



Adding resolution to ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with large fragments of mtDNA

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ABSTRACT

The construction of a stable phylogeny for the Cestoda, indicating the interrelationships of recognised orders and other major lineages, has proceeded iteratively since the group first received attention from phylogenetic systematists. Molecular analyses using nuclear ribosomal RNA gene fragments from the small (*ssrDNA*) and large (*lsrDNA*) subunits have been used to test competing evolutionary scenarios based on morphological data but could not arbitrate between some key conflicting hypotheses. To the ribosomal data, we have added a contiguous fragment of mitochondrial (mt) genome data (mtDNA) of *partial nad1-trnN-trnP-trnI-trnK-nad3-trnS-trnW-cox1-trnT-rnl-trnC-partial rrmS*, spanning 4034–4447 bp, where new data for this region were generated for 18 species. Bayesian analysis of mtDNA and rDNA as nucleotides, and where appropriate as amino acids, demonstrated that these two classes of genes provide complementary signal across the phylogeny. In all analyses, except when using mt amino acids only, the Gyrocotylidae is sister group to all other Cestoda (Nephroposticophora), and Amphilinidea forms the sister group to the Eucestoda. However, an earliest-diverging position of Amphilinidea is strongly supported in the mt amino acid analysis. Amphilinidea exhibit a unique tRNA arrangement (*nad1-trnI-trnL2-trnP-trnK-trnV-trnA-trnN-nad3*), whereas Gyrocotylidae shares that of the derived lineages, providing additional evidence of the uniqueness of amphilinid genes and genomes. The addition of mtDNA to the rDNA genes supported the Caryophyllidae as the sister group to (Spathebothriidea + remaining Eucestoda), a hypothesis consistently supported by morphology. This relationship suggests a history of step-wise evolutionary transitions from simple monozoic, unsegmented tapeworms to the more familiar polyzoic, externally segmented (strobilate) forms. All our data partitions recovered Haplobothriidea as the sister group to Diphyllbothriidae. The sister-group relationship between Diphyllidea and Trypanorhyncha, as previously established using rDNA, is not supported by the mt data, although it is supported by the combined mt and rDNA analysis. With regards to the more derived taxa, in all except the mt amino acid analysis, the following topology is supported: (Bothriocephalidea (Litobothriidea (Lecanicephalidea (Rhinebothriidea (Tetraphyllidea, (*Acanthobothrium*, Proteocephalidea), (Nippotaeniidea, Mesocestoididae, Tetrabothriidea, Cyclophyllidea))))), where the Tetraphyllidea are paraphyletic. Evidence from the mt data provides strong (nucleotides) to moderate (amino acids) support for Tetraphyllidea forming a group to the inclusion of Proteocephalidea, with the latter consistently forming the sister group to *Acanthobothrium*. The interrelationships among Nippotaeniidea, Mesocestoididae, Tetrabothriidea and Cyclophyllidea remain ambiguous and require further systematic attention. Mitochondrial and nuclear rDNA data provide conflicting signal for certain parts of the cestode tree. In some cases mt data offer results in line with morphological evidence, such as the interrelationships of the early divergent lineages. Also, Tetraphyllidea, although remaining paraphyletic with the inclusion of the Proteocephalidea, does not include the most derived cestodes; a result which has consistently been obtained with rDNA.

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1. Introduction

With over 5000 described species and 740 genera, tapeworms (Platyhelminthes: Cestoda) represent one of the most successful

groups of parasitic helminths. Due to their hidden existence in the bowels of many kinds of vertebrates, and as intermediate developmental stages spent within a diversity of invertebrates and vertebrates, the known diversity is likely just a small fraction of the true numbers that exist (Caira and Littlewood, *in press*). Many host species have simply not been sampled and species discovery continues at a pace. The group has received considerable attention from systematists, not least because they are ubiquitously distributed,

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having radiated with their hosts into all their habitats (Khalil et al., 1994), but also because they include causative agents of disease in humans, livestock and wildlife. Additionally, the study of tapeworms provides a lens on the evolution of parasitism, coevolution, and host-parasite interactions. Thus, a resolved phylogeny for the group is an important goal for the interpretation of cestode evolution and for understanding their role in shaping the ecology and evolution of their hosts.

The strategy of combining small and large subunit nuclear ribosomal RNA genes (*ssrDNA* and *lsrDNA*, respectively) has been employed to resolve phylogenies among metazoan lineages over various taxonomic levels (e.g., Medina et al., 2001; Telford et al., 2003). Among the flatworms (phylum Platyhelminthes), complete *ssrDNA* and partial *lsrDNA* fragments have been combined to generate informative phylogenetic frameworks for understanding the systematics of the phylum as a whole (Littlewood et al., 1999a; Larsson and Jondelius, 2008), as well as its major constituent lineages, foremost among them being the parasitic groups: Digenea (Olson et al., 2003), Monogenea (Olson and Littlewood, 2002) and Cestoda (Olson et al., 2001). Separately, and in combination, these genes have yielded considerable phylogenetic resolution within and between the parasite groups, at high and low taxonomic levels, and even for relatively taxon-dense assemblages (e.g., Bray et al., 2009; Palm et al., 2009; Badets et al., 2011). Importantly, a combined *ssrDNA* and partial *lsrDNA* approach to flatworm phylogenetics has provided valuable independent estimates of interrelationships for comparison with morphology-based (cladistic) analyses, and a basis for inferring anatomic character change through evolutionary time. In most cases, character mapping onto molecular phylogenetic frameworks and reconciliation of molecular and morphological phylogenies in a statistical framework, have been fruitful in promoting an integrative approach to flatworm systematics (Littlewood et al., 1999a; Cribb et al., 2003; Kuchta et al., 2008; Healy et al., 2009), and in helping to understand the evolution of parasitism (e.g., Poulin, 2009a,b; Poulin, 2011).

In spite of these advances with nuclear ribosomal DNA (rDNA), a need for additional data has been prompted by (i) relatively poor nodal support for some branches, particularly among deep and mid-level nodes in the trees, (ii) tree instability, as detected by markedly different, but not statistically different, tree topologies from similar data sets, and (iii) occasional conflict with some key, morphologically-based evolutionary relationships and/or evolutionary transitions; e.g., see Lockyer et al. (2003), Olson and Tkach (2005).

One approach to adding more data, advocated convincingly by Mallatt and Winchell (2002), has been to extend the sampling of *lsrDNA* by sequencing beyond the first three variable domains (D1–D3) and capturing most, if not all of the *lsrDNA* domains. This strategy has added considerable insight into the evolution of Metazoa, Ecdysozoa, and deuterostomes (Mallatt and Winchell, 2002, 2007; Mallatt and Giribet, 2006; Mallatt et al., 2010, in preparation). Using this approach, and analysing approximately 6000 bp of complete *lsr + ssrDNA* for each of 26 ingroup and 3 outgroup species, Waeschenbach et al. (2007) provided additional resolution to the interrelationships of tapeworms at the ordinal level. The resulting phylogeny was used to identify the few unequivocal morphological synapomorphies supported by the tree, and to interpret the evolution of proglottization (the formation of serially repeated reproductive organs), external segmentation, and scolex type within the Cestoda. Although hypothesis-testing clearly rejected some notions of interrelatedness suggested by previous studies, other hypotheses could not be supported or rejected with certainty. Of greatest uncertainty were the relative position of the Caryophyllidea and the Spathebothriidea, and the interrelationships of the tetraphyllidean lineages.

Most tapeworm orders have internal proglottization and external segmentation and possess a scolex for anchoring the worm to

its host. These are key innovations, held to be responsible for the group's success. However, the earliest diverging taxa provide a tantalising array of less complex morphotypes that may reveal the origins and evolution of these innovations. Amphilinidea and Gyrocotylidea (collectively termed the 'Cestodaria') do not possess a scolex (although each has an attachment organ), are monozoic (possessing a single set of male and female reproductive organs), and lack external segmentation. All other cestode groups (the Eucestoda) possess a scolex. Caryophyllidea are also monozoic but Spathebothriidea are polyzoic (with multiple, serially repeated gonads), yet neither are externally segmented. All other cestodes show classic proglottization (segmented body parts each with a set of reproductive organs), an innovation that facilitates massive increases in fecundity and the prospect of delivering and protecting eggs into a diversity of habitats (marine, freshwater, land) as they are voided from their definitive vertebrate hosts through defecation.

In spite of the wealth of nucleotide data, the complete *lsr + ssrDNA* tree of Waeschenbach et al. (2007; Figs. 1d and 2) raises a point of controversy. Their results suggest multiple origins of proglottization in the early radiation of the tapeworms, due to the position of the Caryophyllidea and Spathebothriidea. The Spathebothriidea appeared as the earliest diverging eucestodes, which is at odds with morphologically derived scenarios, which place caryophyllids as basal eucestodes (Hoberg et al., 1997). The acquisition and/or expression of genes for proglottization seems to be a major evolutionary innovation and not one likely to be repeated. Additionally, the position of the Caryophyllidea in Waeschenbach et al. (2007) suggests the bothriate scolex condition arose independently at least twice, which again may be improbable. Mackiewicz (2003) went to considerable length to accommodate the competing molecular-based scenarios from a comparative morphological perspective, suggesting in some circumstances that a number of caryophyllidean characters may be secondarily (convergently) derived. This would uphold the complete rDNA tree of Waeschenbach et al. (2007). It must be pointed out, however, that the node positioning Spathebothriidea as the earliest divergent eucestode had somewhat weak support; 94% posterior probability (pp) and 73% likelihood bootstrap support. Furthermore, hypothesis testing failed to refute the alternate position of Caryophyllidea as basal (Waeschenbach et al., 2007; Table 5). This means that the topology of early eucestode evolution, based on complete rDNA is uncertain, and the more traditional, earliest divergent placement of Caryophyllidea as sister to all remaining Eucestoda remains a viable hypothesis requiring further testing.

Additionally, the Amphilinidea has been difficult to place in rDNA-based studies. Both large and small ribosomal subunits for this taxon are very divergent and long, with massive (>1000 bp) inserts, making them difficult to align to other cestodes and contributing to long branches in phylogenetic analyses (e.g., see Lockyer et al., 2003). As such, amphilinid rDNA sequences have been excluded from some estimates of cestode interrelationships (e.g., Olson and Caira, 1999; Olson et al., 2001, 2008; Waeschenbach et al., 2007).

In an attempt to establish a suite of molecular markers capable of resolving the interordinal relationships with consistently high nodal support throughout the cestode tree, we have taken advantage of existing comparative mitogenomic information gathered by us for studies in diagnostics, molecular ecology and phylum-level systematics. Here, complementing data from Waeschenbach et al. (2007), we generate new data from large contiguous fragments of mitochondrial DNA (mtDNA) sequenced from the same taxa, to see whether we can add further stability and improve nodal support to the major branches of the tapeworm tree of life.

Our chosen fragment (*partial nad1-trnN-trnP-trnI-trnK-nad3-trnS-trnW-cox1-trnT-trnL-trnC-partial rrrS*) covers ~30% of the

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