Complete sequence of the mitochondrial genome of the tapeworm *Hymenolepis diminuta*: Gene arrangements indicate that platyhelminths are eutrochozoans

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Abstract

Using “long-PCR” we have amplified in overlapping fragments the complete mitochondrial genome of the tapeworm *Hymenolepis diminuta* (Platyhelminthes: Cestoda) and determined its 13,900 nucleotide sequence. The gene content is the same as that typically found for animal mitochondrial DNA (mtDNA) except that *atp8* appears to be lacking, a condition found previously for several other animals. Despite the small size of this mtDNA, there are two large non-coding regions, one of which contains 13 repeats of a 31 nucleotide sequence and a potential stem-loop structure of 25 base pairs with an 11-member loop. Large potential secondary structures are identified also for the non-coding regions of two other cestode mtDNAs. Comparison of the mitochondrial gene arrangement of *H. diminuta* with those previously published supports a phylogenetic position of flatworms as members of the Eutrochozoa, rather than being basal to either a clade of protostomes or a clade of coelomates.

Introduction

Over 100 complete metazoan mitochondrial genome sequences have been published to date (Boore 1999; Mitochondrial Genomics link at http://www.jgi.doe.gov). Nearly all contain genes for the same13 proteins, 22 tRNAs, and two rRNAs. Among triploblastic animals, there is only one gene, *atp8*, that has been lost from this repertoire, four times independently: one for the mtDNAs of four nematodes (Okimoto et al. 1991, 1992; Keddie, Higazi, and Unnasch 1998); one for the mtDNAs of platyhelminths (Le et al. 2000); one for the mtDNA of the ascidian *Halocynthia roretzi* (Yokobori et al. 1999); and one from the mtDNA of the bivalve mollusk *Mytilus edulis* (Hoffmann, Boore, and Brown 1992). All metazoan mtDNAs have at least one non-coding region that is thought to contain signaling elements for regulating replication and/or transcription; in some cases potential secondary structures are associated with these signals (Shadel and Clayton 1997).

Although metazoan mitochondrial sequences are known to evolve rapidly, their gene arrangements often remain unchanged over long periods of evolutionary time. Comparing the relative arrangements of these genes has several merits as a dataset for reconstructing ancient evolutionary relationships, including their generally slow rate of change and the great number of potential gene orders (Boore and Brown 1998). With some notable exceptions, gene arrangements are relatively stable within major groups, but variable between them, and arrangement comparisons have resolved several evolutionary relationships, including those among arthropods (Boore et al. 1995; Boore, Lavrov, and Brown 1998; Dowton 1999), annelids (Boore and Brown 2000), gastropods

(Kurabayashi and Ueshima 2000), and echinoderms (Smith et al. 1993) and placing brachiopods with the eutrochozoans (Stechmann and Schlegel 1999).

The superphyla Deuterostomia (*e.g.* Chordata, Echinodermata, Hemichordata) and Protostomia (*e.g.* Arthropoda, Annelida, Mollusca) each appear to have evolved from an ancestor with a coelomic body cavity. Based largely on the view that the coelom evolved once, in their common ancestor, they are traditionally united into the clade Coelomata to the exclusion of the (presumably) earlier branching Platyhelminthes (Hyman 1951). Platyhelminths share a number of developmental features with protostomes, such as spiral cleavage, the mesoderm originating from a mesentoblast, and the mouth arising from the blastopore. In the traditional phylogenetic scheme, these shared features are interpreted as primitive for all of the Coelomata, with the Deuterostome variations being later derived for that superphylum. However, others view these shared features as indicating that the Platyhelminthes evolved within the Protostomia, requiring the interpretation that either the coeloms of deuterostomes and protostomes are of separate origins or that Platyhelminthes has lost its coelom secondarily. Most who favor this latter phylogeny place Platyhelminthes as an early offshoot within Protostomia (see Brusca and Brusca 1990), but some recent studies (Aguinaldo et al. 1997; Balavoine 1997, 1998; Carranza, Baguñà, and Riutort 1997) have concluded that Platyhelminthes (or just those other than the Acoela [Ruiz-Trillo et al. 1999]) are, instead, allied specifically with the group of coelomate protostomes dubbed the Eutrochozoa, *e.g.* Mollusca and Annelida, but not Arthropoda (Ghiselin 1988).

Until recently, there were no complete mitochondrial sequences representing the phylum Platyhelminthes; however, these are now available for the cestodes *Taenia*

crassiceps (Le et al. 2000) and *Echinococcus multilocularis* (GenBank record AB018440) and for the trematodes *Fasciola hepatica* (Garey and Wolstenholme 1989; Le et al. 2000; GenBank record AF216697), *Schistosoma mansoni* (Blair et al. 1999; Le et al. 2000), *S. japonicum*, *S. mekongi*, and *Paragonimus westermani* (Le et al. 2000). However, these studies have emphasized specific comparative aspects without yet making a detailed genome description. We analyze and describe here the complete mitochondrial genome sequence of a platyhelminth, the tapeworm (*i.e.* class Cestoda) *Hymenolepis diminuta* and compare aspects of this genome with those already published. Comparison of the mitochondrial gene arrangement of *H. diminuta* with all others available supports the view that platyhelminths are eutrochozoans.

Materials and Methods

Determination of the mtDNA Sequence

Individuals of the tapeworm *Hymenolepis diminuta* were collected from artificially infected rat gut and preserved in hot 70% ethanol. About 10 posterior proglottids from each of five adult cestodes were longitudinally disrupted with sterilized forceps. Eggs were collected from these disrupted proglottids and pelleted by centrifugation in order to separate them from the ethanol supernatant. The eggs were then crushed in 150 µl CTAB buffer using a pestle fitting a 1.5 ml eppendorf tube, followed by addition of 450 µl CTAB buffer supplemented with 50 µg Proteinase K and incubated at 65˚ C for two hours. Proteins were removed by extraction with phenol/chloroform and the DNA was precipitated by adding 50% (v/v) 7.5 M ammonium acetate and an equal volume of 100% ethanol. After centrifugation and washing the pellet with 70% ethanol, it was dried under vacuum and resuspended in 50 µl ddHOH.

Initially, a short fragment (~450 nt) of *H. diminuta cob* was amplified by PCR using AmpliTaq DNA polymerase (Fisher) and 40 cycles of 94º for 15 s, 53º for 30 s, 72º for 2 min, followed by an incubation at 72º for 10 min. Amplifying primers [Cytb-424F (GGW TAY GTW YTW CCW TGR GGW CAR AT) and Cytb-876R (GCR TAW GCR AAW ARR AAR TAY CAY TCW GG)] were designed based on sequences well-conserved in many distantly related taxa. The amplification product was visualized on a 1% agarose gel by ethidium bromide staining and UV irradiation, then purified by three serial passages through Ultrafree spin columns (Millipore; 30,000 NMWL) and used as a template for dRhodamine dye-terminator cycle sequencing reactions (Perkin-Elmer). Sequence was resolved using an ABI 310 automated DNA sequencer.

Six oligonucleotide primers were then designed for "long-PCR" (Barnes 1994). The first two (Hym-Cytb-F [GCA CAA GAG TGG TAG TAA AAA TCC C] and Hym-Cytb-R [CGA ACA AGG GTG AAA CCC GTA ACA G]) match the ends of the *cob* sequence as determined above. Two others (Hym-12S-F [TTT AGG ACT TGA TAG TAG GGT AGA C] and Hym-12S-R [ATC GTC CTT TAT AAC ACA CCT TCC C]) match each end of a previously published fragment of *H. diminuta rrnS* (von Nickisch-Rosenegk, Lucius and Loos-Frank 1999). The remaining pair (Hym-16S-F [TTA TAA ATG GCC GCA GTA TAT TGA C] and Hym-16S-R [AGG CAA TTA ATT ATG CTA CCT TYG C]) was designed from an alignment of three cestode DNA fragments (450 bp of *Echinococcus multilocularis*, *Catenotaenia lobata* and *Schistocephalus solidus*; unpublished data, von Nickisch-Rosenegk). In each case these primers face "out" from the fragments. These were used in all 12 possible combinations with rTth-XL DNA polymerase (Perkin-Elmer). Reaction conditions were optimized for Mg^{++} concentration and annealing temperature as necessary. Visualization, purification, and sequencing of these products were as above, except that an ABI 377 automated sequencer was used for most reactions, with additional oligonucleotides being used for "primer walking". All sequences were determined on both strands.

Analysis

Sequences were produced and assembled using the ABI suite of programs (*e.g.* Sequencing Analysis™ and Sequence Navigator™). Subsequent manipulations used MacVector™ 6.5 (Oxford Molecular Group) and GCG (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI). Twelve protein encoding genes and two

ribosomal RNA genes were easily identified by comparisons with similar published sequences. Twenty-two tRNA genes were identified by eye based on their potential for forming tRNA-like secondary structures; specific identities were assigned by anticodon sequences.

Mitochondrial gene arrangements were compared by eye for gene adjacencies in all pairwise combinations for the following taxa: *H. diminuta*, *Echinococcus multilocularis* (GenBank record AB018440), *Fasciola hepatica* (Garey and Wolstenholme 1989; Le et al. 2000; GenBank record AF216697), *Schistosoma mansoni* (Blair et al. 1999; Le et al. 2000), *S. japonicum*, *S. mekongi*, *Taenia crassiceps* (Le et al. 2000), *Lumbricus terrestris* (Boore and Brown 1995), *Platynereis dumerilii* (Boore and Brown 2000 and GenBank AF178678), *Helobdella robusta* (partial), *Galathealinum brachiosum* (partial) (Boore and Brown 2000), *Limulus polyphemus* (Lavrov, Boore, and Brown 2000), *Tetilla* sp. (partial) (Watkins and Beckenbach 1999), and the gene arrangement previously inferred to be primitive for Vertebrata (Boore, Daehler, and Brown 1999; Boore 1999). Each of the three contending phylogenetic positions of the Platyhelminthes (see above and below) was evaluated by eye for whether these sets of shared gene boundaries are consistent or homoplasious.

Results and Discussion

Amplification of the Complete *H. diminuta* mtDNA

Five combinations of *H. diminuta*-specific primers yielded single fragments from the "long-PCR" reactions: Hym-Cytb-F and Hym-12S-R (9 kb); Hym-Cytb-F and Hym-16S-R (8 kb); Hym-Cytb-R and Hym-16S-F (6 kb); Hym-Cytb-R and Hym-12S-F (5 kb); Hym-16S-F and Hym-12S-R (1 kb). Together, these five fragments and the shorter fragments of *cob* and *rrnS* jointly represent the entire mitochondrial genome in overlapping fragments. The composite sequence reveals a genome of 13,900 nts, among the smallest so far observed (GenBank accession number AF314223).

Gene Content and Organization

The two rRNA genes, 22 tRNA genes, and 12 of the 13 protein encoding genes typical of metazoan mtDNAs were identified for *H. diminuta* by comparisons of sequence similarity and/or potential secondary structures with those of other metazoans. Their arrangement is shown in figure 1. One protein gene, *atp8*, typically found in animal mtDNAs, appears to be missing from this mtDNA (see below), as has been inferred for each of the other completely sequenced flatworm mtDNAs reported (Le et al. 2000; GenBank record AB018440). Gene arrangements are substantially similar among the studied flatworm mtDNAs; details of this are presented below.

All genes are encoded by the same strand, a condition also found in the other flatworms (Le et al. 2000; GenBank record AB018440), annelids, two bivalve mollusks, nematodes, brachiopods, and a sea anemone (see Boore 1999; Mitochondrial Genomics link at http://www.jgi.doe.gov); all other mtDNAs studied so far have these genes

distributed between the two strands. The mitochondrial genes of *H. diminuta* are generally small. Most are comparable to the reduced sizes of those in nematodes (Okimoto et al. 1991, 1992; Keddie, Higazi, and Unnasch 1999), except for *atp6* and *cox3*, which are each smaller in *H. diminuta* by about 100 nucleotides.

Absence of *atp8*

After careful analysis of the *H. diminuta* mtDNA sequence, the only ORF that is a candidate for *atp8* appears to be (mostly) within the 183 nt region otherwise described as a non-coding region (below). Designated ORF52, it is equivalent to 52 codons (plus a stop codon) in length, similar to the typical size of *atp8*, beginning 34 nucleotides after *trnY* and overlapping *trnS2(uga)* by 10 nucleotides (fig. 2). This ORF starts with TTG, but the next codon is ATA, a more commonly used start codon for mitochondrial protein genes. It ends with TAA, although potential abbreviated stop codons (see below) are present that could truncate this ORF by one, two, or nine residues, thus requiring less or no overlap with the downstream *trnS2(uga)*. However, there are several reasons to doubt that it is the *atp8* gene. Blast searches and direct comparisons with published *atp8* sequences and with the sequence of all published flatworm mtDNAs using both nucleotide and inferred amino acids reveal no similarity to this or to any other published mitochondrial gene. This lack of similarity also extends to hydrophilicity profile, which is a well conserved feature of this protein (Watkins and Beckenbach 1999). Characteristic amino acid signatures of the Atp8 protein (*e.g.* WXW near the carboxyl terminus) are not present in ORF52. Comparisons of the entire sequence of *H. diminuta* with published sequences of *atp8* genes as well as with the corresponding protein sequences reveal no

similarity. The gene for *atp8* is therefore assumed to be absent, and it seems unlikely that ORF52 encodes any functional peptide, considering its small size, A+T-richness, and internal repeat (below).

Several other mtDNAs are also missing *atp8*, almost certainly as independent losses (see above). For each of these cases, it is unknown whether *atp8* moved to the nucleus with its protein product imported to function within mitochondria or simply became dispensable.

Initiation and Termination Codons

For *H. diminuta* mtDNA, 10 of the 12 protein genes can be inferred to initiate with ATG without overlap of the upstream gene. The exceptions are *nad4*, which appears to use ATT, and *cox1*, which appears to use GTT. In each case there are no other eligible start codons that are reasonable alternatives, and the inferred amino terminal residues are similar to those of their homologues in other metazoans. The inference for *nad4* requires overlap with the upstream *nad4L*, as is common for these two genes among metazoan mtDNAs. Although GTT is not usually designated as an eligible start codon, *cox1* is most extreme among mitochondrial proteins for initiating with unusual start codons (Wolstenholme 1992).

Abbreviated stop codons (T and TA) are also common among metazoan mitochondrial protein genes, where post-transcriptional polyadenylation forms a complete UAA stop codon (Ojala, Montoya, and Attardi 1981). For *H. diminuta* mtDNA, 11 of the 12 open reading frames end with a complete stop codon (nine are TAG and two are TAA). Only *cox1* is inferred to end with an abbreviated stop codon, although there is

an in-frame TAG stop codon that, if used, would require overlap with the downstream tRNA gene by 10 nts.

Genetic Code

The mitochondrial genomes of most studied invertebrates appear to deviate from the "universal" genetic code in the identities of ATA, TGA, and AGR codons (see Wolstenholme 1992). Further differences have been inferred for Platyhelminthes such that AAA encodes N rather than K, ATA encodes I (as in the universal code) rather than M, and TAA encodes Y rather than termination (Bessho, Ohama, and Osawa 1992).

The identities of AAA and of ATA in *H. diminuta* mtDNA appear to be consistent with these flatworm deviations. Pairwise comparisons using the two best conserved genes, *cob* and *cox1*, identified the codons corresponding to AAA of *H. diminuta* for *Lumbricus terrestris* (AAY=6; AAR=2), *Katharina tunicata* (AAY=4; AAR=0) and *Drosophila yakuba* (AAY=4; AAR=0) homologues. Similar comparisons for *H. diminuta* ATA codons determined their matches in *L. terrestris* (ATY=9; ATR=0), *K. tunicata* (ATY=6; ATR=0), and *D. yakuba* (ATY=6; ATR=0). There are weaknesses of such inferences: the pairwise comparisons are non-independent due to the evolutionary relationships of the taxa, and it is not clear whether mutation bias or selection for amino acid composition can be stronger influences on substitution than the tendency to synonymous substitutions. However, acknowledging these limitations, we tentatively assign AAA and ATA as N and I, respectively, for *H. diminuta* mtDNA.

The third case that has been suggested as a deviation for flatworm mtDNAs, TAA encoding Y rather than termination, does not seem possible for *H. diminuta*. No TAA

codon is found in-frame for any protein-encoding gene. Two genes, *cox2* and *nad6*, are predicted to end on a TAA stop codon and one gene, *cox1*, is predicted to end on an abbreviated stop codon that is completed to UAA post-transcriptionally (fig. 2). Each of these genes would greatly overlap their adjacent downstream gene if they were to extend to the first in-frame TAG codon.

Nucleotide and Amino Acid Composition

H. diminuta mtDNA is very A+T-rich (71%) even for an animal mitochondrial genome. The distribution of the purines vs. pyrimidines between the strands for each TA and GC pair is highly biased, with the reported strand being very rich in T and G. The values calculated for TA-skew ([T-A]/[T+A]) and GC-skew ([G-C]/[G+C]) (Perna and Kocher 1995) are 0.29 and 0.33, respectively. (Skew values range from +1 to -1; the value is zero if the strands have no skew.) The nucleotide composition of the reported strand is 25.4% A, 45.6% T, 19.3% G, and 9.6% C. Each of these values is even more extreme for the subset of nucleotides in the third positions of codons (table 1), which are presumably more free to change.

Transfer RNA Genes

Most of the tRNA gene sequences can be folded into conventional secondary structures with features common to mitochondrial tRNAs (fig. 3). Notable exceptions are tRNA(S1), tRNA(S2), and tRNA(R), which have an unpaired DHU-arm. Additional base pairing for a longer anticodon stem is possible for each of these three tRNAs, a condition found tRNA(S1) in other mtDNAs (*e.g.* Boore and Brown 1994, 2000).

Anticodon nucleotides are identical to those most commonly found for the corresponding tRNA genes in other mtDNAs with two exceptions. tRNA(K) for *H. diminuta* mtDNA, as for each of the other seven flatworms whose mtDNAs have been completely sequenced, has a CTT anticodon rather than the more common TTT. Having C here in the wobble position would be consistent with the inferred genetic code variation such that only AAG (rather than AAR) specifies lysine. However, it is unclear how the GTT anticodon of tRNA(N) for all eight of these flatworm mtDNAs would recognize not only AAY, as for the typical code, but also AAA for flatworms. More unusually, the codon of tRNA(R) is ACG, since A in the wobble position is very uncommon. Arginine is specified by all four CGN codons, requiring this A to pair with all four nucleotides (this anticodon is normally TCG). The genes for tRNA(R) of the other two studied cestodes, *E. multilocularis* (GenBank AB018440) and *T. crassiceps* (Le et al. 2000) also have these two unusual features of A in the wobble position and an unpaired DHU arm (fig. 3). However, all of the five studied trematodes have a tRNA(R) with potential for a standard cloverleaf structure and a TCG anticodon (not shown). Whether any nucleotides of these tRNAs are post-transcriptionally modified is unknown.

Non-coding Sequences

Despite its small size, *H. diminuta* mtDNA contains two non-coding regions of significant size. The larger, of 444 nucleotides, is between *nad5* and *trnG* and consists of 12 identical repeats of a 31 nt sequence (372 nts) plus one repeat with a single nt difference. Part of this thirteenth repeat can form a stem-loop with the remaining 41 nucleotides of this non-coding region. This structure has 25 canonical base pairs and a

loop of 11 nucleotides (fig. 4) and terminates at the beginning of *trnG* with an overlap of two nucleotides. The single nucleotide difference in this thirteenth unit is a T-to-C transition at nucleotide 16 of the repeat creating a perfect match at this position of the stem. This is similar to reported repeat sequences of the nematode *Meloidogyne javanica* which contains 11 identical repeats of a 63 nucleotide unit, with the last copy modified by a single substitution, and which can also potentially form a stem-loop structure (Okimoto et al. 1991). Large potential secondary structures can be found also between *nad5* and *trnG* (as for *H. diminuta*) and between *trnY* and *trnL1* for each of the cestodes *E. multilocularis* and *T. crassiceps* (fig. 4). In some of these cases, there is potential for competing, mutually exclusive hairpins such as those identified in *Lumbricus terrestris* mtDNA (Boore and Brown 1995). No similar structures could be identified for the non-coding regions of any of the trematode mtDNAs. The function of this region, if any, is unknown; however, similar stem-loop structures have been shown to initiate replication in mammals (*e.g.* Monnerot, Solignac, and Wolstenholme 1990), and it is possible that the putative structure in this region serves a similar function in other metazoans.

A second region, of 183 nucleotides, is between *trnY* and *trnS2(uga)*. Although it contains an open reading frame (ORF) of 52 amino acids (discussed above), it seems more likely to be a non-coding region. No significantly stable potential secondary structure could be folded using these 183 nucleotides, which are 81% A+T. A further feature of this non-coding region in *H. diminuta* is a 36-nucleotide tandem repeat. Further small intergenic regions were found, the largest being between *trnW* and *cox1* (28 nts) and between *trnL1(uag)* and *trnL1(uaa)* (26 nts). Whether any of these non-coding regions serve any function awaits further study.

Phylogenetic Reconstruction Using Gene Arrangements

The mitochondrial gene arrangements of two cestodes, *T. crassiceps* and *E. multilocularis* (Le et al. 2000), are identical, and differ from that of *H. diminuta* only by the reversal in order of *trnS2* and *trnL1*. The trematodes *S. japonicum*, *S. mekongi*, and *F. hepatica* (Le et al. 2000) have these two tRNA genes in the same order as in *T. crassiceps* and *E. multilocularis* and otherwise differ from *H. diminuta* only by the positions of *trnE*, *trnV*, and *trnW* for the schistosomes and of *trnE* for *F. hepatica*. The African schistosome, *S. mansoni*, has several additional gene rearrangements (Le et al. 2000); these must be derived independently for this lineage based on the similarity among the cestodes and the other schistosomes, whose shared arrangements are parsimoniously inferred as primitive. Further, the arrangement of the genes within the portion spanning from *trnP* to *trnW* of *H. diminuta* is also identical in three other cestodes whose mtDNAs have been only partially sequenced (*Diphyllobothrium nihonkaiens* [GenBank accession AB006205], *Spirometra erinacei* [AB006204], and *S. proliferum* [AB006203]) except for the lack of *trnS1(gcu)*.

H. diminuta has two pairs of adjacent genes that have been commonly found together among animal mtDNAs (see Boore 1999), *nad4L-nad4* and *trnL1(uag)-trnL2(uaa)*. Although the significance is doubtful, it also shares single gene boundaries with several nematodes: *trnQ-trnF* as in *Caenorhabditis elegans and Ascaris suum* (Okimoto et al. 1992); *cox1-trnT* and *trnV-trnA* as in *Meloidogyne javanica* (Okimoto et al. 1991). The most noteworthy gene arrangement similarities, however, are to several

animals within the proposed higher level group Eutrochozoa (*e.g.* mollusks and annelids; Ghiselin 1988).

The arrangement of the genes in *H. diminuta* from *nad1* through *trnS1(gcu)* is nearly identical to that found among several annelids, including one previously considered to be of the phylum Pogonophora (*Galathealinum brachiosum*) (Boore and Brown 1995, 2000) (fig. 5) and, except for *trnS1(gcu)*, in an echiuran (*Urechis caupo*; J. Boore, unpublished data). Although shared single gene boundaries are less strong as a phylogenetic character (Boore and Brown 1998), it is worth noting that all these taxa also share the gene arrangement *trnG-cox3.*

The three most commonly proposed evolutionary positions for the Platyhelminthes are: 1) basal to a clade of coelomate animals (fig. 5A); 2) basal to protostomes only (fig. 5B); or 3) derived eutrochozoans (fig. 5C) (see above and below for references and significance). For several of the genes whose arrangements are identical among some platyhelminths and annelids (representative of the Eutrochozoa), a different arrangement is shared among chordates and arthropods. The positions for Platyhelminthes depicted in figure 5A or 5B would require either identical gene translocations or reversions to a previous arrangement in independent lineages for multiple genes. Further, a small portion of a poriferan mtDNA has been reported that contains only one of the relevant genes; since this group is indisputably an outgroup to the other taxa in figure 5, the primitive position of *trnK* can be inferred. All possible pairwise comparisons of the gene arrangements for the taxa considered here (see fig. 5 legend) were made. No gene arrangements are shared in a pattern to support the relationships of figure 5A or 5B. There are no homoplasious gene rearrangements for the tree shown in figure 5C. All of

the gene arrangements identified as being informative are found in *H. diminuta*, *T. crassiceps*, *E. multilocularis*, and *F. hepatica*, and all but the single boundary *nad3-trnS1* in *S. japonicum* and *S. mekongi*.

However, none of the informative gene boundaries are found in *S. mansoni* mtDNA except for *trnG-cox3*. This is the basis for the cautionary message of Le et al. (2000). It is important to note that if the rearranged genome of *S. mansoni* had been the only one sampled from platyhelminths, there would have been a lack of phylogenetic resolution, but still no homoplasy in this analysis. Although the generally slow rate of gene rearrangement in animal mtDNAs (Boore 1999) makes it more likely that the signal of ancient relationships will be preserved, occasionally rapid rearrangements are not expected to be misleading in phylogenetic analysis. Even if there are many changes in some lineages, the great number of potential arrangements and the lack of apparent "hotspots" (Boore and Brown 1998) make convergence unlikely, especially for multiple rearrangements (notable exceptions to this may be in apparent movements of non-coding sequence [Mindell et al. 1998] or in exchange of nearest-neighbor tRNA genes [Boore and Brown 1998; Boore 1999]). Cladistic analysis as used here prevents artifactual clustering of taxa based on shared primitive characters (*i.e.* the "short" branches) but rather unites taxa only on the basis of shared, derived gene rearrangements parsimoniously inferred to have occurred only once in their common ancestor.

Arrangements of this set of genes in taxa related to those in figure 5 are consistent with this inference of evolutionary relationships. Echinoderms are thought to be the sister taxon to Chordates on the tree in figure 5C; all echinoderm mtDNAs studied so far share the gene arrangements *cox2-trnK-atp8* and *trnL2(uaa)-nad1-trnI* with chordates. There is

slight similarity in the gene arrangements of *H. diminuta* with a few mollusks, which would also be expected to be included in the clade of Eutrochozoa. In *H. diminuta* the gene arrangement is *nad1-trnN-trnP*, whereas the arrangement is *nad1-trnP* in *Dentalium eboreum*, *Nautilus* sp. (J. Boore, unpublished), *Loligo bleekeri* (GenBank AB009838), and *Katharina tunicata* (but with *trnP* in opposite orientation; Boore and Brown 1994). Lastly, it may be noteworthy that three of the four tRNA genes in the cluster preceding *nad3* in *H. diminuta* are the same, although in different arrangement, to those preceding *nad3* in *K. tunicata* (Boore and Brown 1994) and *Nautilus* sp. (J. Boore, unpublished).

The position of the Platyhelminthes in the system of Metazoa has been debated for decades (Brusca and Brusca 1990). Traditionally, the coelom has been viewed as a feature uniting the protostomes and deuterostomes to the exclusion of Platyhelminthes (Hyman 1951), although this is questioned by those who view the mechanisms of schizocoely (as for protostomes) and enterocoely (as for deuterostomes) as fundamentally different and therefore of separate origins. Some investigators view the simplicity of the Platyhelminthes as a secondary reduction from a coelomate ancestor (Ax 1963; Smith and Tyler 1985). Since platyhelminths share some of the basic characters of protostomes, such as spiral cleavage, the mesoderm originating (at least in part; see [Boyer et al. 1996]) from the 4d mesentoblast, and the mouth arising from the blastopore, some include them in Protostomia, but generally as basal to the other, presumably derived, schizocoelomate phyla (see Brusca and Brusca 1990).

However, an alternative interpretation is that Platyhelminthes is derived within the Eutrochozoa, a group of protostomes including annelids and mollusks, but not arthropods (Ghiselin 1988). This is supported by ultrastructural analysis (Rieger 1986), by some

sequence comparisons (Aguinaldo et al. 1997; Balavoine 1997; Carranza, Baguñà, and Riutort 1997; Ruiz-Trillo et al. 1999), and by our gene arrangement comparisons here. Further work on those flatworms comprising the Acoela may determine whether this group also occupies such a phylogenetic position or instead is basal among animal life as has been inferred by DNA sequence comparisons (Ruiz-Trillo et al. 1999).

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Table 1

Codon usage in the 12 protein-encoding genes of *H. diminuta* mtDNA.

Amino acid	Anti-codon	Codon family	Third codon position				
			All	T	C	A	G
Phe (F)	GAA	TTY	417	393	24	----	----
Leu (L)	TAA	TTR	419	----	----	300	119
Leu (L)	TAG	CTN	87	42	4	27	14
Total Leu	----------		(506)	----	----	----	----
Ile (I)	GAT	ATH	296	172	16	108	----
Met (M)	CAT	ATG	93	----	----	----	93
Val (V)	TAC	GTN	298	144	9	85	60
Ser (S)	TGA	TCN	170	112	7	32	19
Ser (S)	GCT	AGN	194	87	14	56	37
Total Ser	----------		(364)	----	----	----	----
Pro (P)	TGG	CCN	80	52	6	19	3
Thr (T)	TGT	ACN	110	60	5	28	17
Ala (A)	TGC	GCN	100	47	7	30	16
Tyr (Y)	GTA	TAY	215	181	34	----	----
His (H)	GTG	CAY	53	42	11	----	----
Gln (Q)	TTG	CAR	22	----	----	7	15
Asn (N)	GTT	AAH	136	70	12	54	----
Lys (K)	CTT	AAG	46	----	----	----	46

Asp (D)	GTC	GAY	67	57	10	----	----
Glu (E)	TTC	GAR	73	----	----	34	39
Cys (C)	GCA	TGY	131	107	24	----	----
Trp (W)	TCA	TGR	88	----	----	57	31
Arg (R)	ACG	CGN	52	40	1	10	1
Gly (G)	TCC	GGN	190	84	13	43	50
Total ----------			3337	1690	197	890	560

Figure legends

Figure 1: Arrangement of the mitochondrial genome of the tapeworm *Hymenolepis diminuta*. Gene scaling is only approximate. All genes are coded by the same DNA strand and are transcribed clockwise. The origin(s) of transcription remains undetermined. All genes have standard nomenclature except for the 22 tRNA genes, which are designated by only the one letter code for the corresponding amino acid, with numerals differentiating each of the two leucine- and serine-specifying tRNAs (L1 and L2 for codon families CUN and UUR, respectively; S1 and S2 for codon families AGN and UCN, respectively). "NC" refers to a largest non-coding region.

Figure 2: A partly schematic representation of the 13,900 nt mtDNA sequence of *Hymenolepis diminuta*. To conserve space, most of the center portions of the larger genes have been replaced with a numeral indicating the number of omitted nucleotides. For *cox1* and for *nad4* the initiator methionine is placed in parentheses to indicate presumed non-conformity to the genetic code. Asterisks indicate stop codons, either complete or abbreviated (see text).

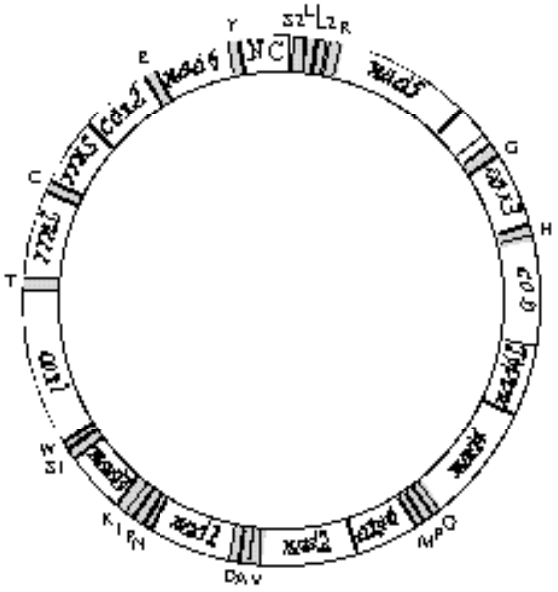
Figure 3: DNA sequences for the 22 tRNA genes of *Hymenolepis diminuta* mtDNA folded into inferred secondary structures. For the three tRNAs with unpaired DHU arms (those for R, S1, and S2), additional potential base pairings are shown with dark lines. The *H. diminuta* tRNA(R) is unusual not only in having an unpaired DHU arm, but in having an A in the first anticodon position; the similar structures predicted for tRNA(R)

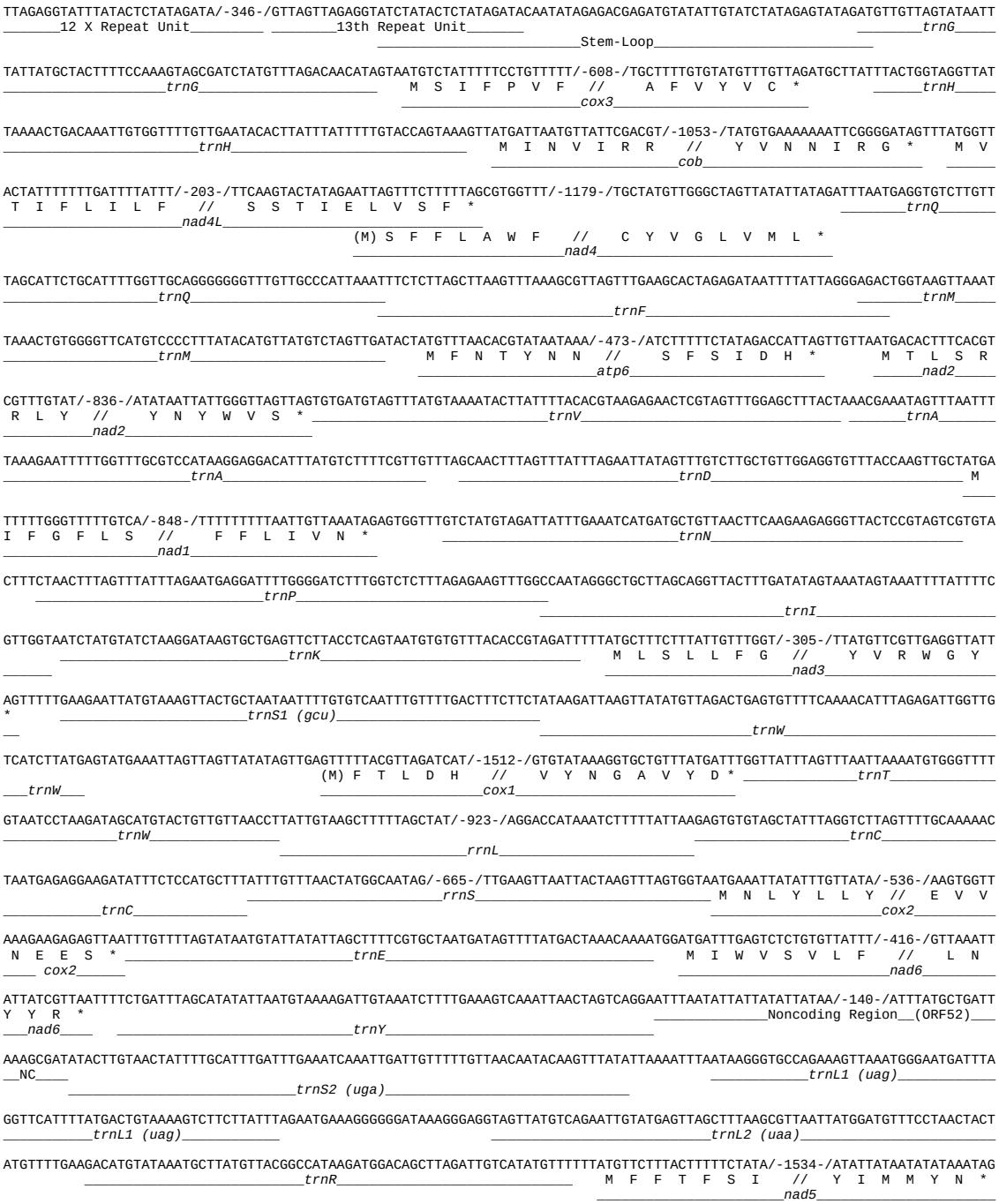
of *T. crassiceps* and *E. multilocularis* mitochondrial genomes are shown in the lower right hand box with nucleotides that differ from *H. diminuta* shown in lower case letters.

Figure 4: Potential secondary structures found for the non-coding regions between either *nad5* and *trnG* or *trnY* and *trnL1* of three cestodes. Foldings presented as triplex or quadriplex indicate alternative, potential hairpins. In the case of *H. diminuta*, this structure includes part of a 13th repeat element (see text) with an arrow marking the only nucleotide difference from the preceding 12 repeat elements.

Figure 5: The three contending hypotheses of the phylogenetic relationships of Platyhelminthes to several other animal phyla, either as basal coelomates (A), as basal protostomes (B), or as eutrochozoans (C). Only the relationship shown in (C) is supported by a comparison of complete mitochondrial gene arrangements. A portion that is similar between the arrangements of annelids and Platyhelminthes is shown. A subset of these genes has an alternative arrangement shared among other phyla; these are shown in boldface along with their flanking genes. No gene arrangements are shared in a pattern to support the relationships in A or B. For these comparisons, Annelida is represented by *Lumbricus terrestris* (Boore and Brown 1995), *Platynereis dumerilii* (Boore and Brown 2000 and GenBank AF178678), *Helobdella robusta* (partial) and *Galathealinum brachiosum* (partial) (Boore and Brown 2000), Arthropoda by *Limulus polyphemus* (Lavrov, Boore, and Brown 2000) [previously inferred to be the primitive arrangement for studied arthropods (Boore et al. 1995; Boore, Lavrov, and Brown 1998; Boore 1999; Lavrov, Boore, and Brown 2000)], and Chordata by the gene arrangement most

commonly observed [previously inferred to be primitive for Vertebrata except for the position of one tRNA that is not part of this subset of genes (Boore, Daehler, and Brown 1999; Boore 1999)]. Platyhelminthes is represented by *H. diminuta*, *E. multilocularis* (GenBank AB018440), *T. crassiceps*, and *F. hepatica* (Garey and Wolstenholme 1989; Le et al. 2000), and Porifera by *Tetilla* sp. (Watkins and Beckenbach 1999). The arrangements of all other non-depicted genes are unknown for the poriferan. Genes are underlined to signify opposite transcriptional orientation. Unconnected gene blocks are not adjacent.

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13th Repeat Unit
trnG
Stem-Loop
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trnG
M S I F P V F // A F V Y V C *
trnH
cox3
TAAAACTGACAAATTGTGGTTTTGTTGAATACACTTATTTATTTTTGTACCAGTAAAGTTATGATTAATGTTATTCGACGT/-1053-/TATGTGAAAAAAATTCGGGGATAGTTTATGGTT
trnH
M I N V I R R // Y V N N I R G * M V
cob
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T I F L I L F // S S T I E L V S F *
trnQ
nad4L
(M) S F F L A W F // C Y V G L V M L *
nad4
TAGCATTCTGCATTTTGGTTGCAGGGGGGGTTTGTTGCCCATTAAATTTCTCTTAGCTTAAGTTTAAAGCGTTAGTTTGAAGCACTAGAGATAATTTTATTAGGGAGACTGGTAAGTTAAAT
trnQ
trnM
trnF
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trnM
M F N T Y N N // S F S I D H *
M T L S R
atp6
nad2
CGTTTGTAT/-836-/ATATAATTATTGGGTTAGTTAGTGTGATGTAGTTTATGTAAAATACTTATTTTACACGTAAGAGAACTCGTAGTTTGGAGCTTTACTAAACGAAATAGTTTAATTT
R L Y // Y N Y W V S *
trnV
trnA
nad2
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trnA
trnD
M
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I F G F L S // F F L I V N *
trnN
nad1
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trnI
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trnK
M L S L L F G // Y V R W G Y
nad3
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trnW
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(M) F T L D H // V Y N G A V Y D *
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trnE
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nad6
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trnY
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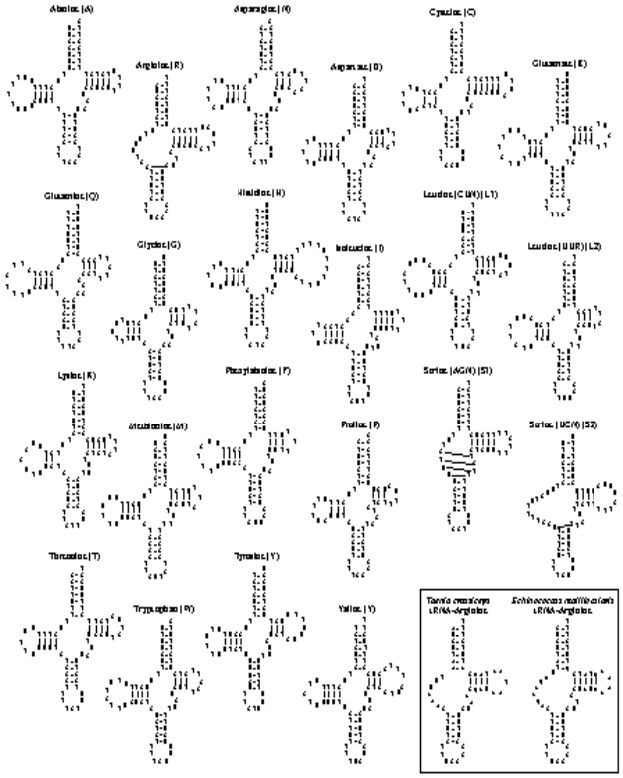
nad5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Histidrna. (H)
Isoleucrna. (I)
Leucrna. (CUN) (L1)
Leucrna. (UUR) (L2)
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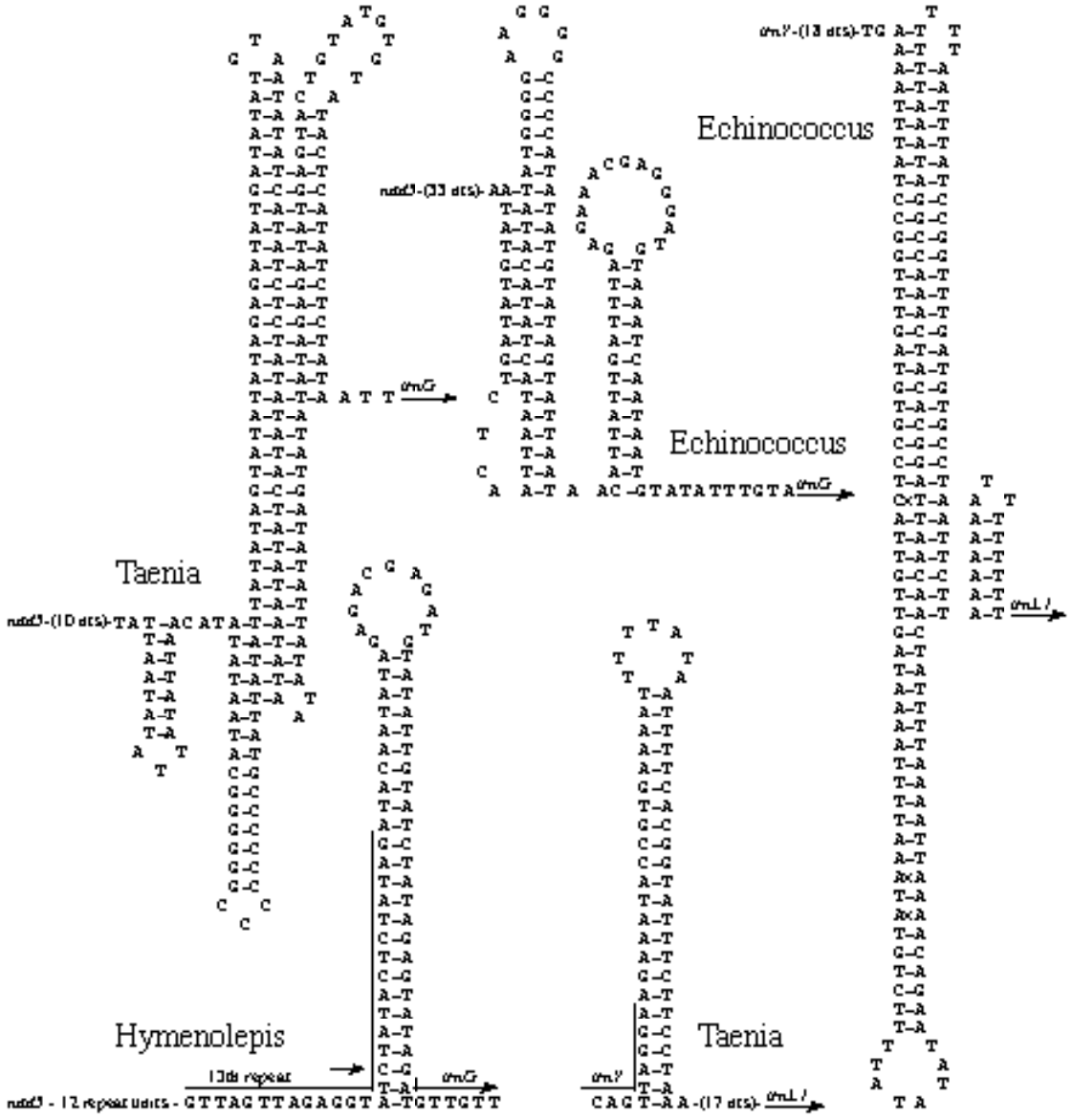
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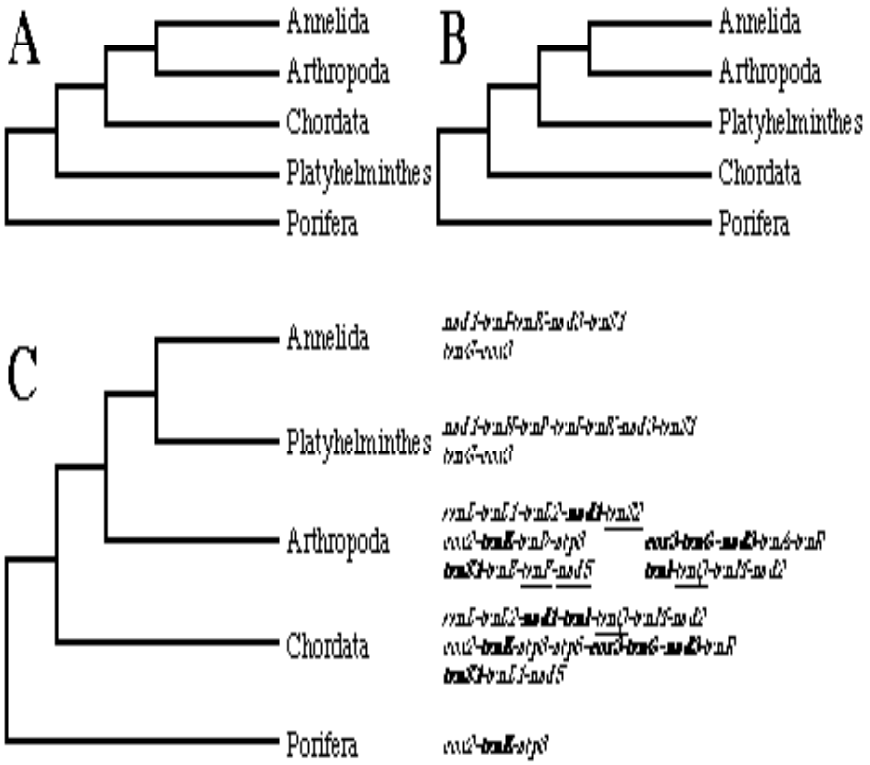
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