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Molecular phylogeny and systematics of anoplocephaline cestodes in rodents and lagomorphs

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Abstract

A molecular phylogenetic hypothesis is presented for the anoplocephaline cestodes of placental mammals based on sequence data from the mitochondrial cytochrome *c* oxidase I (COI) gene, the nuclear-encoded 28S rRNA gene and the internal transcribed spacer region I of rRNA (ITS1). The material consists of 35 species representing nine genera of cestodes, with emphasis on taxa parasitising rodents and lagomorphs in the Holarctic region. The resulting phylogenies show considerable disagreement with earlier systematic and phylogenetic hypotheses derived from morphology. Specifically, the results contradict the view of uterine morphology being the primary determinant of deeper phylogenetic splits within Anoplocephalinae. Also, the role of genital duplication as a means of generic divergence was not found to follow consistently the pattern suggested by earlier hypotheses. Colonisation of novel host lineages has evidently been the predominant mode of diversification in anoplocephaline cestodes of placental mammals; evidence for phyletic co-evolution was obscure. The phylogenies consistently distinguished a large monophyletic group including all species from arvicoline rodents (voles and lemmings), primarily representing the genera *Anoplocephaloides* Baer, 1923 and *Paranoplocephala* Lühe, 1910. Phylogenetic relationships within the "arvicoline clade" of cestodes were generally poorly resolved. Consistent support for nodes above and below the unresolved polytomy indicates a rapid radiation involving a nearly simultaneous diversification of many lineages, a scenario also proposed for the arvicoline hosts.

Introduction

The anoplocephaline cestodes (order Cyclophyllidea, family Anoplocephalidae) represent a diverse group of parasites infecting both terrestrial mammals (placentals and marsupials) and birds. Based on the number of genera present in these hosts, the most important radiation of anoplocephalines has been in rodents and lagomorphs (Spasskii, 1951; Beveridge, 1994). Also, in a broader phylogenetic context, terrestrial mammals are recognised as the basal hosts for cyclophyllidean diversification (Hoberg et al., 1999).

The subfamily Anoplocephalinae Blanchard, 1891 is separated from the other subfamilies of the Anoplocephalidae (*sensu* Beveridge, 1994) (i.e. Thysanosomatinae Skrjabin, 1933, Linstowiinae Fuhrmann, 1907 and Inermicapsiferinae López-Neyra, 1943) by a saccate uterus that persists in gravid proglottides. However, Beveridge (1994) and Hoberg et al. (1999) have provided morphological evidence for the non-monophyly of the Anoplocephalidae, with the Anoplocephalinae and the Thysanosomatinae possibly forming a monophyletic lineage separate from Linstowiinae and Inermicapsiferinae.

Phylogenetic schemes for anoplocephalid cestodes (in various senses) have been proposed by Baer (1927), Spasskii (1951), Tenora (1976) and Beveridge (1994), but none of these have relied on formal methods of phylogeny construction. Spasskii (1951) incorporated the patterns of uterine development in the systematic arrangement of

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Anoplocephalidae (*sensu* Spasskii, 1951) by distinguishing the subfamily Anoplocephalinae with a tubular early uterus and the subfamily Monieziinae Spasskii, 1951 with a reticulated early uterus. This arrangement was later adopted by Tenora (1976), but not by Yamaguti (1959), Schmidt (1986) and Beveridge (1994).

The best substantiated phylogenetic hypothesis for Anoplocephalinae (plus the Thysanosomatinae) is that of Beveridge (1994), who based his view on selected morphological features, uterine morphology being the primary determinant of the deeper phylogenetic splits. Beveridge (1994) also emphasised the importance of genital duplication, previously proposed by Spasskii (1951), Baer (1955) and Rausch (1980), as a mechanism of divergence within the Anoplocephalinae. These authors have indicated several pairs of genera that are separated from each other primarily by the number of genitalia per proglottis (single/double). Beveridge's (1994) hypothesis indicates a certain degree of phyletic coevolution between anoplocephalines and their hosts, but cases of host-switch between rodents, lagomorphs and other mammals are also implied.

The first molecular phylogenetic hypothesis for the anoplocephaline cestodes of placental mammals is presented here, with particular emphasis on taxa parasitising rodents and lagomorphs in the Holarctic region. The hypothesis is based on sequence data from the mitochondrial cytochrome *c* oxidase I (COI) gene, the nuclear-encoded 28S rRNA gene and the internal transcribed spacer region I of rRNA (ITS1). The resulting phylogenies are used for inferring the co-evolutionary history, systematics and character evolution in anoplocephaline cestodes.

Materials and methods

Cestodes

The material consists of 35 species representing nine genera of anoplocephaline cestodes from placental mammals (Table 1). Most of the species belong to *Paranoplocephala* Lühe, 1910 (19 species) and *Anoplocephaloides* Baer, 1923 (7 species) from arvicoline rodents (voles and lemmings, a subfamily within the Muridae). *Paranoplocephala* and *Anoplocephaloides* species included in this study parasitise arvicoline rodents of the genera *Clethrionomys* Tilesius, *Microtus* Schrank, *Chionomys* Miller, *Lemmus* Link, *Synaptomys* Baird and *Dicrostonyx* Gloger. In both cestode genera there are several unnamed species, whose independent status has been confirmed primarily by molecular criteria (Haukisalmi et al., 2004; Wickström, 2004); these more or less cryptic species have been indicated by Roman numerals in Table 1 and Figures 11–14. For example, *Paranoplocephala* cf. *omphalodes* I, II and III have been separated from the true *P. omphalodes* (Hermann, 1783) by COI sequences (Haukisalmi et al., 2004). *Paranoplocephala* spp., as presently conceived (Rausch, 1976; Tenora et al., 1986; Genov et al., 1996), are known only from rodents (also including non-arvicoline species), whereas *Anoplocephaloides* (*sensu* Rausch, 1976 and Beveridge, 1994) includes species from various rodents, lagomorphs and perissodactyls. A single species of *Anoplocephaloides* from a non-rodent host, i.e. *A. mamillana* (Mehlis, 1831) from a horse, was included in the present analysis. The other examined genera are *Andrya* Railliet, 1893 (two species), *Mosgovoyia* Spasskii, 1951 (one species) and *Schizorchis* Hansen, 1948 (one species) from lagomorphs, *Diandrya* Darrah, 1930 from sciurid rodents (marmots, one species), *Monoecocestus* Beddard, 1914 from hystricognath rodents (one species), *Anoplocephala* Blanchard, 1848 from perissodactyls (horses, two species) and *Moniezia* Blanchard, 1891 from artiodactyls (ruminants, one species).

Beveridge (1994) has distinguished three main types of early uteri within the Anoplocephalinae, i.e. tubular, partly reticulated and completely reticulated ones. However, the division into partly and completely reticulated types seems to be fairly artificial, since there are several intermediate/deviating forms within *Paranoplocephala* that cannot clearly be defined as belonging to either (Figures 1–10; see Haukisalmi & Henttonen, 2001). Therefore, each of the species is classified here simply as having either a reticulated or tubular early uterus (Table 1). The number of genitalia per proglottis (single/double) is given in Table 2.

DNA extraction, PCR amplification and sequencing

For DNA extraction, see Wickström et al. (2003). Portions of the mitochondrial cytochrome *c*

Table 1. Cestode specimens included in the present analysis (in alphabetical order). The early uterus is divided into two main types, reticulated (R) and tubular (T). The arvicoline host genera *Microtus, Chionomys, Synaptomys, Lemmus, Dicrostonyx* and *Clethrionomys* abbreviated to the first letter(s), i.e. *M, Ch, S, L, D* and *Cl*, respectively. The other host genera are spelled out. Parasite specimens are numbered (when more than one specimen was examined) and GenBank accession numbers of cytochrome *c* oxidase I mtDNA (COI), 28S rRNA and ITS1 rRNA sequences are given in the corresponding order.

Species	Uterus type	Host species	No	Locality	Country	Accession No (COI, 28S, ITS1) M = missing
Andrya cuniculi	R	*Oryctolagus cuniculus*		Teneriffe	Spain	AY189957, AY569723, AF314409
A. rhopalocephala	R	*Lepus europaeus*		Hódmezövásárhely	Hungary	AY189958, AY569724, AY752647
Anoplocephala magna	T	*Equus burchellii*		Werribee, Victoria	Australia	AY568206, AY586610, M
A. perfoliata	T	*Equus caballus*		Werribee, Victoria	Australia	AY568189, AY569769, AY752646
Anoplocephaloides dentata	T	*Ch. nivalis*	1	Trento	Italy	AY568190, AY569725, M
		Ch. nivalis	2	Bourg-Saint-Maurice	France	AY568191, AY569726, M
*A.*cf. *dentata* I	T	*M. oeconomus*	1	Pallasjärvi	Finland	AY423809, AY569727, AY752640
		M. agrestis	2	Aberdeen	Scotland	AY423834, AY569728, M
*A.*cf. *dentata* II	T	*M. oeconomus*	1	Kolyma River, Siberia	Russia	AY568192, AY569729, M
		M. oeconomus	2	Northern Yukon (BCP[$])	Canada	AY568193, AY569730 AY752641
		M. oeconomus	3	GAAR[$], Alaska (BCP)	USA	AY568194, M, M
A. kontrimavichusi	T	*S. borealis*	1	Fairbanks, Alaska	USA	AY568195, AY569731, AY752642
		S. borealis	2	YUCH[#], Alaska (BCP)	USA	AY568196, AY569732, M
A. lemmi	T	*L. sibiricus*	1	Taimyr, Siberia	Russia	AY568197, AY569733, AY752643
		L. trimucronatus	2	Kolyma River, Siberia	Russia	AY568198, AY569734, M
		L. trimucronatus	3	YUCH[#], Alaska (BCP)	USA	AY568199, M, M
A. mamillana	T	*Equus caballus*			Germany	M, AY569770, M
*A.*cf. *variabilis*	T	*Ch. nivalis*	1	Trento	Italy	AY568207, AY569735, AY752644
		Ch. nivalis	2	Bourg-Saint-Maurice	France	AY568208, AY569736, M
		M. agrestis	3	Pallasjärvi	Finland	AY568209, AY569737, AY752645
		M. miurus	4	Toolik Lake, Alaska	USA	AY586611, AY586607, M
Diandrya composita	R	*Marmota caligata*	1	YUCH[#], Alaska (BCP)	USA	AY181550, AY569739, M
		Marmota broweri	2	GAAR[$], Alaska (BCP)	USA	AY568212, AY569740, M
		Marmota caligata	3	YUCH[#], Alaska (BCP)	USA	AY181551, AY569741, AY752649
Moniezia sp.	R	*Rangifer tarandus*		Paistunturi	Finland	M, M, AY752651
Monoecocestus americanus	R	*Erethizon dorsatum*		YUCH[#], Alaska (BCP)	USA	AY568184, AY569772, AY752652
Mosgovoyia pectinata	T	*Lepus timidus*	1	Häme	Finland	M, M, AY752650
		Oryctolagus cuniculus	2	North Yorkshire	England	AY568211, AY569771, AY752648
Paranoplocephala alternata	R	*D. groenlandicus*	1	Cape Krusenstern, Alaska (BCP)	USA	AY181502, AY569742, AY299551
		D. torquatus	2	Kolyma River, Siberia	Russia	AY181431, AY569743, AF314413*
P. arctica	R	*D. groenlandicus*	1	Wrangel Island	Russia	AY181505, AY569744, AF314412
		D. groenlandicus	2	Alaska	USA	AY181507, AY569745, AY752661
P. blanchardi	R	*M. agrestis*	1	Heinävesi	Finland	AY189955, AY569746, AY752653
		M. agrestis	2	Stilleryd	Sweden	AY189956, AY569747, M
P. etholeni	R	*M. pennsylvanicus*	1	Fairbanks, Alaska	USA	AY568186, AY569773, M
		M. pennsylvanicus	2	Fairbanks, Alaska	USA	AY568214, AY569774, AY752654
P. fellmani	R	*L. lemmus*	1	Finse	Norway	AY568200, AY569748, AY752655
		L. lemmus	2	Finse	Norway	AY586612, AY569749, M
P. gracilis	R	*M. agrestis*	1	Hattusaari, Pielinen	Finland	AY395633, AY569750, AY752656*
		M. agrestis	2	Kielder Forest	Scotland	AY568215, AY569751, M
P. kalelai	R	*Cl. rufocanus*	1	Kilpisjärvi	Finland	AY181512, AY569752, AY752660
		Cl. glareolus	2	Narvik	Norway	AY181513, AY569753, M
		Cl. glareolus	3	Narvik	Norway	AY189959, M, M

Table 1. Continued.

Species	Uterus type	Host species	No	Locality	Country	Accession No (COI, 28S, ITS1) M = missing
P. krebsi	R	*D. groenlandicus*	1	Wrangel Island	Russia	AY568201, AY569754, M
		D. groenlandicus	2	Victoria Island, Nunavut	Canada	AY568216, AY569755, AF314416
P. longivaginata	R	*Cl. rufocanus*	1	Kolyma River, Siberia (BCP)	Russia	AY568202, AY569756, M
		Cl. rufocanus	2	Kolyma River, Siberia (BCP)	Russia	AY568203, M, AY752657
P. macrocephala	R	*M. pennsylvanicus*	1	Fairbanks, Alaska	USA	M, AY569757, AY752658
		M. pennsylvanicus	2	YUCH[#], Alaska (BCP)	USA	AY181517, AY569758, M
		M. pennsylvanicus	3	YUCH[#], Alaska (BCP)	USA	AY181518, AY586608, M
P. nordenskioeldi	R	*D. groenlandicus*		Victoria Island, Nunavut	Canada	AY568204, AY569759 AF314411
P. oeconomi	R	*M. oeconomus*	1	Barbacs	Hungary	AY568217, AY569760, M
		M. oeconomus	2	Barbacs	Hungary	AY568205, AY569761, M
P. omphalodes	R	*M. agrestis*	1	Espoo	Finland	AY181525, AY569762, M
		M. arvalis	2	Dévaványa	Hungary	AY181536, AY569763, M
*P.*cf. *omphalodes* I	R	*M. oeconomus*	1	Pallasjärvi	Finland	AY181520, M, AY752659
		Microtus sp.	2	WRST[‡], Alaska (BCP)	USA	AY181543, AY586609, M
*P.*cf. *omphalodes* II	R	*M. oeconomus*		GAAR[$], Alaska (BCP)	USA	AY181547, M, M
*P.*cf. *omphalodes* III	R	*M. miurus*	1	GAAR[$], Alaska (BCP)	USA	AY189952, AY569764, M
		M. miurus	2	Noatak NP, Alaska (BCP)	USA	AY181541, AY569765, M
P. primordialis	R	*M. oeconomus*		Yukon (BCP)	Canada	AY568218, AY569766, AY752662
P. serrata	R	*D. torquatus*	1	Yamal, Siberia	Russia	AY568220, AY569767, M
		D. groenlandicus	2	Byron Bay, Nunavut	Canada	AY568219, AY569768, AF314414
Paranoplocephala sp.	R	*Ch. nivalis*		Bourg-Saint-Maurice	France	AY568188, M, M
Schizorchis caballeroi	T	*Ochotona collaris*		YUCH[#], Alaska (BCP)	USA	M, AY569775, AY752663

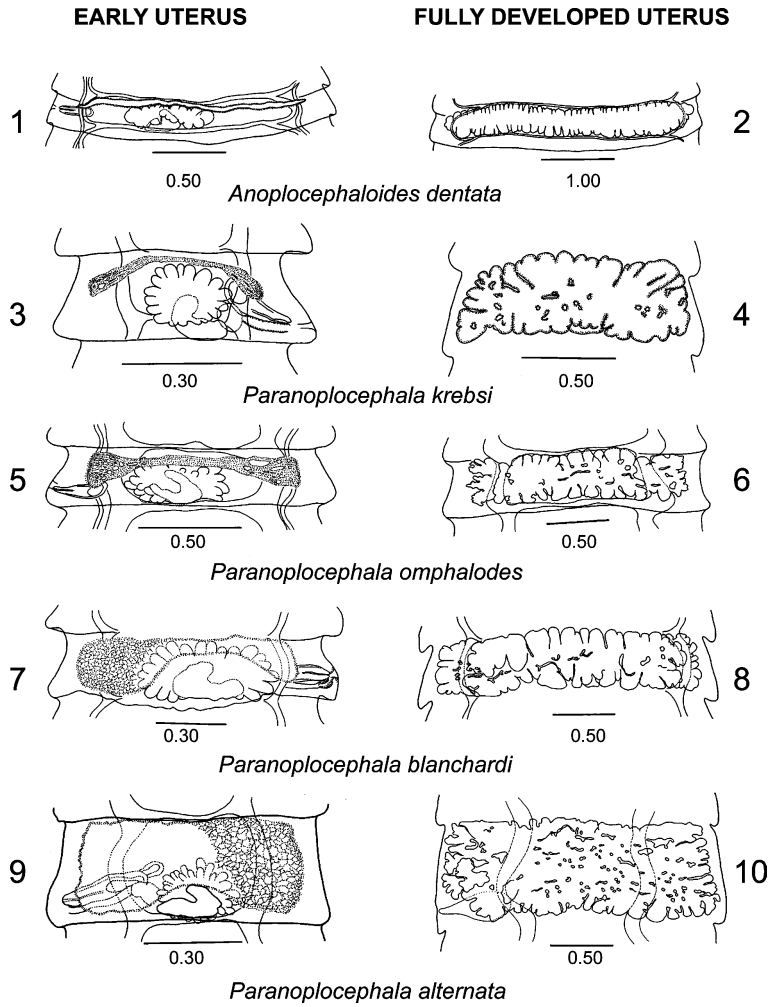
[$] Gates of the Arctic National Park and Reserve; [#] Yukon-Charley Rivers National Preserve; [‡] Wrangel-St.Elias National Park and Reserve; * ITS1 sequence is obtained from a separate individual; [§] Specimen collected in connection with the Beringian Co-evolution Project (BCP, Hoberg et al., 2003).

oxidase I (COI) gene and of 28S ribosomal RNA were amplified and sequenced from all species listed in Table 1. However, some of the species (particularly from non-arvicoline hosts) did not amplify or amplified poorly either with COI or 28S rDNA primers, and therefore these two data-sets are not completely identical with respect to species composition. ITS1 was cloned and sequenced from a smaller subset of species. Most of the species from arvicoline rodents (*Paranoplocephala* spp. and *Anoplocephaloides* spp.) are represented by at least two individuals in the COI and 28S rDNA data-sets, whereas species from non-arvicoline hosts are usually represented by one individual.

A 641 bp long fragment was amplified from COI. Amplification of DNA and sequencing methods for COI are described in Wickström et al. (2003) and Haukisalmi et al. (2004). We are confident that our sequences represent the true partial mitochondrial COI as there were no anomalies of the type commonly associated with pseudogenes (Zhang & Hewitt, 1996; Bensasson et al., 2001) and the translated protein sequences obtained matched previously published data for other cestode species (complete mitochondrial genomes of *Hymenolepis diminuta* (Rudolphi, 1819), GenBank acc. no. NC_002767 and *Echinococcus multilocularis* (Leuckart, 1863), GenBank acc.no. NC_000928). About 1400 bp of domains D1-D3 of 28S rDNA were amplified in a single reaction, as in Lockyer et al. (2003), and directly sequenced with labelled (PCR) primers from both directions, as described in Haukisalmi et al. (2004). For methodological notes on amplifying, cloning and sequencing of ITS1, see Haukisalmi et al. (2001).

Phylogenetic analyses

For the phylogenetic analysis of individual sequence data-sets, the outgroup comprised species



Figures 1–10. Early uterus and fully-developed uterus of five species of anoplocephaline cestodes from arvicoline rodents depicting the variability of uterine structures between the two extremes, tubular (1, 2) *vs* completely reticulated (9, 10). Scale-bars in mm.

from two other cyclophyllidean families, Hymenolepididae and Taeniidae. Representatives of Thysanosomatinae, the putative sister group of Anoplocephalinae, could not be obtained for sequencing. Outgroup sequences were retrieved from GenBank. *Echinococcus multilocularis* (NC_000928), *Rodentolepis nana* (Siebold, 1852) (AB033412) and *H. diminuta* (NC_002767, AF314223) were used in the COI alignment, *Rodentolepis microstoma* (Dujardin, 1845) (AF286918), *H. diminuta* (AY157181) and *Wardoides nyrocae* (Yamaguti, 1935) (AF286919) in the 28S rDNA alignment and *H. diminuta* (AF461125), *R. microstoma* (AY221167) and *Echinococcus granulosus* (Batsch, 1786) (AJ245930, AJ237773) were tested as outgroups for ITS1.

The genera *Mosgovoyia* and *Monoecocestus* were specified as outgroups for the combined sequence data-sets of COI + 28S rDNA based on their basal position in the individual data-sets. Since these analyses showed that *Andrya* spp. form the putative sister group for the terminal "arvicoline clade" (below), the former species were used as an outgroup in the combined data-sets of COI + 28S rDNA + ITS1. The latter analysis aimed at elucidating the phylogenetic relationships within the large "arvicoline clade". Taeniids and hymenolepidids were not used in the combined data-sets, as different individuals were used as outgroups in different data-sets and as they are probably too distant to function well as outgroups.

Table 2. Anoplocephaline genera parasitising placental mammals. Classification and other information are primarily from Beveridge (1994). The genera have been ordered according to their principal host groups.

Genus	Hosts	Early uterus	Genitalia
**Anoplocephaloides* Baer, 1923	rodents (murids, sciurids), perissodactyls	tubular	single
Ctenotaenia Railliet, 1893	rodents (sciurids)	tubular	double
**Diandrya* Darrah, 1930	rodents (sciurids)	reticulated	double
Gallegoides Tenora & Mas-Coma, 1978	rodents (murids)	tubular	single
**Monoecocestus* Beddard, 1914	rodents (mainly hystricognaths), peccaries	reticulated	single
**Paranoplocephala* Lühe, 1910	rodents (mainly murids)	reticulated	single
Pseudocittotaenia Tenora, 1976	rodents (geomyids)	tubular	double
Viscachataenia Denegri, Dopchiz, Elissondo & Beveridge, 2003	rodents (chinchillids)	reticulated	double
**Andrya* Railliet, 1893	lagomorphs (leporids)	reticulated	single
**Cittotaenia* Riehm, 1881	lagomorphs (leporids), rodents (chinchillids)	tubular	double
Leporidotaenia Genov, Murai, Georgiev & Harris, 1990[§]	lagomorphs (leporids)	tubular	single
**Mosgovoyia* Spasskii, 1951	lagomorphs (leporids)	tubular	double
Diuterinotaenia Gvozdev, 1961	lagomorphs (ochotonids)	tubular	double
Ectopocephalium Rausch & Ohbayashi, 1974	lagomorphs (ochotonids)	tubular	double
**Schizorchis* Hansen, 1948	lagomorphs (ochotonids)	tubular	single
Paramoniezia Maplestone & Southwell, 1923	suids, marsupials	tubular	double
Crossotaenia Mahon, 1954	ruminants	spherical	single
**Moniezia* Blanchard, 1891	ruminants, suids, rodents, primates, birds	reticulated	double
**Anoplocephala* Blanchard, 1848	perissodactyls, hyracoids, proboscids, primates	tubular	single
Flabelloskrjabinia Spasskii, 1951[§]	perissodactyls (tapirs)	tubular	single
Bertiella Stiles & Hassall, 1902	primates, rodents, dermopterans, marsupials	tubular	single

* Genus included in the present analysis. [§] Genus not recognised in Beveridge (1994).

Sequences were assembled and edited using Align IR^{TM} Sequence Assembly and Alignment Software (LI-COR Inc., Nebraska, USA) and aligned in ClustalW (Thompson et al., 1994) with default gap penalties. Further minor adjustments to improve alignments were made by eye. Regions where the alignment was ambiguous were excluded from the analyses. Sites at which an insertion affected a single taxon only were also excluded, because they were phylogenetically uninformative. Data on nucleotide substitutions and amino acid replacement were determined using MacClade version 4 (Maddison & Maddison, 2000).

Phylogenetic relationships were reconstructed using the Bayesian approach (Huelsenbeck et al., 2001) implemented in the program MrBayes v.3.0B4 (Ronquist & Huelsenbeck, 2003) and the maximum parsimony (MP) algorithm implemented in PAUP* v. 4.0b10 (Swofford, 2002). A consensus tree was constructed from combined nucleotide sequences for COI + 28S rDNA and COI + 28S rDNA + ITS1, as well as each individual data-set. The substitution model used for the combined data-sets corresponded to the general time-reversible model with gamma-distributed rate variation among sites approximated with five categories (α shape estimated). This model, GTR + γ, was the best model found for ITS1 and 28S rDNA sequences using MrModeltest v.2 (a variant of ModelTest by Posada & Crandall, 1998). The model proposed for the COI sequences additionally allowed for invariant sites (GTR + γ + I). In the Bayesian analysis, base frequencies were estimated, four chains were used (default temperature) and the starting tree was random. The analysis was run for 11 million generations with a sample frequency of 100. The first 10,000 trees were discarded, so that the final consensus was based on 100,000 trees. Support for nodes were expressed as posterior probabilities (calculated by MrBayes) and also as bootstrap support (1,000 replicates). The latter employed the NJ algorithm with maximum likelihood (ML) distances, using the substitution model found by ModelTest (GTR + γ in combined data-

sets) and parameters estimated from the MrBayes tree. Three independent runs (shortest run one million generations) were compared to confirm that the likelihood plateau represented a real optimum and not a local optimum, which might have varied between runs. For comparison, analyses were also performed using the MP algorithm in PAUP*. The parsimony analyses were carried out heuristically with 1,000 random additions, TBR swapping and MulTrees option in effect. Bootstrap analyses were conducted for 1,000 rearrangements (with 10 random additions). The results of the MP analyses are reported only if supported structure that is not evident in the Bayesian analyses is recovered.

Results

Individual sequence data-sets

The two phylogenetic methods used (Bayesian and MP) resulted in trees with similar topologies, and therefore these are not treated separately in the following account.

The phylogenetic trees based on each of the three individual data-sets (Figure 11) consistently distinguished a large monophyletic group including all species of *Anoplocephaloides* and *Paranoplocephala* from arvicoline rodents, *Diandrya composita* Darrah, 1930 from marmots and the two *Andrya* species from lagomorphs. The other, basal taxa included various combinations of species from perissodactyls (*Anoplocephala* spp., *Anoplocephaloides mamillana*), lagomorphs (*Mosgovoyia*, *Schizorchis*), ruminants (*Moniezia*) and non-arvicoline rodents (*Monoecocestus*).

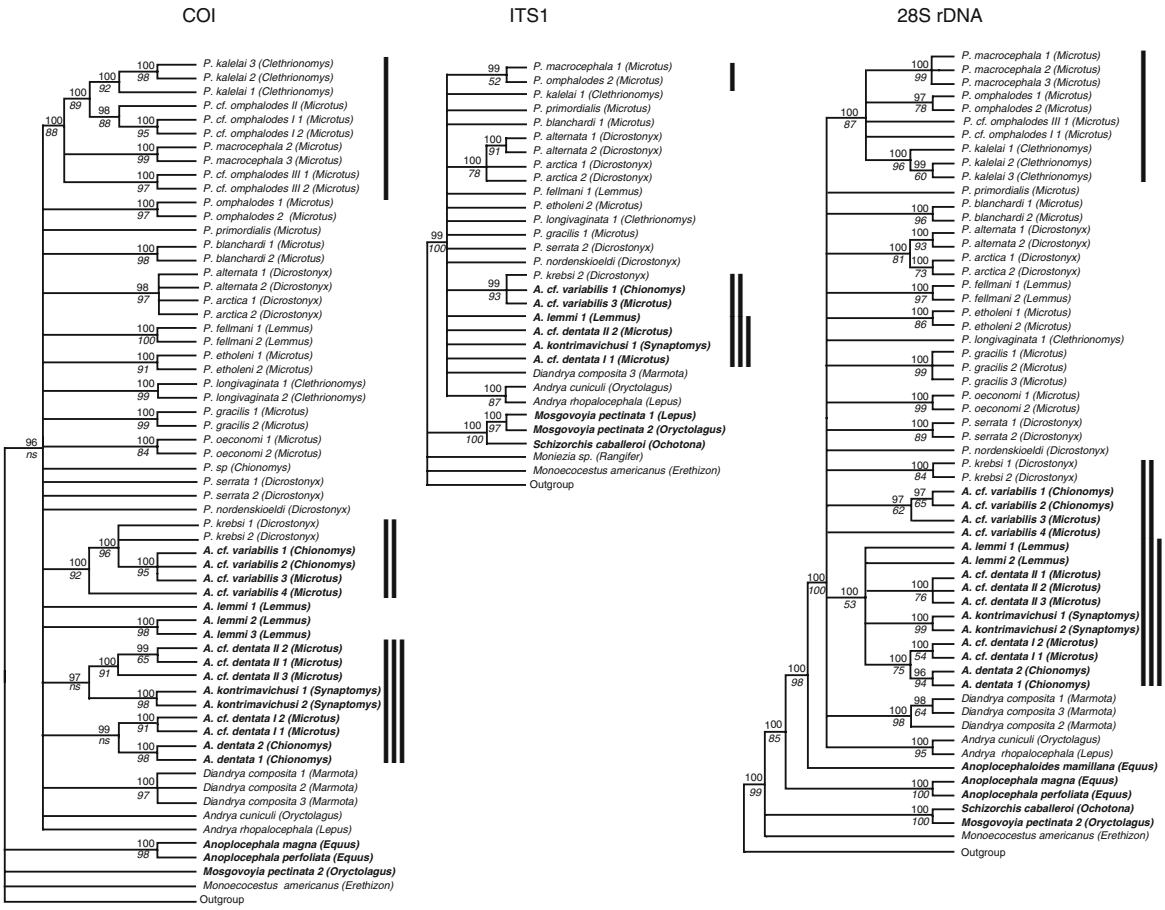
Individual data-sets showed generally poor resolution and a nearly "star-like" (pectinate) topology within the crown clade. However, there was relatively high and consistent support for three monophyletic subgroups within this clade. One of these groups (subclade I) included the earlier recognised clade consisting of four *P. omphalodes*-like species, *P. macrocephala* (Douthitt, 1915) and *P. kalelai* (Tenora, Haukisalmi & Henttonen, 1985) (i.e. *Paranoplocephala* (*sensu stricto*) of Haukisalmi et al. (2004)). The second group (subclade II) comprised *A. variabilis* (Douthitt, 1915)-like species and *P. krebsi* Haukisalmi, Wickström, Hantula & Henttonen, 2001, and the third supported group (subclade III) included *A. dentata*-like species, *A. lemmi* and *A. kontrimavichusi* Rausch, 1976 (i.e. *Anoplocephaloides* (*sensu stricto*)). *Paranoplocephala arctica* (Rausch, 1952) and *P. alternata* Haukisalmi, Wickström, Hantula & Henttonen, 2001 were also strongly associated with each other, and their conspecificity is supported by some of the molecular markers, but not by others (Wickström et al., 2003).

There was no evidence for inclusive monophyly of the *Paranoplocephala* spp. in any of the data-sets, although they tended to remain separate from *Anoplocephaloides*, with the exception of *P. krebsi*, which was closely associated with *A. variabilis*-like species. *Anoplocephaloides* was recognised as a clearly non-monophyletic assemblage, since *A. mamillana* was placed as the sister group of the crown clade (in 28S rDNA).

Combined sequence data-sets

As no conflicting branches with posterior probabilities greater than 95% were found in the individual data-sets, the sequences were combined in further analyses since this has been shown to improve phylogenetic estimation (Cunningham, 1997; Yoder et al., 2001). All three independent runs of the Bayesian analysis of COI + 28S rDNA and COI + 28S rDNA + ITS1 respectively converged on the same optimum, and the long runs of 11 million generations showed no increase in log-likelihood scores, suggesting that the trees found under a particular model were stable. The nodes with high posterior probabilities that were not present in MP topologies were not supported by bootstrap analysis. The relationship between bootstrap support and posterior probability appears to be influenced by branch length, as all short branches with high posterior probability had only low bootstrap support, suggesting that support for these nodes depends upon only a few sites and is therefore not reliable.

The combined sequence data-sets of COI + 28S rDNA, using *Mosgovoyia* and *Monoecocestus* as an outgroup, also distinguished the large, monophyletic crown group, the only difference concerning the position of *Andrya* spp., which were now placed outside this clade as its sister group (Figure 12). The monophyletic group including all *Paranoplocephala* and *Anoplocephaloides* species from arvicoline rodents (plus *D. composita*) is



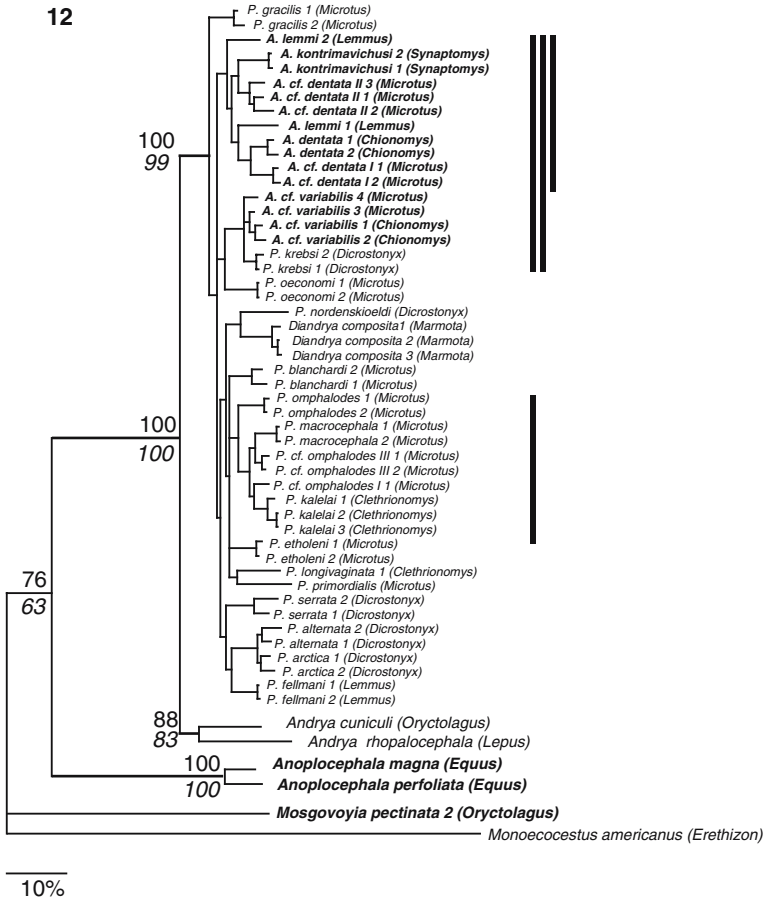
Figures 11. Phylogenetic trees for anoplocephaline cestodes produced by Bayesian analysis of individual sequence data-sets of COI (522 bp of which 204 informative), ITS1 (408 bp of which 227 informative) and 28S rDNA (1158 bp of which 223 informative). Bayesian posterior probabilities are shown above the branches. Branches with posterior probabilities $< 95\%$ have been collapsed. Bootstrap values ($> 50\%$, based on NJ with ML distances) are shown in italics below branches. Other branches have $< 50\%$ bootstrap support (ns). Species with a tubular early uterus are shown in bold, species with a reticulated early uterus in normal font. The three subclades within the 'arvicoline clade' have been indicated by vertical lines (clade I = single line; clade II = double line; clade III = triple line).

hereafter called the ''arvicoline clade''. The combined data-set also supported the non-monophyly of both *Paranoplocephala* and *Anoplocephaloides* and the existence of three monophyletic subgroups within the arvicoline clade (above). The monophyly and sister group status of *Andrya* spp. with respect to the arvicoline clade gives strong support for the separation of *Andrya* and *Paranoplocephala*, a long-standing taxonomic problem. Despite their obvious monophyly, the two *Andrya* species from leporids are genetically very different from each other (Figures 12–14).

The phylogenies based on all three sequence data-sets, using *Andrya* spp. as an outgroup, showed similar topologies in MP and Bayesian reconstructions. The clades supported in the Bayesian phylogeny (Figure 14) by both posterior probabilities and NJ bootstraps based on ML distances were the same as in the COI + 28S rDNA data-set. The combined data-set of COI + 28S rDNA + ITS1 gave further support for the three monophyletic subclades, but otherwise the resolution within the arvicoline clade remained low.

Basal relationships

Since all the basal taxa could not be combined in the same analysis and since their phylogenetic relationships are hard to trace from the individual

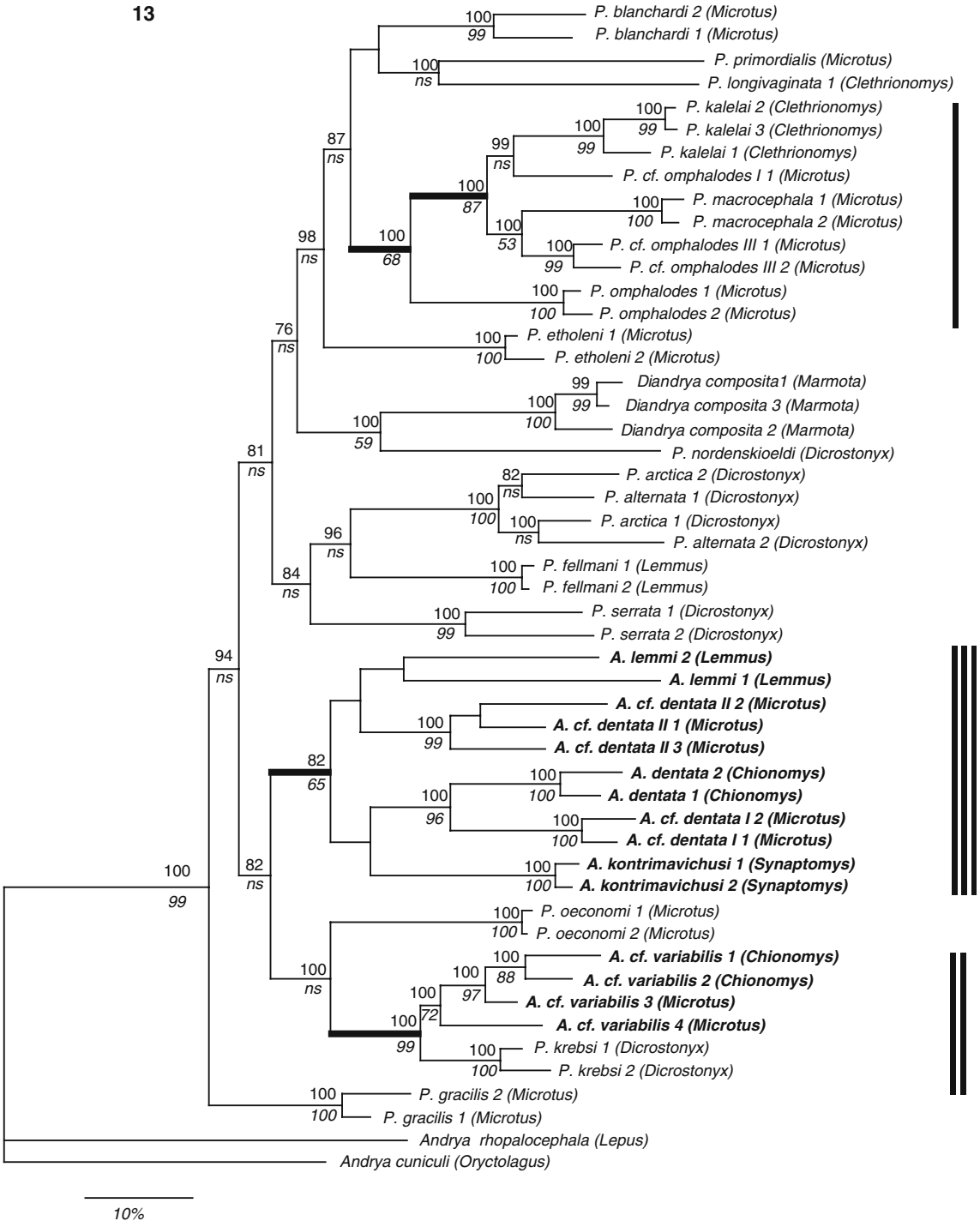


Figures 12–13. Phylogenetic trees for anoplocephaline cestodes based on combined mitochondrial COI and 28S rDNA sequences produced by Bayesian analysis implemented in MrBayes with GTR + γ distances. Posterior probabilities (> 70%) are shown above the branches; bootstrap values (> 50%, based on NJ with ML distances) are shown in italics below the branches; other branches have < 70% (Bayesian) or < 50% (bootstrap) support (ns). 12. All species. 13. The 'arvicoline clade' with *Andrya rhopalocephala* and *A. cuniculi* as outgroups. Symbols and fonts as in Figure 11.

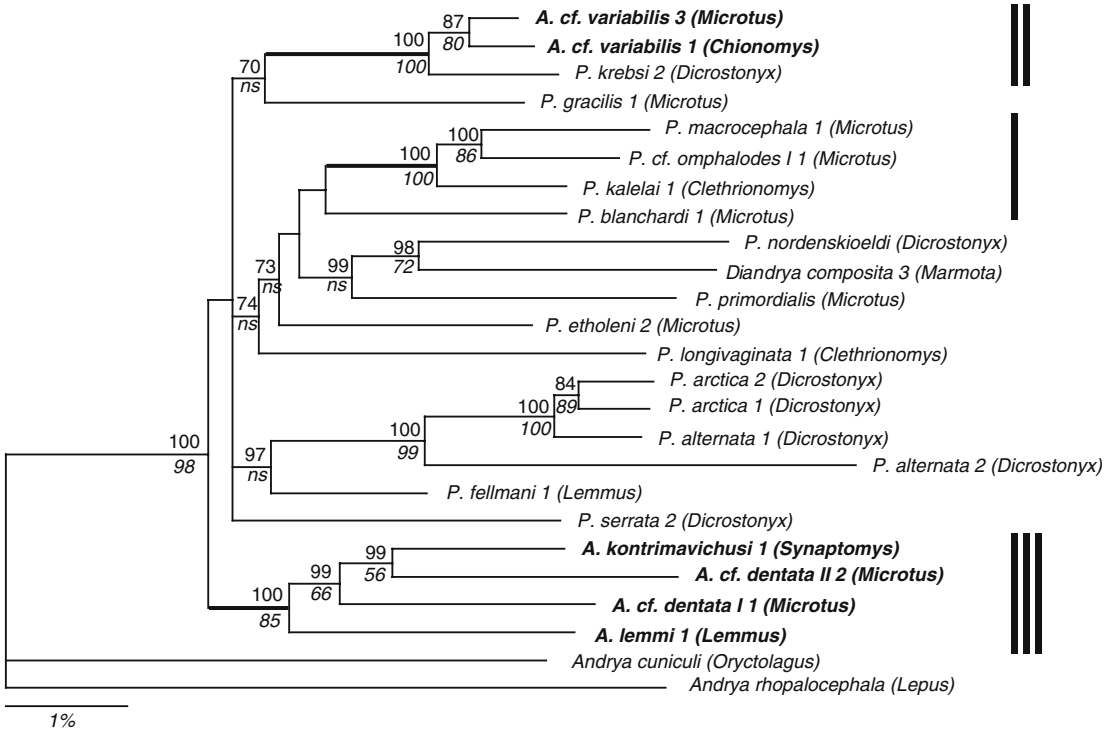
trees, we reconstructed a "super tree" by manually incorporating all supported basal relationships from individual and combined data-sets within the same phylogram (Figure 15). The position of *Andrya* was different in the individual and combined data-sets, probably due to the use of distantly related outgroups in the individual data-sets. Here we followed the combined data that are probably more reliable (placing *Andrya* as the sister group of the arvicoline clade). Otherwise the topology within the resulting consensus tree does not conflict with any of the supported relationships in the individual and combined data-sets. The basal phylogenetic relationships and patterns of character evolution are deduced from this consensus tree.

The consensus tree suggests that the present assemblage comprises four major lineages originating from a polytomy. The arvicoline clade + *Andrya* form the crown group in the lineage that seems to have diversified initially in perissodactyls (*A. mamillana*, *Anoplocephala*). The second lineage includes two genera from lagomorphs (*Mosgovoyia* and *Schizorchis*). Additionally, *Moniezia* (from ruminants) and *Monoecocestus* (from rodents) form two basal lineages with no associations with other genera.

Assuming that the tubular uterus is the ancestral type within the present assemblage (see Discussion), there would have been three or four independent conversions to the reticulated type in *Moniezia*, *Monoecocestus*, *Andrya* and



Figures 12–13. Continued.



Figures 14. Phylogenetic tree for the 'arvicoline clade' of anoplocephaline cestodes based on combined mitochondrial COI, 28S and ITS1 rDNA sequences produced by Bayesian analysis implemented in MrBayes with GTR $+\gamma$ distances. *Andrya rhopalocephala* and *A. cuniculi* were used as outgroups. Posterior probabilities (>70%) are shown above the branches; bootstrap values (>50%, based on NJ with ML distances) are shown in italics below the branches; other branches have <70% (Bayesian) or <50% (bootstrap) support (ns). Symbols and fonts as in Figure 11.

Paranoplocephala within the arvicoline clade (which also includes *Anoplocephaloides* spp. with tubular uteri). However, if the precursor of the arvicoline clade + *Andrya* had a reticulated uterus, then there should have been at least one reversal to the tubular type within the arvicoline clade.

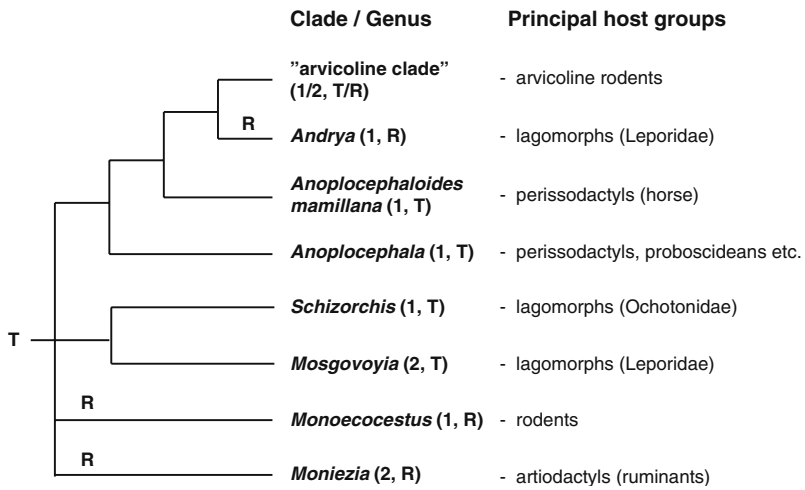
In addition, if the precursor of Anoplocephalinae had a single set of genitalia per proglottis (as is generally assumed), it would imply three occasions of genital doubling in *Moniezia*, *Mosgovoyia* and *D. composita* within the arvicoline clade. There was no indication of reversal to the putative ancestral state.

Discussion

Host-parasite co-evolution

The anoplocephaline cestodes of placental mammals comprise 19-21 genera, most of which parasitise rodents (eight genera) and lagomorphs (six or seven genera) (Table 2). Minor radiations have occurred in perissodactyls, artiodactyls and primates, but not in other orders of placental mammals. The majority of anoplocephaline genera are restricted to a certain family of hosts, with some notable exceptions (*Anoplocephala, Anoplocephaloides*, *Bertiella* Stiles & Hassall, 1902 and *Moniezia*). Due to the limited coverage of the present material and varying phylogenetic resolution, we have not presented a formal co-evolutionary analysis for anoplocephaline cestodes and their hosts. However, some obvious patterns are discussed below.

The recent phylogenetic analyses have consistently supported the monophyly of rodents + lagomorphs, known collectively as 'Glires' (e.g. Murphy et al., 2001; Lin et al., 2002). Perissodactyls and artiodactyls (or more precisely, cetartiodactyls, including whales) represent most recent mammalian radiations, being clearly derived with respect to Glires.



Figures 15. 'Super tree' produced by manually incorporating all supported basal relationships of anoplocephaline cestodes from individual and combined data-sets within the same phylogram. The figures within parentheses show the number of genitalia per proglottis (single; double), and the letters within parentheses show the structure of early uterus (T, tubular; R, reticulated). A possible pathway of uterine evolution portrayed on the tree.

Generally, there is a distinctive lack of basal cophylogenetic associations in the present material. For example, the genera from rodents and lagomorphs do not consistently appear as sister groups, and when they do (arvicoline clade + *Andrya*), they are derived with respect to the genera from perissodactyls and artiodactyls (Figure 15). The present results are in agreement with the 18S (rDNA) sequence data of Foronda et al. (2003), showing that *Anoplocephaloides dentata* and *Andrya cuniculi* (Blanchard, 1891) are more closely related to each other than either is to *Mosgovoyia ctenoides* (Railliet, 1890).

Each of the four major host lineages appears to have been colonised independently in the early history of anoplocephaline cestodes. The arvicoline clade + *Andrya* seems to originate via colonisation from perissodactyls, and their subsequent separation is probably also due to colonisation (from either direction), since the divergence of their hosts (rodents *vs* lagomorphs) must have occurred much earlier. However, the early history of the lineage leading to the arvicoline clade remains partly obscure, since *Anoplocephala* occurs in several mammalian orders, and it is not known which of them is the original host group. The high genetic distance between *Andrya rhopalocephala* (Riehm, 1881) and *A. cuniculi* from leporids suggests that they have a long independent history and that they have diverged soon after the putative colonisation event from perissodactyls.

However, it should be emphasised that the basal phylogenetic pathways suggested above may have to be modified if additional anoplocephaline genera and species become available. Particularly, the inclusion of genera from Australian marsupials, which probably represent a monophyletic lineage with respect to those in placental mammals (Beveridge, 1994), would be needed for a more comprehensive phylogenetic hypothesis of anoplocephaline cestodes. Beveridge (1994) has provided a cladistic hypothesis suggesting that all anoplocephalines in Australian marsupials originate from the wide-spread genus *Bertiella* that colonised Australia via rodents, thus providing a possible link between the lineages parasitising placentals and marsupials and suggesting that anoplocephaline cestodes of marsupials are not necessarily as 'primitive' or ancient as have been assumed (cf. Tenora, 1976).

Conroy & Cook (1999) verified the monophyly of arvicoline rodents (voles and lemmings) and suggested that the observed lack of resolution among arvicoline genera is due to ''pulses of speciation'', i.e. almost simultaneous diversification of multiple lineages without discernible genetic traces. The evolutionary history of the most diverse vole genus *Microtus* (with about 65 extant species) is also characterised by a burst of rapid diversification

(Conroy & Cook, 1999), although the extensive phylogenetic analysis of Jaarola et al. (2005) distinguished several monophyletic species groups within this genus. Thus, concomitant diversification burst(s) in the anoplocephaline cestodes parasitising arvicoline rodents is a likely explanation for the large polytomy observed in our material. Since the species within the arvicoline clade (of cestodes) are closely related (and not all third positions in COI were variable), the observed polytomy cannot be ascribed to saturation. Instead, consistent support for nodes above and below the unresolved polytomy indicate a rapid radiation involving a nearly simultaneous diversification of many cestode lineages (cf. Lessa & Cook, 1998; Conroy & Cook, 1999; Jaarola et al., 2005).

There is almost no evidence for cospeciation of hosts and parasites within the arvicoline clade, but there are several clear instances of host shift associated with specific or generic divergence. Firstly, the position of the monotypic genus *Diandrya* within the arvicoline clade shows that it has diverged through a shift from voles or lemmings (possibly from *Dicrostonyx*) to the Nearctic marmots. The topologies for the subclades I and II also suggest at least a single colonisation event in each case (see Haukisalmi et al., 2004, for molecular phylogeny of the former subclade, i.e. *Paranoplocephala* (*sensu stricto*)). In addition, the evolutionary history of the subclade III (*Anoplocephaloides* (*sensu stricto*)) is characterised by extensive colonisation, but a more detailed molecular phylogeny for this assemblage will be presented elsewhere.

We conclude that colonisation of novel host lineages has evidently been the predominant mode of diversification in anoplocephaline cestodes of placental mammals; evidence for basal cophylogeny is obscure in the present material. However, the basal co-evolution within Anoplocephalinae can ultimately be tested only from a material including species from marsupials and birds. Extensive colonisation, associated with rapid radiation, also characterises the diversification within the large arvicoline clade. Thus, ecological determinants, such as feeding strategies, may have been more important in shaping the extant patterns of host-parasite associations than co-evolutionary processes. Although the evolutionary history of most (if not all) of the larger helminth assemblages studied so far includes various combinations of cospeciation and colonisation (for reviews see Beveridge & Spratt, 1996; Hoberg & Klassen, 2002), colonisation appears to have been the prevailing mode in several disparate endoparasite groups of mammals (e.g. Hoberg & Lichtenfels, 1994; Hoberg, 1995; Hoberg et al., 2000; Brant & Gardner, 2000; Beveridge & Chilton, 2001).

Character evolution and systematics

Uterine morphology has played a key role in the systematic and phylogenetic arrangements within anoplocephaline cestodes. Specifically, taxa with tubular and reticulated early uteri have been suggested as representing different phyletic lineages (Beveridge, 1994), although the systematic rearrangement of Spasskii (1951) based on this dichotomy has not gained general acceptance. The present analysis, which includes most of the anoplocephaline genera with a reticulated uterus, suggests, however, that the reticulated uterus has emerged independently at least three times within the Anoplocephalinae. Thus, the structure of the early uterus does not serve as the main phylogenetic determinant within this subfamily, implying that Spasskii's (1951) separation of the Anoplocephalinae (with a tubular uterus) and the Monieziinae (with a reticulated uterus) is unjustified. Homoplasy of uterine structures, which is evident in the phylogeny of families and subfamilies of cyclophyllidean cestodes (Hoberg et al., 1999), appears to be characteristic also for the genera and species within the anoplocephaline cestodes.

We have assumed here that the tubular condition of the uterus is the ancestral type within the present assemblage, and generally within the Anoplocephalinae. This assumption is supported by the presence of tubular uteri in all anoplocephaline cestodes not included in the present analysis, including those from marsupials and birds, which probably form two independent lineages separate from those in placental mammals (Beveridge, 1994). In addition, the uterus is initially tubular in the Thysanosomatinae (the putative sister group of the Anoplocephalinae), later developing paruterine organs, and also in the outgroups to the Cyclophyllidea, such as the Proteocephalidea and Trypanorhyncha.

Lineages characterised by reticulated uteri originate from both ancient and more recent

divergence events. Although the uterine evolution within the arvicoline clade remains obscure, it is possible that the morphologically heterogeneous reticulated uteri have appeared more than once within this clade. Also, the reticulated uteri of *Andrya* and *Paranoplocephala* probably have an independent origin, although these genera have sometimes been considered morphologically indistinguishable. Interestingly, some of the phylogenies suggest that the divergence (and a host shift) of *A. variabilis*-like species from *P. krebsi* (within the subclade II) has been accompanied by a reversal to the putative ancestral uterine type (tubular).

Due to their multiple origins, the reticulated uteri of anoplocephalines are expected to show structural differences between various lineages. Although this matter has not been studied in a comparative manner, *Moniezia* seems to differ from the other genera, since its uterus retains a reticulated structure throughout development, whereas in other genera these structures are lost or reduced in the fully-developed (sac-like) uterus. In addition, the early uteri of *Paranoplocephala* species exhibit considerable morphological heterogeneity, ranging from the narrow, 'partly' reticulated forms to the 'completely' reticulated uteri that cover most of the medulla (Figures 1-10). Other, deviating types have also been recognised (Haukisalmi & Henttonen, 2001). The partly reticulated uterus is characteristic for all species within the subclade I (*Paranoplocephala* (*sensu stricto*)). However, identical uteri are known from other *Paranoplocephala* species outside this subclade (e.g. *P. gracilis* Tenora & Murai, 1980, *P. nordenskioeldi* Haukisalmi, Wickström, Hantula & Henttonen, 2001 and *P. serrata* Haukisalmi & Henttonen, 2000). Also, *Paranoplocephala* species with a typical completely reticulated uterus (*P. alternata, P. arctica, P. etholeni* Haukisalmi, Henttonen, Niemimaa & Rausch, 2002 and *P. longivaginata* Chechulin & Gulyaev, 1998) do not form an inclusive monophyletic group in any of the phylogenies. Thus, the uterine diversity within *Paranoplocephala* seems to have a limited phylogenetic correspondence, although this may partly be due to the generally low level of resolution within the arvicoline clade.

In various phylogenetic schemes for anoplocephaline cestodes, the genus pairs *Andrya* + *Diandrya*, *Monoecocestus* + *Moniezia* and *Mosgovoyia* + *Schizorchis* have been defined as sister taxa, being separated by the number of genitalia per proglottis (and occasionally by other features). Of these genera, *Mosgovoyia* and *Schizorchis* appeared here as sister groups, suggesting that their divergence has been accompanied by genital duplication in *Mosgovoyia*. The other proposed associations are not supported. However, genital duplication has occurred in *Diandrya* in connection with its divergence from ancestors in arvicoline rodents (all with a single set of genitalia), not from *Andrya*. Although the change in genital number has clearly been a frequent phenomenon in the evolution of the anoplocephaline cestodes (Beveridge, 1994), detailed phylogenetic pathways of this character cannot yet be inferred from the existing, still incomplete, material.

Although we have not systematically screened all morphological characters, it seems that the distribution of testes provides an apomorphic/synapomorphic feature for the clade including *Anoplocephaloides mamillana* + *Andrya* + arvicoline clade. All species within this large clade are characterised by an antiporal or antiporal/anterior position of the testes with respect to the ovary. In most of the other anoplocephaline cestodes of placental mammals, testes have a predominantly posterior position or they are scattered (almost) throughout the medulla (Beveridge, 1994). The only exceptions seem to be *Cittotaenia* Riehm, 1881 and *Ctenotaenia* Railliet, 1893, both with a testicular distribution approaching the pattern described above. However, since the latter species have double genitalia, they cannot be directly compared with species having a single set of genitalia. Based on general morphology, no unambiguous synapomorphies can yet be specified for the arvicoline clade or *Andrya* + arvicoline clade.

Besides refuting the dichotomy into the Monieziinae and the Anoplocephalinae, the present results include a few additional systematic implications. *Anoplocephaloides* (*sensu* Rausch, 1976 and Beveridge, 1994) is clearly paraphyletic, and new genera will need to be erected for *A. mamillana* and *A. variabilis*-like species (subclade II). The name *Anoplocephaloides* should be reserved for the monophyletic subclade III (i.e. *Anoplocephaloides* (*sensu stricto*)). *Anoplocephaloides* species parasitising leporid lagomorphs and tapirs have already been assigned to *Leporidotaenia* Genov, Murai, Georgiev & Harris, 1990 and *Flabelloskrjabinia* Spasskii, 1951, respectively, and Gulyaev (1996) has

proposed *Paranoplocephaloides* Gulyaev, 1996 for two *Anoplocephaloides*-like species from voles (neither included in the present analysis). *Anoplocephaloides* still includes additional phylogenetic lineages, all of which may ultimately warrant generic status (cf. Rausch, 1976; Genov & Georgiev, 1988). However, this will not be possible without a comprehensive taxonomic revision (preferably accompanied by a molecular phylogenetic analysis) of the taxa previously assigned to *Anoplocephaloides*.

Since *Andrya* and *Paranoplocephala* are morphologically very similar, the *Paranoplocephala* species of rodents with an *Andrya*-like, 'completely reticulated' early uterus have variously been assigned to *Andrya*. The present analysis supports the independent status of these genera, but morphological criteria that would unambiguously separate them are still lacking (cf. Rausch, 1976; Tenora et al., 1986; Genov et al., 1996). Tenora (1998) resurrected *Aprostatandrya* Kirshenblat, 1938, previously regarded as a junior synonym of *Paranoplocephala* by Rausch (1976) and others. However, the present data and our previous analyses (Haukisalmi & Henttonen, 2003; Haukisalmi et al., 2004) show unambiguously that the type-species of *Aprostatandrya*, *A. macrocephala* (Douthitt, 1915), belongs to *Paranoplocephala* (*sensu stricto*), thereby supporting the action of Rausch (1976).

Although the *Paranoplocephala* species did not form here an inclusive monophyletic assemblage, we predict that with additional molecular and morphological characters, this genus will prove to be monophyletic with respect to other lineages within the arvicoline clade, as partly suggested by one of the combined data-sets (Figure 13). However, a taxonomic revision, including representatives from other rodent groups, will ultimately be needed for this diverse and morphologically heterogeneous genus.

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