J Mol Evol (1999) 48:586–596

JOURNAL OF MOLECULAR EVOLUTION

© Springer-Verlag New York Inc. 1999

Contributions to the Phylogeny of the Cyclophyllidea (Cestoda) Inferred from Mitochondrial 12S rDNA

Markus von Nickisch-Rosenegk,[1] Richard Lucius,[1] Brigitte Loos-Frank[2]

[1] Humboldt-Universität zu Berlin, Lehrstuhl für Molekulare Parasitologie (Institut für Biologie), Philippstr. 13, 10115 Berlin, Germany
[2] Universität Hohenheim, Institut für Zoologie, Fachgebiet Parasitologie, Emil-Wolf-Str. 34, 70599 Stuttgart, Germany

Received: 1 October 1997 / Accepted: 4 December 1997

Abstract. A 314-bp fragment of the mitochondrial 12S rRNA gene from 21 cestodes species of eight families was synthesized by PCR with specially designed primers. These allowed amplification of parasite DNA without concomitant synthesis of host DNA. Phylogenetic trees were inferred from the sequence data using three methods (maximum parsimony, maximum likelihood, and Fitch–Margoliash). At the major nodes all three trees were similar. For the first time the genus *Mesocestoides* could be arranged into the Cyclophyllidea and a narrow relationship between the Mesocestoididae, Taeniidae, Hymenolepididae, Anoplocephalidae, and Dipylidiidae was shown. Members of the families Catenotaeniidae and a cluster of two families (Hymenolepididae and Dilepididae) form two monophyletic groups which derive prior to the remaining families of this phylogenetic study. A third and a fourth clear monophyletic group were formed by the Taeniidae and by the Mesocestoididae. A high degree of variation within the examined 304-bp fragment was observed between two isolates of *Taenia taeniaeformis,* supporting often discussed genetic heterogeneity within this species. In contrast, only one nucleotide exchange was found in 23 isolates of *Echinococcus multilocularis* of various geographic origin, indicating that this species is genetically homogenous.

Key words: *Mesocestoides* — Phylogeny of cyclophyllideans — 12S rRNA gene — Mitochondrial DNA — cestode-specific primers

Correspondence to: M. von Nickisch-Rosenegk

Introduction

Systemic relationships between cestodes of the order Cyclophyllidea are so far based mainly on morphological criteria, which are mostly of little significance for phylogeny (Mariaux 1996). In the case of cestodes it is often difficult to distinguish between secondary loss and convergence of morphological characters and various authors weigh characters of taxa differently. Such difficulties relate, in particular, to the family Mesocestoididae, which until now did not have an unequivocal position among the Cyclophyllidea (Khalil et al. 1994). This is not only due to the aberrant morphology of its genera, but also due to the fact that the life cycle is not completely known.

Mitochondrial DNA sequences and their high degree of evolutionary variation are suitable for investigations with closely related taxa (Brown et al. 1982). We used the method of Kocher et al. (1989), which was subsequently used by various groups (Ballard et al. 1992; Schliewen et al. 1994; van der Kuyl et al. 1995). The mitochondrial 12S rRNA gene fragment used is suitable for phylogenetic examinations, as the highly variable noncoding regions of this gene are flanked by smaller conserved stretches. These variable regions were amplified in each species with primers constructed from the conserved flanking ones. However, we could not utilize the universal primers for mitochondrial 12S rDNA constructed by Kocher et al. (1989) because of the high homology with vertebrates. The contamination of cestode DNA with host material, inevitable when worms or larvae are isolated, resulted in the predominant amplification of host DNA. With a newly developed cestode-

Table 1. Sources of the 59 DNA isolates used for phylogenetic analysis: hosts, origin, and condition of the adult and larval cestodes

Species	Adults	Metacestodes	Number	Geographic origin	Host
Catenotaenia lobata	Fresh		1	Southern Germany	*Microtus arvalis*
Catenotaenia pusilla	Fresh		1	Southern Germany	*Microtus arvalis*
Dilepis undula	Fresh		1	Southern Germany	*Turdus merula*
Diphyllobothrium sp.	EtOH		1	Northern Germany	*Phoca vitulina*
Diploposthe laevis	EtOH		2	Berlin	*Aythya* sp.
Dipylidium caninum	EtOH		2	Southern Germany	*Vulpes vulpes*
Echinococcus granulosus	EtOH		2	Africa, Spain	*Camelus dromedarius, Capra aegagrus*
Echinococcus multilocularis		Fresh (9), EtOH (14)	23	Austria, France, Germany, USA	*M. arvalis, Meriones unguiculatus*
Hymenolepis diminuta	Fresh		2	Southern Germany	*Rattus norvegicus*
Hymenolepis sp.	Fresh		1	Southern Germany	*Microtus arvalis*
Mesocestoides leptothylacus	Fresh		6	Southern Germany	*Vulpes vulpes*
Mesocestoides lineatus	EtOH		2	Brandenburg	*Vulpes vulpes*
Mesocestoides vogae		Fresh	1	USA	*Rattus norvegicus*
Moniezia expansa	Fresh		2	Southern Germany	*Ovis ammon*
Mosgovoyia pectinata	EtOH		1	Berlin	*Lepus europaeus*
Schistocephalus solidus		Fresh	2	Southern Germany	*Gasterosteus aculeatus*
Taenia martis		Fresh	4	Southern Germany	*Ondatra zibethicus*
Taenia saginata		EtOH	2	Southern Germany	*Bos taurus*
Taenia solium		EtOH	1	Southern Germany	*Sus scrofa*
Taenia taeniaeformis 1		Fresh	1	Southern Germany	*Ondatra zibethicus*
Taenia taeniaeformis 2		EtOH	1	Egypt	*Rattus norvegicus*

EtOH, preserved in 70% ethanol.

specific primer pair, PCR fragments of the 12S mitochondrial rRNA gene from several cyclophyllidean and pseudophyllidean cestodes were amplified. The sequences of these PCR products allowed phylogenetic comparisons by different methods.

This molecular tool revealed for the first time a noteworthy topology of a phylogenetic tree of a large group of eight families of cyclophyllidean cestodes. This is a further step toward the elucidation of the phylogeny of cestodes, which is currently not well understood (Mariaux 1996). The arrangement of all families from which species were tested reveals a pattern which supports the recent taxonomic classification by Khalil et al. (1994).

Materials and Methods

DNA Samples

Fifty-nine DNA samples of 21 cestode species from various geographic origins (Table 1) were analyzed. Most of the specimens were taken from the collection of the Fachgebiet Parasitologie, Universität Hohenheim or were freshly collected. The isolates of *E. multilocularis* from the United States were kindly provided by Dr. R.L. Rausch, University of Washington, Seattle: those from France, by Dr. M. Liance, Centre Universitaire, Laboratoire de Parasitologie, Créteille; and the four isolates from Austria by Dr. H. Auer, Klinisches Institut für Hygiene der Universität Wien. The *E. granulosus* material from Africa was isolated by Dr. T. Romig, Fachgebiet Parasitologie, Universität Hohenheim; and the material from Spain, by Dr. M. Frosch, Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover. *Mesocestoides lineatus, Mosgovoyia pectinata,* and *Diploposthe laevis* were kindly provided by Dr. J. Primer, Institut für Zoo- und Wildtierforschung, Berlin. Metacestodes of *Mesocestoides vogae* (=*M. corti*) (Etges 1991) were originally isolated from the fence lizard *Sceloporus occidentalis* (Specht and Voge 1965) and were kept in our laboratory by passage in mice.

DNA Extraction

A small sample of parasite tissue (about 200 mg) was cut into small pieces and digested for 6 h in digestion buffer (10 m*M* Tris-base/HCl, pH 7.5; 25 m*M* EDTA; 75 m*M* NaCl; 0.1% SDS; 0.5 mg/ml proteinase K) at 50°C. Proteins were removed twice by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and with chloroform/isoamyl alcohol (24:1), respectively (Sambrook et al. 1989). DNA was precipitated from the aequeous phase with 3 *M* sodium acetate, pH 4.8 (1:10), and isopropanol (v/v). After vacuum-drying the precipitate was dissolved in 200 µl TE buffer and the RNA was digested with RNase A. After a further Phenol/chloroform extraction the DNA was again precipitated and dried. The dried pellet was dissolved in 100 µl TE buffer.

Primer Construction

As cestode tissues are in close contact with host tissues, it is difficult to prepare cestode DNA, which is absolutely free of host material. Therefore, universal primer pairs derived mainly from vertebrate sequences (Kocher et al. 1989) proved to be unreliable for the amplification of cestode DNA, as they preferentially amplified trace amounts of host DNA associated with the parasite (v. Nickisch et al., unpublished data). To avoid the amplification of host DNA we constructed cestode-specific primers. Published 12S rRNA sequences of human, mouse, rat, *Xenopus, Drosophila,* and *Ascaris* from the EMBL databank were aligned with the CLUSTAL program (Higgins and Sharp 1988) of the UWGCG multialignment software by the University of Wisconsin Genetic Computer Group (Devereux et al. 1984) and optimized by eye. A primer pair was constructed from conserved regions flanking a large fragment. These primers were designated L00902 (5′-AATTTCGTGCCAGCCATCGCGG-3′) and H01558 (5′-

TTCCAGTACATTTACCTTGTTACGAC-3′) according to their position in the "Anderson sequence" (Anderson et al. 1981). Only under conditions of low stringency [20 × (94°C/1 min; 35°C/2 min; 73°C/2 min)] and a high concentration of primers (150 pmol) did PCR with genomic DNA of our specimens produce extremely small amounts of a second DNA fragment of about 600 bp besides a distinct band of 700 bp. The PCR products were cloned into a plasmid vector with a thymidine overhang at the 3′ position (Mead et al. 1991). After digestion of the plasmids the different insert sizes could be visualized in an agarose gel. Sequence comparison of PCR products obtained from pure host DNA (extracted from blood samples of the intermediate hosts *Ondatra zybeticus* and *Microtus arvalis*) with the 600- and 700-bp products and Genbank sequences revealed that the 700-bp band was derived from contaminating host material, while the 600-bp band was of parasite origin. After sequence analysis homologies between fragments and DNA sequences from Genbank revealed that the obtained 600-bp fragments of three synthesis products were parts of the 12S rDNAs of *E. multilocularis, T. taeniaeformis,* and *T. martis,* respectively. Alignment of these sequences, containing about 440 bp sequenced in both directions, allowed the construction of a pair of cestode-specific primers.

These primers, 60.for. (5′-TTAAGATATATGTGGTACAGGATTAGATACCC-3′) and 375.rev. (5′-AACCGAGGGTGACGGGCGGTGTGTACC-3′), flank a fragment of about 314 bp with homology to the mitochondrial rDNA sequence containing 386 bp, spanned by the primers described by Kocher et al. (1989).

PCR and Direct Sequencing

Amounts between 200 and 800 ng of genomic DNA were mixed with reaction buffer [20 m*M* Tris-base/HCl, pH 8.5; 16 m*M* $(NH_4)_2SO_4$; 2 m*M* $MgCl_2$; 50 m*M* KCl, 150 μg/ml BSA], 50 pmol of each primer, 50 nmol of dNTPs, and 1 U of Taq polymerase (Saiki et al. 1988). The mixture was overlaid with mineral oil and cycled 50 times in a Perkin–Elmer Thermocycler 480 as follows: 1 min at 93°C, 1.5 min at 55°C, and 2 min at 73°C. In cases where the cestode DNA was difficult to amplify and signals were weak or missing, a two-phase PCR was applied: 12 cycles of 1 min at 92°C, 2 min at 45°C, and 2 min at 73°C, followed by 40 cycles of 1 min at 93°C, 1 min at 55°C, and 2 min at 73°C. The synthesis products were separated from the remaining genomic DNA and from the primers with glass milk (Vogelstein and Gillespie 1979). Amounts of less than 1 μg of these purified fragments were optimal for the sequencing reactions.

For sequencing, the template DNA was added to 50–100 pmol primer (one of the primers used for PCR) and annealing buffer (1 *M* Tris-base/HCl, pH 7.6; 100 m*M* $MgCl_2$; 160 m*M* DTT) and heated for 3 min at 96°C. Immediately after heat denaturation of the double-stranded DNA the sample was kept on ice water (Casanova et al. 1990). Labeling mix A (Pharmacia, Uppsala, Sweden), 10 μCi ^{35}S-dATP, 3 U T7 polymerase, and 0.5% Nonidet P40 (Bachmann et al. 1990) were added and mixed by pipetting. Four aliquots were mixed with the respective ddNTPs (Sanger et al. 1979) without delay. Further sequencing steps were performed according to the instructions of the manufacturer (Pharmacia). The samples were electrophoretically separated on denaturating polyacrylamide gels with TBE buffer. This method of direct sequencing after PCR avoids the reading of nucleotide misincorporation caused by Taq polymerase, as mismatches appear only in a weak background.

Phylogenetic Analysis

Sequences obtained from autoradiography films were aligned with the UWGCG software by the University of Wisconsin Genetic Computer Group (Devereux et al. 1984) and the multialignment program CLUSTAL (Higgins and Sharp 1988) available in the HUSAR software package (Geniusnet, DKFZ-Heidelberg) and optimized by eye (Feng and Doolittle 1987). The suitability of the comparison of the 304-bp fragments for phylogenetic studies was calculated by the method of Hillis and Huelsenbeck (1992) on the basis of the distribution of 1000 randomly computed cladogram lengths. The g_1 value was taken from the table constructed by the same authors. The alignment was analyzed by maximum parsimony (Swofford 1993) and the Fitch–Margoliash method (Fitch and Margoliash 1967) derived from a distance matrix (Kimura 1980), and for both algorithms branching orders were evaluated by bootstrapping (Felsenstein 1985) using PHYLIP software (Felsenstein 1988). Additionally, sequences were compared with a further program of the PHYLIP software package (Felsenstein 1988) applying the maximum-likelihood method (Felsenstein 1981; Saitou 1990). The topology of the maximum-likelihood tree was confirmed by computing the significance of the branch lengths. The distance data for each aligned sequence were tested with a nonapproximative decomposition method regarding the significance of grouping according to a distinct branching order using the splits program (Bandelt and Dress 1992) on the HUSAR computer at the DKFZ-Heidelberg. All algorithms were computed on a ConvexOS.

Results and Discussion

Amplification of cestode DNA from various cyclophyllidean families with the primer pair 60.for and 375.rev yielded a sequence of 314 bp of the 12S rRNA gene, overlapping the fragment introduced by Kocher et al. (1989). The amplified fragments of the tapeworms were about 70 bp shorter as estimated from published vertebrate fragments of this location. As these sequences were rather conserved throughout evolution, it was impossible to amplify specifically tapeworm DNA. This motivated us to construct a new pair of tapeworm specific primers.

A portion of the 314-bp fragment, which contains 304 bp, was used for comparison between the specimens. An alignment of the sequences revealed that up to 50% of the nucleotides were conserved between representatives of the Cyclophyllidea and its outgroup (Fig. 1). There was a high degree of homogeneity of nucleotide exchanges and the g_1 value (–0.576543) was negative (Hillis and Huelsenbeck 1992). According to these authors the relative proportion of mutations within the 304-bp gene fragment was sufficient to allow phylogenetic comparisons. In addition, we tested the sequence data with respect to the reliability of grouping families at distinct nodes by the splits test. In this test the splittable percentage for significant biological data should be about 70 to 100%, which was the case at the important nodes. Therefore, sequence comparison could reliably differentiate between most of the species and families. In one case even different strains of one species could be distinguished.

Using the sequences of two pseudophyllidean cestodes as an outgroup, we constructed phylogenetic trees by three methods (maximum parsimony, maximum likelihood, and Fitch–Margoliash). One thousand bootstrap replications were applied to assess the confidence of the branching order of the tree topology by the Fitch–Margoliash and maximum-parsimony methods (Figs. 2

```
M.lep  CATAGACAAAATTATTCTATAATACTTTTAAGTCCTAATTAAAAATTAA-ATTCTCATAATTTATA-ACTAT-AAATGTAACGCATGAAATCTTTTACACG
M.lin  ............C........T.............................................................................A.
M.vog  ..........C.A.......T................A.TT.C..C-T....A...G..GA...T.................................A.
E.mul  ATC.ACA..CTAAT.CAA.C.T...A...........AC.--T.CC.T..---T.C..CAATATTC....-C........A..CA...A..C...T.A.
E.gra  ATC.ACA..CTAAT..AA.C.T...A...........A..-.T..C.T..---T.C..CAACACCCT....T........A..CA...A..C...T.A.
T.ta1  ...TT..T..T.A...AA.C.T...............A.C.TC--..A...A-.T.C....A.ATACT....T..............TA......T.AA
T.ta2  ...TT....C.....GA.C.T................A..TTT--..-.G.A-.T.C..CCA.T.CCT....................TA....C.T.A.
T.mar  ...TT....T.A...AA..T...A.............A.T..T--..-...AT.T-C..CCA.C.T.T...T................TA......T.A.
T.sag  ...TA....C.A...TA.C.T................A..T..--..--GCA..T.C..C.A.A.CTT....C...............T.TA.....T.A.
T.sol  ...CA....C.A...TG...T................A..T.T.A..-.G.A-.T.C..C.A.A.CTT....C..A.........A..TA......T.A.
M.exp  ..ATA....C.A...T..C.T...AC...........C..TTTTAC....A.A...CTTA.A...AC.ATAT.............C........A.T..A.
M.pec  ..C.......C.A....C...T...A.C.......G.A..CCCTA...C.G.A.TTC.CCAA...GTA.TAT.............C...CA...AATT.A.
D.lae  ....A....T.A...T..C.T...A.C..........A..T-TT....C--CT.T.C.TG...A.C.T...C..T...............A...AA.T.A.
D.und  ....T....C.....A..C.T.....C.G........ACCT-TC.C--C--AA.TTC..AGAGA.T.T......................A.....GT...
H.dim  ........C...A.A....C.C...A.C.........A..T-T.--..C--.A.A.C..A...TA.ATT.....................A...AA.T.A.
H.sp.  ....A......A.A.T....T...A.C..........A..T-T.--..C--.A.T.C..A...TAGTTT.......A.............A...AG.T.A.
C.lob  ....A....C.A...T..C.T.....CG......T.A.C..TT.-GTCTCC.A..C..A..T--..T................A...A..CCC.T...
C.pus  ....C....C.C...G..C.T.....CG.........A....TTA-GTCT..ACA.CT.AC.C--.TT......................A..CC..T.T.
D.can  ...TA....C.A.A.TA...T...A.C..........AC...T.A..TCTA.ACACAT...AC--.TA......G...............A..C.A.T.A.
D.sp.  ...CA....T.A...TA...T...A.C..........A..-----...CC-..G.T.C..AG.TA.ACT.TATT................A.C..GG..T.
S.sol  ..CTAC...T.....TAGA.T...A.C..T.......A..----A...C-C.A.T.C.CGGC-A.TCA.TAT..GG..............A.C...A...T.

M.lep  CAGCACATAGACTTGGCTTAAATAA---ATA-TACACAAATTCAG---TTATTAAACAATAATATTAAACCAGATATACACCAACATAATAAAAGTAAAT
M.lin  ....................................................................................................
M.vog  ........................C...A............A.....C..C...G........C...................................
E.mul  .............A......C...A...C-.........C.T.C..A...C.............C........C..............A..G.......
E.gra  ....................C...A...C-.........C...A..ACC......G.......C.......................C..G...G..A
T.ta1  ........................C...C-........C...T....CC...C.G........................T..................
T.ta2  ........................A...C-.........C..........C.C...G.........................CG..............
t.mar  ...............A.......................C.T.AA..CC.....G.............T...........................A
T.sag  .............A......C...A...CC.........C.T.TACA.C.C.C...............................................
T.sol  ....................C...C...C-.........ATAC..-....C..........C.....................................
M.exp  .............A.........CTTCATA.......T..A.-..A.C......................T..................A.T......A
M.pec  .......................CCTCC--...........A.....CC....GG.......C....................ATT.......A
D.lae  ....T...................TA.CTA..........A..CTAC..--..G........CT...................A..CTT........
D.und  ........................TA---A..........A..CTA..---.G........C....................T.C.....C..A
H.dim  ................T.......TC.ATA..........AG.CTA...--..G........C.....................A.T........
H.sp.  ........................TA..TA.G........A.ACTA..---.G..............................G..ACT........
C.lob  .............A......C....TC.CCC..........A..TTAC..---............T...T..................G.......C
C.pus  .....G.......A......C........TC..........A..TCAC..---G...............T.....................GG......
D.can  .............A.......---TTA...A..........A.AATCA.T--.......C.......................A..G.......
D.sp.  .............A.......-G.TTA---A.............GCA--.C...G.............................CC..G.......
S.sol  .............A...-....-..TTA---....G.....G..CGCA--.C...G...........................G.T..G.......

M.lep  TAATAGGGGGATCATCCTTTACAACACACCTTCCCCTAATAAGAATCGCTTACTGCCAAACTATTTTAGTTAAAAAACTAAGA-AAAATTAAACAATACA
M.lin  .......C..........................................C.................................................
M.vog  .......C...C........A...................G..T..A..CG.A...................................T.....TT.ATT.
E.mul  .......T...C.........T.TG..T..........C.......A..C..........C..........C.......GA.C..CC...C.-..ATAT
E.gra  .......T...C.........T.TG..T..........C..........C..........C..........C.....C.G..C..C...TT.A..ATAC
T.ta1  ..T....C...............C..............A..........C.........T...........TTTTT......C.T.-...T.AT.GTAC
T.ta2  ..A....C...A...........C..............A....G.....C.........T...........T.TT.......C...GC..C.AT-GTAC
T.mar  .......C...A...........C..............A.......A..C..........C..........T..........T..T.AAT.CA..ATAC
T.sag  .......C...A...........C..............A.......A..C..........C..........CT........T-..T....G.T-C.TAC
T.sol  .......C...A...........CT.............A.......A..C..........C..........CT.........T..T....C.A-.ATAC
M.exp  .......C...C..............G...........GCT.....A...G.........C..........C..........C.T...AT..T....A.
M.pec  .......C...C.........G..G...........G..TG..T.....CG....G...GG..........T.......GC.T...A...T......
D.lae  ..T....C.....G......................G.CTG..T..A...G....................T..........C...T.AC..T.T..T.
D.und  ..T....C...............C...........C...TG..T......G....................C..........C.....AT..T..CTT.
H.dim  .......C.....G.......T..............G.ACG..T.....CG....................T..........T.....AT..T.TA.T.
H.sp.  ..G....C.....G.........G............G.ATG..T.T....G....................T..........A.TTTTACT.T.TAGTT
C.lob  .......C...C.........AT.T..........CG.....T..AT.CG....................--..T.......CC.C.A..T.TT.GC.C
C.pus  .......C...........................CG...G..T..AT.CG....................--.........T.....ATTCT..CCA.
D.can  ..T..A.C...C.C..T.....TTG...........G.ACG..T..A...G.........C..........................A...G.A...ACTA.AT
D.sp.  .......C.....G.......T..............G...G..G.AA..C.............A.......T.........TT...T.A..TG.T..AT
S.sol  ....G..C.....G.......T..............G...G....AA..C.....................T.........TA.T.T.AC.TAG-CTAG
```

Fig. 1. Alignment of a 304-bp fragment from the mitochondrial 12S rRNA gene of 21 examined tapeworms of eight families. The two lowest sequences are Pseudophyllideans, which are used as outgroup.

and 3). The Fitch–Margoliash method is based on a distance matrix (Table 2). The significance of all branches by the maximum-likelihood method (Fig. 4) was at the 95% confidence level or above.

Our study shows that all taxa were clearly monophyletic within their families except the two genera of the Anoplocephalidae and *Diploposthe laevis* (Hymenolepididae), with lower bootstrap values of about 60 to 70% only. In spite of these relatively low bootstrap values within the Hymenolepididae, as shown at all nodes in the three algorithms, bootstrap values of about 74 to 90% indicate that the Hymenolepididae including *Diploposthe laevis* and *Dilepis undula* (Dilepididae) form a monophyletic group. Even the two most parsimonious trees

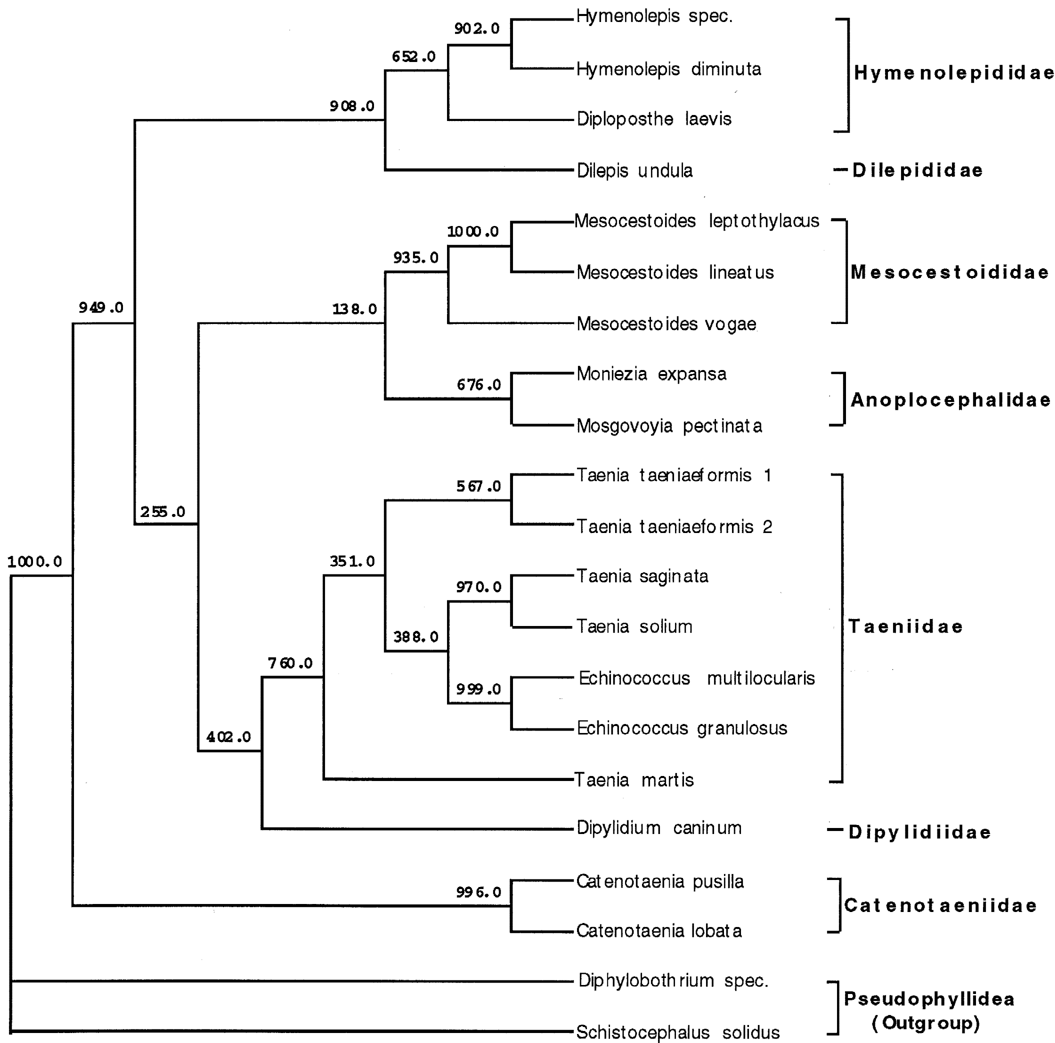

Fig. 2. Phylogenetic tree of 21 12S rDNA sequences of tapeworms inferred by the Fitch–Margoliash method using heuristic search with stepwise-addition bootstrapping and 1000 replications. Numbers at nodes represent the percentage of bootstrap replications.

found by maximum parsimony, which are different only in changing the positions of *D. laevis* and *D. undula,* could alter those findings.

Besides this group there are three other clear monophyletic groups, the Taeniidae, the Catenotaeniidae, and the Mesocestoididae. A nonapproximative decomposition testing (splits) of all the distance data resulted in a significance of grouping of about 99% for each family. Regarding the bootstrap values (72–76%) for Taeniidae there is an increase in evidence with those splits values. Our data on the Taeniidae support the data of Gasser et al. (1995), based on restriction analysis of PCR products.

The dendrograms calculated by the maximum-parsimony and Fitch–Margoliash algorithms show that the Taeniidae, Mesocestoididae, Dipylidiidae, and Anoplocephalidae derived from the same origin. In contrast, the maximum-likelihood calculation derived the Anoplocephalidae from the same node as the Hymenolepididae–Dilepididae complex.

Furthermore, the Catenotaeniidae represented a sister group juxtaposed to the remaining families. All three algorithms form the same tree topology, with the Catenotaeniidae separated from remaining species at the first node.

Using the maximum-parsimony method the hymenolepidid–dilepidid complex derived from the same node as the Mesocestoididae when all 19 cyclophyllidean sequences were used for calculation. In contrast, deletion of the uncertain Anoplocephalidae and Dipylidium sequences (bootstrap values between 13 and 45%) resulted in a separation of the hymenolepidid–dilepidid complex versus a monophyletic group formed by the Taeniidae and Mesocestoididae at the second node. The remaining two algorithms yielded the same topology at this node. The uncertain position of the anoplocephalid and dipylidiid cestodes within the branching orders of all three used algorithms could be due to the absence of more closely related taxa in our sample. Nevertheless, our dendrograms suggest a close phylogenetic relationship between the Taeniidae and the Mesocestoididae, and that

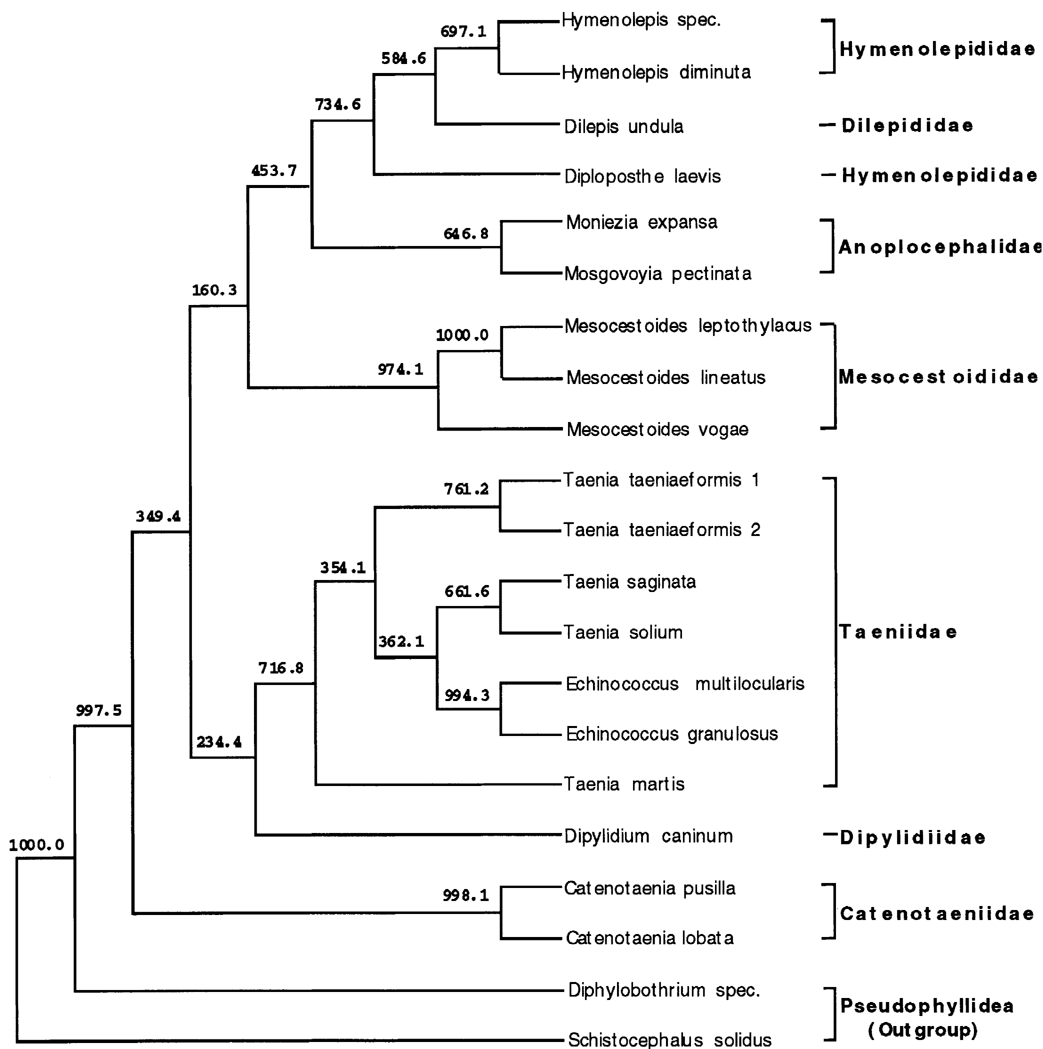

Fig. 3. Phylogenetic tree of 21 12S rDNA sequences of tapeworms inferred by the maximum-parsimony method using bootstrapping and 1000 replications. Numbers at nodes represent the percentage of bootstrap replications.

all other examined taxa derive prior to them. The low bootstrap values and the inconsistency of branching in the above-mentioned groups were due to the uncertain positions of the Anoplocephalidae and of the representative of the Dipylidiidae. The bootstrap values by maximum parsimony for a common node of Taeniidae and Mesocestoididae were less than 35% (25% for Fitch–Margoliash). These values could not be verified by the splits program. In the absence of the Anoplocephalidae and Dipylidiidae, the splittable percentage of a tree structure, which branched the Taeniidae and the Mesocestoididae from the same node, was 72%. This indicates less than 30% of noise in the original distance data. All alternative branchings for the Mesocestoididae or Taeniidae tested by the splits program achieve only 30–50% for the tree-like part of the data. Additionally, the above-mentioned reduction of the data (omission of the Anoplocephalidae and Dipylidiidae) calculated with 1000 bootstrap replications resulted in a branching percentage of between 71 and 75% for the Taeniidae–Mesocestoididae node depending on the algorithm used (maximum parsimony or Fitch–Margoliash). Nevertheless, we have to observe these bootstrap values in further studies, which must compare most of the cyclophyllidean families to confirm such uncertain positions.

All our phylogenetic trees correspond principally to the systematic classification of the Cyclophyllidea by Khalil et al. (1994), which is based on criteria of morphology and biology (Table 3) but, besides taxonomy, does not elucidate the phylogenetic relationship between the families.

The tested branching orders, which separate Catenotaeniidae at the first node and the hymenolepidid–dilepidid complex at the second, yielded a splittable percentage of more than 80 for each node in splits decomposition testing (bootstrapping: more than 90%). The early separations derived at the first and the second nodes of the trees of the monophyletic groups described above reflect the phylogenetic positions of their hosts. These hosts represent phylogenetically old vertebrates

Table 2. Distance matrix as an example calculated by the Kimura two-parameter model of 21 12S rDNA sequences from tapeworms: mean distances (adjusted for missing data)[a]

	E.mul	E.gra	M.lep	M.lin	M.vog	T.ta1	T.ta2	T.mar	T.sag	T.sol	M.exp	M.pec	D.lae	D.und	H.dim	H.sp.	C.lob	C.pus	D.can	D.sp.	S.sol
E.mul	0.0000																				
E.gra	0.1039	0.0000																			
M.lep	0.3137	0.3186	0.0000																		
M.lin	0.2950	0.2998	0.0175	0.0000																	
M.vog	0.3103	0.3443	0.1415	0.1237	0.0000																
T.ta1	0.3393	0.3055	0.2340	0.2122	0.2567	0.0000															
T.ta2	0.2919	0.2761	0.2292	0.2128	0.2536	0.1361	0.0000														
T.mar	0.2726	0.2381	0.2075	0.1871	0.2218	0.1761	0.1786	0.0000													
T.sag	0.2440	0.2691	0.2250	0.2043	0.2454	0.1848	0.1369	0.1508	0.0000												
T.sol	0.2228	0.2320	0.2045	0.1841	0.2078	0.1782	0.1449	0.1334	0.0661	0.0000											
M.exp	0.3478	0.3450	0.2378	0.2262	0.2758	0.3223	0.3388	0.2680	0.2739	0.2925	0.0000										
M.pec	0.4116	0.3751	0.2851	0.2622	0.2661	0.3420	0.3569	0.2908	0.3388	0.3253	0.2199	0.0000									
D.lae	0.3677	0.3602	0.2308	0.2193	0.2304	0.2881	0.2565	0.2665	0.2704	0.2661	0.2558	0.2419	0.0000								
D.und	0.3554	0.3378	0.2292	0.2335	0.2072	0.2633	0.2326	0.2321	0.2789	0.2669	0.2641	0.2889	0.1966	0.0000							
H.dim	0.3497	0.3537	0.1981	0.1826	0.2089	0.2881	0.2669	0.2520	0.2673	0.2495	0.2505	0.2291	0.1394	0.2057	0.0000						
H.sp.	0.3674	0.3856	0.2523	0.2401	0.2621	0.3080	0.2867	0.2709	0.2868	0.2550	0.2881	0.2684	0.1435	0.2328	0.1250	0.0000					
C.lob	0.3290	0.3111	0.2786	0.2676	0.2450	0.3035	0.2916	0.3231	0.2745	0.2713	0.3699	0.3723	0.2610	0.2764	0.2885	0.3118	0.0000				
C.pus	0.3535	0.3385	0.2568	0.2363	0.2523	0.3146	0.2987	0.2673	0.2633	0.2661	0.2916	0.3263	0.2511	0.2257	0.2101	0.2590	0.1826	0.0000			
D.can	0.3325	0.3725	0.3137	0.2998	0.2872	0.3573	0.3197	0.2916	0.3053	0.2770	0.3349	0.3460	0.2773	0.3385	0.2450	0.2334	0.3535	0.2785	0.0000		
D.sp.	0.3609	0.3717	0.2358	0.2204	0.2693	0.2966	0.2734	0.2501	0.2688	0.2841	0.2989	0.3008	0.2107	0.2942	0.2134	0.2101	0.3282	0.2637	0.2933	0.0000	
S.sol	0.3914	0.4151	0.2957	0.2840	0.3151	0.3182	0.3059	0.2894	0.2867	0.3160	0.3260	0.3157	0.2566	0.3463	0.2899	0.2657	0.4076	0.3079	0.3348	0.1545	0.0000

[a] Calculations by other models yielded the same tree topology as the Fitch–Margoliash algorithm or already used in an alternative algorithm (maximum likelihood).

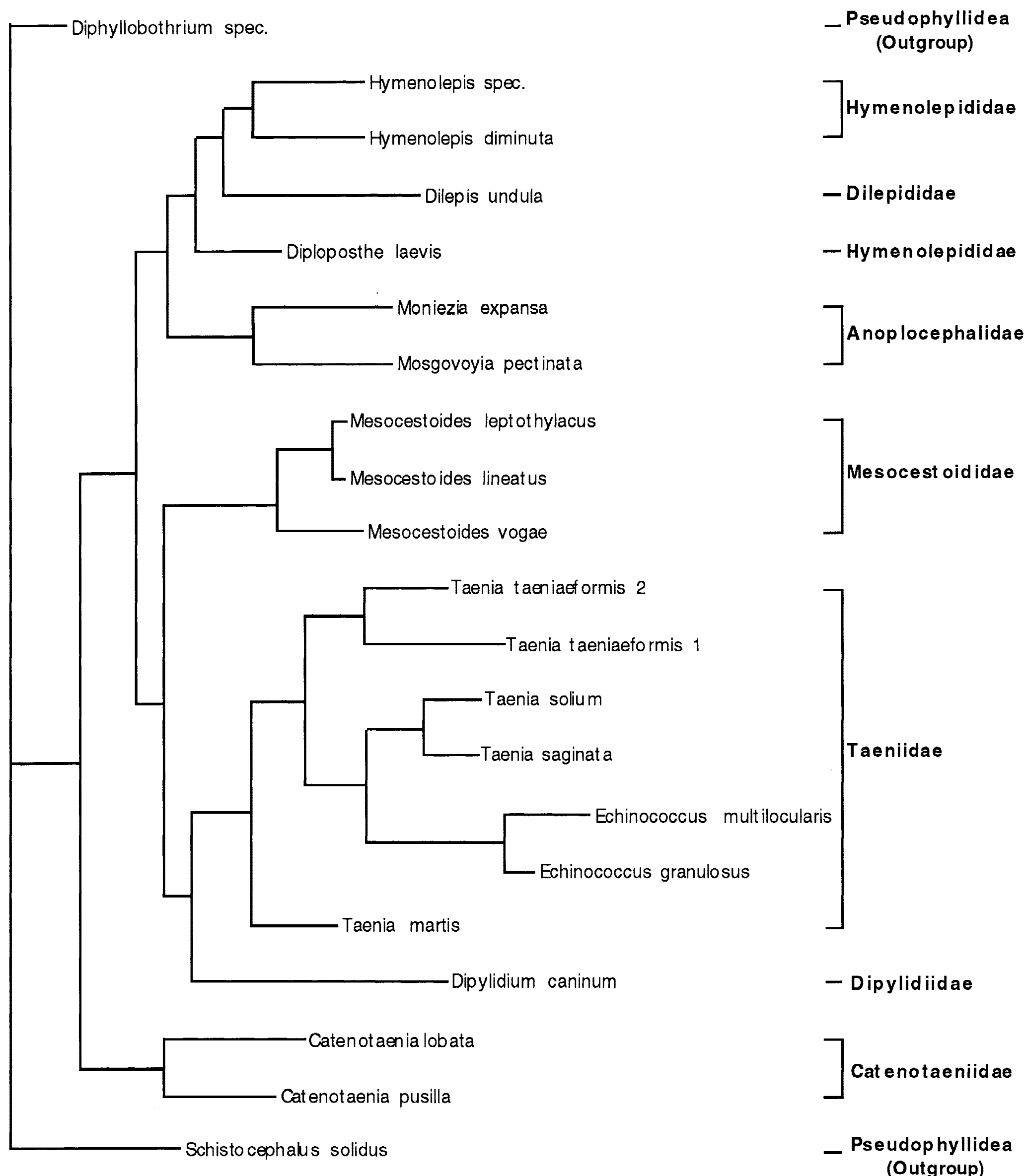

Fig. 4. Phylogenetic tree of 21 12S rDNA sequences of tapeworms inferred by the maximum-likelihood method.

such as birds and rodents (early mammals). Without exception, the remaining taxa at the ends of trees are parasites of carnivores, i.e., phylogenetically younger mammals.

This adds completely new information to the discussion on the systematic position of the genus *Mesocestoides*. Its morphology shows some controversial traits: the genital pore is median, scolex armature and rostellum are lacking, the ovary and vitellarium each consist of two compact masses, and a paruterine organ develops at the posterior end of the tube-like uterus. Wardle et al. (1974) erect an order Mesocestoididea between the Trypanorhyncha and the Tetrabothriidea, while Schmidt (1986) and Khalil et al. (1994) raise the two known genera (*Mesocestoides* and *Mesogyna*) to family rank, with an uncertain systematic relationship within the Cyclophyllidea. Brooks et al. (1991) tentatively suggest a common origin of the Mesocestoididae and the Taeniidae on the basis of tegumental "hairs" of their metacestodes, in contrast to cysticercoids of other cyclophyllideans, having "series of fibrous layers instead." As apparently hairs (microtriches?) on metacestodes were also described in certain proteocephalideans, Brooks et al. (1991) discuss the possibility of the Taeniidae and Mesocestoididae having one monophyletic group within the Cyclophyllidea having paraphyletic origins from various groups of the Proteocephalidea as common ancestors, admitting that this is only one possible phylogenetic development already postulated (Freeman 1973, Brooks 1978). On the other hand, the presence or absence of a primary lacuna in the metacestode is considered by Brooks et al. (1991) as a character which separates the Taeniidae from the Mesocestoididae. A further suggestion of the authors is that if the primary lacuna should be plesiomorphic for all

Table 3. Current classification of the order Cyclophyllidea, consisting of 15 valid families: the examined species (in *italics*) and their main hosts (in parentheses)

Mesocestoididae (carnivores, birds)
Mesocestoides leptothylacus, M. lineatus, M. vogeae
Nematotaeniidae (frogs, toads)
Dioecocestidae (seabirds, grebes)
Pogynotaeniidae (seabirds, flamingoes)
Taeniidae (carnivores, man)
Echinococcus multilocularis, E. granulosus, Taenia martis, T. saginata, T. solium, T. taeniaeformis
Amabiliidae (flamingoes)
Acoleidae (seabirds, rails)
Catenotaeniidae (rodents)
Catenotaenia lobata, C. pusilla
Davaineidae (birds)
Hymenolepididae (birds and mammals)
Hymenolepis diminuta, Hymenolepis sp., Diploposthe laevis
Dilepididae (birds, marsupials)
Dilepis undula
Dipylidiidae (carnivores)
Dipylidium caninum
Paruterinidae (birds)
Metadilepididae (birds)
Anoplocephalidae (primates, rodents, ruminants, kangaroos)
Moniezia expansa, Mosgovoyia pectinata

eucestodes, the Taeniidae would be members at the basis of the cyclophyllidea, and if it is synapomorphic for some cyclophyllideans only, the taeniids would be a highly evolved group, whereas families with paruterine organs (Mesocestoididae) would hold a position at the base of the order. The monophyletic origin of both the latter families resulting from our phylogenetic trees sustains the theory of Freeman (1973), which places both families in close relationship to each other. This suggests that there are also parallels in their biology. The somewhat uncertain positions of the Anoplocephalidae, the Dipylidiidae, and even the Mesocestoididae (see above) require further investigations, which should include more cyclophylidean families and representatives of the order Proteocephalidea.

In addition, the entire life cycle of *Mesocestoides* is not yet known. The number of intermediate hosts remains enigmatic and the mode of infection is not clear. Due to this lack of information on morphological and biological characters, systematic classification has been impossible so far. Since the publication by Soldatova (1944) oribatid mites are believed to be the first intermediate hosts. This finding, however, could not be reproduced in our laboratory and by a number of other investigators despite intensive studies. Attempts to infect any invertebrates and to prove their role as first intermediate hosts always failed. Nor has anyone succeeded in infecting vertebrate intermediate hosts directly with eggs or whole paruterine organs of a *Mesocestoides* species. Tetrathyridia, the metacestodes of the genus, are found in a vast number of vertebrates, from amphibians to mammals, but it is not known how they acquire larvae. We found that the oncospheres of *Mesocestoides leptothylacus* could be activated under conditions corresponding to mammalian intestines (unpublished data). Together with our genetically based findings, this supports the notion of a single mammalian intermediate host as in the taeniids. Another parallel trait in the biology of the Taeniidae and Mesocestoididae which points to a related life cycle is the fact that both have primarily carnivores as definitive hosts (the taeniid species of human and the two known *Mesocestoides* species of birds being exceptions).

Our dendrograms demonstrate a small genetic difference of two *Mesocestoides* species in Germany, in contrast to other authors, who assure a single species only. Since a large number of species from all over the world are called *M. lineatus,* without any proof for this designation, a well-defined species from foxes in southern Germany was described as *Mesocestoides leptothylacus* (Loos-Frank 1980). It has a long, slender cirrus pouch with a straight cirrus. Another species, with a round–oval cirrus pouch and a highly coiled cirrus, also exists. This is called *M. lineatus* (Priemer 1983) and was isolated from foxes in eastern Germany. Although molecular data do not clearly separate two species, we tend to support the existence of two species, *Mesocestoides lineatus* and *M. leptothylacus,* on the basis of clear morphological differences.

The American species *M. corti,* isolated as a larval stage from *Sceloporus occidentalis* by Specht and Voge (1965) was renamed *M. vogae* by Etges (1991) for several reasons. This species is separated in all three phylogentic trees from the other *Mesocestoides* species, which might reflect the unique ability of its metacestodes to reproduce asexually. Nevertheless, its position within the family is undoubtly supported by our data.

Interestingly there is an immediate neighborhood of *Diploposte laevis,* the former usually considered a member of the Acoleidae, with the Hymenolepididae, which sustains the rearrangement (Khalil et al. 1994) within these families. Furthermore, all three methods strongly separate *Dipylidium caninum* (Dipylidiidae) from the Dilepididae, formerly the only family. This supports the view of several authors, among them Khalil et al. (1994), who recognize a family Dipylidiidae on morphological and biological grounds. They are parasites of carnivores and their intermediate hosts are amphibians, reptiles, and arthropods, respectively.

The high degree of sequence variation (13%) found between isolates of *T. taeniaeformis* from Germany and from Egypt supports recent results on the diversity of this species. Intraspecific variation of isoenzymes (Okamoto et al. 1995) and differences in the morphology, infectivity, protein composition, and restriction length polymorphisms (Azuma et al. 1995) within *T. taeniaeformis* were shown to exist. The phylogenetic study on taeniids by Okamoto et al. (1995) compares five isolates of *T. taeniaeformis,* of which up to 9.5% of the nucleotides of the

cytochrome *c* oxidase subunit I differ, and suggest that one of the isolates represents a separate species.

In contrast to *T. taeniaeformis,* the 22 isolates of *E. multilocularis* in our study showed a high degree of sequence conservation. Only one transition (T → C) was found in the 304 base pairs studied, which was independent of the geographic origin and of the intermediate host species from which the metacestodes were isolated. This result is in line with the data of Okamoto et al. (1995), who did not find sequence differences among six isolates of *E. multilocularis,* regardless of their geographical origin or intermediate host. Similarly, Bowles et al. (1992) found only two nucleotide exchanges within 366 base pairs when they compared two isolates of *E. multilocularis.*

This genetic homogeneity of *E. multilocularis* is in marked contrast to *E. granulosus,* where a variety of strains with up to 9.3% sequence variation between different isolates exists (Bowles et al. 1992). This suggests that *E. multilocularis* is a relatively young species which has not yet diversified to the degree that other cyclophyllidean species have. To detect genetic differences between isolates of *E. multilocularis,* methods with a higher sensitivity, as, e.g., study of the variable number of tandem repeats (VNTR), will probably be of more value.

Our study demonstrates that the 12S rRNA gene is a useful tool to differentiate between taxa of helminths by PCR methods, provided primers and PCR conditions allow specific amplification of the parasite, and not of the host DNA. The primer pair developed for our study allows such specific amplification of the DNA of cestodes and trematodes (data not shown). The gene fragment used here was employed in other phylogenetic studies on various taxonomic groups as distant as monkeys (van der Kuyl et al. 1995) and onychophores (Ballard et al. 1992). Therefore, in the context of parasitology, this gene fragment could open the possibility to analyze the phylogenetic relationship of hosts and their parasites in parallel, in order to characterize the coevolution between these organisms.

Acknowledgments. This study was supported by grants from the Grimminger-Stiftung für Zoonoseforschung and the Deutsche Forschungsgemeinschaft.

References

Anderson S, Bankier AT, Barrell BG, et al. (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–465

Avise JC (1994) Molecular markers, natural history and evolution. Chapman and Hall, New York

Azuma H, Okamoto H, Oku Y, Kamiya M (1995) Intraspecific variation of *Taenia taeniaeformis* as determined by various criteria. Parasitol Res 81:103–108

Bachmann B, Lüke W, Hunsmann G (1990) Improvement of PCR amplified DNA sequencing with the aid of detergents. Nucleic Acids Res 18:1309

Ballard JW, Olsen GJ, Faith DP, Odgers WA, Rowell DM, Atkinson PW (1992) Evidence from 12S ribosomal RNA sequences that Onychophorans are modified arthropods. Science 258:1345–1348

Bandelt HP, Dress AWM (1992) Split decomposition: A new and useful approach to phylogenetic analysis of distance data. Mol Phylogenet Evol 1:242–252

Bowles J., D. Blair and D. P. McManus (1992) Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol. Biochem. Parasitol. 54:165–174

Brooks DR (1978) Evolutionary history of the cestode order Proteocephalidea. Syst Zool 27:312–323

Brooks DR, Hoberg EP, Weekes PJ (1991) Preliminary phylogenetic systematic analysis of the major lineages of the Eucestoda (Platyhelminthes: Cercomeria). Proc Biol Soc Wash 104:651–668

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: Tempo and mode of evolution. J Mol Evol 18:225–239

Casanova JL, Pannetier C, Jaulin C, Konvilsky P (1990) Optimal conditions for direct sequencing double-stranded PCR products with sequenase. Nucleic Acids Res 18:4028

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequences analysis programs for the VAX. Nucleic Acids Res 12: 387–395

Etges FJ (1991) The proliferative tetrathyridium of *Mesocestoides vogae* sp. n. (Cestoda). J Helminthol Soc Wash 58:181–185

Felsenstein J (1981) Evolutionary trees from DNA sequences: A maximum likelihood approach. J Mol Evol 17:368–376

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791

Felsenstein J (1988) Phylogenies from molecular sequences: Inference and reliability. Annu Rev Genet 22:521–565

Feng D-F, Doolittle RF (1987) Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J Mol Evol 25:351–360

Fitch WM, Margoliash E (1967) Construction of phylogenetic trees. Science 15:279–284

Freeman RS (1973) Ontogeny of cestodes and its bearing on their phylogeny and systematics. Adv Parasitol 11:481–517

Gasser RB, Chilton NB (1995) Characterisation of taeniid cestode species by PCR-RFLP of IST2 ribosomal DNA. Acta Tropica 59:31–40

Higgins DG, Sharp PM (1988) CLUSTAL: A package for performing multiple sequence alignments on a microcomputer. Gene 73:237–244

Hillis DM, Huelsenbeck JP (1992) Signal, noise and reliability in molecular phylogenetic analysis. J Hered 83:189–195

Khalil LF, Jones A, Bray RA (1994) Keys to the cestode parasites of vertebrates. CAB International, Wallingford, UK/University Press, Cambridge

Kimura M (1980) A simple method for estimating evolutionary rate of bases substitution through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kocher TD, Thomas WK, Meyer A, et al. (1989) Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 86:6196–6200

Loos-Frank B (1980) *Mesocestoides leptothylacus* n. sp. und das nomenklatorische Problem der Gattung *Mesocestoides* Vaillant, 1863 (Cestoda, Mesocestoididae). Tropenmed Parasit 31:2–14

Mariaux J (1996) Cestode systematics: Any progress? Int J Parasitol 26(3):231–243

Mead DA, Pey NK, Herrnstadt C, Marcil RA, Smith LM (1991) A universal method for the direct cloning of PCR amplified nucleic acid. Biotechnology 9:657–663

Okamoto M, Besso Y, Kamiya M, Kurosawa T, Horii T (1995) Phylogenetic relationships within *Taenia taeniaeformis* variants and other taeniid cestodes inferred from the nucleotide sequence of the cytochrome c oxidase subunit I gene. Parasitol Res 81:451–458

Priemer J (1983) On the problem of European *Mesocestoides* species (Cestoda) from mammals. Helminthologica 20:89–95

Saiki RK, Gelfand HD, Stoffel S, et al. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Saitou N (1990) Maximum likelihood methods. Methods Enzymol 183: 584–598

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schliewen UK, Tautz D, Pääbo S (1994) Sympatric speciation suggested by monophyly of crater lake cichlids. Nature 368:629–632

Schmidt GD (1986) Handbook of tapeworm identification. CRC Press, Boca Raton, FL

Soldatova AP (1944) A contribution to the study of the development cycle in the cestode *Mesocestoides lineatus* (Goetze, 1782), parasite of carnivorous mammals. CR (Dokl) Acad Sci URSS 45:310–312

Specht D, Voge M (1965) Asexual multiplication of *Mesocestoides* tetrathyridium in laboratory animals. J Parasitol 51:268–272

Swofford DL (1993) PAUP: Phylogenetic analysis using parsimony, version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign

van der Kuyl AC, Kuiken CL, Dekker JT, Goudsmit J (1995) Phylogeny of African monkeys based upon mitochondrial 12S rRNA sequences. J Mol Evol 40:173–180

Vogelstein B, Gillespie D (1979) Preparative and analytical purification of DNA from agarose. Proc Natl Acad Sci USA 76:615–619

Wardle RA, McLeod JA, Radinovsky S (1974) Advances in the zoology of tapeworms 1950–1970. University of Minnesota Press, Minneapolis