

Research note

On the phylogenetic positions of the Caryophyllidea, Pseudophyllidea and Proteocephalidea (Eucestoda) inferred from 18S rRNA[☆]

I. Kodedová¹, D. Doležel¹, M. Broučková, M. Jirků, V. Hypša, J. Lukeš, T. Scholz^{*}

Institute of Parasitology, Academy of Sciences of the Czech Republic, and Faculty of Biology, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

Received 31 May 2000; received in revised form 13 July 2000; accepted 13 July 2000

Abstract

A phylogenetic analysis of tapeworms (Eucestoda) based on complete sequences of the 18S rRNA genes of 43 taxa (including new sequences of 12 species) was carried out, with the emphasis on the groups parasitising teleost fish and reptiles. Spathebothriidea and Trypanorhyncha (the latter group being paraphyletic) appeared as basal groups of the Eucestoda but their position was not stable. The tetrafoosate orders (Litobothriidea, Lecanicephalidea, Tetraphyllidea, Proteocephalidea, Nippotaeniidea, Tetrabothriidea and Cyclophyllidea) were well separated from the remaining groups. Results supported polyphyly of the Pseudophyllidea formed by two distinct clades: one with diphylobothriids (*Diphylobothrium*, *Schistocephalus*, *Spirometra* and *Duthiersia*) and another including *Abothrium*, *Probothriocephalus*, *Eubothrium* and *Bothriocephalus*. The former pseudophyllidean clade formed a separate branch with the Caryophyllidea (*Khawia* and *Hunterella*) and Haplobothriidea (*Haplobothrium*), the latter taxon being closely related to either caryophyllideans or diphylobothriids in different analyses. Proteocephalideans formed a monophyletic group in all analyses and constituted a clade within the Tetraphyllidea thus rendered paraphyletic. Within the Proteocephalidea, the *Acanthotaeniinae* (*Acanthotaenia* from reptiles in Africa) and *Gangesiinae* (*Gangesia* and *Silurotaenia* from silurid fish in the Palearctic Region) were separated from parasites of freshwater fish and mammals. The family Proteocephalidae was found to be paraphyletic due to the placement of a monticelliid species, *Monticellia* sp., in a clade within the former family. The genus *Proteocephalus* appeared as an artificial assemblage of unrelated taxa which is congruent with previous molecular analyses. © 2000 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Eucestoda; 18S rRNA; Phylogeny; Pseudophyllidea; Caryophyllidea; Proteocephalidea

Recently, considerable progress has been achieved in studies on the phylogeny of tapeworms (Eucestoda) and new hypotheses based on morphological, life-cycle, ultra-structural and molecular data have been proposed [1–4]. The first complete 18S rRNA gene sequence of a tapeworm was provided by Král'ová et al. [5] and partial sequences of the same gene were subsequently used for the evaluation of phylogenetic relationships within several orders of the Eucestoda [3]. The basal position of the Caryophyllidea was congruent with conclusions derived from morphological and life-cycle analyses [1] but the rRNA-based tree [3] indicated paraphyly of the Pseudophyllidea in contrast to previous classifications in which this order was treated as

a monophyletic assemblage [6]. Interestingly, recent evaluation of an enlarged set of morphological characters of the type genera of the pseudophyllidean families also favoured the paraphyly scenario [7].

The most comprehensive phylogenetic analysis of tapeworms based on sequences of the 18S rRNA and elongation factor-1 α genes performed so far included 23 species representing all 14 currently recognised orders of the Eucestoda [8]. In this study, the Spathebothriidea was found to be a sister group of all the remaining eucestodes that were split into the difossate and tetrafoosate lineages. The basal position of the Caryophyllidea was not supported and the Proteocephalidea formed a clade within the Tetraphyllidea that appeared, in accordance with previous analyses [3,9], to be paraphyletic. Possible paraphyly of the Pseudophyllidea was not tested because sequence data from representatives of only the family Diphylobothriidae were available [8].

To date, sequence data of few members of the orders parasitising predominantly teleost fish and reptiles, i.e. the Caryophyllidea, Pseudophyllidea and Proteocephalidea, are

[☆] The nucleotide sequences have been deposited in the GenBankTM under the accession numbers AF267287–AF267298.

^{*} Corresponding author. Tel.: +420-38-777-5431; fax: +420-38-530-0388.

E-mail address: tscholz@paru.cas.cz (T. Scholz).

¹ Present address: Institute of Entomology, Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic

available. In the present analysis, attention has been paid to cestodes of these three orders because their phylogenetic position has remained problematic. Complete sequences of the 18S rRNA gene from 12 eucestode species enlarged the existing data set and enabled us to analyse the evolution of these important parasites.

The organisms sequenced are listed below. Voucher specimens have been deposited in the helminthological collection of the Institute of Parasitology or are taxonomically evaluated if they represent new, hitherto undescribed taxa (marked with an asterisk).

(1) *Khawia sinensis* Hsü of the order Caryophyllidea, 1935 ex *Cyprinus carpio* (Pisces: Cyprinidae), Hamerský pond near Nové Hradky, Czech Republic, collected by T. Scholz (AF267287).

(2) *Bothriocephalus claviceps* of the order Pseudophyllidea (Goeze, 1782) ex *Anguilla anguilla* (Pisces: Anguillidae) from Štědrónín, Orlický reservoir, Czech Republic, T. Scholz (AF267288); (3) *Bothriocephalus* sp. (*) ex *Dorosoma mexicanum* (Pisces: Atherinidae) from Catemaco Lake, Veracruz, Mexico, T. Scholz (AF267289); (4) *Duthiersia fimbriata* (Diesing, 1850) ex *Varanus exanthematicus* (Reptilia: Varanidae) from Ghana, B. Koudela, D. Modrý and T. Scholz (AF267290); (5) *Eubothrium salvelini* (Schränk, 1790) ex *Salvelinus alpinus* (Pisces: Salmonidae), Bourget Lake, France, V. Hanzelová and T. Scholz (AF267291); (6) *Probothriocephalus* sp. ex *Xenodermichthys copei* (Pisces: Alepocephalidae) from Porcupine Bight, North Atlantic, R.A. Bray (AF267292).

(7) *Acanthotaenia* sp. of the order Proteocephalidea from *V. exanthematicus* (Reptilia: Varanidae) from Ghana, B. Koudela, D. Modrý and T. Scholz (AF267293); (8) *Gangesia parasiluri* Yamaguti, 1934 ex *Silurus asotus* (Pisces: Siluridae) from Lake Suwa, Japan, T. Shimazu (AF267294); (9) *Proteocephalus chamelensis* Pérez-Ponce de León, Brooks et Berman, 1995 ex *Gobiomorus maculatus* (Pisces: Eleotridae) from Cuitzmala River, Chamelá, Jalisco, Mexico, T. Scholz (AF267295); (10) Proteocephalinae gen. sp. (*) ex *Didelphis marsupialis* (Mammalia: Didelphidae) from Los Tuxtlas, Veracruz, Mexico, T. Scholz (AF267296); (11) *Monticellia* sp. (*) ex *Ophisternon aenigmaticum* (Pisces: Synbranchidae) from Catemaco Lake, Veracruz, Mexico, T. Scholz (AF267297); (12) *Silurotaenia siluri* (Batsch, 1786) ex *Silurus glanis* (Pisces: Siluridae) from Štědrónín, Orlický reservoir, Czech Republic, T. Scholz (AF267298).

A small piece of an adult specimen stored in 70% ethanol was cut with a razor blade, transferred into phosphate buffered saline, repeatedly washed and mechanically homogenised. Cells were subsequently pelleted by brief centrifugation, resuspended in lysis buffer, and genomic DNA was isolated as described elsewhere [10]. Approximately 10–50 ng of genomic DNA was used for PCR amplification of the 18S rRNA gene that was amplified in two overlapping fragments using the oligonucleotides 81 + 83 and 82 + 84 of Mariaux [3]. The PCR products of expected size were gel-

purified and cloned into either pT7Blue vector (Novagen) or pCRII vector (Invitrogen). Both strands were sequenced using either the Amersham (Termoseq II Terminator) or Perkin–Elmer (ABI Prism BigDye Terminator) sequencing kits. Internal primers designed to match the conserved regions [5,11] were used to complete the sequences.

The sequences obtained in this study were aligned with 18S rRNA genes of cestodes available from the GenBank™, selected to represent all available tapeworm lineages with emphasis on taxa presumed to be related to the analysed groups (i.e. members of Tetraphyllidea). One monogenean and two members of the primitive cestode orders Gyrocotylidea and Amphiliidea were used as outgroups.

The following 18S rRNA sequences retrieved from the GenBank™ were used in this work: *Polystomoides malayi* AJ228792; *Gyrocotyle rugosa* AF124455 (Gyrocotylidea); *Schizocoerus liguloideus* AF124454 (Amphiliidea); *Hunterella nodulosa* AF124457 (Caryophyllidea); *Spathobothrium simplex* AF124456 (Spathobothriidea); *Grillotia erinaceus* GER228781; *Hepatoxylon* sp. AF124462; *Tentacularia* sp. AF124461 (Trypanorhyncha); *Haplobothrium globuliforme* AF124458 (Haplobothriidea); *Abothrium gadi* AGA228773; *Bothriocephalus scorpii* AJ228776; *Diphyllobothrium stemmacephalum* AF124459; *Schistocephalus solidus* AF124460; *Spirometra erinacei* D64072 (Pseudophyllidea); *Echinobothrium fauleyae* AF124464; *Macrobothridium* sp. AF124463 (Diphylloidea); *Cephalobothrium aetobatidis* AF124466; *Eniochobothrium gracile* AF124465 (Lecanicephalidea); *Anthobothrium laciniatum* AF124471; *Calliobothrium* sp. AF124469; *Platybothrium auriculatum* AF124470; *Rhinebothrium maccallumi* AF124476 (Tetraphyllidea); *Litobothrium janovyi* AF124468; *Litobothrium* (syn. *Renyxa*) *amplifica* AF124467 (Litobothriidea); *Proteocephalus exiguus* (= *P. longicollis*) X99976; *P. perplexus* AF124472 (Proteocephalidea); *Amurotaenia decidua* AF124474 (Nippotaeniidea); *Tetrabothrius forsteri* AF124473 (Tetrabothriidea); *Echinococcus granulosus* EGU27015; *Hymenolepis diminuta* AF124475 (Cyclophyllidea).

The sequences were initially aligned using the Megalign program (Dnastar Inc.); several alignments were constructed under a wide range of parameters and analysed by PAUP Version 4.0b [12]. The sequences of taxa that remained monophyletic in all alignments were used to prepare partial subalignments. The subalignments were then aligned together by the more rigorous method of Malign (algorithm: build, score 4, alignswap, alignaddswap, treeswap, treeaddswap, contig) [13]. The final alignment was corrected by eye and ambiguously aligned regions were removed prior to further analysis. The phylogenetic analyses and the calculation of nodal support (maximum parsimony, maximum likelihood, bootstrap and Bremer's indices) were performed using PAUP. For maximum parsimony, 50 replicates of a heuristic search by TBR algorithm with random addition of sequences were performed. The matrix was analysed under assumptions of transversions/

transitions ratio 1:1, 1:3 and 1:5, respectively, and gaps were treated as missing data. The same algorithm was used to calculate bootstrap support (500 replicates) and Bremer (decay) indices. For maximum likelihood, the HKY85 and Felsenstein84 methods were employed.

The length of the complete 18S rRNA gene ranged from 2041 bp (*P. chameleensis*) to 2211 bp (Proteocephalinae gen. sp. ex *Didelphis*). A final alignment contained 2449 positions of which 1998 (382 parsimony-informative) were included into the analyses.

General instability of the tapeworm 18S rRNA matrix, particularly affecting the placement of supposedly primitive Spathebothriidea and Trypanorhyncha, has been demonstrated in an extensive study by Olson and Caira [8]. Similarly, considerable sensitivity of phylogenetic position of these two groups to the extent of the character exclusion was observed in the present study. Thus, the position of spathebothriideans and trypanorhynchs, as well as the monophyly/paraphyly of the latter, varied in different matrices, while the remainder of the taxa retained a relatively stable topology. The definitive phylogenetic position of the trypanorhynchs is unlikely to be resolved until additional molecular data are available.

In Fig. 1, the strict consensus tree obtained after the exclusion of all ambiguously aligned regions is shown. The structure of this tree is broadly similar to the maximum parsimony tree of Olson and Caira (Fig. 2A in Ref. [8]). The Spathebothriidea and Trypanorhyncha are the most primitive eucestode groups branching at the base of the tree. Trypanorhyncha represented by *Tentacularia*, *Hepatoxylon* and *Grillotia* appeared paraphyletic, the latter two genera forming a strongly supported monophyletic group in all analyses. Topologies identical to Fig. 1 were obtained by both maximum likelihood models, the only difference being the placement of Spathebothriidea as a sister group of the Caryophyllidea + Pseudophyllidea + Haplobothriidea lineage (data not shown).

In the crown of the tree, the largest monophyletic group embraced the tetrafossate orders (Litobothriidea, Lecanicephalidea, Tetraphyllidea, Proteocephalidea, Nippotaeniidea, Tetrabothriidea and Cyclophyllidea) and was well separated in all analyses from the remaining eucestode groups consisting of the monofossate and difossate tapeworms.

The principal innovation of this study is the confirmation that the Pseudophyllidea is not a monophyletic group. The polyphyly of pseudophyllidean tapeworms was indicated by previous molecular [3] and morphological analyses [7] and is confirmed by the present analyses. The Pseudophyllidea formed two distinct clades in all obtained trees (Fig. 1); the first clade was represented by *Diphyllobothrium*, *Schistocephalus*, *Spirometra* and *Duthiersia* (all Diphyllobothriidae) whereas other pseudophyllideans (species of *Abothrium*, *Probothriocephalus*, *Eubothrium* and *Bothriocephalus*) formed a separate branch. Under the transition/transversion ratio 1:3 assumption, the bothriocephalid pseudophyllideans were more closely related to members of the Diphyllidea

(*Echinobothrium* and *Macrobothridium*), parasites of elasmobranchs, than to other eucestodes. *D. fimbriata* from reptiles in Africa was a basal taxon to other diphyllobothriid species within the first pseudophyllidean clade (Fig. 1).

The Caryophyllidea, represented by members of two of the four recognised families, i.e. Caryophyllaeidae (*Hunterella*) and Lytocestidae (*Khawia*), did not constitute a basal group of the Eucestoda in our dataset. On the contrary, they grouped with *Haplobothrium* and diphyllobothriids, which supports previous results [8]. A close grouping of caryophyllideans, haplobothriids and one of the two clades of pseudophyllideans corresponds in some aspects with the taxonomic arrangement of Nybelin [14] who placed caryophyllideans as a family within the Pseudophyllidea. The non-basal position of the Caryophyllidea reported by others [8] was supported by the present analyses that included sequences of an additional caryophyllidean taxon from different family and geographical region (Europe). Previously, a complete

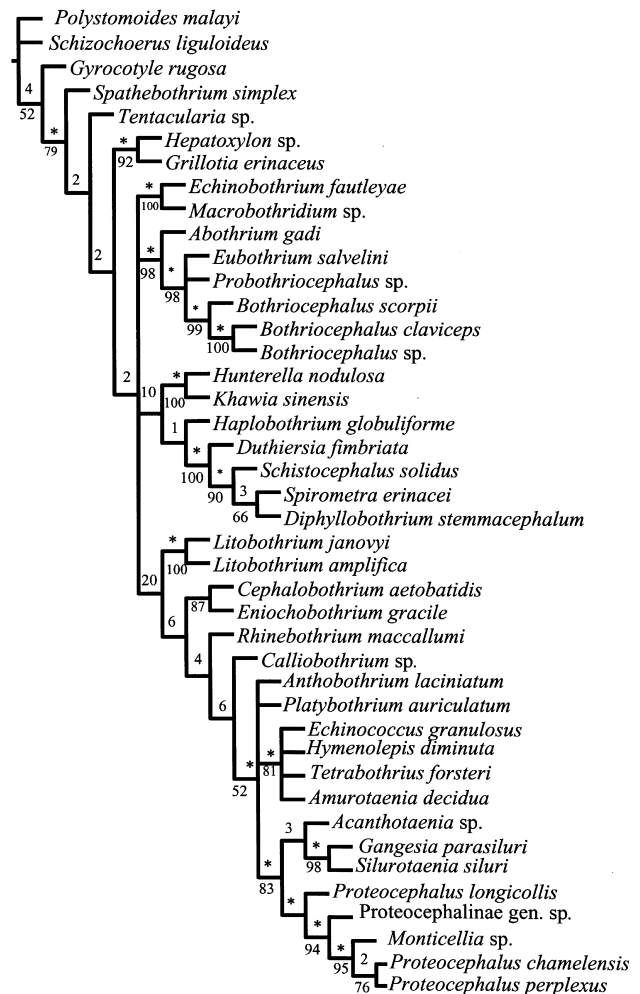


Fig. 1. Maximum parsimony; strict consensus of 48 most parsimonious trees (L = 2027; CI = 0.39; RI = 0.60). Bootstrap values >50% (below the lines) and Bremer's indices (above the lines) shown at the nodes. * = Bremer's index >20.

18S rRNA sequence of only one species (*H. nodulosa*) from North America was available [8].

The ordinal status of haplobothriids [15], that were, according to several authors [16,17], affiliated with the Pseudophyllidea or considered to be closely related to the Trypanorhyncha [18], is only partly supported in this study because this taxon appeared in the pseudophyllidean branch of the family Diphyllbothriidae in some trees (data not shown).

Topology of the second pseudophyllidean clade (Fig. 1) confirmed the monophyletic status of the genus *Bothriocephalus*. Both freshwater species (*B. claviceps* and *Bothriocephalus* sp. from the Palearctic and Neotropical Regions, respectively), were more closely related to each other than to the marine cestode *B. scorpii* from Northeastern Atlantic.

In all trees constructed, the Proteocephalidea appeared to be a monophyletic taxon that formed a clade within the Tetraphyllidea. Paraphyly of the latter group was proposed in a study based exclusively on morphological characters that included only two proteocephalidean taxa (*Proteocephalus exiguus* and *Monticellia lenha*) [9]. The present study supports this hypothesis because five taxonomically unrelated species representing four subfamilies (Proteocephalinae, Gangesiinae, Acanthotaeniinae, Monticelliinae) and originating from different hosts, including reptiles and a mammal, from four continents (Europe, Asia, North America and Africa) were analysed.

Within the Proteocephalidea (Fig. 1), members of the Acanthotaeniinae (*Acanthotaenia* from a monitor lizard from Africa) and Gangesiinae (*Gangesia* and *Silurotaenia* from siluriform fish from the Palearctic Region) constituted a clade distantly related to the remaining proteocephalideans. The separate position of the acanthotaeniine and gangesiine cestodes from other proteocephalideans corresponds with earlier report [19] that studied the phylogenetic relationships within the Proteocephalidea on the basis of the mitochondrial (16S) and nuclear (28S) rRNA gene sequences. The position of the Acanthotaeniinae as the most primitive subfamily of the Proteocephalidea was first demonstrated by an analysis based on morphological characters while the Gangesiinae appeared as one of the most evolved proteocephalidean subfamilies closely related to the Proteocephalinae [20].

The family Proteocephalidae appeared paraphyletic as the only representative of the Monticelliidae (*Monticellia* sp.) grouped with species of the Proteocephalinae. The present data also suggest that the genus *Proteocephalus* is an artificial assemblage of unrelated taxa, this result being in accordance with the conclusions of Zehnder and Mariaux [19]. The Holarctic species *P. longicollis* (synonym *P. exiguus* [21]) was basal to the remaining proteocephalideans from the Neotropical (*Monticellia* sp., *P. chamelensis*) and Nearctic (*P. perplexus*) fish and from the opossum (Proteocephalinae gen. sp.) in Mexico.

The latter species is the first representative of the order found in a homiotherm vertebrate and its taxonomic posi-

tion was a matter of question. However, the present study has demonstrated that it undoubtedly belongs among the Proteocephalidae, which corresponds well with its morphology. Phylogeny derived from the 18S rRNA sequences also supported the association of the nipotaeniid *Amurotaenia* with the Cyclophyllidea and Tetrabothriidea (95% bootstrap) that was observed in previous phylogenetic analyses based on morphological and molecular data [1,3,8].

Results of the present study expand several hypotheses [3] but also question, as did the previous study by Olson and Caira [8], some widely accepted ideas about phylogeny of the Eucestoda that were largely based on morphological, ultrastructural and life-cycle data [1,2,4]. Apparently, more information on members of the cestode groups that are scarcely represented in existing analyses is needed in order to better understand the evolution of major lineages of eucestodes as well as interrelationships of members of individual groups.

Acknowledgements

Thanks to Dr Rodney A. Bray, Natural History Museum, London, UK; Dr Takeshi Shimazu, Nagano Prefectural College, Japan; Drs David Modrý and Břetislav Koudela, Veterinary and Pharmaceutical University, Brno, Czech Republic and Dr Vladimíra Hanzelová, Parasitological Institute, Košice, Slovakia, for providing specimens of *Probothriocephalus* sp., *G. parasiluri*, *Acanthotaenia* sp., *D. fimbriata* and *E. salvelini*, respectively. Stays of one of the authors (T.S.) in Mexico that enabled collection of some specimens for this study was supported by the National Council for Science and Technology of Mexico (CONACyT), Center for Investigation and Advanced Studies of the National Polytechnic Institute (CINVESTAV-IPN) Mérida, Mexico, and Institute of Biology, National Autonomous University of Mexico (UNAM), Mexico City, Mexico. The study was supported by the Grant Agency of the Czech Republic (No. 206/98/0591) and the Grant Agency of the Academy of Sciences of the Czech Republic (No. A6022903).

References

- [1] Hoberg EP, Mariaux J, Justine J-L, Brooks DR, Weekes PJ. Phylogeny of the orders of the Eucestoda (Cercomeromorphae) based on comparative morphology: historical perspectives and a new working hypothesis. *J Parasitol* 1997;83:1128–247.
- [2] Hoberg EP, Brooks DR, Mariaux J. Phylogeny of the orders of the Eucestoda: morphological and molecular evidence. *Syst Parasitol* 1999;42:12–37.
- [3] Mariaux J. A molecular phylogeny of the Eucestoda. *J Parasitol* 1998;84:114–24.
- [4] Justine J-L. Spermatozoa as phylogenetic characters for the Eucestoda. *J Parasitol* 1998;84:385–408.
- [5] Král'ová I, Van de Peer Y, Jirků M, Van Ranst M, Scholz T, Lukeš J. Phylogenetic analysis of a fish tapeworm, *Proteocephalus exiguus*,

- based on the small subunit rRNA gene. *Mol Biochem Parasitol* 1997;84:263–6.
- [6] Bray RA, Jones A, Andersen KI. Order Pseudophyllidea Carus, 1863. In: Khalil LF, Bray RA, Jones A, editors. Keys to the cestode parasites of vertebrates. Wallingford: CAB International, 1994. pp. 205–47.
- [7] Bray RA, Jones A, Hoberg EP. Observations of the phylogeny of the cestode order Pseudophyllidea Carus, 1863. *Syst Parasitol* 1999;42:13–20.
- [8] Olson PD, Caira JN. Evolution of the major lineages of tapeworms (Platyhelminthes: Cestoidea) inferred from 18S ribosomal DNA and elongation factor-1 α . *J Parasitol* 1999;85:1134–59.
- [9] Caira JN, Jensen K, Healy CJ. On the phylogenetic relationships among tetraphyllidean, lecanicephalidean and diphyllidean tapeworm genera. *Syst Parasitol* 1999;42:77–151.
- [10] Jirků M, Kolesnikov AA, Benada O, Lukeš J. Marine fish and ray trypanosomes have large kinetoplast minicircle DNA. *Mol Biochem Parasitol* 1995;73:279–83.
- [11] Cunningham CO, McGillivray DM, MacKenzie K. Phylogenetic analysis of *Gyrodactylus salaris*, Malmberg 1957 based on the small subunit (18) ribosomal RNA gene. *Mol Biochem Parasitol* 1995;71:139–42.
- [12] Swofford DL. PAUP 4.0 - phylogenetic analysis using parsimony. Version 4. Computer Program. Sunderland, MA: Sinauer, 1998.
- [13] Wheeler W, Glatstein D. MALIGN. Version 1.99, 1994 (<http://www.vims.edu/~mes/hennig/software.html>).
- [14] Nybelin O. Anatomisch-systematische studien über Pseudophyllideen. Göteborgs Kungl Vetenskap Vit Samh Handl 1922;26:1–228.
- [15] Jones A. Order Haplobothriidea Joyeux & Baer, 1961. In: Khalil LF, Bray RA, Jones A, editors. Keys to the cestode parasites of vertebrates. Wallingford: CAB International, 1994. pp. 249–51.
- [16] Yamaguti S. *Systema helminthum*, The cestodes of vertebrates, vol. 2. New York: Interscience, 1959.
- [17] Schmidt GD. Handbook of tapeworm identification. Boca Raton, FL: CRC Press, 1986.
- [18] Freeman R. Ontogeny of cestodes and its bearing on their phylogeny and systematics. *Adv Parasitol* 1973;11:481–557.
- [19] Zehnder MP, Mariaux J. Molecular systematic analysis of the order Proteocephalidea (Eucestoda) based on mitochondrial and nuclear rDNA sequences. *Int J Parasitol* 1999;29:1841–52.
- [20] Rego AA, de Chambrier A, Hanzelová V, et al. Preliminary phylogenetic analysis of subfamilies of the Proteocephalidea (Eucestoda). *Syst Parasitol* 1998;40:1–19.
- [21] Scholz T, Hanzelová V. Tapeworms of the genus *Proteocephalus* Weinland, 1858 (Cestoda: Proteocephalidae), parasites of fishes in Europe. *Studie AV ČR. Academia Praha* 1998;2/98:1–119.