# Studies on the mitochondrial genome and rDNA genes from the salmon louse, *Lepeophtheirus salmonis*.

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor scientiarum of the University of Bergen

by

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**CONTENTS**

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## LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

**I.** Tjensvoll K, Hodneland K, Nilsen F, Nylund A (2005)

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

Gene 353: 218-230.

**II.** Tjensvoll K, Glover KA, Nylund A (In press)

**Sequence variation in four mitochondrial genes of the salmon louse, *Lepeophtheirus salmonis*.**

Diseases of Aquatic Organisms.

**III**. Tjensvoll K, Hodneland K, Nylund A (submitted)

**The phylogenetic position of *Lepeophtheirus salmonis* (Copepoda: Siphonostomatoida) in relation to other crustaceans based on the 28S ribosomal RNA sequence.**

Manuscript submitted to Journal of Crustacean Biology.

# INTRODUCTION

## *Historical perspectives*

The salmon louse, *Lepeophtheirus salmonis* (*L. salmonis*), was first mentioned in the literature by the Norwegian bishop Pontoppidan in 1753 (Pontoppidan 1753). However, without reference to several earlier accounts the female louse was first figured, and described by Krøyer in 1837 (Krøyer 1837). Salmon lice are natural parasites on salmonids, and have usually occured in low numbers on wild fishes (Wootten et al. 1982, Berland 1993). Still, White did report heavy infestations of wild salmon already in 1940, but this was considered to be an extreme case (White 1940). The first serious outbreaks of *L. salmonis* infections occurred on Norwegian Atlantic salmon farms during the 1960s, soon after cage culture began (Pike & Wadsworth 1999). Since then the increase in salmon production has been dramatic in the North Atlantic (Pike & Wadsworth 1999), and *L. salmonis* has become a major fish pathogen causing severe skin damage by feeding on mucus, epidermis and blood (Figure1) (White 1940, Brandal et al. 1976, Wootten et al. 1977, Kabata 1979, Wootten et al. 1982). A further consequence of *L. salmonis* infections can be osmoregulatory breakdown, secondary virus or bacterial infections followed by death (Wootten et al. 1982, Nylund et al. 1993). It has been estimated that *L. salmonis* annually causes economical losses for the farming industry in Norway by 500 million NOK (Pike & Wadsworth 1999, Boxaspen & Næss 2000). However, *L. salmonis* is also a major problem in several other countries with salmon farming industry as Scotland, Ireland, Shetland, Faeroe Islands and Canada (Wootten et al. 1982, Todd et al. 1997, Pike & Wadsworth 1999, Boxaspen & Næss 2000, Mustafa et al. 2001).

Since the introduction of salmon farming, a decline in wild salmon and sea trout stocks have become noticeably, and a link between the fish farming activity and the high *L. salmonis* prevalences on wild salmonids have been suggested (Tully & Whelan 1993a, Pike & Wadsworth 1999, Tully et al. 1999, Finstad et al. 2000, Bjørn et al. 2001, Heuch & Mo 2001,

Bjørn & Finstad 2002, Butler 2002, Skilbrei 2004). These assumptions are based on infections of wild salmonids coinciding with *L. salmonis* epidemics on farmed fish following the industry development (Tully et al. 1999, Finstad et al. 2000, Bjørn et al. 2001, Bjørn & Finstad 2002, Butler 2002). Especially sea trouts, which have residence in coastal water, are believed to be under an extremely high *L. salmonis* infection pressure (Tully & Whelan 1993a, Tully et al. 1993b, Birkeland 1996, Skilbrei 2004). Heavily infested sea trout post-smolts have been observed to return to rivers within few weeks after their smolt migration, a phenomenon called premature return (Tully et al. 1993b, Birkeland 1996, Bjørn et al. 2001). This behavior is probably a consequence of the delousing effect achieved in freshwater, and a maintenance of their osmotic balance (Birkeland 1996). Since wild salmon do not have residence in coastal areas, but migrate to the feeding ground in the North Norwegian Sea, it was believed that these smolts perhaps were less exposed to infestations by *L. salmonis* (Skilbrei 2004). However, studies have indicated that migrating salmon smolts become infected by *L. salmonis* in coastal waters, before they reach the open sea, and that subsequent re-infestations also occur in the open ocean (Finstad et al. 1994, Jacobsen & Gaard 1997, Finstad et al. 2000, Todd et al. 2000, Skilbrei 2004). In Norway it is assumed that louse epizootics can cause mortality of 30-50% of all emigrating sea trout smolts, and 48-86% of all wild salmon smolts (Bjørn et al. 2001, Butler 2002).

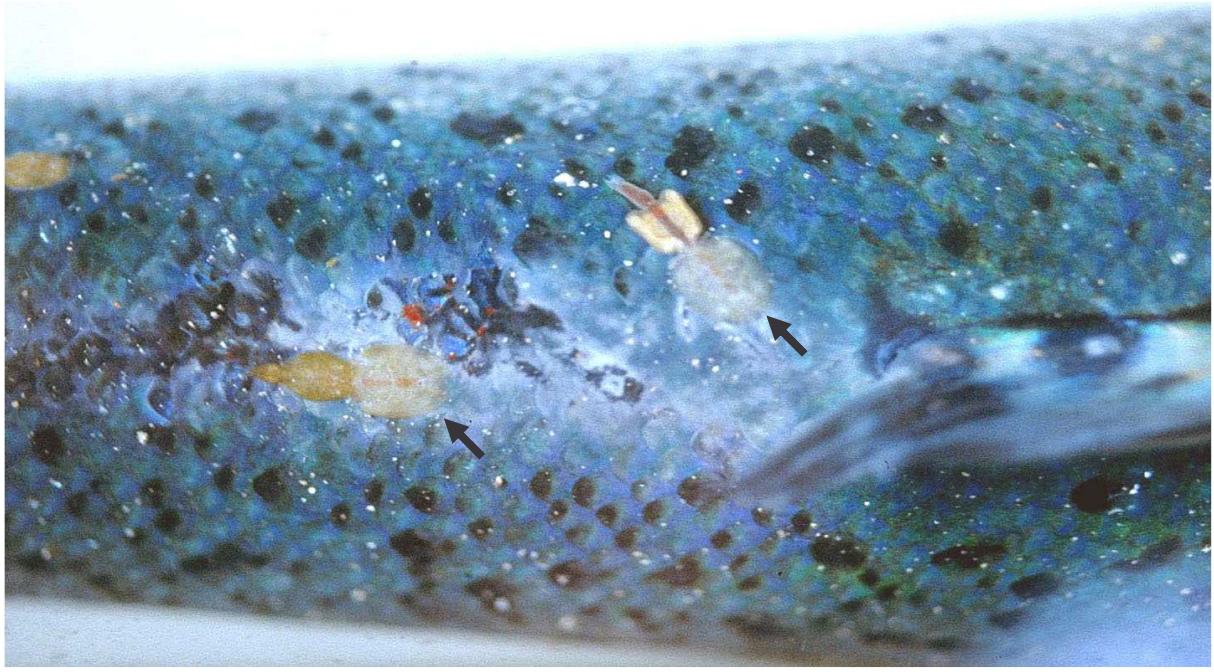

**Figure 1**: An Atlantic salmon infected with *Lepeophtheirus salmonis* feeding on mucus, epidermis and blood.

***Copepod systematics***

*Lepeophtheirus salmonis* is an arthropod belonging to the phylum Crustacea, subclass Copepoda (Siphonostomatoida, Caligidae). In addition to the crustaceans the arthropods also include the hexapods, chelicerates and myriapods (NCBI taxonomic database, http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/). Within Arthopoda it has long been claimed that the hexapods constitute a monophyletic group, and that their closest relatives are found within the myriapods (Nardi et al. 2003). More recently, molecular studies have rejected this relationship in favor of a closer affinity between Hexapoda and Crustacea (e.g. Friedrich & Tautz 1995, Boore et al. 1998a, Garcia-Machado et al. 1999, Giribet et al. 2001, Regier & Shultz 2001, Nardi et al. 2003, Mallatt et al. 2004, Giribet et al. 2005, Regier et al. 2005), also called Pancrustacea (Zrzavy & Stys 1997). Some morphological evidence also support this relationship (Friedrich & Tautz 1995, Giribet et al. 2001). The monophyletic status of the Hexapoda has also been questioned. This is due to the position of the Collembola

(the wingless hexapods) outside the Pancrustacean clade, in some cases, resulting in a paraphyletic Hexapoda (e.g. Nardi et al. 2003, Bitsch & Bitsch 2004, Bitsch et al. 2004, Lavrov et al. 2004).

An extensive fossil record suggests that the crustaceans arose and diversified during Cambrium (ca. 570 mya), and have subsequently undergone a long period of independent evolution (Spears & Abele 1997). Hence, the Crustacea has evolved into a group with extreme morphological diversity (Martin & Davis 2001, Regier et al. 2005). Most crustaceans are aquatic, and today the phylum is classified into the six classes Branchiopoda, Remipedia, Cephalocarida, Maxillopoda, Ostracoda and Malacostraca (Martin & Davis 2001). Copepoda is placed within the Maxillopoda and comprises approximately 11500 species placed in about 200 families and 1650 genera (Humes 1994). The aquatic copepods are found in various habitats from freshwater to marine or hypersaline inland waters, and from polar waters to hot springs (Huys & Boxshall 1991). It is considered to be the largest and most diverse group within Crustacea with life histories ranging from free-living to benthic and parasitic (Kabata 1970, Huys & Boxshall 1991). The copepods are usually small in size, with most species having body lengths of 0.5 to 5mm (Huys & Boxshall 1991). In terms of their size, diversity and abundance they can be regarded as the insects of the seas.

Copepod taxonomy is primarily based on morphological characters relating to modifications of the cephalic feeding structure (mandibles), and body segmentation (Kabata 1979, Huys & Boxshall 1991). It was Kabata (1979) who formally reassessed the phylogenetic relationships of the families of copepods parasitic on fishes, which later resulted in a revised classification for the entire subclass Copepoda (Huys & Boxshall 1991). Kabata (1979) suggested a monophyletic Copepoda with the ancestral “archiecopepod” forms living on or near the sea bottom, which in time split into two copepod groups (Figure 2). One group

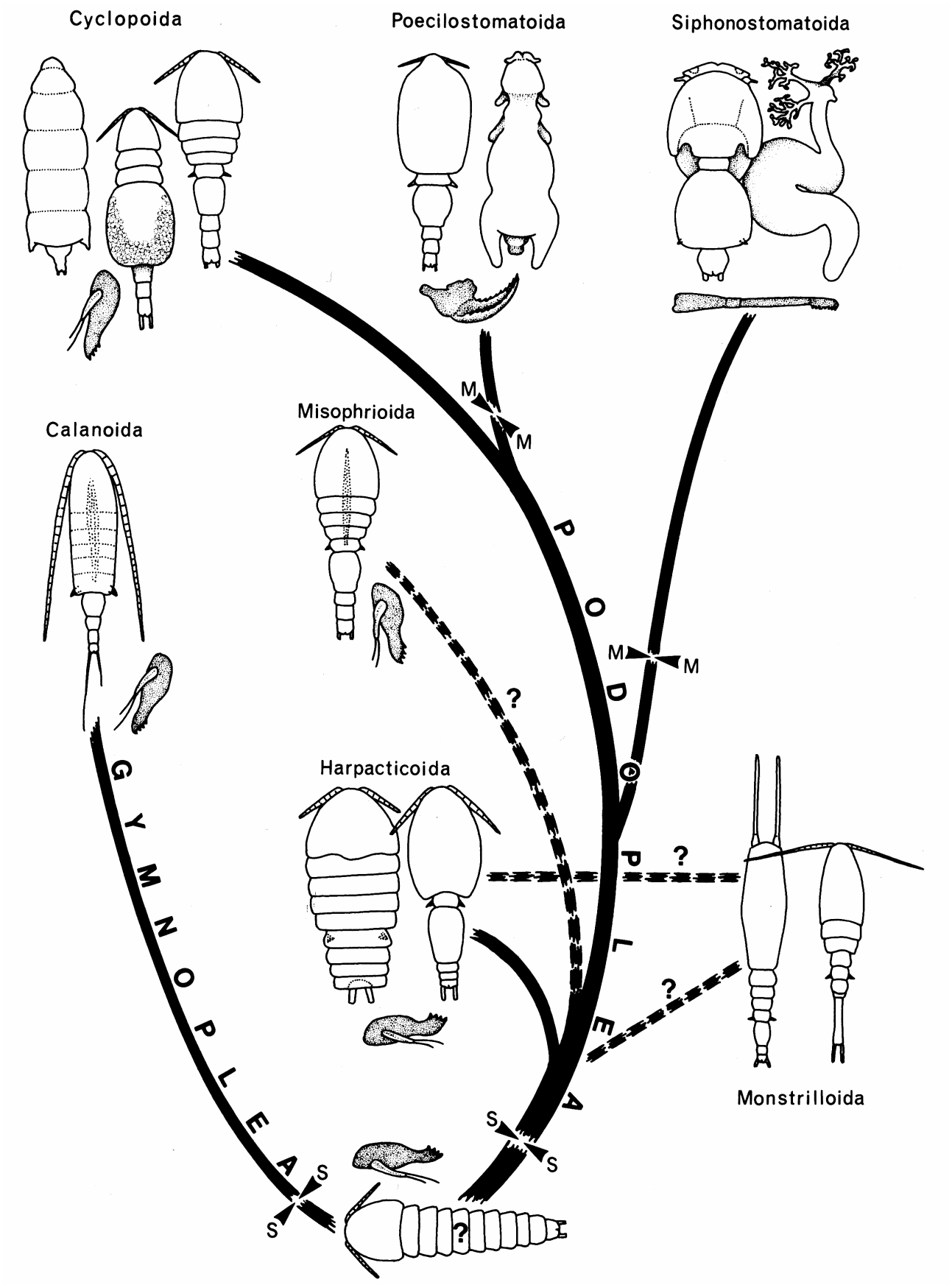


**Figure 2**: Copepod relationships proposed by Kabata (1979).

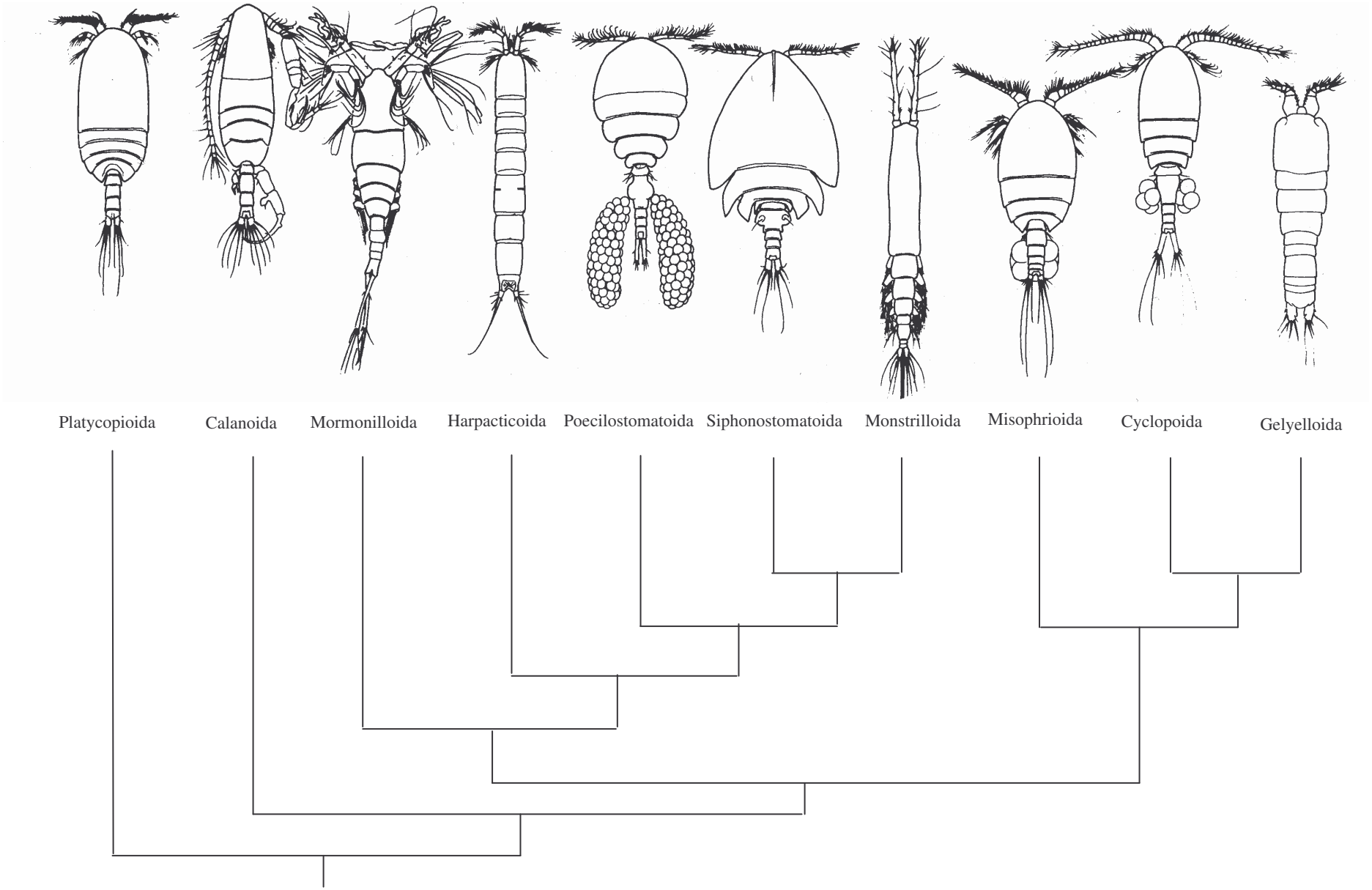


**Figure 3**: Copepod relationships proposed by Huys and Boxshall (1991).

consisting of the planktonic order Calanoida, while the main group consisted of active swimming copepods with the most primitive branches represented by the orders Monstrilloida, Harpacticoida and Misophrioida (Kabata 1979). Within the main group Siphonostomatoida was believed to be of a more ancient origin than the Poecilostomatoida and Cyclopoida, an assumption that was based on the development of the siphon-like mandibles (Kabata 1979). The Poecilostomatoida was believed to branch off from the Cyclopoida not very far back in the evolutionary past, while the Cyclopoida was considered the most recently derived order (Kabata 1979). In 1991 Huys and Boxshall erected several new orders and proposed a new copepod phylogeny suggesting the Mormonilloida, Harpacticoida, Poecilostomatoida, Siphonostomatoida and Monstrilloida as monophyletic (Figure 3), with the Siphonostomatoids as a sister-group to the Monstrilloida (Huys & Boxshall 1991). The cyclopoids belonged to a clade constituting a sister-group to the Siphonostomatoida-Poecilostomatoida clade. Moreover, the Calanoida formed a separate group also in this analysis.

Today, the ten copepod orders suggested by Huys and Boxshall (1991) are still recognized (Martin & Davis 2001). Copepods parasitic on fishes are mostly found within the orders Cyclopoida, Poecilostomatoida and Siphonostomatoida (Kabata 1992). The cyclopoids are the most abundant group of copepods in freshwater. They are primarily planktonic, but some parasitic freshwater cyclopoids are also found as well as free-living marine species (Huys & Boxshall 1991). In contrast, virtually all poecilostomatoids are parasites, and the majority are marine. It is a morphologicaly diverse group of copepods, containing a great number of families with large body sizes and peculiar morphology (Kabata 1979, Huys & Boxshall 1991). The third order, Siphonostomatoida, contains exclusively parasitic species, and about 75% of the parasitic copepods found on fishes belong to this order. These copepods are mostly marine in distribution, but a small number of species are also found in freshwater.

Many highly transformed types of copepods are found within the Siphonostomatoida, i. e. members of the family Nicothoidae that have lost all their appendages (Huys & Boxshall 1991).

*Lepeophtheirus salmonis* is placed within the Siphonostomatoida, family Caligidae (Kabata 1979). All Caligidae have similar morphology, characterized by five tagmata (cephalothorax, the fourth leg-bearing segment, genital complex, the abdomen and the tail). However, the caligids have also acquired several morphological traits as an adaptation to their life as parasites. They have a flattened body and prehensile appendages allowing them to attach to the host surface, as well as making them capable of free movement. Of the 31 Caligidae genera *Caligus* is acknowledged as the largest, whereas *Lepeophtheirus* is the second largest genus. These two genera are morphologically very similar, but one feature distinguish *Lepeophtheirus* spp from *Caligus* spp and that is the lack of lunules on the anterior margin of the adult parasites (Kabata 1979).

### *The biology and life cycle of* L. salmonis

*Lepeophtheirus salmonis* has a circumpolar distribution in the northern hemisphere, occurring on most species in the genera *Salmo, Oncorhynchus* and *Salvelinus* (e.g. Kabata 1979, Wootten et al. 1982, Johnson & Albright 1991a). The life cycle of *L. salmonis* consists of 10 stages, each separated by a moulting phase (Figure 4) (Kabata 1979, Johnson & Albright 1991b, Schram 1993). The first three stages are free-living, including two planktonic naupliar stages (nauplius I and II) and one infective copepodid stage. The copepodid attaches to the host by the second antennae, and once attached it uses the maxillipeds to move on the host surface to find a suitable place to settle (Schram 1993). Most copepodids prefer to settle on the skin and fins, although gills have also been reported as

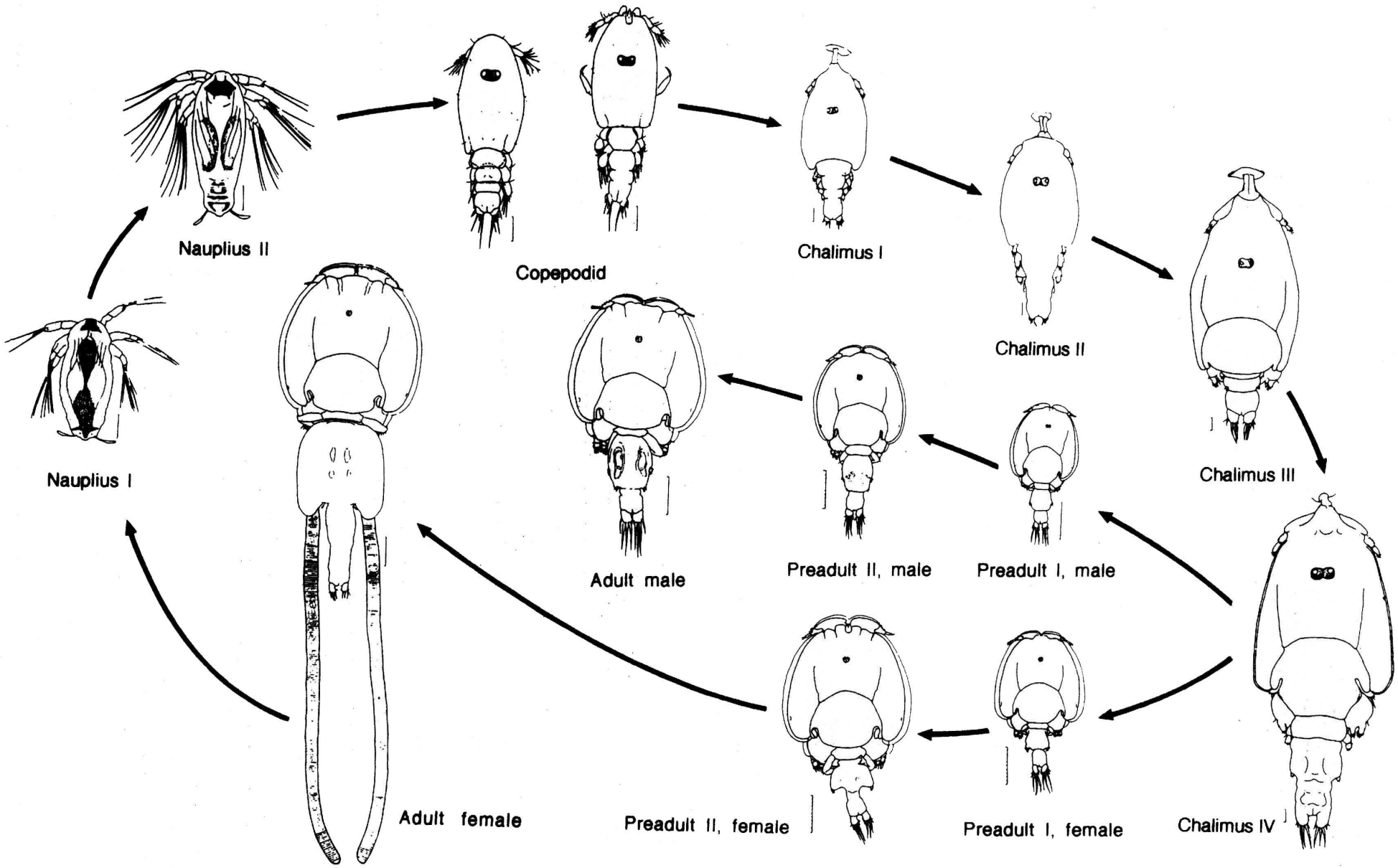


**Figure 4**: Life cycle of *Lepeophtheirus salmonis* (Schram 1993). Scale bars: nauplius-chalimus = 0.1 mm, preadult-adult = 1 mm.

settlement sites (Wootten et al. 1982, Johnson & Albright 1991b, Pike & Wadsworth 1999, Tucker et al. 2000, Treasurer & Wadsworth 2004). After moulting the copepodid transforms into chalimus I. All four chalimus stages (chalimus I-IV) are fixed to the body surface of the host by a frontal filament, and a new filament is produced between each moulting. After molting to the pre-adult stage the parasite is, however, able to move freely on the host surface. It is then attached to the host by the second antennae and the cephalothorax, which now may function as a cupping glass (Schram 1993).

Development, growth and survival of *L. salmonis* are greatly influenced by water temperature. Laboratory experiments have shown that development from the first nauplius to the infectious copepodid takes around 9.3 days at 5°C, 3.6 days at 10°C and only 1.9 days at 15°C (Johnson & Albright 1991a). The duration of the copepodid stage can, however, last as long as 10 days at 10°C (Johnson & Albright 1991a). Moreover, Boxaspen (2005) demonstrated that the last successful settlement of copepodids on salmon at 8°C occured 23 days after the copepodids had been introduced into the tank, and after 30 days at 6°C respectively (Boxaspen 2005). Development from egg to adult male takes around 40 days (5-6 weeks), while the time from egg to adult female takes 52 days (7.4 weeks) at 10°C (Wootten et al. 1982, Johnson & Albright 1991a). Normal development of embryos and larvae may take place at temperatures as low as 4°C (Boxaspen & Næss 2000).

### *The combat against* L. salmonis

Today, two different approaches are used in the combat against *L. salmonis* (Evensen et al. 2004). These include the use of bath treatment with synthetic pyrethroids (Cypermethrin and deltamethrin), as well as oral treatment where in-feed chemicals are being used (Pike & Wadsworth 1999, Roth 2000, Stone et al. 2002, Grave et al. 2004, Westcott et al. 2004). Until recently organophosphates (trichlorfon (Neguvon), dichlorvos (Nuvan) and azamethiphos

(Salmosan)) were the most used pesticides against *L. salmonis* (Ramstad et al. 2002). However, the last three years the use of bath-administered neurotoxins in general (organophosphates and synthetic pyrethroids) have been in decline, while the use of the oral preparation emamectin benzoate (SLICE) has increased considerably (Evensen et al. 2004). The reason for this is that SLICE has a suitable toxic effect on all life stages of *L. salmonis*, while the synthetic pyrethroids have less effect on the chalimus stages (Pike & Wadsworth 1999, Ramstad et al. 2002, Stone et al. 2002, Westcott et al. 2004). Still, synthetic pyrethroids are the predominant treatment for larger fishes (>1 kg), due to the higher costs and the required quarantine period when using SLICE on large fish (Evensen et al. 2004, Grave et al. 2004)**.** Wrasse, or cleaner-fish, is also used as treatment, but in a smaller scale (Frost & Nilsen 2004).

A major concern when using chemicals in the combat against *L. salmonis* is the development of resistance. Most chemicals used against *L. salmonis* are insecticides, and resistant insect populations do exist for these drugs (Waldstein & W.H. 2000, Ahmad et al. 2003, Burgess 2004, Ffrench-Constant et al. 2004). Similar effects have also been observed for *L. salmonis*, particular regarding organophosphates, where reduced treatment efficiency has been reported several times (Jones et al. 1992, Devine et al. 2000, Tully & McFadden 2000, Denholm et al. 2002, Ramstad et al. 2002). In time it is also expected that reduced treatment efficiency will emerge from the use of synthetic pyrethroids (Sevatdal & Horsberg 2003).

### *Epizootic studies of* L. salmonis

Several attempts have been made to obtain polymorphic genetic markers for studying the population genetic structure of *L. salmonis* (Isdal et al. 1997, Todd et al. 1997, Nolan et al. 2000, Tully & Nolan 2002, Dixon et al. 2004, Todd et al. 2004). In Norway, allele frequency

data from four allozymes indicated that differences might exist between northern and southern *L. salmonis* samples (Isdal et al. 1997). However, allozymes did not differentiate between *L. salmonis* collected in different locations throughout Scotland, while random amplification of polymorphic DNA (RAPD) did (Todd et al. 1997). Farm specific markers were also found in this study (Todd et al. 1997). Based on these results an expanded RAPD study was performed on farmed, and wild salmon from Scotland (Dixon et al. 2004). This study did not confirm the genetic differentiation previously found between *L. salmonis* samples in Scotland, although some degree of genetic differentiation was observed (Dixon et al. 2004). Another study, using microsatellites, showed differences between *L. salmonis* collected in Ireland, Scotland and Norway (Nolan et al. 2000). On the other hand, a recent study also using microsatellite markers did not demonstrate genetic differentiation between samples of *L. salmonis* collected on wild and farmed salmonids in Scotland, or between *L. salmonis* collected on salmonids from Scotland, Norway and Canada (Todd et al. 2004). However, significant genetic differentiation between *L. salmonis* from the North Atlantic versus the North Pacific Ocean was observed (Todd et al. 2004).

The rRNA genes coding for 18S rRNA and 5.8S rRNA of *L. salmonis*, in addition to the intergenic spacers (ITS-1 and ITS-2), have also been characterized for use in a population genetic study (Hodneland et al., unpublished). However, no genetic difference was found within any of these sequences when *L. salmonis* from Norway and Japan were compared.

***Candidate genes for studies of epizootiology and phylogeny***

Mitochondrial genes and the ribosomal DNA unit have been used extensively in phylogeny, and population genetic studies (e.g. Hale & Singh 1987, Hillis & Dixon 1991, Garcia-Machado et al. 1999, Saito et al. 2000, Schwenk et al. 2000, Umetsu et al. 2002, Yamauchi et al. 2002, Gantenbein & Largiader 2003, Papetti et al. 2005). While conserved regions of the

rDNA unit can be used in studies of more ancient stages of evolution, other faster evolving regions are used to infer closer genetic relationships (Grechko 2002). The mitochondrial genes are found to evolve at different rates, and thus have been classified according to their properties in resolving phylogenetic relationships among distantly related taxa (Zardoya & Meyer 1996). However, the mitochondrial genes are believed to be most suited for population genetic studies since they accumulate substitutions up to 10 times faster than nuclear genes (Shearer et al. 2002, Ballard & Whitlock 2004). Moreover, due to maternal inheritance the mitochondrial genome is haploid (Moritz et al. 1987), resulting in a population size equal to 1/2 of that for nuclear genes. Mitochondrial DNA (mtDNA) is therefore more subjected to genetic drift and fixation than nuclear DNA (Altukhov & Salmenkova 2002, Ballard & Whitlock 2004). Altogether, analyses of the mtDNA often prove useful in assessing genetic structure, gene flow or phylogenetic relationships among populations, or closely related species, when nuclear markers fail (Shoemaker et al. 2003).

*Mitochondrial genes*

The mitochondrial genome is a circular DNA molecule. It codes for proteins that, together with nuclear encoded products, form enzyme complexes involved in the production of ATP in oxidative phosphorylation, as well as other biochemical functions (Cummins 2001, Saccone et al. 2002). To date 102 mitochondrial genomes of arthropods have been characterized (http://www.ncbi.nlm.nih.gov/genomes/ORGANELLES/mztax_short.html), and of these 30 are within the phylum Crustacea (see Appendix, Table 1). The length of the mtDNAs, within the metazoan, have been reported in the range of 14-42 kb (Crease 1999). Despite this large size variation all metazoan mtDNAs, with few exceptions, contain the same 37 genes; 13 protein-coding genes, two rRNAs and 22 tRNAs in addition to a non-coding control region (table 1) (Boore 1999).

**Table 1**: The genes found in metazoan mitochondrial genomes; 13 protein-coding genes, two ribosomal RNAs and 22 tRNA genes in addition to a control region.

| Genes and regions | Designation |
|---|---|
| Cytochrome oxidase subunit I, II, III | *COI*, *COII*, *COIII* |
| Cytochrome b | *Cyt B* or *Cyt b* |
| NADH dehydrogenase subunits 1-6, 4L | *ND1-6, ND4L* |
| ATP synthase subunits 6, 8 | *A6*, *A8* or *ATP6, ATP8* |
| Large ribosomal RNA subunit | 16S rRNA or *lrRNA* |
| Small ribosomal RNA subunit | 12S rRNA or *srRNA* |
| 18 transfer RNAs each specifying a single amino acid | Corresponding one-letter amino acid or amino acid abbreviation (e.g. tRNA-A or tRNA-Ala) |
| Two transfer RNAs specifying leucine | tRNA-L (CUN), tRNA-L (UUR) or tRNA-Leu (CUN), tRNA-Leu (UUR) |
| Two transfer RNAs specifying serine | tRNA-S (AGN), tRNA-S (UCN) or tRNA-Ser (AGN), tRNA-Ser (UCN) |
| Non-coding control region | D-loop |

In some species all genes are coded from one mtDNA strand, whereas in others the genes are distributed between the two strands (Boore 1999). As opposed to genomic DNA the mitochondrial genes do not contain introns, the genes often overlap, but if not, the intergenic regions are very short (Wolstenholme 1992b). Although most protein-coding genes are relatively conserved, both rRNA genes and the tRNA genes are variable both in size and structure (Wolstenholme 1992a). Gene order may vary between metazoan lineages, but a conservation of gene order is expected among closely related species and genera (Boore 1999, Saccone et al. 2002). A gene organization representative of the Arthropoda is shown in figure 5.

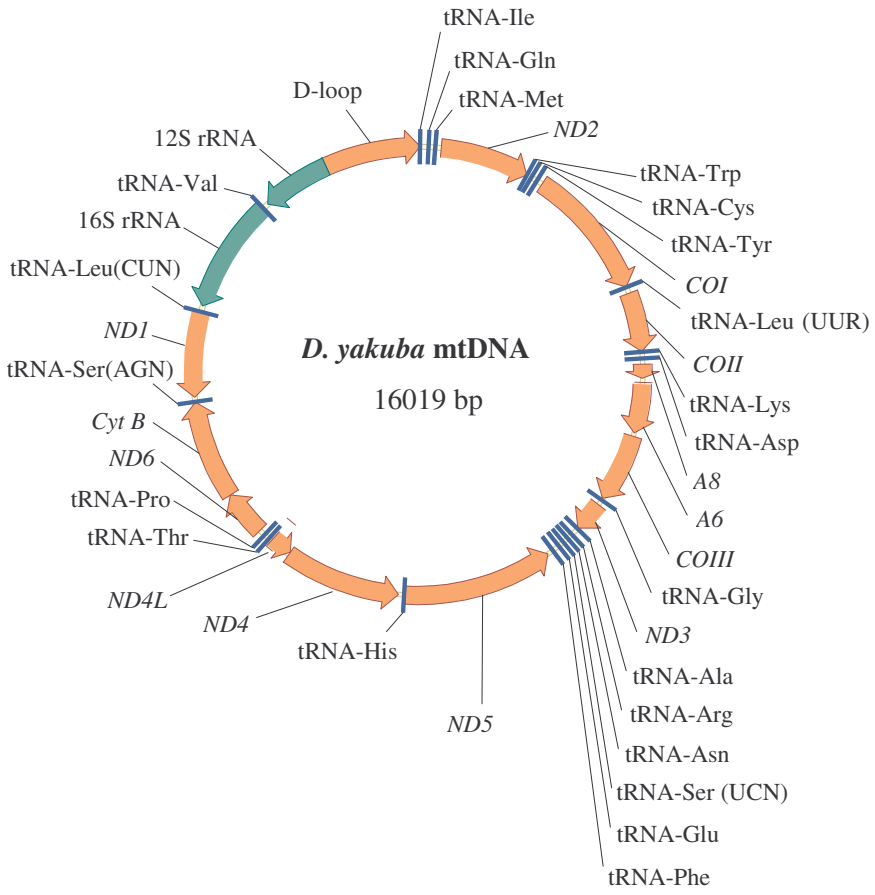


**Figure 5**: Mitochondrial gene organization representative of the Arthropoda, here presented by the genome of *Drosophila yakuba*. Genes coded on the complementary strand are shown by counter-clockwise arrows.

The four mitochondrial genes *COI*, 16S rRNA, *A6* and *Cyt B* are the most often used in population genetic studies, also within Crustacea (e.g. Bucklin et al. 1997, Lee 2000, Gopurenko & Hughes 2002, Jarman et al. 2002, Hurwood et al. 2003, Baratti et al. 2005). Another region considered to be informative for studies of population structure is the control region (D-loop). In a number of metazoan species tandemly arranged repeated sequences are found within the D-loop, and the number of repeated copies have been found to vary between individuals of a species (Wolstenholme 1992b, Saccone et al. 2002). However, it has quite recently been reported that the variability in this region is extremely high, even when

considering members of the same species, making alignment of the D-loop difficult to perform (Altukhov & Salmenkova 2002, Saccone et al. 2002).

*rRNA genes*

Ribosomal RNA (rRNA) constitutes 80-90% of the total cellular RNA in both eukaryotes and prokaryotes. It is represented in the genome by multiple genes where the number varies from 100-200 copies in lower eukaryotes up to several hundred in higher eukaryotes (Lewin 1997). The rRNA genes are arranged as tandem repeats of a nuclear ribosomal gene cluster (rDNA) encoding 18S rRNA, 5.8S rRNA and 28S rRNA, which are separated by the transcribed spacers ITS-1 and ITS-2 (Figure 6) (Hillis & Dixon 1991). An external transcribed spacer (ETS) is located upstream of the 18S rRNA gene (Figure 6). The transcribed spacers (ITS-1, ITS-2 and ETS) contain the processing signals for the rRNA transcripts, as the genes are transcribed as parts of a larger precursor molecule that is subsequently processed in several steps to yield the mature rRNAs (Hillis & Dixon 1991). A nontranscribed spacer (NTS), also called the intergenic spacer (IGS), is located at the 5` end of the rDNA unit separating the 18S-28S tandem repeats (Figure 6).

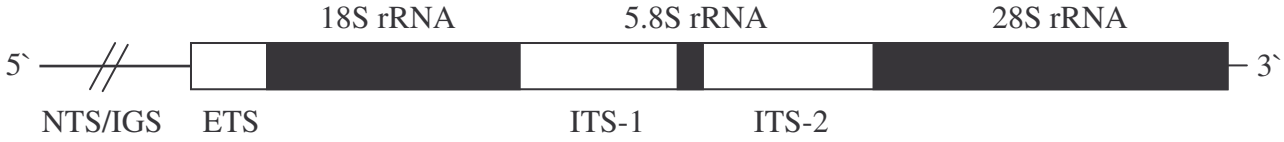


**Figure 6**: Organization of an eukaryotic rDNA tandem repeat. The rRNA genes 18S, 5.8S and 28S are transcribed as a large precursor molecule, also including two internal transcribed spacers (ITS-1 and 2) and the external transcribed spacer (ETS). The nontranscribed spacer (NTS), or the intergenic spacer (IGS), separates the repeated units.

Ribosomal RNA genes consist of both core regions, as well as expansion regions (Clark et al. 1984). Core segments have very conserved nucleotide sequences, which fold into secondary structures that are common to all eukaryotes (Linares et al. 1991). In contrast, the expansion segments can vary considerably in length and sequence between different eukaryotic organisms (Linares et al. 1991). This may be due to the presence of repetitive motifs, insertions or an accumulation of single nucleotide polymorphisms (Hassouna et al. 1984, Hancock & Dover 1988, Tautz et al. 1988, Hillis & Dixon 1991). Of the rRNA genes 18S rRNA is most used in phylogeny, due to its relatively slow evolving sequence (Hillis & Dixon 1991). The 28S rRNA gene, on the other hand, is larger and the expansion regions are much more variable, both in length and sequence than those of 18S rRNA (Hancock et al. 1988). Hence, 28S rRNA has been used in population genetic studies, as well as within molecular systematics (e.g. Crandall et al. 2000, Jarman et al. 2000, Remigio & Hebert 2000, Babbitt & Patel 2002, Stevens et al. 2002, Taylor et al. 2002, Sawabe et al. 2003, Schnabel & Hebert 2003, Duan et al. 2004, Mallatt et al. 2004).

In addition to the rRNA genes, the spacer regions can also be used to infer phylogenetic and population genetic relations (Hillis & Dixon 1991). Among the spacers, the NTS evolves most rapidly, while the transcribed spacers (ITS-1, ITS-2 and ETS) are somewhat more conserved (Hillis & Dixon 1991). A tandemly repeated sequence comprises also part of the NTS region. This sequence generally varies in length of 100-200 bp, resulting in a variable overall length of the NTS region (Jorgensen & Cluster 1988). The number of sub-repeating elements differ among individuals, but it has also been found differences in the NTS repeat within individuals (Jorgensen & Cluster 1988). Despite this, several population genetic studies have been performed using the NTS region (e.g. Cunningham et al. 2003, De Arruda et al. 2003, Printzen et al. 2003, Gupta et al. 2004, Huguet et al. 2004).

## AIMS OF THE STUDY

The main goal of this project was to characterize both mitochondrial and nuclear genes, and use them to study the population genetic structure of *L. salmonis* along the Norwegian coast and throughout the North Atlantic. The ability to provide such data should prove useful in analysing; a) the dispersal potential of *L. salmonis* larvae, b) gene flow between *L. salmonis* populations, c) recurrent infestation on salmon farms, d) the impact of these infestations on wild salmonids and e) development of resistance in *L. salmonis* populations to chemoterapeutants. Since the mitochondrial genes *A6, COI, Cyt B* and 16S rRNA have been reported to be highly informative for population genetic studies in other crustaceans, and arthropods, these genes were chosen for the present study. The nuclear 28S rRNA gene also contains variable regions, and the nontranscribed spacer (NTS) includes tandemly repeated sequences that could be informative for population genetic studies. Furthermore, both mitochondrial and rDNA genes have proved useful for inferring phylogenetic relationships.

Specific aims were to:

1. Characterize the mitochondrial genome of *L. salmonis*.
2. Characterize 28S rRNA, and the nontranscribed spacer (NTS), of *L. salmonis*.
3. Use the mitochondrial genes *A6, COI, Cyt B* and 16S rRNA, in addition to 28S rRNA and the NTS region, to study the population genetic structure of *L. salmonis* along the Norwegian coast and in the North Atlantic.
4. Use sequence information from mitochondrial and rDNA genes to find the phylogenetic position of *L. salmonis*.

## SUMMARY OF RESULTS

***Paper I***

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

The mtDNA from *L. salmonis* is 15 445 bp. It contains 13 protein-coding genes, two ribosomal RNA genes and 22 tRNA genes in addition to a non-coding control region (D-loop). Whereas tRNA-Cys was not identified in the *L. salmonis* mtDNA, two copies of tRNA-Lys were characterized. This has not previously been reported in any crustacean species.

The mitochondrial gene order in *L. salmonis* differs significantly from the gene order found in the three copepods (*Tigriopus japonicus*, *Eucalanus bungii, Neocalanus cristatus*), and the other crustaceans previously characterized. Among the exceptions are the organization of *ND4*/*ND4L* and *A8*/*A6*, which are usually transcribed as one bicistronic mRNA, but are separated by several genes in the *L. salmonis* mtDNA. Furthermore, the two rRNA genes are encoded on opposite strands in *L. salmonis*, and this has not previously been found in any other arthropods. Despite these differences a phylogenetic analysis, based on the mitochondrial protein sequences, did group *L. salmonis* together with *T. japonicus*.

***Paper II***

**A study of single nucleotide polymorphisms (SNPs) in four mitochondrial genes of the salmon louse, *Lepeophtheirus salmonis*.**

The four mitochondrial genes *A6, COI, Cyt B* and 16S rRNA were used to examine the genetic variation in *L. salmonis* collected from seven locations; Norway (Finmark, Sogn og Fjordane and Øst-Agder), Scotland, Canada, Russia and Japan. All genes showed an extremely high level of polymorphisms, leading to an intraspecific variation of 17.5% in *A6*,

15.9% in *COI*, 14.4% in *Cyt B* and 10.6% in 16S rRNA. The majority of the polymorphisms were only observed within single individuals, resulting in a high number of private haplotypes within each gene.

Sequence variation found in the four mitochondrial genes did not reveal genetic differentiation among the three Norwegian samples from Finmark, Sogn og Fjordane and Øst-Agder. Furthermore, no genetic differentiation was observed between *L. salmonis* sampled in Norway, Scotland and Russia. However, pairwise sequence comparisons indicated that a weak degree of differentiation might exist between *L. salmonis* sampled in the northeast Atlantic, and *L. salmonis* from the east coast of Canada. All samples collected in the Atlantic were clearly different from the Pacific sample, as expected. Extensive gene flow due to passive transport of *L. salmonis* larvae along the Norwegian coast, and the migratory pattern of the salmonid host is suggested to explain the lack of distinct populations in the North Atlantic.

***Paper III***

**The phylogenetic position of *Lepeophtheirus salmonis* (Copepoda, Siphonostomatoida) in relation to other crustaceans based on the 28S ribosomal RNA sequence.**

Two separate phylogenetic analyses, based on the sequence of 28S rRNA, were performed in order to find the position of *Lepeophtheirus salmonis* (Copepoda, Siphonostomatoida, Caligidae) in relation to a selection of other copepod, crustacean and arthropod species. The arthropod phylogeny shows monophyly of several accepted groups like Copepoda, Hexapoda (Insecta and Collembola) and Branchiopoda, giving support to the phylogenetic analyses performed. Furthermore, the Hexapoda is placed as sister-group to the Copepoda. The three orders within the Copepoda (Siphonostomatoida, Poecilostomatoida and Cyclopoida) are each monophyletic, with the Poecilostomatoida being the closest relative to Siphonostomatoida.

Within the Siphonostomatoida, *L. salmonis* group together with *Lepeophtheirus pollachius* with 100% support. The examined *Caligus* species constitute the sister-group to *Lepeophtheirus* spp., thus making the Caligidae monophyletic. However, members of the family Lernaeopodidae do not constitute a monophyletic group in our analysis.

# DISCUSSION

## *Characterization of the* L. salmonis *mitochondrial genome*

Very little is known about the population structure of *L. salmonis* (Tully & Nolan 2002), despite the economical losses this pathogen causes for the Atlantic salmon farming industry (Pike & Wadsworth 1999, Mustafa et al. 2001). Several population genetic studies have been performed, but these studies have resulted in contradictory conclusions based on the use of different genetic markers (Isdal et al. 1997, Todd et al. 1997, Nolan et al. 2000, Tully & Nolan 2002, Dixon et al. 2004, Todd et al. 2004). This contributes to a great confusion about the existence of distinct *L. salmonis* populations, and new genetic approaches are therefore needed. Since mitochondrial genes are used extensively in population genetic studies, the mitochondrial genome of *L. salmonis* was characterized (AY625897) (Paper I). The mtDNA contains the usual 37 genes found in metazoan mitochondrial genomes, including 22 encoding tRNAs, but a completely new gene organization was revealed (Figure 7). tRNA-Cys was not identified, neither by the tRNAscan-SE program nor manually anticodon/motifs searches, and this was probably due to large deviations in the secondary structure. However, two copies of tRNA-Lys are present in the mitochondrial genome of *L. salmonis*. Moreover, 12S and 16S rRNA has opposite transcriptional polarity in the *L. salmonis* mtDNA (Paper I).

To date, a total of 30 crustacean mitochondrial genomes have been characterized (see Appendix, Table 1). Within the copepods the mtDNA from *L. salmonis* (Paper I) and *T. japonicus* (Machida et al. 2002) have been completely characterized, while *Eucalanus bungii* and *Neocalanus cristatus* are only partly characterized (Machida et al. 2004). Even if the differences in locations of tRNA genes are ignored, since tRNA genes are frequently involved in gene rearrangements (Wolstenholme 1992b), very limited similarities in gene order are demonstrated between the four copepods (Paper I). In comparison, 21 of the other 28 characterised crustacean mtDNAs have similar gene organization as the mitochondrial

genome of *D. yakuba* (see figure 2 in Paper I; Lavrov et al. 2004, Yamauchi et al. 2004, Miller et al. 2005, Segawa & Aotsuka 2005, Miller et al. Unpublished, Swinstrom et al. Unpublished). These include all the four branchiopods characterized (*Daphnia pulex*, *Artemia franciscana*, *Triops cancriformis* and *Triops longicaudatus*), eight of eleven decapods (*Penaeus monodon*, *Panulirus japonicus*, *Portunus trituberculatus*, *Callinectes sapidus*, *Marsupenaeus japonicus*, *Pseudocarcinus gigas*, *Macrobrachium rosenbergii* and *Geothelphusa dehaani*), five stomatopod (*Squilla mantis*, *Harpiosquilla harpax*, *Squilla empusa, Lysiosquillina maculata* and *Gonodactylus chiragra*), two of three cirripeds (*Tetraclita japonica* and *Pollicipes polymerus*), one cephalocarid (*Hutchinsoniella macracantha*) and one pentastomid (*Armillifer armillatus*). Hence, it is quite conspicuous that the mtDNAs from the four copepods all have different gene organizations (see figure 2 in Paper I). One feature that is shared between *L. salmonis*, *T. japonicus* and *N. cristatus*, but not *E. bungii*, is the separation of *ND4* and *ND4L* by several genes (see figure 2 in Paper I). In vertebrates, *ND4* and *ND4L* are localized together due to transcription of one bicistronic mRNA (Wolstenholme 1992b). This is probably also the general rule for the crustaceans, with exception of the three copepods and one cirriped, *Megabalanus volcano* (Paper I; Begum et al. 2004, Lavrov et al. 2004, Yamauchi et al. 2004, Miller et al. 2005, Segawa & Aotsuka 2005, Sun et al. 2005, Miller et al. Unpublished, Swinstrom et al. Unpublished). Furthermore, *A6 and A8* are also transcribed as one bicistronic mRNA among higher invertebrates, as *A6* has an internal start codon within *A8* (Wolstenholme 1992b, Hickerson & Cunningham 2000). Overlap of *A6* and *A8* are found in all crustacean mtDNAs characterized, with *L. salmonis* being the only exception (Paper I; Begum et al. 2004, Lavrov et al. 2004, Yamauchi et al. 2004, Miller et al. 2005, Segawa & Aotsuka 2005, Sun et al. 2005, Miller et al. Unpublished, Swinstrom et al. Unpublished).

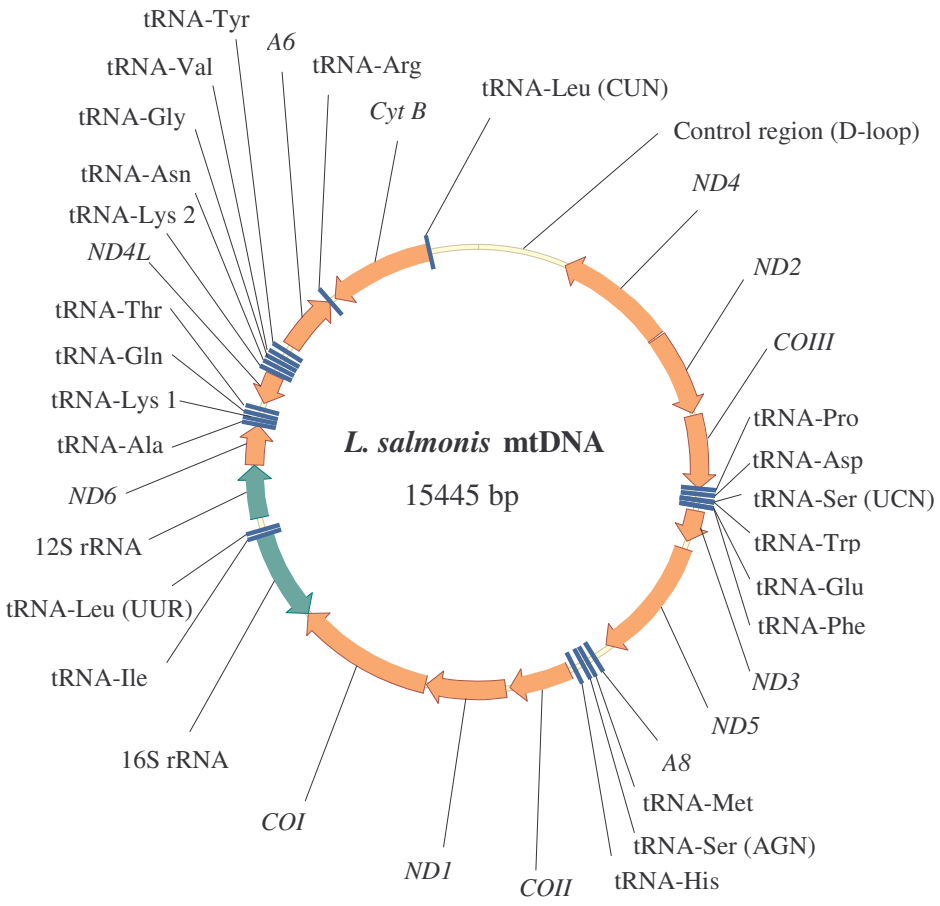


**Figure 7**: The mitochondrial genome of *Lepeophtheirus salmonis* contains 13 protein-coding genes, two rRNA genes and 22 tRNA genes in addition to a control region (AY625897). Two copies of tRNA-Lys are present, while tRNA-Cys was not identified. Both DNA strands contain coding regions, but very few genes overlap.

At present, it is not clear which mechanisms are responsible for the deviating gene organization seen in *L. salmonis* compared to the other crustaceans (see figure 2 in Paper I; Begum et al. 2004, Lavrov et al. 2004, Yamauchi et al. 2004, Miller et al. 2005, Segawa & Aotsuka 2005, Sun et al. 2005, Miller et al. Unpublished, Swinstrom et al. Unpublished). Three mechanisms have, however, been proposed for mitochondrial gene rearrangements 1) the duplication-random loss mechanism (e.g. Moritz et al. 1987, Boore & Brown 1998b, Boore 1999, Lavrov et al. 2002) 2) transposition of genes (e.g. Moritz et al. 1987, Boore &

Brown 1994, Macey et al. 1997, Boore & Brown 1998b, Groth et al. 2000, Saccone et al. 2002, Tomita et al. 2002) and 3) intramitochondrial recombination (e.g. Dowton & Campbell 2001, Machida et al. 2002, Miller et al. 2004). The duplication-random loss mechanism is the most accepted model to explain mitochondrial gene rearrangements (Moritz et al. 1987, Boore & Brown 1998b, Boore 1999, Lavrov et al. 2002). In this model, the gene duplication is either a result of errors in the replication or strand slippage and misparing, followed by a deletion of one gene copy from the genome. The deleted gene copy has then often been inactivated (e.g. Moritz et al. 1987, Stanton et al. 1994, Arndt & Smith 1998, Kumazawa et al. 1998, Boore & Brown 1998b, Lavrov et al. 2002). The second mechanism proposed for mitochondrial gene rearrangements involves transposition of genes that are flanked by two tRNAs (Saccone et al. 2002). The genes are then considered to be similar to a transposable element, with the two tRNAs corresponding to the long terminal repeats (LTRs) (Saccone et al. 1990). Three genes (*Cyt B*, *A6* and *ND4L*) are flanked by tRNAs in the *L. salmonis* mtDNA, and thus might be similar to transposable elements (see Saccone et al. 2002). Intramitochondrial recombination, as well as transposition, may lead to inversion of genes (see Machida et al. 2002, Miller et al. 2004). In *L. salmonis* mtDNA the two rRNA genes have inverted orientation, compared to each other (Figure 7). This phenomenon has not earlier been reported in any crustaceans, but has been found in five starfishes (Smith et al. 1989, Asakawa et al. 1995, Matsubara et al. 2005). Inversion of a fragment containing 16S rRNA has been suggested to result in the different transcriptional directions, of the two rRNA genes, seen in these cases. In conclusion, the ancestral caligid mtDNA organization is at the present time unknown, and it is therefore impossible to say which mechanisms that have resulted in the mitochondrial gene order observed in *L. salmonis*.

### *Two tRNA-Lys genes present in the* L. salmonis *mtDNA*

Two copies of tRNA-Lys, possessing the same anticodon (UUU), are present in the mtDNA of *L. salmonis* (Paper I). A sequence comparison revealed that only 45% of the nucleotides were identical between the two *L. salmonis* tRNA-Lys sequences. Moreover, both sequences gave high tRNA scores, by the tRNAscan-SE program, and distinct secondary cloverleaf structures were proposed (Paper I). Considering this and the fact that the algorithm in the tRNAscan-SE program is far too strict to account for the structural diversity observed in mitochondrial tRNA genes, it reduces the possibility of one tRNA-Lys being a false positive (Paper I).

Generally, two tRNA-Leu (CUN or UUR) and two tRNA-Ser (AGN or UCN) genes are present in animal mitochondrial genomes (Boore 1999). However, within arthropods duplication of other tRNA genes have also been found (e.g. Hoffmann et al. 1992, Gissi & Pesole 2003). Dissimilar anticodons were observed in these cases, and it was therefore concluded that the use of different genetic codes were the reason for these tRNA duplications (Hoffmann et al. 1992, Gissi & Pesole 2003). In the demosponge *Axinella corrugata* (*A. corrugata*) two copies of tRNA-Ala have been described (Lavrov & Lang 2005). Both tRNAs possess the same anticodon (UGC), distinct secondary structures were predicted and the nucleotide sequences had only an identity of 52% (Lavrov & Lang 2005). This is analogous to the tRNA-Lys situation found in the *L. salmonis* mtDNA. In the study published by Lavrov and Lang (2005) a hypothesis called tRNA gene recruitment was introduced. This hypothesis was based on the result from a tRNA phylogeny indicating that one tRNA-Ala, in *A. corrugata*, originated from tRNA-Thr rather than from tRNA-Ala (Lavrov & Lang 2005). An explanaition involving duplication of tRNA-Thr followed by a mutation in the anticodon was implied (see also Higgs et al. 2003, Rawlings et al. 2003). Since mtDNAs from closely related caligid species have not been characterized, it is at present not possible to perform a tRNA

phylogeny to find the origin of the two tRNA-Lys genes in *L. salmonis*. A sequence comparison of all the tRNA genes in the *L. salmonis* mtDNA did, on the other hand, demonstrate that the tRNA- Lys 1 gene is most similar to tRNA-Tyr (50% identity), whereas tRNA-Lys 2 has highest similarity to tRNA-Met (62% identity). Still, the similarity is not particularly high, and no conclusion regarding tRNA gene recruitment can therefore be made from these results.

The duplication of tRNA-Lys in *L. salmonis* might also be explanied by a duplication-random loss mechanism (e.g. Moritz et al. 1987, Boore & Brown 1998b, Boore 1999, Lavrov et al. 2002). This implies that tRNA-Lys has been duplicated, but one gene copy has not yet been deleted from the genome. Moreover, three genes separate the tRNA-Lys copies in the *L. salmonis* mtDNA (Figure 7). It is therefore likely that a transposition of one tRNA-Lys gene must have followed the duplication event, if this is the expected scenario (Moritz et al. 1987, Boore & Brown 1994, Macey et al. 1997, Boore & Brown 1998b, Groth et al. 2000, Tomita et al. 2002). This speculation is based on the suggestion that tRNA genes could be considered as mobile elements, due to the frequently observed tRNA rearrangements seen in mitochondrial genomes (Moritz et al. 1987, Saccone et al. 2002). The fact that the two sequences are very divergent does, however, not support a tRNA-Lys duplication hypothesis. On the other hand, the duplication event could be ancient, and the initial sequence resemblance could therefore have been eroded by substitutions.

In marsupial the mitochondrial tRNA-Lys is not funtional, and a nuclear-encoded version is therefore imported into the mitochondria (Dörner et al. 2001). Several unusual features did, however, indicate that the mitochondrial tRNA-Lys gene found in marsupials was a pseudogene. An alignment of the mitochondrial tRNA-Lys gene sequences revealed that several marsupials do not possess the anticodon (UUU) for lysine-tRNA (Dörner et al. 2001). Furthermore, loss of several conserved nucleotides in the inferred tRNA secondary

structure was also observed. Although this situation is in marked contrast to that found in *L. salmonis* we cannot at present exclude the possibility that one tRNA-Lys gene might not be functional in *L. salmonis*.

### *Population structure of* L. salmonis

Our study on the population genetic structure of *L. salmonis* revealed an extremely high level of intraspecific variation in the four mitochondrial genes *A6, COI, Cyt B* and 16S rRNA (Paper II). Despite this, no genetic differentiation was observed between *L. salmonis* sampled along the Norwegian coast, or between *L. salmonis* from Norway, Scotland and Russia (Paper II). Passive transport of *L. salmonis* larvae by ocean currents and the migratory pattern of the salmonid hosts are the two factors assumed to contribute to the high gene flow observed in the North Atlantic. A weak indication that *L. salmonis* sampled in Canada might be different from *L. salmonis* in the northeast Atlantic was, however, observed when the sequences were pairwise compared (Paper II). Furthermore, *L. salmonis* from the Pacific Ocean (Japan) was clearly distinct from the six Atlantic samples (Paper II).

Data from previous studies have indicated that *L. salmonis* displays population genetic differentiation in both Norway (Isdal et al. 1997) and Scotland (Todd et al. 1997, Dixon et al. 2004). *Lepeophtheirus salmonis* sampled from Norway, Scotland and Ireland have also been demonstrated to be genetically different (Nolan et al. 2000). Phenotypic plasticity where enzymes are differently expressed during different life-stages, or as a consequence of environmental factors may perhaps explain the results obtained by Isdal et al. (1997). Moreover, the possibility for contamination from the epidermal host mucus, host blood, bacterias or from epibionts on *L. salmonis* must be regarded as high in the RAPD studies which differentiated Scottish samples (Todd et al. 1997, Dixon et al. 2004). Contamination of *L. salmonis* samples with foreign DNA certainly would influence on the resulting analysis,

and this could also explain why the RAPD results published by Todd et al. (1997) have not been possible to reproduce (Dixon et al. 2004). One of the two informative microsatellites published by Nolan et al. (2000) has also been used in a more extensive population genetic study, including six microsatellite markers (Todd et al. 2004). The results from this study contradict the existence of distinct *L. salmonis* populations in the North Atlantic (Todd et al. 2004), results that are also supported by our findings using mitochondrial genes (Paper II).

Data from the present study suggests that a high level of genetic exchange exist between *L. salmonis* samples along the Norwegian coast (Paper II). This supports the deduction commonly made for marine invertebrates in that the inclusion of a planktonic larva in the life cycle confers dispersal potential, and extensive gene flow between samples resulting in genetic homogeneity (e.g. Johnson & Black 1982, Hunt & Ayre 1989, Liu et al. 1991, Hunt 1993, Williams & Benzie 1993, Silberman et al. 1994, Ayre 1995). *Lepeophtheirus salmonis* has three free-living stages in its life cycle (Schram 1993), and the copepodids can survive up to 23 days at 8°C and 30 days at 6°C (Boxaspen 2005). Furthermore, both the nauplia and copepodids are presumed to be centered in the upper meters of the water column (Heuch et al. 1995, Costelloe et al. 1996), and the *L. salmonis* larvae are therefore expected to be dispersed over considerable distances due to passive transport by ocean currents (see Costelloe et al. 1996, Costelloe et al. 1998, Asplin et al. 1999, Bucklin et al. 2000, Pedersen et al. 2001, Tully & Nolan 2002). Two streams are dominating along the Norwegian coast, the Norwegian Atlantic Current and the Norwegian Coastal Current (Figure 8). The Norwegian Atlantic Current has one branch that deflects and enters through the Faroe-Shetland channel encountering the Norwegian Coastal Current, while another branch deflects west of Møre going southwards (Figure 8) (Poulain et al. 1996, Fosså 2001, Pedersen et al. 2001). The Norwegian Coastal Current, on the other hand, originates

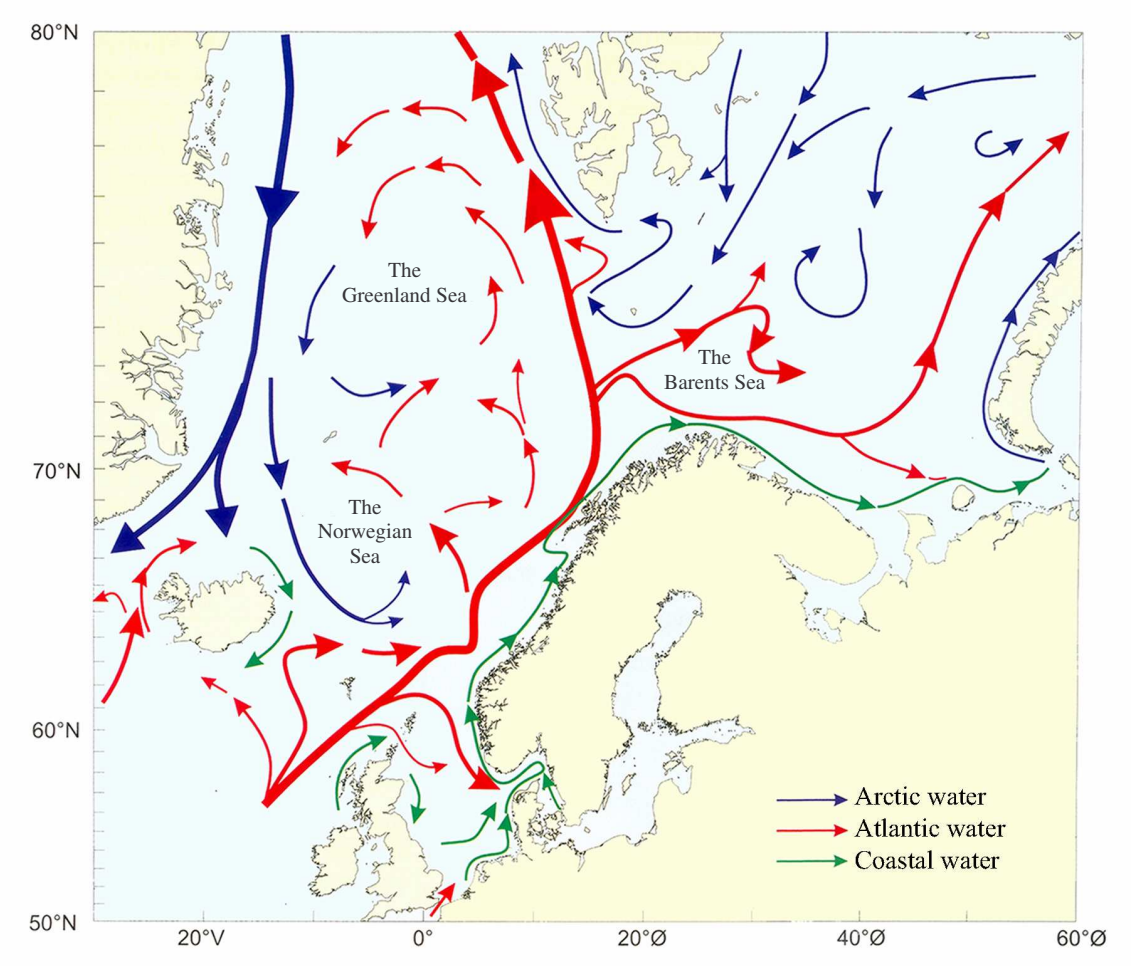


**Figure 8**: The mean current system of the Norwegian Sea, Greenland Sea and Barents Sea (Fosså 2001).

from the Baltic Sea transporting water from Skagerrak northward along the Norwegian coast (Figure 8) (Pedersen et al. 2001, Asplin et al. 2002). It is characterized as a stream being very variable in direction, resulting in a high exchange of water in the fjords (Asplin et al. 2002). Consequently, *L. salmonis* larvae from different fjords are likely to be mixed, and an accumulation of larvae may be achieved by the many gyres resulting from this stream (see Kaartvedt 1993). The Norwegian Coastal Current is also characterized as a very strong current. However, the speed varies extensively, depending on the weather conditions, but can range from 20 cm/s to 1 m/s (Pers. com. Lars Asplin, Institute of Marine Research, Bergen, Norway). In conclusion, the three free-living stages of the *L. salmonis* can last 3-4 weeks making it reasonable to assume that passive transport of nauplius and copepodid larva by the

Norwegian Coastal Current, and the Norwegian Atlantic Current, contributes greatly to the high gene flow observed between *L. salmonis* samples along the Norwegian coast.

Another aspect of *L. salmonis* dispersion to be considered is the contribution from wild salmonids, including escaped farm salmon. Wild salmon are assumed to contribute extensively to the spreading of *L. salmonis* throughout the North Atlantic (Todd et al. 2004). In Norway, *L. salmonis* infestations of wild salmon occur when the post-smolts migrate through the fjords and coastal areas on their way to the feeding grounds (Finstad et al. 2000, Todd et al. 2000). In contrast to this, the migratory pattern of escaped farm salmon is poorly known. Studies indicate that many return to their release location, but a large number (20-40%) also migrate to the oceanic feeding grounds (Hansen et al. 1987, Hansen et al. 1993, Jacobsen & Gaard 1997, Butler 2002). However, prior to seaward migration escaped farm salmon may spend a considerable amount of time in coastal waters, which can result in heavy infestations with *L. salmonis* (Jacobsen & Gaard 1997, Heuch & Mo 2001, Butler 2002). This further leads to the assumption that escaped farm salmon transfer increasing numbers of *L. salmonis* to wild salmon in the open seas (see Jacobsen & Gaard 1997), since high burdens of lice have been found on salmon in this area (Holst et al. 1993, Jacobsen & Gaard 1997). Salmon from most countries bordering the North Atlantic utilize the area north of the Faroe Islands during their oceanic feeding phase (Jacobsen & Gaard 1997). A study examining the distribution, migratory pattern and origin of wild salmon caught outside the Faroes reported that 40% of the recaptured salmon were of Norwegian origin, while 20% originated from both Scotland and Russia (Hansen & Jacobsen 2003). Salmon from Canada were also caught in this same feeding area (Hansen & Jacobsen 2003). This suggests an explanatory mechanism for the overall genetic similarity found, in our study, between *L. salmonis* sampled from Norway, Scotland, Russia and Canada (Paper II). The presence of both chalimus and pre-adult stages of *L. salmonis* on wild salmon throughout the winter months, and the increasing

abundance and density of *L. salmonis* with the sea age of the wild salmon indicate that infestation occurs at the feeding ground (Jacobsen & Gaard 1997). Furthermore, salmon returning from the sea in summer and autumn re-infect the coastline, and in this way contribute to the high gene flow observed between *L. salmonis* samples throughout the North Atlantic (Paper II).

Sea trout spend from 1 to 5 years in freshwater before migrating to coastal waters to feed. Once in sea, the trout are largely coastal in their habitat, but some fish have the ability to migrate over variable distances (Pemberton 1976, Butler 2002). For instance, in Scotland tagged sea trout have been recaptured 126 km from their starting point (Butler 2002). Hence, the possibility that infected sea trout migrating at large distances can contribute to the high gene flow observed between *L. salmonis* samples in the North Atlantic cannot be excluded.

Although the overall result show that there is no differentiation between *L. salmonis* samples throughout the North Atlantic a pairwise sequence comparison indicated that a weak, but significant, differentiation might exist between the European samples and *L. salmonis* sampled from the east-coast of Canada (Paper II). These results contrast with the results published by Todd et al. (2004) based on data from six microsatellite markers. It is, however, expected when applying markers with a high mutation rate, like the mtDNA, that some individual low frequency alleles might appear (see Neigel 1997), and this may explain why a weak difference was observed between *L. salmonis* collected from Canada and several locations in Europe (Paper II). This explanation is supported by the insignificant values displayed when the analysis was performed on haplotypes based only upon the most frequent polymorphisms (Paper II).

Despite the genetic similarity, it is clear that the mitochondrial genes used in this study can detect barriers to gene flow where they exist (Paper II). *Lepeophtheirus salmonis* sampled from wild chum salmon (*Oncorhynchus keta*) in the Pacific Ocean (Ishikari, Japan) was

clearly distinct from the Norwegian, Scottish, Russian and Canadian samples, when all four mitochondrial genes were aligned (Paper II). This suggests geographic isolation or strongly reduced gene flow between *L. salmonis* in the Pacific Ocean and the North Atlantic, as expected, but a possible adaptation to the host *Oncorhynchus* could also partly explain this (Paper II). Wild salmonids found in the North Atlantic are in the genera *Salmo* and *Salvelinus*, while the species present in the Pacific Ocean is mostly within the genera *Oncorhynchus* (e.g. Nagasawa & Takami 1993, Nagasawa 2004)

***High intraspecific variation in* L. salmonis *mitochondrial genes***

The four mitochondrial genes *A6, COI, Cyt B* and 16S rRNA of *L. salmonis* contained extremely high levels of genetic variation (Paper II). Highest intraspecific variation was found within *A6* where 17.5% of the nucleotides were polymorphic followed by 15.9% in *COI*, 14.4% in *Cyt B* and 10.6% in 16S rRNA. This high variation is most likely a consequence of a large *L. salmonis* population size caused by the higher accessibility of hosts, introduced by the salmon farming industry (Paper II). A large population size will result in a higher amount of low frequency haplotypes being present within the sample, and thereby lead to a higher genetic variation. When we compare the intraspecific variation within each of the six locations in the North Atlantic, large differences are observed among them. In *A6* the variation ranged from 5.6-7.1%, in *COI* from 5.2-6.8%, in *Cyt B* from 3.6-5.8% and in 16S rRNA from 2.9-4.6%. However, a comparison of the North Atlantic samples with *L. salmonis* from wild chum salmon (*Oncorhynchus keta*) caught in the Pacific Ocean revealed an intraspecific variation that seems to be noticeably lower (2.5% in *A6*, 1.4% in *COI*, 4.9% in *Cyt B* and 0.9% in 16S rRNA). This support the assumption that the high intraspecific variation found in the North Atlantic is due to a large *L. salmonis* population size. Although *L. salmonis* occurs on farmed coho salmon (*Oncorhynchus kisutch*) and rainbow trout

(*Oncorhynchus mykiss*) in Japan, it is not a serious problem for the farming industry (Nagasawa 2004). This is partly explained by the fact that these salmonids seem less susceptible to *L. salmonis* infestations (Nagasawa 2001, 2004). Still, *L. salmonis* is a common parasite of wild chum and pink salmon, and it is believed that these are the most important hosts for *L. salmonis* in the Pacific Ocean (Nagasawa 2001, 2004). The abundance of the wild salmonids varies, however, greatly from year to year resulting in a large influence on the *L. salmonis* population in this region (Nagasawa 2001).

On the other hand, it cannot be excluded that the high level of intraspecific variation observed within the four mitochondrial genes of *L. salmonis* could also be a consequence of negative selection (Paper II). That is, the high level of low frequency haplotypes present within the *L. salmonis* samples could be a result of an accumulation of slightly deleterious polymorphisms (Fry 1999, Blier et al. 2001, Fay et al. 2001). The use of delousing pesticides, in the combat against *L. salmonis*, may result in an artificial selection where negative, rather than positive traits, could be selected for.

### *Phylogenetic studies of* L. salmonis

The expansion regions within 28S rRNA are highly variable, and sequence variations could therefore be present within this gene from *L. salmonis*. This was, however, not the case. Amplification of 3692 bp of 28S rRNA from *L. salmonis* sampled in Norway and Japan did not reveal any sequence variation, the same as found using 18S rRNA (Hodneland et al. unpublished). Furthermore, characterization of the NTS region, by screening a *L. salmonis* genomic library, was unsuccesful.

The 28S rRNA was used in a phylogenetic study to find the position of *L. salmonis* in relation to other crustacean, and arthropod, species (Paper III). Since 18S rRNA is often used to analyse arthropod relationships (Martin & Davis 2001) two phylogenies based on this gene

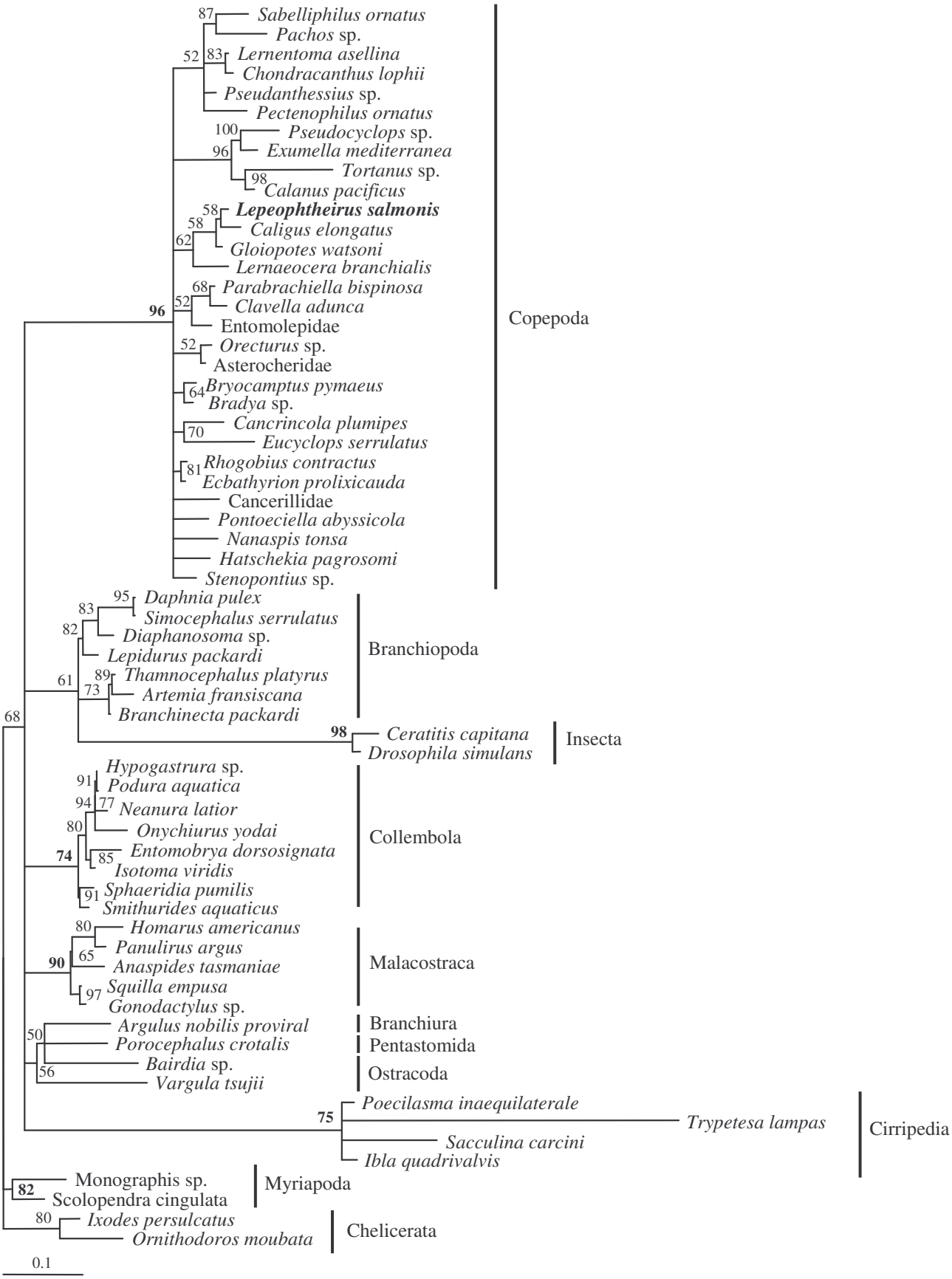


**Figure 9**: Arthropod phylogeny based on 18S rRNA, with Chelicerata as outgroup. The maximum-likelihood tree was constructed in Tree-Puzzle 5.2 using HKY with gamma distribution, and 2500 puzzling steps (values presented above branches). The Copepoda form a highly supported monophyletic group, but their relationship to other arthropods included is not resolved. Other supported groups are the Cirripedia, Malacostraca, Collembola, Insecta and Myriapoda.

were performed to compliment the 28S rRNA phylogeny. Moreover, *COI* has been classified as a reliable marker in resolving distant phylogenetic relationships among vertebrates (Zardoya & Meyer 1996), and a *COI* phylogeny was thus also performed. The 18S rRNA phylogeny included 64 species representative of the four arthropod groups (see Appendix, Table 2), but the phylogeny is not well resolved (Figure 9). Despite this poor resolution it is clearly demonstrated that the copepods included constitute a highly supported monophyletic group with a quartet puzzle (QP) bootstrap value of 96. In addition to this, several other groups are also supported (QP>70%). These include Cirripedia (QP bootstrap=75), Malacostraca (QP bootstrap=90), Collembola (QP bootstrap=74), Insecta (QP bootstrap=98) and Myriapoda (QP bootstrap=82). The position of the copepods in relation to the other arthropods included is, however, not resolved.

To better resolve the relationships within the Copepoda a new 18S rRNA phylogeny was performed (Figure 10) where only members from the Copepoda are included. This phylogeny place all the caligids in one highly supported group (QP bootstrap=84). *Lepeophtheirus salmonis* group together with *C. elongatus* (QP bootstrap=90), with *G. watsoni* as a sister-group to them. However, the relationships within the Siphonostomatoida are not resolved, as is also the case for Cyclopoida, Harpacticoida and Poecilostomatoida. On the other hand, the Calanoida form a group with high support (QP bootstrap=85).

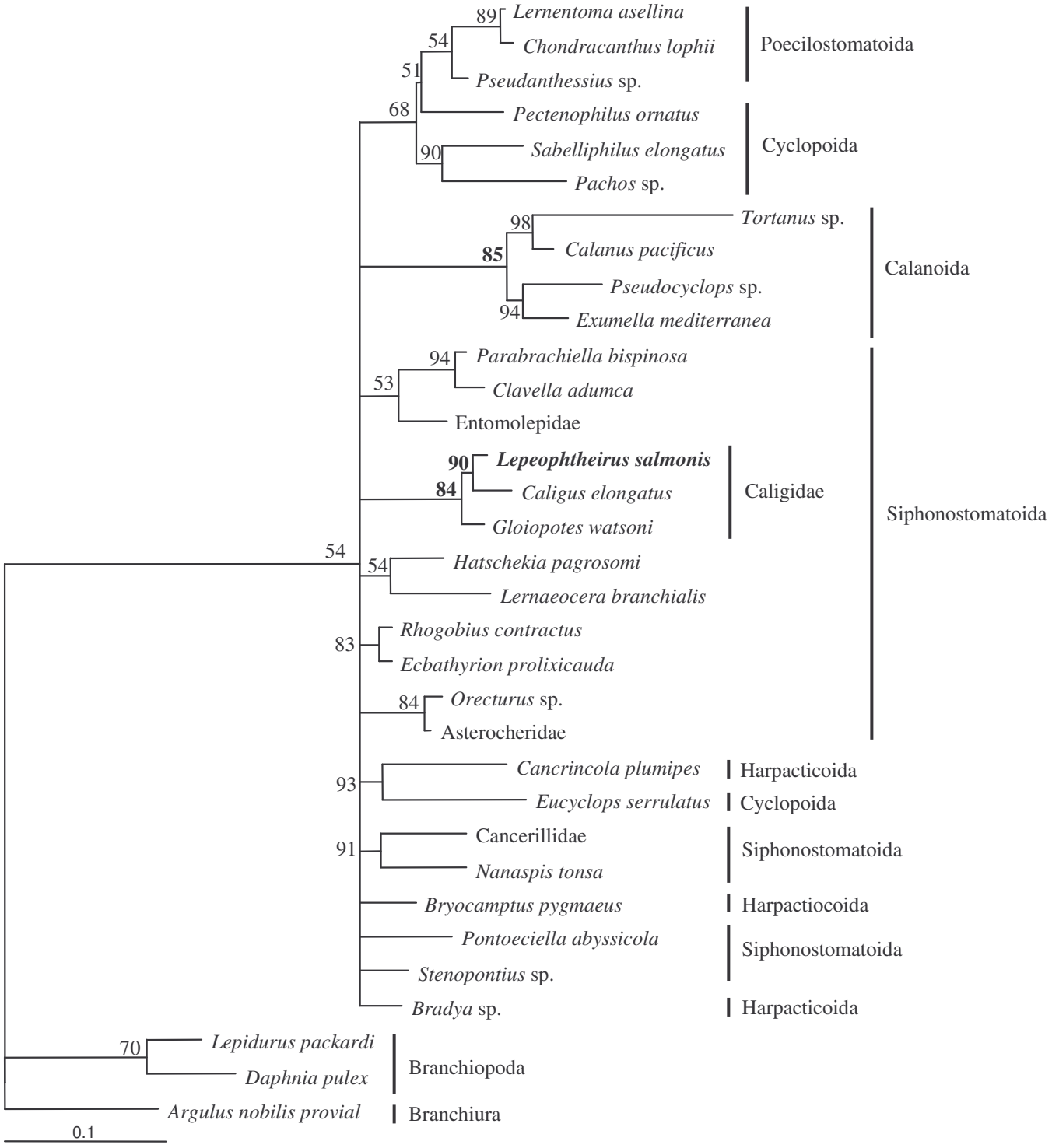


**Figure 10**: Copepod relationships inferred from 18S rRNA, with Branchiura and two branchiopods as outgroups. The maximum-likelihood tree was constructed in Tree-Puzzle 5.2 using the HKY model with gamma distribution, and 10 000 puzzling steps (values presented above branches). The tree is not well resolved, but the three caligids are placed in one distinct group. Moreover, the Calanoida constitute a highly supported group.

Low resolution have also been the result of previous phylogenetic studies using 18S rRNA (e.g. Turbeville et al. 1991, Abele et al. 1992, Spears & Abele 1997, Mallatt et al. 2004). Within Crustacea a phylogenetic analysis of 18S rRNA from selected maxillopodan suggested that the copepods are more closely related to the cirripeds than previously thought (Abele et al. 1992). However, the data set were analyzed in different ways, and only one of the four trees supported this relationship (Abele et al. 1992). Another phylogenetic study using 18S rRNA made it impossible to speculate in maxillopodan, crustacean or arthropod relationships due to low resolution (Spears & Abele 1997). Still, species from the same crustacean taxa grouped together with high support, as was also the case in this study (see figure 9). Consequently, the phylogenetic performance of the rRNA genes has been questioned (e.g. Spears & Abele 1997, Giribet & Ribera 2000). On the other hand, it has often been concluded within arthropod phylogeny, based on rDNA sequences, that inclusion of more taxa or more data per taxon might result in improved resolution (Spears & Abele 1997). This was, however, not the case in our analysis where inclusion of a substantial number of arthropod species in the 18S rRNA phylogeny did not improve the resolution (Figure 9). In comparison the copepod and arthropod phylogenies based on 28S rRNA were much better resolved (Paper III). Both 28S rRNA phylogenies place *L. pollachius* as the closest relative to *L. salmonis*. *Caligus elongatus* and *C. curtus* constitute a sister-group to the two *Lepeophtheirus* species, forming a monophyly of the Caligidae. Siphonostomatoida is also supported using 28S rRNA, with the Poecilostomatoida being the closest relative of the taxa represented followed by Cyclopoida. The Copepoda form a monophyletic group, with the Hexapoda as their closest relative. This phylogeny also supports the morphological copepod phylogeny proposed by Huys and Boxshall (1991) (Figure 3) in placing the Cyclopoida as a sister-group to Siphonostomatoida and Poecilostomatoida. In contrast, the copepod phylogeny proposed by Kabata (1979) is not supported. They placed the Siphonostomatoida as the most

acient group, which constituted a sister-group to the Poecilostomatoida-Cyclopoida clade (Figure 2).

*COI* did not give any phylogenetic resolution for the arthropods included in this study (see Appendix, Table 3). This applied to phylogenies based on both nucleotide and amino acid sequences (data not presented), although the latter did group the copepods as monophyletic with a support of 82%.

*COI* also resulted in relatively low-resolution phylogeny when only the copepods were included in the analysis (Figure 11). However, the caligids are placed in one group with high support (QP bootstrap=97), where *L. salmonis* group together with *L. pectoralis* (QP bootstrap=92). Furthermore, the three *Caligus* species (*C. curtus*, *C. centrodonti* and *C. elongatus*) constitute a sister-group to *Lepeophtheirus* spp. The closest relative to the Caligidae is the Harpacticoid species, *Cletocamptus helobius*, but the support for this relationship low (QP bootstrap=65). Two other harpacticoids is placed as outgroup to the Calanoida, also with low support (QP bootstrap=57), resulting in a polyphyletic Harpacticoida. Still, Calanoida forms a group with high support (QP bootstrap=91). Due to low support for many of the branches, it is not possible to compare this analysis with the copepod relationships proposed by Kabata (1979) (Figure 2), or Huys and Boxshall (1991) (Figure 3). The caligid phylogeny proposed by Øines et al (2005), based on *COI*, is not well supported, and many of the species were not identified. Hence, a comparison with this study is of little value.

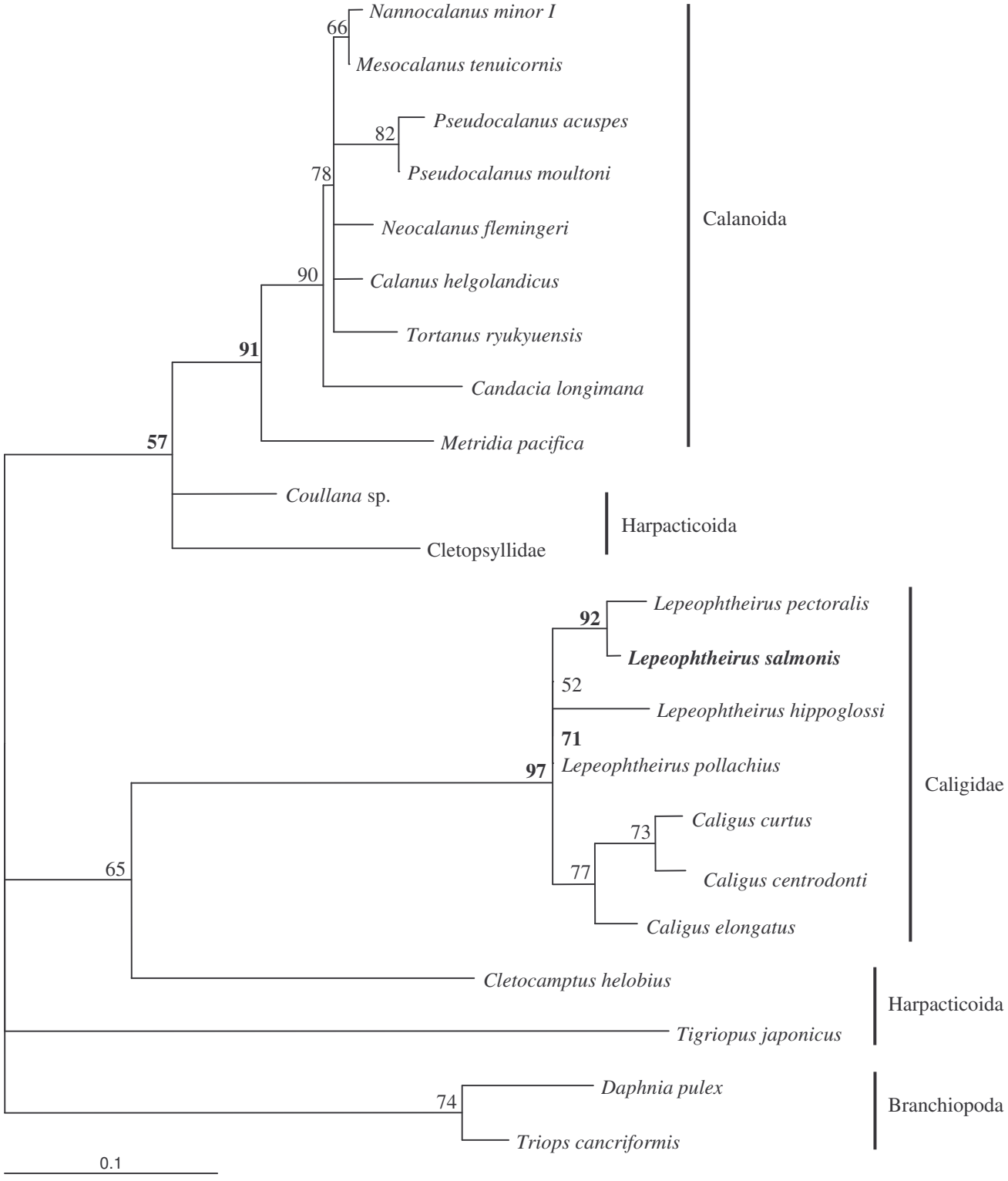


**Figure 11**: The relationships within Copepoda inferred from the amino acid sequences of *COI* with two branchiopods as outgroup. The maximum-likelihood tree was constructed in Tree-Puzzle 5.2 using the HKY model with gamma distribution, and 1000 puzzling steps (values presented above branches). The phylogeny groups *L. salmonis* together with *L. pectoralis*, being close relatives to *Caligus* spp.

For more than a century, phylogenetic analyses of crustaceans have shown a high degree of diversity. It has therefore been implied that crustacean phylogeny can only be sensibly discussed in the framework of arthropod relationships (Giribet et al. 2005). However, only a few of the molecular phylogenies that deal with arthropods use a broad sample of crustacean taxa (Giribet et al. 2001, Regier & Shultz 2001, Nardi et al. 2003, Bitsch et al. 2004, Lavrov et al. 2004, Mallatt et al. 2004, Regier et al. 2004, Giribet et al. 2005, Regier et al. 2005). Within Copepoda very few molecular systematic studies have been published (Bucklin et al. 1992, Bucklin et al. 1995, Braga et al. 1999, Bucklin et al. 1999, Hill et al. 2001, Bucklin et al. 2003, Øines & Heuch 2005), and the position of copepods in relation to other crustaceans have only been inferred in a few cases (Abele et al. 1992, Spears & Abele 1997). Furthermore, the molecular phylogenies based on 18S rRNA (Figure 9 and 10) and *COI* (Figure 11) presented in this thesis, in addition to 28S rRNA (Paper III), showed quite different results. Although *COI* is considered to be a very conserved protein, and has been classified as a good phylogenetic marker for resolving distant vertebrate relationships (Zardoya & Meyer 1996), a high intraspecific variation resulting in several amino acid substitutions was found within this gene in *L. salmonis* (Paper II). It was therefore not unexpected that the phylogenies based on *COI* resulted in low resolution, since this gene seems to be too polymorphic to resolve deeper copepod relationships (Figure 11). While the analysis performed on 18S rRNA was not able to resolve the relationships within Copepoda or copepods relationship to other crustaceans (Figure 9 and 10), the analysis of 28S rRNA resulted in a better resolution (Paper III). This is in agreement with a previous phylogenetic study where the performance of 18S and 28S rRNA was compared, and higher resolution was achieved using 28S rRNA due to the contribution of more phylogenetic signal (Mallat et al 2004). The copepod phylogeny based on 28S rRNA (Paper III) also supported the morphological phylogeny proposed by Huys and Boxshall (1991). However, the support for

Cyclopoida is low, and more taxa must be included to better infer the relationships within the Copepoda.

# CONCLUSION

The main goal of this project was to characterize both mitochondrial and nuclear genes, and use them to study the population genetic structure of *L. salmonis* throughout the North Atlantic. Whereas the nuclear 28S rRNA gene was not suited for detecting any differentiation between *L. salmonis* samples, the four mitochondrial genes *A6, COI, Cyt B* and 16S rRNA clearly differentiated between *L. salmonis* sampled in the North Atlantic and the Pacific Ocean, as expected. No genetic differentiation was, however, demonstrated between *L. salmonis* sampled throughout the North Atlantic (Norway, Scotland, Russia and Canada). The migratory pattern of the salmonid hosts and passive transport of *L. salmonis* larvae by ocean currents are believed to contribute to this high gene flow. These results have implications for the salmon farming industry in different ways. In order to use pesticides in the combat against *L. salmonis*, delousing must be synchronised over a broader geographic area than what is currently done. The most important implication is, however, that the potential for spreading of genes associated with possible resistance is extensive, considering that *L. salmonis* can be dispersed over large geographic distances.

## FUTURE PERSPECTIVES

Based on the results presented in this thesis, several aspects warrant further investigation:

- Characterization of the *L. salmonis* mitochondrial genome revealed a completely new gene organization within Crustacea (Paper I). It is currently difficult to specify which mechanisms are responsible for this novel gene order, but characterization of more mitochondrial genomes from closely related caligid species may provide some answers.
- Two tRNA-Lys genes are present within the mitochondrial genome of *L. salmonis* (Paper I). One way of investigating whether the tRNA-Lys duplication is caused by duplication of tRNA-Lys itself, or if it is a result of tRNA gene recruitment, would be to perform a phylogentic comparison of mitochondrial tRNAs from several closely related caligid species. Knowledge about the origin of the two tRNA-Lys genes found in *L. salmonis* may give insight into the mechanisms resulting in the frequently observed mitochondrial tRNA rearrangements. Furthermore, it will also give support to previously proposed mechanisms involved in tRNA evolution.
- In this study, weak genetic differentiation was observed between the European and the Canadian *L. salmonis* samples (Paper II). This indication contrast with the recent data published by Todd et al. (2004) using microsatellites. It would therefore be interesting to use their microsatellite markers on our *L. salmonis* samples, for an evaluation of these results. Markers associated with genes that are targets for development of resistance could perhaps reveal differentiation between *L. salmonis* samples. There are already some indications that a sodium channel gene, which is the target gene for pyrethroids, might differentiate between North Atlantic *L. salmonis* samples (Pers. com Anders Fallang, Norwegian School of Veterinary Science, Oslo, Norway), and this approach should be tested.

- The 28S rRNA gene seems very promising for resolving copepod relationships (Paper III). Nevertheless, this needs to be verified by including more copepods representative of several orders, families and genera. Moreover, the mitochondrial gene order has been found to vary across the Metazoa, generating interest in using this for phylogenetic inference (see Boore et al. 1998a, Boore & Brown 1998b, Wilson et al. 2000, Lavrov et al. 2004). So far, different mitochondrial gene orders have been found within the four copepods characterized, or partially characterized. A phylogeny based on mitochondrial gene orders from different copepods could therefore be interesting to perform, for inferring copepod relationships, when more genomes have been sequenced.

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## APPENDIX

**Table 1:** Mitochondrial genomes characterized within the phylum Crustacea.

| Species | Accession number |
|---|---|
| *Argulus americanus* | NC_005935 |
| *Armillifer armillatus* | NC_005934 |
| *Artemia franciscana* | NC_001620 |
| *Callinectes sapidus* | NC_006281 |
| *Cherax destructor* | NC_011243 |
| *Daphnia pulex* | NC_000844 |
| *Eriocheir sinensis* | NC_006992 |
| *Geothelphusa dehaani* | NC_007379 |
| *Gonodactylus chiragra* | NC_007442 |
| *Harpiosquilla harpax* | NC_006916 |
| *Hutchinsoniella macracantha* | NC_005937 |
| *Lepeophtheirus salmonis* | NC_007215 |
| *Lysiosquillina maculata* | NC_007443 |
| *Macrobrachium rosenbergii* | NC_006880 |
| *Marsupenaeus japonicus* | NC_007010 |
| *Megabalanus volcano* | NC_006293 |
| *Pagurus longicarpus* | NC_003058 |
| *Panulirus japonicus* | NC_004251 |
| *Penaeus monodon* | NC_002184 |
| *Pollicipes polymerus* | NC_005936 |
| *Portunus trituberculatus* | NC_005037 |
| *Pseudocarcinus gigas* | NC_006891 |
| *Speleonectes tulumensis* | NC_005938 |
| *Squilla empusa* | NC_007444 |
| *Squilla mantis* | NC_006081 |
| *Tetraclita japonica* | NC_008974 |
| *Tigriopus japonicus* | NC_003979 |
| *Triops cancriformis* | NC_004465 |
| *Triops longicaudatus* | NC_006079 |
| *Vargula hilgendorfii* | NC_005306 |

**Table 2**: Sequences included in the phylogenies based on 18S rRNA, in alphabetical order.

| **Species** | **Accession numbers** | **Species** | **Accession numbers** |
|---|---|---|---|
| *Anaspides tasmaniae* | L81948 | *Lepidurus packardi* | L34048 |
| *Argulus nobilis proviral* | M27187 | *Lernaeocera branchialis* | AY627030 |
| *Artemia franciscana* | AJ238061 | *Lernentoma asellina* | AY627003 |
| Asterocheridae sp. | AY627018 | Monographis sp. | AY596371 |
| Bairdia sp. | L81943 | *Nanaspis tonsa* | AY627029 |
| Bradya sp. | AY627016 | *Neanura latior* | AY037172 |
| *Branchinecta packardi* | L26512 | Orecturus sp. | AY627017 |
| *Bryocamptus pygmaeus* | AY627015 | *Onychiurus yodai* | AY037171 |
| *Calanus pacificus* | L81939 | *Ornithodoros moubata* | L76355 |
| *Caligus elongatus* | AY627020 | Pachos sp. | AY627014 |
| *Cancerillidae sp.* | AY627021 | *Panulirus argus* | AY743955 |
| *Cancrincola plumipes* | L81938 | *Parabrachiella bispinosa* | AY627027 |
| *Ceratitis capitata* | AH006961S1 | *Pectenophilus ornatus* | AY627032 |
| *Chondracanthus lophii* | L34046 | *Podura aquatica* | AF005452 |
| *Clavella adunca* | AY627028 | *Poecilasma inaequilaterale* | AY520654 |
| *Daphnia pulex* | AF014011 | *Pontoeciella abyssicola* | AY627031 |
| Diaphanosoma sp. | AF144210 | *Porocephalus crotali* | M29931 |
| *Drosophila simulans* | AY037174 | Pseudanthessius sp. | AY627007 |
| *Ecbathyrion prolixicauda* | AY627024 | Pseudocyclops sp. | AY626994 |
| *Entomobrya dorsosignata* | AY596360 | *Rhogobius contractus* | AY627023 |
| Entomolepidae sp. | AY627025 | *Sabelliphilus elongatus* | AY627010 |
| *Eucyclops serrulatus* | L81940 | *Sacculina carcini* | AY520656 |
| *Exumella mediterranea* | AY629259 | *Scolopendra cingulata* | U29493 |
| *Gloiopotes watsoni* | AY627019 | *Simocephalus serrulatus* | AF144216 |
| Gonodactylus sp. | L81947 | *Sminthurides aquaticus* | AY596364 |
| *Hatschekia pagrosomi* | AY627026 | *Sphaeridia pumilis* | AY145140 |
| *Homarus americanus* | AY743945 | *Squilla empusa* | L81946 |
| Hypogastrura sp. | AY596362 | Stenopontius sp. | AY627022 |
| *Ibla quadrivalvis* | AY520655 | *Thamnocephalus platyurus* | AF144218 |
| *Isotoma viridis* | AY596361 | Tortanus sp. | AY626995 |
| *Ixodes persulcatus* | AY274888 | *Trypetesa lampas* | L26520 |
| *Lepeophtheirus salmonis* | AF208263 | *Vargula tsujii* | DQ096577 |

**Table 3**: Protein sequences included in the phylogeny of *COI*, in alphabetical order.

| **Species** | **Accession numbers** | **Species** | **Accession numbers** |
|---|---|---|---|
| *Artemia parthenogenetica* | AAX54683 | *Gonodactylaceus caldwelli* | AAG29090 |
| *Calanus helgolandicus* | AAT99460 | *Heterosaccus lunatus* | AAY59885 |
| *Caligus centrodonti* | AAW81049 | *Ixodes persulcatus* | BAC22597 |
| *Caligus curtus* | AAW81045 | *Lepeophtheirus hippoglossi* | AAW81041 |
| *Caligus elongatus* | AAW81050 | *Lepeophtheirus pollachius* | AAW81042 |
| *Candacia longimana* | AAN16096 | *Lepeophtheirus salmonis* | YP_271852 |
| *Ceratitis capitata* | AAY34455 | *Lirceolus bisetus* | AAW32901 |
| *Cherax destructor* | AAR37034 | *Lithobius forficatus* | CAC69937 |
| *Cletocamptus helobius* | AAK63000 | *Mesocalanus tenuicornis* | AAL68664 |
| Cletopsyllidae sp. | AAQ97371 | *Metridia pacifica* | AAL84604 |
| Coullana sp. | AAK63001 | *Nannocalanus minor I* | AAG53450 |
| *Cyamus erraticus* | AAZ05871 | *Neocalanus flemingeri* | AAG53442 |
| *Dahlella caldariensis* | AAC47571 | *Neoverruca brachylepadoformis* | BAD98495 |
| *Daphnia magna* | AAV67773 | *Ornithodoros moubata* | BAC22581 |
| *Daphnia pulex* | AAQ90458 | *Panulirus japonicus* | NP694520 |
| *Daphniopsis pusilla* | AAM47518 | *Penaeus monodon* | NP038289 |
| *Drosophila yakuba* | CAC14066 | *Pseudocalanus acuspes* | AAL68665 |
| *Gammarus oceanicus* | AAX22163 | *Pseudocalanus moultoni* | AAG53453 |
| *Gomphiocephalus hodgsoni* | AAO43659 | | |

# Paper I

# Paper II

# Paper III