* sp. II** [1S], ***Joyeuxiella* sp. IV** [1E], ***Mesocestoides* sp. III** [2E]
Felis lybica	African Wildcat	1 [S]	*Hydatigera taeniaeformis* [1S], ***Joyeuxiella* sp. I** [1S], ***Joyeuxiella* sp. III** [1S]
Genetta genetta	Small-spotted Genet	2 [S]	*Hydatigera parva* [2S], ***Joyeuxiella* sp. I** [1S]
Genetta maculata	Rusty-spotted Genet	5 [S]	*Hydatigera parva* [3S], ***Joyeuxiella* sp. I** [3S]
Ichneumia albicauda	White-tailed Mongoose	1 [S]	***Pseudandrya* cf. *mkuzii*** [1S]
Leptailurus serval	Serval	5 [S]	*Hydatigera taeniaeformis*[1S], *Hydatigera* sp.[3S], ***Mesocestoides* sp. I** [1S], ***Mesocestoides* sp. II** [2S]
Lupulella mesomelas	Black-backed Jackal	12 [N] + 4 [S]	*Echinococcus equinus* [2N], *Echinococcus ortleppi* [2N], *Taenia hydatigena* [1N], *Taenia* sp. (f) [8N,4S]
Lycaon pictus	African Wild Dog	1 [N] + 1 [S]	*Taenia* sp. (g) [1N,1S]
Panthera leo	Lion	9 [N] + 4 [S]	*Echinococcus equinus* [5N], *Hydatigera taeniaeformis*[1N], *Taenia regis* [5N,4S], *Taenia* spp. (h [1N,1S], i [1S])
Panthera pardus	Leopard	2 [S]	*Taenia* spp. (j[1], k[1])[S]
Proteles cristata	Aardwolf	1[S]	***Dipylidium* sp.** [1S]

[a] Species as listed in Terefe et al. (2014).

rRNA sequence, PCRs of *cob*, *nad1* and 18S rRNA genes remained negative. The phylogenetic positioning of this species among 18 hymenolepidids from mammals, derived from *cox1* sequences truncated to 518 bp, is shown in Fig. 2. Based on the *cox1* data the new species is embedded within a statistically unsupported clade of *Hymenolepis* spp., *Rodentolepis* spp., *Staphylocystis* spp. and *Pseudanoplocephala crawfordi*. Analysis of complete *cox1* sequence length places the novel entity basal to *P. crawfordi* and *Hymenolepis* spp. but also unsupported (Fig. S2). Comparison of the 12S rRNA fragment with GenBank entries did not show a close match.

Morphology was examined based on various worm fragments from a single host. Segment size and maturity suggest them to derive from at least two specimens. No scoleces were available. Total strobila length could not be determined. The width of mature and gravid proglottids does not exceed 1.5 mm. The proglottids are craspedote, their shape is wider than long or almost square in the case of gravid proglottids. Segments have unilateral genital pores. Up to 12 testes could be counted per mature proglottid, six or seven of which are located aporally. The cirrus sack is club-shaped and 200–300 μm in length, starting from the genital pore. The internal vesicula seminalis takes up about half of the space within the cirrus sac. An external vesicula seminalis is not visible. The cirrus of 9 μm diameter is unarmed and cylindrical. Beginning just posterior of the cirrus sac opening, the vagina slopes towards the anterior end of the proglottid and opens into a prominent seminal receptacle, which reaches the middle of the segments and is twisted or curved. The large seminal receptacle persists in gravid proglottids. Within mature segments, the vitellarium can be spotted posterior of the lobed ovary. The uterus starts as an irregular, patchy network in pregravid segments, reaching both anterior and posterior margins. Gravid proglottids are completely filled with eggs, with uterine septa faintly remaining. The rounded oncospheres (26–32 μm diameter) are wrapped in three thin envelopes, of which the outermost has a diameter of 58–65 μm. The diameter of the middle envelope varies between 40 and 50 μm. The innermost membrane surrounding the oncosphere is up to 36 μm wide. Fig. 3 illustrates the morphology of the eggs, as well as a mature proglottid of the species from the dorsal side.

These morphological characteristics were compared with those of previously described species of the family Hymenolepididae. Most species from mammals could be excluded based on the number of testes in mature proglottids. The morphology closely matches descriptions of three *Pseudandrya* species. *Pseudandrya mkuzii*, a tapeworm species that was described by Ortlepp in 1963 and the only hymenolepidid previously known from white-tailed mongoose, is a plausible match based on morphology and host information.

3.1.2. *Mesocestoididae - Mesocestoides spp.*

Three distinct sequences could be assigned to the family of Mesocestoididae: *Mesocestoides* sp. I was found in a serval (*Leptailurus serval*) from South Africa, *Mesocestoides* sp. II in two servals from South Africa, and *Mesocestoides* sp. III in two domestic cats from Ethiopia.

The complete *nad1* (888 bp) and *cox1* (1599 bp) genes were obtained from specimens of *Mesocestoides* spp. II and III, and of a European *M. litteratus* for comparative purposes, whereas only a 1006 bp long fragment of *cox1* could be obtained from *Mesocestoides* sp. I. For the construction of a concatenated sequence tree including data from the NCBI data base, the *nad1* and *cox1* sequence lengths were reduced to 258 (plus one codon gap) bp and 373 bp, respectively (Fig. 4). The reference species were *M. litteratus* (sequences obtained in this study), *M. lineatus*, *M. corti* and *Mesocestoides* spp. M1, M2 and M3 (Varcasia et al., 2018) retrieved from GenBank. It should be noted that *nad1* and *cox1* sequences of *M. lineatus* are not derived from the same specimen, because the *nad1* sequence corresponding to the *cox1* isolate was too short (202 bp) (MH463537). However, this sequence and the *nad1* sequence included in the phylogenetic tree, which was 261 bp, were 100% identical and both originated from *V. vulpes* in Slovakia (Hrčkova et al., 2011). In the phylogenetic tree, which is based on the complete genes and allows for gaps and missing data, these two sequences of *M. lineatus* were included as separate isolates (Fig. S3). Accepting missing data in the tree calculation also enabled the inclusion of *Mesocestoides* sp. I, whose close relationship with the Mediterranean M1 and M2 was confirmed, and the distinctness of the new *Mesocestoides* species II and III was emphasized.

Parts of the 12S rRNA (283 bp) and *cob* (479 bp) genes could only be sequenced from *Mesocestoides* sp. II and did not match closely with any *Mesocestoides* sequence in the NCBI GenBank. Fragments of the 18S rRNA gene were sequenced from both *Mesocestoides* sp. I (506 bp) and II (509 bp), both sequences are identical and because of its highly conserved nature, the comparison with GenBank entries remained inconclusive; the sequences matched several *Mesocestoides* species by >99%.

The morphological description of *Mesocestoides* sp. I is based on early proglottids of a single incomplete specimen. Seven specimens of *Mesocestoides* sp. II and eight of *Mesocestoides* sp. III were available for examination. In all three species, the testes are located in the space between the longitudinal excretory vessels and are separated into two flanks by the female sexual organs. The testes may meet in the midline at the anterior end of the proglottid. Testes numbers varied depending on

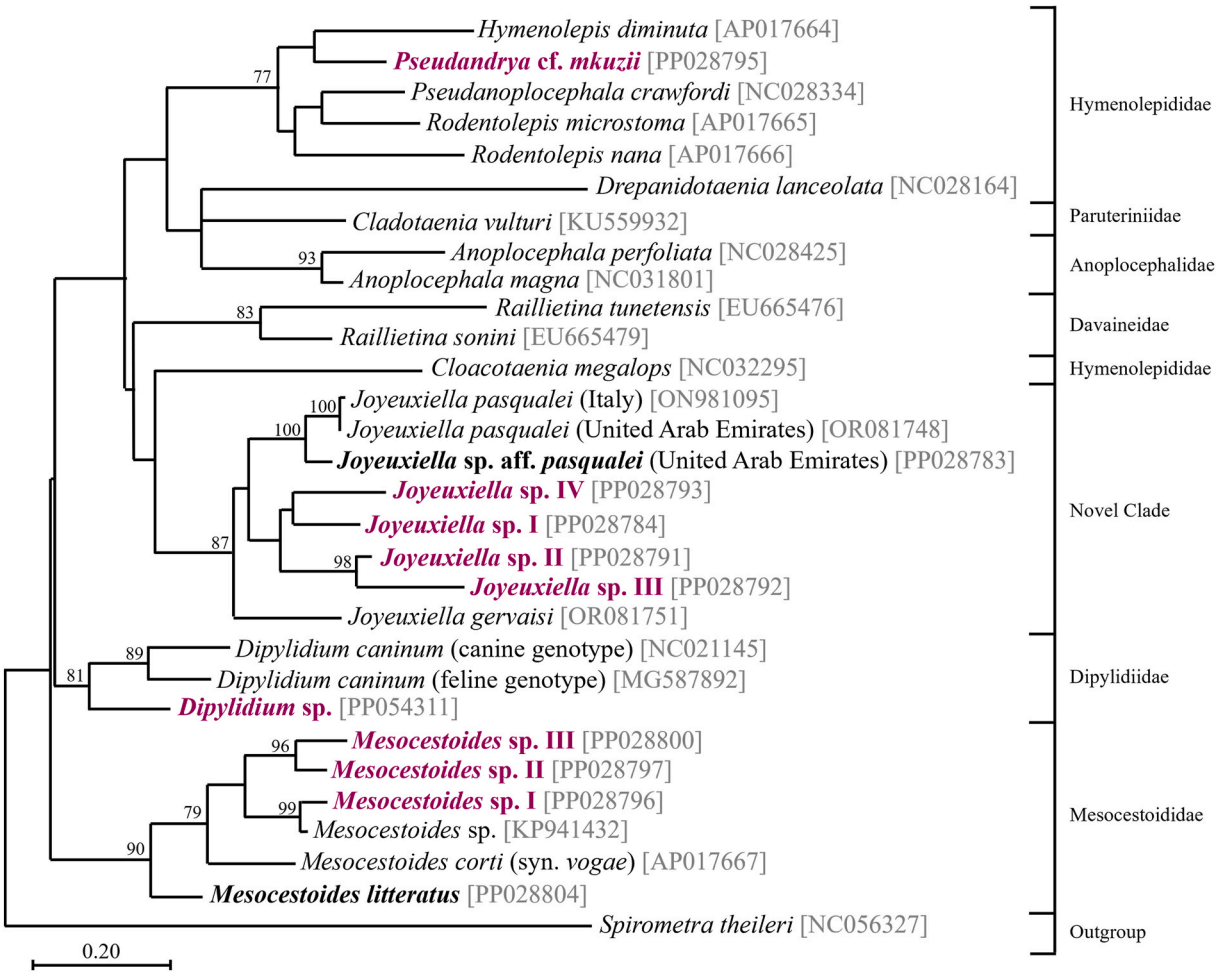

Fig. 1. Maximum likelihood (GTR + G + I) phylogenetic tree based on a 355 bp long fragment of *cox1*. 28 species of cyclophyllidean cestodes are included, *Spirometra theileri* is used as an outgroup. Respective GenBank accession numbers are added after the species name. Novel lineages are written in bold and colour. Bootstrap values > 70 (%) are included. The scale bar represents the estimated number of substitutions per site.

the maturity level of the segments. Between 24 and 30 testes per proglottid were counted in *Mesocestoides* sp. I, 40–57 for *Mesocestoides* sp. II and 28–34 in *Mesocestoides* sp. III. Cirrus sacks of all three species are round. Ovaries and vitellaria may reach the posterior margins of the craspedote segments in all three species. Worms of species II have short and stout gravid proglottids, reaching 1.4 mm width and 2 mm length. Those of species III are in comparison more slender, flatter and elongated in shape, with up to 1 mm width and 5 mm length. *Mesocestoides* sp. II tends to have distinctly serpentine uteri, those of *Mesocestoides* sp. III are mostly narrowly twisted around a linear axis in a corkscrew manner. A typical shape of the uterus of *Mesocestoides* sp. I could not be determined due to the singularity and prematurity of the material at hand.

3.1.3. Dipylidiidae - Dipylidium sp.

From the *Dipylidium* specimen found in an aardwolf (*Proteles cristata*) from South Africa, a *cox1* (1647 bp) and *nad1* (894 bp) sequence as well as the front part of *cob* (472 bp) and a fragment of the 18S rRNA gene (468 bp) were obtained. The novel sequence represents the earliest branching lineage within the *Dipylidium* clade in all the phylogenetic trees (Figs. 1 and 6, Supplementary Figs. S1, S4, and S5). Based on the phylogenetic tree constructed with concatenated mitochondrial sequences, the novel *Dipylidium* sp. and *D. caninum* (canine and feline genotypes) form a sister clade to two *Joyeuxiella* spp. (q.v. 3.1.4.) which is, however, not strongly supported (Fig. 6 and S4). This sister-clade relationship cannot be seen when only *cox1* is analysed (Fig. 1 and S1), nor in the 18S rRNA tree (Fig. S5).

Two gravid proglottids of a single poorly conserved worm were available for morphological examination. No scolex was found. Width of the gravid proglottids is between 1.8 and 2.1 mm, length up to 5 mm. The two genital openings are equatorial, the cirrus sack (about 130 µm long within gravid proglottids) is positioned anterior to the female genital opening. Seminal receptacles are regressed but still visible in the gravid proglottids. The eggs fill the complete proglottid, crossing the longitudinal excretory ducts. However, in contrast to *Dipylidium caninum*, they are not clearly sorted into multi-ovular packets. Hexacanth embryos are 15–18 µm in diameter and are encapsulated in two visible envelopes. The inner membrane surrounding the embryo measures 18–22 µm in diameter. The very thin and oval outer membrane (egg capsule) ranges 26–37 µm by 24–28 µm. Apart from being more oval and slightly smaller in size, the egg capsules are similar to those found in *Joyeuxiella* spp. (Fig. 5).

3.1.4. Dipylidiidae - Joyeuxiella spp.

Four different species of *Joyeuxiella* were identified. *Joyeuxiella* sp. I was the most common and found in six different hosts from South Africa (one African wildcat, one African civet, one small-spotted genet and three rusty-spotted genets). Fragments of *Joyeuxiella* sp. II, obtained from a single domestic cat from South Africa, did not provide sufficient material suitable for morphological examination. The descriptions of *Joyeuxiella* sp. III are based on two worms isolated from an African wildcat from South Africa. Morphological information on *Joyeuxiella* sp.

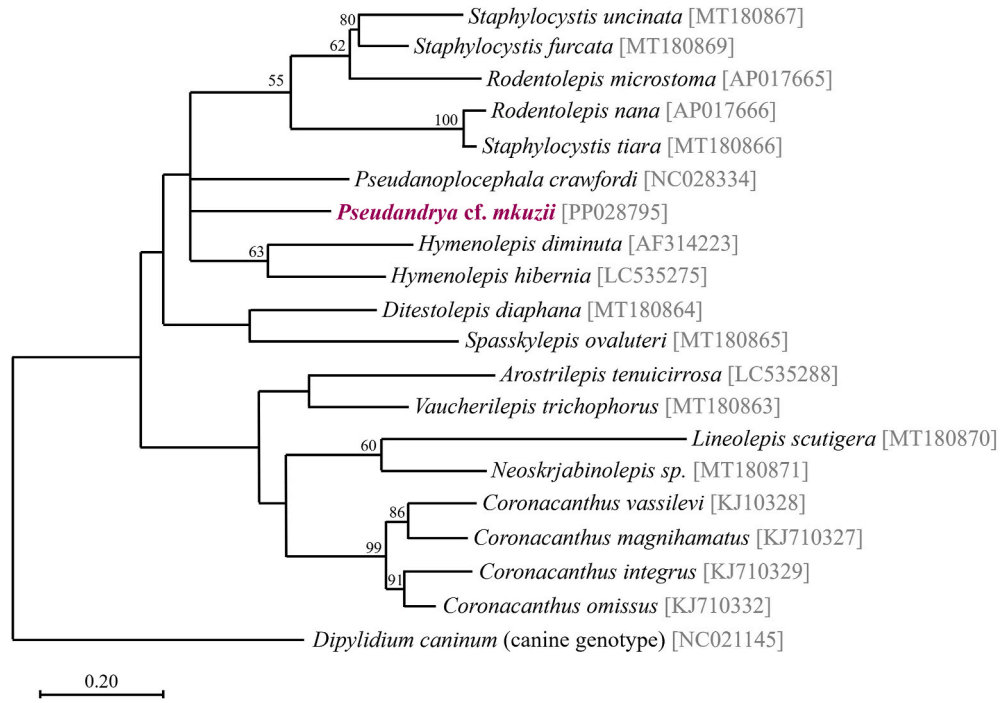

Fig. 2. Maximum likelihood (GTR + G + I) phylogenetic tree based on a 518 bp long fragment of *cox1*. 19 species of hymenolepidids from mammals are included. The canine genotype of *Dipylidium caninum* is used as an outgroup. Respective GenBank accession numbers are added after the species name. *Pseudandrya* cf. *mkuzii* is a novel lineage. Bootstrap values > 50 (%) are included. The scale bar represents the estimated number of substitutions per site.

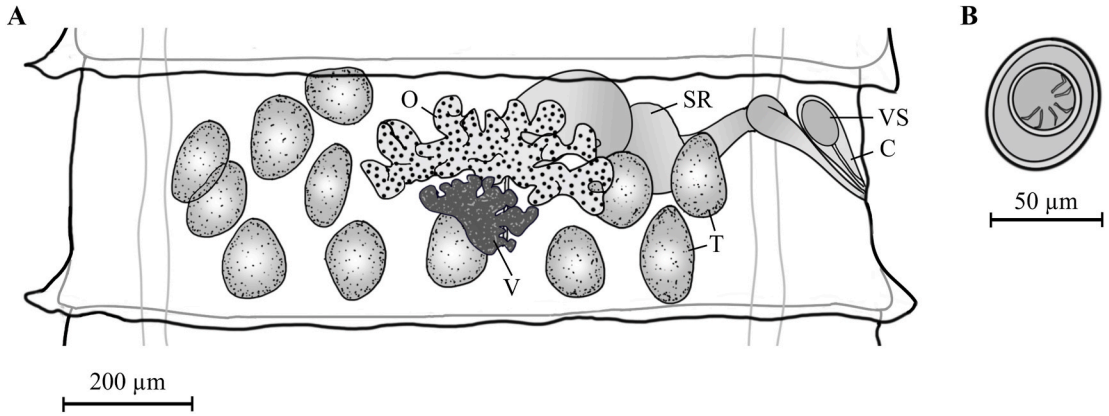

Fig. 3. Morphological illustrations of *Pseudandrya* cf. *mkuzii*. A: Dorsal view of a mature proglottid. O = ovary, V = vitellarium, T = testes, SR = coiled seminal receptacle, C = cirrus sack, VS = internal vesicula seminalis. B: Egg with three thin envelopes around the oncosphere.

IV is based on four incomplete specimens found in a domestic cat and two African wolves from Ethiopia.

The complete *cox1* gene was obtained from *Joyeuxiella* spp. I and III (1629 bp), whereas only a 964 bp long *cox1* fragment of *Joyeuxiella* sp. II, a 1600 bp sequence of *Joyeuxiella* sp. IV and a 955 bp fragment of *J.* sp. aff. *pasqualei* could be sequenced. The complete *nad1* gene was received from *J.* sp. aff. *pasqualei* (891 bp), as well as partial sequences from *Joyeuxiella* spp. I (741 bp) and III (833 bp). An additional 468 bp fragment of *cob* was obtained from *Joyeuxiella* spp. I, III and *J.* sp. aff. *pasqualei*. This allowed the construction of a concatenated sequence tree with *Joyeuxiella* spp. I and III, combining the sequences of these three mitochondrial genes for a more precise phylogenetic positioning of *Joyeuxiella* among other genera (Fig. 6). For alignment, the length of the *cox1* and *nad1* sequences of *Joyeuxiella* spp. was reduced to 1554 bp and 737 bp, respectively. The final dataset of Fig. 6 uses 2768 positions, including gaps from insertions or deletions. While *Joyeuxiella* spp. had been positioned separately from *Dipylidium* spp. based on a short *cox1* fragment in Fig. 1, they are placed as a sister clade to *Dipylidium* spp. when the combined sequences are used (Fig. 6). As seen in Fig. 1, *J.* sp. aff. *pasqualei* clusters with sequences of *J. pasqualei* published by Bezerra-Santos et al. (2022) and Schuster et al. (2023), but only correspond to a maximum of 93.3% (with OR081744 - Schuster et al., 2023). The sequence of *J. gervaisi* (OR081751), synonymised with *J. fuhrmanni*, that was published by Schuster et al. (2023) takes the most basal position in the *Joyeuxiella* clade. This can also be seen when using untrimmed sequences. However, the placement of *Joyeuxiella* IV, which moves to the second most basal position of the *Joyeuxiella* clade, changed (Supplementary Fig. S1).

In addition to protein coding genes, partial sequences of the 18S rRNA gene of the species I (534 bp), III (422 bp) and *J.* sp. aff. *pasqualei* (534 bp) and the 12S rRNA (289, 287 and 298 bp respectively) could be sequenced. Only the 12S rRNA sequence of *Joyeuxiella* sp. aff. *pasqualei* correlated to an entry in GenBank. It was 100% identical to an undescribed *Mesocestoides* species from Turkey (MH992704.1).

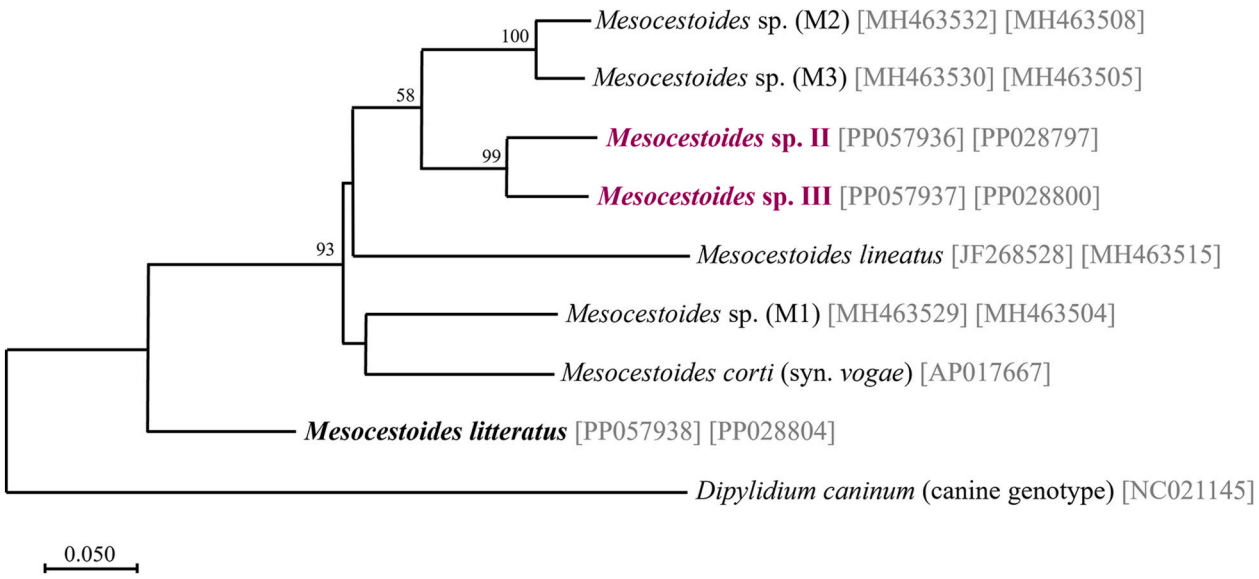

Fig. 4. Maximum likelihood (GTR + G + I) phylogenetic tree based on 634 bp long concatenated sequences of *nad1* (261 bp) and *cox1* (373 bp). Eight lineages of *Mesocestoides* are included. The canine genotype of *Dipylidium caninum* is used as an outgroup. Respective GenBank accession numbers are added after the species name. Novel lineages are written in bold and colour. Bootstrap values > 50 (%) are included. The scale bar represents the estimated number of substitutions per site. *Mesocestoides* sp. I was omitted, as no *nad1* sequence could be obtained.

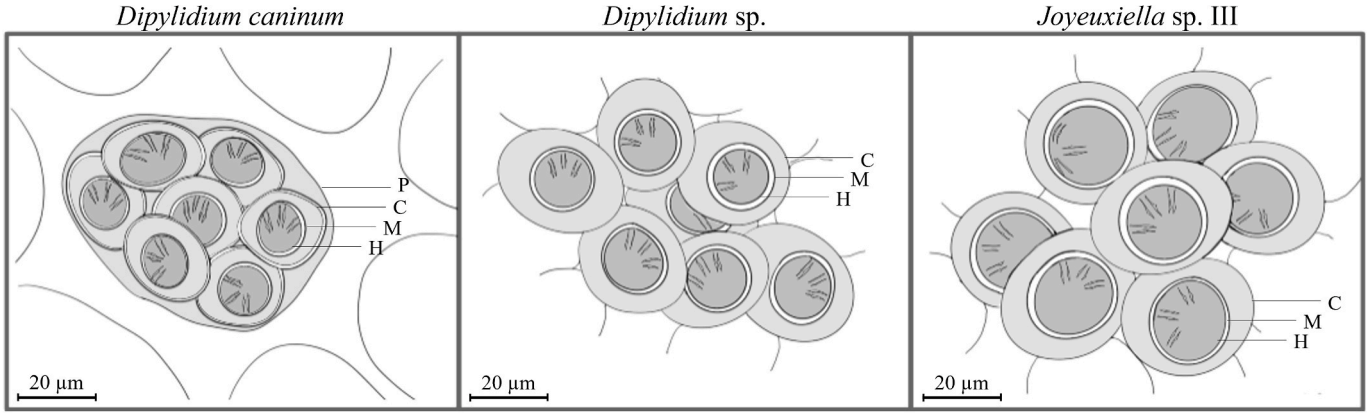

Fig. 5. Morphological illustrations of different ovarious structures in gravid proglottids of *Dipylidium caninum*, *Dipylidium* sp. and *Joyeuxiella* sp. III. P = egg packet, C = egg capsule, M = oncospheral membrane, H = hexacanth embryo.

To investigate the phylogenetic position of *Joyeuxiella*, especially with respect to *Dipylidium*, based also on ribosomal DNA, a phylogenetic tree was constructed using 402 bp long sequences, including alignment gaps, of the 18S rRNA gene from 23 species of different cyclophyllidean families. In this phylogram, *Joyeuxiella* spp. and *Anonchotaenia* spp. (of the family Paruterinidae) form sister clades, neighbouring the *Dipylidium* and *Raillietina* (Davaineidae) clades and *Catenotaenia pusilla* (Catenotaeniidae) (Supplementary Fig. S5).

The comparative numbers and measurements of various morphological features observed in adult *Joyeuxiella* spp. are listed in Table 4.

Most *Joyeuxiella* specimens were incomplete or fragmented, thus the full strobila length can only be estimated. *Joyeuxiella* sp. I attains a length of at least 5 cm. Two specimens of *Joyeuxiella* sp. III were 7.5 cm and approximately 12 cm long. Merely short fragments were available of both *Joyeuxiella* spp. II and IV. Based on the size of the proglottids of *Joyeuxiella* sp. IV, the full strobila length was estimated to be > 15 cm. The proglottids of *Joyeuxiella* sp. IV are considerably larger in both length and width than those of the other *Joyeuxiella* spp. I-III and measured up to 2 mm in width in mature segments. Gravid and pregravid proglottids of *Joyeuxiella* spp. grow more slender and longer than the posterior mature segments, which are wider than long (Fig. 7B–F). The width of the strobilae of *Joyeuxiella* sp. I specimens range between 0.1 and 0.3 mm at the neck and 0.5–1 mm at the widest part of the posterior mature proglottids. For *Joyeuxiella* sp. III, similar measurements ranging from 0.17 mm (anterior) to 1 mm (posterior) were made. Rostellar hooks of *Joyeuxiella* spp. I, III and IV are thorn-shaped and decrease in size towards the base of the rostellum (Fig. 7D and E). No scolex was available of *Joyeuxiella* sp. II and the small fragments could not be stained due to their poor condition. The stained specimens of *Joyeuxiella* spp. I and III reveal two vasa deferentia that are located close to the anterior margin of the mature proglottid and each coil toward the cirrus sacks, ending in the genital pores at the upper third of the proglottids. Some mature proglottids of one specimen of *Joyeuxiella* sp. III have long cirri protruding from the genital openings (Fig. 7B). The testes are found in the space posterior to the vasa deferentia and between the longitudinal excretory vessels (Fig. 7A and B). In contrast to *Joyeuxiella* spp. I and III, the vasa deferentia of *Joyeuxiella* sp. IV do not reach the anterior margins of the segments and the genital pores are located closer to mid proglottid (Fig. 7C). Exact numbers of testes could not be determined for *Joyeuxiella* sp. IV due to the condition of the specimens,

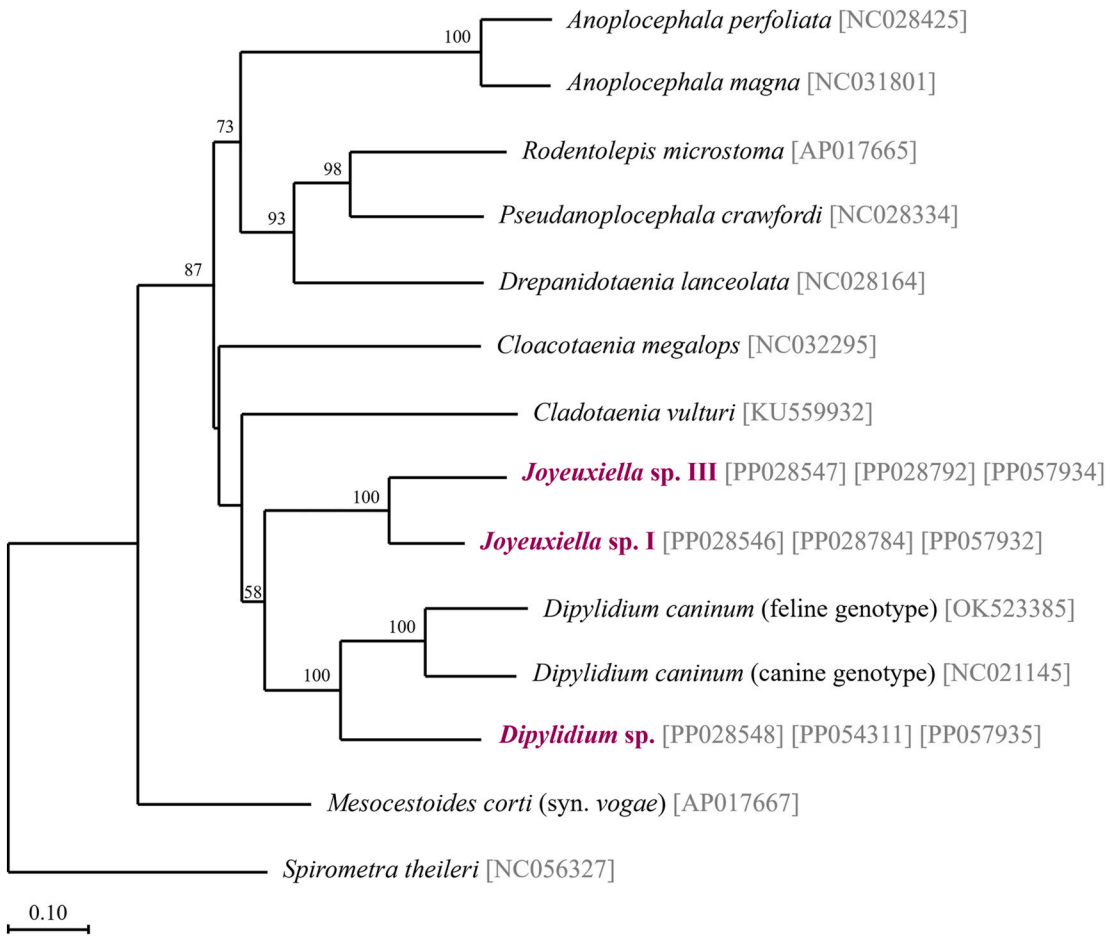

Fig. 6. Maximum likelihood (GTR + G + I) phylogenetic tree based on 2768 positions of concatenated sequences of *cob* (469 bp), *cox1* (1560 bp) and *nad1* (739 bp). 13 species of cyclophyllidean cestodes are included, *Spirometra theileri* is used as an outgroup. Respective GenBank accession numbers are added after the species name. Novel lineages are written in bold and colour. Bootstrap values > 50 (%) are included. The scale bar represents the estimated number of substitutions per site.

Table 4
Comparison of morphological features of *Joyeuxiella* spp. Descriptions and measurements of *J. pasqualei*, *J. gervaisi* (syn. *J. fuhrmanni*) and *J. echinorhyncoides* are as published by Jones (1983) and Schuster et al. (2023).

	Joyeuxiella sp. I	*Joyeuxiella* sp. III	*Joyeuxiella* sp. IV	*Joyeuxiella pasqualei*	*Joyeuxiella gervaisi*	*Joyeuxiella echinorhyncoides*
Hook size (length in µm)	7–11	6–10	7–13	7–17	8–17	10–26
Hook blade distinctly longer than hook base	no	no	no	no	no	yes
Number of testes per proglottid	27–57	35–40	–	40–130	20–60	25–120
Testes present anterior to vasa deferentia	no	no	yes	yes	no	yes
Egg capsule size (diameter in µm)	32–38	30–42	–	42–94	35–71	52–83
Embryo size (diameter in µm)	16–29	19–30	–	23–40	20–40	30–45
Egg capsules present lateral to l.e.v.	no	no	–	yes	no	no

l.e.v. = longitudinal excretory vessels.

though testes could be spotted anterior to the vasa deferentia sporadically. No gravid proglottids were available of *Joyeuxiella* sp. IV. Gravid proglottids of both *Joyeuxiella* spp. I and III are similar in appearance, with eggs that are confined to the space between the longitudinal excretory ducts (Fig. 7F). The egg capsules of both species are round or slightly oval in shape with average diameters ranging between 30 and 40 µm (Fig. 5, Table 4).

Fig. 7 depicts the general structure of the scolex, hooks and mature proglottid of *Joyeuxiella* sp. I (A, D and E), a younger mature segment and a gravid proglottid of one specimen of *Joyeuxiella* sp. III (B and F) and a mature proglottid of *Joyeuxiella* sp. IV found in this study (C).

4. Discussion

The importance of biodiversity for our ecosystems, and the impact of biodiversity loss is becoming increasingly evident. This explicitly includes parasitic organisms whose role in terms of biomass and ecological functions has only recently begun to be appraised (Wood and Johnson, 2015; Cable et al., 2017; Carlson et al., 2020). Yet, even more than with free-living organisms, there are severe gaps of knowledge in particular on the diversity of parasites of wildlife. Recent description of new parasite species - often discovered by molecular characterization - even from well-known host animals indicates a vast number of hidden taxa.

The magnitude of the unsurfaced part of the iceberg that is

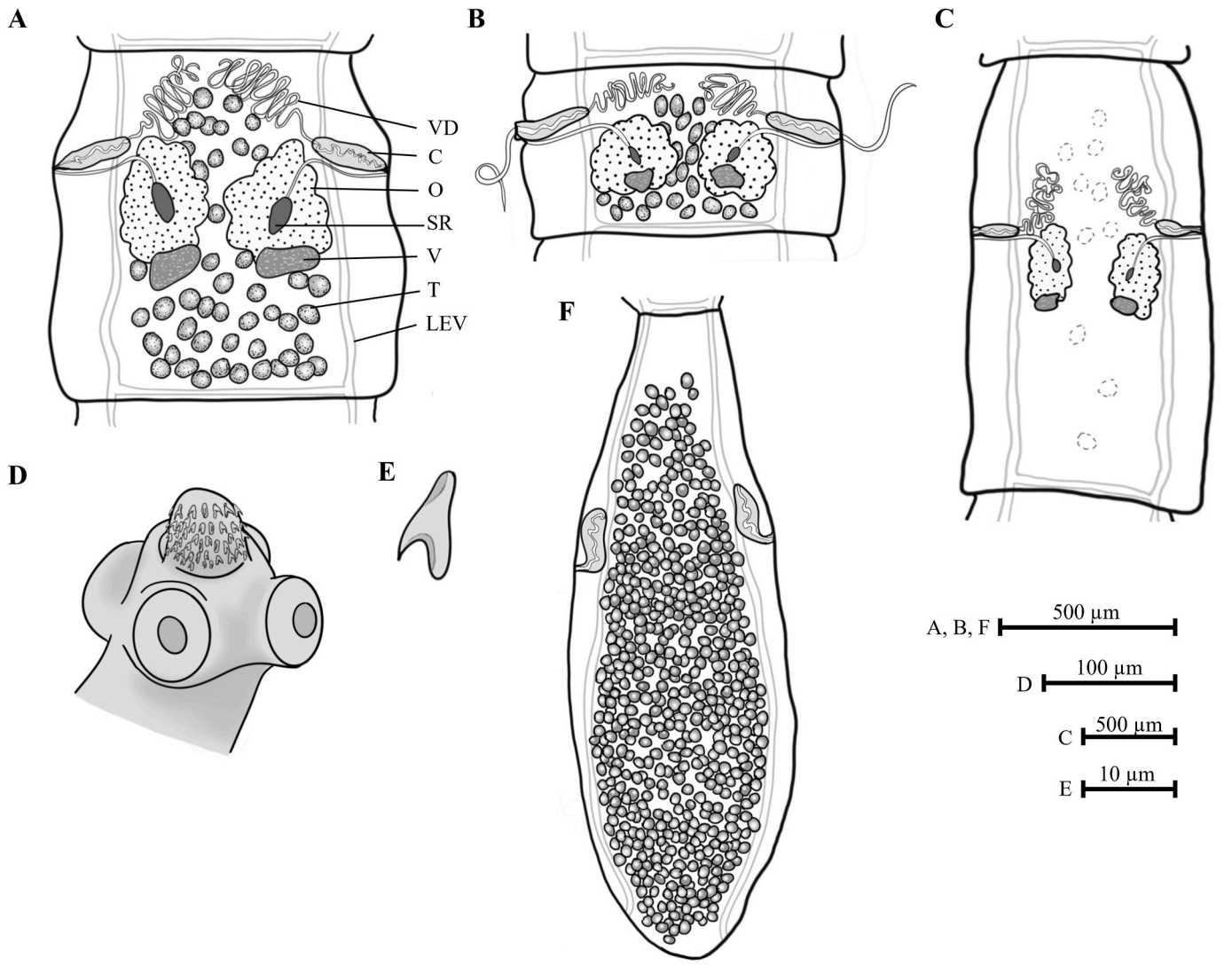

Fig. 7. Morphological illustrations of *Joyeuxiella* spp. I (A,D,E), III (B,F) and IV (C). A, B and C: Mature proglottids; D: Scolex. E: Hook. F: Gravid proglottid. VD = vasa deferentia, C = cirrus sack, O = ovary, SR = seminal receptacle, V = vitellarium, T = testes, LEV = longitudinal excretory vessel.

cyclophyllidean biodiversity, which was elaborately featured by the Planetary Biodiversity Inventory of tapeworms in vertebrates (Caira and Jensen, 2017), could be reaffirmed in this study. Within the 77 host animals examined, 21 out of 32 cyclophyllidean cestode species found (~65.6 %) are genetically novel. Considering only the non-taeniid cestodes treated here, eight out of eleven discovered lineages could not be attributed to any described species. The only cestodes that could be identified both morphologically and genetically belonged to two strains of *Dipylidium caninum*, which are currently under debate to be two separate species and can therefore also serve as examples of cryptic species (Labuschagne et al., 2018; Jesudoss Chelladurai et al., 2023). One other specimen could be determined to species level based on morphology and host species, *Pseudandrya* cf. *mkuzii*, while genetic data had not been obtained previously. For the remaining eight species, neither morphological nor molecular data allowed identification further than to genus level. These results emphasize the gaps of our knowledge on the true biodiversity of cestodes even from well-known terrestrial mammal hosts.

Based on previous DNA barcoding, most novel lineages of Cyclophyllidea in mammals were members of the Hymenolepididae family, which are highly abundant on the African continent (Mariaux et al., 2017). This exceptionally large family of cestodes comprises several genera of neglected wildlife tapeworms, many of them in need of revision. Adult hymenolepidids parasitise a large number of African bird, rodent and insectivore species and are less frequently also found in hosts belonging to the orders Chiroptera and Carnivora (Czaplinski and Vaucher, 1994; Haukisalmi et al., 2010; Dimitrova et al., 2019; Neov et al., 2021).

The hymenolepidid species isolated from a white-tailed mongoose could be assigned to the genus *Pseudandrya* based on the number of testes, which are diagnostic for this genus. The distinguishing characters of the three described species, *P. mkuzii*, *P. straeleni* and *P. monardi*, are based on the initial descriptions (Fuhrmann, 1943; Baer and Fain, 1955; Ortlepp, 1963). Data on the variability of these characters are not available, so their diagnostic values are uncertain. *Pseudandrya monardi*, in contrast to *P. mkuzii*, *P. straeleni* and our specimen, has visible external seminal vesicles and can therefore be excluded as a candidate for our species (Fuhrmann, 1943; Baer and Fain, 1955; Ortlepp, 1963). Although the size of the eggs of our species is more similar to those of *P. straeleni* than to those of *P. mkuzii*, we tentatively allocate it to *P.* cf. *mkuzii*, based on the host and to a lesser extent the geographical origin: *Pseudandrya mkuzii* was also isolated from a white-tailed mongoose by Ortlepp (1963) in South Africa, whereas *P. straeleni* was found in a gerbil (*Tatera* sp.) in former Belgian Congo (Baer and Fain, 1955). For this uncertainty, we allocate our specimen to *P.* cf. *mkuzii*. All three *Pseudandrya* species are morphologically very similar, and even Ortlepp

(1963) discussed the possibility of their co-specificity. For a conclusion, more specimens of *Pseudandrya* from different hosts and regions would need to be analysed.

Phylogenetically, *Pseudandrya* cf. *mkuzii* is positioned in a clade with *Hymenolepis* spp., *Rodentolepis* spp., *Staphylocystis* spp. and *Pseudanoplocephala crawfordi* (Figs. 1 and 2 and S2). Most nodes of the hymenolepidid tree are not strongly supported by the bootstrap values, but the topology is largely in agreement with the major clades recognized by Neov et al. (2021), in which similar *cox1* sequences generated high posterior probability values. Considering the morphology, a common feature of *P.* cf. *mkuzii* and *P. crawfordi* can be observed. The large number (>3) of testes per proglottid, a defining character (Czaplinski and Vaucher, 1994). Also the host ranges of *P.* cf. *mkuzii* and *P. crawfordi* include mammals of the Ferungulata grandorder (i.e., carnivores and ungulates), which is a rarity among hymenolepids. This suggests that both species may be closely related, but further phylogenetic studies are needed to confirm this.

None of the Mesocestoididae in our study could be assigned to a known species. Morphological identification within this family is notoriously unsatisfactory due to phenotypic plasticity of most structures. Characters regarded to be most useful for species delimitation are the length and structure of the cirrus and the shape of the vitellaria and ovaries, in some cases also the number and positioning of the testes. However, measurements may overlap (Certkova and Kospuko, 1975; Loos-Frank, 1990; Rausch, 1994; Gubányi and Eszterbauer, 1998; Hrčkova et al., 2011). Phenotypically, *Mesocestoides* spp. I and III are closest to *M. lineatus*. *Mesocestoides* sp. II is larger, has a higher number of testes and corresponds in some respects to the description of *M. zacharovae*, a species found in domestic cats and canines and reported from eastern Europe to Siberia (Certkova and Kospuko, 1975, 1978).

Various *Mesocestoides* species are known to parasitise small cats, such as *M. lineatus*, *M. petrovi* and *M. zacharovae* (Certkova and Kospuko, 1975; Hrčkova et al., 2011). Cestodes identified as *M. lineatus* in South Africa had been reported by Verster (1979) and were gathered from a single domestic dog. *Mesocestoides* spp. I and III resemble morphological descriptions of *M. lineatus* and therefore may have been assigned to this species in the past. In the present study, *Mesocestoides* spp. I and II were found in servals in South Africa. Specimens of *Mesocestoides* sp. II from two servals were well-developed, while *Mesocestoides* sp. I, from one serval, was represented by a single, frail specimen. The only report of *Mesocestoides* from a serval is mentioned by Hudson (1934) from Kenya. Several small specimens of this helminth, designated as *Mesocestoides* sp., were described by Hudson to resemble the morphology of *M. longistriatus* Setti (1897), a species found in the African wildcat (*Felis lybica*) and considered by Witenberg (1934) to be identical to *M. lineatus* forma *litterata*, ergo *M. litteratus*. *Mesocestoides* sp. III was found in two domestic cats from Ethiopia, where *Mesocestoides* sp. have previously been reported in dogs (Gebremedhin et al., 2020).

Due to their morphological differences and geographic origins, the novel lineages II and III are herein tentatively listed as separate species, though it is noted that they are genetically very close. The genetic analyses of the *Mesocestoides* species I-III showed that they are most closely related to species previously found in the Mediterranean area (Varcasia et al., 2018). In Fig. 1 (and Figs. S1 and S3), *Mesocestoides* sp. I is positioned next to an unidentified *Mesocestoides* species (KP941432), which correlates with the Mediterranean *Mesocestoides* sp. M2 clade represented in Fig. 4 (Häußler et al., 2016; Varcasia et al., 2018). *Mesocestoides* lineages II and III are placed next to the Mediterranian entities M2 and M3 (Fig. 4), which may have their origin in Northern Africa (Varcasia et al., 2018). However, in the phylogenetic tree based on the untrimmed sequences (Fig. S3), *Mesocestoides* spp. II and III are positioned basal to the others, with the exception of *M. litteratus*, which remains the most basal species. This could be an indication that there is a separate African clade within the family. To confirm this hypothesis, more samples would be needed and, in particular, more complete reference gene sequences.

Three members of the cosmopolitan cestode family Dipylidiidae were found in the examined animals. Both the feline and canine genotypes of *Dipylidium caninum* could be isolated from domestic cats and the latter also from an African wolf. As the names suggest, the two genotypes seem to be adapted to dogs and cats, respectively. Due to this adaptation and their genetic differences (only 78.7 % of their complete mitochondrial genomes are identical) they may warrant species status (Labuschagne et al., 2018; Jesudoss Chelladurai et al., 2023), which is supported by our data (Figs. 1 and 6).

The third *Dipylidium* species found in an aardwolf from South Africa is genetically unknown and has some unique morphological characteristics not described so far for this genus. Although genetically and morphologically close to *D. caninum*, it differs in a feature of the genus previously considered determinant of the genus, the multi-egg packets. Three to thirty eggs are surrounded by an outer membrane and form egg packets that generate a distinct pattern, the outline of which can be seen early in mature proglottids (Witenberg, 1932). This character is used to differentiate between *Dipylidium* and *Joyeuxiella* (Schmidt, 1970; Khalil et al., 1994; Schuster, 2020). The eggs of the *Dipylidium* species found in the aardwolf closely resemble those of *D. caninum* but are separate and not found in multi-ovular packets (Fig. 5). Both the terms ‘egg packet’ and ‘egg capsule’ are being used synonymously in literature on *Dipylidium* and *Joyeuxiella* species, even though the structures differ between the genera. The definition of the word “capsule” or how an “egg” is characterised varies among different publications, leading to confusion when comparing morphological structures. Here, the term ‘egg packet’ is used to describe the structure that encapsulates multiple eggs in *Dipylidium caninum*, whereas the term ‘egg capsule’ refers to the outermost envelope of singular eggs.

An additional genus of the Dipylidiidae, *Diplopylidium* Beddard (1913), also possesses mono-ovular capsules. However, the arrangement of the genital pores of *Diplopylidium* is reversed compared to *Joyeuxiella* and *Dipylidium* and they have a rostellum armed with hooks that are shaped reminiscent of taeniid hooks (López-Neyra, 1927; Witenberg, 1932; Jones, 1994; Schuster, 2020). No scolex of the new *Dipylidium* sp. was available, but the genital openings of the novel species are positioned according to *Dipylidium* rather than *Diplopylidium* morphology. Also, a comparison of the *Dipylidium* sequence found in the aardwolf with the feline and canine genotypes of *D. caninum* and the only available 100 bp long fragment of the *cox1* gene of *Diplopylidium noelleri* (Poon et al., 2017) showed higher similarities to *D. caninum* (92 % and 93 %, respectively) than to *D. noelleri* (87 %). Based on the phylogenetic analyses, the new species was therefore tentatively placed in the genus *Dipylidium*, despite the morphological peculiarities regarding the egg packets. Further studies are required to decide if this morphological feature justifies the establishment of a new genus.

The only previous report of Dipylidiidae in aardwolves can be found in Baer (1926). The species is therein identified as *Dipylidium caninum*, but the morphological basis for this allocation is not given.

The last group of non-taeniid cestodes found in the present study belongs to the genus *Joyeuxiella*. Three species of *Joyeuxiella* are currently considered to be valid: *J. pasqualei*, *J. gervaisi* (syn. *J. fuhrmanni*) and *J. echinorhyncoides*, all of them previously reported from the African continent (Jones, 1983; Schuster, 2020; Schuster et al., 2023). The phylogenetic analyses conducted in the present study revealed four distinct species. For morphological comparisons, all characteristics listed in Table 4 were selected for their consistency across the stages of proglottid maturation and for their resistance to damage by preservation techniques and condition. This reduces the chance of comparing and possibly misinterpreting features that may have different appearances due to timing and type of preservation. *Joyeuxiella* sp. I and *Joyeuxiella* sp. III are morphologically very similar and correspond to the descriptions of *J. gervaisi* (Setti, 1895)/*J. fuhrmanni* (Baer, 1924), the most distinctive characteristics being the positions of eggs and testes (Table 4). Whilst the morphology of *Joyeuxiella* sp. II could not be examined in detail, it can be surmised from the close genetic relationship

to *Joyeuxiella* sp. III that species II might have similar features. Although *Joyeuxiella* spp. I and III can be morphologically identified as *J. gervaisi*, they are genetically clearly distinguishable from each other and from *J. gervaisi*. Based on the short *cox1* sequences used in Fig. 1, *Joyeuxiella* spp. I-III form a sister clade to *J. pasqualei*, while *J. gervaisi* is positioned basal to the other species. Consequently, *J. gervaisi* can be regarded as a complex of cryptic species that can only be differentiated genetically.

The type specimen of *J. gervaisi* described by Setti (1895) was found in an Abyssinian genet (*Genetta abyssinica*) in Eritrea. Other hosts of this species (initially identified as *J. fuhrmanni*) are various feliforms from southern Africa, including Serval (*Leptailurus serval*), African wildcat (*Felis lybica*), the domestic cat (*Felis sylvestris domesticus*), the Rusty-spotted genet (*Genetta maculata*) and an undetermined species of genet, which would be in accordance with our findings (Table 3) (Mettrick and Beverley-Burton, 1961; Jones, 1983; Schuster, 2020, Schuster et al., 2023).

Not all morphological characteristics of *Joyeuxiella* sp. IV could be examined, but some features differ from the descriptions of *J. gervaisi* and the appearance of *Joyeuxiella* I and III. The position of the testes does not correspond to theirs but agrees with the two other known species. However, *J. echinorhyncoides* can be excluded by the shape of the rostellum, which is comparatively long with a bulbous tip, and hooks which have proportionately longer blades (Jones, 1983). This leaves *J. pasqualei*; and indeed, the strobila of this species equals the dimensions of that of *Joyeuxiella* sp. IV, which is noticeably larger in size compared to the other species.

Joyeuxiella sp. IV was the only *Joyeuxiella* species in this study found in the north of Africa, in Ethiopia, and furthermore in a canid host. Canids have been documented to be suitable hosts for *J. pasqualei* (Jones, 1983; Schuster, 2020; Bezerra-Santos et al., 2022). Although Joyeuxiella sp. IV and *J. pasqualei* resemble each other morphologically, and can both utilise canid hosts, they are genetically distinct species (Fig. 1 and S1). It is interesting to note that the two GenBank *J. pasqualei* sequences from Italy and the United Arab Emirates, isolated from a canid and two cats respectively, do not differ from each other except for two bases but are clearly different from the *J.* sp. aff. *pasqualei* specimen analysed here, which originated also from the United Arab Emirates (Bezerra-Santos et al., 2022; Schuster et al., 2023). The *J. pasqualei* sequences from GenBank show only ~93% identity to *J.* sp. aff. *pasqualei*. Curiously, the 12S rRNA sequence of *J.* sp. aff. *pasqualei* matches the GenBank sequence MH992704, which is assigned to a *Mesocestoides* sp. The corresponding sample material consisted of tetrathyridia found in the abdominal cavity of a dog from Turkey. To clarify this discrepancy further investigation would be necessary.

Our data therefore suggest that both *J. gervaisi* and *J. pasqualei* are complexes of cryptic species.

Considering the genus *Joyeuxiella* as a whole, the genetic data raise questions about the currently assumed close relationship to *Dipylidium* and therefore the affiliation to the family of Dipylidiidae. *Joyeuxiella* had been assigned to the Dipylidiidae family, which currently consists of the three genera *Dipylidium*, *Diplopylidium* and *Joyeuxiella*, merely based on similar morphology.

The first phylogenetic analysis based on a 355 bp long *cox1* sequence clearly separated the *Joyeuxiella* group from *Dipylidium* spp. and positioned it closer to members of the Hymenolepididae and Davaineidae. However, by analysing a longer fragment of the mitochondrial DNA concatenated of three fragments of the *cox1*, *nad1* and *cob* gene with 2768 bp in length, *Joyeuxiella* is placed next to the *Dipylidium* species, but the bootstrap value is low and not strongly supported. In another phylogenetic tree based on a fragment of the nuclear 18S rRNA gene, the *Dipylidium* clade is not closer to *Joyeuxiella* than it is to *Anonchotaenia* and *Raillietina* clades. This would suggest a more distant relationship between *Joyeuxiella* and *Dipylidium*. However, it must be considered that the alignment on which this calculation is based had a length of only 402 bp including gaps, and the bootstrap value at the position of separation was very low (Fig. S5). Due to these somewhat contradictory results, we hesitate to make a definite statement about the taxonomic status of *Joyeuxiella* at this time. Larger data sets, including *Diplopylidium*, are required to clarify this. Nonetheless, the common feature of two sets of reproductive organs in each segment, shared by all members of the current family Dipylidiidae, indicates a common ancestry of the group.

Assigning a genetically novel cestode to a described species rests on various factors. Some are derived from the specimen itself, others from the type-material. If genetic determination is not possible, identification relies on the quality of the worm material. Cestodes recovered from carcasses of road-killed animals are rarely in an ideal condition for morphological investigation, sometimes not even for DNA analyses. Decomposition processes begin almost immediately after the death of the host, causing damage to the worm tissue and making later staining and evaluation of characteristics difficult or even impossible. On the side of the type-material, usually only morphological descriptions and drawings are available as reference. Often, text descriptions leave room for interpretation, and there is usually no information about intraspecific variations of the diagnostic features, since often only one specimen is used as a basis for descriptions. In case of cryptic species with similar morphology, sequence analysis of the type-material would be required for unambiguous taxonomic treatment – which harbours difficulties of its own. Apart from the problem of locating the specimen and obtaining permission, the main difficulty lies in the DNA analysis of the usually old sample material itself. However, new sequencing techniques will certainly help to solve the latter in the future and further studies will add to the library of species and thereby provide new insights into the complexity of speciation and evolution.

Ethical aspects

Permissions were issued for the South African study by the Limpopo Department of Economic Development, Environment & Tourism (LEDET) (permit number ZA/LP/87586) and the Department of Agriculture and Rural Development, Gauteng Provincial Government (permit number CPFG-0136). Research permits for the Namibian study were issued by the Ministry of Environment and Tourism (Permit No.: 1740/2012) as well as the National Commission on Research, Science and Technology (Authorization No.: AN202101126) in line with Namibian regulations. No research permits were required for the Ethiopian study.

CRediT authorship contribution statement

S. Dumendiak: Formal analysis, Investigation, Visualization, Writing – original draft. **A. Halajian:** Investigation, Resources. **Y.T. Mekonnen:** Investigation, Resources. **O. Aschenborn:** Investigation, Resources. **G.J. Camacho:** Resources. **R.K. Schuster:** Resources. **U. Mackenstedt:** Supervision, Writing – review & editing. **T. Romig:** Conceptualization, Supervision, Writing – review & editing. **M. Wassermann:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2024.100929.

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