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VERSITA

The phylogeny of the Lepocreadioidea (Platyhelminthes, Digenea) inferred from nuclear and mitochondrial genes: Implications for their systematics and evolution

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Abstract

The phylogenetic relationships of representative species of the superfamily Lepocreadioidea were assessed using partial *lsr*DNA and *nad1* sequences. Forty-two members of the family Lepocreadiidae, six putative members of the Enenteridae, six gyliauchenid species and one Gorgocephalidae, were studied along with 22 species representing 8 families. The Lepocreadioidea is found to be monophyletic, except for the two species of the putative enenterid genus *Cadenatella*, which are found to be only distantly related to the lepocreadioids. The Lepocreadioidea is formed of five clades in a polytomy, the Gorgocephalidae, a clade containing the Enenteridae and Gyliauchenidae, a small clade of atypical lepocreadiines and the deep-sea lepidapedine lepocreadiids, a small clade consisting of a freshwater form and a group of shallow-water putative lepidapedines and the final clade includes the remaining lepocreadiids. Thus, the generally accepted concept of the Lepocreadiidae is polyphyletic. The Enenteridae (minus *Cadenatella*) and the Gyliauchenidae are jointly and individually monophyletic, and are sister groups. The *nad1* gene on its own places a deep-sea lepocreadiine with the deep-sea lepidapedines, whereas *lsr*DNA, combined sequences and morphology place this deep-sea lepocreadiine within a group of typical lepocreadiids. It could not be demonstrated that a significant proportion of sites in the *nad1* gene evolved under positive selection; this anomalous relationship therefore remains unexplained. Most deep-sea species are in a monophyletic group, a few of which also occur in shallow waters, retaining some characters of the deep-sea clade. Many lepocreadioid species infect herbivorous fish, and it may be that the recently discovered life-cycle involving a bivalve first intermediate host and metacercariae encysted on vegetation is a common life-cycle pattern. The host relationships show no indication of co-speciation, although the host-spectrums exhibited are not random, with related worms tending to utilize related hosts. There are, however, many exceptions. Morphology is found to be of limited value in indicating higher level relationships. For example, even with the benefit of hindsight the gyliauchenids show little morphological similarity to their sister group, the Enenteridae.

Keywords

Lepocreadiidae, Plagiorchiida, Digenea, deep-sea, phylogeny, *lsr*DNA, *nad1*, *dN/dS*, selection

Introduction

Morphological systematists have encountered a problem resulting from the great similarity of many of the digenean parasites of teleost fishes, the so called ‘allocreadioid problem’ (Cable 1956). Several hundred current genera can be included in this somewhat homogeneous group, many of which were, in the first half of the 20th century, placed in the superfamily Allocreadioidea Looss, 1902 (see Cribb 2005b). More recently, the superfamily Lepocreadioidea Odhner, 1905 has generally been considered the best depository for those taxa with a spiny tegument which are involved in this problem. It has been clear to morphologists, nevertheless, that this group is not monophyletic, but it has taken the introduction of mo-

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lecular phylogenetics to begin to clarify the relationships. When Bray (2005d) produced a morphological key to the superfamily he included eleven families 'for convenience in identification'. Molecular results have shown that three of these families (Acanthocolpidae Lühe, 1906, Apocreadiidae Skrjabin, 1942 and Brachycladiidae Odhner, 1905) are not closely related to the Lepocreadiidae Odhner, 1905 (Cribb *et al*. 2001, Olson *et al*. 2003, Bray *et al*. 2005). Three other families await molecular studies, namely the Deropristidae Cable et Hunninen, 1942; Liliatrematidae Gubanov, 1953 and Megaperidae Manter, 1934. Molecular phylogenetic analysis indicates that the four remaining families form a monophyletic group, the group now known under the name Lepocreadioidea.

As presently understood the Lepocreadioidea contains only fish parasites, overwhelmingly marine forms. They are of considerable interest biologically as they comprise important groups of worms in a range of marine habitats. Lepocreadioids (lepocreadiids in particular) are common in a wide range of pelagic and benthic species. A particular concentration and radiation is a group seemingly adapted to members of the fish order Tetraodontiformes. Another radiation of lepocreadioids occurs in and dominates the digenean fauna of tropical herbivorous fishes. Lepocreadioids also include the group of worms which dominate the digenean fauna of really deep-sea (>1,000 m) fishes (Bray 1995, 2004; Klimpel *et al*. 2001). Evidence presented by Bray *et al*. (1999) indicated that a large clade of lepocreadiids has radiated in the deep-sea and that within this clade, shallow-water life-styles are secondarily derived. Considering the size of the superfamily, with about 95 genera, little is known of such important biological characteristics as the life-cycle, development and distribution. Nothing is known of the life-cycle of the exclusively herbivorous fish-parasites groups, the Enenteridae Yamaguti, 1958, Gyliauchenidae Ozaki, 1933 and Gorgocephalidae Manter, 1966. The life-cycles of various lepocreadiid species have been studied, and are discussed in detail below, but it is clear that their pattern varies considerably, with both gastropods and bivalves described as first intermediate hosts.

In order to resolve further the interrelationships of the Lepocreadiidae, we chose to add to the existing published data for partial (D1-D3) nuclear large subunit ribosomal RNA (*lsr*DNA) and partial mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*) genes (Bray *et al*. 1999). The *lsr*DNA fragment has provided substantial resolution for a number of phylogenetic estimates of digeneans (e.g. acanthocolpids, Bray *et al*. 2005; schistosomatids, Webster *et al*. 2006). In order to add finer phylogenetic resolution amongst closely related taxa we chose a mitochondrial gene for its relatively faster rate of evolution (e.g. Brown *et al*. 1979) and because we have had some success with *nad1* in previous studies, thus providing us with established PCR primers; e.g. amongst echinostomes (Kostadinova *et al*. 2003). However, we were also mindful that choosing a mitochondrial gene for resolving interrelationships of animals from relatively deep or distant phylogenetic lineages might be problematical.

Mitochondrial genes are integral components of oxidative phosphorylation (OXPHOS) pathways whereby animal cells release energy, and one might expect that continued environmental or ecological sources of hypoxia (e.g. oxidative stress through oxygen deprivation as a direct result of parasitism and/or living at considerable depth) might be reflected in positive natural selection on key mt genes (Hochachka 1986). Such evidence has been reported recently in cytochrome *c* oxidase genes of mammals living in cold, high habitats (see also da Fonseca *et al*. 2008, Luo *et al*. 2008), and a recent review has shown how sequence variation in mtDNA is not always selectively neutral (Dowling *et al*. 2008). Identifying genes under positive directional selection may indicate how parasites adapt to new survival or reproductive challenges. In order to elucidate any non-neutral evolution in the fragment of *nad1*, the *dN/dS* ratio (non-synonymous substitutions per site divided by synonymous substitutions per synonymous site) was determined in order to scan for evidence of positive selection in the phylogenetic tree. In particular, we wished to determine whether *dN/dS* ratios were significantly elevated for *nad1* in lineages of deep-sea taxa (parasites found in fish deeper than 500 m). Beyond curiosity in the present study, the need to establish these ratios arose from the peculiar clustering of all deep-sea parasites in the *nad1* only tree (see below, comments on *Prodistomum priedei*), but not in the *lsr*DNA or combined evidence tree. Establishing non-neutral selection in mt genes of deep-sea parasites has important implications for their use in building phylogenies (e.g. see Dowling *et al*. 2008). Specifically, we aimed to estimate whether *dN/dS* ratios across deep-sea lineages were significantly different to all other lineages, and showed evidence of evolution under positive selection.

Materials and methods

Choice of taxa and outgroups

Table I indicates the taxa chosen, including GenBank accession numbers for published and new sequences. Fifty three putative lepocreadioid species (representing 36 genera) and 24 species from 8 families were sampled. The sampling encompassed those basal and sister taxa 'bracketing' the Lepocreadioidea within the Plagiorchiida, as indicated in a previous molecular study (Olson *et al*. 2003). In this way, the best possible estimate of the position of these and related taxa was possible and the monophyly of the Lepocreadioidea could be tested. In order to root the phylogenetic trees, species of the following outgroups were chosen: *Paragonimus westermanii* Kerbert, 1878, *Echinostoma revolutum* (Fröhlich, 1802) and *Fasciola hepatica* Linnaeus, 1758.

Molecular analysis

Total genomic DNA (gDNA) was extracted from ethanol preserved specimens using the DNeasy tissue kit (QIAGEN) fol-

Table I. Primers used for amplification and sequencing of (a) large subunit (*lsr*DNA) Domains D1-D3 and (b) partial *nad1*; all primers are 5'-3'.

Primers and target genes	Forward (F) or Reverse (R)	Primer sequence (5'-3')
(a) *lsr*DNA primers		
PCR and sequencing primers		
LSU5'	F	TAGGTCGACCCGCTGAAYTTAAGCA
ZX-1[a]	F	ACCCGCTGAATTTAAGCATAT
1500R[b]	R	GCTATCCTGAGGGAAACTTCG
Additional sequencing primers		
420R	R	GGTTTCACGCACTGTTTACTC
300F	F	CAAGTACCGTGAGGGAAAGTTG
ECD2	R	CTTGGTCCGTGTTTCAAGACGGG
900F	F	CCGTCTTGAAACACGGACCAAG
1090F	F	TGAAACACGGACCAAGG
(b) *nad1* primers		
PCR and sequencing primers		
NDJ1[c]	F	AGATTCGTAAGGGGCCTAATA
ND1J2A[d]	R	CTTCAGCCTCAGCATAATC
Additional sequencing primers		
ND3bc	R	CNGCCTCRGCATAATC
ND3b	R	GGRGTNCGRTTACACTCACA

[a]Modified from Van der Auwera *et al*. (1994); original ZX-1: ACCCGCTGAA**Y**TTAAGCATAT; **Y** was replaced with T. [b]Tkach *et al*. (2003); [c]Bray *et al*. (1999); [d]Morgan and Blair (1998).

lowing the manufacturer's instructions. The eluate was concentrated to a volume of 20 µl using Microcon YM–100 (Millipore) columns. PCR reactions were carried out in 25 µl volumes using illustra™ puReTaq Ready-To-Go™ PCR beads (GE Healthcare), 10 µM of each primer (see Table I for list of primers) and 1–2 µl gDNA. Partial *lsr*DNA (1767–1895 bp) was amplified using ZX-1 or LSU5' and 1500R; partial *nad1* (456–510 bp) was amplified using NDJ1 and NJ1J2A; difficult templates were amplified using PCR with NDJ1 and the nested primer ND3b. Cycling conditions for partial *lsr*DNA were as follows: denaturation for 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, 2 min at 72°C; and 7 min extension at 72°C. Cycling conditions for partial *nad1* (NDJ1+ NJ1J2A) were as follows: denaturation for 3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 52°C, 1 min at 72°C; and 7 min extension at 72°C. For nested PCRs (NDJ1+ ND3b), cycling conditions were the same as for NDJ1+NJ1J2A, except the annealing step was at 50°C. PCR amplicons were either gel-excised using a QIAquick™ Gel Extraction Kit (QIAGEN) or purified directly using QIAquick™ PCR Purification Kit (QIAGEN) following the standard manufacturer-recommended protocol. Cycle-sequencing from both strands was carried out on an ABI 3730 DNA Analyser, Big Dye version 1.1. using ABI BigDye™ chemistry. Problematic products for *nad1* were cloned using a TOPO TA Cloning® Kit with pCR®2.1-TOPO® vector (Invitrogen), following the manufacturer's instructions. Positive clones were grown for 15 h in 3 ml volumes of LB at 37°C at 200 rpm in a shaking incubator. Plasmid DNA was purified using QIAprep Spin Miniprep Kit (QIAGEN) following the standard manufacturer-recommended protocol, and cycle-sequenced from both strands using M13 primers. Contiguous sequences were assembled and edited using Sequencher™ (GeneCodes Corp., Ver. 4.6) and sequence identity checked using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nih.gov/BLAST/).

Alignment and phylogenetic analysis

Alignments were performed using ClustalX (Thompson *et al*. 1997) with default settings and penalties as follows: gap opening = 10, gap extension = 0.20, delay divergent sequences = 30%, DNA transition weight = 0.5. The alignment was adjusted by eye in MacClade (Maddison and Maddison 2005). Regions that could not be aligned unambiguously were excluded from the analysis. The full alignments for *lsr*DNA and *nad1* gene partitions are available in Supplementary Table SI (see **Annex:** http://www.actaparasitologica.pan.pl/), with an indication of exclusion sets. Phylogenetic trees were constructed using Bayesian inference (BI) and Maximum Likelihood (ML). Modeltest version 3.7 (Posada and Crandall 1998) was used to select a model of evolution using the Akaike Information Criterion; GTR+I+G was chosen for *lsr*DNA and Kimura-3-parameter with unequal base frequency+I+G for *nad1*. BI was performed with P4 (Foster 2004); http://code.google.com/p/p4-phylogenetics/. Model settings in P4 were set equivalent to the GTR+I+G. Base compositional heterogeneity amongst lineages was examined using posterior predictive simulations (Bollback 2002) of the χ^2 statistic generated under a model with a given number of composition vectors (CV) (Foster 2004). A 'polytomy prior' (Lewis *et al*. 2005) also was implemented in the model. Parameters were estimated separately for each gene. Analyses were run for 2,000,000 generations and sampled

every 200 generations; 400,000 generations were discarded as 'burnin'. Log marginal likelihoods were calculated using equation 16 by Newton and Rafferty (1994), as implemented in P4.

ML analyses were performed in PAUP* version 4.0b10 (Swofford 2002) using successive approximation: model parameters were estimated based on a starting tree determined by neighbor-joining (NJ). A heuristic search was performed implementing the estimated model parameters using nearest-neighbor-interchange (NNI) branch swapping. Model parameters were estimated on the best tree and a heuristic search performed using subtree-pruning-regrafting (SPR) branch swapping. After estimating model parameters, heuristic searches using tree-bisection-reconnection (TBR) branch swapping were performed until the topology remained unchanged. Final ML model settings were as follows: nucleotide frequencies (π {A} = 0.1871; π {C} = 0.1801; π {G} 0.3071; π {T} 0.3257); rate matrix ({A,C} = 0.6639; {A,G} = 4.3815; {A,T} = 2.0647; {C,G} = 0.5882; {C,T} = 5.9562; {G,T} = 1.0000); invariable sites = 0.3943; Gamma shape parameter = 0.722402.

In addition to posterior probabilities from BI, nodal support was estimated by ML bootstrapping (100 replicates) using Genetic Algorithm for Rapid Likelihood Inference (GARLI) Version 0.942 (Zwickl 2006) under default settings, except setting 'Genthreshfortopoterm' to 10,000 generations. Clades were considered to have high nodal support if BI posterior probability was ≥95% and ML bootstrap resampling was ≥70%.

A partition homogeneity test (incongruence length difference test; Farris *et al*. 1995), as implemented in PAUP* (Swofford 2002), was conducted to determine whether *lsr*DNA and *nad1* data partitions were significantly heterogeneous from one another. Additionally, selected Shimodaira-Hasegawa (S-H) tests were run in order to determine whether individual and combined data partitions were compatible with the various different phylogenetic solutions. S-H tests were run under a RELL distribution with 1000 bootstrap replicates, using likelihood parameters taken from the ML analysis for each individual test data set; e.g. for *nad1* data set, likelihood scores for trees were estimated using the model determined for the *nad1* ML analysis.

Detecting positive selection

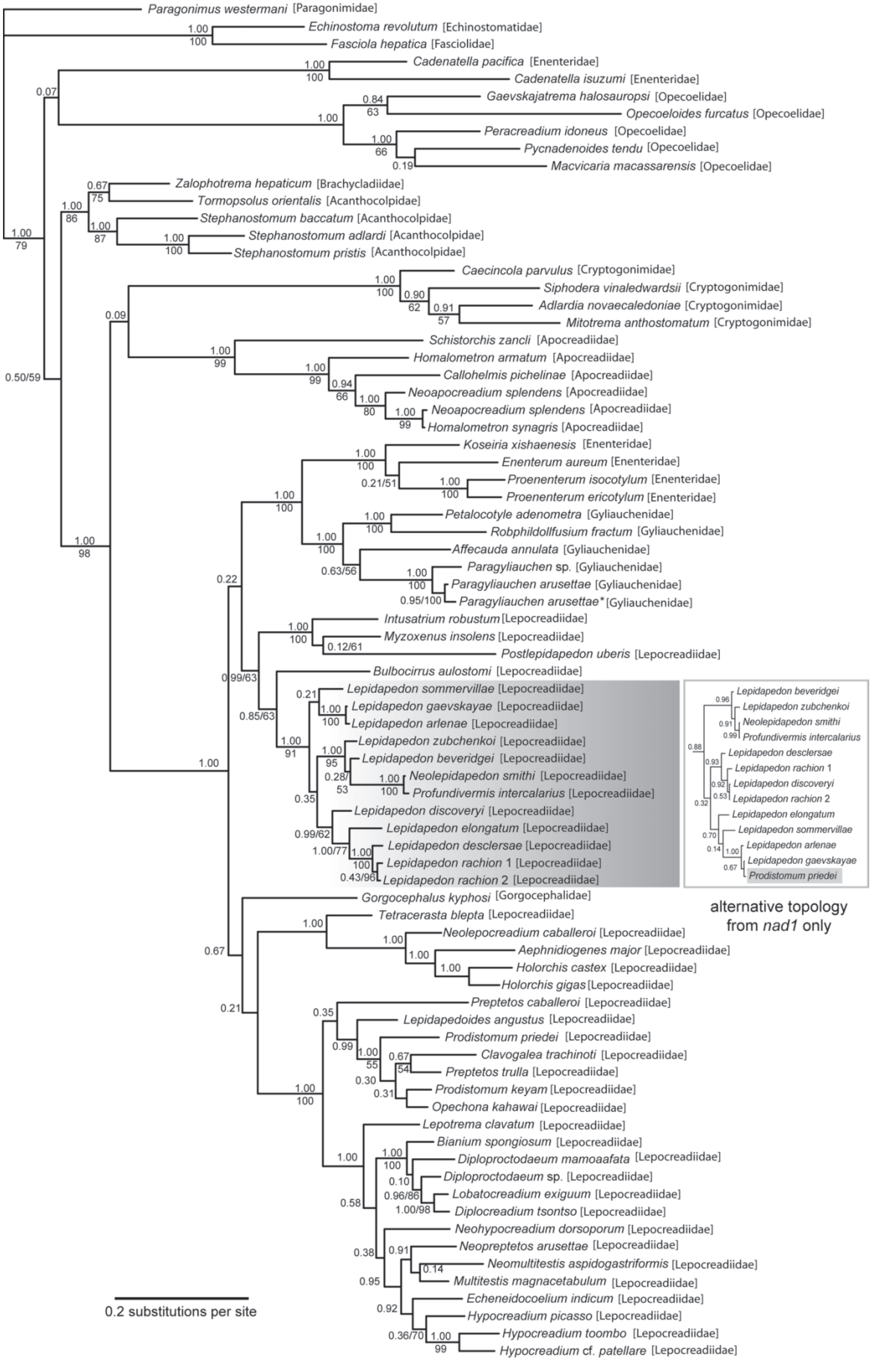
The combined evidence (*lsr*DNA+*nad1*) BI tree was used as the backbone phylogeny to detect positive selection on nucleotide alignments of the *nad1* gene. Those taxa for which no *nad1* data were available (see Table II) were pruned from the phylogeny. ML analysis was applied in order to test the hypothesis of positive directional selection in individual lineages of the gene phylogeny (Yang 2001). A model of codon evolution in which the *dN/dS* ratio is averaged across all codons and all lineages (the fixed ratio model, M0) was compared to an alternative model incorporating variation in the *dN/dS* ratio between lineages of the phylogeny (branch model). A model in which the *dN/dS* ratio varied between codon positions in the alignment and lineages (branch-sites model) was also tested. Pairs of models which allow or disallow positive selection on some lineages and sites were compared using the Likelihood Ratios Test: twice the difference in the log likelihood values was compared to a χ^2 squared distribution with degrees of freedom equal to the difference in the number of estimated parameters between the two models compared.

Analyses were implemented using the CODEML program in the PAML (version 3.14) package (Yang 1997) (http://abacus.gene.ucl.ac.uk/software/paml.html). Nucleotide frequencies at each codon position were used to estimate equilibrium codon frequencies. Branch lengths estimated under the simplest model of evolution, M0 (where the *dN/dS* ratio was averaged across all lineages and codons) were used as initial branch length estimates to speed up more computationally intensive branch and branch-site models. Computations were run 3 times with different combinations of initial ts/tv rate ratio and *dN/dS* ratio values: 1 and 1; 0.1 and 10; 10 and 0.1. If outcomes varied, the results with the highest log likelihood were used.

Results

Interrelationships

Partition homogeneity tests indicated that *nad1* and *lsr*DNA were significantly heterogeneous (P = 0.01). Although each gene partition was analysed individually, we present here only the combined analysis, and use it as a source of reference in discussing individual gene trees. For many taxa, *nad1* could not be amplified and therefore was not available, and so we prefer not to compare data sets with different taxon inclusion sets, or to compare topologies derived from only a subset of taxa. All the methods treated missing characters (and gaps from indels) as missing data, thus missing characters (*nad1* for some taxa) do not contribute to any phylogenetic information; therefore where *nad1* is missing for some taxa their placement relies entirely on *lsr*DNA. In analyses using either all three codon positions or omitting the third position for *nad1*, the mitochondrial gene provided the least amount of information as evidenced by poor nodal support and short branches. Indeed, given the small size of this partition and the large number of taxa, only poorly resolved trees could be estimated from *nad1* alone. Most of the structure of the combined analysis appears to come from the phylogenetic signal provided by partial *lsr*DNA, although the topology of the partial *lsr*DNA tree differs only slightly from the combined analysis. Similar tree topologies were found with each of the phylogeny reconstruction methods. Shimodaira-Hasegawa (S-H) tests showed that when constrained against the combined evidence (*lsr*DNA+*nad1*) tree topology, the *lsr*DNA data was not significantly different from an unconstrained solution (P = 0.108). In contrast, S-H tests showed that when constrained against the *lsr*DNA


Paragonimus westermani [Paragonimidae]
Echinostoma revolutum [Echinostomatidae]
Fasciola hepatica [Fasciolidae]
Cadenatella pacifica [Enenteridae]
Cadenatella isuzumi [Enenteridae]
Gaevskajatrema halosauropsi [Opecoelidae]
Opecoeloides furcatus [Opecoelidae]
Peracreadium idoneus [Opecoelidae]
Pycnadenoides tendu [Opecoelidae]
Macvicaria macassarensis [Opecoelidae]
Zalophotrema hepaticum [Brachycladiidae]
Tormopsolus orientalis [Acanthocolpidae]
Stephanostomum baccatum [Acanthocolpidae]
Stephanostomum adlardi [Acanthocolpidae]
Stephanostomum pristis [Acanthocolpidae]
Caecincola parvulus [Cryptogonimidae]
Siphodera vinaledwardsii [Cryptogonimidae]
Adlardia novaecaledoniae [Cryptogonimidae]
Mitotrema anthostomatum [Cryptogonimidae]
Schistorchis zancli [Apocreadiidae]
Homalometron armatum [Apocreadiidae]
Callohelmis pichelinae [Apocreadiidae]
Neoapocreadium splendens [Apocreadiidae]
Neoapocreadium splendens [Apocreadiidae]
Homalometron synagris [Apocreadiidae]
Koseiria xishaenesis [Enenteridae]
Enenterum aureum [Enenteridae]
Proenenterum isocotylum [Enenteridae]
Proenenterum ericotylum [Enenteridae]
Petalocotyle adenometra [Gyliauchenidae]
Robphildollfusium fractum [Gyliauchenidae]
Affecauda annulata [Gyliauchenidae]
Paragyliauchen sp. [Gyliauchenidae]
Paragyliauchen arusettae [Gyliauchenidae]
Paragyliauchen arusettae* [Gyliauchenidae]
Intusatrium robustum [Lepocreadiidae]
Myzoxenus insolens [Lepocreadiidae]
Postlepidapedon uberis [Lepocreadiidae]
Bulbocirrus aulostomi [Lepocreadiidae]
Lepidapedon sommervillae [Lepocreadiidae]
Lepidapedon gaevskayae [Lepocreadiidae]
Lepidapedon arlenae [Lepocreadiidae]
Lepidapedon zubchenkoi [Lepocreadiidae]
Lepidapedon beveridgei [Lepocreadiidae]
Neolepidapedon smithi [Lepocreadiidae]
Profundivermis intercalarius [Lepocreadiidae]
Lepidapedon discoveryi [Lepocreadiidae]
Lepidapedon elongatum [Lepocreadiidae]
Lepidapedon desclersae [Lepocreadiidae]
Lepidapedon rachion 1 [Lepocreadiidae]
Lepidapedon rachion 2 [Lepocreadiidae]
Gorgocephalus kyphosi [Gorgocephalidae]
Tetracerasta blepta [Lepocreadiidae]
Neolepocreadium caballeroi [Lepocreadiidae]
Aephnidiogenes major [Lepocreadiidae]
Holorchis castex [Lepocreadiidae]
Holorchis gigas [Lepocreadiidae]
Preptetos caballeroi [Lepocreadiidae]
Lepidapedoides angustus [Lepocreadiidae]
Prodistomum priedei [Lepocreadiidae]
Clavogalea trachinoti [Lepocreadiidae]
Preptetos trulla [Lepocreadiidae]
Prodistomum keyam [Lepocreadiidae]
Opechona kahawai [Lepocreadiidae]
Lepotrema clavatum [Lepocreadiidae]
Bianium spongiosum [Lepocreadiidae]
Diploproctodaeum mamoaafata [Lepocreadiidae]
Diploproctodaeum sp. [Lepocreadiidae]
Lobatocreadium exiguum [Lepocreadiidae]
Diplocreadium tsontso [Lepocreadiidae]
Neohypocreadium dorsoporum [Lepocreadiidae]
Neopreptetos arusettae [Lepocreadiidae]
Neomultitestis aspidogastriformis [Lepocreadiidae]
Multitestis magnacetabulum [Lepocreadiidae]
Echeneidocoelium indicum [Lepocreadiidae]
Hypocreadium picasso [Lepocreadiidae]
Hypocreadium toombo [Lepocreadiidae]
Hypocreadium cf. patellare [Lepocreadiidae]
0.2 substitutions per site
Lepidapedon beveridgei
Lepidapedon zubchenkoi
Neolepidapedon smithi
Profundivermis intercalarius
Lepidapedon desclersae
Lepidapedon rachion 1
Lepidapedon discoveryi
Lepidapedon rachion 2
Lepidapedon elongatum
Lepidapedon sommervillae
Lepidapedon arlenae
Lepidapedon gaevskayae
Prodistomum priedei
alternative topology
from nad1 only

tree topology, the combined data was significantly different from the unconstrained solution (P = 0.004), thus demonstrating that *nad1* did contribute something to the combined evidence solution. The most notable difference between estimates using individual *nad1* and *lsr*DNA data partitions was that with *nad1* alone (including or excluding third positions), *Prodistomum priedei* clustered in the deep sea clade (see inset in Fig. 1).

Figure 1 shows the tree inferred using BI analysis of combined *lsr*DNA and *nad1* sequences, with the third codon position omitted, including also species where only *lsr*DNA was available. In Figure 2 the poorly supported clades are collapsed and some taxa are summarised at the family level. There appeared to be greater congruence between the BI and ML analyses when third codon position is excluded; the third position seems to add noise, rather than signal. Support values at many nodes, particularly those at deeper (earlier) nodes are poor, and this should be borne in mind when considering the following discussions. In this section we will discuss the overall patterns evident in this tree with some comment when this conflicts with other results. Later, we will discuss the inferred relationships in more detail, taking into consideration morphological and biological factors.

The putative lepocreadioids do not form a monophyletic assemblage, as the two putative enenterids, *Cadenatella pacifica* and *C. isuzumi* cluster with the opecoelids with very low support (Fig. 1). We are not treating this relationship with opecoelids as significant, but the exclusion from the Lepocreadioidea certainly is.

The remaining lepocreadioids are monophyletic with good support, but the Lepocreadiidae is polyphyletic. The Enenterinae and Gyliauchenidae are each monophyletic with good support and together they form clade I, again with good support. Clade II consists of the lepidapedines and four lepocreadiines. Three of these lepocreadiines form Group III, whereas one of the lepocreadiids (*Bulbocirrus*) clusters with the lepidapedines forming clade IV.

Clade V is a small group with a basal freshwater form (*Tetracerasta*) and a group of morphologically similar putative shallow-water marine lepidapedines whilst the remaining lepocreadiines form the clade VI which is divided into an isolated *Preptetos* species, a clade of *Lepocreadium*-like forms (clade VII) and a largish clade of worms many of which are from tetraodontiform fishes (clade VIII).

Taxa designated as deep-sea species are indicated in Table II by the inclusion of the depth data of collection, but the bulk of the species in this study are from shallow inshore waters, often associated with coral reefs. In the case of *Lepidapedon rachion* and *L. elongatum* the collection depth is given, to show that these are relatively shallow-water members of the predominantly deep-water genus. In global terms, shallow-water *Lepidapedon* species are unusual (Bray and Gibson 1995).

Selection

Maximum likelihood analysis of codon evolution can be used to detect signatures of positive selection, a high rate of non-synonymous substitutions per non-synonymous site (*dN*) relative to synonymous substitutions per synonymous site (*dS*). These analyses can also predict individual codons or individual lineages of a phylogeny which have undergone positive selection (Yang 2001). These tests showed no strong statistical evidence for positive selection (*dN/dS* >1) between lineages. However, the average *dN/dS* ratio was higher in deep-sea lineages (*dN/dS* = 0.0196) than in others (*dN/dS* = 0.0094) suggesting either relaxed purifying selection in these lineages or that a very small number of sites may have evolved under positive selection within the lineages. In order to identify such sites, a 'branch-site' model was applied to identify a subset of sites in a subset of lineages that may be evolving under positive selection. However, the model was not a significantly better fit to the data than an equivalent null model (rate variation between sites and lineages but with no sites under positive selection) and no subset of sites showed *dN/dS* >1 (Supplementary Table SII; see **Annex:** http://www.actaparasitologica.pan.pl/). In conclusion, it could not be demonstrated that a significant proportion of sites in the *nad1* gene evolved under positive selection, although a greater proportion of sites do appear to have evolved under no stronger selective constraint in deep-sea lineages than in non deep-sea lineages.

Discussion

Although we aimed to provide greater phylogenetic resolution by combining partial nuclear *lsr*DNA with a partial mt gene, it is clear that little phylogenetic signal was afforded by *nad1* alone. The length of the fragment and the difficulty in amplifying it for all taxa suggest that this would not be the mitochondrial gene of choice in future studies. However, the combined evidence tree (with *nad1* third positions excluded) was somewhat better resolved than that estimated from *lsr*DNA alone, and provided a suitable framework with which to consider the evolutionary radiation and systematics of the Lepocreadiidae.

In this section, we explore morphological and biological characters in the light of the results from our molecular phylogenetic analysis. We will base our discussion on the two-gene tree illustrated in Figure 1 and simplified in Figure 2, pointing

Fig. 1. Bayesian inference trees for *lsr*DNA+*nad1* (GTR+I+G) with 2 CV for partition *lsr*DNA (χ^2 statistic p = 0.999) and 3 CV for partition *nad1* (χ^2 statistic p = 0.970); arithmetic means of log likelihood scores –18906.903), showing posterior probabilities (all nodes) and ML bootstrap values (for nodes where values >50%). Inset shows region of Bayesian inferred tree for *nad1* alone (with third codon positions excluded)

Table II. List of taxa with host and locality data, and gene sequences used in this study. Depth refers to depth at which deep-sea taxa were collected. Sequences marked with* are new. Unless specified all host taxa were taken from less than 50 m. Abbreviations: GBR – Great Barrier Reef

Species and classification	Depth (m)	Source: Host/locality	GenBank accession	
			*lsr*DNA	*nad1*
Order Plagiorchiida				
Family Lepocreadiidae				
Aephnidiogenes major Yamaguti, 1933		*Diagramma labiosum*, Lizard Island, GBR	FJ788468*	–
Bianium spongiosum Bray et Cribb, 1998		*Ostracion cubicus*, Lizard Island, GBR	FJ788469*	FJ788429*
Bulbocirrus aulostomi Yamaguti, 1965		*Aulostomus chinensis,* Heron Island, GBR	FJ788470*	FJ788430*
Clavogalea trachinoti (Fischthal et Thomas, 1968)		*Trachinotus coppingeri,* Heron Island, GBR	FJ788471*	FJ788431*
Diplocreadium tsontso Bray, Cribb et Barker, 1996		*Balistoides conspicillum,* Heron Island, GBR	FJ788472*	FJ788432*
Diploproctodaeum sp.		*Arothron stellatus*, Lizard Island, GBR	FJ788473*	FJ788433*
Diploproctodaeum mamoaafata Bray, Cribb et Barker, 1996		*Ostracion cubicus*, Heron Island, GBR	FJ788474*	FJ788434*
Echeneidocoelium indicum Simha et Pershad, 1964		*Echeneis naucrates*, Swain Reefs, GBR	FJ788475*	FJ788435*
Holorchis castex Bray et Justine, 2007		*Diagramma pictum*, New Caledonia	FJ788476*	FJ788436*
Holorchis gigas Bray et Cribb, 2007		*Plectorhynchus chrysotaenia*, Lizard Island, GBR	FJ788477*	FJ788437*
Hypocreadium cf. *patellare* Yamaguti, 1938		*Balistoides viridescens*, Lizard Island, GBR	FJ788478*	FJ788438*
Hypocreadium picasso Bray, Cribb et Justine, 2009		*Rhinecanthus aculeatus,* Lizard Island, GBR	FJ788479*	–
Hypocreadium toombo Bray et Justine, 2006		*Pseudobalistes fuscus,* New Caledonia	FJ788480*	FJ788439*
Intusatrium robustum Durio et Manter, 1968		*Bodianus perditio,* New Caledonia	FJ788481*	FJ788440*
Lepidapedoides angustus Bray, Cribb et Barker, 1996		*Epinephelus cyanopodus,* Heron Island, GBR	FJ788482*	FJ788441*
Lepidapedon arlenae Bray et Gibson, 1995	1500	*Trachyryncus scabrus*, Goban Spur, NE Atlantic	AJ405262	AJ405276
Lepidapedon beveridgei Campbell et Bray, 1993	3965	*Coryphaenoides armatus*, Porcupine Abyssal Plane, NE Atlantic	AJ406263	AJ405277
Lepidapedon desclersae Bray et Gibson, 1995	985	*Mora moro*, Goban Spur, NE Atlantic	AJ405264	AJ405278
Lepidapedon discoveryi Bray et Gibson, 1995	3965	*Coryphaenoides armatus*, Porcupine Abyssal Plane, NE Atlantic	AJ405265	AJ405279
Lepidapedon elongatum (Lebour, 1908)	52	*Gadus morhua*, North Sea	AJ405266	AJ405280
Lepidapedon gaevskayae Campbell et Bray, 1993	2890	*Coryphaenoides armatus*, Rockall Trough, NE Atlantic	AJ405267	AJ405281
Lepidapedon rachion (Cobbold, 1858) [1 on Figure]	64	*Gadus morhua*, North Sea	AJ405260	AJ405274
Lepidapedon rachion (Cobbold, 1858) [2 on Figure]	142	*Melanogrammus aeglefinus*, North Sea	AJ405261	AJ405275
Lepidapedon sommervillae Bray et Gibson, 1995	2000	*Coryphaenoides guentheri,* Goban Spur, NE Atlantic	AJ405268	AJ405282
Lepidapedon zubchenkoi Campbell et Bray, 1993	4100	*Coryphaenoides leptolepis*, Goban Spur, NE Atlantic	AJ405269	AJ405283
Lepotrema clavatum Ozaki, 1932		*Acanthochromis polyacanthus*, Lizard Island, GBR	FJ788483*	FJ788442*
Lobatocreadium exiguum (Manter, 1963)		*Pseudobalistes fuscus*, New Caledonia	FJ788484*	FJ788443*
Multitestis magnacetabulum Mamaev, 1970		*Platax teira,* Heron Island, GBR	FJ788485*	FJ788444*
Myzoxenus insolens (Crowcroft, 1945)		*Notolabrus tetricus*, Tasmania	FJ788486*	FJ788445*
Neohypocreadium dorsoporum Machida et Uchida, 1987		*Chaetodon flavirostris*, Heron Island, GBR	FJ788487*	FJ788446*
Neolepidapedon smithi Bray et Gibson, 1989	985	*Mora moro*, Goban Spur, NE Atlantic	AJ405270	AJ405284
Neolepocreadium caballeroi Thomas, 1960		*Trachinotus blochii*, Lizard Island, GBR	FJ788488*	FJ788447*
Neomultitestis aspidogastriformis Bray et Cribb, 2003		*Platax teira,* Heron Island, GBR	FJ788489*	FJ788448*
Neopreptetos arusettae Machida, 1982		*Pomacanthus sexstriatus*, Ningaloo, W. Australia	FJ788490*	–
Opechona kahawai Bray et Cribb, 2003		*Arripis trutta*, Tasmania	FJ788491*	FJ788449*
Postlepidapedon uberis Bray, Cribb et Barker, 1997		*Choerodon venustus*, Heron Island, GBR	FJ788492*	FJ788450*
Preptetos caballeroi Pritchard, 1960		*Naso vlamingii*, Heron Island, GBR	AY222236	AJ405269
Preptetos trulla (Linton ,1907)		*Ocyurus chrysurus*, Kingston, Jamaica	AY222237	–
Prodistomum keyam Bray et Cribb, 1996		*Monodactylus argenteus*, Stradbroke Island, Queensland, Australia	FJ788493*	FJ788451*
Prodistomum preidei Bray et Merrett, 1998	747	*Epigonus telescopus*, Goban Spur, NE Atlantic	AJ405272	AJ405286
Profundivermis intercalarias Bray et Gibson, 1991	4143	*Coryphaenoides armatus*, Porcupine Abyssal Plane, NE Atlantic	AJ405271	AJ405285
Tetracerasta blepta Watson, 1984		*Posticobia brazieri*, Brisbane River, Queensland, Australia	FJ788494*	FJ788452*

Family Acanthocolpidae			
Stephanostomum adlardi Bray, Cribb, Waeschenbach et Littlewood, 2007	*Plectropomus leopardus*, Lizard Island, GBR	EF506761	FJ788453*
Stephanostomum baccatum (Nicoll, 1907)	*Eutrigla gurnardus*, North Sea, UK	AY222256	–
Stephanostomum pristis (Deslongchamps, 1824)	*Phycis phycis*, Scandola, Corsica	DQ248222	–
Tormopsolus orientalis Yamaguti, 1934	*Seriola dumerili*, Scandola, Corsica	DQ248217	–
Family Apocreadiidae			
Callohelmis pichelinae Cribb et Bray, 1999	*Hemigymnus melapterus*, Heron Island, GBR	FJ788495*	–
Homalometron armatum (MacCallum, 1895)	*Lepomis microlophus*, Pascagoula River, Mississippi, USA	AY222241	–
Homalometron synagris (Yamaguti, 1953)	*Scolopsis monogramma*, Heron Island, GBR	AY222243	–
Neoapocreadium splendens Cribb et Bray, 1999	*Scolopsis monogramma*, Lizard Island, GBR	AY222242	FJ788454*
Schistorchis zancli Hanson, 1953	*Zanclus cornutus*, Opunohu Bay, Moorea, French Polynesia	AY222240	–
Family Brachycladiidae			
Zalophotrema hepaticum Stunkard et Alvey, 1929	*Zalophus californianus*, California, USA	AY222255	–
Family Cryptogonimidae			
Adlardia novaecaledoniae Miller, Bray, Goiran, Justine et Cribb, 2009	*Nemipterus furcosus*, New Caledonia	FJ788496*	FJ788455*
Caecincola parvulus Marshall et Gilbert, 1905	*Micropterus salmoides*, Pascagoula River, Wilkerson's Ferry, Mississippi, USA	AY222231	–
Mitotrema anthostomatum Manter, 1963	*Cromileptes altivelis*, Heron Island, GBR	AY222229	–
Siphodera vinaledwardsii (Linton, 1901)	*Sciaenops ocellatus*, Gulf of Mexico, South of Horn Island, Mississippi, USA	AY222230	–
Family Echinostomatidae			
Echinostoma revolutum (Fröhlich, 1802)	*Mesocricetus auratus,* laboratory infection	AY222246	AF025832
Family Enenteridae			
Cadenatella isuzumi Machida, 1993	*Kyphosus vaigiensis*, Heron Island, GBR	FJ788497*	FJ788456*
Cadenatella pacifica (Yamaguti, 1970)	*Kyphosus vaigiensis*, Heron Island, GBR	FJ788498*	FJ788457*
Enenterum aureum Linton, 1910	*Kyphosus vaigiensis*, Moorea, French Polynesia	AY222232	FJ788458*
Koseiria xishaense Gu et Shen, 1983	*Kyphosus vaigiensis*, Heron Island, GBR	AY222233	FJ788459*
Proenenterum ericotylum Manter, 1956	*Aplodactylus arctidens*, Tasmania	FJ788499*	FJ788460*
Proenenterum isocotylum Manter, 1954	*Aplodactylus arctidens*, Tasmania	FJ788500*	FJ788461*
Family Fasciolidae			
Fasciola hepatica Linnaeus, 1758	*Capra hircus,* Saudi Arabia	AY222244	AF216697
Family Gorgocephalidae			
Gorgocephalus kyphosi Manter, 1966	*Kyphosus vaigiensis*, Lizard Island, GBR	AY222234	FJ788462*
Family Gyliauchenidae			
Affecauda annulata Hall et Chambers, 1999	*Naso tuberosus*, Lizard Island, GBR	FJ788501*	FJ788463*
Paragyliauchen sp.	*Centropyge bicolor*, Lizard Island, GBR	FJ788502*	FJ788464*
Paragyliauchen arusettae Machida, 1984	*Pomacanthus sexstriatus*, Ningaloo, W. Australia	AY222235	–
Paragyliauchen arusettae Machida, 1984*	*Pomacanthus sexstriatus*, Ningaloo, W. Australia	FJ788503*	FJ788465*
Petalocotyle adenometra Hall et Cribb, 2000	*Prionurus microlepidotus*, Stradbroke Island, Queensland, Australia	FJ788504*	FJ788466*
Robphildollfusium fractum (Rudolphi, 1819)	*Sarpa salpa*, Mediterranean Sea at Perpignan, France	FJ788505*	–
Family Opecoelidae			
Gaevskajatrema halosauropsi Bray et Campbell, 1997	*Halosauropsis macrochir*, Goban Spur, NE Atlantic Ocean	AY222207	–
Macvicaria macassarensis (Yamaguti, 1952)	*Lethrinus miniatus*, Heron Island, GBR	AY222208	–
Opecoeloides furcatus (Bremser in Rudolphi, 1819)	*Mullus surmuletus*, off Corsica, France	AF151937	–
Peracreadium idoneum (Nicoll, 1909)	*Anarhichas lupus*, North Sea, UK	AY222209	–
Pycnadenoides tendu Bray et Justine, 2007	*Pseudobalistes fuscus*, New Caledonia	FJ788506*	FJ788467*
Family Paragonimidae			
Paragonimus westermanii Kerbert, 1878	*Canis lupus familiaris* (dog), Hyogo, Japan (LSU)	AY116874	AF063783

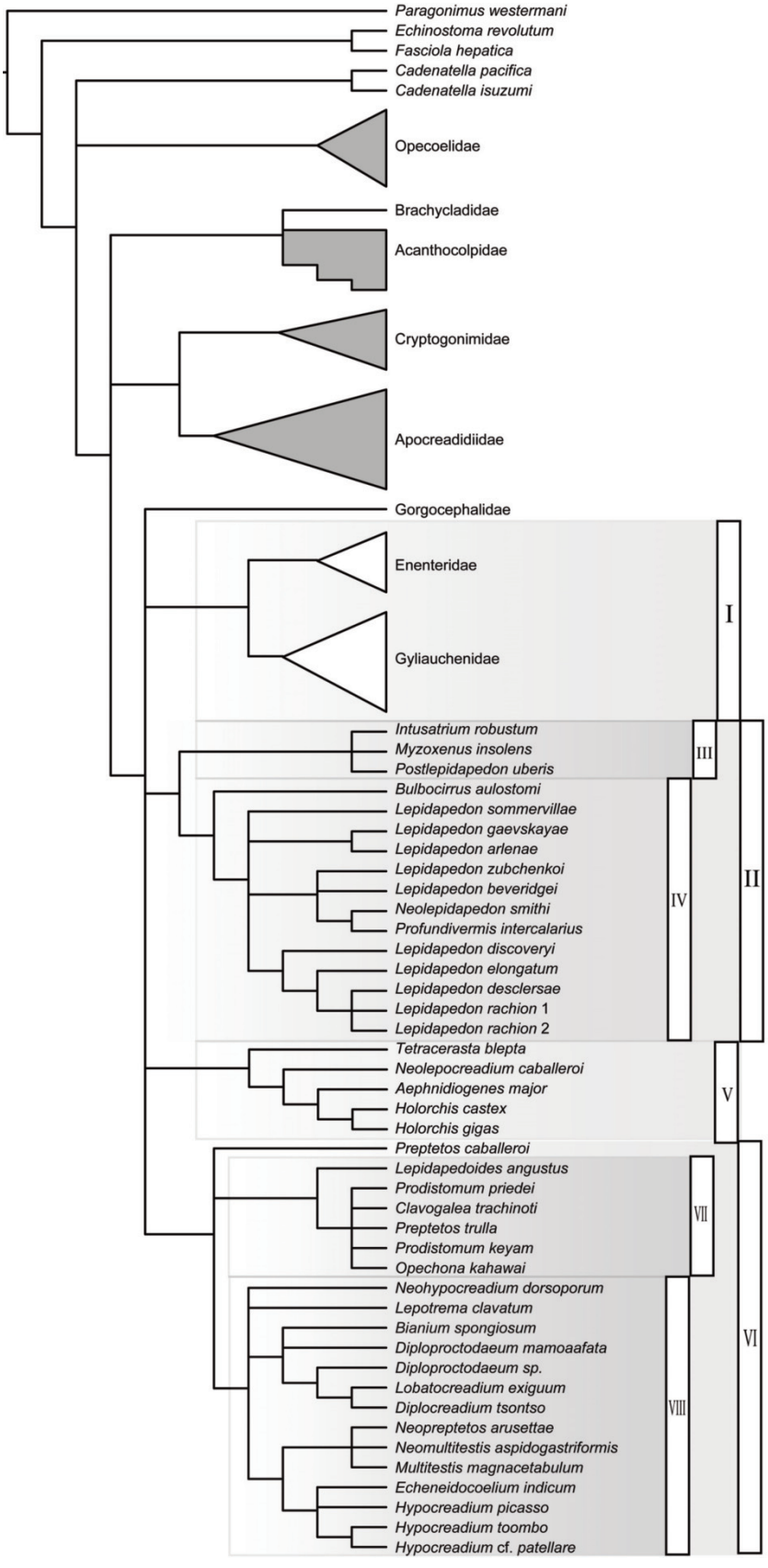


Fig. 2. Simplified schematic of Figure 1 with poorly supported nodes collapsed and some families summarized

out the discrepancies found in other trees, but taking only well supported nodes as the basis for discussion.

Cadenatellinae Gibson et Bray, 1982: This taxon was erected (Gibson and Bray 1982) for enenterids (*sensu lato*) without a distinct cirrus-sac and a canalicular seminal receptacle, but possessing a naked seminal vesicle and a uterine seminal receptacle, to include *Cadenatella* Dollfus, 1946, *Jeancadenatia* Dollfus, 1946 and possibly *Pseudozakia* Machida et Araki, 1977, *Sphincteristoma* Yamaguti, 1966 and *Sphincteristomum* Oshmarin, Mamaev et Parukhin, 1961. The latter two genera are now considered apocreadiids (Cribb 2005a). The recent attempts to develop a morphological phylogeny of the enenterids (or enenterines) by Brooks *et al*. (2000) and Bray and Cribb (2001, 2002a) found the genus *Cadenatella* (as recognised here) monophyletic. Nahhas and Cable (1964), Overstreet (1969) and Bray and Cribb (2001) considered *Jeancadenatella* synonymous with *Cadenatella*. Brooks *et al*. (2000) retained the genus *Jeancadenatia* but pointed out that their results supported the synonymy. They found that (*Cadenatella*, *Jeancadenatia*) was the sister-group to the remaining enenterids, i.e. *Koseiria* and *Enenterum*. They did not recognise *Proenenterum* as an enenterid. Bray and Cribb (2001, 2002a) found that *Cadenatella* was the sister taxon to *Enenterum* Linton, 1910, and that *Koseiria* Nagaty, 1942 and *Proenenterum* Manter, 1954, along with *Pseudozakia* were in a separate clade, with *Koseiria* being paraphyletic. All molecular evidence presented here indicates that *Cadenatella* is not closely related to the remaining enenterids. In fact, *Cadenatella* is morphologically rather distinct from the well-established enenterid genera *Enenterum* and *Koseiria*, and *Proenenterum* (which according to our molecular studies is clearly an enenterid). With the advantage of hindsight, the distinctness of *Cadenatella* appears convincing. *Cadenatella*, represented in this study by two of its nine species, differs from the three undoubted enenterid genera in the single testis, the uroproct (an anus was described in *C. dollfusi* Hafeezullah, 1980, see Hafeezullah 1980), the naked seminal vesicle (an apocreadiid-like feature) and a uterine (rather than a canalicular) seminal receptacle. *Cadenatella* shares host group (the herbivorous fish genus *Kyphosus* Lacepède, 1801) with many enenterids and with *Enenterum* it shares an ornamented oral sucker. However, the detail of the ornamentation of the oral sucker differs distinctly from that of *Enenterum* (see figures 2 and 7 *vs* 14, 15, 18 and 19 of Bray and Cribb 2001). It seems likely that the Cadenatellinae will be recognised as a distinct monogeneric family. Nothing is known of the life-cycle of this taxon.

Lepocreadioidea Odhner, 1905: All of the taxa which previous molecular studies (e.g. Cribb *et al*. 2001, Olson *et al*. 2003) have shown to be in this superfamily are included in the monophyletic group in our study. Bray (2005d) included several other families in the superfamily key 'for reasons of convenience in identification'. Molecular studies (Cribb *et al*. 2001, Olson *et al*. 2003, this study) have shown that the families Apocreadiidae Skrjabin, 1942, Acanthocolpidae Lühe, 1906 and Brachycladiidae Odhner, 1905 do not group with the lepocreadioids, and we await molecular studies of Megaperidae Manter, 1954, Liliatrematidae Gubanov, 1953 and Deropristidae Cable et Hunninen, 1942.

***Gorgocephalus* Manter, 1966:** Is resolved as a lepocreadioid, but its relationship to the other clades within the superfamily is not resolved. Manter (1966) erected Gorgocephalinae Manter, 1966 as a subfamily of the Lepocreadiidae. Later authors (e.g. Yamaguti 1971, Bray 2005b) have considered the taxon to have family rank. *Gorgocephalus* is the only genus in the family and includes just three species, all of which are parasites of herbivorous fishes of the genus *Kyphosus*. *Gorgocephalus* has many unique or unusual features including a single caecum, extensible tentacles on the oral sucker, a ventral 'anus' associated with the oesophagus and a spacious genital atrium with a dorsal genital pore. Some enenterids have distinct lobes on the oral sucker, but none could be interpreted as elongate tentacles, so that morphologically *Gorgocephalus* is not clearly related to any particular lepocreadioid group. Nothing is known of the life-cycle of these worms.

Lepocreadiidae Odhner, 1905: This family is polyphyletic, formed of the clades II, V and VI which are discussed later.

Gyliauchenidae and Enenteridae (clade I): These families are found to be sister taxa. They are almost solely parasites of herbivorous, mainly reef, fishes. Although there are no obvious morphological similarities, a couple of points can be made relating to their shared site of infection and probable algal diet (Hughes-Stamm *et al*. 1999, Jones *et al*. 2000). In life, worms of both families are bright orange, red or yellow and clearly detectable in the gut, sometimes indeed they are visible through the gut wall. It seems possible that members of both families sequester algal pigment. The gyliauchenids often have an extremely long coiled oesophagus, sometimes distinctly longer than the body-length (e.g. *Ptychogyliauchen* Hall et Cribb, 2004; see Hall and Cribb 2004). In three *Enenterum* Linton, 1910 species (*E. elsti* Bray, 1978, *E. prudhoei* Bray, 1978 and *E. tongaatensis* Bray, 1986) the prepharynx is elongate and coiled in a similar fashion to the oesophagus in gyliauchenids (Bray 1978, 1986). Functionally it appears that the prepharynx in these enenterids (lying between the oral sucker and the pharynx) and the oesophagus in the gyliauchenids (between the pharynx, which replaces the oral sucker, and the oesophageal bulb) are similar, although their actual function is not known (Jones *et al*. 2000). No data are available on the life-cycle of these two families, a glaring gap in our knowledge of marine digenean biology.

Gyliauchenidae: This group is always recovered as monophyletic. The family is divided into two clades, one, poorly supported, includes the amphistomatous species and the other includes species with the ventral sucker in the conventional position on the mid- to anterior ventral surface. It is now considered that the muscular structure associated with the distal part of the alimentary system in gyliauchenids is a pharynx, not an oral sucker (Pearson 1992, Hall and Cribb 2005).

Petalocotyle Ozaki, 1934 and *Robphildollfusium* Paggi et Orecchia, 1963 are always sister taxa. Their non-amphistomatous condition (Paggi and Orecchia 1963, Hall and Cribb 2000, Pérez-del Olmo *et al*. 2007) may be a symplesiomorphy or a synapomorphy, the former condition being the intuitively more satisfying. Despite the similarities between these genera, their relationship has only recently been recognised (Hall and Cribb 2005).

Amphistomatous gyliauchenids: *Affecauda* Hall et Chambers, 1999 and *Paragyliauchen* Yamaguti, 1934. The monophyly, albeit poorly supported, of the amphistomatous genera implies synapomorphy of this condition. *Paragyliauchen* is always monophyletic in all analyses and never directly related to the non-amphistomatous clade, despite the fact that it does not exhibit extreme amphistomy, in that the ventral sucker is placed non-terminally with the testes largely posterior to it (Yamaguti 1934, Machida 1984).

Cable and Hunninen (1942), Cable (1956) and Yamaguti (1971) all considered the gyliauchenids close to the lepocreadiids and the latter included them within the Lepocreadiidae, whereas other authors have considered gyliauchenids closer to paramphistomes or microscaphidiids, due to the lack of an oral sucker (Ozaki 1937a, b; Pearson 1992). Much was made by Manter (1940) and Cable and Hunninen (1942) of the shared presence of the lymphatic (or paranephridial) system in gyliauchenids and lepocreadiids. Pearson (1986) has, however, shown that the paranephridial system is widespread amongst digeneans suggesting that this cannot be considered a synapomorphy for these two families. Cable and Hunninen (1942) said 'The genital complex and excretory system of the gyliauchenids agree well with those of the lepocreadiids and certainly are unlike those of typical amphistomes', but gave little other evidence of close morphological relationships between these families. Despite the lack of obvious morphological similarities, our molecular results are unambiguous in placing this taxon within the Lepocreadioidea, as presently recognised.

Enenteridae (*sensu stricto*): Bray (1978) and Brooks *et al*. (2000) have summarised the history of the ideas on the status and position of this family. The latter stated, after a cladistic analysis of the group, that 'we consider the Enenteridae to be nested well within the Lepocreadiidae'. Bray (2005a) updated the history to include early molecular results, but while retaining the family 'for ease of identification', pointed out that Cribb *et al*. (2001) found the Enenteridae embedded within the Lepocreadiidae. Olson *et al*. (2003) used only two congeneric lepocreadiids (*Preptetos* Pritchard, 1960) in their phylogeny, so the relationships within the Lepocreadioidea were not resolved. Our results reinforce the view that the family status of the Enenteridae is not sustainable, unless the Lepocreadiidae is itself split into several families. Few previous authors have seriously considered *Cadenatella* as non-enenterids – indeed, the taxon was originally erected as a subgenus of *Enenterum* (Dollfus 1946) – but in our study the Enenteridae contains only *Koseiria*, *Enenterum* and *Proenenterum*. The poorly supported relationships indicate that the plesiomorphic condition in the family is an unornamented oral sucker, a common, dorsal anus and parasitism in fishes of the genus *Kyphosus*. The lobed oral sucker is, in any case, an autapomorphy of *Enenterum*. *Proenenterum* is always recovered as monophyletic, with the cyclocoel lacking an anus and parasitism in *Aplodactylus arctidens* Richardson, 1839 (marblefish) as synapomorphies (Bray and Cribb 2002a).

Deep-sea lepidapedines and related lepocreadiines (clade II): The deep-sea lepidapedines are the sister to *Bulbocirrus* Yamaguti, 1965 forming clade IV, which in turn is sister to clade III containing *Intusatrium* Durio et Manter, 1968, *Myzoxenus* Manter, 1934 and *Postlepidapedon* Zdzitowiecki, 1993.

***Intusatrium*, *Myzoxenus* and *Postlepidapedon* (clade III):** There appears to be no major morphological synapomorphy for this well-supported group. *Intusatrium* and *Postlepidapedon* share a coiled, tubular internal seminal vesicle, a striking distinction from the saccular internal seminal vesicle of typical lepocreadiids. The condition is, however, variable in other related forms such as the enenterids and gyliauchenids. Biologically, it is notable that the three species utilized in this study are parasites of labrid fishes. The autapomorphy of *Myzoxenus* is longitudinal muscular lamellar lips on the ventral sucker. Nothing is known of the life-cycle of these forms.

***Bulbocirrus* and deep-sea lepidapedines (clade IV):** *Bulbocirrus* is the sister for the 'deep-sea lepidapedine' clade but support for this relationship is not strong. *B. aulostomi* Yamaguti, 1965 is found only in the trumpetfish *Aulostomus chinensis* (Linnaeus, 1766) (Syngnathiformes, Aulostomidae) (Yamaguti 1965, Bray and Cribb 1998), which is found strictly in shallow-water usually over reefs into which it retires when disturbed. There seems no host or trophic similarity between this worm and either the deep-sea lepidapedines or the enenterids. The cirrus-sac is certainly not typically lepocreadiine, and the cylindrical or bulbous internal seminal receptacle may be related to the cylindrical, thick-walled seminal vesicle of the typical lepidapedine. On the other hand, Brooks *et al*. (2000) chose a group of related genera including *Bulbocirrus*, *Neoallolepidapedon* Yamaguti, 1965, *Callogonotrema* Oshmarin, 1965 (considered a synonym of *Neoallolepidapedon* by Bray 2005c) and *Allolepidapedon* Yamaguti, 1940, all parasites of syngnathiforms, as the outgroup for their cladistic study of the Enenteridae. The characters that were considered as synapomorphies for this group of genera and the enenterids are a long prepharynx, well-developed metraterm, vitellarium not extending into the forebody and ani or uroprocts. The occurrence of these characters in the enenterids is, in fact, sporadic, with several having a short prepharynx (see matrices in Brooks *et al*. 2000; Bray and Cribb 2001, 2002a), the vitellarium reaching into the forebody in *Koseiria xishaense* Gu et Shen, 1983, *K. huxleyi* Bray et Cribb, 2001 and *Proenenterum isocotylum* Manter, 1954 (Manter 1954; Gu and Shen 1983; Bray and Cribb 2001, 2002a), and species of *Proenenterum*, which the current study has shown to be enenterids, have a cyclocoel, without ani or uroproct (Manter 1954, Bray and Cribb 2002a).

The placing of *Bulbocirrus* in these trees is not easily explained, and may illustrate either the poor taxon sampling in parts of the tree or, indeed, its overall complexity.

***Lepidapedon* Stafford, 1904, *Neolepidapedon* Manter, 1954 and *Profundivermis* Bray et Gibson, 1991:** *Lepidapedon* is recovered as monophyletic in the analyses not including *nad1* data, but in the combined analyses the other genera are embedded in it, reflecting the results found before with these data (Bray *et al*. 1999). In the *nad1*-only tree the lepocreadiine *Prodistomum priedei* Bray et Merrett, 1998 is embedded within *Lepidapedon* (Fig. 1, inset). This finding will be discussed elsewhere, when discussing the position usually recovered for this species.

***Neolepidapedon* and *Profundivermis*:** Even when embedded within *Lepidapedon*, these genera are found as sisters. These two genera share non-delimited external gland-cells around the external seminal vesicle, in contrast to the membrane delimited gland-cell sheath in *Lepidapedon*. Both species used in this study are found in gadiforms, but this is also the case for all species of *Lepidapedon* used here. When *Neolepidapedon smithi* Bray et Gibson, 1989 and *Profundivermis intercalarias* Bray et Gibson, 1991 are embedded in *Lepidapedon* they are associated most closely with *Lepidapedon beveridgei* Campbell et Bray, 1993 and *L. zubchenkoi* Campbell et Bray, 1993, both from macrourid fishes of the genus *Coryphaenoides* Gunner, 1765. *Profundivermis intercalarias* and *L. beveridgei* are both found in *Coryphaenoides armatus* (Hector, 1875). A notable similarity between the four species discussed here is the vitellarium extending well into the forebody, a condition not found in the other *Lepidapedon* species (Bray and Gibson 1989, 1991, 1995; Campbell and Bray 1993). The life-cycle of *Neolepidapedon* and *Profundivermis* is not known.

***Lepidapedon*:** Only one sister species relationship within the *Lepidapedon* is well-supported, that is (*L. arlenae*, *L. gaevskayae*). *Lepidapedon arlenae* Bray et Gibson, 1995 and *L. gaevskayae* Campbell et Bray, 1993 are both deep-sea forms in macrourid hosts. They are both considered to be in the 'Elongatum-group, subgroup i' of Bray and Gibson (1995), and *L. gaevskayae* was considered to be the most similar species to *L. arlenae* by Bray and Gibson (1995), being distinguished only by minor morphological features.

Other well-supported relationships include the paraphyletic relationship between *L. beveridgei* and *L. zubchenkoi* and *Neolepidapedon* and *Profundivermis*. Morphologically *L. beveridgei* and *L. zubchenkoi* are similar, with the vitellarium reaching into the forebody and both species occur in *Coryphaenoides* spp.

The relationship of *L. discoveryi*, *L. elongatum* and *L. desclersae* with *L. rachion* is well-supported although not all the internal details of the clade are robustly resolved. The former three species are morphologically very similar and only distinguishable by multiple characters (Bray and des Clers 1992) and belong in the 'Elongatum-group' of species (Bray and Gibson 1995).

Apart from these relationships it seems that the resolution within the genus is too poor for it to be worth discussing in detail.

Life-cycles in clade IV: The life-cycles of *Lepidapedon elongatum*, and possibly of *L. rachion*, have been studied. Køie (1985b) described the life-cycle of *L. rachion*, but it was not completed experimentally, and there must be doubt as to whether the larval stages described actually refer to this species. The postulated first intermediate host is the gastropod *Nassarius reticulatus* (Linnaeus, 1758), which harbours rediae producing oculate (!) cercariae with straight, setiferous tails. The metacercaria is found in planktonic cnidarians, ctenophores, chaetognaths and polychaetes. This is a puzzling suite of hosts, as the main final host, the haddock *Melanogrammus aeglefinus* (Linnaeus, 1758), feeds almost exclusively on benthic organisms, such as brittle stars, worms, molluscs and small fish. On the other hand, the life-cycle of *L. elongatum* has been studied in detail by Amosova (1955) and Køie (1985a). The rediae are reported in the digestive gland of the rissoid gastropod *Onoba aculeus* (Gould, 1841). Most notably the cercaria is atypical for the family Lepocreadiidae in that eyespots are lacking and the tail is short, stumpy and lacks setae. On emergence from the snail the cercaria crawls, using the tail and suckers in a fashion similar to cotylomicrocercous opecoelid cercariae. Metacercariae encyst usually in annelids, but occasionally in molluscs and echinoderms. The only other evidence we have of the life-cycle of this clade is the report of metacercariae of *Paralepidapedon hoplognathi* (Yamaguti, 1938) in echinoid echinoderms (Shimazu and Shimura 1984).

(*Tetracerasta* (*Neolepocreadium* (*Aephnidiogenes* (*Holorchis castex*, *H. gigas*)))) (clade V): This clade is recovered with good support, but the relationship of *Tetracerasta* Watson, 1984 to the remaining taxa is not clear. *T. blepta* Watson, 1984 is a parasite of mainly freshwater eels in eastern Australia and is characterised by a complex, lobed oral sucker and the lack of an external seminal vesicle (Watson 1984). This form appears to have no obvious relationship with its sister clade based on morphology.

Life-cycles in clade V: The life-cycles of *Tetracerasta blepta* Watson, 1984, a freshwater species used in our study, and *Holorchis pycnoporus* Stossich, 1901, a congener of two species in our study, have been described (Bartoli and Prévôt 1978, Watson 1984). The first intermediate hosts of both species are reported to be rissooidean prosobranchs. *T. blepta* is found in the hydrobiid *Posticobia brazieri* Smith, 1882 and *H. pycnoporus* in the barleeiid *Barleeia rubra* (Adams, 1795). Cercariae of both species develop in rediae, are ophthalmotrichocercous, i.e. they have straight tails with numerous setae and distinct eyespots are present, and are positively phototactic. The cercariae of *T. blepta* penetrate and encyst in muscles and viscera of small freshwater fishes and tree frog tadpoles. On the other hand, the natural second intermediate hosts of *H. pycnoporus* are prosobranch gastropods and the cardiid bivalve *Parvicardium papillosum* (Poli, 1795).

(*Neolepocreadium* (*Aephnidiogenes* (*Holorchis castex*, *H. gigas*))): These species, whose relationships are strongly

supported, have a distinct morphological character in common, i.e. the ovary is distant from the anterior testis and usually close to the ventral sucker. In *Neolepocreadium caballeroi* Thomas, 1960 the space between the ovary and anterior testis is filled with vitelline follicles, whereas in *Aephnidiogenes* Nicoll, 1915 and *Holorchis* Stossich, 1901 the uterus fills the bulk of this space. Bray and Cribb (1997) summarised the history of the subfamily Aephnidiogeninae Yamaguti, 1934 and pointed out that those authors (e.g. Prudhoe 1956, Skrjabin and Koval 1960, Paggi and Orecchia 1974) who based their concept of this subfamily on the anterior position of the ovary included (*inter alia*) *Holorchis* and *Aephnidiogenes* in this subfamily, and 'some would include *Neolepocreadium*'. Another concept, based on the male terminal genitalia and adopted by Yamaguti (1971) and Bray (2005c), included only the type-genus. The molecular results presented here support the former concept and throw doubt on two aspects of the latter concept. Clearly, the ovary position has better phylogenetic signal than the male terminal genitalia in this case. Bray and Cribb (1997) and Bray (2005c) considered that the male terminal genitalia of *Holorchis* was closest to those exhibited by members of the subfamily Lepidapedinae and placed it in that taxon. In this case, therefore, the terminal genitalia can be a confusing factor. On the other hand, Bray and Cribb (1997) and Bray (2005c) thought that the terminal genitalia of *Neolepocreadium* suggested a placement of the genus within the subfamily Lepocreadiinae, although with 'a vestigial pars prostatica'. It seems likely that the more inclusive concept of the Aephnidiogeninae is valid. *Neolepocreadium caballeroi* is found almost exclusively in carangids of the genus *Trachinotus* (see Thomas 1960). *Aephnidiogenes major* Yamaguti, 1933, *Holorchis castex* Bray et Justine, 2007 and *H. gigas* Bray et Cribb, 2007 are all parasites of haemulid fishes, which is clearly a major host group for these genera (Bray and Cribb 1997). Bray and Cribb (2007) commented on the finding of yet more similar species in haemulid fishes and suggested that *Aephnidiogenes* may have arisen from within *Holorchis*. Our molecular results do not confirm this, but show that the genera are certainly close, and that the peculiar, reduced male terminal genitalia of *Aephnidiogenes* arose from within the Aephnidiogeninae in its broader concept

Typical Lepocreadiinae, clade VI: This well-supported clade includes all the species that have archetypical lepocreadiine terminal genitalia and are found, almost exclusively in shallow-water hosts.

***Preptetos caballeroi*:** Molecules do not satisfactorily place this species in either of the two major clades (VII or VIII) into which the typical lepocreadiines (clade VI) are divided, although morphology clearly places it in clade VII.

Clade VII: This clade includes 6 species, whose internal relationships are not resolved. Members of this group have a rather uniform morphology. The two *Prodistomum* species are not resolved as monophyletic. A possibly significant finding is that in the tree based solely on *nad1* sequences, *Prodistomum priedei* is found amongst the deep-sea lepidapedines, in a well-supported clade with two *Lepidapedon* species (*L. arlenae* and *L. gaevskayae*). *P. priedei* parasitizes the deep-water cardinalfish *Epigonus telescopus* (Risso, 1810) (Perciformes, Epigonidae) (Bray and Merrett 1998, Køie 2000, Bray and Kuchta 2006). As Bray and Merrett (1998) pointed out, it is unusual to find a lepocreadiine parasite in a perciform host in deep-water. In a sample of nearly 58,000 deep-sea fishes only about 5% were perciforms (Merrett *et al*. 1991a, b) and almost all records of deep-sea lepocreadiids are of lepidapedines. It is, therefore, striking that the *nad1* gene allies this species with deep-sea forms, rather than with the morphologically similar shallow-water lepocreadiines which are found to be its relatives in all other studies.

Life cycles in clade VII: This clade probably has the most data available on life-cycles, although this is by extrapolation to morphologically related forms. The life-cycles of several *Opechona* species, congeners of one species utilized in our study, have been elucidated and there have been many observations of various life-cycle stages attributed to *Opechona* spp. (listed in Bray and Gibson 1990). Ophthalmotrichocercous cercariae develop in rediae in nassariid (Køie 1975, Martorelli 1991) and collumbellid (Stunkard 1969, 1980b) prosobranchs. The metacercariae and juveniles of *Opechona* spp. are reported unencysted in a wide range of cnidarians, ctenophores, chaetognaths, annelids and fish larvae and have been reported free-swimming (see Bray and Gibson 1990, and references therein; Martorelli 1991, 2001; Gómez del Prado-Rosas *et al*. 2000; Morandini *et al*. 2005; Øresland and Bray 2005). In none of the other genera in clade VII are the parthenogenetic parts of the life-cycle known, nor the first intermediate host. *Prodistomum polonii* (Molin, 1859) is reported to utilise bivalves as second intermediate host (see Bray and Gibson 1990) and is found progenetic and unencysted in the stomach of crabs (Mordvinova 1985, Gaevskaya and Mordvinova 1996). All we know of the non-adult life-cycle of *Lepidapedoides* are reports of 'immature or juvenile forms which seem to belong to *L. nicolli*', free in the intestine of a wide variety of teleost species. The hosts of these forms '... probably serve as at least facultative intermediate hosts' (Manter 1934). Similar data on the life-cycle of morphologically similar genera, in particular *Lepocreadium* Stossich, 1903, show similar cercarial morphology and parthenogenetic parasitism in nassariid, muricid and conid gastropods. Metacercariae are said to occur in cnidarians, ctenophores, annelids, bivalves and 'turbellarians' (see Yamaguti 1975, Stunkard 1980a, Bartoli 1983). In summary, as far as we know all members of clade VII have parthenitae parasitizing related (Sorbeoconcha) prosobranch gastropods, in the form of redial generations. The sexual phase begins as an ophthalmotrichocercous cercaria, which penetrates a wide range of invertebrate, or occasionally vertebrate, second intermediate hosts. The final hosts are exclusively marine teleosts.

Clade VIII: This includes a group of species found mainly in tetraodontiform hosts. There is a basal polytomy with the species *Lepotrema clavatum* Ozaki, 1932 and *Neohypocrea-*

dium dorsoporum Machida et Uchida, 1987 not associated with other taxa. *L. clavatum* is a parasite of a range of coral reef fishes, including tetraodontiforms of the families Balistidae and Monacanthidae, and pomacentrids (Bray and Cribb 1996c). An unusual feature of this worm is the dorsal position of the excretory pore, which occurs at about the level of the caecal terminations. *N. dorsoporum*, found more or less exclusively in coral reef fishes of the family Chaetodontidae, is unusual in its dorsal genital pore (Machida and Uchida 1987, Bray *et al*. 1994, Hassanine and Gibson 2005, Hassanine 2006b).

The remaining taxa are divided into two resolved clades. One is basically the taxon which has been known as the subfamily Diploproctodaeinae Ozaki, 1928, plus *Lobatocreadium exiguum* (Manter, 1963). Members of the 'Diploproctodaeinae' are characterised by a scoop-like feature of the anterior part of the body, sometimes in the form of a flat plate and sometimes an incised groove (Bray *et al*. 1996). Most have the caeca abutting the posterior body wall, a situation that has been interpreted as ani, but apparently do not often actually perforate the body wall (Shimazu 1994, Bray *et al*. 1996). *Lobatocreadium exiguum* has neither of these characters, but is resolved as the sister taxon of *Diplocreadium tsontso* Bray, Cribb et Barker, 1996, which has an anterior groove, but not terminally abutting caeca. These two species share several morphological features as well as both being parasites of balistids. Two striking shared features are the relative positions of the testes, which are situated with one almost directly dorsal to the other, and the multilobate ovary (Manter 1963, Bray and Cribb 1996a, 2002b; Bray *et al*. 1996). In both species the vitellarium is extensive, reaching to the oral sucker.

The remaining taxa divide into two fairly well-supported clades. In one *Neopreptetos arusettae* Machida, 1982, *Neomultitestis aspidogastriformis* Bray et Cribb, 2003 and *Multitestis magnacetabulum* Mamaev, 1970 form a polytomy. The latter two species both have multiple testes (Mamaev 1970, Bray and Cribb 2003). In these three species the uterus extends posteriorly beyond the testes, an unusual feature in lepocreadiids. *Neopreptetos arusettae* is a parasite of pomacanthid fishes (Machida 1982, Bray and Cribb 1996b), while the species with multiple testes are found in ephippids of the genus *Platax*. The other clade includes *Echeneidocoelium indicum* and three *Hypocreadium* species. *Echeneidocoelium indicum*, which shows no morphological similarity to *Hypocreadium*, is found in remoras (Simha and Pershad 1964, Madhavi 1970, Bray and Cribb 1998), and is presumably only in this position due to poor sampling in this clade. Morphologically *Hypocreadium* species form a convincing clade of highly flattened, more or less circular worms, which are parasites of balistids (Bray and Cribb 1996a, Bray and Justine 2006), but this monophyly is not significantly reflected in our study.

Life-cycles in clade VIII: As far as we are aware there is only one study of the life-cycle of a congener of the species in clade VIII; i.e. the intriguing recent study of the life-cycle of *Diploproctodaeum arothroni* Bray et Nahhas, 1998 by Hassanine (2006a). Hassanine found that the trichocercous cercariae developed in rediae in the ostreid bivalve *Crassostrea cuccullata* (Born, 1778) in mangrove thickets in lagoons of the Egyptian coast of the Gulf of Aqaba. The cercariae lack penetration glands (and no eyespots are described) and emerge at night and encyst on vegetation. Their definitive final host, the tetraodontid white-spotted puffer fish *Arothron hispidus* (Linnaeus, 1758) feeds on algae and benthic animals, particularly sessile ones such as bivalves (Froese and Pauly 2009). It is, of course, too soon to say whether this type of life-cycle is likely to be common to all of the species in clade VIII, but it should be noted that many of them are parasites of related tetraodontiforms with similar trophic habits.

Conclusions

***Deep-sea*:** Bray *et al*. (1999) studied the evolution of clades of digeneans apparently adapted to the deep-sea. They used the fellodistomid genus *Steringophorus* Odhner, 1905 and the lepocreadiid genus *Lepidapedon* along with outgroups. They found that it appears that these genera have radiated in the deep-sea and the relatively shallow-water forms in these clades have secondarily returned to shallower waters. The current study adds further data to the *Lepidapedon* study, in that a much wider group of related taxa have been analysed. The conclusion is the same, i.e. the members of *Lepidapedon* and the closely related genera *Profundivermis* and *Neolepidapedon* form a monophylum adapted to deep-sea fishes. The frequent finding of members of this clade (not sequenced) in Antarctic continental shelf fishes (see Zdzitowiecki 1997) indicate that the adaptation may also be for cold waters. One strange result, repeating the finding by Bray *et al*. (1999), is that in contrast to the combined *lsr*DNA+*nad1* analysis and *lsr*DNA only analysis, in the *nad1* only tree the lepocreadiine *Prodistomum priedei* is clustered within *Lepidapedon*. Why nuclear and mitochondrial genes would yield such strikingly different results is not clear. Although we have ruled out any biases arising from positive selection in *nad1*, more molecular markers are needed to understand whether the *nad1* result is anomalous, a case of convergence, or shared by other mitochondrial (or even nuclear) genes. We used only short fragments of *nad1* in this study, and although there was no convincing evidence of positive selection within and between lineages of parasites that occur over such an extensive range of depths, we remain cautious and curious as to how other protein-coding genes (especially those involved in oxidative phosphorylation), might be affected by depth. Other *Prodistomum* species reported from deep-sea hosts, such as *P. hynnodi* (Yamaguti, 1938), *P. alaskensis* (Ward et Fillingham, 1934) and *P. lichtenfelsi* Raychard, Blend et Dronen, 2008, should be examined for similar molecular characteristics.

***Life-cycles*:** It has generally been considered that the ophthalmotrichocercous cercariae developing in rediae in prosobranch gastropods, penetrating invertebrate or, occasionally

small vertebrate, second intermediate hosts is the typical, common, lepocreadiid life-cycle (Cribb *et al*. 2003). The recent finding of lepocreadiid trichocercous cercariae lacking penetration glands and eyespots, which develop in rediae in bivalves and encyst on substrate, including algae (Hassanine 2006a), has thrown into question the universality of the 'typical' life-cycle. The phylogeny inferred in our study indicate that this 'typical' life-cycle may be restricted to clades V and VII. As so many of the lepocreadioid species studied are parasites of herbivorous or benthic grazing fishes, it may be that the 'bivalve life-cycle' is the plesiomorphic pattern for the superfamily. It is not clear, however, how widespread is the parasitization of bivalves by lepocreadiids. There are two further supposed recent records of unidentified lepocreadiid cercariae in bivalves (Hanafy *et al*. 1997, Aguirre-Macedo and Kennedy 1999). The deep-sea forms, represented by *Lepidapedon elongatum*, have developed a distinct cycle adapted to lack of light and the bathybenthic habit of many of its hosts. Its cercariae, which develop rediae in prosobranch gastropods, lack eyespots and have a short, stumpy tail lacking setae. The cercariae crawl, rather than swim, and encyst in annelids (usually). The loss of eyespots parallels the situation in isopods (Hessler *et al*. 1979), where shallow-water forms secondarily derived from deep-water forms lack eyespots in contrast to most shallow-water species. The life-cycle of lepocreadiids is being revealed as less uniform than has been generally believed. At least three distinct patterns occur, each clearly adapted to the environmental conditions and the trophic patterns of their definitive hosts, and probably representative of monophyletic clades.

***Host-relations*:** There is some congruence between higher taxa of hosts, i.e. orders and families, and monophyletic clades in the tree. At least some of this congruence is artificial, in that some of the species represented here are not oioxenic; i.e. they also apparently occur in other hosts, sometimes of distantly related groups (the possibility of as yet unrecognised oioxenic or stenoxenic cryptic species complicates the issue). For example, the *Lepotrema clavatum* specimens utilised are from a perciform host, but many records of this species are from balistid and monacanthid tetraodontiforms (Bray and Cribb 1996c, 2002b; Machida and Kuramochi 1999). Another problem is the relatively small coverage of the group: many of the missing taxa may not reinforce the findings. Nevertheless, it is clear that the host-relationships within the group are not random. All of the monophyletic group of deep-sea species and their relatives occur in gadiforms, mostly in macrourids. Gadiforms, and macrourids in particular, are a dominant group in deep-water, but are not the only dominant group (Merrett and Haedrich 1997). None of these lepidapedines are found, for example, in ophidiiforms, aulopiforms or scorpaeniformes, the other dominant deep-sea fish orders. Clades V and VII are restricted to perciforms, but occur in a wide variety of families. Clade VIII has many species found exclusively in tetraodontiforms, along with some perciform parasites. Other notable relationships of monophyletic groups with closely related hosts are the *Cadenatella* species in *Kyphosus*, the *Proenenterum* species in *Aplodactylus*, clade III in Labridae, *Aephnidiogenes* + *Holorchis* in Haemulidae, *Lobatocreadium* + *Diplocreadium* in Balistidae and *Hypocreadium* species in Balistidae. It is noteworthy that the Gyliauchenidae and Enenteridae partition the available herbivorous hosts, with practically no apparent overlap. Gyliauchenids have a wider range of hosts, with acanthurids, siganids and scarids predominating, whereas enenterids are, more or less, restricted to kyphosids.

***Morphological characters*:** Recent studies of the Lepocreadiidae have stressed the importance of the male terminal genitalia as an indicator of relationships (Bray and Gibson 1997, Bray 2005c). The monophyly of the *Lepidapedon*-like species indicates that some value remains to this feature, but other results indicate that interpretations of changes to the standard patterns are not straightforward. For example, the terminal genitalia of *Aephnidiogenes* have been used to validate the subfamily Aephnidiogeninae (Yamaguti 1971, Bray and Cribb 1997, Bray 2005c) and the male terminal genitalia of *Holorchis* have been interpreted as of the lepidapedine type (Bray and Cribb 1997, Bray 2005c). The results of this study show that a close relationship between *Aephnidiogenes* and *Holorchis* is well-supported, and that they are not close to the lepidapedines. The position of the ovary, distant from the anterior testis, from which it is separated by the bulk of the uterus, and close to the ventral sucker is clearly an important phylogenetic character, a point reinforced by the well-supported phylogenetic proximity of *Neolepocreadium*, with a similar situation of the ovary, although with a somewhat different uterine configuration.

In *Lepidapedon* the vitelline distribution is shown to outweigh details of the terminal genitalia in the monophyly of (*Lepidapedon zubchenkoi*, *L. beveridgei* (*Neolepidapedon*, *Profundivermis*)).

The non-homology of other characters is indicated by our results. The lobation of the oral suckers of *Cadenatella*, *Enenterum*, *Gorgocephalus* and *Tetracerasta* is clearly not an indicator of close relationships. Close examination of the structure of these lobes supports the findings of non-homology (Manter 1966, Watson 1984, Bray and Cribb 2001).

In summary, despite the relatively small samples used and the low support in many parts of the trees, this study has highlighted several points which are worthy of further study. The relationships of the Cadenatellinae needs further investigation and the group probably should be considered a distinct family. The Lepocreadiidae is polyphyletic. It may be found that it is more natural to remove the Lepidapedinae and related forms from the Lepocreadiidae as a distinct family. Perhaps life-cycle information supports this view. In general there is a pressing need for more data on life-cycles, as it is becoming clear that the Lepocreadioidea does not have one uniform strategy, but it is not clear how widespread different patterns are.

Relationships revealed here suggest that the present major classification levels for the Enenteridae, Gorgocephalidae and

Gyliauchenidae are broadly appropriate. In contrast, the Lepocreadiidae as envisaged until now appears to be an unnatural assemblage that will require some division.

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