Substitution saturation and nuclear paralogs of commonly employed phylogenetic markers in the Caryophyllidea, an unusual group of non-segmented tapeworms (Platyhelminthes) 

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ABSTRACT

Caryophyllidean cestodes (Platyhelminthes) represent an unusual group of tapeworms lacking serially repeated body parts that potentially diverged from the common ancestor of the Eucestoda prior to the evolution of segmentation. Here we evaluate the utility of two nuclear and two mitochondrial molecular markers (ssrDNA and lsrDNA, nad3 and cox1) for use in circumscribing generic boundaries and estimating interrelationships in the group. We show that these commonly employed markers do not contain sufficient signal to infer well-supported phylogenetic estimates due to substitution saturation. Moreover, we detected multiple trnK + nad3 + trnS + trnW + cox1 haplotypes within individuals, indicating a history of gene exchange between the mitochondrial and nuclear genomes. The presence of such nuclear paralogs (i.e. numts), to our knowledge described here in cestodes for the first time, together with the results of phylogenetic, saturation and split-decomposition analyses all suggest that finding informative markers for estimating caryophyllidean evolution is unusually problematic in comparison to other major lineages of tapeworms.

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1. Introduction

The Caryophyllidea (Platyhelminthes: Cestoda) is an unusual group of tapeworms lacking serially-repeated body structures. Whether their unsegmented condition represents divergence prior to the evolution of segmentation in tapeworms, or alternatively, secondary loss of segmentation, remains controversial. Molecular phylogenetic analyses have identified the group as either the sister group to the other true tapeworms (i.e. Eucestoda), or in a more derived position as a sister lineage to the segmented Diphyllobothriidea (Olson et al., 2001, 2008; Brabec et al., 2006; Waeschenbach et al., 2007; for reviews see Mackiewicz, 2003 and Olson and Tkach, 2005). In addition to lacking the hallmark feature of tape-worms, they are also unique among extant groups in parasitising benthic feeding siluriform and cypriniform fishes, are modestly diverse in comparison to other cestode orders (41 genera, 150 species) and are nearly cosmopolitan in distribution (Mackiewicz, 1972, 1994; Oros et al., 2008, 2010).

Although their phylogenetic position has been assessed using both molecular and morphological data (e.g. Hoberg et al., 1997, 2001; Olson et al., 2001), they remain one of the few tapeworm groups to date whose interrelationships have been largely unexplored, with the first morphological cladistic-based estimate published only recently by Oros et al. (2008). Their assessment, based on 30 morphological characters of all known genera, differed considerably from the most recent classification of the group (i.e. Mackiewicz, 1994) and demonstrated that most characters commonly used to circumscribe taxa exhibit high levels of homoplasy. Moreover, there appeared to be little geographic structure related to their phylogenetic history, suggesting that either the extensive movement of taxa through time has obscured their centre of origin, or that morphology-based phylogenetic estimates are misleading.

Here we evaluate the suitability of four commonly used nuclear and mitochondrial (mt) markers for phylogenetic inference and taxonomic circumscription: the large and small nuclear ribosomal RNA subunits (lsrDNA and ssrDNA) and the mt cytochrome c
oxidase subunit 1 (barcoding region) and nicotinamid dehydrogenase subunit 3 (cox1 and nad3) genes. Of these, the ribosomal genes have been used most extensively for estimating interrelationships within and among tapeworm orders, having been found to provide informative characters across a broad range of divergences (e.g. Lockyer et al., 2003; de Chambrier et al., 2004; Brabec et al., 2006; Healy et al., 2009; Olson et al., 2010). In contrast, mt genes have been employed with less frequency, particularly for studies aimed at resolving interrelationships above the level of genus, and have been used most extensively for studies of the highly derived cyclophyllidean cestodes of medical importance (e.g. Taeniidae; see Olson and Tkach, 2005 for review). In a study of these markers in cyclophyllidean cestodes we found intra-individual variation, but were able to demonstrate the existence of multiple mt haplotypes that most likely represent paralogs that have become incorporated into the nuclear genome (i.e. numts; Bensasson et al., 2001).

2. Materials and methods

2.1. DNA amplification and sequencing workflow

Table 1 lists 25 specimens representing 19 species sequenced. Genomic DNA was extracted using a standard phenol chloroform extraction method (Sambrook and Russell, 2001). The following extraction method (Sambrook and Russell, 2001). The following extraction method (Sambrook and Russell, 2001). The following extraction method (Sambrook and Russell, 2001). The following extraction method (Sambrook and Russell, 2001). The following extraction method (Sambrook and Russell, 2001).

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automated alignments were checked in MacClade ver. 4.08 (Maddison, D.R., and Maddison, W.P., 2005. MacClade 4: Analysis of phylogeny and character evolution. Version 4.08a. Analysis of phylogeny and character evolution. Version 4.08a. http://www.macclade.org) and ambiguously aligned positions were manually excluded prior to phylogenetic analyses. This resulted in the removal of 16.6%, 23.1%, 4.4%, and 0% of positions from the ssrDNA, lsrDNA, cox1 and nad3 alignments, respectively.

2.2. Estimating saturation in the data partitions

To examine the degree of substitution saturation in our datasets, we adapted two distinct methodologies. For the rDNA, we made use of the methodology specifically designed for this type of data described by Struck et al. (2008) in which we first examined the sequence variation across both rDNA alignments by performing sliding window analyses, and based on those results, grouped the alignment positions into classes of identity (at 10% increments in degree of variation). For each class, we determined its C factor (i.e. the ratio of the standard deviation of the transition/transversion distribution to that of the uncorrected p (i.e. patreric distance distribution)), and O/E ratio, which is calculated from the ratio of the consistency index (CI) of the complete dataset to the CI of the reduced datasets (i.e. each identity class). To determine CI values, equally weighted heuristic searches with 1,000 repetitions of CI the consistency index (i.e. the ratio of the standard deviation of the transition/transversion ratios and uncorrected p distances.

For test for substitution saturation within the individual codon positions of the mt genes we employed the entropy-based index of substitution saturation approach of Xia et al. (2003). In short, this approach tests whether the observed entropy in the sequences is significantly smaller than the entropy of “full substitution saturation”. The ratio of observed entropy to the entropy of full substitution saturation is the index of substitution saturation (I3S) and when this is not significantly smaller than the critical I3S value (the value at which the sequences start to fail to recover the correct tree: I3S(C)), one can conclude that the sequences have experienced severe substitution saturation (see Xia and Lemey, 2009). All of the information entropy-based index analyses were done using the software DAMBE (Xia and Xie, 2001), following the practice of Xia and Lemey (2009).

2.3. Phylogenetic analyses

To produce phylogenetic estimates from individual datasets and to compare their contributions with the overall phylogenetic evidence, we ran a set of analyses based on maximum likelihood (ML) and Bayesian inference (BI) criteria. ML and BI were conducted on each individual dataset, both complete and with saturated positions excluded, and in case of mt genes, also including automated alignments. This resulted in the removal of 16.6%, 23.1%, 4.4%, and 0% of positions from the ssrDNA, lsrDNA, cox1 and nad3 alignments, respectively.

2.4. Estimating the strength of the phylogenetic signal

To visualise the number of positions that support bipartitions within the ML trees constructed, and to identify other strongly supported splits contradicting those found in the phylogenetic trees (i.e. to test the level of the background noise in the alignments), we performed split decomposition analysis (SDA) using the software Splits Analyses Methods (SAMS; Wägele and Mayer, 2007). SAMS analyses were run under default settings with the following exceptions: gaps were evaluated as a new state and the search for the total number of supporting characters of each split was determined with splits = search option. Each time, 50 highly supported splits were plotted and only those actually present in the tree with highest likelihood were depicted on corresponding phylograms. To make the SDA as well as the phylogenetic analyses of mt data more straightforward, we included only one randomly chosen “functional” haplotype representative of each of the Caryophyllidean species that bear multiple mt variants, so that the final datasets to be analysed consisted of 28 sequences including three outgroup taxa. In order to choose a single haplotype for each taxon, we first excluded haplotypes with coding regions interrupted by one or more in-frame stop codons, and then chose randomly among the remaining presumed “functional” haplotypes for each taxon, which always grouped together in phylogenetic analyses (Supplementary Fig. S1) and exhibited little intra-specific divergence (Supplementary Table S1).

3. Results and discussion

3.1. Primary sequence characteristics

The combined data (nuclear ssrDNA, D1–D3 lsrDNA, and mt trnK + nad3 + trnS + trnW + cox1) produced 4,277 unambiguously aligned coding positions for analysis. The length of the amplified fragments varied from 2,074 to 2,190 bp in ssrDNA, 1,539 to 1,665 bp in lsrDNA and 1,198 to 1,324 bp in the trnK + nad3 + trnS + trnW + cox1 mt region. Comparison of the latter region with available tapeworm mt genomes (Lupi et al., 2010) showed the codon GTG to serve as an additional initiation codon to the universal ATG in the nad3 and cox1 genes of 12 of the 25 samples. Moreover, based on the inferred termination codons of our nad3 data, we found no evidence to support the idea that TAA codes for tyrosine in caryophyllidean tapeworms, rather than the standard stop codon in the universal genetic code. These features of the flatworm mt genetic code support the previous findings of Nakao et al. (2000) in cestodes and Telford et al. (2000) in the rhabditophoran plathyhelminths. Thus, the “echinoderm mt translation code” (GenBank’s translation table 9) should be used to translate cestode mt nucleotide sequences as opposed to the “flatworm translation code” (translation table 14) available in common bioinformatic resources.

3.2. Nuclear copies of mitochondrial genes (numts)

Cloned sequences showed a total of 59 distinct mt haplotypes in 13 of the 25 samples, with 2–8 unique mt haplotypes per individ-
Phylogenetic analyses (Fig. 1) showed no clustering of the “numt” haplotypes. Instead, with just two exceptions (Carophyllaeus laticeps 04/112 hpt1 and Carophyllaeus fennica 04/112 hpt1), all of the identifiable “numt” haplotypes grouped with their respective “functional” ones, indicating the transfer of mtDNA to mitochondria in cells (i.e. heteroplasmy), and (iii) nuclear mt pseudogenes, or numts (e.g. Bensasson et al., 2001; Song et al., 2008; Struck et al., 2008).

Table 2 shows the number and sequence characteristics of trnK + nad3 + trnS + trnW + cox1 mtDNA haplotypes observed within the scope of this study.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Alla</th>
<th>Numtsb</th>
<th>Indelsc</th>
<th>Stopsd</th>
<th>Mutationsf</th>
<th>Substitutions (non-numts)g</th>
<th>Substitutions (non-numts/numts)h</th>
<th>Identicalib</th>
<th>Non-umb length/ATIc</th>
<th>Numt length/ATIc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carophyllaeides fennica</td>
<td>4</td>
<td>3</td>
<td>1–2</td>
<td>1–7</td>
<td>0–98/1–46</td>
<td>NA</td>
<td>2–141</td>
<td>1,151</td>
<td>1,299/60.8</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>1,303/60.2</td>
<td>1,300/60.5</td>
</tr>
<tr>
<td>Carophyllaeides fennica</td>
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<td>1</td>
<td>1</td>
<td>1–2</td>
<td>0–2/1–2</td>
<td>2–4</td>
<td>1–3</td>
<td>1,294</td>
<td>1,299/60.4</td>
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</tr>
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<td>finland</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,300/60.5</td>
<td></td>
</tr>
<tr>
<td>Diphyllobothrium sp.</td>
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<td>3</td>
<td>1–10</td>
<td>1–12</td>
<td>4–85/12–111</td>
<td>24–211</td>
<td>943</td>
<td>1,303/59.3</td>
<td>1,302/60.4</td>
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<tr>
<td>Hunterella nodulosa</td>
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<td>0</td>
<td>0–0/0–14</td>
<td>2–51</td>
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<td>1,320–1,321/57.7</td>
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<tr>
<td>Lytocetus indicus</td>
<td>2</td>
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<td>0</td>
<td>0–0/1–4</td>
<td>1–5</td>
<td>NA</td>
<td>1,300</td>
<td>1,308/60.6</td>
<td>1,325</td>
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<tr>
<td>Wenyonia viridis</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>2–11</td>
<td>0–87/1–39</td>
<td>8</td>
<td>3–127</td>
<td>1,097</td>
<td>1,297–1,298/59.2%</td>
<td>1,288–1,296/59.3</td>
</tr>
<tr>
<td>Caryophyllaeides laticeps</td>
<td>6</td>
<td>8</td>
<td>1–4</td>
<td>2–11</td>
<td>0–87/1–39</td>
<td>8</td>
<td>3–127</td>
<td>1,097</td>
<td>1,297–1,298/59.2%</td>
<td>1,288–1,296/59.3</td>
</tr>
<tr>
<td>Caryophyllaeides laticeps</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>14</td>
<td>2–113/0–49</td>
<td>4–36</td>
<td>173–176</td>
<td>1,107</td>
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<td>1,296/60.5</td>
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<tr>
<td>sp. sk97</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>1,296/60.5</td>
<td></td>
</tr>
<tr>
<td>Gladicris catostomi</td>
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<td>1</td>
<td>1</td>
<td>1–9</td>
<td>0–0/0–1</td>
<td>1</td>
<td>1–2</td>
<td>1,319</td>
<td>1,322/58.7</td>
<td>1,321/58.8</td>
</tr>
<tr>
<td>Hunterella nodulosa</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0–0/0–14</td>
<td>3–9</td>
<td>NA</td>
<td>1,284</td>
<td>1,300/58.9</td>
<td>1,322/58.8</td>
<td></td>
</tr>
<tr>
<td>Monobothrium hunteri</td>
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<td>2</td>
<td>1</td>
<td>3–5</td>
<td>1–40/1–17</td>
<td>4</td>
<td>6–68</td>
<td>1,245</td>
<td>1,324/58.7</td>
<td>1,322/59.4</td>
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<tr>
<td>Wenyonia viridis</td>
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<td>1–2</td>
<td>4–6</td>
<td>0–3/2–5</td>
<td>NA</td>
<td>4–7</td>
<td>1,273</td>
<td>1,288/56.6</td>
<td>1,287/56.6</td>
</tr>
</tbody>
</table>

**a** Number of all unique haplotypes observed.

**b** Number of observed putative nuclear mitochondrial pseudogenes (“numt” haplotypes).

**c** Number of indels within nad3 + cox1 coding regions of “numt” haplotypes.

**d** Number of in-frame stop codons within nad3 + cox1 coding regions of “numt” haplotypes.

**e** Number of synonymous/non-synonymous substitutions within nad3 + cox1 coding regions.

**f** Substitution differences between all pairs of “non-numt” haplotypes.

**g** Substitution differences between all pairs of both “non-numt” and “numt” haplotypes.

**h** Number of identical sites (all “non-numt” and “numt” aligned).

**i** Ungapped length of “non-numt” haplotypes and their mean AT content.

**j** Ungapped length of “numt” haplotypes and their mean AT content.

The aligned ssrDNA and lsrDNA data contained 2,245 and 1,828 positions of which 1,872 and 1,406 remained after the exclusion of ambiguously aligned positions, respectively. Sliding window analyses revealed that the positions could be divided into the classes of identity of 0–40% in the ssrDNA and 0–60% in the lsrDNA data parts, with the vast majority of positions belonging to the lowest 0–10% variability class in both genes. For the purposes of the C factor and O/E ratio calculations, lsrDNA classes of identity with variability of 30–60% were pooled to avoid estimates based on small sample sizes. Omitting the ambiguously aligned positions from our ssrDNA and lsrDNA datasets resulted in total exclusion of highly variable classes (i.e. 40–60% for ssrDNA and 70–80% for lsrDNA), as well as a significant decrease in the number of positions belonging to other classes (see Supplementary Table S2).

Our C factor and O/E ratio values (Supplementary Table S2) resemble those of the analysis of Struck et al. (2008). In their study we observed a sharp drop by an order of a magnitude in C factor values linked to the growing genetic distances between pairs of taxa and thus growing variability of classes of identity. By analyzing their datasets, Struck et al. (2008) found the C factor of 20 to represent the value below which the classes of identity should be treated as saturated. Based on our data, the C factor drops to the boundary value of 20 (30–40% class of ssrDNA) or even slightly below it (19 of 30–60% class in lsrDNA), indicating that the highly variable classes of identity are reaching saturation levels.
Even if the statistical power of our analyses is slight due to the relatively small number of taxa and positions in the classes, we can conclude that by excluding the ambiguously aligned positions from our datasets we also remove the most variable, clearly saturated characters left for subsequent phylogenetic analyses, and that omission would result in a significant decrease of parsimony-informative characters and saturation does not play a major role. We used those data for phylogenetic reconstruction.

Compared with the rDNA data, the mtDNA genes appear to be highly saturated as evaluated by the entropy-based index of substitution saturation values of Xia et al. (2003). Third codon positions of both mt genes and first codon positions of first and second codon positions of nad3 showed substantial saturation (illustrated by their C factor values of the classes of identity remaining in each of the rDNA data partitions would result in a significant decrease of parsimony-informative characters left for subsequent phylogenetic analyses, and while the O/E ratio and C factor values of the classes of identity remaining in the datasets (0–40% class of SSRDNA, 0–60% class of lsrDNA) indicate that homoplasy across these classes of identity does not differ substantially and saturation does not play a major role, we used those data for phylogenetic reconstruction.

FIG. 1. Phylogenetic analysis of putative nuclear mitochondrial pseudogenes (numts) distribution in the Caryophyllidea based on the analysis of the entire trnK+nad3+trnS+trnW+cox1 mitochondrial DNA region by maximum likelihood. Mitochondrial haplotypes (hpt) in bold type represent the “functional” (“non-numt”) haplotypes without in-frame stop codons or frame shift mutations.
Fig. 2. Phylograms resulting from concatenated ribosomal, mitochondrial and all four genes combined analyses by maximum likelihood (ML) with bootstrap and Bayesian posterior probability nodal support values indicated at the branches (right) and the split-support spectra for the respective datasets estimated with Splits Analyses Methods program (left). Column height of a split represents the number of supporting positions counted for each partition of a split separately (in- and outgroup of a split shown above and below the horizontal axis, respectively): binary positions (two character states only); asymmetrical positions (one partition with one character state, the other with more than one state); noisy positions (more than one state in each partition, but the majority state within a group can still be identified). Compatible splits are depicted on the corresponding ML tree with their number. Results of ML and split-decomposition analyses of individual genes are provided in Supplementary Figs. S2–S4.
spectra on Supplementary Figs. S2 and S3 indicate that there is present on the best tree on the corresponding phylogram. Split datasets) and Fig. 2 (combined datasets) outline the 50 most highly supported splits. Whereas split support spectra are meant to detect only a fraction of the ITS population actually present within an individual (Králová-Hromadová et al., 2010), the presence of multiple ITS variants calls not just for the need of cloning and characterisation of multiple clones, but may also bias correct phylogenetic inference by detecting only a fraction of the ITS population actually present within an individual (Králová-Hromadová et al., 2010).

To date, the most taxon-rich evaluation of caryophyllidean phylogeny based on molecular data has been that of Olson et al. (2008), and although not identical in terms of the taxa sequenced, their results are highly congruent with our concatenated phylograms (Fig. 2). These molecular studies, together with the comprehensive morphological analysis of the Caryophyllidea by Oros et al. (2008), illustrate the conflict between phylogenetically-derived estimates of their evolution and traditional classification schemes that divide the group according to the position of inner longitudinal muscles relative to the reproductive organs (e.g. Mackiewicz, 1994). Using our consensus topology (Fig. 2, bottom), we searched the morphological matrix of Oros et al. (2008) for synapomorphies congruent with our molecular-based estimates and found only two characters (10 and 17; see Oros et al., 2008): the presence of scolex grooves uniting Wenyonia and Monobothrioides, and the presence of an external seminal vesicle uniting Cladidaris, Hunterella and Mono- bothrium. Although we do not have a fully resolved molecular phylogeny with which to be able to speculate on the validity of the morphological characters, the current insight foresees the need for re-evaluation of the utility of these characters in systematic studies and underlines the difficulty in evaluating this unusual and early-branching tapeworm lineage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2012.01.005.

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