Characterization of the mitochondrial genome of *Diphyllobothrium latum* (Cestoda: Pseudophyllidea) – implications for the phylogeny of eucestodes

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SUMMARY

The complete nucleotide sequence of the mitochondrial genome was determined for the fish tapeworm *Diphyllobothrium latum*. This genome is 13 608 bp in length and encodes 12 protein-coding genes (but lacks the *atp8*), 22 transfer RNA (tRNA) and 2 ribosomal RNA (rRNA) genes, corresponding to the gene complement found thus far in other flatworm mitochondrial (mt) DNAs. The gene arrangement of this pseudophyllidean cestode is the same as the 6 cyclophyllidean cestodes characterized to date, with only minor variation in structure among these other genomes; the relative position of *trnS2* and *trnL1* is switched in *Hymenolepis diminuta*. Phylogenetic analyses of the concatenated amino acid sequences for 12 protein-coding genes of all complete cestode mtDNAs confirmed taxonomic and previous phylogenetic assessments, with *D. latum* being a sister taxon to the cyclophyllideans. High nodal support and phylogenetic congruence between different methods suggest that mt genomes may be of utility in resolving ordinal relationships within the cestodes. All species of *Diphyllobothrium* infect fish-eating vertebrates, and *D. latum* commonly infects humans through the ingestion of raw, poorly cooked or pickled fish. The complete mitochondrial genome provides a wealth of genetic markers which could be useful for identifying different life-cycle stages and for investigating their population genetics, ecology and epidemiology.

Key words: *Diphyllobothrium latum*, diphyllobothriasis, Pseudophyllidea, Cestoda, mitochondrial genome, molecular phylogeny.

INTRODUCTION

All species of the pseudophyllidean tapeworm genus *Diphyllobothrium* infect fish-eating vertebrates, with 14 being causative agents of diphyllobothriasis in humans (Ashford and Crewe, 2003). Of these, *D. latum* is the most geographically widespread, occurring in Northern Europe (Peduzzi and Boucher-Rodoni, 2004), North America (Rausch and Hilliard, 1970), South America (Santos and de Faro, 2005), and possibly also in Asia, including Japan and Korea (Lee *et al*. 1989; Yamane *et al*. 1996). It is also recognized as an emerging disease in some countries (e.g. in Brazil) (Sampaio *et al*. 2005), as developments in mariculture, the world trade in fish and fish products, and the trend for humans to eat raw fish (e.g. see Macpherson, 2005), increases the incidence of diphyllobothriasis. The movement of fish and fish products, and the multiple species of *Diphyllobothrium* causing diphyllobothriasis drive the need to rapidly and accurately identify the larval forms of cestodes to species. The identification of plerocercoids of *Diphyllobothrium* from marine and freshwater fishes, using morphology alone, is considered a task for experts (Anderson and Gibson, 1989). Recently, molecular tools have provided some progress. For example, Yera *et al*. (2006) reported the use of 2 mitochondrial genes for identifying *Diphyllobothrium nihonkaiense*, and Skerikova *et al*. (2006) assessed the validity of *D. pacificum* as a valid species using the sequences of the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA). There is a need to develop reliable diagnostic tests for species of *Diphyllobothrium*. The mitochondrial genome provides a rich source of molecular markers for the specific identification of helminths and for investigating population genetic

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structures (e.g. see Hu *et al.* 2004; Hu and Gasser, 2006; Le *et al.* 2006).

To date, the complete mitochondrial genomes of 13 flatworm species (6 from cestodes and 7 from trematodes, respectively) have been published. The sequences determined for *Taenia crassiceps* (see Le *et al.* 2000), *T. solium* (see Nakao *et al.* 2003), *T. asiatica* (see Jeon *et al.* 2005), *Hymenolepis diminuta* (see von Nickisch-Rosenegk *et al.* 2001), *Echinococcus multilocularis* (see Nakao *et al.* 2002), and *E. granulosus* (see Le *et al.* 2002*b*) all represent the order Cyclophyllidea. Comparative mitogenomics has rapidly become established as an approach for differentiating species and host-specific strains of *Echinococcus* (reviewed by McManus, 2006).

Only 2 orders of cestodes are known to include species that infect humans. Cyclophyllideans, including *Taenia*, *Echinococcus* and *Hymenolepis* and the Pseudophyllidea, with *Diphyllobothrium* as the commonest of 6 genera; *Diplogonoporus*, *Ligula*, *Pyramicocephalus*, *Schistocephalus*, and *Spirometra* are the other 5 (Ashford and Crewe, 2003). Pseudophyllidea and Cyclophyllidea are very different orders, both in terms of morphology, habitat, life-cycle and life-history strategies (see Caira and Littlewood, 2001). Each represents distinct lineages in the evolutionary history of the cestodes, with cyclophyllideans recognized as representing one of the most derived lineages (Hoberg *et al.* 2001; Olson *et al.* 2001), and pseudophyllideans variously placed as a basal difossate lineage (morphology: Hoberg *et al.* 2001) or a polyphyletic difossate assemblage (molecular data: Olson *et al.* 2001). A recent molecular study by Brabec *et al.* (2006) formally recognized and circumscribed 2 lineages of pseudophyllideans. The first, including the families Diphyllobothriidae and Cephalochlamyidae, giving rise to the 'Diphyllobothriidea', which was resolved as a basal lineage amongst the difossate tapeworm lineages, and the second, more derived clade, the 'Bothriocephalidea' including the remaining 4 pseudophyllidean families. Sequencing the mitochondrial genome of *D. latum* may also provide improved insights into the use of mitogenomics for resolving cestode relationships.

Metazoan mitochondrial genomes are typically circular DNA molecules, ranging from 14 kb to 18 kb in size and contain 37 genes: 13 protein-encoding genes (*cox1*-*cox3*, *cytb*, *nad1*-*nad6*, *nad4L*, *atp6* and *atp8*), 2 ribosomal RNA genes (*rrnS* and *rrnL*), 22 transfer RNA (*trn*) genes (Boore, 1999), but the *atp8* gene is lacking from most nematode and flatworm species reported thus far (Le *et al.* 2002*a*; Kim *et al.* 2006). Due to the uniformity of gene content across the diverse metazoan groups, the mitochondrial DNAs (mtDNAs) have attracted attention as one of the most useful genetic markers employed both for phylogenetic studies and genome evolution in a variety of animal groups. Another idiosyncratic feature of metazoan mtDNA is to evolve more rapidly than nuclear genomes, and there is a varying degree of substitution rates in different gene loci (e.g. Brown *et al.* 1982). These characteristics meet well with attempts to resolve deep-branching phylogeny of the major groups of metazoans (Lavrov *et al.* 2004; Lavrov and Lang, 2005) and to employ useful genetic markers for many phylogenetic (biogeographical, molecular ecological) studies of taxa with relatively recent origins (Avise, 2000). Accordingly, complete mitochondrial genome sequences have become increasingly popular among contemporary molecular 'phylogeneticists', and the number of genome sequences published has been increasing during the last decade. In the present study, we determined the complete mitochondrial genome sequence of *D. latum* as a basis for the future definition of strain and species-specific markers, and for assessing mitogenomics in resolving the interrelationships of cestodes.

MATERIALS AND METHODS

Sampling and molecular techniques

An adult worm of *Diphyllobothrium latum* was obtained from a 31-year-old Russian female patient after anthelmintic treatment. The specimen was stored in 70% ethanol prior to DNA extraction. Total genomic DNA of *D. latum* was extracted using a QIAamp tissue kit (Qiagen Co.), according to the manufacturer's instruction and used as a template DNA for PCR amplification. Initially, 5 small fragments of *D. latum* mtDNA, ranging in size from ~360 to 670 bp, were PCR-amplified using their corresponding primer sets for each of 5 gene regions (*cob* [CytbF/CytbR], *nad1* [PLND1-F/PLND1-R], *cox1* [p-1F/p-1R], *rrnL* [PL16S-F/PL16S-R], and *rrnS* [PL12S-F/PL12S-R]; see Table 1). PCR reactions (50 μl volume) were performed in 10 mM Tris-HCl (pH 8·4), 50 mM KCl; 2·5 mM $MgCl_2$; 200 μM of each dNTP; 100 pmol of each primer and 2·5 U *Taq* polymerase (TaKaRa, Japan) under the following cycling conditions: 1 cycle (94 °C for 3 min), 35 cycles (94 °C for 1 min, 50 °C to 60 °C for 30 sec, 72 °C for 1 min 30 sec), and 1 cycle (72 °C for 10 min). The sequences obtained from the 5 amplicons were then used to design *D. latum*-specific primer sets for long PCR (see Table 1 for details of the primers). Five overlapping fragments (ranging in size from 450 bp to 5·5 kb) and covering the entire mitochondrial genome of *D. latum* were amplified using the Expand Long Template PCR System (Roche, USA) under the following conditions: 1 cycle of initial denaturation (45 s at 94 °C), 35 cycles of denaturation-primer annealing-elongation (10 s at 92 °C, 30 s at 63 °C, and 8 min at 68 °C), and 1 cycle of the final extension (12 min at 72 °C). A negative

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Table 1. The sequences and their relative positions of the PCR primers used in the present study

(The binding sites of the primers correspond to the relative positions in the mtDNA of *Diphyllobothrium latum*. The IUPAC codes were used for R (A, G), Y (C, T), W (T, A), K (T, G), S (C, G), H (A, T, C) and D (A, T, G).)

Primers	Binding site	Primer sequence (5′–3′)	Reference*
CytbF	1141–1166	GGWTAYGTWYTWCCWTGRGGWCARAT	Boore and Brown (2000)
CytbR	1549–1592	GCRTAWGCRAAWARRAARTAYCAYTCWGG	Boore and Brown (2000)
PLND1-F	5250–5273	KCGTAAGGGBCCWAAHAAGGTTGG	Present study
PLND1-R	5945–5965	AATCATAACGAAYACGHGGHA	Present study
p-1F	7501–7526	TGGTTTTTTGTGCATCCTGAGGTTTA	Miyadera *et al*. (2001)
p-1R	7918–7944	AGA AAGAACGTA ATGAAAATGAGCAAC	Miyadera *et al*. (2001)
PL16S-F	8913–8951	WYYGTGCDAAGGTAGCATAAT	Present study
PL16S-R	9317–9338	AWAGATAAGAACCRACCTGGCT	Present study
PL12S-F	9608–9633	CAGTGCCAGCAKCYGCGGTTADWCTG	Present study
PL12S-R	9547–9571	AYCSWGRKTGWCGGGCGRTRTGTAC	Present study
Dl/n-cob-F1	1415–1446	TCTTATTTTACAACTAAGGATTTTTTC	Present study
Dl-ND1-R1	5357–5414	AATAAACAATAAAGTACAACAATTAAA	Present study
Dl-ND1-F1	5609–5632	GCGGCTTGTGTTATAAAAGATATT	Present study
Dl-CO1-R	7682–7705	AAACAGCCGTCTTTACATCTAAAC	Present study
Dl-CO1-F	7682–7705	GTTTAGATGTAAAGACGGCTGTTT	Present study
Dl-16S-R	9064–9087	TAAAGATCCTAGGGTCTTTCCGTC	Present study
Dl-16S-F	9064–9087	GACGGAAAGACCCTAGGATCTTTA	Present study
Dl-12S-R	9547–9571	GCTTACGCTTAATAAATACTACCG	Present study
Dl/n-12S-F1	9730–9760	GTGATTAGGTTATGA AAGGGATTAGATACC	Present study
Dl/n-cob-R2	1378–1407	CACGTCACCATAACCAAATGAAGAAAATAA	Present study

* The sources for the primers cited from previous studies.

control (no template) was also included in every PCR run. The amplicons were separated on 1% agarose gels, excised and purified using a QIAquick PCR purification kit (Qiagen, Germany). Sequencing of the amplicons was performed in both directions by ‘primer walking’ using a Big Dye Terminator Cycle-Sequencing Kit (Applied Biosystems), according to the manufacturer’s instructions. Overlapping fragments were assembled to complete the sequence of the mt genome.

Gene annotation and phylogenetic analyses

With the aid of automatic organelle genome annotation program (DOGMA; Wyman *et al*. 2004), 12 protein-coding and 2 rRNA genes of *Diphyllobothrium latum* were identified by sequence comparison with those of other flatworm mtDNAs. Putative secondary structures of 22 tRNA genes were identified using DOGMA or *via* the recognition of potential secondary structures and anticodon sequences. Secondary structures of non-coding regions were predicted using the RNAdraw program (Matzura and Wennborg, 1996). For phylogenetic analysis, 7 species of cestode (including *D. latum*) for which complete mitochondrial genome sequences are available were included. The mitochondrial genome sequences used are as follows: *D. latum* (DQ985706; this study), *Echinococcus granulosus* (NC_008075), *E. multilocularis* (NC_000928), *Hymenolepis diminuta* (NC_002767), *Taenia asiatica* (NC_004826), *T. crassiceps* (NC_002547) and *T. solium* (NC_004022). The amino acid sequences were inferred from the 12 protein-coding genes of *D. latum* using the codon translation table #9 of the GenBank, and their identities verified by database searches. Inferred amino acid sequences were then subjected to sequence alignment using the program ClustalX (Thompson *et al*. 1997). The alignment of gene sequences was performed using the following options: gap opening penalty = 10, gap extension penalty = 1·0 with a ‘delay divergent sequence’ setting of 30% using the BLOSUM similarity matrix. A conserved block of the concatenated alignment was selected employing the program Gblocks (Castresana, 2000). The most recent molecular phylogeny of flatworm mtDNAs found the Trematoda as a sister group to the Cestoda (Park *et al*., 2007). Thus, *Fasciola hepatica* (NC_002546) and *Paragonimus westermani* (NC_002354) were used as outgroups for all phylogenetic analyses in this study. Bayesian analysis was performed using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). Four Markov Chain Monte Carlo (MCMC) chains were run for 10^6 generations, sampled every 100 generations. Bayesian posterior probability values representing the percentage of samples recovering particular clades were estimated after the initial 1000 trees (the first 10^5 generations) were discarded. Phylogenetic analyses were conducted using the maximum parsimony (MP) and neighbour-joining (NJ) methods, employing the program PAUP* 4.0b10 (Swofford, 2002). The MP analysis was performed with the exhaustive search option. The

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confidence level for each branch was estimated by nonparametric bootstrap analysis with 1000 random replications using a heuristic search option. The maximum likelihood mapping method (Strimmer and von Haeseler, 1997) was conducted to assess the amount of phylogenetic structure in the amino acid dataset, and the maximum likelihood (ML) tree was reconstructed using the TREE-PUZZLE 5.2 program (Schmidt *et al.* 2002) with the mtREV24 matrix (Adachi and Hasegawa, 1996) as an evolution model for mitochondrial proteins.

RESULTS AND DISCUSSION

Long-PCR amplification of D. latum *mtDNA*

The long-PCR amplification using each *D. latum* mtDNA-specific primer sets consistently generated a single amplicon. Primer sets (amplicon sizes) were as follows: Dl/n-cob-F1 and Dl-ND1-R1 (4 kb); Dl-ND1-F1 and Dl-CO1-R (2 kb); Dl-CO1-F and Dl-16S-R (1·3 kb); Dl-16S-F and Dl-12S-R (450 bp); and Dl/n-12S-F1 and Dl/n-cob-R2 (5·5 kb) (see Table 1 for details). The sequence identity in overlapping regions for these 5 long-PCR fragments was verified by comparison with the sequences of the 5 initial shorter amplicons from *cob*, *nad1*, *cox1*, *rrnL* and *rrnS*. All of the sequences were assembled to obtain the complete sequence of the mitochondrial genome of *D. latum*.

Gene content and organization

The complete mtDNA sequence determined for *D. latum* is 13 608 bp in length (GenBank Accession number; DQ985706), and it encodes 12 protein-coding genes (lacking *atp8*), 22 *trn* genes and 2 rRNA genes (Fig. 1), like other flatworm mtDNAs. All genes are transcribed in the same direction, a common feature of the flatworm mtDNAs reported thus far (Johnston, 2006; Littlewood *et al.* 2006). The gene arrangement for the mitochondrial genomes of cestodes published to date, including that of *D. latum*, is the same, with the exception of that of *Hymenolepis diminuta* where the relative position of *trnS2* and *trnL1* is switched (von Nickisch-Rosenegk *et al.* 2001). The relative position and length of each gene locus in the mt genome of *D. latum* are given in Table 2. The nucleotide composition of the entire mtDNA sequences is 23·6% A, 44·6% T, 19·8% G, and 12·0% C and is thus biased toward A and T (the A+T content of 68·2%; Table 3), similar to those of *E. multilocularis* (69%; Nakao *et al.* 2002) and *E. granulosus* (67%; Le *et al.* 2002*b*), but distinct from those of *H. diminuta* (71%; von Nickisch-Rosenegk *et al.* 2001), *T. crassiceps* (74%; Le *et al.* 2000) and *T. asiatica* (71·4%; Jeon *et al.* 2005).

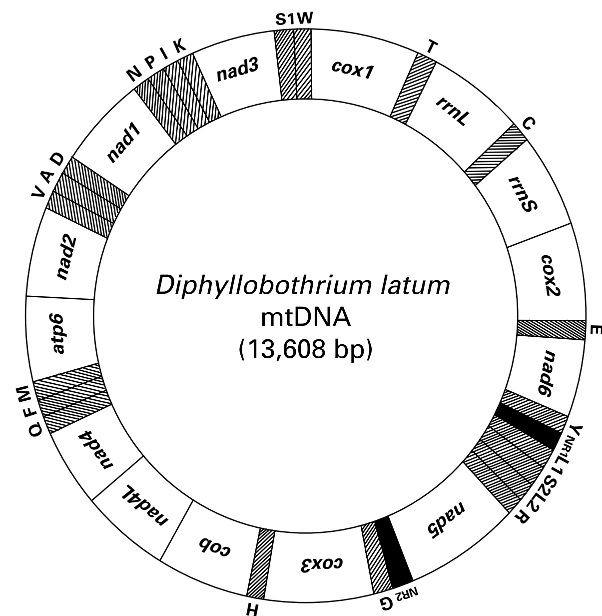

Fig. 1. Circular representation of the mitochondrial genome of *Diphyllobothrium latum*. Genes are not drawn to scale, and the tRNA genes are indicated by hatched areas.

Protein-coding genes

It is widely acknowledged that the nucleotide composition of protein-coding genes of metazoan mtDNAs is not randomly distributed (Saccone *et al.* 2002). This compositional bias is often associated with the amino acid sequence composition and unequal usage of synonymous codons within amino acid families (Herbeck and Novembre, 2003). For the protein-coding genes of *D. latum* mtDNA, amino acids inferred from T-, G-, and A-rich codons are very abundant: T-rich codons with more than 2 Ts in a triplet represent Phe (10·18% TTT and 1·37% TTC), Leu (8·00% TTA, 3·18% TTG, and 2·08% CTT), Ile (4·43% ATT), Val (4·61% GTT), Ser (3·27% TCT), Tyr (4·67% TAT) and Cys (2·56% TGT). The proportion of G-rich (more than 2 Gs in a triplet) and A-rich codons (more than 2 As in a triplet) are 12·36% and 9·75%, respectively, whereas C-rich codons account for 4·07% (Table 4). These 3 classes of codons, abundant for a specific nucleotide, represent 66·46% of all amino acids. The codon usage bias avoiding C is also particularly prominent at the third position of synonymous codons (Scouras and Smith, 2001). In almost all cases, the frequency of a specific codon is decreased when the third codon position is replaced with a C: The relative frequencies of Phe are 10·18% for TTT and 1·37% for TTC. The relative frequencies for Val are 4·61% for GTT, 2·11% for GTA, 2·14% for GTG, and 0·86% for GTC. The start and termination codons for the 12 protein-coding genes were inferred by comparing their sequences with those inferred from previously

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Table 2. Mitochondrial genome organization of *Diphyllobothrium latum*

Genes/ regions	No. of		Codons		Positions (5′–3′)
	Nucleotides	Amino acids	Initiation	Termination	
trnG	66				1–66
cox3	651	216	GTG	TAG	70–720
trnH	67				711–777
cob	1107	368	ATG	TAA	781–1887
nad4L	261	86	ATG	TAA	1889–2149
nad4	1251	417	ATG	TAG	2110–3360
trnQ	63				3361–3423
trnF	67				3420–3486
trnM	67				3483–3549
atp6	510	169	ATG	TAG	3553–4062
nad2	879	292	ATG	TAG	4065–4943
trnV	64				4945–5008
trnA	63				5016–5078
trnD	64				5082–5145
nad1	891	296	ATG	TAG	5146–6036
trnN	66				6036–6101
trnP	65				6109–6173
trnI	63				6183–6245
trnK	66				6252–6317
nad3	357	118	ATG	TAG	6318–6674
$trnS1_{(AGN)}$	59				6664–6722
trnW	63				6725–6787
cox1	1566	522	ATG	TAG	6796–8361
trnT	62				8352–8413
rrnL	968				8414–9381
trnC	64				9382–9445
rrnS	740				9446–10185
cox2	570	189	ATG	TAA	10186–10755
trnE	71				10759–10829
nad6	459	152	ATG	TAG	10826–11284
trnY	65				11288–11352
NR1	222				11353–11574
$trnL1_{(CUN)}$	67				11575–11641
$trnS2_{(UGN)}$	66				11652–11717
$trnL2_{(UUN)}$	64				11730–11793
trnR	56				11794–11849
nad5	1578	525	ATG	TAA	11844–13421
NR2	187				13422–13608

Table 3. Nucleotide composition of the mitochondrial genome of *Diphyllobothrium latum*

Description	Length (bp)	A	C	T	G	A+T
Entire sequence	13 608	23·6	12·0	44·6	19·8	68·2
Protein-coding sequence	10 080	21·6	12·0	46·4	20·0	68·0
Codon position: 1st	3360	23·5	10·7	41·6	24·2	65·1
2nd	3360	17·4	15·6	47·2	19·8	64·6
3rd	3360	23·9	9·8	50·4	15·9	74·3
Ribosomal RNA gene sequence	1708	29·2	11·7	39·9	19·2	69·1
Transfer RNA gene sequence	1418	29·6	11·2	36·6	22·6	66·2
Non-coding region	409	31·1	10·5	43·3	15·2	74·4

published mtDNAs of flatworm species. The 12 protein-coding genes of *D. latum* mtDNA were translated using the flatworm mitochondrial genetic code (translation table #9 in GenBank). All open reading frames (11 out of 12) are inferred to use ATG as an initiation codon, with the exception of *cox3* for which GTG is used as the start codon. All 12 protein-coding genes are predicted to end with complete codons. Eight of 12 genes (*cox3*, *nad4*, *atp6*, *nad2*, *nad1*, *nad3*, *cox1* and *nad6*) are inferred to terminate with TAG, whereas the others (*cob*, *nad4L*, *cox2*, and *nad5*) have TAA (see Table 2).

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Table 4. Codon usage for 12 protein-coding genes of the *Diphyllobothrium latum* mitochondrial genome

NC	AA	No.	%	NC	AA	No.	%	NC	AA	No.	%	AA	Ab.	No.	%
TTT	Phe	342	10·18	CCA	Pro	17	0·51	GAT	Asp	48	1·43	Ala	A	119	3·54
TTC	Phe	46	1·37	CCG	Pro	5	0·15	GAC	Asp	19	0·57	Cys	C	116	3·45
TTA	Leu	269	8·01	ACT	Thr	69	2·05	GAA	Glu	31	0·92	Asp	D	67	1·99
TTG	Leu	107	3·18	ACC	Thr	7	0·21	GAG	Glu	36	1·07	Glu	E	67	1·99
CTT	Leu	70	2·08	ACA	Thr	33	0·98	TGT	Cys	86	2·56	Phe	F	388	11·55
CTC	Leu	10	0·30	ACG	Thr	9	0·27	TGC	Cys	30	0·89	Gly	G	233	6·93
CTA	Leu	44	1·31	GCT	Ala	78	2·32	TGA	Trp	56	1·67	His	H	51	1·52
CTG	Leu	22	0·65	GCC	Ala	9	0·27	TGG	Trp	41	1·22	Ile	I	262	7·80
ATT	Ile	149	4·43	GCA	Ala	22	0·65	CGT	Arg	46	1·37	Lys	K	52	1·55
ATC	Ile	26	0·77	GCG	Ala	10	0·30	CGC	Arg	3	0·09	Leu	L	522	15·54
ATA	Ile	87	2·59	TAT	Tyr	157	4·67	CGA	Arg	0	0·00	Met	M	87	2·59
ATG	Met	87	2·59	TAC	Tyr	50	1·49	CGG	Arg	6	0·18	Asn	N	106	3·15
GTT	Val	155	4·61	TAA	*	4	0·12	AGT	Ser	81	2·41	Pro	P	86	2·56
GTC	Val	29	0·86	TAG	*	8	0·24	AGC	Ser	24	0·71	Gln	Q	23	0·68
GTA	Val	71	2·11	CAT	His	41	1·22	AGA	Ser	42	1·25	Arg	R	55	1·64
GTG	Val	72	2·14	CAC	His	10	0·30	AGG	Ser	17	0·51	Ser	S	365	10·86
TCT	Ser	110	3·27	CAA	Gln	13	0·39	GGT	Gly	151	4·49	Thr	T	118	3·51
TCC	Ser	11	0·33	CAG	Gln	10	0·30	GGC	Gly	21	0·63	Val	V	327	9·73
TCA	Ser	69	2·05	AAT	Asn	62	1·85	GGA	Gly	19	0·57	Trp	W	97	2·89
TCG	Ser	11	0·33	AAC	Asn	18	0·54	GGG	Gly	42	1·25	Tyr	Y	207	6·16
CCT	Pro	48	1·43	AAA	Asn	26	0·77						*	12	0.36
CCC	Pro	16	0·48	AAG	Lys	52	1·55								

* Stop (termination) codon.
Ab, Abbreviation.

Transfer RNA and ribosomal RNA genes

Twenty-two nucleotide segments, ranging in size from 56 to 71 nucleotides are predicted to fold into clover leaf-like secondary structures (Fig. 2), which are almost the same as those reported thus far for other cestode species. An amino-acyl stem of 7 nucleotide pairs and an anticodon stem of 5 nucleotide pairs are the common characteristics of 22 tRNAs found in *D. latum* mtDNA. Of 22 inferred tRNA secondary structures, 19 display the typical clover leaf-like configuration with a dihydrouridine (DHU)-arm, but this arm is lacking for the other 3 (*trnS1*, *trnS2*, and *trnR*). The *D. latum trnC* possesses a DHU arm as found in *Hymenolepis diminuta* and some other trematodes (*Fasciola hepatica* and *Paragonimus westermani*), but it is missing from other cestodes characterized to date (i.e. *Taenia solium*, *T. asiatica*, *Echinococcus granulosus* and *E. multilocularis*) and from all 5 schistosome species published thus far (see details in Littlewood *et al.* 2006). The feature of *trnC* lacking a DHU arm, which is shared among some of cyclophyllidean cestodes and trematode groups, is likely to reflect an independent, homoplastic trait as it is absent from *D. latum* and *H. diminuta* (see Fig. 3). Based on sequence comparison with those of other neodermatan groups, 2 rRNA genes were identified: *rrnL* (968 bp) and *rrnS* (740 bp) are separated by the *trnC* (Table 1), and this gene arrangement is common to all other cestode and trematode mtDNAs reported thus far, but this is not the case for some monogenean mitochondrial genomes (Park *et al.*, 2007).

Non-coding regions

Eighteen intergenic sequence regions, representing a total length of 484 bp and varying from 1 to 222 bp, were detected in the mtDNA genome of *D. latum*. Of these, 2 non-coding regions (NR1 and NR2) were particularly prominent. Located between *trnY* and *trnL1* (222 bp for NR1) and between *nad5* and *trnG* (187 bp for NR2), respectively, these non-coding regions are still relatively small compared with those in trematodes (e.g. Littlewood *et al.* 2006). In some schistosomes, however, the long non-coding region (conventionally called 'LNR') is A+T-rich and generally known to range in size up to 5–7 kb, showing the considerable length variation among strains (Le *et al.* 2001). The A+T contents of the NR1 and NR2 of *D. latum* mtDNA are 77·0% (35·1% for A, 41·9% for T, 11·7% for G, and 11·3% for C) and 71·1% (26·2% for A, 44·9% for T, 19·3% for G, and 9·6% for C), respectively; the value for the NR1 is considerably higher than the average of the entire sequence (A+T content of 68·2%). Hairpin-like secondary structures of these non-coding regions were predicted. The secondary structures of 2 non-coding regions were inferred to contain 2 (NR1) and 4 (NR2) stem-loop structures, respectively (Fig. 4A and B). A hairpin-like stem-loop secondary structure is often found in the non-coding regions of cestode

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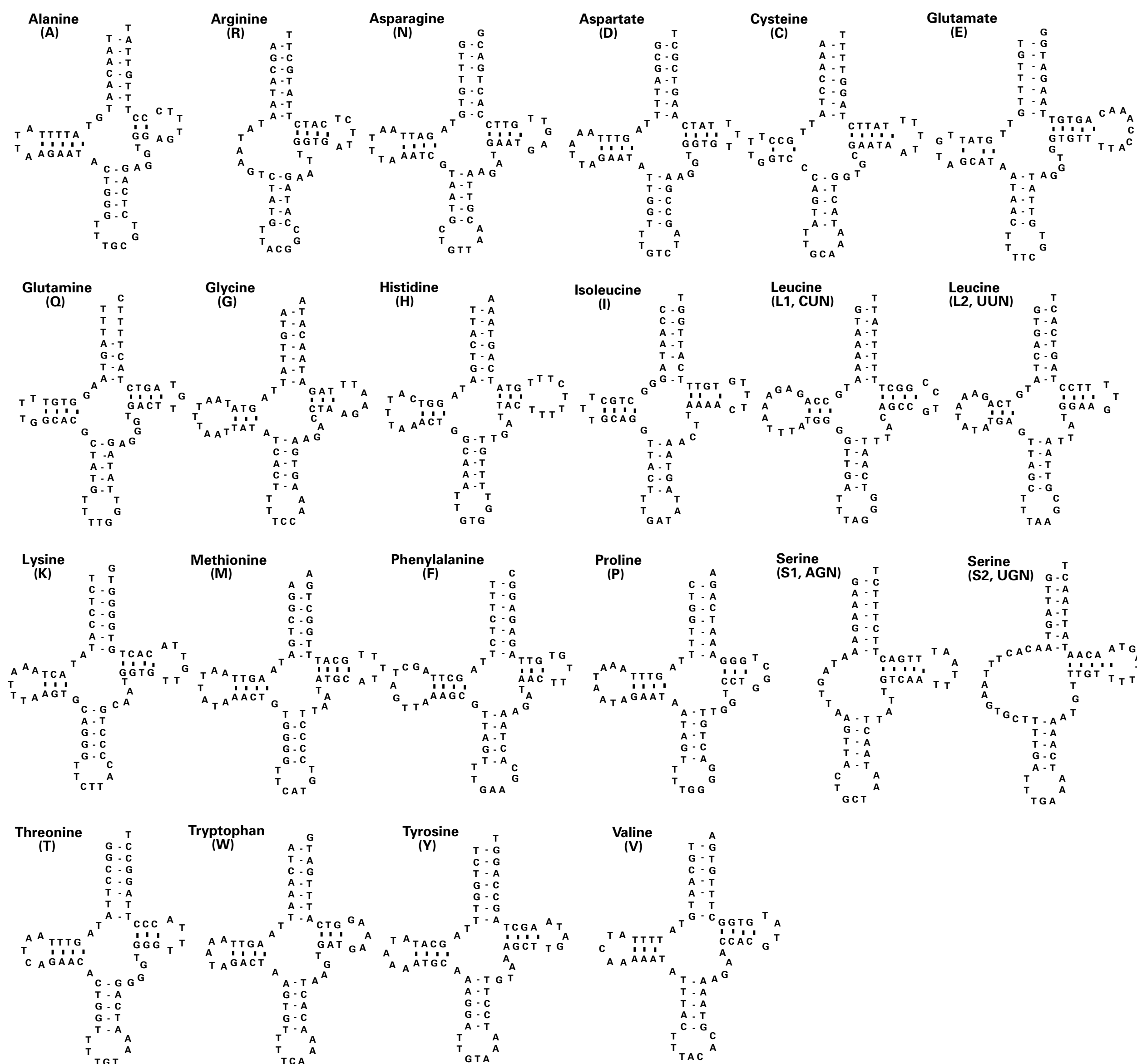

Fig. 2. Predicted secondary structures of the 22 mitochondrial transfer RNAs of *Diphyllobothrium latum*.

mtDNAs, but its functional role is not yet clear (*cf.* von Nickisch-Rosenegk *et al.* 2001; Littlewood *et al.* 2006).

Mitochondrial molecular phylogeny of eucestodes

To assess the phylogenetic position of *D. latum* and the utility of mt genomes in resolving the interrelationships of cestode orders, an analysis of amino acid sequence data representing all 12 mitochondrial protein-coding gene loci for 9 selected flatworm species (including *D. latum*) was performed. A concatenated alignment set of 2980 homologous amino acid positions from conserved blocks was used. Based on maximum likelihood mapping analysis, more than 99·9% of all random samples of the quartet (33·3%, 28·6% and 38·0% in each trapezoid) were fully resolved. Of the 2980 homologous positions, 1280 variable sites were phylogenetically informative under the MP criterion. The exhaustive search option of the MP method yielded a single tree (length = 5024 steps, CI = 0·850, RI = 0·645; Fig. 3). Phylogenetic relationships among the eucestodes using different analytical approaches (MP, Bayesian, NJ, and ML methods) are the same in their topology (see Fig. 3). Phylogenetic relationships among species are well resolved with maximal nodal support throughout. Monophyly of *Taenia*, *Echinococcus* (the family Taeniidae and the order Cyclophyllidea) is well supported. *Hymenolepis diminuta*, a member of the family Hymenolepididae was resolved as a sister taxon to the Taeniidae. This mitogenomic prediction is concordant with morphology-based phylogenetic hypothesis (Brooks *et al.* 1991; Hoberg *et al.* 1997, 1999) and also with molecular estimates of eucestode phylogeny based on nuclear rDNA sequences (Mariaux, 1998; Olson *et al.* 2001). This information suggests that mitogenomic data sets provide a useful

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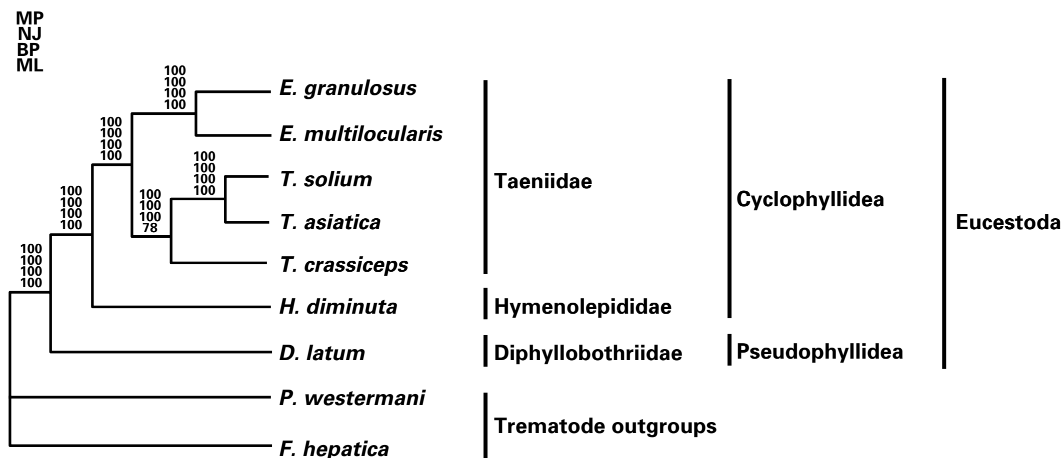

Fig. 3. Phylogenetic relationships among eucestode species based on inferred amino acid sequence data selected from 12 mitochondrial protein-coding gene loci for 9 flatworm species. The topology of the trees constructed using different analytic approaches (MP, NJ, Bayesian, and ML methods) was the same. The numbers above the branches represent bootstrap percentages for maximum parsimony (MP), neighbour joining (NJ), posterior probability values for Bayesian phylogeny (BP), and the quartet puzzling supporting values for maximum likelihood (ML), respectively.

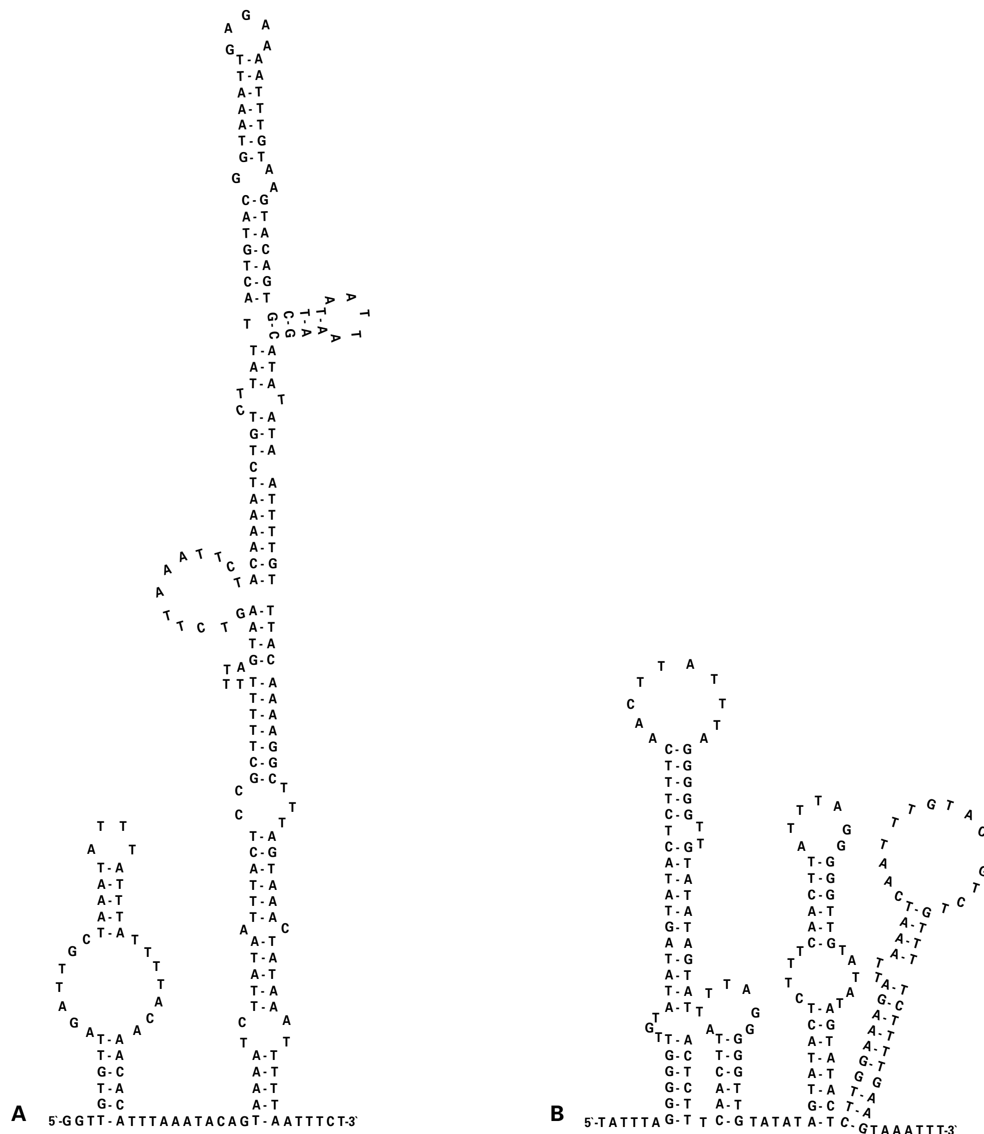

Fig. 4. Secondary structures predicted for the non-coding regions identified in the mtDNA of *Diphyllobothrium latum*. The two non-coding regions NR1 (A) and NR2 (B) are located between *trnY* and *trnL1* and between *nad5* and *trnG*, respectively.

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means of resolving cestode interrelationships. The traditional works for cestode classification have recognized dichotomous grouping within the class Cestodaria, represented by the monozoic orders Gyrocotylidea and Amphilinidea, and Eucestoda (Cestoidea *sensu* Ehlers, 1985), comprising all other cestode groups. Although there is little doubt about the monophyly of Eucestoda, its internal phylogeny, particularly phylogenetic relationships among major ordinal groups have not yet been fully resolved (Mariaux, 1996; Hoberg *et al*. 1997; Littlewood *et al*. 2001). In the present study, each of the genes is readily alignable, and there is considerable sequence variation between *Diphyllobothrium* and the cyclophyllidean mt genes available for phylogenetic analysis (with over 40% of the alignable sites being phylogenetically informative under parsimony). A recent study (Hardman and Hardman, 2006) suggests that most of the amino acid sequences inferred from neodermatan mt genes performed well in resolving relationships among taxa for which mt genomes were available, with *nad2* being most useful and *nad5* being of least value. These authors concluded that a minimum of 4 kb of sequence (whether concatenated, or 40 samples of randomly selected 100 bp fragments) was needed to resolve expected nodes. Thus, further mitochondrial genome information from a broad range of major eucestode groups, including primitive orders (e.g. Caryophyllidea and Spathebothriidea) will likely provide a deeper insight into outstanding issues concerning cestode evolution, such as the origins and radiation of scolex morphology, patterns of strobilation and proglottization (e.g. see Olson *et al*. 2001). Considering the relative ease with which entire genomes can now be characterized, we suggest sequencing additional exemplar taxa from other cestode orders, in particular the basal lineages, in order to select optimum gene regions and develop PCR-based protocols for mitochondrial DNA-based estimates of cestode phylogeny.

As revealed in earlier mitochondrial genome surveys (von Nickisch-Rosenegk *et al*. 2001; Le *et al*. 2002*b*; Nakao *et al*. 2002; Jeon *et al*. 2005), the gene arrangement of cestode mtDNAs is conserved, based on current information, and the pseudophyllidean *D. latum* mtDNA displays the same arrangement. The only exception is *Hymenolepis diminuta* for which the relative position of 2 tRNA genes (*trnS2-trnL1*) is switched, compared with those reported in all other eucestode studied to date. It is also anticipated that further mitochondrial genome surveys will improve our understanding of mitochondrial genome evolution, particularly of gene arrangements within the context of the phylogeny of major eucestode groups. Additional mitochondrial genome sequences for species of *Diphyllobothrium* will allow the identification of conserved gene regions for the design of PCR primers flanking regions of high variability for species and/or 'strain' identification. Of particular interest will be genes and non-coding regions offering higher rates of mutation for investigating population variation.

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