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Phylogeny of hymenolepidids (Cestoda: Cyclophyllidea) from mammals: sequences of 18S rRNA and COI genes confirm major clades revealed by the 28S rRNA analyses

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Research Paper

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Abstract

The aim of the study is to test a hypothesis for the phylogenetic relationships among mammalian hymenolepidid tapeworms, based on partial (D1–D3) nuclear 28S ribosomal RNA (rRNA) genes, by estimating new molecular phylogenies for the group based on partial mitochondrial cytochrome *c* oxidase I (COI) and nuclear 18S rRNA genes, as well as a combined analysis using all three genes. New sequences of COI and 18S rRNA genes were obtained for *Coronacanthus integrus*, *C. magnihamatus*, *C. omissus*, *C. vassilevi*, *Ditestolepis diaphana*, *Lineolepis scutigera*, *Spasskylepis ovaluteri*, *Staphylocystis tiara*, *S. furcata*, *S. uncinata*, *Vaucherilepis trichophorus* and *Neoskrjabinolepis* sp. The phylogenetic analyses confirmed the major clades identified by Haukisalmi *et al.* (*Zoologica Scripta* 39: 631–641, 2010): *Ditestolepis* clade, *Hymenolepis* clade, *Rodentolepis* clade and *Arostrilepis* clade. While the *Ditestolepis* clade is associated with soricids, the structure of the other three clades suggests multiple evolutionary events of host switching between shrews and rodents. Two of the present analyses (18S rRNA and COI genes) show that the basal relationships of the four mammalian clades are branching at the same polytomy with several hymenolepidids from birds (both terrestrial and aquatic). This may indicate a rapid radiation of the group, with multiple events of colonizations of mammalian hosts by avian parasites.

Introduction

Among cyclophyllidean cestodes, Hymenolepididae Perrier, 1897 is the most species-rich family comprising more than 920 species parasitic in birds and mammals (Mariaux *et al.*, 2017). The number of species from mammalian hosts exceeds 366 (Mariaux *et al.*, 2017; Makarikov *et al.*, 2018, 2020; Makarikova, 2018; Tkach *et al.*, 2018; Gardner *et al.*, 2020; Makarikov & Georgiev, 2020). These are parasitizing mostly insectivores (Eulipotyphla), rodents (Rodentia) and bats (Chiroptera) (Vaucher, 1971; Czaplinski & Vaucher, 1994; Georgiev *et al.*, 2006; Mariaux *et al.*, 2017). Phylogenetic relationships among hymenolepidids, including among the taxa occurring in mammals, remain unresolved. The pioneer study by Haukisalmi *et al.* (2010) proposed the first phylogenetic hypothesis for the relationships among mammalian hymenolepidids, which was based on sequencing partial (D1–D3) 28S ribosomal RNA (rRNA) gene; it revealed the presence of four major phyletic lineages in the group, which were named '*Ditestolepis* clade', '*Arostrilepis* clade', '*Hymenolepis* clade' and '*Rodentolepis* clade'. Subsequently, Neov *et al.* (2019) analysed the phylogenetic relationships of this group based on partial (D1–D3) 28S rRNA gene of 12 selected taxa as well as sequences obtained by Haukisalmi *et al.* (2010) and other authors (Greiman & Tkach, 2012; Greiman *et al.*, 2013; Tkach *et al.*, 2013, 2018; Binkienė *et al.*, 2015, 2019; Makarikov *et al.*, 2015, 2018), comprising a total of 40 taxa. This study confirmed the same major clades but also added more details on the evolution of the host–parasite associations and the main morphological characteristics of the members of this group. Recently, using also 28S rRNA gene, Kornienko *et al.* (2019) analysed the phylogenetic relationships within the *Ditestolepis* clade, involving seven out of the eight genera belonging to it. All these studies used homologous regions of the 28S rRNA gene. However, these results are only the beginning of understanding the evolutionary history of mammalian hymenolepidids. Generally, for more robust phylogenetic hypotheses of cestode groups, it is necessary to implement denser taxon sampling and the inclusion of additional genes (Mariaux & Olson, 2001; Littlewood *et al.*, 2008; Waeschenbach & Littlewood, 2017; Kornienko *et al.*, 2019).

The aim of the present study is to test the hypothesis for the phylogenetic relationships among mammalian hymenolepidids (based on the 28S rRNA gene) by examining the

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Table 1. Cestode species sequenced and used in the phylogenetic analyses in the course of the present study.

Cestode species	Host species	Locality (Bulgaria)	GenBank accession no.		Vouchers[a]
			18S rRNA	COI	
Ditestolepis diaphana (Cholodkowski, 1906)	*Sorex araneus*	Kalimok	MT193127	MT180864	C0128.1.1
Coronacanthus integrus (Hamann, 1891)	*Neomys fodiens*	Boyana River	KJ710320	KJ710329	C0128.1.5
Coronacanthus magnihamatus Vasileva, Tkach & Genov, 2005	*Neomys fodiens*	Boyana River	KJ710321	KJ710327	C0128.1.7
Coronacanthus omissus Baer & Joyeux, 1943	*Neomys fodiens*	Boyana River	KJ710319	KJ710330	C0128.1.6
Coronacanthus vassilevi Genov, 1980	*Neomys fodiens*	Boyana River	KJ710322	KJ710328	C0128.1.8
Lineolepis scutigera (Dujardin, 1845)	*Sorex araneus*	Kalimok	MT193133	MT180870	C0128.1.2
Neoskjrabinolepis sp.	*Sorex araneus*	Kalimok	MT193134	MT180871	C0128.1.3
Spasskylepis ovaluteri Schaldybin, 1964	*Neomys fodiens*	Boyana River	MT193128	MT180865	C0128.1.9
Staphylocystis tiara (Dujardin, 1845)	*Crocidura suaveolens*	Kalimok	MT193129	MT180866	C0128.1.11
Staphylocystis furcata (Stieda, 1862)	*Sorex araneus*	Kalimok	MT193132	MT180869	C0128.1.4
Staphylocystis uncinata (Stieda, 1862)	*Crocidura suaveolens*	Kalimok	MT193130	MT180867	C0128.1.12
Vaucherilepis trichophorus Tkach, Vasileva & Genov, 2003	*Neomys fodiens*	Boyana River	MT193126	MT180863	C0128.1.10

[a]Accession numbers of the specimens used for DNA extraction ('hologenophores', see Pleijel *et al.*, 2008) in the IBER–BAS Helminthological Collection are presented.

phylogeny of the group on the basis of cytochrome *c* oxidase I (COI) and 18S rRNA genes as well as a combined analysis using these two genes and the previously published sequences of 28S rRNA genes (Neov *et al.*, 2019).

Materials and methods

Cestode sampling and identification

The materials used in the present study were collected and analysed for 28S rRNA gene in a previous study (Neov *et al.*, 2019). Shrews were collected by trapping from Boyana River, Vitosha Mts. (42.637°, 23.260°) and Kalimok Field Station (44.012°, 26.440°) near Nova Cherna, Bulgaria. The helminthological study of host individuals was permitted by the Ministry of Environment and Waters of Bulgaria and followed the instructions presented in the permission. Adult cestodes were isolated from intestines. Specimens were preserved in 70% ethanol permitting both morphological and molecular study. Each cestode included in the analysis was divided into two parts. The anterior part (containing the scolex) was stained with iron acetocarmine (Georgiev *et al.*, 1986) and dehydrated in alcohol series, cleared in dimethyl phthalate and mounted in Canada balsam or Berlese's medium (Swan, 1936) for morphological identification. Specimens used for DNA extraction were deposited as voucher slides in the Helminthological Collection of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences (IBER–BAS), Sofia (table 1). The posterior parts of the specimens were used as tissue samples for DNA extraction.

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Total DNA was isolated using Single Worm PCR Protocol (Williams *et al.*, 1992). The amplification of a region of 18S rRNA gene was accomplished using the primers WormA 5′-GCGAATGGCTCATTAAATCAG-3′ (forward) and WormB 5′-CTTGTTACGACTTTTACTTCC-3′ (reverse) as suggested by Littlewood & Olson (2001). A section of the mitochondrial COI gene was amplified using the primers PBI-cox1F_PCR 5′-CATTTTGCTGCCGGTCARCAYATGTTYTGRTTTTTTGG-3′ (forward) and PBI-cox1R_PCR 5′-CCTTTGTCGATACTGC CAAARTAATGCATDGGRAA-3′ (reverse) (Scholz *et al.*, 2013). The PCR mixtures contained 25 µL of NZYTaq II 2× Green Master Mix (Cat. No. MB358; Nzytech, Lisbon, Portugal), 1 µM of each primer (FOR/REV), 10 ng template DNA and PCR-grade water to a total volume of 50 µL. PCR reactions for the 18S rRNA amplicon were carried out under the following conditions: initial denaturation at 94°C for 5 min, 30 cycles (denaturation at 94°C for 30 s; primer annealing at 50°C for 30 s; extension at 72°C for 120 s) and final extension at 72°C for 10 min. PCR reactions for amplification of the fragment of the COI gene were identical, with the difference that the extension phase was reduced to 30 s. PCR products were visualized on 1% agarose gel with GreenSafe staining (NZYTech, Lisbon, Portugal) under ultraviolet light. Fragment size was determined using GeneRulerTM 100 bp Ladder Plus (Fermentas, Thermo Scientific, Waltham, USA). All amplicons were purified using the PCR/DNA Clean-Up Purification Kit (EURx Sp. z o.o. Gdansk, Poland) and sequenced in both directions by a PlateSeq kit (Eurofins Genomics, Ebersberg, Germany) using the PCR primers (for 18S rRNA and COI genes) and two additional internal primers (for 18S rRNA gene): 1270F 5′-ACTTAAAGGAATTGACGG-3′ and 1270R 5′-CCGTCAATTCCTTTAAGT-3′.

Phylogenetic analyses

The newly obtained 12 18S rRNA sequences and 12 COI sequences (table 1) were manually edited and then aligned using MEGA software, version 7.0 (Kumar *et al.*, 2016) and

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Table 2. Published sequences of 18S rRNA gene of dilepidid (*Dilepis undula*, outgroup) and hymenolepidid cestodes deposited in GenBank used in the present phylogenetic analysis.

Cestode species	GenBank accession no.	Host species	Host family	Geographic origin	Source
Dilepididae					
Dilepis undula (Schrank, 1788)	AF286981	*Turdus merula*	Turdidae	UK	Olson *et al.* (2001)
Hymenolepididae					
Fimbriaria sp.	AF286982	*Anas platyrhynchos*	Anatidae	USA	Olson *et al.* (2001)
Hymenolepis (*sensu lato*) *microps* (Diesing, 1850)[a]	KY403995	*Lagopus lagopus*	Phasianidae	Norway (Kattfjord)	Pistone *et al.* (2017)
Rodentolepis microstoma (Dujardin, 1845)[b]	AJ287525	*Mus musculus* (laboratory mouse)	Muridae		Littlewood & Olson (2001)
Rodentolepis nana (Siebold, 1852)	AY193874	*Mus musculus*	Muridae	USA (Nebraska)	Olson *et al.* (2003)
Hymenolepis diminuta (Rudolphi, 1819)	AF286983	*Rattus norvegicus* (laboratory rat)	Muridae		Olson *et al.* (2001)
Wardoides nyrocae (Yamaguti, 1935)	AJ287587	*Cygnus olor*	Anatidae	?	Littlewood & Olson (2001)

[a]The generic allocation of this species is uncertain. Pistone *et al.* (2017) mentioned it as a member of *Hymenolepis*; this genus currently includes mammalian cestodes only (e.g. Binkienė *et al.*, 2019) and the affiliation of '*H. microps*' requires further studies. Therefore, we designate it as a member of '*Hymenolepis* (*sensu lato*)'.
[b]For morphological and biological characteristics of the laboratory strain ('Nottingham strain'), see Cunningham & Olson (2010).

Table 3. Published sequences of COI of dilepidid (*Dilepis undula*, outgroup) and hymenolepidid cestodes deposited in GenBank used in the present phylogenetic analysis.

Cestode species	GenBank accession no.	Host species	Host family	Geographic origin	Source
Dilepididae					
Dilepis undula (Schrank, 1788)	EU665471	*Turdus merula*	Turdidae	UK	Littlewood *et al.* (2008)
Hymenolepididae					
Citrilolepis citrili Dimitrova, Georgiev, Mariaux & Vasileva, 2019	MK463853	*Crithagra citrinelloides*	Fringillidae	Ethiopia	Dimitrova *et al.* (2019)
Cloacotaenia megalops (Nitzsch in Creplin, 1829)	KU641017	'Duck'	Anatidae	China	Guo (2016)
Drepanidotaenia lanceolata (Bloch, 1782)	KR817910	*Anser anser domesticus*	Anatidae	China	Gao *et al.* (2017)
Fimbriaria sp.	EU665472	*Anas platyrhynchos*	Anatidae	USA	Littlewood *et al.* (2008)
Hymenolepis diminuta (Rudolphi, 1819)	AF314223	Laboratory rat	Muridae	–	von Nickisch-Rosenegk *et al.* (2001)
Hymenolepis hibernia Montgomery, Montgomery & Dunn, 1987[a]	LC063179	*Apodemus agrarius*	Muridae	South Korea	Nkouawa *et al.* (2016)
Pseudanoplocephala crawfordi Baylis, 1927	KR611041	'Pig'	Suidae	China	Zhao *et al.* (2016)
Rodentolepis microstoma (Dujardin, 1845)[b]	AP017665	*Mus musculus* (laboratory mouse)	Muridae	–	Tsai *et al.* (2013)
Rodentolepis nana (Siebold, 1852)	AP017666	?	?	Japan	Kikuchi *et al.* (2019)

[a]The identification of this sample from the Korean Peninsula requires further confirmation by morphological and molecular studies, since the original description of *Hymenolepis hibernia* is from *Apodemus sylvaticus* from Northern Ireland (Montgomery *et al.*, 1987).
[b]For morphological and biological characteristics of the laboratory strain ('Nottingham strain'), see Cunningham & Olson (2010).

version X (Kumar *et al.*, 2018). An analysis using Basic Local Alignment Search Tool (BLAST analysis, see www.ncbi.nlm.nih.gov/BLAST) was applied for comparison and possible identification with sequences available in GenBank for the family Hymenolepididae. For phylogenetic analyses, we used published sequences of 18S rRNA gene (table 2) and COI gene (table 3) from several previous studies (Littlewood & Olson, 2001; Olson *et al.*, 2001, 2003; Littlewood *et al.*, 2008; Guo, 2016; Nkouawa *et al.*, 2016; Zhao *et al.*, 2016; Pistone *et al.*, 2017; Dimitrova *et al.*, 2019). GenBank sequences with less than 90%

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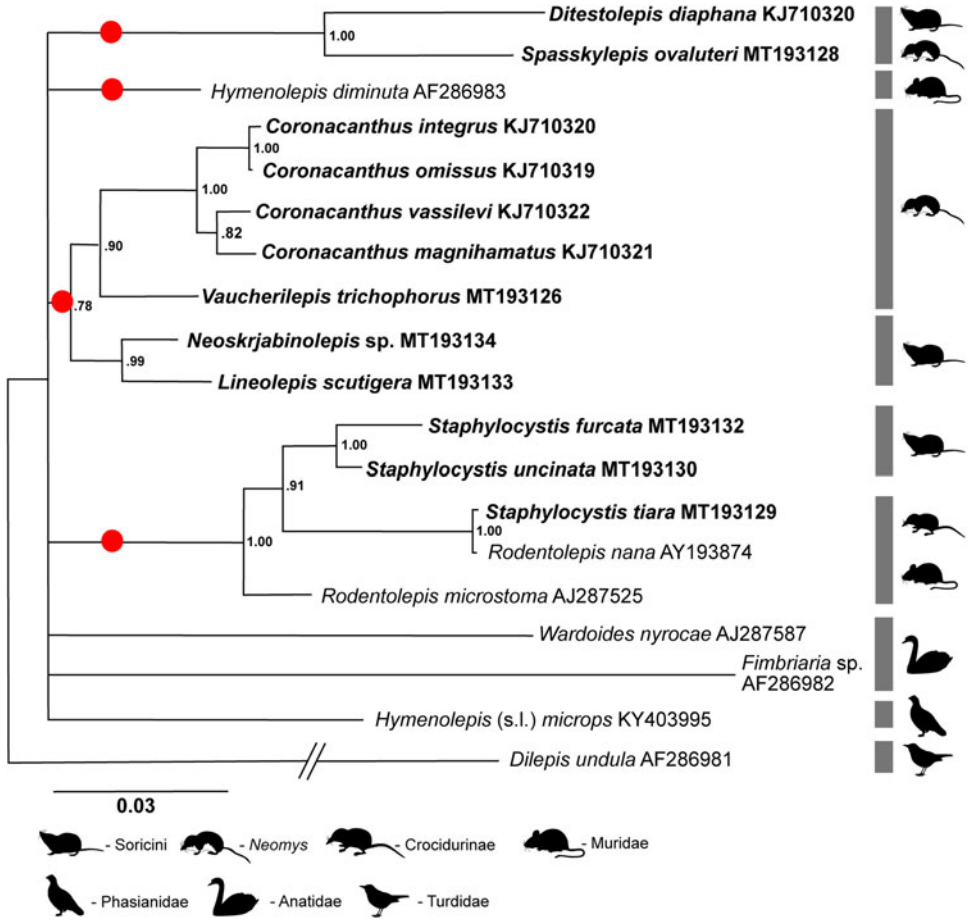

Fig. 1. Bayesian inference tree of phylogenetic relationships among 18 species of hymenolepidid cestodes (15 species from mammals and three species from birds) based on analysis of 18S rRNA gene. *Dilepis undula* (family: Dilepididae) is used as outgroup. The GenBank numbers are added after the binomial name of each species. Newly sequenced taxa are in bold. The major clades recognized by Haukisalmi *et al.* (2010), confirmed by Neov *et al.* (2019) and outlined by the present study are marked by circles. Nodal support is given by posterior probabilities. Scale bar shows the number of substitutions per site.

length coverage compared to our dataset were excluded. The analyses involved 19 sequences for 18S rRNA gene and 22 sequences for the COI gene. The combined analysed involved all taxa originally sequenced for the purposes of the present study (table 1) as well as three additional taxa (*Dilepis undula*, *Hymenolepis diminuta* and *Rodentolepis microstoma*). These were totally 15 taxa, for which sequences with sufficient coverage for the three genes were available – that is, 18S rRNA and COI (tables 2 and 3) and 28S rRNA (Neov *et al.*, 2019).

Phylogenetic analyses were performed using Bayesian inference with MrBayes, version 3.2.7 (Ronquist *et al.*, 2012). Prior to analysis, the best model of nucleotide substitution was selected using MrModeltest 2.4 (Nylander *et al.*, 2004); in all the three cases, this was the general time reversible model, with gamma-distributed estimate of site rate variation and a portion of invariant sites (GTR + G + I). The analyses were each run for 1.5×10^7 generations, two separate runs, each with four chains, discarding 33% (5×10^6) of resulting trees as burn-in. As outgroup for phylogenetic reconstruction analyses of genetic data for the three genes, we used sequences of *D. undula* (Schrank, 1788), a species of the family Dilepididae, believed to represent the most closely related family-group taxon, for which matching molecular data were available (Mariaux *et al.*, 2017).

For clade groups revealed by the present analysis, we used the names proposed by Haukisalmi *et al.* (2010) and adopted in our previous article (Neov *et al.*, 2019); however, in the majority of cases, additional taxa were added based either on our new data or published sequences by other authors. Average standard deviation of split frequencies below 0.01 was observed at the end of each run and served as a proof of chains reaching convergence. Branches persisting in less than 60% of post burn-in samples were treated as polytomies. Nodal support was expressed as posterior probabilities. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). There were a total of 2098 positions in the final dataset for the 18S rRNA analysis, 558 positions for the COI analysis and 3802 positions for the combined analysis.

Results

Based on the 18S rRNA gene, basal relationships of the main phyletic lineages remained unresolved, with a polytomy of the main 'mammalian' clades forming with other hymenolepidids from birds, as well as with the only representative of the mammalian *Hymenolepis* clade (i.e. *H. diminuta*) included in the analysis (fig. 1). The two members of the *Ditestolepis* clade – that is, *Ditestolepis diaphana* and *Spasskylepis ovaluteri* – were revealed as sister taxa. The monophyly of the *Arostrilepis* clade was also well supported, with the genera *Neoskrjabinolepis*, *Lineolepis*, *Vaucherilepis* and *Coronacanthus* included in the present analysis.

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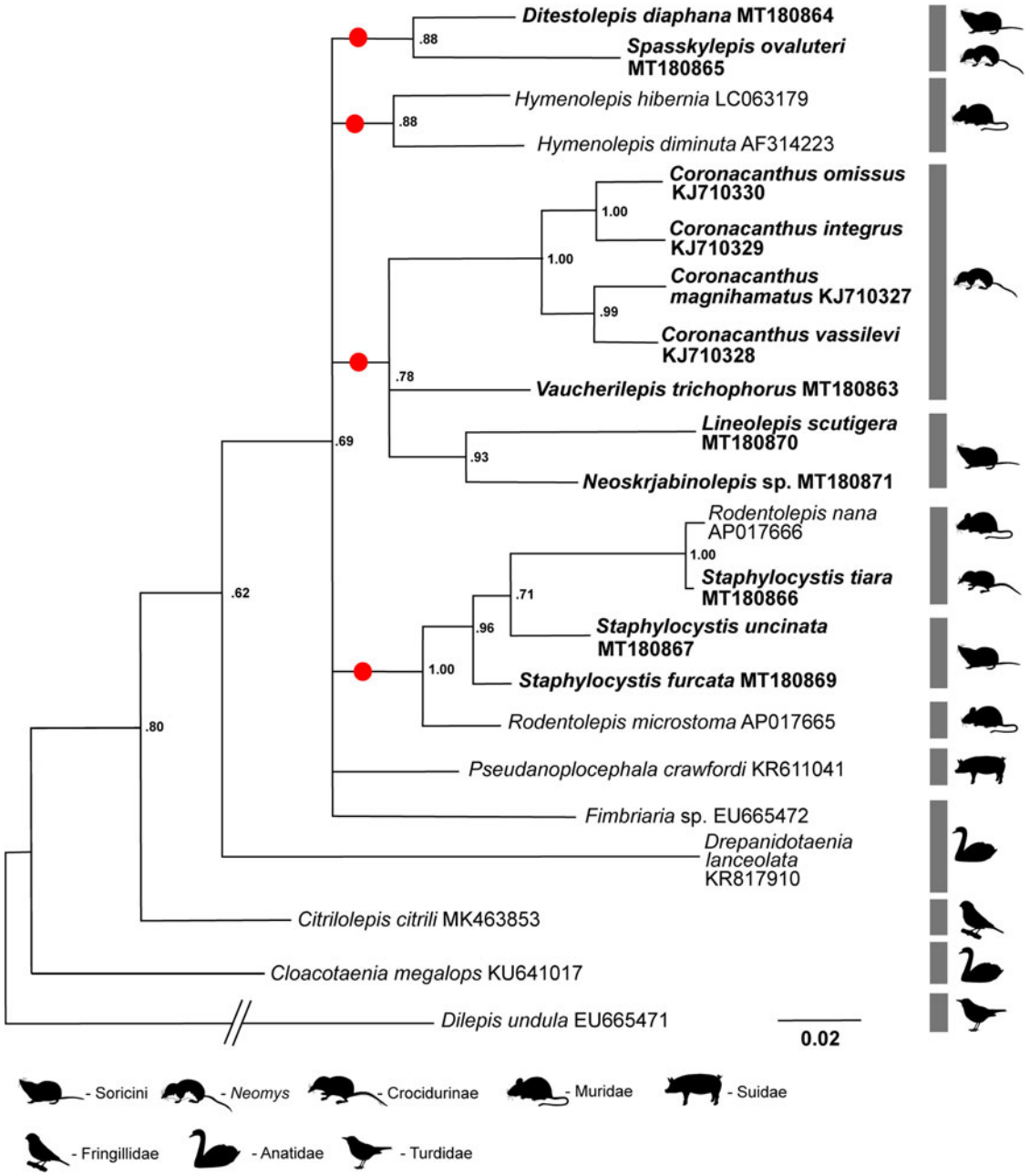

Fig. 2. Bayesian inference tree of phylogenetic relationships among 21 species of hymenolepidid cestodes (17 species from mammals and four species from birds) based on analysis of COI gene. *Dilepis undula* (family: Dilepididae) is used as outgroup. The GenBank numbers are added after the binomial name of each species. Newly sequenced taxa are in bold. The major clades recognized by Haukisalmi *et al*. (2010), confirmed by Neov *et al*. (2019) and outlined by the present study are marked by circles. Nodal support is given by posterior probabilities. Scale bar shows the number of substitutions per site.

The monophyly of the genus *Coronacanthus* was also strongly supported, with another parasite of water shrews (*Vaucherilepis*) being its sister taxon. The *Rodentolepis* clade, represented by the genera *Staphylocystis* (parasitic in shrews) and *Rodentolepis* (parasitic in rodents) in the analysis, is also a monophyletic lineage, with *R. microstoma* basal to the remaining taxa and *Rodentolepis nana* (from rodents) and *Staphylocystis tiara* (from insectivores) revealed as sister taxa.

The COI analysis (fig. 2) revealed each of the four main clades of mammalian hymenolepidids as a monophyletic group. However, the basal branching of cestodes from small mammals remained unresolved, forming a polytomy with a hymenolepidid species parasitic in birds (*Fimbriaria* sp.) and the only hymenolepidid species parasitic in pigs (*Pseudanoplocephala crawfordi*). The remaining hymenolepidids from birds included in the analysis were basal to the polytomy that included taxa from mammals as well as *Fimbriaria* sp. The *Ditestolepis* clade and the *Hymenolepis* clade were well supported. The *Arostrilepis* clade was also well supported but the basal relationships in it were not resolved and the cestodes from water shrews did not form a monophyletic group; however, the genera *Neoskrjabinolepis* and *Lineolepis* were confirmed as sister taxa. In the *Rodentolepis* clade, *R. microstoma* was the basal taxon, and *Staphylocystis furcata* was basal to its congeners and *R. nana*. The strong

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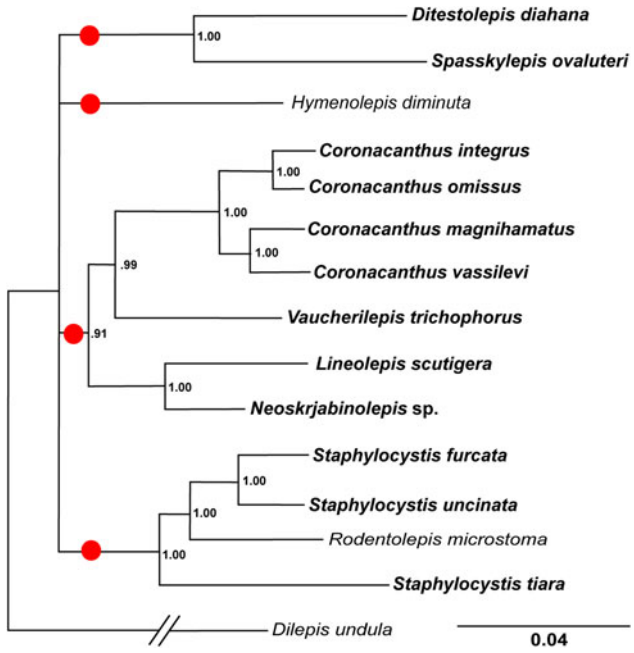

Fig. 3. Bayesian inference tree of phylogenetic relationships among 14 species of mammalian hymenolepidid cestodes based on combining sequences of COI, 18S rRNA and 28S rRNA genes. *Dilepis undula* (family: Dilepididae) is used as outgroup. Taxa represented partially by new sequences are in bold. The major clades recognized by Haukisalmi *et al.* (2010), confirmed by Neov *et al.* (2019) and outlined by the present study are marked by circles. Nodal support is given by posterior probabilities. Scale bar shows the number of substitutions per site.

phylogenetic relationship between *R. nana* (from rodents) and *S. tiara* (from insectivores), as revealed by the 18S rRNA gene analysis, was confirmed.

The combined analysis based on sequences of 28S rRNA, 18S rRNA and COI genes (fig. 3) was characterized by the presence of a basal polytomy. However, the *Ditestolepis* clade, the *Arostrilepis* clade and the *Rodentolepis* clade were each strongly supported. The arrangement of the taxa in the *Arostrilepis* clade was identical with that revealed by the 18S rRNA gene analysis. In the *Rodentolepis* clade, *S. tiara* was basal to the remaining taxa, which included both *Staphylocystis* spp. and *R. microstoma*.

Discussion

The phylogenies based on the 18S rRNA and COI genes confirm the main monophyletic groups among mammalian hymenolepidids revealed by sequencing of 28S rRNA genes (Haukisalmi *et al.*, 2010; Neov *et al.*, 2019). The presence of three of the major clades (*Ditestolepis* clade, *Arostrilepis* clade and *Rodentolepis* clade) is supported by all the analyses performed in the course of the present study. The *Hymenolepis* clade is also supported by the COI-based analysis; however, the 18S rRNA analysis and the combined analysis are not informative about this lineage, since it is represented by a single species only. Of the four major clades, only the *Ditestolepis* clade has a strong host–parasite association with shrews of the family Soricidae (Haukisalmi *et al.*, 2010; Kornienko *et al.*, 2019; Neov *et al.*, 2019). As previously shown (Neov *et al.*, 2019), each of the remaining three clades includes mostly parasites from rodents and soricids, and sometimes from other mammalian orders. This distribution of host–parasite associations across the phylogenetic trees indicates multiple events of host switching in the course of the diversification of hymenolepidids in mammals (Neov *et al.*, 2019). More detailed discussions on the distributions of the various patterns of rostellar apparatus, host–parasite associations and lifecycle peculiarities across the clades confirmed by the present study have been presented by Neov *et al.* (2019).

The relationships within the *Ditestolepis* clade and the *Hymenolepis* clade cannot be discussed confidently due to the limited number of taxa included in each. The relationships within the *Arostrilepis* clade, especially those based on 28S rRNA gene (Neov *et al.*, 2019) and 18S rRNA gene as well as the combined analysis, confirm the close relationships of cestodes from water shrews of the genus *Neomys*, represented in the present dataset by two morphologically dissimilar (Genov, 1980; Tkach *et al.*, 2003; Vasileva *et al.*, 2005) genera – that is, *Vaucherilepis* and *Coronacanthus*. This might be a basis to speculate that armed hymenolepidids from water shrews of the genus *Neomys* are a monophyletic group. However, this hypothesis needs to be tested on the basis of more diverse sample of species.

The position of *S. tiara*, a parasite from crocidurine shrews, varies across the performed analyses, although always positioned in the *Rodentolepis* clade. Two of the analyses (28S rRNA and combined; fig. 3) place it in basal position but the 18S rRNA and COI analyses group it with *R. nana* as a sister taxon, both having a derived position (figs 1 and 2). The variable position of *S. tiara* revealed by the present study and the polyphyletic character of the genera *Staphylocystis* Villot, 1877 and *Rodentolepis* Spasskii, 1954 revealed by the 28S rRNA analysis (Neov *et al.*, 2019) suggest the need for further studies to resolve the phylogenetic relationships of these taxa and to revise the generic concepts in the group.

The monophyly of the hymenolepidids from 'rodents and shrews' was postulated by Haukisalmi *et al.* (2010) and adopted by Neov *et al.* (2019). However, two of the present analyses (18S rRNA and COI) show that the basal relationships of the four mammalian clades are branching at the same polytomy with several hymenolepidids parasitic in birds (both terrestrial and aquatic). This questions the monophyly of the hymenolepidids from mammals and may indicate multiple events of colonizations of mammalian hosts. Therefore, the position and the evolutionary history of mammalian hymenolepidids require a more comprehensive consideration involving taxa of this family from both birds and mammals.

Though differing by the number of the taxa included, the phylogenetic trees based on 28S rRNA gene (Neov *et al.*, 2019), 18S rRNA gene and the combined tree have similar topologies. Although partial (D1–D3) large (28S) and complete small (18S) nuclear ribosomal genes form part of a tandem array (Hillis & Dixon, 1991), together and in combination with partial COI, this combination of genes has been shown to demonstrate utility in resolving intra- and intergeneric relationships among cestode orders (e.g. Waeschenbach & Littlewood, 2017). However, in this study, in spite of adding additional molecular phylogenetic signal from genes known to evolve faster (COI) and slower (18S rRNA) than 28S rRNA D1–D3 (e.g. Machida & Knowlton, 2012), no further resolution was added to the overall molecular phylogenetic hypothesis of hymenolepidids from mammals. Whilst denser, perhaps phylogenomic, gene sampling may be required to resolve the early branching patterns of hymenolepidid lineages, unresolved basal lineages and short internal branches are also considered signatures of rapid bursts of speciation and phenotypic evolution (Schluter, 2000). Undoubtedly, as the

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most species-rich clade of tapeworms, hymenolepidid cestodes have been highly successful in parasitizing birds and mammals, and it may be that their appearance was followed by rapid expansion and radiation through these host lineages.

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Conflicts of interest. None.

Ethical standards. Methodology of the small mammal examination was approved by the Scientific Council of Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Decision 7/16.10.2012.

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