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Genetic characterization of the mitochondrial DNA from *Lepeophtheirus salmonis* (Crustacea; Copepoda). A new gene organization revealed

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Abstract

The mitochondrial DNA (mtDNA) from the salmon louse, *Lepeophtheirus salmonis*, is 15445 bp. It includes the genes coding for cytochrome *B* (Cyt *B*), ATPase subunit 6 and 8 (*A6* and *A8*), NADH dehydrogenase subunits 1–6 and 4L (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5* and *ND6*), cytochrome *c* oxidase subunits I–III (*COI*, *COII* and *COIII*), two rRNA genes (12S rRNA and 16S rRNA) and 22 tRNAs. Two copies of tRNA-Lys are present in the mtDNA of *L. salmonis*, while tRNA-Cys was not identified. Both DNA strands contain coding regions in the salmon louse, in contrast to the other copepod characterized *Tigriopus japonicus*, but only a few genes overlap. In vertebrates, *ND4* and *ND4L* are transcribed as one bicistronic mRNA, and are therefore localized together. The same organization is also found in crustaceans, with the exceptions of *T. japonicus, Neocalanus cristatus* and *L. salmonis* that deviate from this pattern. Another exception of the *L. salmonis* mtDNA is that *A6* and *A8* do not overlap, but are separated by several genes. The protein-coding genes have a bias towards AT-rich codons.

The mitochondrial gene order in *L. salmonis* differs significantly from the copepods *T. japonicus*, *Eucalanus bungii, N. cristatus* and the other 13 crustaceans previously characterized. Furthermore, the mitochondrial rRNA genes are encoded on opposite strands in *L. salmonis*. This has not been found in any other arthropods, but has been reported in two starfish species. In a phylogenetic analysis, using an alignment of mitochondrial protein sequences, *L. salmonis* groups together with *T. japonicus*, being distant relatives to the other crustaceans.

Keywords: *Lepeophtheirus salmonis*; Salmon louse; Copepoda; Crustacea; Mitochondrial DNA; Gene order

1. Introduction

The mitochondrial genome is a circular, double-stranded DNA molecule (mtDNA). In animals, the gene content is very conserved with two rRNA genes, 22 tRNA genes and 13 protein-coding genes in addition to a non-coding control region (D-loop) (Wolstenholme, 1992b). As opposed to genomic DNA the intergenic regions are very short, and the genes often overlap. These overlaps include genes encoded on opposite DNA strands, as well as genes on the same strand (Wolstenholme, 1992b). Within the metazoans the mitochondrial genomes range in size from 14–42 kb (Crease, 1999). This variation in size can to some extent be due to differences in gene length, but in most cases it is a result of size difference in the D-loop (Wolstenholme, 1992b). In the crustaceans, completely characterized so far, the mitochondrial genome size varies from 14.6 to 16.2 kb.

Mitochondrial DNA has traditionally been used in genetic studies at the population level. However, during the last decade the use has been extended to include studies of phylogeny between closely and more distantly related taxa, and patterns of gene flow (e.g. Burton and Lee, 1994; Bucklin et al., 1996; Bucklin and Wiebe, 1998; Wilson et al., 2000; Yamauchi et al., 2002; Ketmaier et al., 2003; Munasinghe et al., 2003; Sacherova and Hebert, 2003).

Abbreviations: *A6*, ATPase subunit 6; *A8*, ATPase subunit 8; *COI–III*, cytochrome *c* oxidase subunits I–III; Cyt *B*, Cytochrome *B*; *ND1–6* and *4L*, NADH dehydrogenase subunit *1–6* and *4L*; AT/D-loop, AT-rich control region; rRNA, ribosomal RNA; tRNA, transfer RNA.

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Since intermolecular recombination is unlikely in the mitochondrial genome, due to maternal inheritance, the gene order itself can also be used to infer phylogenetic relationships (Boore et al., 1998; Hickerson and Cunningham, 2000; Wilson et al., 2000).

So far, the complete mitochondrial genome has been sequenced from 14 crustacean species; four branchiopods [*Daphnia pulex* (Crease, 1999), *Artemia franciscana* (Valverde et al., 1994), *Triops cancriformis* (Umetsu et al., 2002) and *Triops longicaudatus* (NC_006079)], six decapods [*Pagurus longicarpus* (Hickerson and Cunningham, 2000), *Penaeus monodon* (Wilson et al., 2000), *Panulirus japonicus* (Yamauchi et al., 2002), *Portunus trituberculatus* (Yamauchi et al., 2003), *Cherax destructor* (Miller et al., 2004) and *Callinectes sapidus* (Place et al., unpublished)], one stomatopod [*Squilla mantis* (NC_006081)], one cirriped [*Tetraclita japonica* (Begum et al., unpublished)], one ostracod [*Vargula hilgendorfii* (Ogoh and Ohmiya, 2004)] and one copepod [*Tigriopus japonicus* (Machida et al., 2002)]. In addition, the two copepods *Eucalanus bungii* and *Neocalanus cristatus* have been partly characterized (Machida et al., 2004). Between these crustacean groups there are large variation in genetic organization. *P. longicarpus, T. japonicus, V. hilgendorfii* and *C. destructor* have completely different mitochondrial gene orders (Hickerson and Cunningham, 2000; Machida et al., 2002; Ogoh and Ohmiya, 2004). This is also the case for *E. bungii* and *N. cristatus* (Machida et al., 2004). The other ten characterized crustaceans reveal only minor deviations from the insect, *Drosophila yakuba* (Clary and Wolstenholme, 1985). The general assumption is nevertheless, that gene arrangements are quite conserved in phylogenetically related organisms, and variable between distantly related species (Wolstenholme, 1992a; Boore, 1999; Gissi et al., 2004). tRNA rearrangements are most frequently observed, and it has been suggested that tRNA genes can be regarded as mobile elements important in gene shuffling (Saccone et al., 2002).

The salmon louse, *Lepeophtheirus salmonis* (Copepoda; Siphonostomatoida, Caligidae), is a major pathogen on farmed Atlantic salmon (*Salmo salar*) resulting in large economical losses for the farming industry (Mustafa et al., 2001). Despite this, very little is known about the population structure of this parasite (Tully and Nolan, 2002), and so far no genetic markers suitable for population studies have been detected. This paper presents the characterized mitochondrial genome from *L. salmonis*, and the organization of the mitochondrial genes is described. The phylogenetic position of *L. salmonis* within Crustacea is also analyzed based on a selection of mitochondrial protein-coding genes.

2. Materials and methods

2.1. DNA purification and partial mtDNA amplification

The gut from the adult salmon lice (preserved in 70% ethanol) was removed before DNA was extracted, followed by RNase treatment, using the DNeasy DNA Tissue kit (Qiagen). Primers for amplification of partial mtDNA sequences for Cyt *B* and 16S rRNA were designed based on alignment of the crustacean mtDNA sequences available at the time (*Acartia clausi* (AF295332), nine *Calanus species* (AF295334, AF226668, AF227974, AF227975, AF227971, AF295335, AF295333, AF293441 and AF227970), three *Metridia species* (AF227973, AF293440 and AF227972), *Nannocalanus minor* Type I and II (AF293884 and AF293885)). To distinguish between mtDNA from the host and the salmon louse, the 3′-end of the primers were different from the *S. salar* sequence (AF133701). PCR was performed on 300 ng DNA in 50 μl reaction mixture containing 10 × PCR buffer including 1.5 mM MgCl2, 25 mM of each dNTP (Promega), 0.2 μM of each primer (Table 1) (Invitrogen) and 1U Taq DNA polymerase (Amersham Pharmacia Biotech Inc.). Amplification was carried out in a Mastercycler gradient (Eppendorf) at 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min; 72 °C for 7 min.

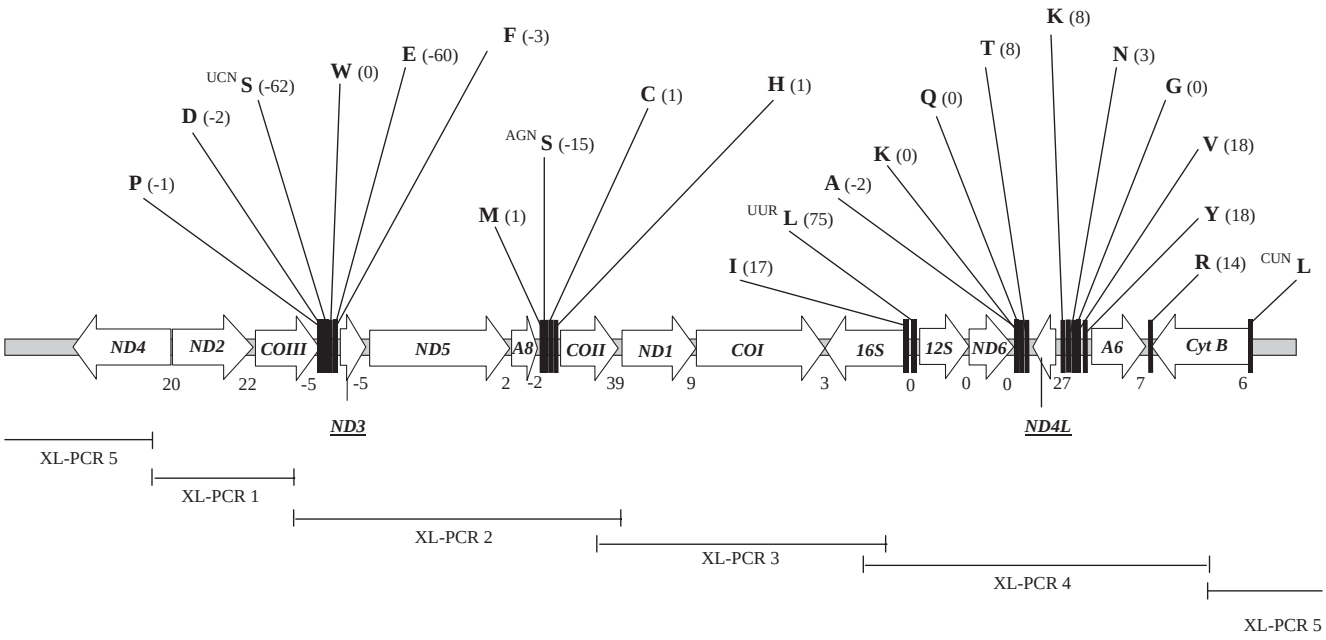
In addition, ESTs encoding *COI*, *COII*, *COIII*, *ND1* and *ND4* were obtained from a cDNA library constructed from an adult female *L. salmonis* (Kvamme et al., 2004).

2.2. XL-PCR

Primers were designed for XL-PCR (Table 1) within the partial mtDNA sequences (see Section 2.1), and the mtDNA was amplified in five fragments (Fig. 1). The PCR reaction mixtures (50 μl) contained 10 × ThermalAce PCR buffer (Invitrogen), 50 × dNTP mix (Invitrogen), 0.2 μM of each primers, 5 × Q-solution (Qiagen), 3U ThermalAce DNA

Table 1
Primers for amplification of the mitochondrial DNA from *L. salmonis*

Primers	Forward (5′→3′)	Reverse (5′→3′)
16S rRNA	AGGTAGCATARTAATTWGTT	TAATTCAACATCGAGGTCAC
Cyt *B*	ATTTTAACTGGATTATTTTTAGCT	AGCAAATAAAAARTATCATTC
XL-PCR 1	GAGATTGGAAGTAGGAAAAGGAG	AAGTGCCCTCAACCGAAACATC
XL-PCR 2	GATGTTTCGGTTGAGGGCACTT	CCTTTACGTGCTTGAGAGTAACC
XL-PCR 3	GTTTATGCCTATTTTACTAGAGATAG	GTAAACCTGTATGAAGCTAGCAT
XL-PCR 4	CATGGTAAAGGTCTTAGGGTCTT	TATCCGTAGACCATATCATGCGG
XL-PCR 5	CCGCATGATATGGTCTACGGATA	TATGGGGTCTATAATCCTGGCAG



Lepeophtheirus salmonis mitochondrial DNA (15 445 bp)

Fig. 1. Genetic organization of the mitochondrial DNA from *L. salmonis*. All tRNAs are indicated by the amino acid they encode. Numbers correspond to the nucleotides separating adjacent genes, negative numbers indicating overlap. The amplification strategy is shown below.

polymerase (Invitrogen) and 500 ng total DNA. Amplification was performed in a Mastercycler gradient (Eppendorf) using the cycling program: 95 °C for 5 min; 15 cycles of 95 °C for 30 s, 48 °C for 30 s, 68 °C for 1 min/kb; 25 cycles of 95 °C for 30 s, 48 °C for 30 s, 68 °C for 1 min/kb with 15 s auto extension; 72 °C for 7 min.

2.3. Sequencing, cloning and sequence analysis

PCR products were purified using "QIAquick PCR purification kit" (Qiagen) before sequencing by primer walking with ABI PRISM BigDye terminator chemistry, version 2 (Applied Biosystems). XL-PCR products, that were difficult to sequence directly, were cloned into the pCR®4-TOPO vector following the procedure described in the "TOPO TA Cloning® Kit for Sequencing" (Invitrogen). Plasmids were purified using the "Wizard® Plus Minipreps DNA Purification System" (Promega) followed by sequencing of a minimum of three clones.

All the mtDNA sequences were edited and analyzed using the Vector NTI Suite 7.0 software package (InforMax Inc.). Protein-coding genes were localized by an open reading frame scan, and then identified by BLAST search followed by both DNA and protein alignment of other known arthropod mtDNA sequences [*D. pulex* (NC_000844), *A. franciscana* (NC_001620), *T. cancriformis* (NC_004465), *T. longicaudatus* (NC_006079), *P. longicarpus* (AF150756), *P. monodon* (NC_002184), *P. japonicus* (NC_004251), *P. trituberculatus* (NC_005037), *C. destructor* (AY383557), *C. sapidus* (AY363392), *S. mantis* (NC_006081), *T. japonica* (NC_008974), *V. hilgendorfii* (NC_005306), *T. japonicus* (AB060648), *E. bungii* (AB091772), *N. cristatus* (AB091773) and *D. yakuba* (X03240)]. Putative initiation and termination codon for the protein-coding genes were predicted, and compared to other crustacean mitochondrial encoded proteins. Codon usage was predicted from amino acid sequence alignments of different mitochondrial protein-coding genes from various arthropods, and tRNA anticodons. Moreover, the frequency of each codon was determined by the "Count-codon output" program (http://www.kazusa.or.jp/codon/countcodon.html) by analyzing a DNA sequence consisting of all 13 protein-coding genes of *L. salmonis*. The overall A+T content was determined in GeneDoc (Nicholas et al., 1997). Transmembrane regions were identified using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html).

2.4. Identification of rRNA and tRNA genes

The *L. salmonis* 16S rRNA and 12S rRNA were inferred by Blast search followed by alignment with other arthropod sequences, and by comparison to the secondary structures accessible on the Comparative RNA website (http://www.rna.icmb.utexas.edu/) (Cannone et al., 2002) and the rRNA Web Server (http://www.psb.ugent.be/rRNA/) (Wuyts et al., 2001). The numbering of helices in the 12S and 16S rRNA structures corresponds to the helix annotations of Cannone et al. (2002) and Wuyts et al. (2001), respectively.

A preliminary screening for tRNA genes was done using the tRNAscan-SE 1.21 program (Lowe and Eddy, 1997). The same parameters as described by Hickerson and Cunningham (2000) were used, except for a cut-off score of four in the present study. Five tRNA genes were

identified manually based on their anticodons, and conserved secondary structure motifs.

2.5. Phylogeny

In the present study concatenated amino acid sequences including Cyt *B*, *COI*, *COII*, *ND1*, *ND5*, *ND6*, *A6*, *COIII*, *ND2*, *ND4*, *ND3* and *ND4L* have been chosen for phylogenetic analysis. The longest contains 12 proteins, and the length of the alignment is 3020 amino acids after the deletion of variable and gapped positions. *A8* was excluded from the analysis. We have also chosen one shorter concatenated amino acid sequence, for phylogenetic analysis, based on studies of the phylogenetic performance of mitochondrial protein-coding genes in resolving relationships among vertebrates (Zardoya and Meyer, 1996). This consists of the five proteins (*ND4*, *ND5*, *ND2*, Cyt *B*, and *COI*, 1950 amino acids), which are rated as good performers in phylogenetic analysis. The chosen amino acid sequence from *L. salmonis* was compared with homologous sequences from other crustaceans [*D. pulex* (NC_000844), *A. franciscana* (NC_001620), *T. cancriformis* (NC_004465), *T. longicaudatus* (NC_006079), *P. longicarpus* (AF150756), *P. monodon* (NC_002184), *P. japonicus* (NC_004251), *P. trituberculatus* (NC_005037), *C. destructor* (AY383557), *C. sapidus* (AY363392), *S. mantis* (NC_006081), *T. japonica* (NC_008974), *V. hilgendorfii* (NC_005306) and *T. japonicus* (AB060648)] and two insects, *D. yakuba* (X03240) and *Locusta migratoria* (X80245). *Ixodes persulcatus* (AB073725) and *Ornithodoros moubata* (AB073679) were chosen as outgroups. In addition to the concatenated amino acid sequences, the amino acid sequences of *COI* were used in a separate phylogenetic comparison. In these analyses two additional copepods, *E. bungii* (AB091722) and *N. cristatus* (AB091773), were added.

Multiple alignments of the protein sequences were made using the Vector NTI Suite software package (InforMax Inc.), and the alignments were manually adjusted by excluding the most variable/gapped positions using GeneDoc (Nicholas et al., 1997). Phylogenetic analysis was then performed using TREE-PUZZLE 5.0 with default settings, and the mtREV24 matrix derived from a vertebrate dataset (http://www.tree-puzzle.de) (Strimmer and Haeseler von, 1996). Gamma distribution rate was used as a model of rate heterogeneity. The gamma distribution parameter alpha was estimated from the data set, and the number of rate categories was eight. TREE-PUZZLE is recommended for analysis of amino acid sequences (Hall and Massachusetts, 2001).

3. Results

3.1. Genome size, content and organization

The mitochondrial genome of the salmon louse is 15 445 bp (Accession number AY625897). Thirteen protein-coding genes (Cyt *B*, *A6*, *A8*, *ND1–6*, *ND4L*, *COI–COIII*), 22 tRNAs and two rRNA genes (12S rRNA and 16S rRNA), in addition to a control region (D-loop) have been characterized (Fig. 1). The gene for tRNA-Cys has, however, not been identified. The mitochondrial gene content is conserved across the different animal phyla, but the gene order may differ strongly between distantly related groups. We have compared the organization of the mitochondrial genome of *L. salmonis* to other crustacean species (Fig. 2). The arrangement of the protein-coding genes is different from any pattern previously observed. However, the gene orders within a few short segments (A, B, C and D) in the *L. salmonis* mtDNA are similar to that observed in other crustaceans (Fig. 2). Segment A (*COIII*, *ND3* and *ND5*) has a similar organization as several other crustaceans (*D. pulex*, *A. franciscana*, *T. cancriformis, T. longicaudatus, P. monodon*, *P. japonicus, P. trituberculatus*, *C. destructor, C. sapidus, S. mantis* and *T. japonica*) and the insect *D. yakuba*. The genes in segment B (*COII* and *ND1*) are also present in *T. japonicus*, but in reverse order compared to *L. salmonis*. Segment C (*ND6* and *ND4L*), which is present in several crustaceans, has the same gene order in *T. japonicus* and *L. salmonis*, but is reversed compared to *D. pulex*, *A. franciscana*, *T. cancriformis, T. longicaudatus, P. monodon*, *P. japonicus, P. trituberculatus*, *C. destructor, C. sapidus, S. mantis* and *T. japonica, V. hilgendorfii, P. longicarpus* and *D. yakuba. ND4* and *ND2*, which constitute segment D, are present in both *L. salmonis* and *C. destructor*. However, the genes are reversed in *C. destructor* compared to *L. salmonis* (Fig. 2).

Eighteen of the 22 tRNA genes in *L. salmonis* are arranged in four main clusters (PDSWEF, MSH, AKQT and KNGVY) (Fig. 1). There are few similarities between the organizations of tRNA genes in *L. salmonis* compared to that observed in other crustaceans.

In *L. salmonis* the mtDNA genes are encoded on the same strand except for Cyt *B*, *ND4*, *ND4L*, 16S rRNA, tRNA-Ser, tRNA-Leu, tRNA-Thr, tRNA-Ile and tRNA-Glu. Most of the genes do not overlap, and the number of nucleotides separating the genes is 1–45 bp. An extreme case is, however, tRNA-Ser (UCN)/tRNA-Trp and tRNA-Phe/tRNA-Glu where almost a complete overlap exists due to coding on opposite strands. Furthermore, *COIII* overlaps tRNA-Pro by five nucleotides, *A8* overlaps tRNA-Met by two nucleotides and *ND3* overlaps *ND5* by five nucleotides and tRNA-Phe by three nucleotides. All these overlapping genes are coded on the same strand.

3.2. Base composition and codon usage

The overall A+T content of the mtDNA sequence of *L. salmonis* is 0.65. However, if only the protein-coding genes are considered the A+T content is 0.63, almost the same as in *A. fransiscana* (0.64) but higher than *T. japonicus* (0.59). The two rRNA genes of *L. salmonis* have on the other hand the highest number of A and T nucleotides (0.71).

Daphnia pulex, Artemia fransiscana, Triops cancriformis, Triops langicaudatus, Penaeus monodon, Panulirus japonicus, Portunus trituberculatus, Callinectes sapidus, Squilla mantis, Tetraclita janponica and Drosophila yakuba (15-16.2 kb)

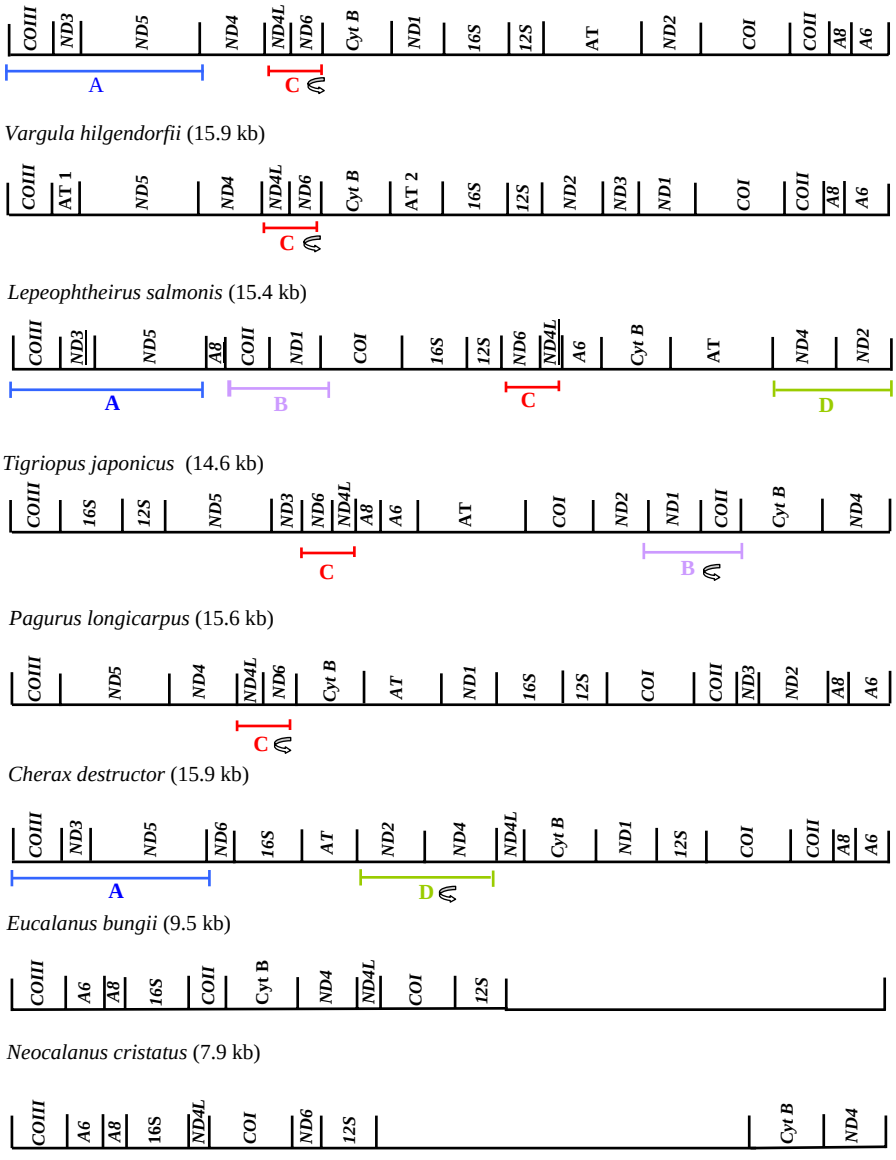


Fig. 2. The genetic organization of the mitochondrial genome from *L. salmonis* compared to other known crustaceans, and *D. yakuba*. AT denotes the control region (D-loop). Genes underlined are uncertain due to low BLAST scores. The tRNA genes are not included, while *E. bungii* and *N. cristatus* are only partly characterized.

L. salmonis use the same mitochondrial genetic code as other arthropods, but the codon frequencies vary (data not shown). The most frequent amino acid in *L. salmonis* is leucine (Leu) followed by phenylalanine (Phe) and valine (Val), while arginine (Arg) is the least frequent (data not shown). If we calculate the ratio of GC/AT occurring in both the first and second codon position, excluding stop codons, *L. salmonis* has a ratio of 0.45. When synonymous codons are compared the AT-rich codons, within each codon family, are most frequently used in translation of the mitochondrial proteins of *L. salmonis* (data not shown). Altogether this implies that *L. salmonis* has a bias towards AT-rich codons in the 13 protein-coding genes identified.

3.3. Protein-coding genes in *L. salmonis* mtDNA

Five putative initiation codons have been identified in *L. salmonis* mtDNA, while TAA and TAG are the only stop codons (Table 2). Ten out of the 13 presumptive protein-coding genes terminate with TAA, while three genes use TAG as a stop codon (Table 2). The lengths of the putative

Table 2
Size comparison of the putative mitochondrial protein-coding genes from *L. salmonis* with other completely characterized crustaceans, and *D. yakuba*

Protein	*L. salmonis*		Number of amino acids															
	Start codons	Stop codons	Ls	Tj	Dp	Af	Tc	Pl	Pm	Pj	Pt	Vh	Cd	Tej	Tl	Sm	Cs	Dy
COI	ATA	TAA	512	509	512	512	513	513	512	511	511	517	511	514	513	512	511	512
COII	ATG	TAA	229	227	226	228	226	229	229	229	228	234	231	227	226	235	228	227
COIII	ATG	TAG	274	265	262	257	262	263	263	263	263	260	262	262	262	262	263	262
Cyt *B*	ATG	TAA	390	376	377	381	378	378	378	378	378	378	378	379	377	378	378	378
A6	ATG	TAA	217	228	224	219	223	224	224	225	225	216	224	221	226	225	224	224
A8[a]	GTT	TAA	31	32	53	52	49	52	52	52	53	50	52	52	49	50	53	53
ND1	ATC	TAA	295	306	311	298	305	310	312	314	318	319	304	306	305	313	312	324
ND2	ATT	TAA	309	321	329	298	333	336	333	333	335	316	334	332	332	314	335	341
ND3[a]	ATA	TAA	118	126	117	111	116	117	117	117	117	116	117	117	114	117	117	117
ND4	ATT	TAG	431	432	440	386	439	446	446	446	444	430	446	443	448	446	444	446
ND4L[a]	ATG	TAG	110	91	91	85	105	93	99	100	100	96	99	96	105	99	100	96
ND5	ATT	TAA	559	580	569	541	575	570	574	576	575	544	575	567	568	570	575	572
ND6	ATT	TAG	150	151	170	219	166	167	173	171	168	149	159	161	165	168	168	174

Ls: *Lepeophtheirus salmonis*, Tj: *Tigriopus japonicus*, Dp: *Daphnia pulex*, Af: *Artemia fransiscana*, Tc: *Triops cancriformis*, Pl: *Pagurus longicarpus*, Pm: *Penaeus monodon*, Pj: *Panulirus japonicus*, Pt: *Portunus trituberculatus*, Vh: *Vargula hilgendorfii*, Cd: *Cherax destructor*, Tej: *Tetraclita japonica*, Tl: *Triops longicaudatus*, Sm: *Squilla mantis*, Cs: *Callinectes sapidus*, Dy: *Drosophila yakuba*.

[a] The exact location of these genes is uncertain due to low score by BLAST search.

proteins in *L. salmonis* are similar to other crustaceans (Table 2).

Blast search with two ORFs resulted in weak similarities with *ND3* and *ND4L* in GenBank (not significant *e*-values). Thus, the putative protein sequences were compared to *ND3* and *ND4L* from other crustaceans, and *D. yakuba* (Table 3). Several conserved stretches were seen within *ND3*, while *ND4L* had fewer invariable sites in its protein sequence. Based on these comparisons it is suggested that these two ORFs encodes the proteins *ND3* and *ND4L*.

Table 3
Protein sequence comparisons (%) of *ND3* and *ND4L* from crustaceans, and *D. yakuba*

	Ls	Tj	Dp	Af	Tc	Pl	Pm	Pj	Pt	Vh	Cd	Tej	Tl	Sm	Cs	Dy
Ls	–	32	38	28	33	33	33	36	34	30	38	36	36	33	35	30
Tj	18	–	28	29	29	28	31	31	28	26	28	27	29	28	27	27
Dp	21	19	–	41	50	51	54	46	47	38	48	43	44	48	46	56
Af	20	22	43	–	46	41	42	45	45	37	44	41	47	41	45	47
Tc	18	17	41	32	–	54	58	56	55	43	52	42	70	53	55	54
Pl	18	23	52	36	47	–	75	70	69	45	64	48	50	69	71	58
Pm	20	22	53	32	45	63	–	67	70	48	69	51	55	75	68	66
Pj	18	24	48	37	53	60	59	–	68	46	63	52	55	62	70	59
Pt	18	25	40	29	44	57	56	51	–	47	67	48	50	65	89	56
Vh	17	22	34	28	32	37	35	39	33	–	44	39	41	48	46	41
Cd	17	22	47	33	42	54	54	54	48	32	–	49	52	63	64	57
Tej	15	17	35	28	34	43	49	38	37	30	37	–	42	48	49	46
Tl	20	18	40	30	70	45	43	49	40	29	38	34	–	52	53	52
Sm	21	15	46	29	46	55	64	49	50	31	46	45	47	–	67	59
Cs	18	25	40	31	47	55	55	52	88	35	47	38	41	50	–	58
Dy	20	21	45	38	43	46	49	50	39	37	47	46	40	49	38	–

ND3 similarity values are given above the diagonal, while *ND4L* values are below the diagonal.

Ls: *Lepeophtheirus salmonis*, Tj: *Tigriopus japonicus*, Dp: *Daphnia pulex*, Af: *Artemia fransiscana*, Tc: *Triops cancriformis*, Pl: *Pagurus longicarpus*, Pm: *Penaeus monodon*, Pj: *Panulirus japonicus*, Pt: *Portunus trituberculatus*, Vh: *Vargula hilgendorfii*, Cd: *Cherax destructor*, Tej: *Tetraclita japonica,* Tl: *Triops longicaudatus*, Sm: *Squilla mantis*, Cs: *Callinectes sapidus*, Dy: *Drosophila yakuba*.

No ORF indicated that *A6* and *A8* overlap in the *L. salmonis* mtDNA. Identification of a putative gene for *A8* was therefore achieved by translating all non-coding sequences, found in the *L. salmonis* mtDNA, in all six reading frame. Sequences consisting of 30–50 amino acids, which correspond to the length of *A8* (see Table 2), were compared to other crustacean *A8* sequences to identify conserved domains. This resulted in a candidate region between *ND5* and tRNA-Met containing 6 of 31 conserved residues. When a search for transmembrane regions was performed two possible transmembrane helices were also found within this sequence. Based on these analyses the region of 93 bp between *ND5* and tRNA-Met is suggested to code for the *A8* protein in *L. salmonis*.

3.4. rRNA genes

The two mitochondrial ribosomal genes, 12S and 16S rRNA, of *L. salmonis* are coded on opposite strands separated by tRNA-Leu and tRNA-Ile. This opposite transcriptional polarity has not been described earlier in arthropods.

Many of the distinct helices in domain I and III were relatively straightforward to recognize in *L. salmonis* 12S rRNA, despite low sequence similarity to other 12S rRNA genes. Helices H9 and H106 mark the gene boundaries resulting in the 12S rRNA 5′-end starting 76 nucleotides downstream of the tRNA-Leu, whereas the 3′-end extends up to the nucleotide adjacent to the *ND6*. Assuming these termini, the 12S rRNA of *L. salmonis* is 590 bp.

A number of conserved 16S rRNA sub-structures could be identified in *L. salmonis*, especially in the 3′ half of the molecule. Helices G1, G2, G16 and G17 in Domain V, and helix H2 in Domain VI were easily

recognized by sequence comparison with the *Drosophila melanogaster* 16S rRNA molecule (Cannone et al., 2002). However, the degree of sequence conservation in the 5′-end of 16S rRNA is extremely low when compared to other 16S rRNA genes, precluding the precise location of its 5′-end. Based on the secondary structure model, the presumptive region containing the 5′-end is likely localized between nucleotide 10 801 and 10 891. If we assume that the 16S rRNA gene occupies all of the available space between its adjacent genes

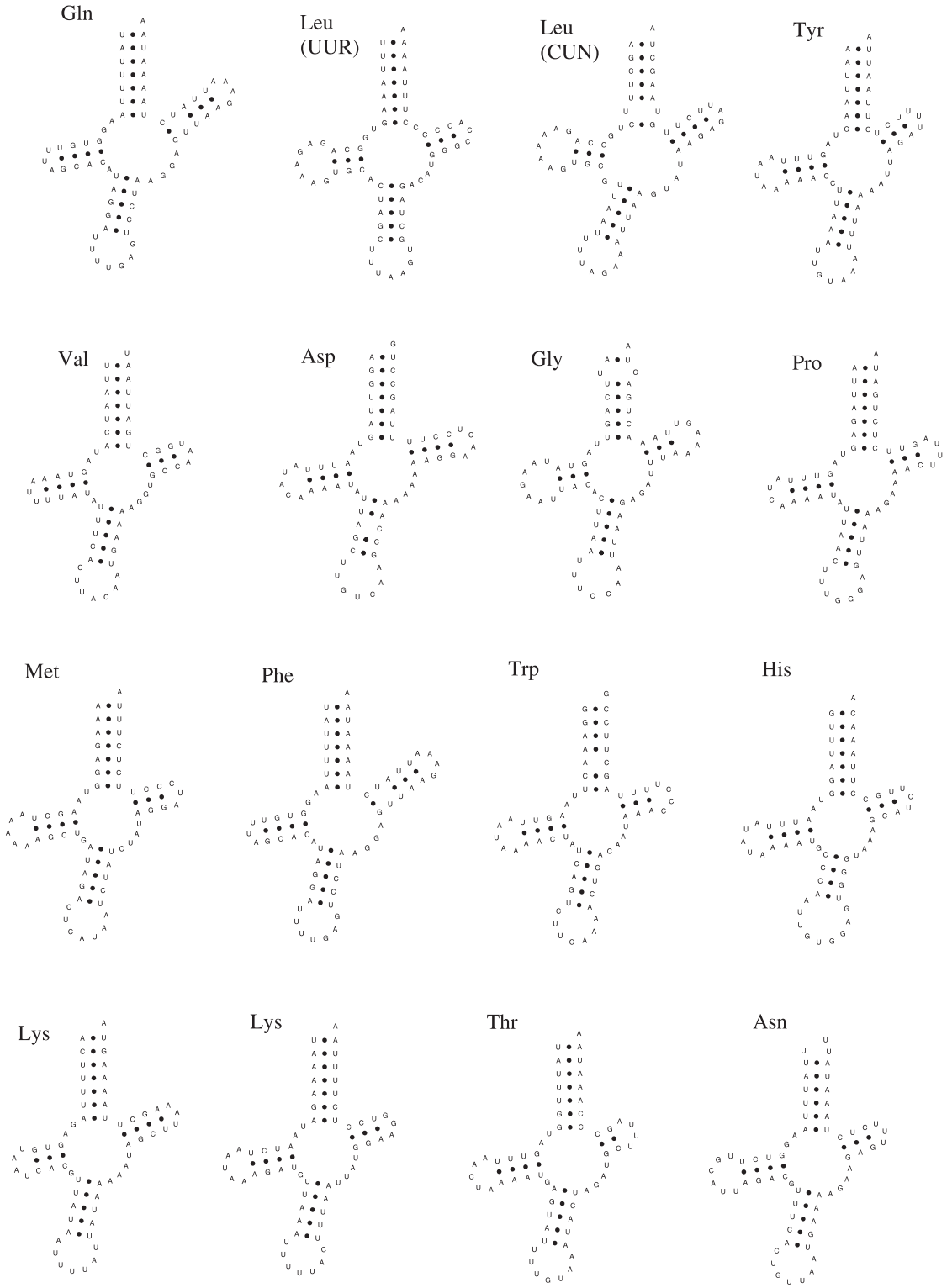


Fig. 3. Secondary structures of the 22 tRNAs found in the mitochondrial DNA of *L. salmonis*.

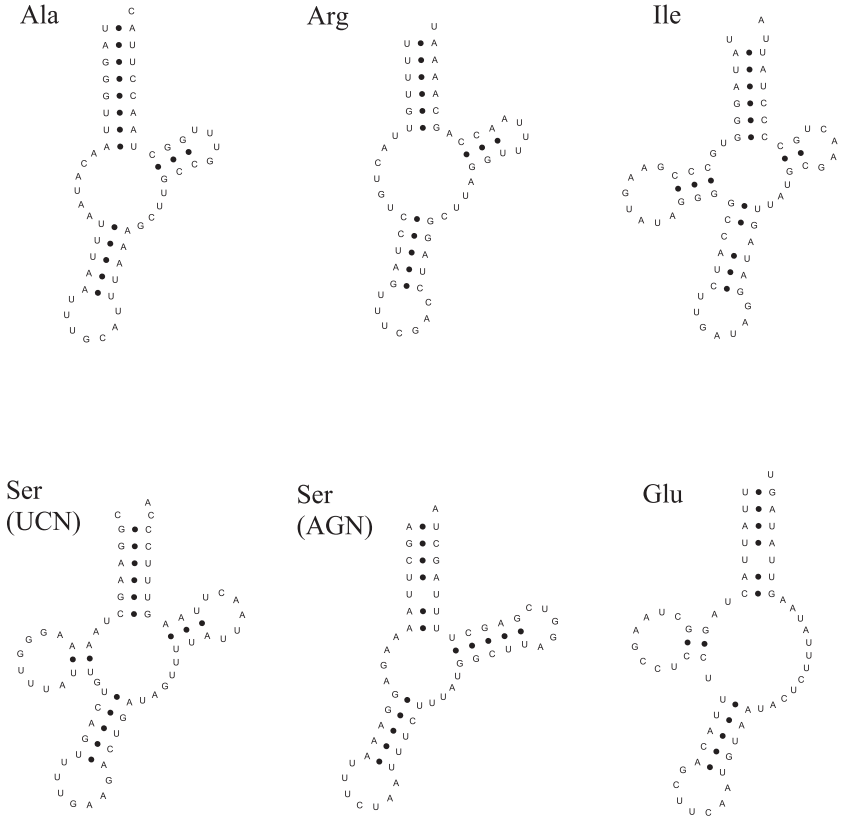


Fig. 3 (*continued*).

(*COI* and tRNA-Ile) the gene length can be estimated at 1009 bp.

3.5. tRNA genes

Seventeen tRNAs were identified by tRNAscan-SE 1.21, and their scores varied from 4.64 to 19.65 with tRNA-Asn having the lowest score. Five tRNA were identified manually based on anticodon sequences, and secondary structures. tRNA-Cys was, however, not identified in the mtDNA of *L. salmonis*. The tRNA genes were small with a size range of 44–64 bp. All anticodons were identical to those described for *D. yakuba* (Clary and Wolstenholme, 1985), except for tRNA-Lys which possesses the anticodon UUU instead of CUU (Fig. 3). Two copies of tRNA-Lys are present in the *L. salmonis* mtDNA, both with relatively high tRNA scan scores (11.62 and 13.15, respectively) (Fig. 1).

A common feature among the metazoans is that the mitochondrial tRNA structures can be very diverse (Wolstenholme, 1992b). In *L. salmonis* 18 of the 22 tRNAs had the characteristic cloverleaf structure (Fig. 3). However, the T-arm seem in most cases to consist of a base pairing between only three nucleotides instead of five, which is the general rule (Wolstenholme, 1992b). In contrast the D-arm do follow the typical base pairing rules by binding between 3–4 bases, except for tRNA-Ser (UCN), tRNA-Glu and some tRNAs (tRNA-Arg, tRNA-Ser (AGN) and tRNA-Ala) that had lost their D-arm completely (Fig. 3).

3.6. Phylogeny

Three phylogenies based on the putative amino acid sequence of a) twelve (Cyt *B, COI, COII, ND1, ND5, ND6, A6*, *COIII*, *ND2*, *ND4*, *ND3* and *ND4L*), b) five (Cyt *B, COI, ND5, ND2* and *ND4)* different protein-coding genes and c) the single protein, *COI,* have been constructed to find the phylogenetic position of *L. salmonis* in relation to a selection of other crustaceans, and two insects. The three analyses all gave similar phylogenies, but with slightly different QP bootstrap values (Fig. 4).

The analyses resulted in phylogenies where all the decapods and the stomatopod group together (QP bootstrap=92–100). Highest support value was obtained when only the five protein-coding genes were used (QP bootstrap=100). Only three (*D. pulex*, *T. cancriformis* and *T. longicaudatus*) of the four branchiopods group together (QP bootstrap=75–95), leaving *A. fransciscana* in an unresolved position. The copepods, *L. salmonis* and *T. japonicus*, group together (QP bootstrap=72–90). Furthermore, the phylogeny based on alignments of *COI* proteins do not resolve the relationships within the copepoda, i. e. *L. salmonis* and *T. japonicus* group together (QP bootstrap=90) while the positions of *E. bungii* and *N. cristatus* are uncertain in relation to the former. In the two phylogenies based on the concatenated amino acid sequences the two insects group together (QP bootstrap=90–96), but the phylogenetic analysis based on *COI* does not give

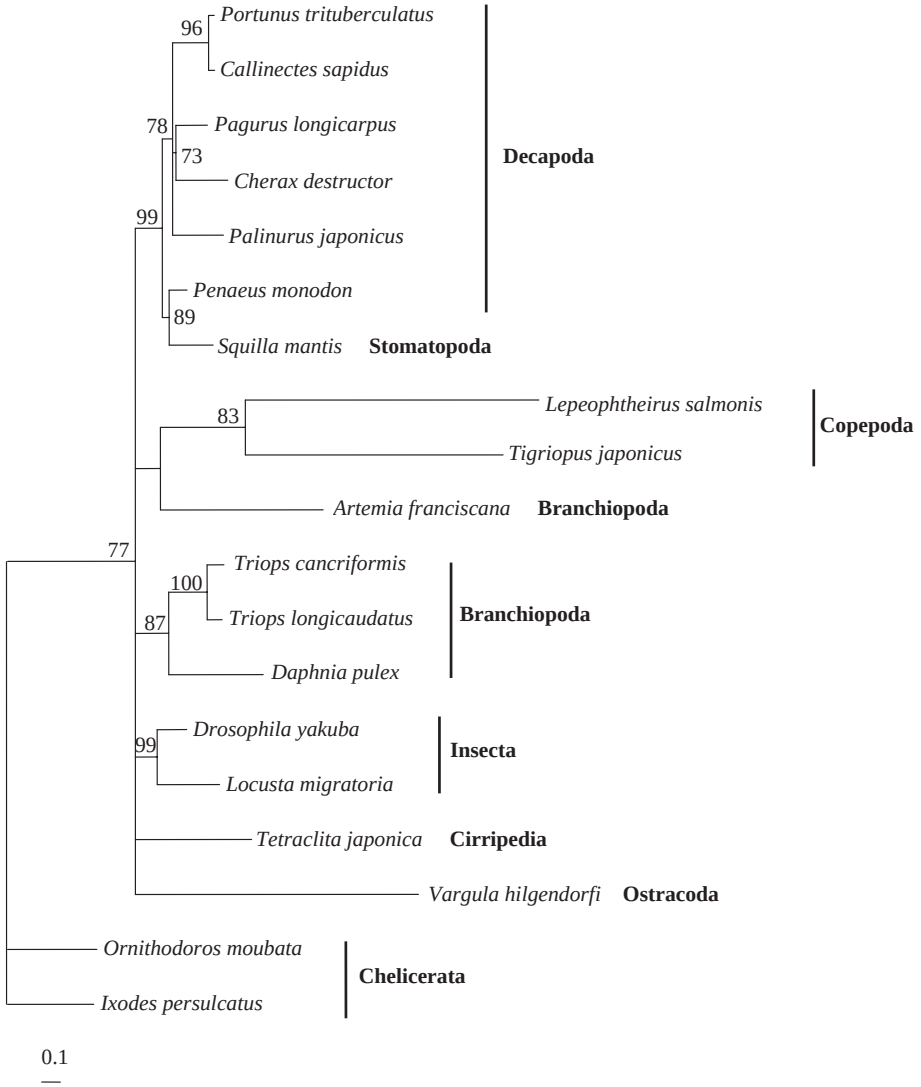


Fig. 4. The phylogenetic position of *L. salmonis* compared to a selection of other crustaceans, and two insects. The evolutionary relationship is presented as a maximum likelihood tree based on an alignment of selected amino acid sequences from mitochondrial genes (Cyt *B*, *COI*, *COII*, *ND1*, *ND5*, *ND6*, *A6, COIII, ND2, ND4, ND3* and *ND4L*). Two Chelicerata were used as an outgroup. Branch lengths represent relative phylogenetic distances according to maximum likelihood estimates based on mtDNA24 matrix (Adachi and Hasegawa, 1996).

significant support to a grouping of these. Moreover, the phylogenetic relationship between the above mentioned groups are not resolved (Fig. 4).

4. Discussion

The mtDNA of *L. salmonis* is 15 445 bp, which is within the size range of other mitochondrial genomes characterized from crustaceans (Valverde et al., 1994; Crease, 1999; Hickerson and Cunningham, 2000; Wilson et al., 2000; Machida et al., 2002; Umetsu et al., 2002; Yamauchi et al., 2002, 2003; Ogoh and Ohmiya, 2004; Begum et al., unpublished; NC_006079; NC_006081; Place et al., unpublished). In the present paper 13 protein-coding genes, two rRNA genes and 22 tRNAs, in addition to a control region (D-loop), have been characterized. Two copies of tRNA-Lys are present in the mtDNA of *L. salmonis*, while tRNA-Cys was not identified.

The mitochondrial gene order in *L. salmonis* is very different from that observed in other crustaceans (Valverde et al., 1994; Crease, 1999; Hickerson and Cunningham, 2000; Wilson et al., 2000; Machida et al., 2002; Umetsu et al., 2002; Yamauchi et al., 2002, 2003; Ogoh and Ohmiya, 2004; Begum et al., unpublished; NC_006079; NC_006081; AY363392), and *ND4*/*ND4L* and *A8*/*A6* do not overlap. In addition, the rRNA genes have opposite transcriptional polarity, which has previously been found in two starfish species (Smith et al., 1989; Asakawa et al., 1995).

4.1. Gene order

The mtDNA of *L. salmonis* displays a completely new gene order. Several major rearrangements are seen in the

organization of the protein-coding genes of *L. salmonis* when compared to other known mtDNAs from arthropods (Clary and Wolstenholme, 1985; Valverde et al., 1994; Crease, 1999; Hickerson and Cunningham, 2000; Wilson et al., 2000; Machida et al., 2002; Umetsu et al., 2002; Yamauchi et al., 2002, 2003; Ogoh and Ohmiya, 2004; Begum et al., unpublished; NC_006079; NC_006081; Place et al., unpublished). However, there appears to be gene order similarities between *L. salmonis* and other crustaceans when comparing short gene segments (segments A, B, C and D) (Fig. 2). Two of these segments (B and C) do resemble the gene order seen in *T. japonicus*, while segments A and D have the same organization as the branchiopods, decapods, one stomatopod, one maxillopod and *D. yakuba* (Fig. 2). Hence, the gene order of *T. japonicus* does not resemble *L. salmonis* more than any of the other arthropods. The two copepods *E. bungii* and *N. cristatus*, which have only been partly characterized, have also completely different gene orders compared to *L. salmonis* (Fig. 2). Thus, different gene organizations seem to be the rule rather than the exception.

If we compare the organization of the tRNA genes, which are frequently involved in rearrangements, *L. salmonis* differs completely from other crustaceans and *D. yakuba* (Clary and Wolstenholme, 1985; Valverde et al., 1994; Crease, 1999; Hickerson and Cunningham, 2000; Wilson et al., 2000; Machida et al., 2002; Umetsu et al., 2002; Yamauchi et al., 2002, 2003; Ogoh and Ohmiya, 2004). The copepods *T. japonicus* and *E. bungii*, the decapods *P. longicarpus* and *C. destructor* and the ostracod *V. hilgendorfii* are also unique, while *A. franciscana* and *P. trituberculatus* have only minor deviations from the tRNA gene order seen in *D. yakuba* (Clary and Wolstenholme, 1985; Valverde et al., 1994; Hickerson and Cunningham, 2000; Machida et al., 2002; Yamauchi et al., 2003).

Two models, a replication termination error and slipped-strand mispairing, are most commonly accepted to explain changes in the mitochondrial gene order (Levinson and Gutman, 1987; Boore and Brown, 1998). Generally, a small number of rearrangements can be reconstructed manually, but this is very difficult in cases with numerous differences (Boore and Brown, 1998). Due to the extensive gene rearrangements in *L. salmonis*, and the lack of mtDNA sequences from close relatives, it is not possible to speculate on the mechanisms generating these differences at the present time.

4.2. The protein-coding genes

The most frequent initiation codon used in mtDNAs from crustaceans is ATG, followed by ATT and ATA. However, several unusual start codons have been proposed for *COI* in different arthropods (ATTA in *D. pulex*, ATCA in *P. longicarpus*, ACG in *C. destructor* and *S. mantis*, GTT in *N. cristatus* and ATAA in *D. yakuba)* (Clary and Wolstenholme, 1985; Crease, 1999; Hickerson and Cunningham, 2000; Machida et al., 2004; Miller et al., 2004; NC_006081). In *L. salmonis* the five start codons ATG, ATT, ATA, ATC and GTT are being used, while TAA and TAG are the only stop codons (Table 2). Incomplete termination codons, such as TA or T, which is completed to TAA by adding A-nucleotides during polyadenylation of transcripts, have been reported in 12 of the 14 completely characterized mtDNA from crustaceans (Valverde et al., 1994; Crease, 1999; Hickerson and Cunningham, 2000; Wilson et al., 2000; Machida et al., 2002; Umetsu et al., 2002; Yamauchi et al., 2002, 2003; Ogoh and Ohmiya, 2004; Begum et al., unpublished; Place et al., unpublished). However, there are no indications of incomplete termination codons in the mtDNA sequence of *L. salmonis*.

When we compare the putative *ND3* protein sequence from *L. salmonis* it has a higher degree of similarity to the other 14 crustaceans characterized (28–38%) than *ND3* from *T. japonicus* (26–32%) (Table 3). Sixteen conserved amino acids of 118 were observed in *L. salmonis* when comparing the crustacean *ND3* sequences. The putative *ND4L* from *L. salmonis* has a protein sequence similarity of 15–21% compared to the other 14 crustaceans, while *T. japonicus* displayed a similarity of 15–25% (Table 3). Only five of 110 amino acids in *L. salmonis ND4L* are conserved, which is in agreement with this gene being conserved to a lesser extent (Wolstenholme, 1992b). Based on this, and the fact that little intergenic sequences are expected within the mitochondrial genome, we conclude that the two ORFs encode the proteins *ND3* and *ND4L*. In vertebrates, *ND4* and *ND4L* are transcribed as one bicistronic mRNA, and are therefore localized together (Wolstenholme, 1992b). The same organization is also found in crustaceans, with the exceptions of *T. japonicus* and *N. cristatus* where six and four genes separate *ND4* and *ND4L*, respectively (Fig. 2) (Machida et al., 2002, 2004). A similar arrangement also exists for *L. salmonis* where *ND4L* is localized between *ND6* and *A6* (Fig. 2).

Among higher invertebrates mtDNAs *A6* and *A8* overlap (Wolstenholme, 1992b; Hickerson and Cunningham, 2000). The same organization is also found within all crustaceans characterized so far (Fig. 2). *A8* is generally initiated from a 5′ start site, while *A6* has an internal start codon within *A8* (Wolstenholme, 1992b; Hickerson and Cunningham, 2000). If *A6* and *A8* were bicistronic in *L. salmonis A8* must have been incorporated in what we here refer to as *A6*, considering the fact that tRNA-Tyr is located close to the initiation site of *A6.* However, only one ORF was identified in this region. By searching all the non-coding regions in the *L. salmonis* mtDNA a more likely localization of *A8* was found between *ND5* and tRNA-Met. This sequence was most similar (21%) to *T. japonicus*, and it was also of similar length as *A8* from this copepod (Table 2). A search for transmembrane regions did also support the localization of *A8*.

4.3. 12S and 16S rRNA

The mitochondrial 12S and 16S rRNA genes in *L. salmonis* contain only 590 and 1009 bp respectively, and are among the smallest metazoan rRNA genes. Both genes have a low sequence similarity compared to other metazoan 12S and 16S rRNA sequences. However, since ribosomal function in all animal taxa is largely determined by its structure, it is generally accepted that its secondary structural arrangement is more conserved than the underlying nucleotide sequence. In order to retain a correctly folded rRNA, mutations in basepairing regions are frequently of the compensatory or semi-compensatory type to retain the correct folding (Gutell et al., 2002). Several examples of compensatory substitutions are evident throughout the *L. salmonis* rRNA genes supporting our secondary structure based alignments, and putative gene sizes. However, the 5′ and 3′-ends of *L. salmonis* 12S rRNA and 16S rRNA must be considered tentative.

In *L. salmonis* the rRNA genes have opposite transcriptional polarity. This phenomenon has not been previously reported in any crustaceans, but has been found in the two starfishes *Asterina pectinifera* and *Pisaster ochraceus* (Smith et al., 1989; Asakawa et al., 1995). In both these cases a fragment containing the 16S rRNA has been inverted compared to 12S rRNA, resulting in different transcriptional directions of the two rRNA genes.

4.4. tRNAs

The tRNA anticodons in *L. salmonis* were identical to those described for *D. yakuba* (Clary and Wolstenholme, 1985), except from tRNA-Lys using UUU. The same anticodon for lysine has also previously been reported for six other crustaceans (Hickerson and Cunningham, 2000; Wilson et al., 2000; Machida et al., 2002; Yamauchi et al., 2002, 2003). However, in *L. salmonis* the tRNA-Lys (UUU) is duplicated. In arthropods both tRNA-Leu (CUN or UUR) and tRNA-Ser (AGN or UCN) genes are duplicated, but in these cases the tRNAs contain different anticodons. Both tRNA-Lys genes in *L. salmonis* gave high scores (11.62 and 13.15) in the tRNA-scan reducing the possibility of a false positive.

The secondary structure of the mitochondrial tRNAs, encoded by metazoans, is very diverse (Wolstenholme, 1992b). Several authors have reported tRNAs that have lost either the T or D-arm (Wolstenholme, 1992b; Mitchell et al., 1993; Terrett et al., 1996; Yamazaki et al., 1997; Noguchi et al., 2000; Machida et al., 2002). This is probably the reason why the tRNAscan-SE 1.21 program used in this paper did not identify six tRNAs in the mtDNA of *L. salmonis*, one tRNA in *C. destructor* (Miller et al., 2004) and seven tRNAs in *V. hilgendorfii* (Ogoh and Ohmiya, 2004). Two sets of tRNA, Ser (UCN)/Trp and Phe/Glu, are complementary and therefore completely overlap in the mtDNA of *L. salmonis*. Thus, these sequences seem capable of folding into different structures (Fig. 3), which has not earlier been described within crustaceans. However, a 36 bp overlap between tRNA-Cys and tRNA-Tyr, coded on the same strand, has been observed in *P. japonicus* (Yamauchi et al., 2002).

4.5. Phylogeny

The evolutionary relationships among the arthropods are controversial. Based on morphological traits it has long been claimed that the Atelocerata, Insecta and Myriapoda, constitute a monophyletic group (c.f. Friedrich and Tautz, 1995; Bitsch and Bitsch, 2004). However, molecular studies indicate that the Atelocerata is not monophyletic, but that an insect-crustacean clade is supported (Nardi et al., 2003; Bitsch and Bitsch, 2004). Results from molecular studies have also questioned the monophyletic status of the Hexapoda by claiming that the majority of insects group together with the Crustacea positioning the Collembola outside the crustacean-insect clade (Friedrich and Tautz, 1995; Boore et al., 1998; Bitsch and Bitsch, 2004). In the present study, analysis of concatenated amino acid sequences, based on two different combinations of 12 mitochondrial genes, doses not resolve the position of the insects with respect to the crustaceans (Fig. 4). More sequences from crustaceans and other arthropods are needed before a robust phylogeny can be generated.

COI has been recommended as the core of a global bio-identification system for animals (Hebert et al., 2003). In the present study the putative amino acid sequence from this gene did not resolve the position of insects in relation to the crustaceans that were included. It should also be pointed out that when Hebert et al. (2003) used their system on the arthropods the two crustaceans included (*Cephalocarida* and *Maxillopoda*) fell outside the arthropod group. Hence, the reliability of *COI* in phylogenetic studies of arthropods can be questioned.

It is not the aim of this study to resolve the possible position of the insects within the Crustacea, but finding the closest relative to *L. salmonis* based on the mitochondrial genes. In all three phylogenies the two copepods (*L. salmonis* and *T. japonicus*) group together, but genetic distance trees show that they are further apart from the other crustaceans than the two chelicerates used as outgroup. Hence, the position of these two copepods in relation to other arthropods is highly uncertain.

The gene order of the mitochondrial genes has not been used for phylogenetic analysis in the present study since little is known about the gene order within crustacean and arthropod genera, families and orders. The large differences found in the gene order between the few species included in this study indicate that the knowledge about the evolution of gene order patterns are too fragmented for a reliable phylogenetic analysis to be performed.

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