Université de Neuchâtel, Faculté des Sciences, Institut de Zoologie

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Titre français:
Analyse systématique moléculaire des Proteocephalidea Mola, 1928 (Eucestoda) basée sur des séquences d'ADN ribosomal

Thèse presentée par Marc P. Zehnder

Forme réduite de la thèse pour l'obtention du grade de docteur ès sciences de l'Université de Neuchâtel

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Molecular systematic analysis of the Proteocephalidea Mola, 1928 (Eucestoda) based on mitocchondrial and nuclear ribosomal DNA sequences

de M. Marc Zehnder

UNIVERSITÉ DE NEUCHÂTEL

FACULTÉ DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel sur le rapport des membres du jury,

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Molecular systematic analysis of the order Proteocephalidea (Eucestoda) based on mitochondrial and nuclear rDNA sequences☆

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Abstract

Two ribosomal DNA sequences were used to infer phylogenetic relationships among the Eucestoda order Proteocephalidea. A 437 bp portion of the 16S mitochondrial and a 1149 bp 5′ portion of the nuclear large sub-unit rRNA molecule were sequenced for 53 proteocephalidean cestodes (representing nine subfamilies and 22 genera) and for one outgroup species. Parsimony and distance-based analyses of the two databases, alone and combined, failed to support the monophyly of the two traditionally accepted families, of numerous subfamilies (with the exception of the Rudolphiellinae and Othinoscolescinae which were validated in our analysis) and of various genera, including the genus *Nomimoscolex* (Woodland), *Ophiotaenia* (La Rue) as well as the type genus *Proteocephalus* (Weinland). Palacarctic *Proteocephalus* species nevertheless constituted a well-defined clade. The two genes globally yielded compatible results; however, the nuclear ribosomal gene provided a better resolution of relations among Proteocephalidea.

Keywords: Eucestoda; LsrRNA; Molecular systematics; Proteocephalidea; *Proteocephalus*; Phylogeny: 16S rRNA

1. Introduction

Tapeworms of the order Proteocephalidea are parasitic in freshwater fishes, amphibians and reptiles. They are diagnosed by the following features: distinct external segmentation, one set of gonads per segment, numerous testes (between 20 and 400), a lateral genital pore, a bilobate ovary at the posterior end of proglottis, follicular and lateral vitellaria, and a scolex with four suckers. An apical organ, such as a sucker, or rarely an armed rostellum, may be present. Both Rego et

☆Note: Nucleotide sequence data reported in this paper are available in the EMBL, GenBank® and DDJB databases under the accession numbers AJ 238826 to 238829, AJ 23881 to 238832, AJ 238834 to 238837, AJ 388590 to 388638, AJ 389477 to 389524. Alignments are available from the following URL: http://www.herbaria.harvard.edu/treebase. Under the study accession number 5389 and the matrix accession numbers M543 and M544. This is part of the PhD thesis of the first author.

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Table 1
List of taxa included in the dataset with host, geographical origin and reference of mounted vouchers from the Geneva Natural History Museum (INVE) collection, when available. Outgroup: Tetraphyllidea Gen. sp. ex *Squalus acanthias*, North Sea

From Brazil, Itacoatiara, Province Amazonas:
Amphoteromorphus parkamoo Woodland, 1935 ex *Paulicea luetkeni*, 11.10.1995. INVE 22245
Amphoteromorphus piraeeba Woodland, 1934 ex *Brachyplatystoma filamentosum*, 07.10.1995. INVE 22239
Crepidobothrium eirasi Rego and de Chambrier, 1995 ex *Phractocephalus hemioliopterus*, 17.10.1995
Endorchis piraeeba Woodland, 1934 ex *Brachyplatystoma filamentosum*, 7.10.1995. INVE 21738
Ephedrocephalus microcephalus Diesing, 1850 ex *Phractocephalus hemioliopterus*, 5.10.1995. INVE 21910
Goezeella siluri Fuhrmann, 1916 ex *Pinirampus pirinampu*, 2.10.1995. INVE 21887
Proteocephalus pirarara (Woodland, 1935) ex *Phractocephalus hemioliopterus*, 1.10.1995. INVE 22106
Megathylacus brooksi Pavanelli and Rego, 1985 ex *Paulicea luetkeni*, 2.10.1995. INVE 21874
Proteocephalus hemioliopteri (Rego, 1984) ex. *Phractocephalus hemioliopterus*, 13.10.1995. INVE 21889
Nomimoscolex admonticellia (Woodland, 1934) ex *Pinirampus pirinampu*, 30.9.1995. INVE 21870
Nomimoscolex dorad (Woodland, 1935) ex *Brachyplatystoma flavicans*, 11.10.1995. INVE 22269
Nomimoscolex lenha (Woodland, 1933) ex *Sorubimychthys planiceps*, 2.10.1995. INVE 21740
Nomimoscolex piraeeba Woodland, 1934 ex *Brachyplatystoma filamentosum*, 13.10.1995. INVE 22284
Nomimoscolex sp. ex *Brachyplatystoma vaillanti*, 2.10.1995. INVE 22298
Nomimoscolex sudobim Woodland, 1935 ex *Pseudoplatystoma fasciatum*, 12.10.1995. INVE 21969
Othinoscolex lenha Woodland, 1933 ex *Sorubimichthys planiceps*, 19.10.1995. INVE 22021
Peltidocotyle sp. specimens ex *Paulicea luetkeni*, 2,4. 10.1995. INVE 21912 and INVE 22373
Rudolphiella piracatinga (Woodland, 1935) ex *Calophysus macropterus*, 1.10.1995. INVE 19868
Zygobothrium megacephalum Diesing, 1850 ex *Phractocephalus hemioliopterus*, 1.10.1995. INVE 21846
From Brazil, Domingos Martins, Espiritu Santo Province:
Ophiotaenia jarara Fuhrmann, 1927 ex *Bothrops jararaca*, 16.10.1989. INVE 12393
From Argentina:
Rudolphiella sp. ex *Luciopimelodus pati*, Rio Parana, Corrientes, Corrientes Province, 30.07.1997. INVE 24668
From Paraguay:
Ageneiella brevifilis de Chambrier and Vaucher, 1999 ex *Ageneiosus brevifilis*, Rio Paraguay, San Antonio, Central Province, 3.11.1995. INVE 21841
Choanoscolex abscisus (Riggenbach, 1896) ex *Pseudoplatystoma coruscans*, San Antonio, Rio Paraguay, Central Province, 14.10.1989
Gibsoniela meursaulti de Chambrier and Vaucher, 1999 ex *Ageneiosus brevifilis*, San Antonio, Central Province, 3.11.1995. INVE 21839
Monticellia coryphicephala (Monticelli, 1891) ex *Salminus maxillosus*, General Diaz, Neembucu Province, 18.10.1989. INVE 17984
Nomimoscolex chubbi (Pavanelli and Takemoto, 1995) ex *Gymnotus carapo*, 18 km south of San Lorenzo, Neembucu Province, 17.10.1989. INVE 20351
Nomimoscolex lopesi Rego, 1989 ex *Pseudoplatystoma fasciatum*, San Antonio, Central Province, 06.11.1995. INVE 21963
Nomimoscolex matogrossensis Rego and Pavanelli, 1990 ex *Hoplias malabaricus*, Arroyo Tapiracuayi, San Pedro Province, 24.10.1989. INVE 17913
Peltidocotyle rugosa Diesing, 1850 ex *Pseudoplatystoma fasciatum*, Rio Paraguay, San Antonio, Central Province, 2.10.1995. INVE 22374
Ophiotaenia paraguayensis (Rudin, 1917) ex *Hydrodynastes gigas*, Chaco Province, 1.11.1989. INVE 16927
Proteocephalus renaudi de Chambrier and Vaucher, 1994 ex *Platydoras armatulus*, 7.11.1991. INVE 17894
Ophiotaenia sanbernardinensis (Rudin, 1917) ex *Helicops leopardinus*, Estancia Santa Sofia, Conecpcion Province, 4.10.1989. INVE 18951
Spatulifer maringaensis Pavanelli and Rego, 1989 ex *Sorubim lima*, San Antonio, Rio Paraguay, Central Province, 14.10.1989
From the United States:
Proteocephalus ambloplitis (Leidy, 1887) ex *Lepomis macrochirus*, Pearl River, Mississipi, 6.6.1996
Ophiotaenia grandis La Rue, 1911 ex *Agkistrodon piscivorus*, Biloxi, Mississipi, 4.9.1997
From Europe:
Ophiotaenia europaea Odening, 1963 ex *Natrix maura*, Hérault, France, 16.5.1990
Proteocephalus exiguus La Rue, 1911 ex *Coregonus* sp. Lake of Bienne, Switzerland, 6.1996
Proteocephalus filicollis (Rudolphi, 1802) ex *Gasterosteus aculeatus*, Stirling, Scotland

Proteocephalus macrocephalus (Creplin, 1852) ex *Anguilla anguilla*, Ohre River, Czech Republic, 3.10.1994
Proteocephalus osculatus (Goeze, 1782) ex *Silurus glanis*, Orlik Water Reserve, Stedronin, Czech Republic, 12.9.1996
Proteocephalus percae (Müller, 1780) ex *Perca fluviatilis*, Lake of Neuchâtel, Switzerland, 6.1996
Proteocephalus pollanicola Gresson, 1952 ex *Coregonus pollan*, Toome Bay, Northern Ireland, 1.8.1996
Proteocephalus torulosus (Batsch, 1786) *ex Leuciscus cephalus*, Rokytná River, Czech Republic,13.5.1995
Silurotaenia siluri (Batsch, 1786), ex *Silurus glanis*, Orlik water reserve, Czech Republic, 13.5.1998

From Japan:

Gangesia parasiluri Yamaguti, 1934 ex *Silurus asotus*, Lake Suwa, Nagano Prefecture, 28.6.1996
Paraproteocephalus parasiluri (Zmeev, 1936) ex *Silurus asotus*, Lake Suwa, Nagano Prefecture, 28.6.1996
Proteocephalus midoriensis Shimazu, 1990 ex *Lefua echigonia*, Midori, Iiyama City, Nagano Prefecture, 20.7.1996
Proteocephalus plecoglossi Yamaguti, 1934 *ex Plecoglossus altivelis*, Lake Biwa, Shiga Prefecture, 2.5.1992
Proteocephalus tetrastomus (Rudolphi, 1810) *ex Hypomeusus nipponensis*, Lake Suwa, Nagano Prefecture, 31.5.1996

From Australia:

Ophiotaenia gallardi (Johnston, 1911) *ex Notechis scutatus*, Melbourne, 12.6.1996

From Africa:

Ophiotaenia ophiodex Mettrick, 1960 ex *Causus maculatus*, Ivory Coast, 6–7.1998
Acanthotaenia sp. ex *Varanus exanthematicus*, Ghana, 1998

al. [1] and Hoberg et al. [2] with morphological data, and Mariaux [3] with molecular data have defined a number of synapomorphies for the order and assessed its monophyly. Over 300 species have been described in two families: Proteocephalidae and Monticelliidae, containing 12 subfamilies and 46 genera [4].

The first proteocephalidean representative, *Taenia* (= *Proteocephalus*) *percae* was described by Müller in 1780. The genus *Proteocephalus* was erected by Weinland in 1858 for fish and amphibian parasites. La Rue [5, 6] erected two families: Proteocephalidae and Monticelliidae in his account of the group. He established the latter family to comprise the species *Tetracotylus* (= *Monticellia*) *coryphicephala* Monticelli, 1891 characterised by the cortical position of uterus, ovary and testes. Woodland [7–15] described numerous species and genera of Monticelliidae from South America and Africa. His system included eight subfamilies, the taxonomy of which was based on the disposition of reproductive organs relative to the longitudinal muscle bundles. Freze [16] subdivided this group more extensively, especially in adding a third family Ophiotaeniidae, parasitic in amphibians and reptiles. In a widely accepted move, however, Brooks [17] synonymised the Ophiotaeniidae with the Proteocephalidae. Schmidt [18] followed the classification of Freze with some exceptions: he did not recognise the Ophiotaeniidae, and the Monticelliidae was characterised by the cortical location of vitelline follicles and thus included the Zygobothriinae. Rego [4] accepted the current classification in general terms but stressed that the finding of intermediate forms between Monticelliidae and Proteocephalidae makes Woodland's scheme of classification inadequate. He thus re-examined the taxonomy of the group, suppressed the Monticelliidae, and included all taxa within the single family Proteocephalidae according to criteria based on holdfast structure [19].

Several phylogenetic investigations of Proteocephalidea based on cladistic analyses of morphological characters have been performed by Brooks [17, 20], Brooks and Rassmussen [21], and Brooks et al. [22] (at a higher level that included Proteocephalidea). More recently, Rego et al. [1] examined the order at the subfamilial level. Results supported the monophyly of the Proteocephalidea but neither family, as currently conceived, was monophyletic. These cladistic analyses, however, were limited by the lack of information on the life-cycles of Monticelliidae, the uneven quality of morphological and biological data in the literature and a shortage of reliable and complete data for many taxa, mainly those described by Woodland [23]. The monophyly of the order nevertheless remains uncontested [1–3].

The purpose of the present work is to examine phylogenetic relationships among representatives

of Proteocephalidea, and within the genus *Proteocephalus* in particular, using DNA sequences as an independent source of information to avoid the constraints mentioned above. This should provide a basis for inferring their phylogeography and the history of their host associations. The mitochondrion-encoded 16S rRNA and the nuclear lsrRNA genes were investigated since they were assumed to evolve at an adequate rate, and since they are both easily amplified by PCR [24]. The lsrRNA was selected as its rate of evolution is greater than that of ssrRNA, which has been shown to be uninformative within the order Proteocephalidea [3].

2. Materials and methods

2.1. Deoxyribonucleic acid preparation, PCR and sequencing

Cestodes collected in this study (Table 1) were either frozen or preserved in 70% or 95% ethanol. Extraction was done by incubating the tissue (two to three segments for the larger forms, up to whole individuals for the smallest forms) in 1 ml of extraction buffer (10 mM Tris, 100 mM Na_2EDTA, 100 mM NaCl, pH 8.0, 0.05% SDS, 200 μg Proteinase K) for 4 h at 55°C. A treatment with RNAse A at a final concentration of 100 $\mu g\ ml^{-1}$ for 30 min and a phenol–chloroform–isoamyl-alcohol (49.5:1:49.5) extraction was then performed. Deoxyribonucleic acid was precipitated by adding one volume of 70% ethanol, dried and re-suspended in 50 μl TE (10 mM Tris, 1 mM Na_2EDTA, pH 8.0). Primers used to amplify approximately 440 bp of the large mitochondrial rRNA (16S) molecule were designed on the basis of conserved residues among taxa from different phyla. We used the following PCR primers: 16S-5′ (5′-*CAG GAA ACA GCT ATG AAC* GNC TGT TTA YYA AAA ACA-3′) and 16S-3′ (5′-*TGT AAA ACG ACG GCC AGT* CGG TCT TAA CTC ARN TCA-3′). The nucleotides in italics correspond to the sM13 reverse and sM13 (−21) forward primer sites of M13 bacteriophage, added to facilitate automated sequencing. Polymerase chain reaction fragments were obtained using a MJ-Research PTC-100 thermo-cycler with the following parameters: 35 cycles (45 cycles when yield was low) with 30 s DNA denaturation at 94°C, 30 s primer annealing at 45°C, and 1 min at 72°C for primer extension. The following primer pair was used to amplify a 5′ portion of approximately 1170 bp of the lsrRNA gene: 28S-5′ (5′-TAC CCG CTG AAC TTA AGC ATA T-3′) and 28S-3′ (5′-CTC CTT GGT CCG TGT TTC AAG AC-3′) (the primer sequences were available in the literature [24, 25]). Cycling conditions were as for 16S, but annealing temperature in this case was 60°C. Polymerase chain reaction conditions were the following: 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 100 μM dNTPs. *Taq* EXTRA-POL 1 (Eurobio) DNA polymerase (1.5 U) was used per reaction. Polymerase chain reaction products were checked for size on 0.8% w/v agarose gel and purified on QIAquick columns (Qiagen). Polymerase chain reaction fragments were directly sequenced for both strands by cycle sequencing using Thermosequenase (Amersham) and a LI-COR 4000 (MWG) automated sequencer. All sequence data from the present research are deposited with EMBL/Genbank data library under accession numbers AJ 238826 to 238829, AJ 238831 to 238832, AJ 238834 to 238837, AJ 388590 to 388638, AJ 389477 to 389524, and the alignments are available from the following URL: http://www.herbaria.harvard.edu/treebase.

2.2. Phylogenetic onalyses

Multiple alignment of a fragment of the 16S and lsrRNA genes was computed using CLUSTAL W [26]. Alignments were slightly modified by hand in GDE 2.2 [27]. All regions of ambiguous alignment were removed from the dataset prior to phylogenetic analysis. Gaps were treated as missing data. Phylogenies based on the principle of parsimony were computed using PAUP* version 4.0b2 (DL Swofford. Phylogenetic analysis using parsimony*, and other methods. Sunderland, MA: Sinauer Associates, 1999). Heuristic searches were performed and the tree bisection–reconnection

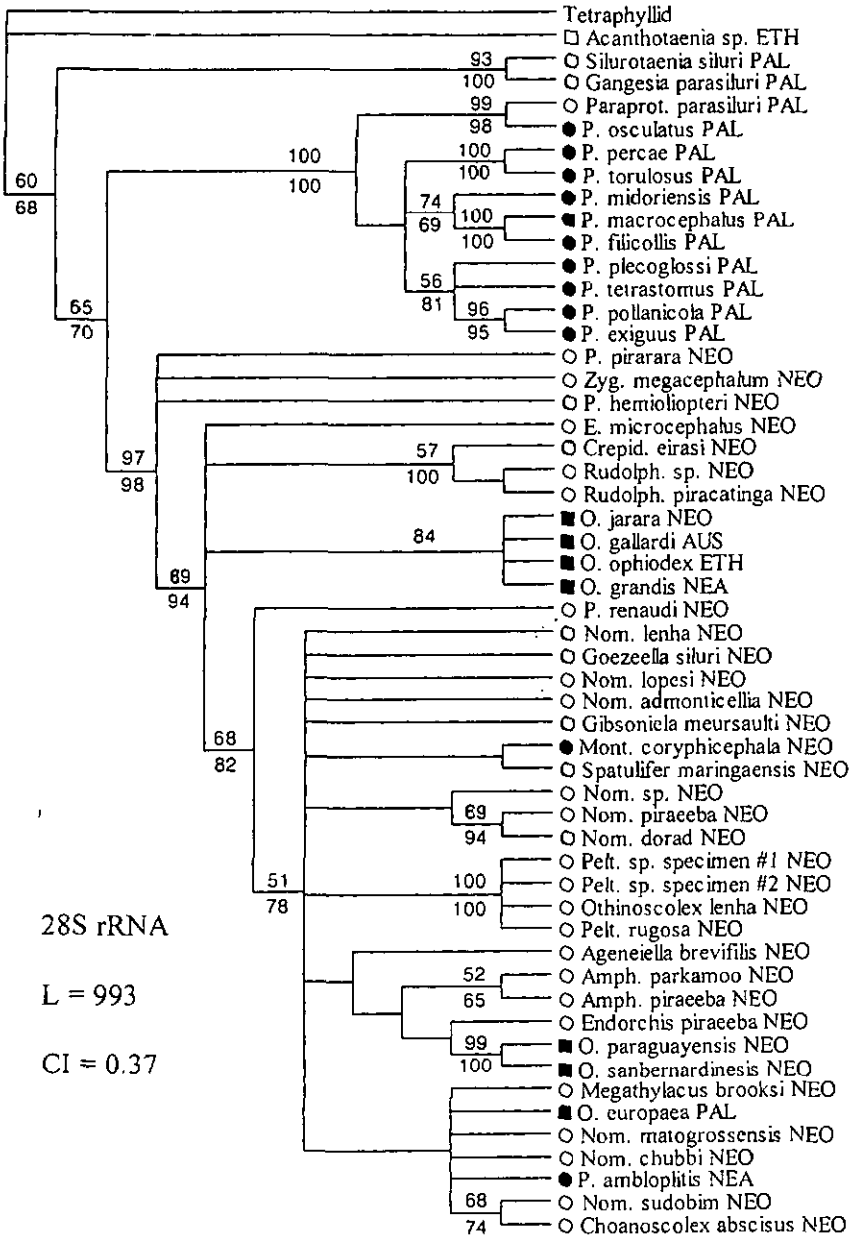

Fig. 1. Strict consensus tree of 75 equally parsimonious trees obtained from a parsimony analysis of the nuclear 28S rRNA data set. Alignment gaps were treated as missing. One-hundred heuristic searches were conducted, retaining 50 minimal trees per replicate. The shortest trees were swapped in a second heuristic search. A tetraphyllidean was used as outgroup. Bootstrap values obtained for 100 replicates of 10 heuristic searches are indicated above the internodes. Figures under the internodes indicate the bootstrap values obtained from 100 replicates using the neighbor-joining distance algorithm. Geographical origin is designated as follows: NEO = Neotropical; NEA = Nearctic; PAL = Palaearctic; AUS = Australian; ETH = Ethiopian. Hosts are designated as follows: ○ = siluriform fishes; ● = other teleost fishes, ■ = snakes, □ = saurians. Abbreviations: Amph. = *Amphoteromorphus*; Cr. = *Crepidobothrium*; E. = *Ephedrocephalus*; Mont. = *Monticellia*; Nom. = *Nomimoscolex*; O. = *Ophiotaenia*; Paraprot. = *Paraproteocephalus*; Pelt. = *Peltidocotyle*; P. = *Proteocephalus*; Rudolph. = *Rudolphiella*; Zyg. = *Zygobothrium*.

(TBR) algorithm was in effect. Indications of support for various clades were obtained by bootstrapping [28]. One-hundred re-sampled datasets were analysed using the heuristic tree searching option of PAUP. Distance-based phylogenies were produced using the DNADIST and NEIGHBOR programs of PHYLIP version 3.572 (J. Felsenstein. PHYLIP—Phylogeny inference package, version 3.2). Sequence data were reduced to a distance matrix using the Kimura 2-parameter model for nucleotide substitution [29]. The default transition/transversion ratio of 2 was used. Bootstrap analyses were done using SEQBOOT of PHYLIP: 100 bootstrapped datasets were constructed and analysed with DNADIST and NEIGHBOR programs. A consensus tree was obtained using CONSENSE. The partition-homogeneity test (PHT) implemented with PAUP* 4.0b2 was used to assess the concordance of the 16S and 28S datasets, using 100 replicates with MAXTREES set to 100. Ambiguously aligned and uninformative characters were excluded from the PHT [30, 31]. The Templeton compatibility test, implemented with PAUP*, was used to evaluate the significance of the difference in length observed when comparing constrained and unconstrained most parsimonious trees.

3. Results

Proteocephalidean cestodes of 53 species, representing nine subfamilies and 22 genera, were analysed in this study. We included a tetraphyllidean species for tree-rooting purposes. A 1149 bp 5′ fragment of the nuclear lsrRNA gene was thus sequenced for 54 species. Due to problematic alignment, 131 sites were excluded from the analysis. Among the remaining 1018 positions, 378 were variable and 248 were phylogenetically informative. Using this gene in parsimony analyses, we found 75 shortest trees with a length of 993 steps and a consistency index (CI) of 0.37 (Fig. 1). For the mitochondrial 16S rRNA fragment, a partial sequence of 437 nucleotides was obtained for 53 cestodes (*Proteocephalus hemioliopteri* was not included in this data matrix). Seventeen positions were removed due to problematic alignment. The resulting matrix thus comprised 420 bp, of which 168 positions were variable and 130 were informative. This database yielded two shortest trees of 861 steps with a CI = 0.28, using parsimony. In addition to this lower consistency index, weaker bootstrap values were observed (Fig. 2).

The overall tree resolution and topologies in both distance and parsimony trees are highly congruent, regardless of the database considered. All analyses are consistent in yielding the following results: the monophyly of the order Proteocephalidea is supported; however, neither of the two families as currently conceived, the Proteocephalidae and Monticelliidae [4], appears as a natural group, both are paraphyletic. Moreover, the monophyly of most subfamilies is not supported in our analyses.

The subfamily Othinoscolescinae, with *Peltidocotyle rugosa* from *Pseudoplatystoma fasciatum*, *Othinoscolex lenha* from *Sorubimichthys planiceps*, and two additional *Peltidocotyle* species from *Paulicea luetkeni* is, nevertheless, well supported by 28S sequences. Genetic distances, measured as the percentage of divergent residues, demonstrate a strong similarity among the three latter species (two *Peltidocotyle* spp. and *Othinoscolex*). Analyses of yet another DNA sequence comprising the 5.8 rRNA and adjacent internal transcribed spacer 2 (ITS2) yielded highly compatible results (data not shown).

The subfamily Rudolphiellinae, which comprises a single genus, likewise appears as a natural group on the basis of analyses of the two following species: *Rudolphiella* sp. and *Rudolphiella piracatinga*.

4. Discussion

The two genes we used in the present systematic analysis of Proteocephalidea performed differently in solving phylogenetic relations within the order. Overall tree resolution, bootstrap values and consistency indices were higher using lsrRNA (CI = 0.37) compared with 16S sequences (CI = 0.28). The number of phylogen-

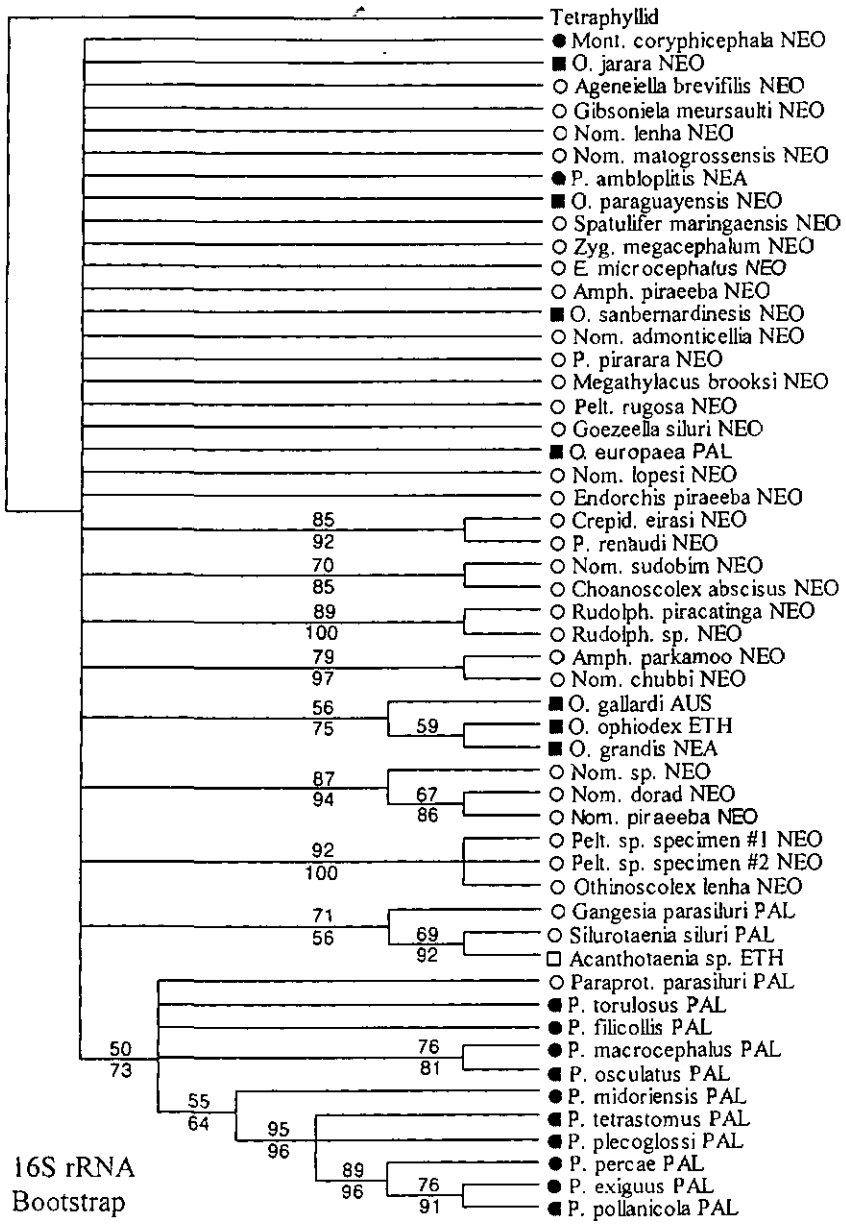

Fig. 2. Fifty per cent bootstrap tree inferred from the 16S rRNA sequences using parsimony. Bootstrap values obtained for 100 replicates of 10 heuristic searches are shown above the internodes. Figures under the internodes indicate the values obtained from 100 bootstrap replicates using the neighbor-joining distance algorithm. See Fig. 1 for names of species, geographical origins and host designations.

etically informative characters was larger and significantly higher transition–transversion rate ratios for nucleotide substitution were found in the former molecule, suggesting that positions were less saturated with multiple substitutions than in 16S. We performed a partition-homogeneity test [30,31] on the two datasets, which indicated that they contained conflicting

information and that, consequently, we should be cautious when combining them for phylogenetic analyses. Among the discrepancies that appear between the trees produced from the 16S and the 28S ribosomal sequences are the phylogenetic relationships between *Gangesia parasiluri*, *Silurotaenia siluri* (both of the subfamily Gangesiinae and found in siluriform fish) and *Acanthotaenia* sp. (an Acanthotaeniinae found in monitors). The former gene suggests the grouping: {[*Gangesia*, (*Silurotaenia*, *Acanthotaenia*)], other Proteocephalidea}, whereas the latter favours the following combination: {*Acanthotaenia*, [(*Gangesia*, *Silurotaenia*), other Proteocephalidea]}. These Proteocephalids displaying a rostellum and/or spines may be considered as the most primitive Proteocephalidea considering their basal position in our phylogenetic reconstructions with both genes. The combined dataset was nevertheless analysed: tree-resolution and consistency index were intermediate to those found when analysing the separate components (data not shown) but, wherever significant conflict occurred between both partitions, the combined dataset generally confirmed the results yielded by the lsrRNA partition. Thus, when resolving the relations between Gangesiinae and Acanthotaeniinae, it strongly supported the [*Acanthotaenia*,(*Gangesia*, *Silurotaenia*)] grouping.

For the reasons cited above, the lsrRNA sequence database was considered as the more reliable, but phylogenetic relationships among several taxa, most of them from South America, could not be solved when using this gene. Some interesting facts were observed nevertheless. Our results did not support the most recent cladistic analysis of Proteocephalidea at the subfamilial level on the basis of 27 morphological characters [1]. Both works question the monophyly of Monticelliidae and Proteocephalidae. The present analysis, however, suggests that most subfamilies, and even most genera, may not be natural groups.

The monophyly of *Rudolphiella* would need to be confirmed by including additional species in the analysis. *Rudolphiella* is a morphologically well-characterised genus for which new synapomorphies, such as egg-membrane peculiarities, have been recently described [32].

In regard to the remaining genera considered in this work, the monophyly of *Amphoteromorphus* is supported only by nuclear ribosomal sequences, and with weak bootstrap values. Our phylogenetic reconstructions, moreover, question the monophyly of *Ophiotaenia*, *Nomimoscolex* and *Prateocephalus*. The case of the latter genus, 14 representatives of which were included in this study, is interesting. A strongly supported clade comprising *Proteocephalus* spp. and including *Paraproteocephalus parasiluri*, all parasitic in teleost fish of Palaearctic origin (from Europe and Japan), is consistently found whatever tree reconstruction method or gene we considered. The remaining *Proteocephalus* specimens of Nearctic and Neotropical origin are invariably excluded from this group, suggesting that the genus *Proteocephalus* is indeed a polyphyletic group. Constraining this genus to be monophyletic in parsimony analyses requires 83 (28S) and 39 (16S) additional steps. A Templeton test indicated that both values were significantly different at $P < 0.05$ (calculated P values < 0.0001). Within this genus, we note the high degree of similarity between *Proteocephalus exiguus* and *Proteocephalus pollanicola* (with 0.2% and 0.5% differing sites between them for the nuclear and mitochondrial dataset, respectively), two species recently placed in synonymy on the basis of morphological, biometrical and genetic (Random Amplified Polymorph DNA–RAPD) studies [33]. Hence we have evidence to confirm the polyphyletic nature of the genus *Proteocephalus* which accounts for over 40% of the nominal species of Proteocephalidea [22]. This genus has previously been suspected of being a composite group, the species of which "... may be more closely related to a variety of different groups than to each other" [22] (p. 663). Indeed, they are scattered to such extent in our reconstructions that the designation *Proteocephalus* will need to be used in a more restrictive sense in future, for example when referring to the Palaearctic species, and thus excluding species from the New World, to which they seem distantly related. Morphological features that would characterise these two groups

of *Proteocephalus* species remain to be established. Recent life-cycle studies of *Proteocephalus* species parasites of fish from the Palaearctic, however, showed that they exhibited differences from the North American species *P. ambloplitis*. No parenteral location of plerocercoid larvae within a second intermediate host has been reported in Palaearctic species, in contrast to this Nearctic species [34]. Therefore, life-cycle data which are completely lacking for proteocephalideans parasitising South American fish and reptiles, including species of Monticelliidae [1], may be useful as an additional source of information to morphological and biometrical studies [34].

Our analyses include nine members of the genus *Nomimoscolex*, which has recently been revised by de Chambrier and Vaucher [23]: they invariably fail to cluster in our reconstructions. We none the less observe a well-supported clade comprising *Nomimoscolex* sp. and two species synonymised on the basis of morphological and biometrical studies by de Chambrier and Vaucher [23]: *Nomimoscolex piraeeba* and *Nomimoscolex dorad*. Our results suggest that the latter two species are indeed close but may not represent a single species, considering that 1% and 5.5% of their positions differed in the lsrRNA and 16S rRNA sequences, respectively. The fact that the genus *Nomimoscolex* did not appear as a monophyletic group was not completely unexpected either. The variations found in uterine position and in the anatomy of the excretory system within this genus are such that its monophyly was also questioned based on anatomical features [23]. A different grouping of these species remains to be defined: a more extensive analysis of these species will be published elsewhere.

Within the Peltidocotylinae, *Peltidocotyle rugosa*, although exhibiting striking morphological similarities with *Peltidocotyle* specimens found in *P. luetkeni* and *Othinoscolex lenha*, displayed genetic distances that are within the range that can be expected between distinct species. The synonymy of the genera *Othinoscolex* and *Peltidocotyle* has been discussed and established in a separate work, using both molecular and morphological approaches [35].

Concerning the genus *Ophiotaenia*, we find a clade supported strongly by nuclear ribosomal sequences that comprises proteocephalids parasitic in snakes: *Ophiotaenia ophiodex* from *Causus maculatus* (Ivory Coast), *Ophiotaenia gallardi* from *Notechis scutatus* (Australia), *Ophiotaenia grandis* from *Agkistrodon piscivorus* (USA) and *Ophiotaenia jararo* from *Bothrops jararaca* (Brazil) (Fig. 1). All are found in Viperidae with the exception of *O. gallardi*, which is found in Elapidae. Furthermore, *Ophiotaenia paraguayensis* and *Ophiotaenia sanbernardinensis*, both parasites of South American snakes (Colubridae), form a robust, yet separate clade in trees produced from the 28S rRNA database exclusively. This latter group is curiously not well supported by the mitochondrial rRNA database. *Ophiotaenia europaea* from *Natrix maura* (Colubridae), France is not closely related to any of the six ophidian species above. *Ophiotaenia* species were thus distributed in three distinct clades which could be characterised by some morphological features: in *Ophiotaenia gallardi*, *Ophiotaenia grandis*, *Ophiotaenia jarara* and *Ophiotaenia ophiodex*, testes are distributed in two lateral fields and the uterine stem is medullary. On the other hand, *Ophiotaenia paraguayensis* and *Ophiotaenia sanbernardinensis* also have testes distributed in two lateral fields, but in this case the uterine stem is cortical and uterine development is of the monticelliid type (see de Chambrier and Rego [36] and Scholz et al. [37] for details). *Ophiotaenia europaea* (with testes in two separate fields and a medullary uterus) did not group with any other species from its genus. This result was confirmed by the fact that two specimens of this species, one from Ukraine (found in *Natrix natrix*) and one from France (found in *N. maura*) were analysed and proved to be identical, in their 16S and lsrRNA sequences.

The question of the geographical origin of Proteocephalidea has been addressed previously: Brooks [17] suggested a South American origin, whereas Euzet [38] and Rego et al. [1] postulated to an African origin. In our present work, the most basal taxa are *Acanthotaenia* from Ghana and Gangesiinae species from the Palaearctic, followed by a clade of Palaearctic *Proteocephalus*

and the remnant species, mostly from South America but also from the Palaearctic, Nearctic, Africa and Australia. Our phylogenetic analyses thus suggest that the hypothesis favouring a South American origin for these cestodes is the less likely. We also observed a remarkable cluster comprising ophidian proteocephalids of various geographical origins (Neotropical, Nearctic, Australian and Ethiopian), suggesting a Gondwanan distribution for the common ancestor of these parasites. Our data additionally suggest that colonisation of reptiles occurred several times during the evolution of this group of parasites.

As far as host–parasite relationships and the geographical origin of the Proteocephalidea are concerned, our conclusions converge with those of Rego et al. [1]. We failed, however, to confirm their phylogenetic hypotheses, and any previous classification based on morphological characters for that matter, when basing our analyses on DNA sequences. We performed parsimony analyses based on our molecular data, constraining tree topologies to fit the morphological phylogeny of proteocephalidean subfamilies proposed by Rego et al. [1] and found that, compared with the unconstrained trees, an additional 76 and 187 steps were required for the 16S and lsrRNA sequences, respectively. A Templeton test indicated the difference in tree length for both genes was significant ($P < 0.0001$). Congruent with some of the present findings were the phylogenetic reconstructions we performed using yet another gene system: nuclear 5.8S rRNA and ITS-2 sequences on a subset of species (data not shown). In particular, the basal position of *Gangesia* and the split between Palaearctic and other species of *Proteocephalus* were supported by these sequences.

The most recent cladistic analysis of Proteocephalidean subfamilies based on a list of morphological characters performed by Rego et al. [1] resulted in a cladogram with CI of 0.571 that revealed a considerable amount of parallel evolution: 14 characters out of the 27 listed showed some level of homoplasy. We likewise encountered some level of homoplasy in the 16S rRNA and, to a lesser extent, in the lsrRNA datasets, and concur in stressing the need to analyse more data to better understand relationships within the order. In particular, an improved sampling of proteocephalideans would provide a better overview of the phylogeny of the group: Neotropical species were abundant, but few Nearctic, Asian, and Australian taxa were available for our study. Moreover, no Nupeliinae, Marsipocephalinae (parasitic in African siluriform fish and displaying cortical testes) or Sandonelliinae (parasitic in African Clupeiformes, and possessing compact vitelline follicles and the taxonomic position of which remains problematic) were represented. Rego et al. [1] recently discussed the status of the two latter subfamilies and found them to be basal in their respective families, but noted a general lack of information on these taxa.

In addition to a wider sampling of species, the use of genes characterised by a slower rate of evolution, and that remain yet to be identified, may prove useful in deciphering relationships within this cestode order. As a result, the systematics of these parasites which display a wide diversity in scolex morphology as well as in internal arrangement of gonads, vitelline follicles and uterus within the proglottis still remains problematic.

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Morphological and molecular analyses of the genera *Peltidocotyle* Diesing 1850 and *Othinoscolex* Woodland 1933, and a morphological study of *Woodlandiella* Freze, 1965 (Eucestoda, Proteocephalidea), parasites of South American siluriform fishes (Pimelodidae)

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Abstract

Morphological studies of type-species of the genera *Peltidocotyle* Diesing, 1850 and *Othinoscolex* Woodland, 1933 showed that these genera are synonyms. We therefore redescribe the type and fresh material of *Peltidocotyle rugosa* Diesing, 1850 and *P. lenha* (Woodland, 1933) [= *Othinoscolex lenha* Woodland, 1933], which appears to constitute two distinct species. They differ in the number and number of layers of testes, the diameter of the metascolex and the field occupied by the vitelline follicles. Phylogenetic analyses based on partial DNA sequences of 16S rDNA, 5.8S-ITS2 and 28S rDNA support these results. *P. lenha* and *Woodlandiella myzofer* (Woodland, 1933) are considered conspecific following a thorough morphological examination. With regard to host-parasite relationships, *P. lenha* has been reported from two host species, which is unusual as South American proteocephalideans generally display an oioxenous type of host-specificity.

Introduction

Peltidocotyle Diesing, 1850, *Woodlandiella* Freze, 1965 and *Othinoscolex* Woodland, 1933 represent three of the five genera comprising the monticelliid subfamily Peltidocotylinae. Each contain a single species and are identified primarily by the structure of their suckers which are biloculate in *Peltidocotyle rugosa* Diesing, 1850 and uniloculate in *Woodlandiella myzofer* Woodland, 1933; *Othinoscolex lenha* Woodland, 1933, on the other hand, was characterised as being devoid of suckers. The recent report of biloculate suckers in *O. lenha* by de Chambrier & Vaucher (1999), and the fact that *W. myzofer*, described from the same host species, has never been found since Woodland's description (1933), raised the question of the validity of these genera. It therefore seemed appropriate to re-examine the type-material of the three genera in order to assess their status. Moreover, morphological and molecular analyses based on DNA sequences were carried out on recently collected material of *O. lenha* from *Sorubimichthys planiceps* (Agassiz), *Peltidocotyle rugosa* from *Pseudoplatystoma fasciatum* (L.) and *Peltidocotyle* specimens from *Paulicea luetkeni* (Steindachner).

Materials and methods

Specimen preservation and fixation

Specimens were fixed directly after dissection of the host's intestine in hot 4% (v/v) neutral formaldehyde solution and were stored subsequently in 75% (v/v) ethanol. Cestodes were stained with Weigert's haematoxylin or Mayer's hydrochloric carmin solution, dehydrated in ethanol, cleared with Eugenol (clove oil) and mounted in Canada balsam. Pieces of strobila were embedded in paraffin wax, sectioned transversely at 12–15 μm, stained with Weigert's hematoxylin and counterstained with 1% eosin B. Eggs were mounted in distilled water for drawing. Voucher specimens were deposited at the Natural History Museum,

* This work is a part of the PhD thesis of the first author

Geneva, Switzerland. For genetic purposes, additional specimens were placed in fresh water in order to clean the strobila, and were preserved in 80% (v/v) ethanol. All measurements are given in micrometres unless otherwise indicated. Abbreviations used in descriptions are as follows: $\bar{X}$ = mean, n = number of measurements, OV = ovary width versus proglottis width ratio, PG = position of genital pore as percentage of proglottis length, PC = cirrus-pouch length versus proglottis width ratio, TM = type-material.

Material examined in molecular analysis

DNA sequences were obtained and aligned for the following species: *Peltidocotyle rugosa* ex *Pseudoplotystoma fasciatum* from San Antonio, Central province, Paraguay, 06.xi.1995: 22374 INVE; two *Peltidocotyle* [= *Othinoscolex*] *lenha*, 21912, 22373 INVE ex *Paulicea luetkeni* (from two separate host specimens) and *P. lenha*, 22021 INVE ex *Sorubimichthys planiceps*, all from Itacoatiara, Amazonas, Brazil, x.1995. *Crepidobothrium* sp. ex *Phractocephalus hemioliopterus* (Schneider) from Itacoatiara, Amazonas, Brazil, 1.x.1995: 21108 INVE and *Monticellia coryphicephala* (Monticelli, 1891) ex *Salminus maxillosus* Valenciennes (in Cuvier & Valenciennes), General Diaz Neembucu, Neembucu province, Paraguay, 18.x.1989: 17984 INVE, are representatives of the subfamilies Proteocephalinae and Monticelliinae, respectively, and were used as outgroup species for tree-rooting purposes, since the structure of the family Monticelliidae is uncertain. All host species belong to the siluroid fish family Pimelodidae, except for *Salminus maxillosus* which is a member of the family Characidae.

DNA preparation, PCR amplification and sequencing

DNA extraction was done by incubating the tissue (2–3 proglottids) in 1 ml extraction buffer (10 mM Tris, 100 mM Na_2EDTA, 100 mM NaCl, pH 8.0, 0.05% w/v SDS, 200 μg Proteinase K) for 4 hours at 55 °C. Then treatment with RNAse-A at a final concentration of 100 μg/ml for 30 minutes and a phenol:chloroform: isoamyl-alcohol (49.5:1:49.5) extraction were performed. DNA was precipitated by adding one volume 70% v/v ethanol, dried and re-suspended in 50 μl TE (10 mM Tris, 1 mM Na_2EDTA, pH 8.0). Primers used to amplify 440 base pairs of the 16S mitochondrial rDNA and the 5.8S rDNA-ITS2 fragments were designed on the basis of conserved residues among taxa from different phyla. The following PCR primers were used: 16S-5′ (5′-*CAG GAA ACA GCT ATG AAC* GNC TGT TTA YYA AAA ACA-3′) and 16S-3′ (5′-*TGT AAA ACG ACG GCC AGT* CGG TCT TAA CTC ARN TCA-3′) for the 16S rDNA gene (the 5′-most 18 nucleotides in italics correspond to the sM13 reverse and sM13 (−21) forward primer sites of M13 bacteriophage, added to facilitate automated sequencing); 5.8S–5′ (5′-CGG TGG ATC ACT CGG CTC-3′) and ITS2–3′ (5′-TCC TCC GCT TAT TGA TAT GC-3′) for the 5.8S rDNA-ITS2 fragment; and 28S–5′ (5′-TAC CCG CTG AAC TTA AGC ATA T-3′) and 28S–3′ (5′-CTC CTT GGT CCG TGT TTC AAG AC-3′) to amplify a 5′ 1000 bp portion of the 28S rDNA gene (Hillis & Dixon, 1991; Barker et al., 1993). PCR reactions were performed on a MJ-Research PTC-100 thermo-cycler with the following parameters: 35 cycles (45 cycles when yield was low) with 30 seconds DNA denaturation at 94 °C, 30 seconds primer annealing at 40 °, 55 ° and 60 °C for the three primer pairs respectively, and 1 minute at 72 °C for primer extension. Ten mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X100, 100 μM dNTP and 1.5 U Taq EXTRA-POL 1 (Eurobio) DNA polymerase were used per reaction. Products were checked for size on 0.8% w/v agarose gel and purified using QIAquick columns (Qiagen). PCR fragments were sequenced directly for both strands by cycle-sequencing using Thermosequenase (Amersham) and a LI-COR 4000 (MWG) automated sequencer. All sequence data from the present research are deposited with EMBL/Genbank data library under the accession numbers AJ238826 to AJ238843, and a complete alignment is available from the following site: http://www.herbaria.harvard.edu/treebase/search.html under the study accession number S371 and the matrix accession numbers M507–M509.

Phylogenetic analyses

Multiple alignment of portions of the 16S, 5.8S rDNA-ITS2 and 28S rDNA genes was computed using Clustal W (Thompson et al., 1994). Alignments were modified slightly by hand using GDE 2.2 (Smith et al., 1994). All regions of ambiguous alignment were removed from the dataset prior to phylogenetic analyses. Gaps were treated as missing data. Phylogenies based on the principle of parsimony were computed using PAUP 3.1.1 (Swofford, 1993). Exhaustive searches were performed. Nodal support was estimated by bootstrapping (Felsenstein, 1985). One hundred resampled datasets were analysed in each case using the exhaustive tree searching option of PAUP. The partition-homogeneity test implemented with PAUP*

4.0b1 (Swofford, 1998) was used to estimate compatibility of the three gene databases. Additionally, maximum likelihood phylogenies were produced using DNAML and distance-based phylogenies were produced using DNADIST and NEIGHBOR programs of PHYLIP version 3.572 (Felsenstein, 1989). Sequence data were reduced to a distance matrix using the Kimura 2-parameter model for nucleotide substitution, which assumes equal base frequencies and the common observation that transitions and transversions occur at different rates (Kimura, 1980). The default transition/transversion ratio of 2 was used (Figure 1). Bootstrap analyses were executed using SEQBOOT of PHYLIP: 100 bootstrapped datasets were constructed and analysed with DNADIST and NEIGHBOR programs. The consensus tree was obtained using CONSENSE.

Peltidocotyle Diesing, 1850

Syns *Othinoscolex* Woodland, 1933; *Woodlandiella* Freze, 1965

Diagnosis

Monticelliidae, Peltidocotylinae. Scolex with four biloculate suckers forming two separate cavities. Metascolex (*sensu* de Chambrier & Paulino, 1997) present. Ovary medullar. Uterus cortical, ventral, with diverticula occasionally protruding across longitudinal musculature into medulla. Testes in dorsal cortex. Vitelline follicles cortical, in two bands, dorsal and ventral. In South American siluroid fishes. Type-species *P. rugosa* Diesing, 1850.

Peltidocotyle rugosa Diesing, 1850

Material examined

From *Pseudoplatystoma coruscans* (L.) (Pimelodidae). Brazil, Mato Grosso, 3.xii.1826: type-material, 2 whole mounts, 8 transverse sections, 35/33–42 (coll. Institut de Zoologie, Neuchâtel). From *Pseudoplatystoma fasciatum* (L.) (Pimelodidae). Paraguay, Dep. Central, Rio Paraguay, San Antonio, 6.xi.1995: 22374, 23839 INVE.

Description (Figures 10, 14)

Strobila acraspedote, longer than 19 mm (TM = 27–36 mm). Mature and gravid proglottides wider than long. Scolex 1,160 in diameter (TM = 1,170–1,240). Suckers biloculate 530 long (TM = 480–650, $\tilde{X}$ = 545, n = 4) and 370 wide (TM = 330–425, $\bar{X}$ = 365, n = 4). Metascolex 3,710–4,870 in diameter (TM = 2,600–3,800). Internal longitudinal musculature well developed, consisting of strong bundles of lightly anastomosed fibres.

Testes cortical, dorsal, 203–231 in number, $\tilde{X}$ = 225, n = 4 (TM = 166–206, $\bar{X}$ = 191, n = 3), in two or three layers, in one dense field, extending to vitelline follicles, 28–33 in diameter (TM = 28–31) (Figure 14). Cirrus-pouch elongate, pyriform, thin-walled, 390–450 long, $\bar{X}$ = 412, n = 4, (TM = 285–325, $\bar{X}$ = 315, n = 8). PC = 13–17%, $\tilde{X}$ = 15%, n = 5 (TM = 18–26%, $\bar{X}$ = 22%, n = 9). Cirrus occupies 51–55%, $\tilde{X}$ = 53%, n = 4 (TM = 44–56%, $\bar{X}$ = 50%, n = 7) of cirrus-pouch length. Genital pore irregularly alternate, PG = 15–20%, $\tilde{X}$ = 17.5%, n = 4, (TM = 8–17%, $\bar{X}$ = 12%, n = 8). Vas deferens reaches median part of proglottis. Vagina with subterminal sphincter, always anterior to cirrus-pouch (Figure 14). Ovary bilobate, medullary, OV = 54–62%, $\bar{X}$ = 56%, n = 6 (TM = 54–62%, $\bar{X}$ = 59%, n = 4).

Vitelline follicles cortical, in two lateral fields, ventral and dorsal, uninterrupted at cirrus-pouch level, occupying 73–85% ($\tilde{X}$ = 80%) (TM = 84–87%, $\bar{X}$ = 86%) of proglottis length on the poral side, and 70–83% ($\tilde{X}$ = 78%) (TM = 83–92%, $\bar{X}$ = 88%) on the aporal side (Figure 14). Ventral follicles spreading slightly further towards median part of segment. Ventral osmoregulatory canals internal with respect to the vitelline follicles; dorsal canals internal with respect to ventral canal. Uterus pre-formed with 4–7 (TM = 4–8) lateral diverticula on each side, occupying up to 45% of width of proglottis at this stage. Uterus occupies up to 55% of width of pregravid proglottis. Oncospheres 11–13 in diameter (TM in whole preparation = 12–14), with hooks 6–8 long (TM = 6–8). Embryophores 20–24 in diameter (TM = 20–22). Outer hyaline envelope up to 60 in diameter (collapsed in TM) (Figure 10).

Remarks

We noted some differences between the fresh and type-material of *P. rugosa*: the cirrus pouch versus proglottis width ratio was smaller ($\bar{X}$ = 15%) in the fresh material than in the type-material ($\bar{X}$ = 22%) (the value also found in *P. lenha*). The position of the genital pore was also closer to the anterior limit of the proglottis in the type-material than in the fresh speci-

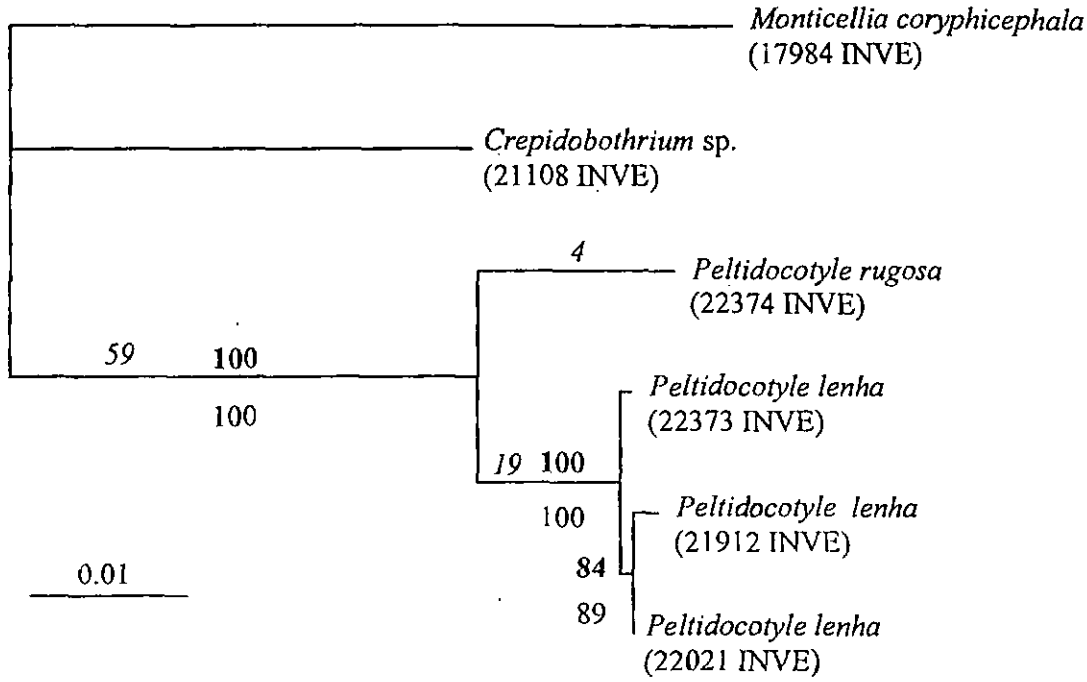


Figure 1. Neighbor-joining tree constructed from the combined dataset of 16S rDNA, 5.8S rDNA-ITS2 and 28S rDNA sequences using the Kimura-two-parameter model for nucleotide substitution. Parsimony analysis yielded an identical a single shortest tree with a length of 100 steps and a consistency index (CI) of 0.89. The number of apomorphies for various branch sections is indicated in italics. Bootstrap values obtained in performing 100 parsimony bootstrap replicates are indicated in bold above the branches; indices obtained in performing 100 NJ bootstrap replicates are indicated below. Specimen voucher numbers are indicated under taxa names.

mens. These differences could be due to the poor state of the very old type-material, which was collected in 1826 (Southwell, 1925)!

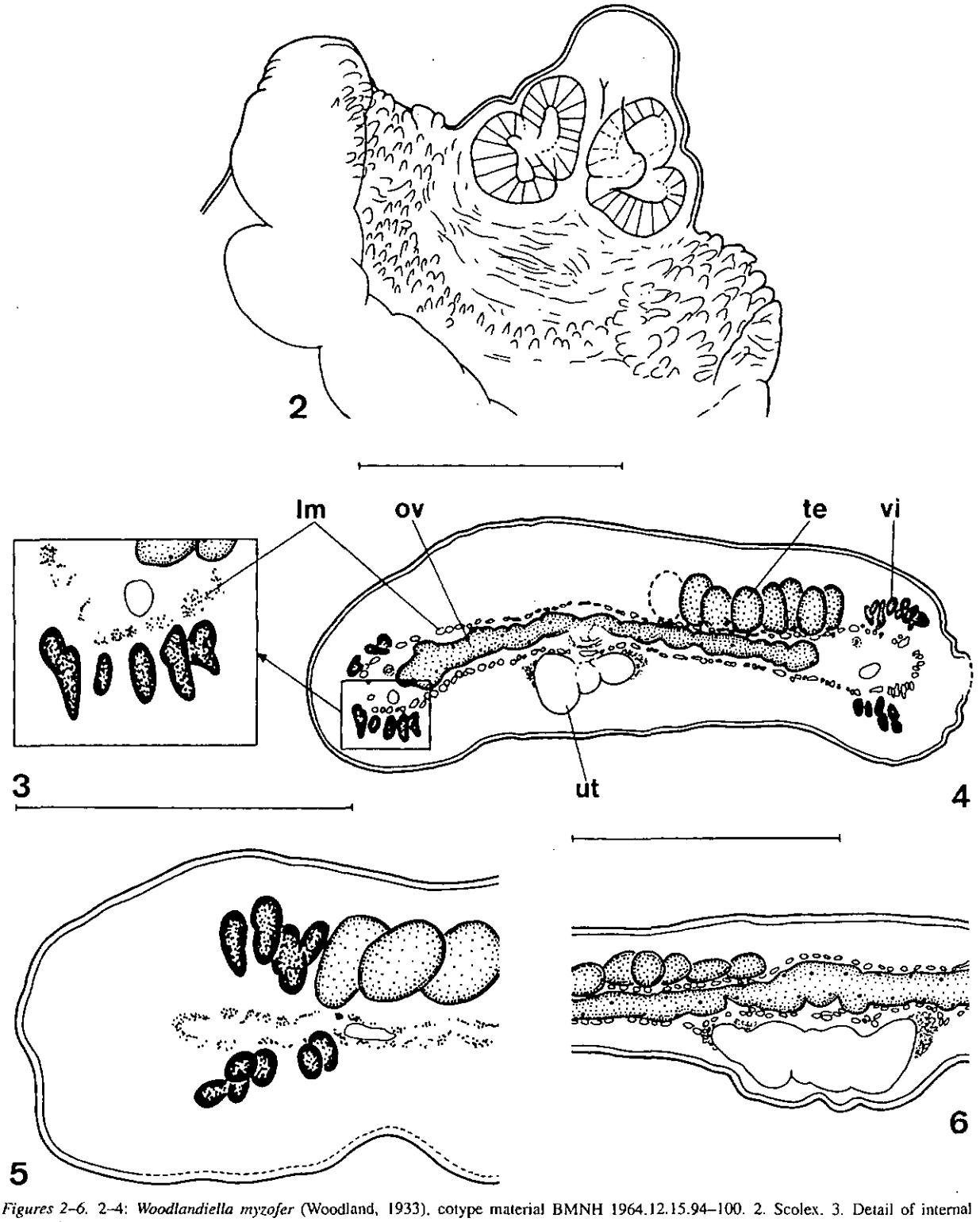
***Peltidocatyle lenha* (Woodland, 1933) Freze, 1965**
Syns *Othinoscolex lenha* Woodland, 1933;
Woodlandiello myzofer (Woodland, 1933);
Peltidocotyle rugosa of Rego & Pavanelli (1987) nec Diesing, 1850

Materiol examined
From *Sorubimichthys planiceps* (Agassiz) (Pimelodidae): Brazil, Amazonas, Itacoatiara, ix.1992 and x-xi.1995: 19537, 20499, 22021, 21882, 21883, 21884, 22080, 22380 INVE.
From *Paulicea luetkeni* (Steindachner) (Pimelodidae):
– Brazil, Amazonas, Itacoatiara, ix.1992 and x-xi.1995: 20498, 21912, 22023, 22024, 22086, 22373, 25369 INVE;
– Brazil, Paranà, Guaira, 31.viii.1993, 21886, 23689 INVE;
– Paraguay, Dep. Central, Rio Paraguay, San Antonio, 27.xi.1993, 22903 INVE.

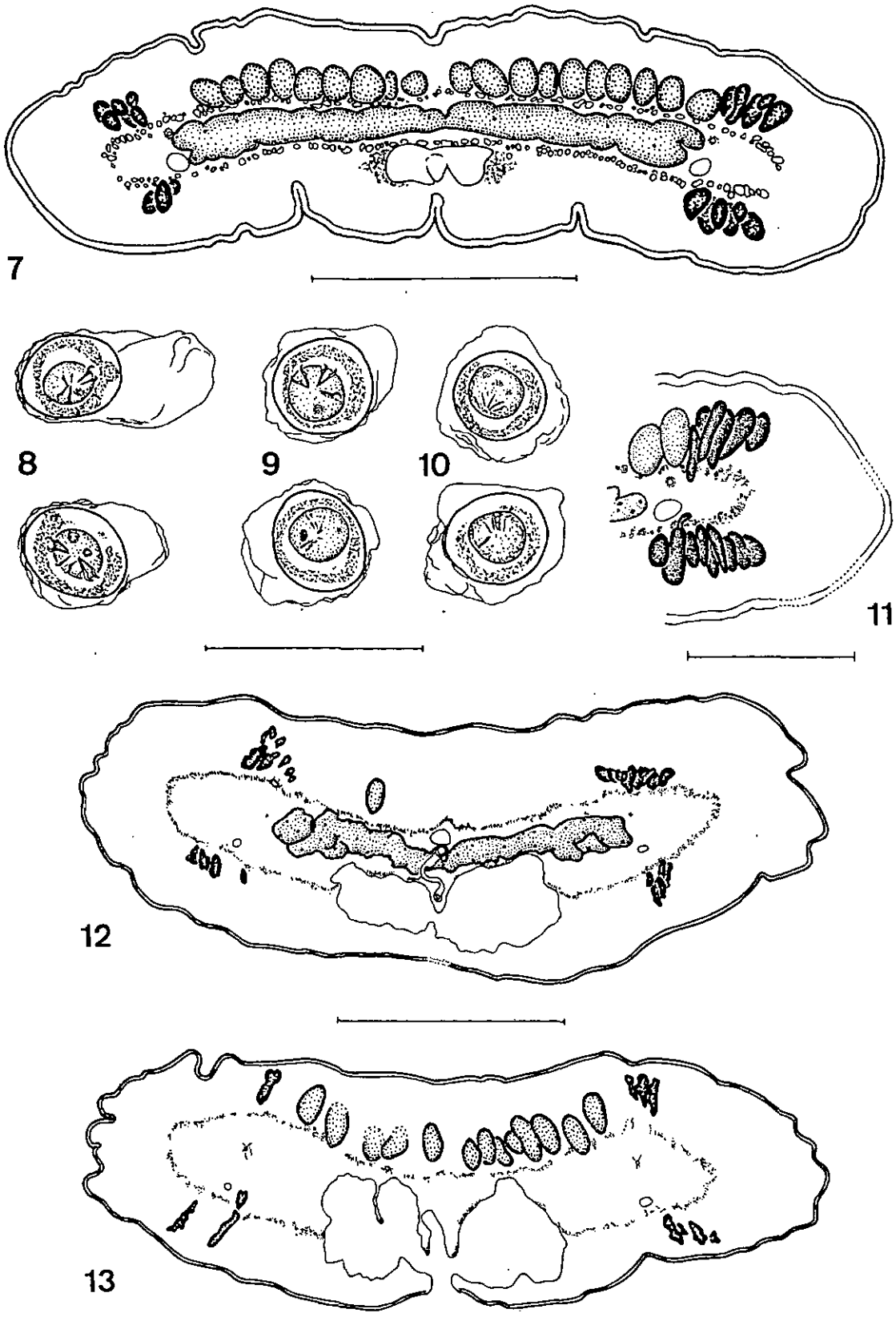
Description (Figures 2–9, 11–13, 15)

Strobila acraspedote, 31–130 mm long and 1,040–2,400 wide. Immature, mature and gravid proglottides wider than long; gravid proglottides occasionally longer than wide. Scolex 600–1,420 in diameter ($\bar{X}$ = 920, n = 15). Suckers biloculate, 380–620 long ($\bar{X}$ = 490, n = 16). Metascolex developed, 980–2,700 in diameter ($\bar{X}$ = 2,000, n = 16). Internal longitudinal musculature consisting in small bundles of parallel fibres, weakly anastomosed (Figures 7, 11–13).

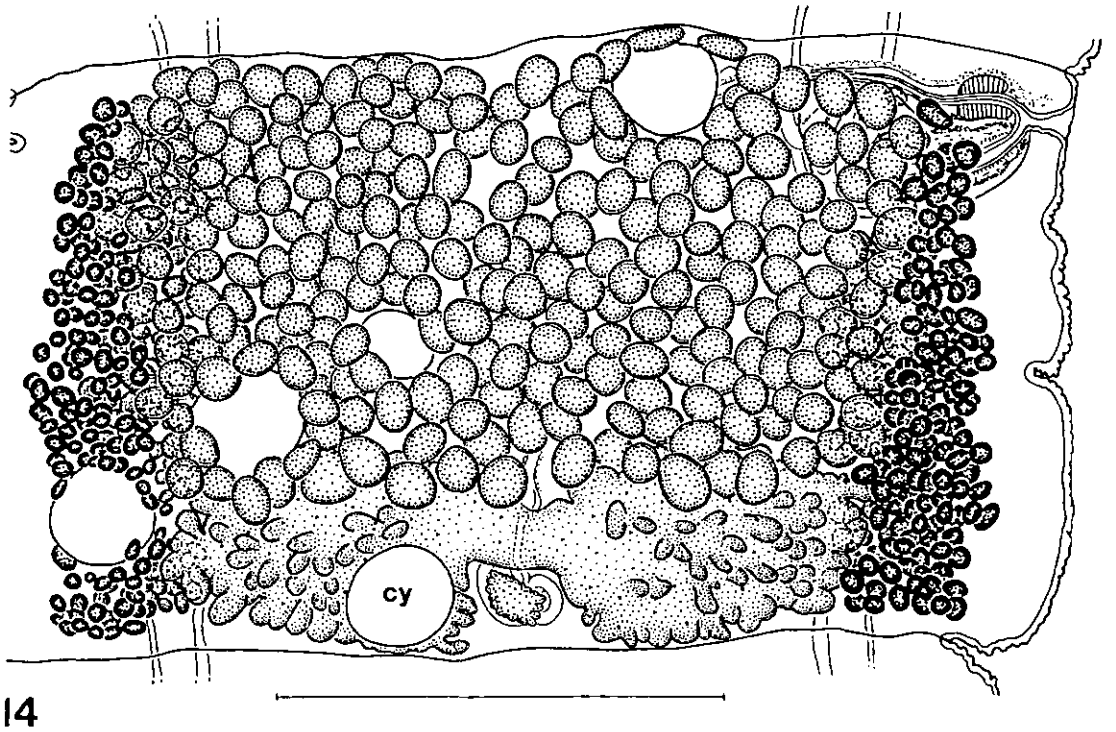
Testes cortical, numbering 117–201 ($\bar{X}$ = 142, n = 19), in one or 2 layers, in one uniform field limited laterally by vitelline follicles and posteriorly by ovary (Figure 15). Testes 32–47 in diameter ($\bar{X}$ = 39, n = 46). Genital pore very anterior, PG = 12–24% ($\bar{X}$ = 18%, n = 48), irregularly alternate. PC = 17–29% ($\bar{X}$ = 23%, n = 81). Cirrus-pouch pyriform, very elongate, thin-walled, 255–530 long ($\bar{X}$ = 385, n = 63). Cirrus occupying 50–63% of cirrus pouch-length ($\bar{X}$ = 56%, n = 13). Ejaculatory duct long and very coiled. Vas deferens reaching median part of proglottis. Vagina always anterior to cirrus-pouch (n = 160), with thicker terminal portion surrounding subterminal sphincter. Ovary medullar, bilobate,



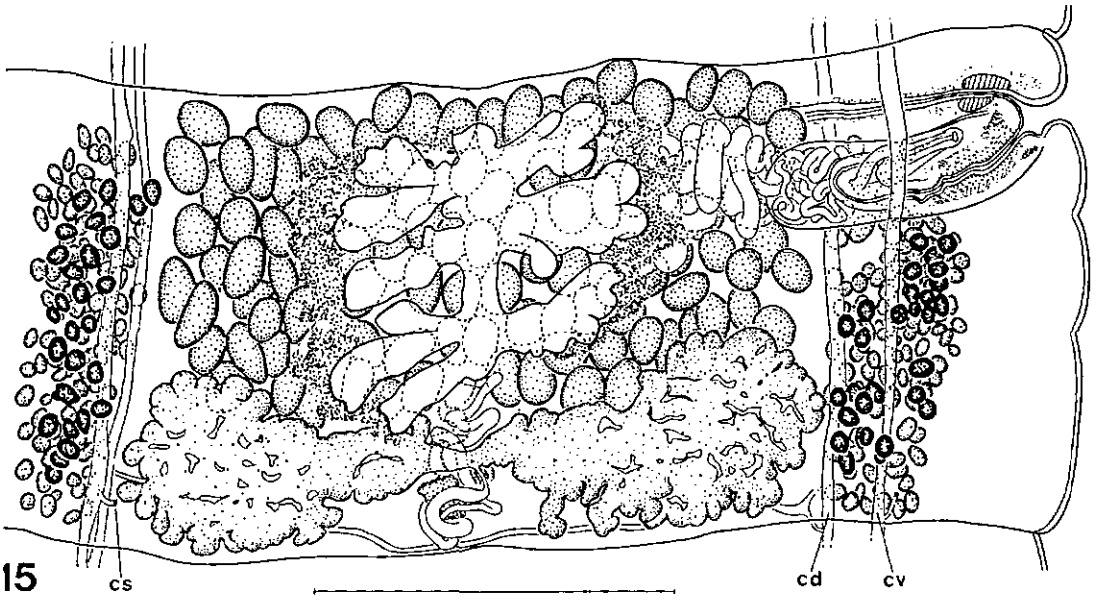
Figures 2–6. 2–4: *Woodlandiella myzofer* (Woodland, 1933), cotype material BMNH 1964.12.15.94–100. 2. Scolex. 3. Detail of internal longitudinal musculature. 4. Transverse section at ovarian level, mature proglottis. 5–6. *Othinoscolex lenha* Woodland, 1933, cotype material BMNH 1964.12.15. 87–92. 5. Transverse section of the posterior part, mature proglottis, detail of internal longitudinal musculature. 6. Transverse section at ovarian level, mature proglottis. *Abbreviations*: lm, internal longitudinal musculature; ov, ovary; te, testes; ut, uterus; vi, vitelline follicles. *Scale-bars*: 2,4,6, 500 μm; 3,5, 250 μm.



Figures 7–13. 7. *Peltidocotyle lenha* (Woodland, 1933) from *Sorubimichthys planiceps*. Transverse section at ovarian level, mature proglottis, 21882 INVE. 8–10. Eggs drawn in distilled water. 8. *P. lenha* from *S. planiceps*. 9. *P. lenha* from *Paulicea luetkeni*, 25369 INVE. 10. *P. rugosa* Diesing, 1850, from *Pseudoplatystoma fasciatum*, 23839 INVE. 11. *P. lenha* from *S. planiceps*. Transverse section at ovary level, mature proglottis, detail of internal longitudinal musculature. 12–13. *P. lenha* from *Paulicea luetkeni*, pregravid proglottis, 21912 INVE. 12. Transverse section at ovarian level. 13. Transverse section of posterior part. *Scale-bars*: 7,12,13, 500 μm; 8–10, 50 μm; 11, 250 μm.



14



15

ɨ. 14. *Peltidocotyle rugosa* Diesing, 1850. Mature proglottis, dorsal view, 23689 INVE. Five cysticercoids are figured. 15. *P. lenha* 933) from *Paulicea luetkeni*. Mature proglottis, ventral view, 23839 INVE. *Abbreviations*: cd, dorsal osmoregulatory canal; cs, ıal; cv, ventral osmoregulatory canal; cy, cysticercoid. *Scale-bar*: 500 μm.

2. Pairwise distance matrix from the three amplified fragments: 16S rDNA/5.8S rDNA and ITS2/28S rDNA ively. Numbers of nucleotide differences are shown below the diagonal and percentages of nucleotide differences wn above.

	Peltidocotyle rugosa 22374 INVE	*Peltidocotyle lenha* 22373 INVE	*Peltidocotyle lenha* 21912 INVE	*Peltidocotyle lenha* 22021 INVE
gosa 22374 INVE	–	2.3/5/0.7	2.6/5/0.8	2.3/5/0.6
ıha 22373 INVE	10/31/7	–	0.23/0/0.2	0/0/0.2
ıha 21912 INVE	11/31/8	1/0/2	–	0.23/0/0.2
ıha 22021 INVE	10/31/6	0/0/2	1/0/2	–

ts obtained from hyperparasitised ces- was impossible to read a "clean" DNA

he observations of Woodland (1933, wrote that the scolex of *Othinoscolex* arge extent resembles that of *Peltido-*, but it is entirely devoid of the biloc- lar or rather biporal suckers figured by)", recent studies have demonstrated the ickers in this species (de Chambrier & 9, figure 85). Indeed, the absence of ie main criterion that lead Woodland to genus *Othinoscolex*. Our present study aterial of these two species convinced are congeneric, and we consequently *Othinoscolex* is a junior synonym of

logical study, together with DNA analy- Figure 1), showed that the three *P.* ns from *Sorubimichthys planiceps* and *keni* exhibited a high level of resem- ver, we found that *P. lenha* had distinc- gical features that differentiated it from ble 1). We noted differences: (1) in the of testes which was greater in *P. rugosa* a; (2) in metascolex diameter which was *ugasa*; (3) in the arrangement of testes . 1–2 layers in *P. lenha* and in 2–3 layers and (4) in the length of the field occu- telline follicles which was shorter on the the proglottis than on the aporal side in reas no such disparity was observable in

data corroborated our findings at the l level regarding the status of the three *P. lenha* specimens, as could be observed from their sequence divergence (Table 2, Figure 1). On the other hand, *P. rugosa* appeared to be a close to but distinct from *P. lenha*. The most useful DNA sequence that supported our conclusions based on anatomy was the ITS2 fragment; it yielded identical sequences for the three *P. lenha* samples. *P. rugosa*, on the other hand, had 31 positions that differed over a total sequence length of 617 nt from the latter group (Table 2), thus supporting our taxonomic decision.

Regarding host-parasite relationships, *P. lenha* has been reported from two Amazonian siluriform fish species, *Paulicea luetkeni* and *Sorubimichthys planiceps*. This finding is unusual considering the oioxenous host-specificity generally encountered in South American proteocephalidean cestodes.

Despite the fact that the distribution and topography of vitelline follicles are generally not considered, we regard them as representing reliable discriminating characters, as indicated above (see also de Chambrier, 1989, p. 375; de Chambrier & Vaucher, 1997, p. 231; Rego et al., 1998, p. 5). Likewise, the use of suitable DNA sequences, i.e. with an appropriate rate of evolution, proved equally effective in distinguishing *P. rugoso* from *P. lenha*, two closely related species, thus illustrating the benefit in using both molecular and morphological approaches (Mariaux, 1996).

Acknowledgements

The authors thank David I. Gibson (The Natural History Museum, London) who kindly lent Woodland's type-material, and are grateful to Claude Vaucher and Jean Mariaux (Natural History Museum, Geneva) for fruitful suggestions and constructive remarks. They also wish to thank two anonymous referees who provided judicious comments on an earlier version of this manuscript.

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Nomimoscolex suspectus n. sp. (Eucestoda: Proteocephalidea: Zygobothriinae) with morphological and molecular phylogenetic analyses of the genus

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Abstract

We describe a new species of *Nomimoscolex* from the Amazon siluriform fishes *Brachyplatystoma filamentosum*, *B. flavicans* and *B. vaillanti*. It differs from *N. piraeeba* in a lower mean number of testes, the paramuscular position of the vitelline follicles, the ovarian width/proglottis width ratio and the cirrus-pouch length/proglottis width ratio. Protein electrophoresis assays performed for 25 enzymatic systems showed that specimens of *N. suspectus* n. sp. from the three host species form a homogenous population which was genetically isolated from *N. piraeeba* and *N. dorad*. Moreover, the latter two species, synonymised by Rego (1991) because of their close morphological similarity, could be separated at eight loci. We thus restore *N. dorad* as a valid species. We finally examined the composition of the genus *Nomimoscolex* using DNA sequences from the 5.8S rRNA, ITS-2 and 28S rRNA nuclear ribosomal genes and a matrix of 24 morphological characters. Phylogenetic relationships were inferred for nine species of the genus, five members of other monticelliid genera and two outgroup species. The results of the phylogenetic analyses performed on morphological and molecular characters converged with those from allozyme studies and showed that *N. suspectus*, *N. piraeeba* and *N. dorad* clustered in a distinct clade that excluded other members of the genus. We therefore recognised them as an aggregate of species to reflect an isolation supported by both morphological and genetic data. Because relationships among the remaining *Nomimoscolex* representatives and other genera were generally poorly resolved, regardless of the database analysed, no action was taken to reorganise them into alternative groupings.

Introduction

Woodland (1934), in the original description of *Nomimoscolex piraeeba* (the type-species of the genus), figured a proglottis (figure 7, plate 10) which could not be assigned to this species on the basis of differences in cirrus-pouch length in relation to proglottis width, in the disposition of vitelline follicles and in the field occupied by the vas deferens (de Chambrier & Vaucher, 1997). The latter authors could not relate it, for that matter, to any other known species of *Nomimoscolex* Woodland, 1934. However, after a recent examination of abundant material collected from Neotropical siluriform fishes, we discovered a new species of *Nomimoscolex*, which matched the figure of Woodland (1934b), parasitising *Brachyplatystoma filamentosum*, *B. flavicans* and *B. vaillanti*. We consequently examined its taxonomic status, including using allozyme electrophoretic methods, of *Nomimoscolex suspectus* n. sp. compared with *N. piraeeba* Woodland, 1934 recorded from *B. filamentosum* and *N. dorad* (Woodland 1935) recorded from *B. flavicans*. Thus, we are able to discuss with new arguments the synonymy of *N. dorad* with *N. piraeeba* proposed by Rego (1991) and followed by de Chambrier & Vaucher (1997).

We also analysed the composition of the genus *Nomimoscolex*, recently revised by de Chambrier & Vaucher (1997). Indeed, these authors showed it to represent a heterogeneous taxon: the variability which

*This work is a part of the PhD thesis of the first author.
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they encountered in the position of osmoregulatory canals and the position of the uterine stem and diverticula within the genus *Nomimoscolex* was such that they emphasised the need for its reorganisation into a number of smaller groupings. In relation to this, we infer phylogenetic relationships for nine of the 11 species presently comprising the genus using morphological characters and DNA sequences from the nuclear 5.8S rDNA, ITS-2 and 28S rDNA genes.

Materials

Material is deposited at the Natural History Museum (INVE), Geneva, Switzerland and Oswaldo Cruz Institute, Rio de Janeiro, Brasil (CHIOC).

Monticelliidae, Endorchiinae:

Endorchis piraeeba Woodland, 1934
From *Brachyplatystoma filamentosum* (Pimelodidae): Brazil, Amazonas State, Itacoatiara, 21738** INVE, 07.10.1995.

Monticelliidae, Zygobothriinae:

Amphoteromorphus parkamoo Woodland, 1935
From *Paulicea luetkeni* (Pimelodidae): Brazil, Amazonas State, Itacoatiara, 22245** INVE, 11.10.1995.

Gibsoniela meursaulti de Chambrier & Vaucher, 1999
From *Ageneiosus brevifilis* (Auchenipteridae): Paraguay, San Antonio, Central Province, 21839** INVE, 03.11.1995.

Harriscolex kaparari (Woodland, 1935)
From *Pseudoplatystoma tigrinum* (Pimelodidae): Brazil, Amazonas State, Itacoatiara, 22018** INVE, 09.10.1995.

Nomimoscolex admonticellia (Woodland, 1934)
From *Pinirampus pirinampu* (Pimelodidae): Brazil, Amazonas State, Itacoatiara, 21870** INVE 30.09.1995.

Nomimoscolex chubbi (Pavanelli & Takemoto, 1995)
From *Gymnotus carapo* (Gymnotidae): Paraguay, Neembucu Province, 18 km south of San Lorenzo, 20351** INVE, 17.10.1989.

Nomimoscolex dorad (Woodland, 1935)
All samples from Brazil, Amazonas State, Itacoatiara.
From *Brachyplatystoma flavicans* (Pimelodidae): 22254*, 22255*, 22257*, 22259* INVE, 03.10.1995; 22260*, 22261*, 22262* INVE, 04.10.1995; 27152, 22263*, 23828* INVE, 06.10.1995; 22265*, 22267*, 27155 INVE, 07.10.1995; 22268*, 22269*/** INVE, 11.10.1995.

Nomimoscolex lenha (Woodland, 1933)
From *Sorubimichthys planiceps* (Pimelodidae): Brazil, Amazonas State, Itacoatiara, 21740** INVE, 02.10.1995.

Nomimoscolex lopesi Rego, 1989
From *Pseudoplatystoma fasciatum* (Pimelodidae): Paraguay, Central Province, San Antonio, 21963** INVE, 06.11.1995.

Nomimoscolex matogrossensis Rego & Pavanelli, 1990
From *Hoplias malabaricus* (Erythrinidae): Paraguay, San Pedro Province, Arroyo Tapiracuayi, 17913** INVE, 24.10.1989.

Nomimoscolex piraeeba Woodland, 1934
From *Brachyplatystoma filamentosum* (Pimelodidae): Brazil, Amazonas State, Itacoatiara, 22283*, 22284*/**, 22288 INVE, 13.10.1995.

Nomimoscolex sudobim Woodland, 1935
From *Pseudoplatystoma fasciatum* (Pimelodidae): Brazil, Amazonas State, Itacoatiara, 21969** INVE, 12.10.1995.

Nomimoscolex suspectus n. sp.
All samples from Brazil, Amazonas State, Itacoatiara. From *Brachyplatystoma filamentosum* (Pimelodidae): Holotype 34212 CHIOC, 6 paratypes 27139, 27143, 27144, 27145 INVE, 34212b-c CHIOC 13.10.1995; 1 paratype 22302* INVE, 07.10.1995; 1 paratype 22319 INVE, 20.10.1995. Other material: 22291 INVE, 18.09.1992; 22301*, 07.10.1995; 22289, 22304*, 22305, 22306*, 22307*/** INVE, 08.10.1995; 22311*, 22312*, 22313*, 22314*, 22315 INVE, 13.10.1995; 22316*, 22317*, 22318 INVE, 15.10.1995; 22320, 20.10.1995; 27142, 13.10.1995. From *Brachyplatystoma flavicans* (Pimelodidae): 27146 INVE, 08.10.1995; 23829* INVE, 06.10.1995; 22308*/**, 22309* INVE, 08.10.1995; 22310* INVE, 11.10.1995. From *Brachyplatystoma vaillanti* (Pimelodidae): 22292, 22293, 22294, 22297*, 22298**, 22299*, 22300* INVE, 02.10.1995.

Ophiotaenia jarara Fuhrmann, 1927
From *Bothrops jararaca* (Viperidae): Brazil, Domingos Martins, Espiritu Santo State, 12393** INVE, 16.10.1989.

Monticelliidae, Monticelliinae:

Choanoscolex cf. *abscisus* (Riggenbach, 1895)
From *Pseudoplatystoma fasciatum* (Pimelodidae): Brazil, Amazonas State, Itacoatiara, 25102** INVE, 11.10.1995.

Proteocephalidae, Proteocephalinae:
Proteocephalus pirarara (Woodland, 1935)
From *Phractocephalus hemioliopterus* (Pimelodidae): Brazil, Amazonas State, Itacoatiara, 22106** INVE, 01.10.1995.
*Specimen used for protein electrophoresis studies
**Specimen used for DNA sequence analyses

All specimens used for protein electrophoresis or DNA sequencing were identified beforehand using conventional methods.

Methods

Specimen preservation and fixation for morphological studies

Specimens were fixed directly after dissection of the host's intestine in hot 4% v/v neutral formaldehyde solution and subsequently stored in ethanol 75% v/v. They were stained with Weigert's haematoxylin or Mayer's hydrochloric carmine solution, dehydrated in ethanol, cleared with Eugenol (clove oil) and mounted in Canada balsam. Pieces of strobila were embedded in paraffin wax, sectioned transversely at 12-15 μm, stained with Weigert's haematoxylin and counterstained with 1% eosin B. Eggs were studied in distilled water. All measurements are given in micrometres unless otherwise indicated. Abbreviations used in descriptions are as follows: x = mean, n = number of measurements, OV = ovarian width / proglottis width ratio, PG = position of genital pore in % of proglottis length, PC = cirrus-pouch length / proglottis width ratio, TM = type-material.

Isoenzyme analyses

Material subject to analysis by protein electrophoresis was placed in an Eppendorf-like tube and stored in liquid nitrogen. Tissue samples were subsequently homogenised in 0.1–0.2 ml of distilled water. A Whatman No. 3 (10 × 2.0 mm) paper wick was dipped into the homogenate and stored at −80°C. This was directly used as a source of enzymes for electrophoresis. Electrophoresis was carried out on a starch gel with a voltage of 90–100 V and a maximum intensity of 73 mA. Enzymatic assays were performed according to the techniques described by Pasteur et al. (1987). Twenty-five enzymes were tested: AAT (Aspartate Aminotransferase, EC 2.6.1.1), ACOH (Aconitase Hydratase, EC 4.2.1.3), ACP (Acid Phosphatase, EC 3.1.3.2), AK (Adenylate Kinase, EC 2.7.4.3), AKP (ALP) (Alkaline Phosphatase, EC 3.1.3.1), ALD (Aldolase, EC 4.1.2.13), CK (Creatin Kinase, EC 2.7.3.2), FK (Fructose Kinase, EC 2.7.1.4), GCDH (Glucose Dehydrogenase, EC 1.1.1.118), GLC (NAD-Glucose Dehydogenase, EC 1.1.1.47), G6PDH (Glucose-6-phosphate Dehydrogenase, EC 1.1.1.49), GPI (Glucose-6-phosphate Isomerase, EC 5.3.1.9), HK (Hexokinase, EC 2.7.1.1), IDH (Isocitrate Dehydrogenase, EC 3.1.1.42), LAP (Leucine Aminopeptidase, EC 3.4.11.1), LDH (L-Lactate Dehydrogenase, EC 1.1.1.27), MDH (Malate Dehydrogenase, EC 1.1.1.37), ME (Malic enzyme, EC 1.1.1.40), MPI (Mannose-6-phosphate Isomerase, EC 5.3.1.8), NP (Purine Nucleoside Phosphorylase, EC 2.4.2.1), PEP (Peptidases, EC. 3.4.-.-: Pep-A, Pep-B, Pep-C and Pep-D, correspond to the following substrates: Val-Leu, Leu-Gly-Gly, Lys-Leu, Phe-Pro) and PGM (Phosphoglucomutase, EC 2.7.5.1). The nomenclature follows that of Richardson et al. (1986), Pasteur et al. (1987) and Durand et al. (1998).

DNA preparation, PCR amplification and sequencing

Specimens used in DNA sequence analyses were either frozen or preserved in 95% ethanol after host dissection. DNA extraction was done by incubating the tissue (2-3 proglottides) in 1 ml extraction buffer (10 mM Tris, 100 mM Na_2EDTA, 100 mM NaCl, pH 8.0, 0.05% w/v SDS, 200 μg Proteinase K) for 4 hours at 55°C. Then a treatment with RNAse-A at a final concentration of 100 μg/ml for 30 minutes and a phenol-chloroform-isoamyl-alcohol (49.5:1:49.5) extraction were performed. DNA was precipitated by adding one volume 70% v/v ethanol, dried and resuspended in 50 μl TE (10 mM Tris, 1 mM Na_2EDTA, pH 8.0). The 5.8S-5′ (5′-CGG TGG ATC ACT CGG CTC-3′) and ITS2-3′ (5′-TCC TCC GCT TAT TGA TAT GC-3′) PCR primers were used to amplify the 5.8S rDNA-ITS2 fragment and were designed on the basis of conserved residues among taxa from different phyla. The 28S-5′ (5′-TAC CCG CTG AAC TTA AGC ATA T-3′) and 28S-3′ (5′-CTC CTT GGT CCG TGT TTC AAG AC-3′) PCR primers were used to amplify an adjacent 5′ 1000 nt portion of the 28S rDNA gene (these primer-sequences were availaible from Hillis & Dixon, 1991, and Barker et al., 1993). The two amplified regions are not contiguous but separated by a 28 nucleotide sequence. PCR reactions were performed on a MJ-Research PTC-100 thermocycler or, altematively, on a Primus (MWG-Biotech) thermocycler, with the following parameters: 35 cycles (45 cycles when yield was low) with 30 seconds

DNA denaturation at 94°C, 30 seconds primer annealing at 55° and 60°C for the two primer pairs respectively, and 1 minute at 72°C for primer extension. Ten mM Tris-HCl pH 9.0, 1,5 mM $mgCl_2$, 50 mM KCl. 0.1% TritonX100, 100 μM dNTP and 1.5 U Taq EXTRA-POL I (Eurobio) DNA polymerase were used per reaction. Products were checked for size on 0.8% w/v agarose gel and purified on QIAquick columns (Qiagen). PCR fragments were directly sequenced for both strands by cycle sequencing using Thermosequenase (Amersham) and a LI-COR 4000 (MWG-Biotech) automated sequeocer. All sequence data from the present research are deposited with EMBL/GenBank data library under accession numbers AJ251340–AJ251355, AJ275064, AJ275067, AJ275068, AJ275227, AJ288595, AJ288597, AJ288602, AJ288603, AJ288607–AJ288608, AJ288611, AJ288613, AJ288614, AJ288616, AJ288618, AJ288625, AJ288628, AJ288631.

Phylogenetic analyses of morphological and molecular characters

Morphological characters

Cladistic analyses of morphological characters were performed to infer phylogenetic relationships among nine *Nomimoscolex* species, one representative for five genera of the subfamily Zygobothriinae and two species used as taxonomic outgroups: *Proteocephalus pirarara* (Woodland, 1935) and *Ophiotaenia jarara* (Fuhrmann, 1927). Character polarisation was based on outgroup comparison with reference to the two latter species. We relied on morphological data compiled from the literature (Woodland, 1933a,b, 1934a,b, 1935a,b, de Chambrier et al., 1992, 1996; de Chambrier & Vaucher, 1997, 1999), but characters were also developed from direct morphological examination of specimens when necessary. A list of 24 binary and multi-state characters representing 59 character states was used in this study; they were defined as follows: $\ll 0 \gg$= plesiomorphic state; $\ll 1 - n \gg$= apomorphic states, $\ll ? \gg$= undefined. All multi-state characters were run as unordered. The numerical data matrix was written with MacClade 3.04 (Maddison & Maddison, 1992).

Molecular characters

Multiple alignment of sequences from the 5.8S rDNA-ITS2 and 28S rDNA genes was computed using Clustal W (Thompson et al., 1994). Alignments were modified slightly by hand in GDE 2.2 (Smith et al., 1994). All regions of ambiguous alignment were removed from the dataset prior to phylogenetic analyses. Gaps were treated as an additional character state (5^{th} base). However tree topologies were identical when gaps were treated as missing characters.

Analyses

Phylogenetic analyses were conducted using PAUP* version 4.0b2 (Swofford, 1996). Branch & Bound searches were performed for finding the most parsimonious trees; heuristic searches were conducted when performing bootstrap and partition-homogeneity test analyses. The phylogenetic trees are presented with the following associated statistics: consistency index (CI), rescaled consistency index (RC), retention index (RI) and homoplasy index (HI), as defined by Maddison & Maddison (1992). Nodal support was estimated by bootstrapping (Felsenstein, 1985): 100 re-sampled datasets were analysed in each case using the heuristic tree searching option of PAUP. The partition-homogeneity test (PHT) implemented with PAUP* was used to estimate compatibility of the two molecular and the morphology-based partitions. Alternative constrained topologies were analysed, and the significance of the difference in the lengths of the strict consensus tree compared to the unconstrained most parsimonious tree was established using the Templeton compatibility test (Templeton, 1983) implemented with PAUP*. For distance-based phylogenies, sequence data were reduced to a distance matrix using the Kimura 2-parameter model for nucleotide substitution which assumes equal base frequencies and the common observation that transitions and transversions occur at different rates (Kimura, 1980). The default transition/transversion ratio of 2 was used.

Nomimoscolex suspectus **n. sp.** (Figures 1–7)

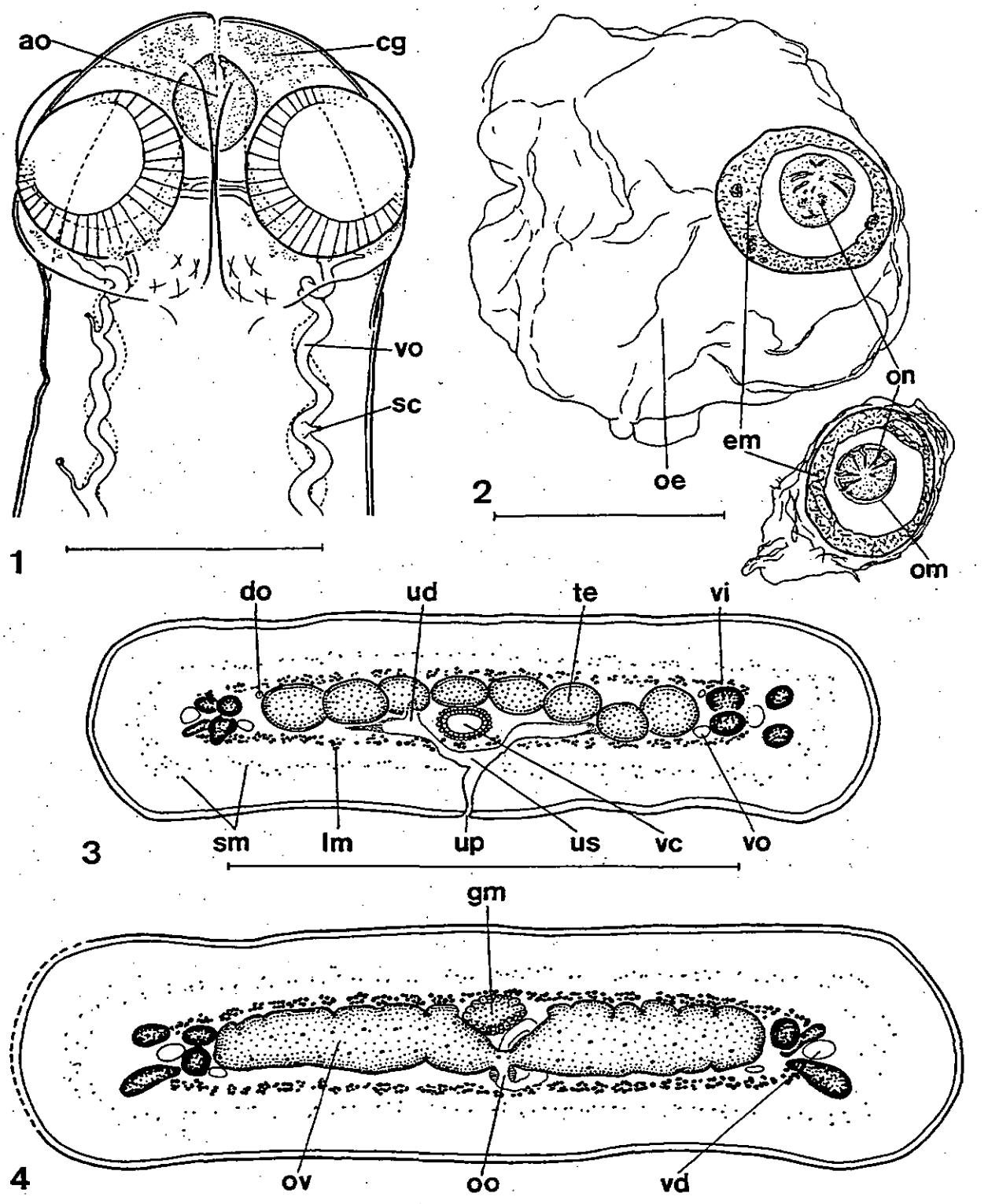
Hosts: Brachyplatystoma filamentosum (Lichtenstein) (type-host), *B. flavicans* (Castelnau) and *B. vaillanti* (Cuvier & Valenciennes).

Type-locality: Itacoatiara, Amazonas State, Brazil, 18.09.1992, 02–20.10.1995.

Site: Posterior part of intestine.

Holotype: 34212 CHIOC.

Paratypes: 22302, 22319, 27139, 27143, 27144, 27145 INVE; 34212b-c CHIOC.



Figures 1–4. Nomimoscolex suspectus n. sp. 1. Scolex, ventral view, holotype, 34212 CHIOC. 2. *Nomimoscolex suspectus* n. sp. Eggs drawn in distilled water, paratype, 22302 INVE. 3. *Nomimoscolex suspectus* n. sp. Transverse section of posterior part, mature proglottis, paratype, 27139 INVE. 4. *Nomimoscolex suspectus* n. sp. Transverse section at ovarian level, mature proglottis, paratype. 27139 INVE. *Abbreviations*: ao, apical organ; cg, glandular cells; do, dorsal osmoregulatory canal; em, embryophore; gm, Mehlis' gland; lm, internal longitudinal musculature: oe, outer envelope; om, oncospheric membrane; on, oncosphere; oo, oöcapt; ov, ovary; sc, secondary canals; sm, secondary musculature; te, testes; ud, uterine diverticula; up, uterine pore; us, uterine stem; vc, vaginal canal; vd, vitelloduct; vi, vitelline follicles; vo, ventral osmoregulatory canal: *Scale-bars*: 1, 250 μ; 2, 50 μm; 3,4, 500 μm.

Description

Monticelliidae, Zygobothriinae. Medium-sized worms, 51–123 mm long (x = 78 mm), up to 1.35 mm wide. Strobila craspedote with transverse folds. Mature and gravid proglottides wider than long. Scolex small, 320–450 (x = 370, n = 19) in diameter, with 4 triangular bulges, each bearing one shallow uniloculate sucker (Figure 1). Scolex covered with spiniform microtriches on its anterior part; those present on the rim of suckers about twice as long. Spiniform microtriches not extending beyond level of posterior margin of suckers (Figure 7). Internal cavity of suckers devoid of spiniform microtriches (Figure 7). Suckers 320–450 in diameter (x = 370, n = 19). Apical structure 60–120 long and 55–100 wide, representing c. 20–30% of scolex width, with an os emerging under apex (Figure 1). Presence of numerous elongate cells with granular cytoplasm extending posteriorly behind the suckers.

Internal longitudinal musculature dense, interrupted laterally, in well-separated, anastomosed longitudinal bundles. Secondary muscle layer not well delimited, situated in cortex (Figures 3, 4).

Testes medullary, spherical to ovoid, numbering 47–93 (x = 69, n = 33), in one dorsal field, in one or 2 layers, separated from vitelline fields by osmoregulatory ducts (Figure 6). Genital pore irregularly alternate, opening at 38–58% (x = 49%, n = 42) of proglottis length. Genital atrium present. Cirrus-pouch elongate, thick-walled, 255–425 (x = 354, n = 54) long. Cirrus pouch length / proglottis width ratio 28–43% (x = 35%, n = 54). Very long cirrus, occupying > 70% of cirrus-pouch length. Ejaculatory duct coiled. Vas deferens situated between base of cirrus-pouch and median part of proglottis and extending beyond latter.

Vagina anterior (70%) or posterior (30%) (n = 217) to cirrus-pouch, with thickened terminal portion surrounding muscular sub-terminal sphincter (Figures 5, 6).

Ovary medullary, massive, bilobate producing rare dorsal lobules. Ovarian width / proglottis width ratio 54–67% (x = 61, n = 46).

Vitelline follicles paramuscular, arranged on either side of internal longitudinal musculature, situated laterally in transverse sections, occupying nearly entire proglottis length (Figures 3, 4).

Uterus cortical, pre-formed in immature proglottides. Formation: presence in immature proglottides on either side of uterine stem of concentration of numerous intensely-staining cells foreshadowing location of future lateral digitate diverticula, still unformed at this stage; lumen present in uterine stem in mature proglottides; uterus growing from cortical stem into medullary region with transversally and dorsally ramified diverticula and lumen in first mature segments; uterus with 10–18 long and ramified diverticula on each side, occupying up to 60% of gravid proglottis width.

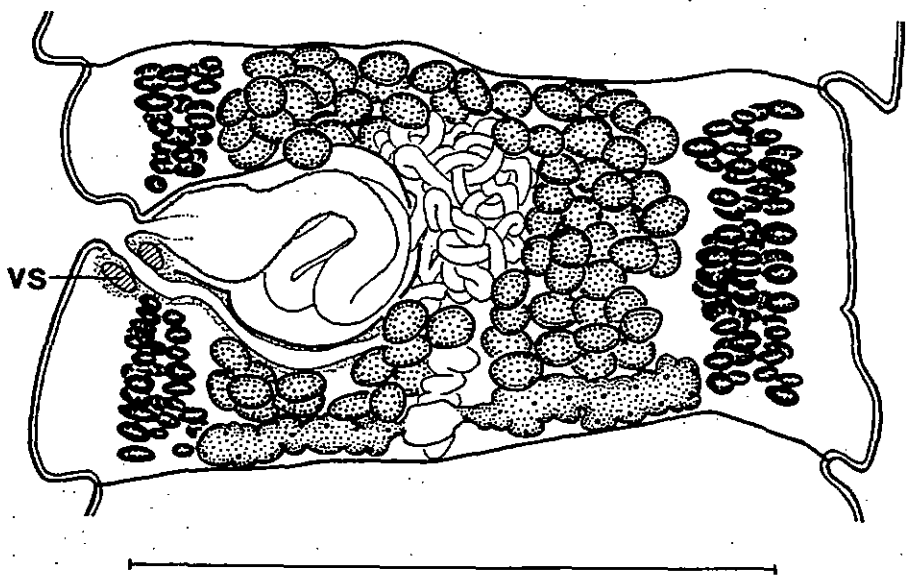
Ripe eggs laid through one or 2 ventral uterine pore-like openings, formation of which is indicated by concentration of chromophilic cells in last immature segments.

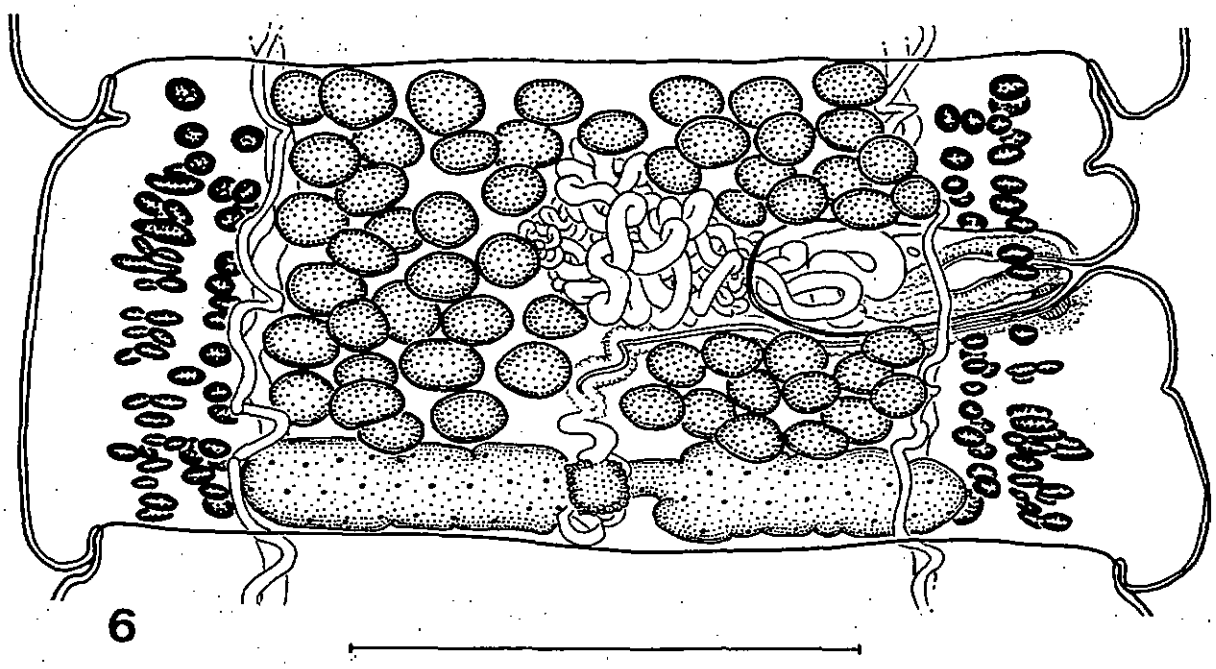
Oncospheres spherical, 14–15 in diameter, with 5.5-6.5 long books, surrounded by oncospheric membrane (Figure 2). Embryophores round or ovoid consisting of 2 layers; outer layer larger than nuclei-containing envelope, 31–35 × 28–30 in diameter; outer hyaline layer > 90 in diameter. Ventral and dorsal osmoregulatory ducts without anastomoses, situated between vitelline follicles and testes. Ventral osmoregulatory duct twice width of dorsal duct, sometimes overlapping marginal testes.

Protein electrophoresis

The population structure of *Nomimoscolex suspectus* specimens collected from three species of the siluriform fish genus *Brachyplatystoma* and the taxonomic status of *Nomimoscolex piraeeba* and *N. dorad* were examined using allozyme electrophoresis methods. Of 25 enzymatic systems tested for allelic polymorphism, nine (AAT, ACP, AK, ALD, GPI, MDH, ME, MPI and NP) yielded genetically interpretable zymograms that showed some level of allelic variation. Seventeen specimens of *Nomimoscolex suspectus* n. sp. (10 from *Brachyplatystoma filamentosum*, four from *B. flavicans* and three from *B. vaillanti*), two specimens of *N. piraeeba* from *B. filamentosum* and 11 specimens of *N. dorad* from *B. flavicans* were included in our analysis.

The nine enzymes above demonstrated a very similar electrophoretic mobility between all the specimens of *N. suspectus* we examined, regardless of their host species, thus strongly indicating they belong to the same species. The AAT locus was, however, peculiar in exhibiting an allele with a faster electrophoretic mobility in one specimen of *N. suspectus* from *B. flavicans*. The two *N. piraeeba* samples behaved identically in all systems. The 11 specimens of *N. dorad* likewise form a homogenous group; however, certain samples displayed exceptionally elongated stains that





Figures 5–6. Nomimoscolex suspectus n. sp. 5. Mature proglottis, dorsal view, from syntype material of *N. piraeeba* Woodland (1934, plate 10, figure 7), BMNH 1964.12.15.111-122. *Abbreviations: vs.* vaginal sphincter. 6. *Nomimoscolex suspectus* n. sp. Holotype. 34212 CHIOC, dorsal view, mature proglottis. *Scale-bar*: 500 μm.

represented somewhat slower alleles for the GPI locus (Figure 8, lane 13). We have no explanation as to the possible causes of these smeared stains: they did not systematically occur in other enzymatic systems. We additionally found that the NP zymogram revealed two zones of enzymatic activity in all specimens examined. We interpreted this result as the presence of two loci for this enzyme. The possible simultaneous revelation of another enzyme was ruled out, since no such cases were reported in the literature for this specific enzyme. The HK zymogram (data not shown) was informative but not clearly interpretable: the activity of another enzyme (GCDH) may, in this case, have been revealed simultaneously (Hillis & Morris, 1990). The results of enzyme electrophoresis assays are summed in Table 1.

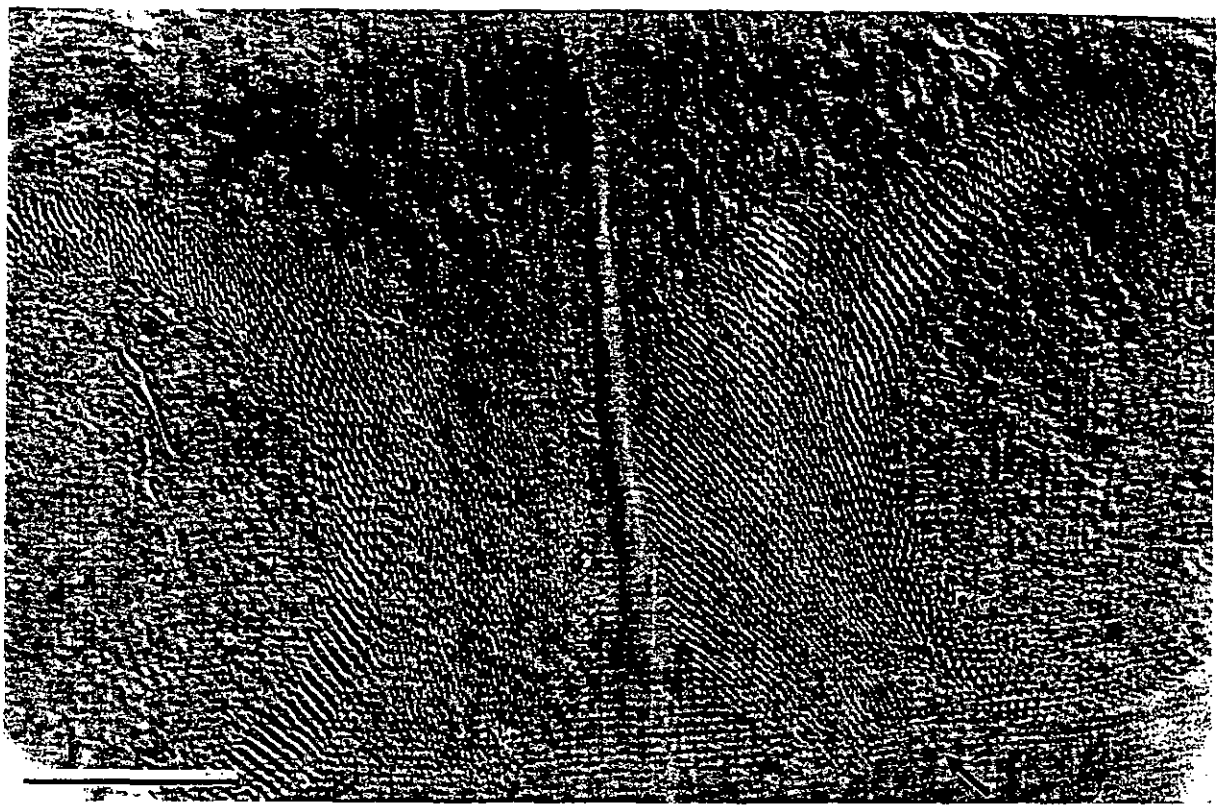


Figure 7. Nomimoscolex suspectus n. sp. Photomicrograph of scolex. Detail of suckers showing the numerous spiniform microtriches on their rim and the smaller spiniform microtriches present on the scolex. The spiniform microtriches end at the level of the posterior margin of the sucker (arrow). Holotype 34212 CHIOC. *Scale-bar*: 50 μm.

Table 1. Alleles detected in enzyme electrophoresis analyses of three species of *Nomimoscolex*.

Loci	*N. suspectus* n. sp. 17 samples	*N. dorad* 11 samples	*N. piraeeba* 2 samples
MPI	Mpi^a	Mpi^a	Mpi^b
AK	Ak^a	Ak^a	Ak^b
ACP	Acp^a	Acp^b	Acp^c
ALD	+*	−*	+*
MDH	Mdh^a	Mdh^b	Mdh^b
NP-1	$Np\text{-}1^a$	$Np\text{-}1^b$	$Np\text{-}1^a$
NP-2	$Np\text{-}2^a$	$Np\text{-}2^b$	$Np\text{-}2^a$
GPI	$Gpi^{a,b}$	$Gpi^{a,c,d}$	Gpi^e
ME	Me^a	Me^a	Me^b

*The alleles for the *ALD* locus are characterised by their migration: + towards the cathode; − towards the anode.

Discussion

Nomimoscolex suspectus n. sp. is placed in the genus *Nomimoscolex* Woodland, 1934 (subfamily Zygobothriinae) due to the cortical or paramuscular position of the vitelline follicles as well as the presence of four simple unilobate suckers. Among the nine species currently placed in the genus (see de Chambrier & Vaucher, 1997), only *N. piraeeba* shares the presence of a glandular apical organ, a craspedote strobila and spiniform microtriches on the suckers. *N. suspectus* differs from the latter in:

– a lower mean number of testes (69 in *N. suspectus*, ranging between 92 and 113 in *N. piraeeba*);

– a greater cirrus-pouch length / proglottis width ratio (28-43 *vs* 21-28);

– a lower ovarian width / proglottis width ratio (54-67 *vs* 67-78);

– the position of the vitelline follicles: paramuscular in *N. suspectus* and cortical in *N. piraeeba* (Table 2).

Owing to the differences mentioned above, the present material is considered to represent a new species, *Nomimoscolex suspectus*. Etymology (Latin): admired, revered, also fallen under suspicion.

Our description matches that of the proglottis figured by Woodland (1934b, figure 7, pl. 10 and figure 5 in the present study) and verifies the observation made

Figure 8. Zymogram of the GPI (Glucose-phosphate Isomerase) enzyme for three *Nomimoscolex* species. Lanes 1, 3–11, 13: *N. dorad*: lanes 2 and 12: *N. piraeeba*: lanes 14 – 21: *N. suspectus* n. sp. An unusually elongate stain with a lower electrophoretic mobility is visible in a sample of *N. dorad* in lane 13.

Table 2. Morphological comparison of three *Nomimoscolex* species.

	N. suspectus n. sp.	*N. piraeeba* TM	*N. piraeeba* Recently collected	*N. dorad* TM	*N. dorad* Recently collected
Scolex diameter	320–450	325	555	395–475	475–590
PC	28–43	21–28	21–24	26–33	16–22
OV	54–67	70–78	67–71	73–74	64–73
GP	38–58	29–46	33–54	32–54	36–51
Position of vitelline follicles	paramuscular	cortical	cortical	cortical	cortical
Number of uterine diverticula	10–18	7–12	7–13	9–13	6–11
Testes number	47–93, x = 69	77–116, x = 92	90–133, x = 113	c. 120	95–162. x = 117
Longitudinal musculature interrupted laterally	yes	no	no	yes	yes

PC, Cirrus-pouch length / proglottis width ratio; OV, Ovarian width / proglottis width ratio; GP, Positioo of genital pore as % of proglottis length; TM, Type-material, x = mean.

by de Chambrier & Vaucher (1997, p. 224) that it cannot be assigned to *N. piraeeba*.

N. suspectus is also present, sympatrically, in *Brachyplatystoma vaillanti* and *B. flavicans*: specimens from these hosts are morphologically identical to those from *B. filamentosum*. Regardless of the host species, the worms are frequently concentrated on a small area of the posterior portion of the intestine and rectum and the scolex often penetrates deeply into the intestinal mucosa.

On the basis of protein electrophoresis studies, *N. suspectus* could be differentiated from *N. piraeeba* at the ACP, AK, GPI, ME, MDH and MPI loci, and from *N. dorad* at the ACP, ALD, GPI, MDH, NP-1 and NP-2 loci. On the other hand, *N. piraeeba* and *N. dorad* displayed allelic polymorphism at eight loci: ACP, AK, ALD, GPI, MPI, ME, NP-1 and NP-2 (Table 1).

Considering *N. piraeeba*, *N. dorad* and *N. suspectus*, each show distinct alleles for several loci, and considering their reproductive isolation as assessed by the apparent lack of hybrids, we conclude that the three taxa above constitute three genetically distinct species of *Nomimoscolex*. A very low rate of heterozygocity was detected, which is a common observation in hermaphroditic organisms, but the GPI locus nevertheless revealed the simultaneous presence of one heterozygote in each of the *N. suspectus* and *N. dorad* populations. They both showed three stains of enzymatic activity due to the dimeric structure of this enzyme (Figure 8). The *N. dorad* heterozygote may be interpreted as being a hybrid between *N. dorad* and *N. suspectus*; however, no additional enzyme yielded results that support this hypothesis. A careful examination of the gel also showed that *N. suspectus* had alleles that migrate slightly slower than that of *N. dorad*.

Phylogeny

Phylogenetic analyses based on morphological and molecular data

Phylogenetic analyses based on morphological and molecular characters were conducted in order to examine the structure of the genus *Nomimoscolex* and its relationships with other genera in the subfamily Zygobothriinae. The analyses thus included nine *Nomimoscolex* species of the 11 recognised species of the genus, one of the four genera of the Zygobothriinae and two outgroup species, *Proteocephalus pirarara* and *Ophiotaenia jorara* which were selected because of their basal position respective to the above species in a previous phylogenetic study of the Proteocephalidea based on partial 28S rDNA sequences (Zehnder & Mariaux, 1999). *Choanoscolex* sp., although belonging to the Monticelliinae, was included in the present analysis since it appeared to be related to *Nomimoscolex sudobim* in the same work.

Morphological analysis

Phylogenetic analyses, based on the morphological matrix listing 24 characters and 59 character states (see Appendix and Table 3), resulted in a single most parsimonious tree with L = 60 steps (minimum length = 35; CI = 0.58; HI = 0.42; RI = 0.58; RC = 0.34; CI excluding uninformative characters = 0.56). Three characters (12, 14 and 24) were uninformative. Some degree of homoplasy was associated with 16 of the 24 characters analysed, as can be seen by the consistency indices given for individual characters in Table 4. The genus *Nomimoscolex* is not monophyletic in our construction and overlaps with the other genera (Figure 9). Constraining *Nomimoscolex* to be monophyletic requires four additional steps, which is a significant difference as evaluated by a Templeton's test (P value = 0.014). A well-defined clade, however, comprising *N. piraeeba*, *N. dorad* and *N. suspectus*, was supported by high bootstrap values and seven character changes: three unambiguous synapomorphies (the presence of spiniform microtriches on the rim of suckers, an apical organ of glandular nature and a uterine pore-like structure; characters 3, 5 and 21) and four characters that showed apparent homoplasy (a craspedote strobila, an internal longitudinal musculature consisting of two layers, a cirrus-pouch length of between 20% and 36% of proglottis width, and an ovary of follicular structure; characters 7, 8, 15 and 16). There was little character support for the remaining internodes (except for internode 1, see Figure 9): the number of changes ranged from one to a maximum of three. As a consequence, relationships among the majority of the species analysed remained largely unsupported. This result was confirmed by bootstrap analyses (Figure 9) that supported no more than the small clade described above.

DNA analyses

Two regions of the nuclear rDNA gene array were sequenced for the same 18 species as in the morphological analysis above: a 714 nt region comprising part of the 5.8S rDNA gene and the Internal Transcribed Spacer 2 (ITS2), the '5.8S-ITS2' partition; and a 1026 nt 5′ region of the adjacent 28S rDNA gene, the '28S' partition.

From '5.8S-ITS2', 249 positions were discarded prior to the analysis due to their problematic alignment. Of the remaining 465 characters, 169 were variable and 137 were phylogenetically informative. Parsimony analyses found three nearly identical most parsimonious trees with L = 336 steps (CI = 0.66, RI = 0.77, RC = 0.51) (data not shown). *Ophiotaenia jorora* and *Nomimoscolex lenha* were not included in this database due to sequencing and alignment difficulties, respectively. The 28S rDNA fragment, on the other hand, was sequenced and aligned for all 18 taxa. Seventeen sites were excluded due to their problematic alignment. The resulting matrix comprised 170 variable positions, of which 93 were informative. Parsimony analyses yielded 15 shortest trees of 213 steps (CI = 0.55, RI = 0.68, RC = 0.38) (data not shown). A partition homogeneity test (PHT) indicated that the two molecular matrices contained no conflicting information and could be combined for phylogenetic analyses (PHT test value: P = 0.13). Analysis of the combined DNA dataset yielded seven shortest trees with L = 523, CI = 0.63, RI = 0.74, RC = 0.46 and a tree resolution intermediate to that of the separate partitions (Figure 10).

Both partitions, alone or combined, failed to confirm the monophyly of the genus *Nomimoscolex*. When applying this topological constraint, 17 additional steps were required, using the 28S database. This difference in tree length was not significant, as demonstrated by a Templeton's test (P = 0.31), and may be a consequence of the low tree-resolution exhibited by this partition. However, when applying the same constraint to the 5.8S-ITS2 partition, which produced trees with a superior resolution and consis-

Table 3. Morphological character matrix for *Nomimoscolex* and other genera included in the phylogenetic analyses.

Species	Characters																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Proteocephalus pirarara	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Ophiotaenia jarara	0	0	0	1	0	0	1	0	0	1	1	0	2	1	2	0	0	1	0	0	0	0	0	0
Amphoteromorphus parkamoo	1	0	0	0	0	1	1	0	0	0	?	0	0	1	1	1	0	0	0	1	0	3	1	1
Choanoscolex sp.	0	0	0	0	0	1	1	0	0	1	1	1	0	1	1	0	2	2	2	1	0	2	0	0
Endorchis piraeeba	1	0	0	0	0	0	1	1	0	1	1	0	0	1	0	0	1	0	1	1	0	3	1	0
Gibsoniela meursaulti	2	0	0	0	0	0	1	0	0	1	1	0	1	1	1	0	1	0	1	1	0	1	1	0
Harriscolex kaparari	0	1	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1	1	0	2	1	0
Nomimoscolex admonticellia	0	0	0	0	0	0	0	1	0	1	2	0	0	1	1	0	1	0	1	1	0	2	1	0
Nomimoscolex chubbi	0	0	0	0	0	0	1	0	0	1	1	0	1	1	1	0	0	0	1	1	0	2	2	0
Nomimoscolex dorad	0	0	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0	0	1	1	1	2	0	0
Nomimoscolex lenha	0	1	0	0	0	0	1	0	0	1	1	0	1	1	1	0	1	0	0	0	0	2	2	0
Nomimoscolex lopesi	0	0	0	0	0	0	1	0	0	2	2	0	0	1	1	0	0	2	0	1	0	2	1	0
Nomimoscolex matogrossensis	0	0	0	1	0	0	1	0	0	1	1	0	0	1	2	0	0	0	1	1	0	2	1	0
Nomimoscolex piraeeba	0	0	1	1	1	0	0	1	0	1	1	0	0	1	1	1	0	0	1	1	1	2	0	0
Nomimoscolex sudobim	0	0	0	0	0	0	0	0	0	2	1	0	0	1	1	0	1	0	2	1	0	2	1	0
Nomimoscolex suspectus n. sp.	0	0	1	1	1	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1	1	1	0	0

Table 4. Consistency indices for individual characters used in analysis.

Character number	Character	CI
1	Suckers structure	0.67
2	Suckers papillae	0.5
3	Suckers spiniform microtriches	1.0
4	Apical organ	0.5
5	Apical organ-structure	1.0
6	Metascolex	0.5
7	Strobila-structure	0.25
8	Longitudinal musculature-layers	0.5
9	Longitudinal musculature-structure	1.0
10	Osmoregulatory ducts-position	0.67
11	Testes-number	0.5
12	Testes-position	1.0
13	Testes-distribution	1.0
14	Cirrus-'spination'	1.0
15	Cirrus-pouch-length	0.5
16	Ovary-structure	0.5
17	Ovary-position	0.4
18	Ovary-width	0.5
19	Uterus-position	0.5
20	Uterus-development	0.5
21	Uterine-'pore-like' structure	1.0
22	Vitelline follicles – position	0.6
23	Vagina-position relative to cirrus-pouch	0.67
24	Eggs-shape	1.0

tency index, and the combined molecular database, the differences in tree length (30 and 47 steps, respectively) were significant in a Templeton's test ($P < 0.0001$). Nevertheless, the molecular databases supported strongly a split among the ingroup species and defined a clade comprising *Nomimoscolex piraeeba*, *N. dorad* and *N. suspectus* which was also present on the morphological tree (Figure 9), and a sister group including the other *Nomimoscolex* representatives, the other genera of the Zygobothriinae and *Choanoscolex* sp. from the Monticelliinae. The '5.8S-ITS2' partition resolved relations efficiently within this clade but the different genera overlapped nevertheless: *N. chubbi* and *Harriscolex kaparari* were strongly associated, as well as *N. sudobim* and *Choanoscolex* sp. Trees from '28S' on the other hand supported little more than the affinity of *H. kaparari* with *N. chubbi*.

The three specimens of *N. suspectus*, each caught from a different species of the Amazon siluriform fish genus *Brachyplatystoma*, were found to be closely related: 28S sequences showed 0.1% differing nucleotides among them. Their 5.8S-ITS2 sequences diverged somewhat more but they all exhibited a $[TG]_{10}$ microsatellite inserted in two regions of ITS2, a feature lacking in the other taxa.

The two molecular and the 'morphology' partitions were ultimately tested for conflicting information in a partition homogeneity test that indicated they could

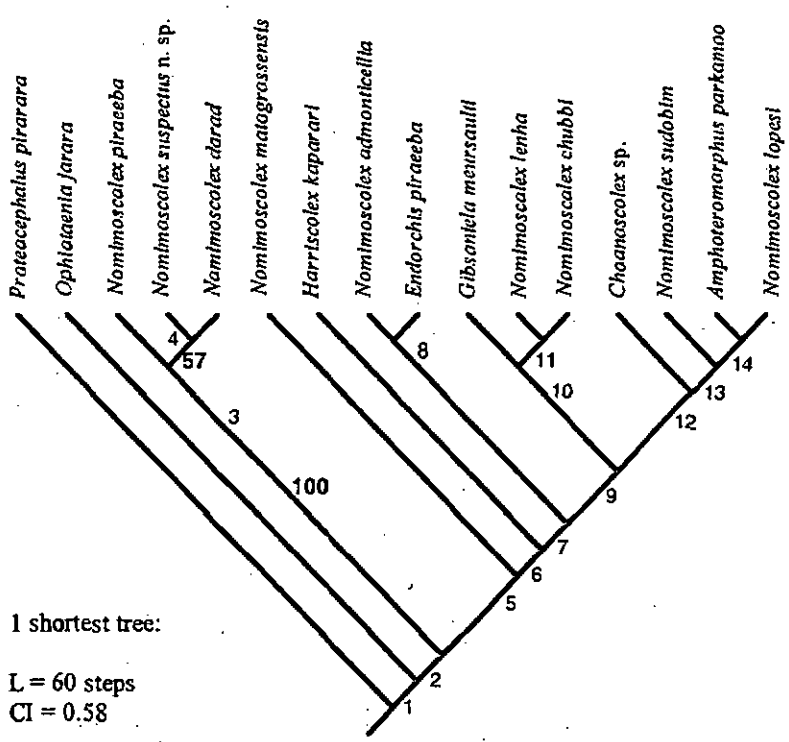


Figure 9. Phylogenetic hypothesis based on 24 morphological characters for the nine species of *Nomimoscolex*, representatives of four additional Zygobothriinae genera and *Choanoscolex* sp., using *Prateocephalus pirarara* and *Ophiotaenia jarara* as outgroup species. Single most parsimonious tree obtained with the Branch and Bound searching algorithm: minimum length = 35 steps, 60 required, CI = 0.58, HI = 0.41 (if uninformative characters are included). Character support for each taxon and internode (numbered) is defined below and includes apomorphy (A), homoplasy as convergence or parallelism (H), reversal (R) and polymorphism or change in terminal taxa (P). Terminal taxa are labelled accordingly: *Ophiotenia jarara* (A: 13; H: 18); *Nomimoscolex suspectus* n. sp. (H: 18, 22; R: 11); *Harriscolex kaparari* (H: 2); *Endorchis piraeeba* (A: 1; H: 22); *Nomimoscolex admonticellia* (H: 11; R: 7); *Gibsoniela meursaulti* (A: 1; H: 22); *N. lenha* (H: 2, 19, 20); *N. chubbi* (R: 17); *Choanosolex* sp. (A: 12, 17; H: 6, 18; R: 23); *N. sudobim* (R: 7); *Amphoteromorphus piraeeba* (A: 24; H: 1, 6, 16, 22; R: 10); *N. lopesi* (H: 18). Nodes numbered from the base are designated as 1-14: 1: (A: 4, 7, 10, 11, 14, 15; R: 17); 2: (A: 19, 20, 22); 3: (A: 3, 5, 21; H: 8, 16; R: 7, 15); 4: (A: 9); 5: (A: 23); 6: (R: 4, 15); 7: (A: 17); 8: (H: 8); 9: (A: 15); 10: (A: 13); 11: (A: 23); 12: (A: 19); 13: (A: 10); 14: (H: 11, 19; R: 17). Bootstrap values obtained for 100 heuristic replicates of 10 heuristic searches are indicated in bold.

be combined for phylogenetic analyses. The combined dataset produced one shortest tree using parsimony (L = 576, CI = 0.60, RI = 0.61, RC = 0.32) (Figure 11) that was highly compatible with the molecular tree.

Discussion

Regarding the structure of the genus *Nomimoscolex*, our present study verifies previous statements made by Woodland (1935a) and de Chambrier & Vaucher (1997) that *Nomimoscolex* may represent a heterogeneous taxon, the members of which would need to be grouped into different genera. Our morphological and molecular data partitions deciphered phylogenetic relationships within this genus to various degrees but were nevertheless congruent in showing that *Nomimoscolex* was not a monophyletic taxon. Brooks (1984, 1995) came to similar conclusions in his phylogenetic analyses based on morphological characters. Moreover, our partitions all strongly supported the grouping of *N. piraeeba*, *N. dorad* and *N. suspectus* n. sp. in a robust cluster. The remaining *Nomimoscolex* spp. were invariably excluded from this clade and blended with the other genera. Their relationships, while resolved on the '5.8S-ITS2' tree, were only partly apparent when combining this partition to the '28S' and morphological datasets, which both produced less resolved trees. Thus, no clear alternative structure for the genus *Nomimoscolex* came forth from our phylogenetic analyses.

The association of the monticelliine *Choanoscolex* sp. with the Zygobothriinae, and its preferential grouping with *N. sudobim* in our analyses of 5.8S-ITS2 sequences, in particular, remains puzzling: the internal arrangement of their reproductive organs relative to the internal longitudinal muscle sheath differs consid-

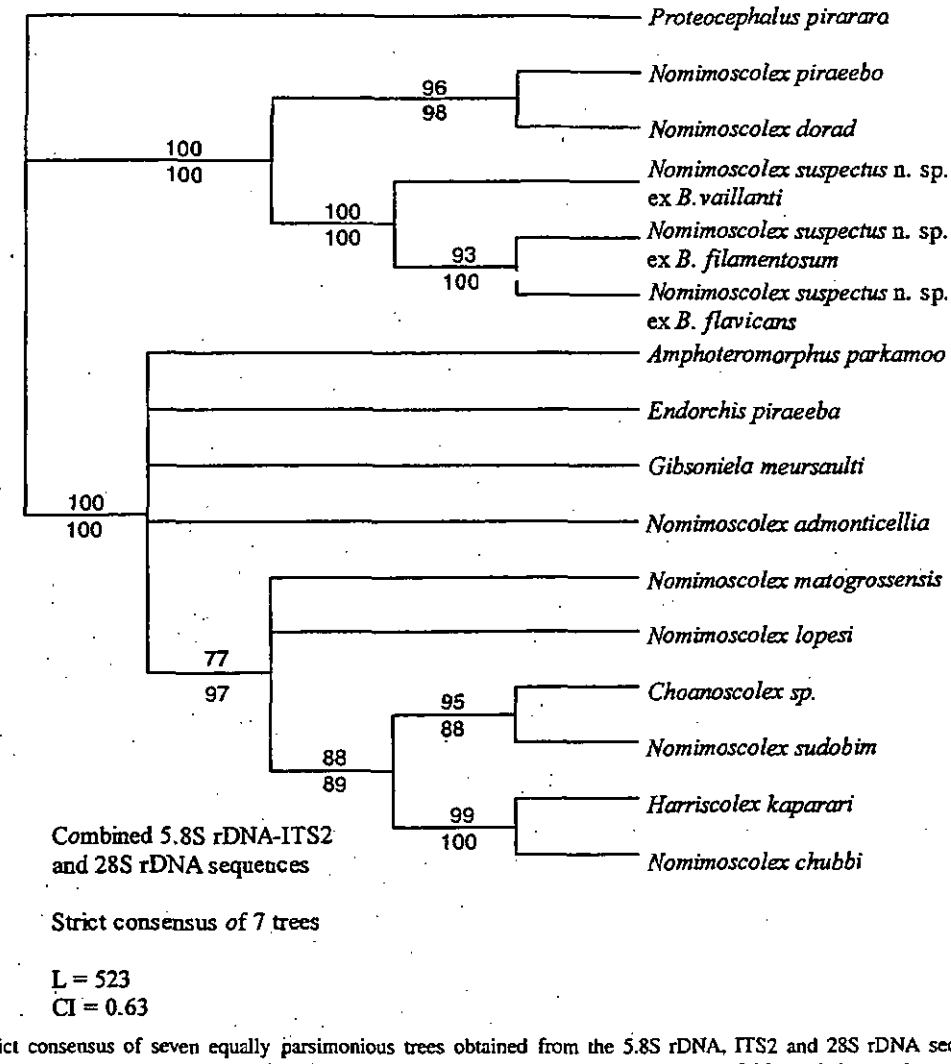


Figure 10. Strict consensus of seven equally parsimonious trees obtained from the 5.8S rDNA, ITS2 and 28S rDNA sequence data set. One hundred heuristic searches were performed. Values obtained from 100 Bootstrap replicates of 10 heuristic searches are shown above internodes. The figures below are obtained from 100 Bootstrap replicates using the neighbor-joining algorithm. The Kimura 2-parameter model for nucleotide substitution was used. *Abbreviations*: *B.*, *Brachyplatystoma*.

erably. In the Monticelliinae the reproductive organs are all cortical, whereas in the Zygobothriinae only the vitelline glands lie in the cortex. So far we have no explanation for this association, but this result once again questions the morphological characters traditionally used in the group.

Conclusions

The simultaneous use of different means of investigation, i.e. morphological examination, isoenzyme and DNA sequence analyses, yielded converging results indicating that *Nomimoscolex suspectus* n. sp. specimens collected from the Amazon siluriform fishes *Brachyplatystoma filamentosum*, *B. flavicans* and *B. vaillanti* belong to one same species.

At the morphological level, *N. suspectus* could be separated from *N. piraeeba* and *N. dorad* by a few features only, i.e. the paramuscular position of vitelline follicles, the cirrus-pouch length / proglottis width ratio, the ovarian width / proglottis width ratio and the number of testes. Considering the resemblance of the three taxa and the degree of morphological overlap they exhibit, these criteria alone would not suffice to confidently state that *N. suspectus* represents another species. At the molecular level, however, allozyme electrophoresis assays revealed allelic polymorphism at six loci between *N. suspectus* and both, *N. piraeeba* and *N. dorad*, suggesting that it is a biologically dis-

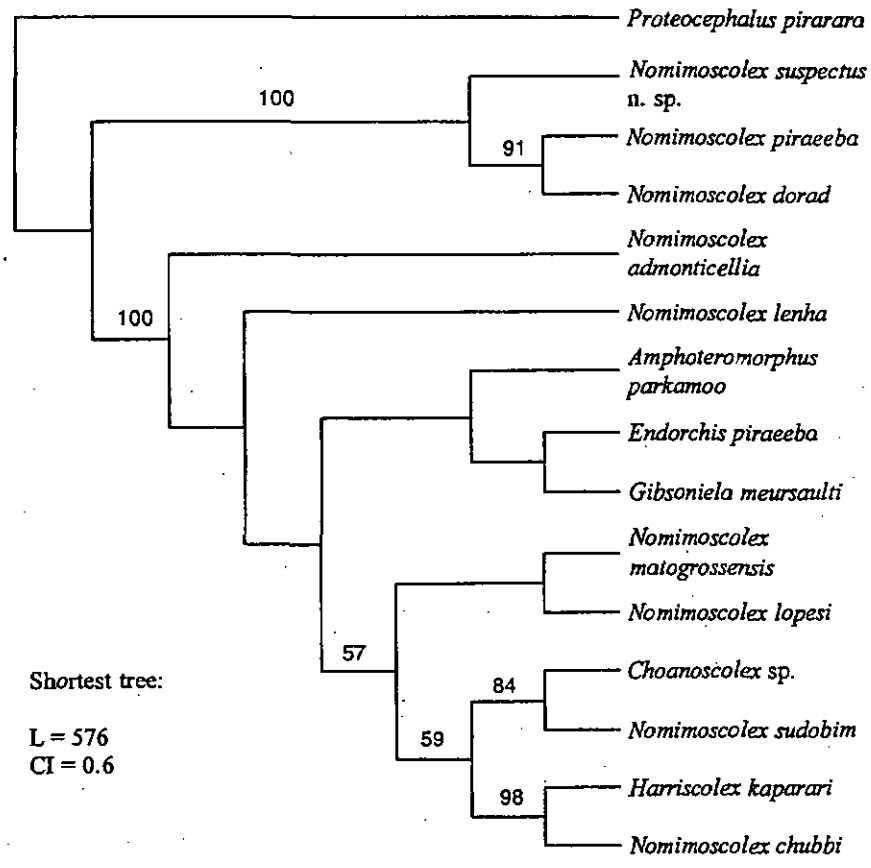


Figure 11. Most parsimonious tree obtained from the combined DNA sequences (5.8S rDNA, ITS2 and 28S rDNA) and morphological partitions. Values obtained from 100 Bootstrap replicates of 10 heuristic searches are shown above internodes. *Nomimoscolex lenha* was included in this analysis even though sequences for 5.8S rDNA and ITS2 were not available for this taxon.

tinct taxon. DNA sequence data were congruent with this conclusion: pairwise sequence divergence rates, for the whole 5.8S rDNA, ITS2 and 28S rDNA region, were of 3.5% between *N. suspectus* and both *N. piraeeba* and *N. dorad*. In comparison, sequence divergence rates ranged only from 0% to 0.8% in samples of *N. suspectus* from different hosts.

N. piraeeba and *N. dorad*, which differed only in the cirrus-pouch length / proglottis width ratio (21–24% and 16–22%, respectively) and the lateral interruption of the internal longitudinal musculature (present exclusively in *N. dorad*, Table 2), appeared as two separate species in isoenzyme studies. Genetic distances, expressed as the percentage of differing nucleotides within the combined 5.8S rDNA, ITS2 and 28S rDNA sequences, likewise indicated that *N. piraeeba* and *N. dorad*, with 2.2% divergence, may represent two species, considering that *N. chubbi* and *Harriscolex kaparari* have diverged at a comparable rate of 2.3%.

Allozyme and DNA sequence data thus supported the re-establishment of *N. dorad*, which had previously been synonymised with *N. piraeeba* (see Rego, 1991; de Chambrier & Vaucher, 1997). Due to the difficulty in distinguishing *N. dorad* from *N. piraeeba*, other than relying on molecular data, we consider it to represent a cryptic species. Curiously, while the expected grouping ((*N. piraeeba*, *N. dorad*), *N. suspectus*) was obtained when using molecular data for phylogenetic analyses, the morphological data favoured the ((*N. dorad*, *N. suspectus*), *N. piraeeba*) combination (by only one step, however).

The results of our phylogenetic studies demonstrated the need for a reorganisation of the genus *Nomimoscolex*. We favour the establishment of an aggregate of species, complying with Art. 6b of the International Code of Zoological Nomenclature (1999), formed by *N. piraeeba*, *N. dorad* and *N. suspectus*, in order to reflect the high degree of morphological identity displayed by the three species and their isolation into a distinct and robust clade that excluded

other members of the genus in phylogenetic analyses. Consequently, the aggregate comprises:
Nomimoscolex (piraeeba) piraeeba Woodland, 1934
Nomimoscolex (piraeeba) dorad (Woodland, 1935)
Nomimoscolex (piraeeba) suspectus n. sp.
Relationships among the remaining species of this genus were not satisfactorily resolved in our analyses: neither the morphological nor molecular trees produced robust, congruent groupings. These species clearly belong to independent lineages and the concept of the genus to which they are presently attributed is artificial. However, due to the lack of satisfactorily supported hypotheses, we are currently unable to propose a complete reorganisation the group.

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Appendix

Description of characters

(1) Suckers (structure). Three states: 0 = normal unilocular type; 1 = unilocular bilobate; 2 = triloculate. Character state 2 is autapomorphic for the genus *Gibsoniela*.

(2) Horny projections (= papillae) on suckers. Two states: 0 = absent; 1 = present.

(3) Spiniform microtriches on suckers. Two states: 0 = absent; 1 = present.

(4) Apical organ. Two states: 0 = absent; 1 = present.

(5) Apical organ (structure). Two states: 0 = not glandular; 1 = glandular.

(6) Metascolex. Two states: 0 = absent; 1 = present.

(7) Strobila (segment structure). Two states: 0 = craspedote; 1 = acraspedote.

(8) Longitudinal musculature. Two states: 0 = presence of one layer; 1 = presence of two layers.

(9) Longitudinal musculature. Two states: 0 = uninterrupted laterally, 1 = interrupted laterally.

(10) Osmoregulatory ducts (position in lateral view). Three states: 0 = overlapping vitelline follicle field; 1 = situated between vitelline follicles and testes; 2 = overlapping testes field.

(11) Testes (mean number per proglottis). Three states: 0 = < 76; 1 = between 76 and 238*; 2 = > 238. *Values comprised within the X − s and X + s limits, where X represents the mean value for all measures, and s is the standard deviation.

(12) Testes (position). Two states: 0 = medullary; 1 = cortical. The cortical position of testes is an autapomorphy of the genus *Choanoscolex*.

(13) Testes (distribution). Three states: 0 = in one field; 1 = in two lateral fields, anteriorly confluent; 2 = in two separate lateral fields.

(14) Spines on cirrus. Two states: 0 = present; 1 = absent. A spinose cirrus is only found in *Proteocephalus pirarara*, used as outgroup.

(15) Cirrus-pouch length / proglottis width ratio. Three states: 0 = > 36%; 1 = between 20% and 36%*; 2 = < 20%. *(see character 11).

(16) Ovary (structure). Two states: 0 = follicular; 1 = compact.

(17) Ovary (position). Three states; 0 = medullary; 1 = medullary with projections into dorsal cortex; 2 = cortical.

Remark: although character polarisation was based on outgroup comparison, our most basal taxon, *Proteocephalus pirarara*, was given the derived character state ≪ 1 ≫ instead of ≪ 0 ≫ for character 17. The state ≪ 1 ≫ - ovary medullary with projections into dorsal cortex - is found only exceptionally in proteocephalid species that are basal to our ingroup. Our other outgroup taxon, *Ophiotaenia jarara*, bears the expected ancestral state ≪ 0 ≫ (medullary position of ovary), which is thereby restored, at the base of the ingroup species in our phylogenetic reconstructions.

(18) Ovarian width / proglottis width ratio. Three states: 0 = between 60% and 78%*; 1 = < 60%; 2 = > 78%. *(see character 11).

(19) Uterus (position). Three states: 0 = entirely medullary; 1 = uterine stem cortical with diverticula projecting into medulla; 2 = entirely cortical.

(20) Uterus (development). Two states: 0 = normal type; 1 = 'monticelliid' type.

(21) Uterine 'pore-like' structure. Two states: 0 = absent; 1 = present.

(22) Vitteline follicles (distribution, as seen in cross-sections). Four states: 0 = medullary; 1 = paramuscular and lateral; 2 = cortical and lateral; 3 = cortical, in two dorsal and two ventral bands.

(23) Position of vagina relative to the cirrus-pouch. Three states. 0 = alternating; 1 = anterior in over 95% of cases; 2 = posterior in over 97% of cases.

(24) Eggs (shape). Two states: 0 = spherical; 1 = elongate. The elongate egg shape is an autapomorphy of the genus *Amphoteromorphus*.