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# Phylogeny of the Monopisthocotylea and Polyopisthocotylea (Platyhelminthes) inferred from 28S rDNA sequences<sup>☆</sup>

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## Abstract

This study focuses on the phylogenetic relationships within the Polyopisthocotylea and Monopisthocotylea, two groups that are often grouped within the monogeneans, a group of disputed paraphyly. Phylogenetic analyses were conducted with multiple outgroups chosen according to two hypotheses, a paraphyletic Monogenea or a monophyletic Monogenea, and with three methods, namely maximum parsimony, neighbour joining and maximum likelihood. Sequences used were from the partial domain C1, full doXmain D1, and partial domain C2 (550 nucleotides, 209 unambiguously aligned sites) from the 28S ribosomal RNA gene for 16 species of monopisthocotyleans, 26 polyopisthocotyleans including six polystomatids, and other Platyhelminthes (61 species in total, 27 new sequences). Results were similar with outgroups corresponding to the two hypotheses. Within the Monopisthocotylea, relationships were: (((*Udonella*, capsalids), monocotylids), (diplectanids, ancyrocephalids)); each of these families was found to be monophyletic and their monophyly was supported by high bootstrap values in neighbour joining and maximum parsimony. Within the Polyopisthocotylea, the polystomatids were the sister-group of all others. Among the latter, *Hexabothrium*, parasite of chondrichthyans, was the most basal, and the mazocraeids, mainly parasites of clupeomorph teleosts, were the sister-groups of all other studied polyopisthocotyleans, these, mainly parasites of euteleosts, being polytomous. © 2000 Australian Society for Parasitology Inc. Published by Elsevier Science. All rights reserved.

**Keywords:** Monogenea; Monopisthocotylea; Phylogenetic analysis; Platyhelminthes; Polyopisthocotylea; 28S rDNA

## 1. Introduction

The phylogenetic relationships among the major groups of parasitic Platyhelminthes (flatworms), which comprise the Monogenea, Trematoda and Cestoda, are controversial. Monophyly of the Monogenea has been recently rejected in a molecular analysis [1], and a pre-

vious analysis based on sperm structure found no synapomorphy for the Monogenea [2–4]. The Monogenea have been considered a monophyletic group in analyses based on morphology [5,6] and a recent analysis listed several synapomorphies [7]. The presence of eyes is one of the characters generally proposed as a synapomorphy for the group, but a recent review has questioned the validity of this character because of the lack of ultrastructural evidence of homology [8], and concluded that a reappraisal of morphological synapomorphies should be undertaken. Although analyses based on 18S [9] or 28S rDNA [1,10] sequences found the Monogenea paraphyletic, a combined morphological and molecular (18S rDNA) analysis recently claimed [11] monophyly of the group.

<sup>☆</sup> Note: New nucleotide sequence data reported in this paper will be available in the GenBank database under the accession numbers AF131706–AF131733. Alignment will be available on request to the authors or from <http://www.mnhn.fr/mnhn/bpph/Data/IndexData.html>.

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Monogeneans classically comprise two groups, the Monopisthocotylea and Polyopisthocotylea (but see Table 1 for problems of terminology). Monophyly of each of these groups has been ascertained on morphological characters [7, 12, 13] and was also indicated by spermatological [2–4] and molecular [1, 9–11, 14] analyses. However, the relationships demonstrated between these two groups and the other parasitic Platyhelminthes vary according to the gene used. Trees based on 28S rDNA (domains C1, D1, C2 [1], or domain D3 [10]) found the Monopisthocotylea sister-group to all other parasitic Platyhelminthes, with topologies such as (Monopisthocotylea, (Polyopisthocotylea, (Digenea, Eucestoda))) [1] or (Monopisthocotylea, (Polyopisthocotylea, Trematoda, Cestoda)) [9], but trees based on 18S rDNA [9] found the Polyopisthocotylea as sister-group for all others, with the topology (Polyopisthocotylea, (Monopisthocotylea-Trematoda, Cestoda)).

Trees based on morphological characters [5–7] favoured the Trematoda as sister-group for the Monogenea + Cestoda, a topology found in no molecular analyses [1, 10, 11, 14] but one [15]. Two main hypotheses of relationships within the parasitic Platyhelminthes, one in which the Monopisthocotylea is the sister-group to all other parasitic Platyhelminthes, and making the Monogenea paraphyletic, and one in which the Monogenea are monophyletic and sister-group of the Cestoda, are summarised in Fig. 1.

In this study, we used 28S rDNA sequences to evaluate the internal phylogenetic relationships within the Monopisthocotylea and Polyopisthocotylea. Sixty-one sequences (27 new), including 42 species (39 genera) of monogeneans and comprising 550 nucleotide sites, were analysed with three methods: maximum parsimony, maximum likelihood, and neighbour joining. Although both groups are monophyletic and thus can be analysed independently, the conflicts between the various topologies of parasitic Platyhelminthes led us to consider the problem of choice of outgroup in undertaking the phylogenetic analysis of each group. For each group, two different analyses were conducted,

one corresponding to the hypothesis of paraphyletic Monogenea, with Monopisthocotylea sister-group to other Neodermata (Fig. 1a), and one corresponding to the hypothesis of a monophyletic Monogenea, within which the Monopisthocotylea and Polyopisthocotylea are sister-groups (Fig. 1b).

## 2. Materials and methods

### 2.1. Material and extraction of DNA

A list of hosts, localities and sources of the new species sequenced for this analysis is presented in Table 2. The species and accession numbers of the sequences used are presented with a classification in Table 3. Specimens were rehydrated in STE (Sodium, Tris, EDTA, pH 8) buffer for 2 h. They were then submitted to a shaking bath at 55°C for 16 h in 500 µl of a solution of 5% Chelex in water, added with 5 µl of proteinase K at 10 µg ml<sup>-1</sup>. In order to deactivate any remaining enzyme that could inhibit the PCR reaction, the solution was heated to 95°C for 10–30 min, before commencing the reaction.

### 2.2. PCR and sequencing

A portion of the 28S rDNA was amplified by PCR. The PCR primers were universal primer C1 (ACCCGCTGAATTTAAGCAT at 5′–3′ position 25 [16]), and reverse primer C3 (CTCTTCAGAGTACTTTTCAAC at 5′–3′ position 390 [16]) which was designed by us and expected to be specific to Platyhelminthes. The amplified portion contained the partial domain C1, full domain D1, and partial C2. However, the sequence for *Stylochus* sp. was obtained with reverse primer D2 [1]. The PCR and sequencing reactions were processed with Perkin Elmer reagents and protocol. The automated sequencing was performed on gel purified PCR products, with the same primers as for PCR, on an ABI automatic sequencer.

Table 1  
Terminology used in this paper and equivalencies in the alternative terminology used by Boeger and Kritsky [7, 12, 13]

Terminology used in this paper	Alternative terminology
Monogenea	Monogenoidea <sup>a</sup>
Monopisthocotylea	Polyonchoinea
Polyopisthocotylea (including Polystomatidae and Sphyanuridae <sup>b</sup> )	Heteronchoinea
Polystomatidae and Sphyanuridae	Polystomatoinea
Polyopisthocotylea (excluding Polystomatidae and Sphyanuridae <sup>b</sup> )	Oligonchoinea

<sup>a</sup>The use of Monogenoidea was rejected by Wheeler and Chisholm [47].

<sup>b</sup>No data about the Sphyanuridae were considered in the present paper.

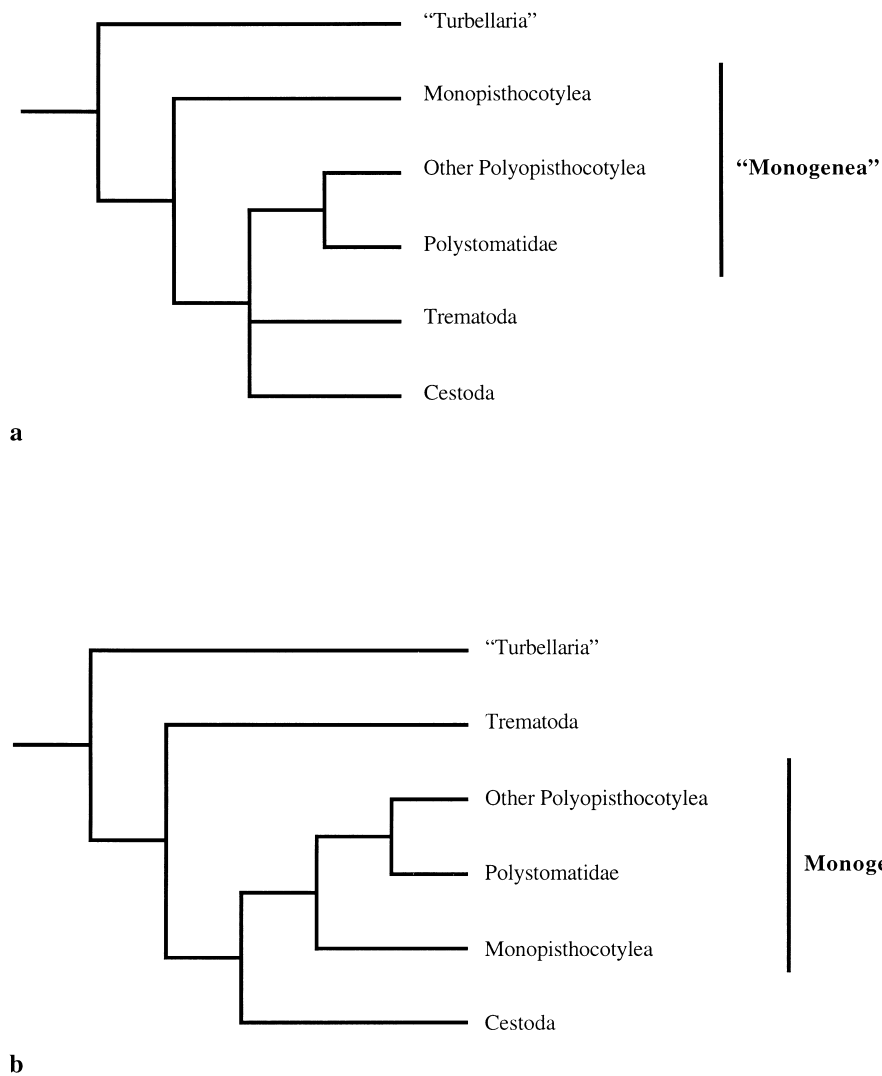


Fig. 1. Two hypotheses for the relationships of the major groups of parasitic Platyhelminthes. (a) Relationships from a molecular analysis with 28S rDNA sequences, according to Mollaret et al. [1] and Littlewood et al. [9]. (b) Relationships with the Monogenea monophyletic [6, 7, 11]. Polystomatids displayed to show sister-group relationships with other Polyopisthocotylea.

### 2.3. Sequence alignment

Although an alignment of 28S rDNA sequences of Platyhelminthes has already been used for phylogenetic analyses [1, 9], a new alignment was done with CLUSTAL W [17], then manually edited using the software Se-AL (Rambaut A, 1996. Se-AL, Sequence Alignment Editor. Version 1.0 alpha 1. Software distributed by the Author, Department of Zoology, University of Oxford, Oxford. Available from <http://evolve.zoo.ox.ac.uk/Se-AL/Se-AL.html>).

### 2.4. Ingroup and outgroup for a general phylogenetic analysis

The final nucleotide matrix constituted 61 species (Table 3, column 0). It comprised 550 sites from

domains C1, D1, C2, including alignment gaps. Ambiguously aligned sites were removed prior to analysis and corresponded partly to previously published analyses [1, 9]. *Stenostomum*, a catenulid, was used as an outgroup. Catenulids have been considered either as basal Platyhelminthes [5, 18] or as a group separated from all Platyhelminthes [19].

### 2.5. Ingroups and outgroups used for the separate phylogenetic analyses

The choice of outgroup has been decided according to the advised principle of rooting with multiple outgroups [20, 21] and justified by the three reasons of: (a) minimising an inappropriate choice; (b) raising the level of generality; and (c) testing the monophyly of the ingroup [22]. In addition, for each analysis we used

Table 2  
Source of Platyhelminthes species sequenced in this study

Platyhelminthes species	Host	Locality and source
<i>Turbellaria</i>		
<i>Pseudomonocelis ophiocephala</i>	Free living	Italy <sup>a</sup>
<i>Stylochus</i> sp.	Free living	Australia <sup>b</sup>
<i>Aspidogastrea</i>		
<i>Multicalyx elegans</i>	<i>Callorhynchus milii</i> (H)	Hobart, Australia <sup>c</sup>
<i>Monopisthocotylea</i>		
<i>Furnestinia echeneis</i>	<i>Sparus aurata</i> (T)	Sète, France
<i>Ligophorus mugilinus</i>	<i>Mugil cephalus</i> (T)	Sète, France
<i>Trochopus pini</i>	<i>Trigla lucerna</i> (T)	Sète, France
<i>Capsala onchidiocotyle</i>	<i>Thunnus thynnus</i> (T)	Sète, France
<i>Tristoma integrum</i>	<i>Xiphias gladius</i> (T)	Sète, France
<i>Calicotyle palombi</i>	<i>Mustelus mustelus</i> (Co)	Ghar el Melh, Tunisia <sup>d</sup>
<i>Polyopisthocotylea</i>		
<i>Polystoma integerrimum</i>	<i>Rana temporaria</i> (A)	Porté, France <sup>e</sup>
<i>Polystomoides malayi</i>	<i>Cuora amboinensis</i> (C)	Kuala Lumpur, Malaysia <sup>e</sup>
<i>Hexabothrium appendiculatum</i>	<i>Scyliorhinus canicula</i> (Co)	Sète, France
<i>Kuhnia scombrus</i>	<i>Scomber scombrus</i> (T)	Sète, France
<i>Grubea cochlear</i>	<i>Scomber scombrus</i> (T)	Sète, France
<i>Hexostoma thynni</i>	<i>Thunnus thynnus</i> (T)	Sète, France
<i>Plectanocotyle</i> sp.	<i>Trigla</i> sp. (T)	Sète, France
<i>Choricotyle</i> cf. <i>chrysophrii</i>	<i>Pagellus acarne</i> (T)	Banyuls, France
<i>Cyclocotyla bellones</i>	Crustacean from <i>Boops boops</i> (T)	Sète, France
<i>Diclidophora luscae capelanus</i>	<i>Trisopterus luscus capelanus</i> (T)	Sète, France
<i>Octomacrum lanceatum</i>	<i>Catostomus catostomus</i> (T)	USA <sup>f</sup>
<i>Gastrocotyle trachuri</i>	<i>Trachurus mediterraneus</i> (T)	Sète, France
<i>Pseudaxine trachuri</i>	<i>Trachurus mediterraneus</i> (T)	Sète, France
<i>Atrispinum acarne</i>	<i>Pagellus acarne</i> (T)	Sète, France
<i>Polylabris heterodus</i>	<i>Diplodus annularis</i> (T)	Banyuls, France
<i>Microcotyle mugilis</i>	<i>Mugil cephalus</i> (T)	Banyuls, France
<i>Metamicrocotyla cephalus</i>	<i>Mugil cephalus</i> (T)	Banyuls, France
<i>Cemocotyle trachuri</i>	<i>Trachurus mediterraneus</i> (T)	Sète, France

Hosts are Teleostei (T), Chondrichthyes (Co), Amphibia (A), Chelonia (C). Specimens were collected by <sup>a</sup>M.C. Curini-Galletti, <sup>b</sup>A. Hugall, <sup>c</sup>K. Rohde, <sup>d</sup>L. Euzet, <sup>e</sup>N. Sinnappah and L.H.S. Lim, <sup>f</sup>R. Hathaway; all others were collected by I. Mollaret.

two outgroups (Table 4), one according to the hypothesis of paraphyletic monogeneans (Fig. 1a), based on the results obtained in previous 28S rDNA analyses [1,9], and one according to the hypothesis of monophyly of the Monogenea (Fig. 1b), based on morphology ([7] and references therein). The use of several outgroup sets may help ascertaining relationships within the ingroup [23]. For analysis of the Monopisthocotylea, the ingroup included all investigated monopisthocotyleans. A first multiple outgroup, corresponding to the paraphyletic monogeneans hypothesis (Fig. 1a, Table 4) was constituted by two taxa in each of the digeneans, cestodes, non-polystomatids polyopisthocotyleans, polystomatids and one gyrocotylean (Table 3, column 1). A second multiple outgroup, corresponding to the monophyletic monogeneans hypothesis (Fig. 1b, Table 4) consisted of all polyopisthocotyleans, the sister-group of the Monopisthocotylea in this hypothesis (Table 3, column 2). For the Polyopisthocotylea, the ingroup included all investigated polyopisthocotyleans. A first multiple outgroup,

corresponding to the paraphyletic monogeneans hypothesis (Fig. 1a, Table 4), was constituted by the digeneans, cestodes and gyrocotyleans (Table 3, column 3). A second multiple outgroup, corresponding to the monophyletic monogeneans hypothesis (Fig. 1b, Table 4) consisted of all monopisthocotyleans, the sister-group of the Polyopisthocotylea in this hypothesis (Table 3, column 4). Within the Polyopisthocotylea, because all results in this analysis and previously published works [7,9,12,13] found the group composed of two sister-groups, the Polystomatidae and the non-polystomatid Polyopisthocotylea, a further study was performed within the non-polystomatid Polyopisthocotylea with the Polystomatidae used as outgroup (Table 3, column 5, Table 4).

## 2.6. Phylogenetic analysis

xUsing PAUP\* version 4.0b2a [Swofford DL, 1998, PAUP\*, phylogenetic analysis using parsimony (\* and other methods). Sunderland, MA: Sinauer Associates],

Table 3

Species in ingroup and outgroup for each analysis. Numbered columns indicate taxa used as ingroups (i) or outgroups (o) for a general analysis (column 0), an analysis of the monopisthocotyleans (column 1, paraphyletic monogenean hypothesis; column 2, monophyletic monogenean hypothesis), an analysis of polyopisthocotyleans (column 3, paraphyletic monogenean hypothesis; column 4, monophyletic monogenean hypothesis), and an analysis of the non-polystomatid polyopisthocotyleans (column 5)

Species and classification	Ingroup (i) and outgroup (o) sets					Accession No.	
	0	1	2	3	4		5
<b>Catenulida</b>							
<i>Stenostomum leucops</i>	o						AJ228801
<b>“Turbellaria”</b>							
<i>Kronborgia isopodicola</i>	i						AJ228800
<i>Polycelis</i> sp.	i						AF026105
<i>Bipalium kewense</i>	i						AF026119
<i>Bdelloura candida</i>	i						AJ228798
<i>Stylochus</i> sp.	i						AF131707 <sup>a</sup>
<i>Pseudomonocelis ophiocephala</i>	i						AF131706 <sup>a</sup>
<i>Temnocephala</i> sp.	i						AJ228802
<b>Aspidogastrea</b>							
<i>Multicalyx elegans</i>	i	o		o			AF131708 <sup>a</sup>
<i>Multicotyle purvisi</i>	i	o		o			AF023115
<b>Digenea</b>							
<i>Heterobilharzia americana</i>	i	o		o			Z46506
<i>Schistosoma mansoni</i>	i	o		o			X13836
<i>Schistosoma haematobium</i>	i			o			Z46521
<i>Schistosoma japonicum</i>	i			o			Z46504
<i>Schistosoma spindale</i>	i			o			Z46505
<i>Echinostoma caproni</i>	i			o			AF026104
<b>Cestoda</b>							
<i>Proteocephalus neglectus</i>	i	o		o			AF026116
<i>Caryophyllaeus</i> sp.	i	o		o			AF026117
<b>Gyrocotylidea</b>							
<i>Gyrocotyle urna</i>	i	o		o			AJ228799
<b>Monopisthocotylea</b>							
<b>Diplectanidae</b>							
<i>Acleotrema</i> sp.	i	i	i		o		AF026118
<i>Furnestinia echeneis</i>	i	i	i		o		AF131711 <sup>a</sup>
<b>Ancyrocephalidae</b>							
<i>Tetrancistrum</i> sp.	i	i	i		o		AF026114
<i>Haliotrema chrysoaenae</i>	i	i	i		o		AF026115
<i>Ligophorus mugilinus</i>	i	i	i		o		AF131710 <sup>a</sup>
<b>Capsalidae</b>							
<i>Trochopus pini</i>	i	i	i		o		AF131714 <sup>a</sup>
<i>Encotyllabe caballeroi</i>	i	i	i		o		AF026112
<i>Benedenia lutjani</i>	i	i	i		o		AF026106
<i>Capsala onchidiocotyle</i>	i	i	i		o		AF131712 <sup>a</sup>
<i>Tristoma integrum</i>	i	i	i		o		AF131715 <sup>a</sup>
<i>Entobdella australis</i>	i	i	i		o		AF026108
<b>Monocotylidae</b>							
<i>Troglocephalus rhinobatidis</i>	i	i	i		o		AF026110
<i>Neoheterocotyle rhinobatidis</i>	i	i	i		o		AF026107
<i>Calicotyle palombi</i>	i	i	i		o		AF131709 <sup>a</sup>
<i>Merizocotyle icopae</i>	i	i	i		o		AF026113
<b>Udonellidae</b>							
<i>Udonella caligorum</i>	i	i	i		o		AJ228803
<b>Polyopisthocotylea</b>							
<b>Polystomatidae</b>							
<i>Polystoma integerrimum</i>	i	o	o	i	i	o	AF131719 <sup>a</sup>
<i>Polystomoides malayi</i>	i		o	i	i	o	AF131718 <sup>a</sup>
<i>Polystomoides australiensis</i>	i	o	o	i	i	o	Z83012
<i>Polystomoides asiaticus</i>	i		o	i	i	o	Z83008
<i>Neopolystoma spratti</i>	i		o	i	i	o	Z83006
<i>Neopolystoma chelodinae</i>	i		o	i	i	o	Z83004

(continued on next page)

Table 3 (continued)

Species and classification	Ingroup (i) and outgroup (o) sets					Accession No.	
	0	1	2	3	4		5
Hexabothrii?dae							
<i>Hexabothrium appendiculatum</i>	i	o	o	i	i	i	AF131724 <sup>a</sup>
Mazocraeidae							
<i>Kuhnia scomбри</i>	i		o	i	i	i	AF131725 <sup>a</sup>
<i>Grubea cochlear</i>	i		o	i	i	i	AF131730 <sup>a</sup>
Hexostomatidae							
<i>Hexostoma thynni</i>	i		o	i	i	i	AF131721 <sup>a</sup>
Plectanocotylidae							
<i>Plectanocotyle</i> sp.	i		o	i	i	i	AF131733 <sup>a</sup>
Diclidophoridae							
<i>Choricotyle</i> cf. <i>chrysophrii</i>	i		o	i	i	i	AF131729 <sup>a</sup>
<i>Cyclocotyla bellones</i>	i		o	i	i	i	AF131731 <sup>a</sup>
<i>Diclidophora luscae capelanii</i>	i		o	i	i	i	AF131732 <sup>a</sup>
Octomacridae							
<i>Octomacrum lanceatum</i>	i		o	i	i	i	AF131723 <sup>a</sup>
Gastrocotylidae							
<i>Gastrocotyle trachuri</i>	i		o	i	i	i	AF131727 <sup>a</sup>
<i>Pseudaxine trachuri</i>	i		o	i	i	i	AF131728 <sup>a</sup>
Neothoracocotylidae							
<i>Pricea multae</i>	i		o	i	i	i	AF026111
Gotocotylidae							
<i>Gotocotyla secunda</i>	i		o	i	i	i	AF026109
Microcotylidae							
<i>Atrispinum acarne</i>	i		o	i	i	i	AF131713 <sup>a</sup>
<i>Polylabris heterodus</i>	i		o	i	i	i	AF131716 <sup>a</sup>
<i>Microcotyle mugilis</i>	i	o	o	i	i	i	AF131722 <sup>a</sup>
<i>Metamicrocotyla cephalus</i>	i		o	i	i	i	AF131720 <sup>a</sup>
<i>Bivagina pagrosomi</i>	i		o	i	i	i	Z83002
Heteraxinidae							
<i>Cemocotyle trachuri</i>	i		o	i	i	i	AF131726 <sup>a</sup>
Axinidae							
<i>Zeuxapta seriola</i>	i		o	i	i	i	AF026103

<sup>a</sup>New sequences. The familial classification used for monogeneans is based on Lebedev [25] and for recently described genera on Spencer Jones and Gibson [32]; *Udonella* (Udonellidae) included within the monopisthocotyleans according to Baer and Euzet [27], confirmed by Littlewood et al. [9].

trees were constructed: (1) with the neighbour-joining (NJ) method [24] with the options of uncorrected distances; (2) by maximum parsimony (MP) with heuristic search command, options gaps treated as fifth base, starting trees obtained by stepwise addition with closest addition sequence; and (3) with maximum likelihood (ML) method with default settings (using Hasegawa–Kishino–Yano HKY85 model). Bootstrap values were calculated for NJ and MP methods, with 1000 replicates.

### 3. Results

#### 3.1. General phylogenetic analysis

An analysis was performed with all 61 species (Table 3, column 0), with 209 sites included, comprising 62 constant, 27 parsimony uninformative, and 120 parsimony informative sites. The relatively small number of informative sites did not allow this analysis to provide reliable

results on phylogeny within the Neodermata, but results confirmed monophyly of the Monopisthocotylea, of the Polyopisthocotylea excluding polystomatids, and of the Polyopisthocotylea including polystomatids, all conclusions previously supported by morphological and molecular analyses. The MP heuristic analysis resulted in polytomy within the Neodermata. The NJ analysis indicated paraphyly of the Monogenea. Relationships were (Monopisthocotylea, ((Polystomatidae, Polyopisthocotylea), (Digenea, Cestoda))). The same topology was found in MP and NJ bootstrap analyses, but bootstrap values were low.

#### 3.2. Analysis of the Monopisthocotylea

The Monopisthocotylea matrix used in this analysis contained six more partial 28S rDNA sequences than previous analyses [1,9]. The alignment included 547 sites for both outgroups. For the outgroups corresponding to the hypotheses of paraphyletic monogeneans

(Fig. 1a, Table 3, column 1) and monophyletic monogeneans (Fig. 1b, Table 3, column 2), respectively, 204/195 sites were unambiguously aligned, gaps included, of which 83/84 sites were parsimony informative.

In all analyses, the families Capsalidae, Monocotylidae, Diplectanidae and Ancyrocephalidae were each found to be monophyletic and thus results are presented as interfamilial relationships.

The analysis of the Monopisthocotylea with the outgroup corresponding to the paraphyletic monogenean gave the same topology with ML, MP bootstrap, NJ, and NJ bootstrap. This topology is presented in Fig. 2a. Families were arranged in two monophyletic groups. One contained the Udonellidae, Capsalidae and Monocotylidae, with Monocotylidae sister-group to the two other families, and the other consisted of the Diplectanidae and Ancyrocephalidae. Relationships at the species level for this topology are given in Fig. 3. A different topology (Fig. 2b) was found in the consensus trees computed, with heuristic MP search, from the six equally most parsimonious trees [length 390; consistency index (CI) excluding uninformative characters 0.464]; this topology did not differ in the first group, but found the second group (Diplectanidae and Ancyrocephalidae) paraphyletic.

The analysis of the Monopisthocotylea with the outgroup corresponding to the monophyletic monogenean, with ML and MP bootstrap methods, gave the same topology as the one found with the other outgroup (Fig. 2a). However, two other topologies were found. The MP heuristic analysis found 360 equally most parsimonious trees (length 361; CI excluding uninformative characters 0.432), and the consensus tree (Fig. 2c) included the Monocotylidae with the second group. The NJ analysis and its bootstrap led to a fourth topology, with Udonellidae sister-group to the other families (Fig. 2d).

Within the Capsalidae, all analyses found the same internal relationships. Within the Monocotylidae, *Troglocephalus* and *Neoheterocotyle* were always grouped in all analyses, but their relationships with other members were variable and did not show high sup-

port values. Within the Ancyrocephalidae, *Ligophorus* and *Haliotrema* were grouped in all analyses.

### 3.3. Analysis of the Polyopisthocotylea

The nucleotide sequences used in the analysis of Polyopisthocotylea contained 18 more 28S rDNA sequences than previous analyses [1,9]. For the outgroups corresponding to the hypotheses of paraphyletic monogeneans (Fig. 1a, Table 3, column 3) and monophyletic monogeneans (Fig. 1b, Table 3, column 4), the alignment included 547 sites, of which 210 were unambiguously aligned, gaps included, with respectively 67/97 parsimony informative sites.

First steps of analysis were to find the general topology within the Polyopisthocotylea including the Polystomatids. All analyses but one, including MP, MP bootstrap, NJ, and NJ bootstrap with the two outgroups corresponding to the paraphyletic monogenean and monophyletic monogenean hypotheses, and ML with outgroup corresponding to the paraphyletic monogenean, resulted in a single topology (Fig. 4a). In this topology, the Polystomatidae was monophyletic and was the sister-group to all other taxa, in which *Hexabothrium* was the most basal group. The single alternative topology was found with ML with outgroup corresponding to the monophyletic Monogenea (Fig. 4b); in this topology, the Polystomatidae and *Hexabothrium* formed a sister-group to all other taxa.

Within the Polystomatidae, *Polystoma* was found to be the sister-group to a group including all other polystomatid species, in all analyses with both outgroups, with the single exception of the MP analysis with the outgroup corresponding to the paraphyletic monogenean.

Because the Polystomatidae was the sister-group for all other taxa in all but one analysis, a detailed analysis of the relationships of the non-polystomatid polyopisthocotyleans was performed with the Polystomatidae as the only outgroup (Table 3, Column 5), thus reducing the risk of taking a too distant outgroup. The alignment contained 266 unam-

Table 4

Ingroup and outgroup for each analysis. For the analysis of the Monopisthocotylea and Polyopisthocotylea, respectively, two hypotheses were considered, Monogenea paraphyletic or monophyletic. Column numbers refer to Table 3

Ingroup	Outgroup	
	Paraphyletic Monogenea hypothesis	Monophyletic Monogenea hypothesis
Monopisthocotylea	Polyopisthocotylea <sup>a</sup> + Digenea + Gyrocotylidae + Eucestoda <i>Column 1</i>	Polyopisthocotylea <sup>a</sup> <i>Column 2</i>
Polyopisthocotylea	Digenea + Gyrocotylidae + Eucestoda <i>Column 3</i>	Monopisthocotylea <i>Column 4</i>
Polyopisthocotylea excluding Polystomatidae	Polystomatidae <i>Column 5</i>	

<sup>a</sup>Including Polystomatidae.

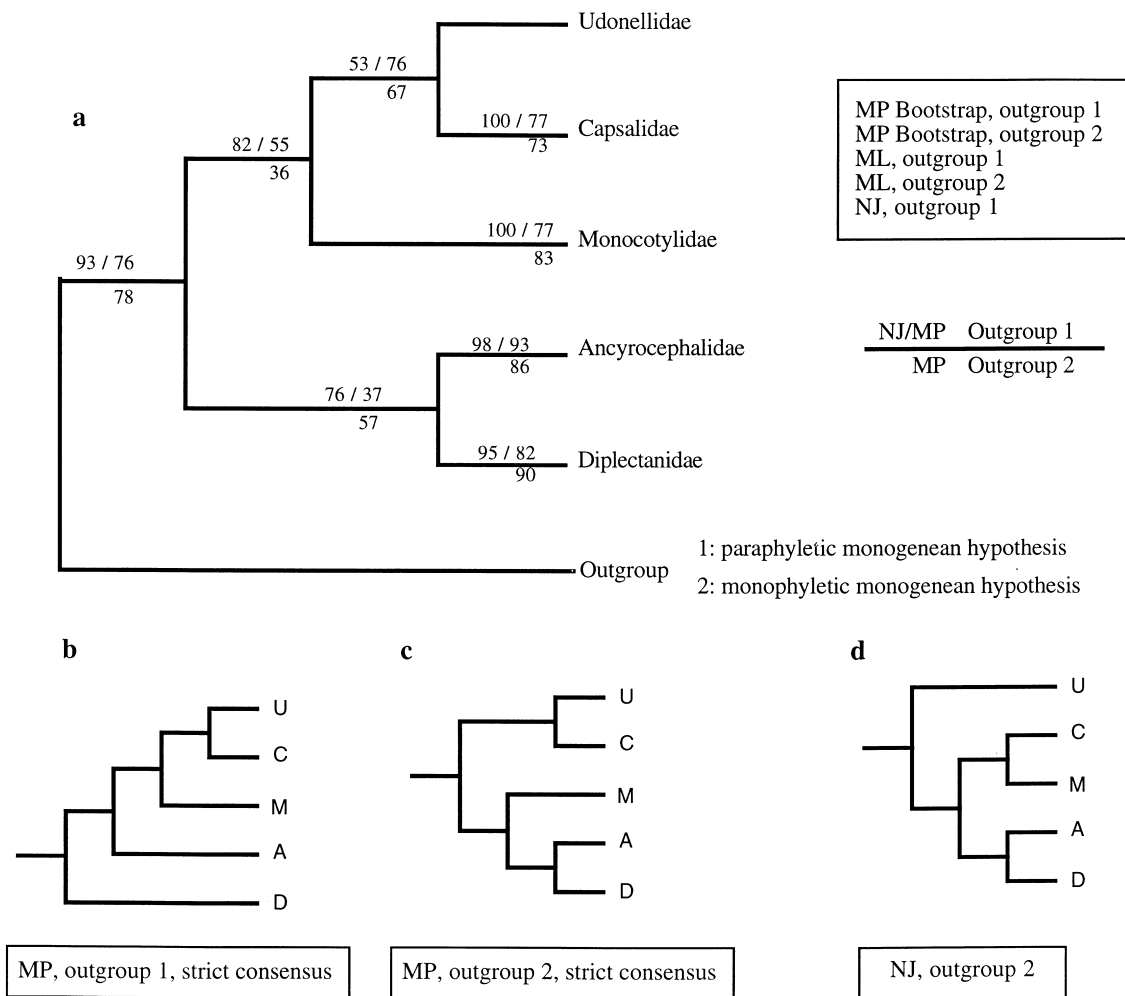


Fig. 2. Monopisthocotylea: topologies found at the family level, with various methods and outgroups. Methods and outgroups are indicated in boxes. (a) Topology found with several methods and outgroups: MP bootstrap analysis with both outgroups, ML with both outgroups, NJ and NJ bootstrap analysis with outgroup corresponding to the paraphyletic monogenean analysis; bootstrap values are indicated, NJ/MP values with outgroup corresponding to the paraphyletic monogenean analysis above branch, and MP values with outgroup corresponding to the monophyletic monogenean analysis under branch. See Fig. 3 for species nodes. (b) Alternative topology, strict consensus of equally most parsimonious MP trees, with outgroup corresponding to the paraphyletic monogenean analysis. (c) Alternative topology, strict consensus of equally most parsimonious MP trees, with outgroup corresponding to the monophyletic monogenean analysis. (d) Alternative topology, NJ tree and NJ bootstrap analysis, with outgroup corresponding to the monophyletic monogenean analysis.

biguously aligned sites, gaps included, of which 75 were parsimony informative. The MP heuristic search led to four equally most parsimonious trees (tree length 298, CI excluding uninformative characters 0.508). The analysis of the Polyopisthocotylea (poly-stomatids excluded), with the polystomatids used as outgroup, gave a similar topology with all methods used (MP, NJ, their bootstraps, and ML), with *Hexabothrium* basal to all others, and the two mazo-craeids *Kuhnia* and *Grubea* sister-groups to all other taxa (Fig. 5). This topology was similar to that found in the analyses including more taxa.

All analyses found a low resolution, indicated by low bootstrap values and short branch length, within a terminal group including all other taxa of the Polyopisthocotylea. Moreover, different topologies were

found with different methods, and the monophyly of this terminal group was not sustained by reliable bootstrap values. We present in Fig. 6 the NJ analysis. Within this terminal group, several groups were found in all analyses, but phylogenetic relationships among these groups were not resolved. One group included *Diclidophora*, *Cyclocotyla*, and *Choricotyle*, and thus corresponds to the family Diclidophoridae. A second group included the Gastrocotylidae *Gastrocotyle* and *Pseudaxine*, the Gotocotylidae *Gotocotyla* and the Neothoracocotylidae *Pricea*; this corresponds to the Gastrocotylina in Lebedev's classification [25]. A third group included the Microcotylidae *Atrispinum*, *Polylabris*, *Microcotyle*, *Metamicrocotyla*, and *Bivagina*, the Heteraxinidae *Cemocotyle*, and the Axinidae *Zeuxapta*; this corresponds to the Microcotylina in



Lebedev’s classification [25], but the Microcotylidae was never found monophyletic within this assemblage.

**4. Discussion**

*4.1. General analysis*

The sensitivity of phylogenetic analysis depends on many factors, among these alignment, data sampling, outgroup sampling, and rooting method. The present analysis was based on a relatively small number of

sites. The analysis of the whole data set showed low resolution. This probably depended on two main factors: (1) the choice of too distant outgroup taxa for testing relationships of monogeneans, that could be explained by a cascade effect [23]; and (2) the increased number of ingroup taxa without a corresponding increase in the number of sites [26]. Nevertheless, the results appear informative. Paraphyly of Monogenea was suggested, and monophyly of the Monopisthocotylea and of the Polyopisthocotylea was constantly found, thus confirming observations based on more reduced data sets [1, 9, 10]. Therefore, separ-

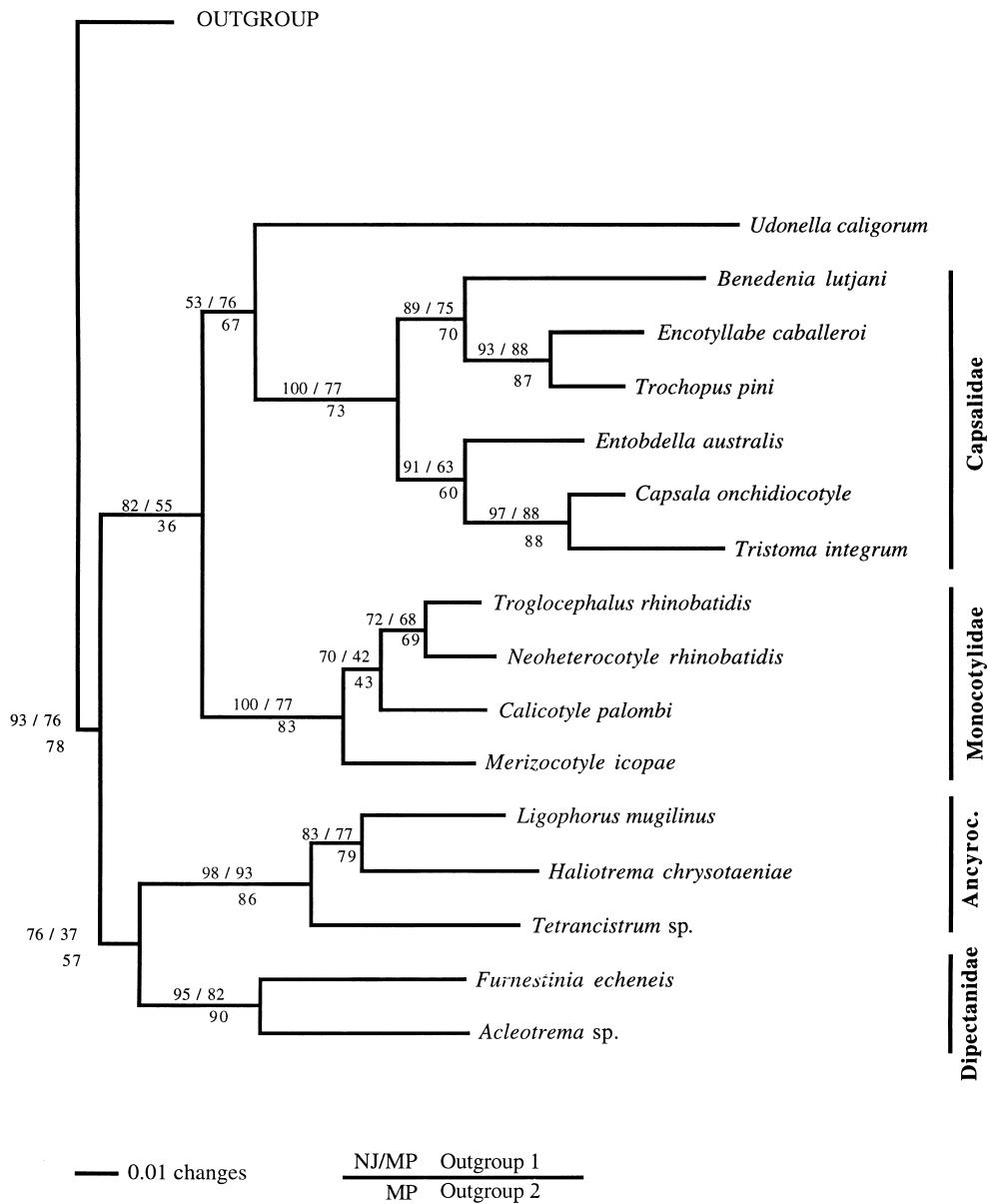


Fig. 3. Monopisthocotylea: phylogenetic relationships at the species level. Tree obtained in neighbour-joining analysis with 16 partial 28S rDNA sequences of Monopisthocotylea; the same topology was found in MP bootstrap and NJ bootstrap. Bootstrap values are indicated, NJ/MP values with outgroup corresponding to the paraphyletic monogenean analysis above branch, and MP values with outgroup corresponding to the monophyletic monogenean analysis under branch. Ancyroc.: Ancyrocephalidae.

ate analyses of the two groups Monopisthocotylea and Polyopisthocotylea could be accurately performed.

#### 4.2. Implications for monopisthocotylean phylogeny

The present analysis confirmed monophyly of each of the families Capsalidae, Monocotylidae, Ancyrocephalidae, and Diplectanidae.

For the Capsalidae our sampling comprised six genera belonging to four of the five subfamilies recognised [27]; *Benedenia lutjani* and *Entobdella australis* belong to Benediniinae; *Trochopus pini* to Trochopodinae; *Capsala onchidiocotyle* and *Tristoma integrum* to Capsalinae; and *Encotyllabe caballeroi* to

Encotyllabinae. In our analysis, the Capsalinae grouped together. *Entobdella australis* did not group with the other Benediniinae, *B. lutjani*, but appeared closer to Capsalinae. Regarding the unique features of *E. australis* comparing with other Benediniinae, such as absence of septa in the discs or shape and length of the disc, Bychowsky [28] suggested that the unification of *Benedenia* and *Entobdella* in the same subfamily was not justified and that therefore *Entobdella* should be in another subfamily. Our molecular analysis was congruent with this suggestion and therefore not with Whittington and Kearns's interpretation of the subfamily Benediniinae [29]. Considering that this subfamily contains 15 other genera [29], additional molecular

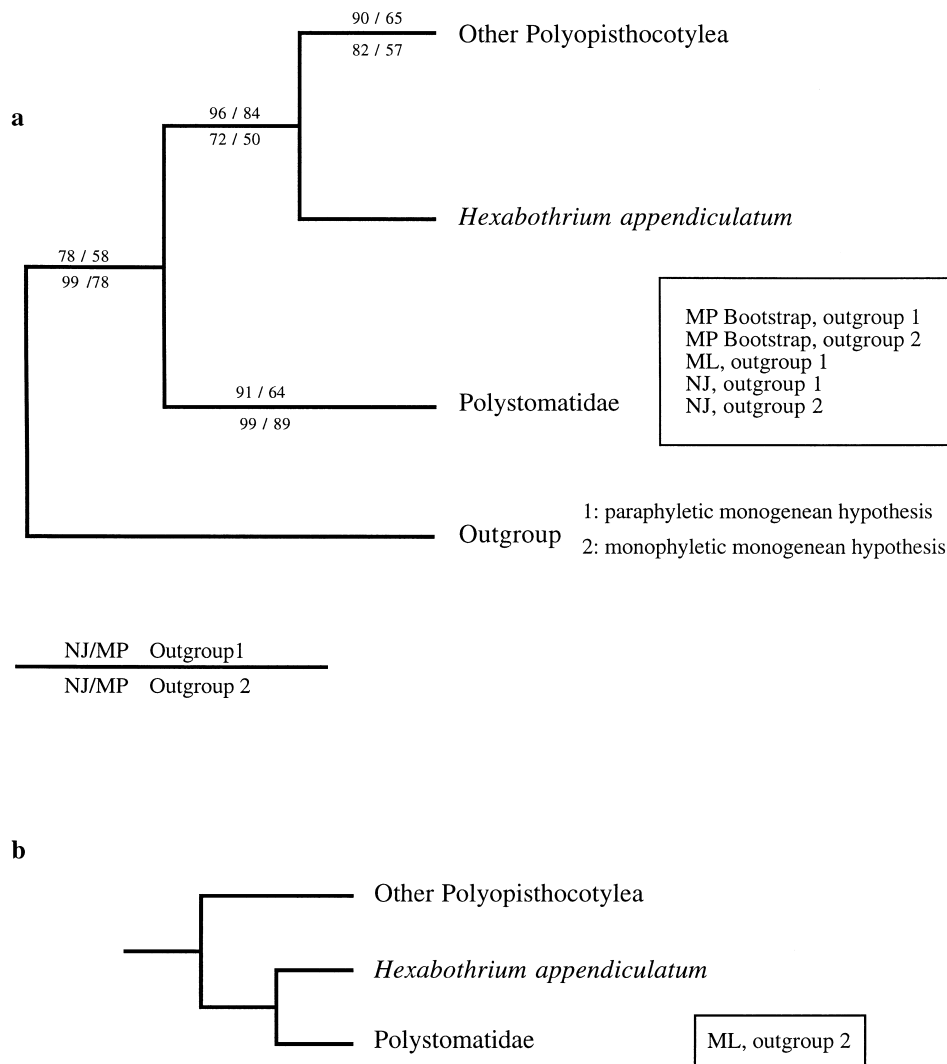


Fig. 4. Polyopisthocotylea: relationships between basal groups with various methods and outgroups. Methods and outgroups are indicated in boxes. (a) Topology obtained in all analyses but one. Bootstrap values are indicated, NJ/MP values with outgroup corresponding to the paraphyletic monogenean analysis above branch, and NJ/MP values with outgroup corresponding to the monophyletic monogenean analysis under branch. (b) Single alternative topology, found with ML analysis and with outgroup corresponding to the monophyletic monogenean analysis.

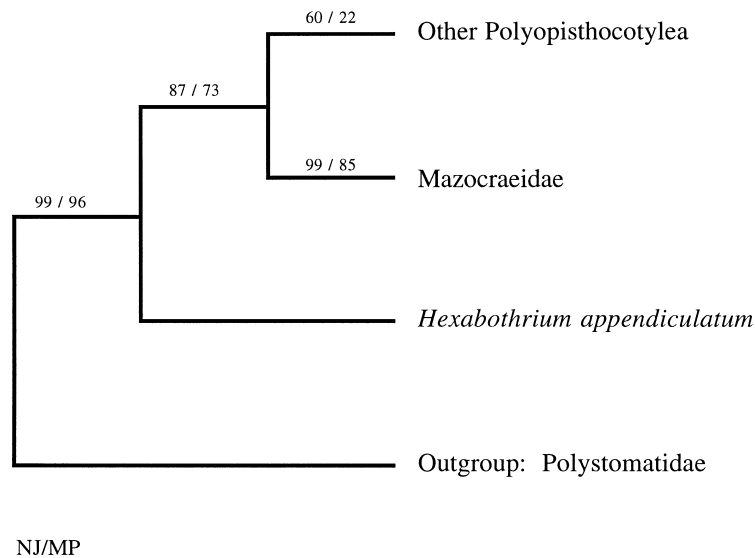


Fig. 5. Polyopisthocotylea: relationships between basal groups obtained with outgroup limited to the Polystomatidae. Same topology found with all methods (NJ, MP, their bootstraps, and ML). Bootstrap values are indicated, NJ/MP values.

data should be obtained to confirm the present analysis.

For the Monocotylidae, our sampling comprised four genera belonging to four of the six subfamilies recognised [30], with *Neoheterocotyle rhinobatidis* (Heterocotylinae), *Calicotyle palombi* (Calicotylinae), *Merizocotyle icopae* (Merizocotylinae), and *Troglocephalus rhinobatidis* (Dasybatotreminae). Our analysis showed monophyly of the Monocotylidae and therefore removes the uncertainty of a previous molecular analysis [1]. Although our analysis supported a grouping of Dasybatotreminae and Heterocotylinae, uncertainty remains for relationships of *C. palombi* and *M. icopae*; similarly, a morphological analysis indicated a basal polytomy of the various subfamilies constituting the Monocotylidae [30].

The Ancyrocephalidae Bychowsky, 1937 appeared a monophyletic group in the present analysis, but with only three genera sampled. Kritsky and Boeger [31] reduced the ancyrocephalids to subfamilial status within the family Dactylogyridae Bychowsky, 1933. The Ancyrocephalidae is considered as a large “catch-all” group [31] and has been augmented recently by more than 100 genera [32]. In view of the high probability of invalid phylogenetic reconstruction when only a small sample of a large group is analysed [33], an error increased if the group is likely to be paraphyletic [31], discussion about phylogenetic relationships within the ancyrocephalids is here avoided.

The Diplectanidae, represented only by two taxa, appeared monophyletic.

Previous studies of monogenean systematics have utilised various types of characters. Schemes have been established according to morphological charac-

ters [25, 27, 28, 34–38], spermatological characters [2, 3], chaetotaxy of larvae [39], combined morphological and spermatological characters in cladistic studies [7, 12, 13], and molecular characters [1, 9].

With regard to the relative positions of families in the phylogeny, our molecular study found four different topologies (Fig. 2) with different outgroups and methods, but one topology (Fig. 2a) was found with both outgroups and displayed relatively high bootstrap values. This common topology shows the following relationships: (((Udonellidae, Capsalidae), Monocotylidae), (Diplectanidae, Ancyrocephalidae)), i.e. a grouping of udonellids, capsalids and monocotylids, sister-group to a grouping of diplectanids and ancyrocephalids. A comparison of the present analysis with certain previous classifications is hampered by the usual problems of translation of traditional classifications into cladistic schemes. Moreover, comparisons of schemes within the monogenean are biased by (a) the non-monogenean status of *Udonella* in most previous classifications; (b) inclusion of Udonellidae in the functional outgroup in certain analyses [12, 13]. All classifications cited above are in accordance with the grouping of Capsalidae and Monocotylidae except [2, 3, 12, 13, 35, 39]. It is noteworthy, as Littlewood et al. [9] recognised, that the grouping of Udonellidae, Capsalidae and Monocotylidae corresponds to pro parte Capsaloidea Price, 1936 as interpreted by Sproston [38]. The Gyrodactylidae (not studied here) were also included in the grouping of Udonellidae, Capsalidae and Monocotylidae after an analysis of 18S rDNA [9], but not in the Capsaloidea. The Microbothriidae (not studied here) were included in the Capsaloidea, but lack molecular information;

assessment of the validity of the Capsaloidea therefore requires additional sequences.

Boeger and Kritsky [12] have grouped the Diplectanidae and Dactylogyridae (corresponding partly to our Ancyrocephalidae) with the Pseudomurraytrematidae in the most terminal branch of their monopisthocotylean tree. The grouping of Ancyrocephalidae and Diplectanidae is in accordance with their scheme.

In terms of spermatozoal structure, the Capsalidae and *Udonella* correspond to type 2 as defined by Justine et al. [40] (two axonemes, no microtubules),

and the Monocotylidae correspond to type 3 (one complete axoneme and one incomplete axoneme, plus microtubules). Watson [41] has suggested that types 2 and 3 should be combined in a type 2/3. Therefore, the grouping Udonellidae, Capsalidae and Monocotylidae would correspond to sperm structure 2/3, and the grouping Ancyrocephalidae and Diplectanidae to sperm structure 4 (one axoneme, no microtubules). The topology with Udonellidae, Capsalidae and Monocotylidae sister-group to Ancyrocephalidae and Diplectanidae (Fig. 2a) thus presents good agreement with spermatological data. In

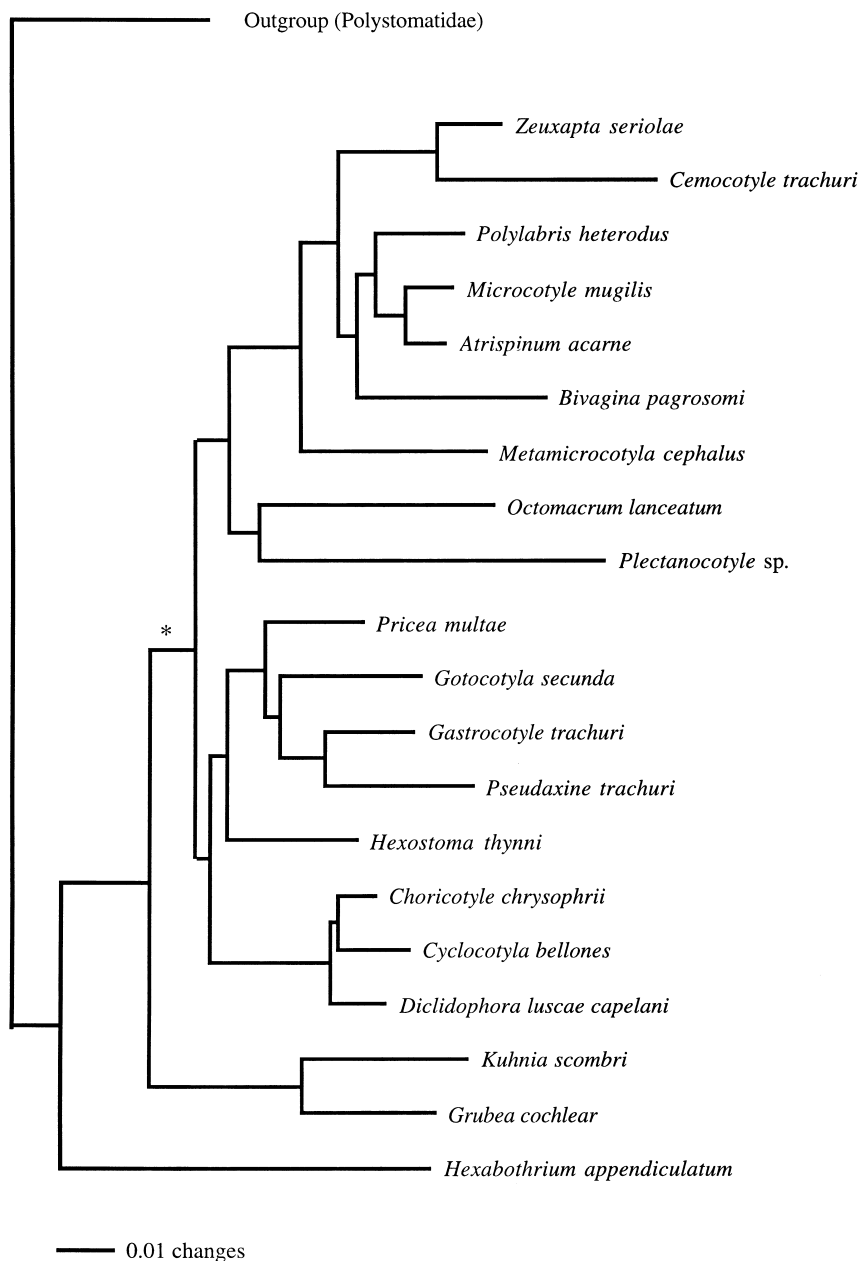


Fig. 6. Polyopisthocotylea: tree obtained with outgroup limited to the Polystomatidae. Tree obtained in neighbour-joining analysis with 20 partial 28S rDNA sequences of Polyopisthocotylea. Relationships between the clades within the node (\*) higher than the mazocraeids were not resolved.

contrast, the topology in Fig. 2b, with Ancyrocephalidae and Diplectanidae making a paraphyletic group basal to the grouping Udonellidae, Capsalidae and Monocotyliidae, contradicts spermatozoological data, because the single axoneme structure cannot be interpreted as plesiomorphic within the Monopisthocotylea, and contradicts morphological data for which the grouping Ancyrocephalidae and Diplectanidae are terminal taxa [12].

#### 4.3. Implications for polyopisthocotylean phylogeny

Our analysis indicated that the Polystomatidae are the sister-group of the remaining Polyopisthocotylea, which is in accordance with morphological cladistic analyses [12, 13] and previous molecular studies [9]. Lebedev [25] and Boeger and Kritsky [12, 13] divided the monogeneans into three subclasses, namely the Polyonchoinea Bychowsky, 1937 (corresponding to our Monopisthocotylea), the Oligonchoinea Bychowsky, 1937 (our Polyopisthocotylea pro parte) and the Polystomatoinea Lebedev, 1986 (Polystomatidae and Sphyrnauridae). Recently, Boeger and Kritsky [7] proposed the new subclass Heteronchoinea to include the Polystomatoinea and Oligonchoinea; this is in perfect agreement with the results of our molecular analysis. In Table 1 we tried to summarise the equivalencies between the various terminologies used for the higher taxa of monogeneans.

Our results on the internal relationships for the Polystomatidae suggested *Polystoma integerrimum* as sister-group of the other Polystomatidae sampled (genera *Polystomoides* and *Neopolystoma*) and thus established sister-group relationships between *Polystoma*, parasite of amphibians, and the two other genera, parasites of chelonians. A study using 18S rDNA found relationships similar to our results, with two sister-groups, respectively polystomatids of chelonians and hippopotamus, and polystomatids of amphibians and lungfish (Sinnappah ND, 1998. Thesis. University of Perpignan, France). Internal relationships of polystomatids of chelonians have been detailed [42].

*Hexabothrium appendiculatum*, a parasite of Chondrichthyes, presented an early divergence among Polyopisthocotylea as shown by its basal position in all analyses. This hexabothriid appeared as the sister-group of all other non-polystomatid Polyopisthocotylea. Although this species was the single representative of the Hexabothriidae in our study, it is nomenclaturally significant as the type species of the genus. A morphological cladistic analysis [43] placed *Hexabothrium* at a basal position in the tree of Hexabothriidae. Our analysis confirms the basal position of the Hexabothriidae in Boeger and Kritsky's scheme [7, 12, 13], in which the

Chimaericolidae (not treated in our study) was the only more basal group.

The two mazocraeids, *Kuhnia* and *Grubea*, were found sister-group to all other polyopisthocotyleans. It is of general knowledge that most members of the family Mazocraeidae are parasitic in the relatively early divergent teleost fish family Clupeidae [28, 37] and a mazocraeid-clupeid co-evolution has been suggested [28]. However, the two species sampled in our study for the family Mazocraeidae are parasites of Scombridae. As scombrids and clupeids, although phylogenetically distant, are both pelagic fishes, this suggests that mazocraeids are primarily parasites of clupeids, with a later host-switching to the scombrids. Clupeids are relatively early divergent, but scombrids belong to the most derived teleosts [33, 44]. In our further analysis, for host-parasite discussions, the Mazocraeidae are here accepted as primarily parasites of Clupeidae.

Within the terminal group, our analysis could not resolve phylogenetic relationships. However, some groups were found in all analyses and were supported by relatively high bootstrap values: these are the Dicliphoridae, the Gastrocotylinea, and the Microcotylinea. Relationships between these groups were not resolved. The monophyly of the suborders Gastrocotylinea and Microcotylinea was recently confirmed in a morphological cladistic analysis [7].

Our results are closely similar to the bootstrap analysis, based on morphological data, presented in fig. 4 of Boeger and Kritsky [7], in which Hexabothriidae and Mazocraeidae were basal groups and a terminal polytomy included all other families. It differs, however, for the position of *Plectanocotyle*, considered a member of the mazocraeid branch in their analysis, but placed within the terminal polytomy in our analysis.

Non-monophyly of the Microcotylidae was suggested by our results; this confirms a result of morphological analyses [7, 12].

#### 4.4. Implications for host-parasite co-evolution

Congruence between our results on polyopisthocotyleans and known host phylogeny (Fig. 7) allows identification of two major nodes. The hexabothriid node corresponds to the Chondrichthyes/Osteichthyes node, and the mazocraeid node to a major node within the Teleostei, the Clupeomorpha/Euteleostei node [33, 45].

A low degree of resolution has been obtained for the nodes of Polyopisthocotylea more terminal than the Hexabothriidae and Mazocraeidae, irrespective of outgroup sampling. It has been suggested that a radiation process may be identified by low bootstrap values in all the internal branches surrounding the radiation point in the tree [46]; thus, the low resolution

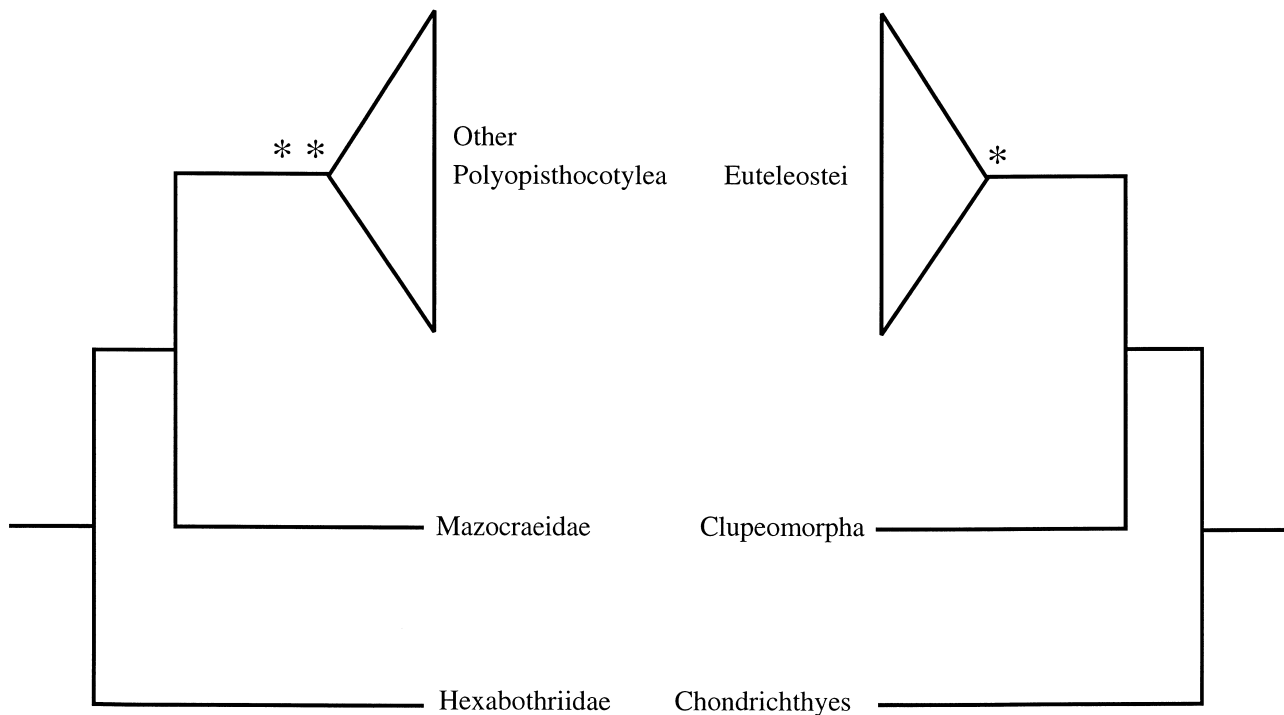


Fig. 7. Congruence between parasite polyopisthocotylean molecular phylogeny (this study), left, and host vertebrate phylogeny, right. \*Radiation event of hosts, dated Cretaceous; \*\*Possible radiation event of parasites.

found for the terminal polyopisthocotyleans could suggest radiation of this group. The cladistic morphological analysis of Boeger and Kritsky [13] (fig. 4 in [7]) also presents a polytomy in the branches more terminal than the Mazocraeidae, and this is also seen in previous, non-cladistic, schemes [27,28]. Classical authors [27,28,35] often claimed that terminal polyopisthocotyleans and teleosts co-evolved. Cladistic morphological and molecular analyses of the teleosts [33,44] showed lack of resolution in the Euteleostei and particularly in the Percomorpha which was attributed to a radiation event, dated by palaeontological data to the Cretaceous. Thus, it may be that two radiation events took place, both in the Euteleostei and in the terminal Polyopisthocotylea after the Cretaceous, but our molecular evidence does not support any co-evolution scheme within these branches. Also, instead of signalling a radiation process, lack of resolution could simply indicate lack of phylogenetic signals for these terminal branches.

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