Molecular Studies on *Sphaerospora truttae* and Other Freshwater Myxozoans

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Doctor of Philosophy

By

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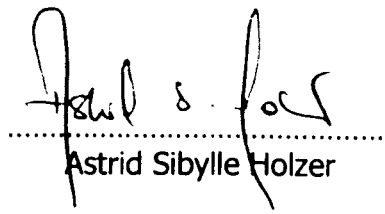
DECLARATION

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted, nor submitted for any other degree.

All sources of information have been duly acknowledged.



Astrid Sibylle Holzer

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ABSTRACT

This study investigates the life cycle of *Sphaerospora truttae,* a myxozoan parasite of the Atlantic salmon, using molecular methods based on the 18S rDNA. DNA sequencing showed that the 18S rDNA of *S. truttae* differs substantially from the sequence obtained from its proposed alternate actinosporean life cycle stage, *Echinactinomyxon* type 5. With more than 90% sequence identity *Echinactinomyxon* type 5 is closely related to *Myxobolus portucalensis* whereas *S. truttae* with an extraordinary long 18S sequence (2541 bp), with inserts in the variable regions of the gene, does not relate closely to any myxozoans. On the basis of the obtained sequence for *S. truttae,* a single round nested PCR assay was developed which allows low-level detection and specific identification of *S. truttae* in all life cycle stages. Furthermore, two of the primers from the PCR assay were successfully used on tissue sections in an optimised *in situ* hybridisation (ISH) protocol. ISH experimentally identified the gills as the predominant entry locus of *S. truttae* into the fish host and it detected the spatio-temporal migration of the parasite via the vascular system into the target organ, the kidney. The ISH protocol and the PCR assay were also used to screen oligochaetes and other co-occurring invertebrates for *S. truttae* infection but an alternate host for *S. truttae* could not be identified. However, 12 actinosporean stages were found and they were characterized on the basis of their 18S rDNA, together with 9 further myxosporean species from wild fish in the same riverine habitat. Three actinosporeans were found to be genetically identical with three myxosporeans (*Myxidium truttae, Chloromyxum truttae* and *Chloromyxum* sp.) and thus represent alternate life cycle stages of these species. Phlyogenetic analysis of the myxozoans identified a very basal position of *S. truttae* and *S. elegans,* as a sister group to the marine species. All other species were nested in the freshwater clades and clustered according to host tissue localization, but independent from host species or myxozoan spore taxonomy.

SUMMARY

This thesis investigates different aspects of the life cycle of the myxozoan *Sphaerospora truttae* Fischer-Scherl, El-Matbouli & Hoffmann, 1986, a kidney parasite of Atlantic salmon, *Salmo salar* L., using molecular methods on the basis of 18S rDNA sequences, as well as molecular relationships between *S. truttae* and other myxozoans sharing the same riverine habitat in the Scottish highlands.

The development of a new method for the separation of myxosporean spores from fish tissues, employing an aquaeous bi-phase system of 5% polyethylene glycol and 4% dextran, allowed the successful isolation of myxosporean spores of *S. truttae* from kidneys of Atlantic salmon for 18S rDNA sequencing. The molecular characterisation of these spores and the proposed actinosporean stage of *S. truttae,* i.e. *Echinactinomyxon* type 5, released from the oligochaete host, proved that these two organisms share less than 50% of identical base pairs and they therefore have to be regarded as two different species. *Echinactinomyxon* type 5 is closely related to *Myxobolus portucalensis* Saraiva & Molnar, 1990 (92.8% sequence identity) and other members of the genus *Myxobolus* (80% sequence identity), whereas *S. truttae* with its extraordinarily long 18S rDNA sequence, does not show a close relationship with other myxozoans (≤50% sequence identity) although a phylogenetic analysis clearly confirmed the myxozoan identity of *S. truttae* (100% bootstrap support).

The 18S sequence of *S. truttae* was subsequently used to design four specific primers in the variable regions of the sequence. These primers were used in a single round nested PCR assay which proved a valuable tool for the specific identification, early and low-level detection of *S. truttae* in its host.

Furthermore, two of the four primers were biotin-labelled and used for hybridisation of the specific probes to parasite DNA *in situ* on sections of infected fish tissues (*in situ* hybridisation, ISH). A rapid, high contrast ISH protocol based on a

biotin-streptavidin detection system was developed for *S. truttae.* The protocol was found to be species specific under the given conditions of stringency and it allowed the detection of all parasite stages down to single cell level. Subsequently, the methodology was used in an experimental infection study and enabled successful visualisation of the entry of *S. truttae* into the Atlantic salmon and its early development within the host: The gills were identified as the predominant entry locus of *S. truttae* into its host from where the parasite migrated into the blood system within only 3 days. During the following 12 days *S. truttae* was found to proliferate in the blood forming blood stages consisting of a primary cell harbouring up to 120 secondary cells. After 12 days of proliferation, blood stages were found to accumulate in the capillaries of the spleen, kidney and liver, from where they entered these organs by penetrating the capillary walls. Quantification of *S. truttae* in these organs showed that the number of parasite stages in the blood vessels of these organs was higher than the number in the tissue itself, which assumes that proliferation is not continued once the histozoic stage is reached. The invasion of kidney as a target organ for spore formation was not strongly favoured (37.3±3.6%) in comparison with the proportion of parasites in the spleen (35.3±4.7%) and the liver (27.4±5%). However, all histozoic stages in the spleen and the liver were found to degenerate and further development of *S. truttae* into sporogony was found to take place exclusively in the kidney. This is the first complete description of the development of a myxozoan in its fish host, using ISH, and together with *Myxobolus cerebralis* Hofer, 1903, the cause of whirling disease in salmonids, it is the only completely elucidated developmental cycle of a myxozoan in its fish host.

The ecological part of the study concentrated on the identification of the myxozoan fauna of vertebrates and invertebrates present in a *S. truttae*-enzooic highland stream habitat and the potential involvement of the actinosporeans and their

hosts in the life cycle of *S. truttae*. Nine myxosporeans (*Chloromyxum truttae* Léger, 1906, *Myxidium truttae* Léger, 1930, *Sphaerospora elegans* Thélohan, 1892, *Myxobilatus gasterostei* Davis, 1944, *Chloromyxum* sp., *Myxidium giardi* Cépède, 1906, *Zschokkella* sp., *Hoferellus gilsoni* (Debaisieux, 1925) and *Myxobolus portucalensis* Saraiva & Molnar, 1990) were found to share the riverine habitat with *S. truttae*. As vertebrate hosts, salmon, brown trout, eel and stickleback were identified, but six frogs examined for the presence of myxosporeans were found to be uninfected.

Twelve actinosporean types belonging to the collective groups of aurantiactinomyxon, echinactinomyxon, raabeia, synactinomyxon and neoactinomyxum were identified from 5 of the 11 oligochaete species identified in the habitat. Apart from the oligochaetes, various insect larvae were found in the sedimentary habitat of the river system investigated. These were monitored for actinosporean release and submitted to PCR using the *S. truttae* specific assay designed in this study. Two chironomid specimens were found to be PCR positive for myxozoans, harbouring *S. truttae* and *Chloromyxum* sp. However, ISH proved that an infection was not present and that the PCR signals were probably the result of intestinal passage of myxosporean spores.

Infection studies involving the actinosporeans and their hosts did not result in *S. truttae* infection in Atlantic salmon fry. Furthermore, it was impossible to infect oligochaetes with kidney homogenates containing viable *S. truttae* myxosporean spores or by co-habitation of oligochaetes with infected salmon smolts. However, Atlantic salmon were successfully infected by co-habitation with sediments from the outflow of an *S. truttae* infected fish farm, without successful repetition of this experiment. The alternate host or actinosporean stage of *S. truttae* could not be identified.

The molecular characterisation of the community of myxozoans on the basis of their 18S rDNA sequences resulted in 20 new sequences entered into the myxozoan

database, and the genera *Chloromyxum, Hoferellus* and *Myxobilatus* were entered for the first time. Study of the molecular relationships between the different taxa and with other myxozoan sequences available on GenBank showed that the first myxozoans to invade the freshwater were gall bladder parasites, they then invaded the urinary system and finally became histozoic. This general organization of the freshwater myxozoans and the close clustering of the myxosporeans sequenced from the urinary system, independently of host species or spore morphology, suggests that tissue trophism plays an important role in the evolution of the Myxozoa.

Three actinosporeans, i.e. *Neoactinomyxum eiseniellae, Aurantiactinomyxon pavinsis* and *Raabeia* type 3 were found to represent alternate life cycle stages of *Chloromyxum* sp., *Chloromyxum truttae* and *Myxidium truttae*, respectively (approx. 1400 identical base pairs each). Three other actinosporeans encountered (two echinactinomyxon and one raabeia type) showed over 92% sequence identity with myxosporeans from GenBank, whereas all other actinosporeans formed a closely related group devoid of any known myxosporeans.

The sequences of *S. truttae* and *S. elegans* were considerably different from all other freshwater myxosporeans, and they were found to occupy a basal phylogenetic position, with closest relationships to the marine species. The only two life cycles elucidated from species of typically marine genera have been shown to involve alternate polychaete hosts, although freshwater polychaetes are rare, they should be the first target in future studies on the extrapiscine development of *S. truttae.*

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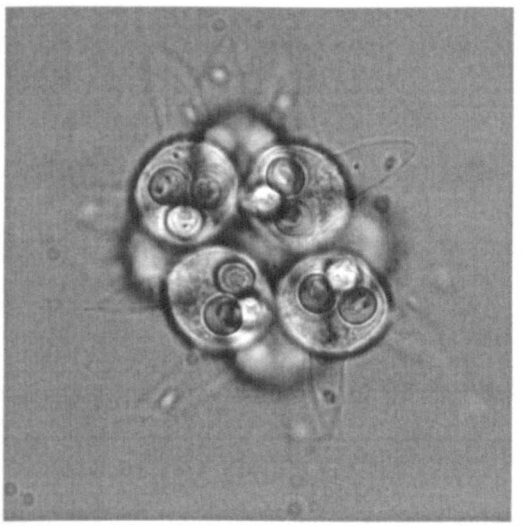
CHAPTER 1

General Introduction



1 General Introduction

1.1 General Characteristics of the Myxozoa

The Myxozoa Bütschli, 1881 are a species-rich and economically important group of parasites utilising aquatic vertebrate and invertebrate hosts. Although best known for the diseases they cause in commercially important fish hosts, myxozoans causing serious pathology represent only a small fraction of the approximately 1400 species and 40 genera described (Lom & Noble 1984). The most important documented pathogens of commercial impact are *Myxobolus cerebralis* Hofer, 1903, the cause of whirling disease in salmonid fish; *Tetracapsuloides bryosalmonae* Canning, Tops, Curry, Wood & Okamura, 2002 (=PKX), the cause of proliferative kidney disease in salmonids; *Kudoa thyrsites* Gilchrist, 1924, the cause of post-mortem myoliquefaction in various marine fishes, particularly cage-cultured Atlantic salmon *Salmo salar*; *Ceratomyxa shasta* Noble, 1950, the cause of salmonid intestinal ceratomyxosis in the Pacific Northwest of the USA and Canada.

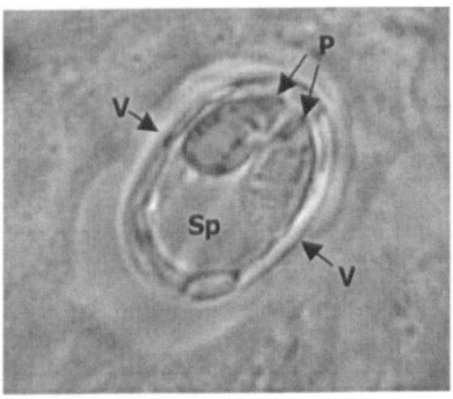


Figure 1.1 A characteristic myxosporean spore: **V**=shell valves, **P**=polar capsules with coiled polar filaments, **Sp**=sporoplasm

The feature by which myxozoan infections in fish are commonly recognised are the characteristic and unmistakable spores which represent a highly organised, multicellular unit (Figure 1.1). Myxozoan spores consist of protective shell valves, organelles known as polar capsules and a usually binucleate sporoplasm which represents the infective germ and which

invades the host after attachment of the spore. The most characteristic structures of the myxozoan spore are the polar capsules. Each polar capsule contains a coiled, eversible polar filament which, on contact with a suitable host, is everted to anchor the spore and allow the infective germ to enter the host. The multicellular nature of spores led Stolc (1899), Emery (1909) and Ikeda (1912) to propose that Myxozoa were in fact Metazoa, although they had been classified as Protozoa. Weill (1938) made the comparison between myxozoan polar capsules and coelenterate (cnidarian) nematocysts. The remarkable similarity between polar capsules and nematocysts during development and in their mature structure suggests a cnidarian origin for myxozoans. Finally, molecular analyses provided compelling evidence of the status of myxozoans as a metazoan phylum and led to the phylogenetic replacement from the protozoan to the metazoan kingdom in the 1990s (Smothers, von Dohlen, Smith & Spall 1994).

1.2 Myxozoan Hosts and Life Cycles

Apart from 20 species occurring in amphibians and reptiles (Lom 1990, Upton, Freed, McAllister & Goldberg 1992, Upton, McAllister & Trauth 1995, Hill, Green & Lucke 1997), one report of a myxozoan-like parasite in the brain of a mole *Talpa europea* L. (Friedrich, Ingolic, Freitag, Kastberger, Hohmann, Skofitsch, Neimeister & Kepka 2000) and the recent discovery in anatid ducks (Lowenstine, Rideout, Gardner, Busch, Mace, Bartholomew & Gardiner 2002), myxozoan vertebrate hosts are exclusively represented by fish where the parasites invade a variety of tissues and organ cavities. The characteristic mature spores are easily released from skin and gills, organs in contact with the ambient water or from the excretory system (kidney tubules,

ureters, urinary bladders) and the digestive system (including the gall bladder with its connection to the gut via the *Ductus choledocus*). Spores trapped in internal organs are only released after death of the fish host.

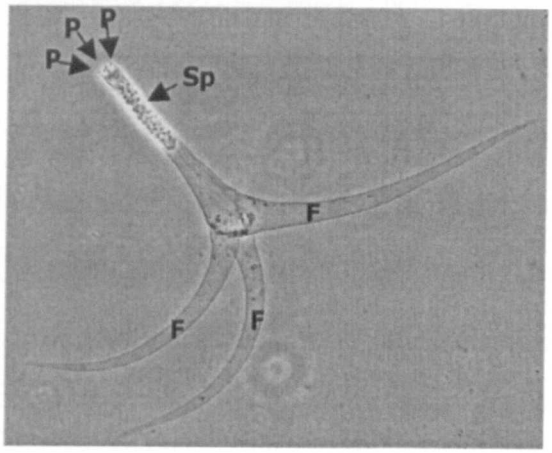


Figure 1.2 A characteristic actinosporean spore: **P**=polar capsules, **Sp**=sporoplasm, **F**=floating appendices.

Until the beginning of the eighties a direct development in the vertebrate host was supposed to be the common scheme in the myxozoans. Failure to experimentally infect fish by oral administration of infected fish tissues was, without questioning, ascribed to insufficient maturation of the spores in the sediment. Only in 1984, did Wolf & Markiw discover that *Myxobolus cerebralis*, the causative agent of whirling disease in salmonids, requires an obligatory alternate host, the oligochaete *Tubifex tubifex* L. (Wolf, Markiw & Hiltunen 1986), in order to complete its life cycle. The sensation of this discovery was the nature of the fish-infecting agent released from the oligochaete. This organism represents a morphologically completely different spore type of the class Actinosporea Nobel, 1980 (Figure 1.2), and therefore an organism that had been described as a separate species in a sister-class of the fish-infecting class Myxosporea Bütschli, 1881. Following the pioneering work of Wolf and Markiw, reports for 25 species of 7 different genera have presented evidence that myxozoan life cycles include oligochaetes as alternate hosts (El-Matbouli & Hoffmann 1989, Ruidisch, El-Matbouli & Hoffmann 1991, Styer, Harrison & Burtle 1991, El-Matbouli, Fischer-Scherl & Hoffmann 1992, Grossheider & Körting 1992, Benajiba & Marques 1993, El-Matbouli & Hoffmann

1993; Kent, Whitaker & Margolis 1993, Yokoyama, Ogawa & Wakabayashi 1993a, Yokoyama, Ogawa & Wakabayashi 1993b, Urawa 1994, Uspenskaya 1995, Yokoyama, Ogawa & Wakabayashi 1995, El-Mansy & Molnar 1997a, El-Mansy & Molnar 1997b, Yokoyama 1997, El-Mansy, Molnar & Szekely 1998, Szekely, El-Mansy, Molnar & Baska 1998, Lin, Hanson & Pote 1999, Molnar, El-Mansy, Szekely & Baska 1999a, Molnar, El-Mansy, Szekely & Baska 1999b, Özer 1999, Szekely, Molnar, Eszterbauer & Baska 1999, Eszterbauer, Szekely, Molnar & Baska 2000, Özer & Wootten 2000, Pote, Hanson & Shivaji 2000). Additionally, polychaetes (Bartholomew, Whipple, Stevens & Fryer 1997, Køie, Whipps & Kent 2004) and several freshwater bryozoans (Anderson, Canning & Okamura 1999, Longshaw, Feist, Canning & Okamura 1999) were identified as alternate hosts for three fish infecting myxozoan species.

The discovery of the morphological "transformation" of myxosporea into actinosporea widened the understanding of the myxozoans markedly and had a revolutionary influence on systematics due to the proposed suppression of the class Actinosporea (Kent, Margolis & Corliss 1994). This suggestion resulted in an extensive controversy which resulted in an ongoing debate (Lester, Hallett, El-Matbouli and Canning (1998, 1999) and Kent & Lom (1999)) because the rules set by Kent *et al.* (1994) contravene the International Code of Zoological Nomenclature in certain cases and they do not allow for new actinosporean types to be named according to the binominal nomenclature. Most of Kent's rules (1994) are being followed today, however, as myxozoan life cycles involve a variety of hosts and not all life cycles are fully understood to date, decisions about the suppression of specific genera and species names might have to be made on the basis of new findings.

1.3 Identification of Actinosporean and Myxosporean Counterparts

Twenty years after the first discovery of a myxozoan life cycle involving an actinosporean and a myxosporean stage, the number of elucidated life cycles is still low. It is not yet clear how the small number of reported actinosporeans can be related to over 1400 species of myxosporeans. It might be speculated that one actinosporean type is able to transform into different myxosporeans depending on the host or tissue in which it develops. On the other hand, the molecular characterisation of two aurantiactinomyxon phenotypes in Germany recently revealed a single genotype (Hallett, Atkinson & El-Matbouli 2002). Thus, phenotypic descriptions and identifications of myxosporean counterparts by cross infections of vertebrate and invertebrate hosts only, do not seem sufficient to conclusively prove the relationships between myxosporeans and actinosporeans. Recent studies on the molecular relationships of the Myxozoa (Smothers *et al.* 1994, Siddall, Martin, Bridge, Desser & Cone 1995, Schlegel, Lom, Stechmann, Bernhard, Leipe, Dykova & Sogin 1996) have not only drastically changed the taxonomy of this group of organisms, but have resulted in techniques for confirming myxosporean life cycles using the small subunit ribosomal RNA gene sequence (18S rDNA). This method was successfully used to identify or confirm the alternating myxosporean and actinosporean stages of *Myxobolus cerebralis* (Andree, Gresoviac & Hedrick 1997), *Ceratomyxa shasta* (Bartholomew *et al.* 1997), *Henneguya exilis* Kudo, 1929 (Lin *et al.* 1999), *Thelohanellus hovorkai* Achmerov, 1960 (Anderson, Canning, Schäfer, Yokoyama & Okamura 2000), *Henneguya ictaluri* Pote, Hanson & Shivaji, 2000 (Pote *et al.* 2000) and the PKX organism *Tetracapsuloides bryosalmonae*

(Anderson *et al.* 1999). 18S sequence data thus allow the identification of genetic homogeneity of variable morphotypes.

1.4 Taxonomy and Species Identification

Intra-phylum classification of the Myxozoa is mainly based on spore morphology (Lom 1987, Moser & Kent 1994, Lom, McGeorge, Feist, Morris & Adams 1997). This system has been commonly criticised as morphological features of the spores were found to show high structural variability resulting in problems with assigning species. However, non-morphological data only became available in the 1990s. When 18S rDNA sequences were first applied to the taxonomy of myxosporeans, discrepancies soon arose between the traditional taxonomic scheme based on spore morphology and the findings from molecular data (Kent *et al.* 1994, Smothers *et al.* 1994, Siddall *et al.* 1995, Schlegel *et al.* 1996). Recent molecular studies employing increasing numbers of species agree on the polyphyly of almost all of the traditional myxozoan "genera" (Kent, Andree, Bartholomew, El-Matbouli, Desser, Devlin, Feist, Hedrick, Hoffmann, Khattra, Hallett, Lester, Longshaw, Palenzeula, Siddall & Xiao 2001, Palenzuela, Redondo & Alvarez-Pellitero 2002, Molnar, Eszterbauer, Szekely, Dan & Harrach 2002, Dyková, Fiala & Nie 2002, Negredo, Dillane & Mulcahy 2003). However, molecular data of several genera are still unavailable and other genera are only poorly represented at the myxozoan molecular database.

The sequence data presently available indicate that there was an ancient split in myxozoan evolution (Kent, Khattra, Hervio & Devlin 1998, Anderson *et al.* 1999). As a result, a new class of myxozoans, the Malacosporea was established (Canning, Curry, Feist, Longshaw & Okamura 2000). The malacasporeans are characterised by soft-

walled spores and use bryozoans as invertebrate hosts, and they form a distinct clade of myxozoans that diverged before the radiation of the myxosporean species which use annelids as alternate hosts.

Molecular data have also added to the means of species identification. The variable regions in the 18S ribosomal gene were found to have some potential for genus- and even species-specific investigations. Assays based on PCR amplification of this gene have been developed for *Ceratomyxa shasta* (Bartholomew, Rodriguez & Arakawa 1995, Palenzuela, Trobridge & Bartholomew 1999), *Myxobolus cerebralis* (Andree, MacConnell & Hedrick 1998, Baldwin & Myklebust 2002), *Kudoa thyrsitis* (Hervio, Kent, Khattra, Sakanari, Yokoyama & Devlin 1997), *Kudoa amamiensis* Egusa & Nakajima, 1980 (Yokoyama, Inoue, Sugiyama & Wakabayashi 2000) and *Tetracapsuloides bryosalmonae* (Saulnier & De Kinkelin 1997, Anderson *et al.* 1999, Morris, Morris & Adams 2002a). The use of PCR assays contributes significantly to the diagnosis and research into myxozoan infections as they allow the detection and identification of infections prior to the development of mature spores in the target organs and they permit the screening of potential alternate hosts.

1.5 Detection of the Early Development in the Fish Host

Information concerning the entry and early development of myxozoans in their fish hosts is rare. For a few species, the epithelia of skin, gills and buccal cavity have been identified as portals of entry into fish using electron microscopy, fluorescent labelling of spores and indirect fluorescence antibody testing (Yokoyama & Urawa 1997, El-Matbouli & Hoffmann 1998, Belem & Pote 2001). Only one published report describes in detail the ultimate attachment process of a myxozoan, i.e. the *Myxobolus*

cerebralis related triactinomyxon spore to its salmonid host, using scanning electron microscopy (El-Matbouli, Hoffmann, Schöl, McDowell & Hedrick 1999a). Likewise, complete details of presporogonic development have to date been resolved only in *Myxobolus cerebralis* (El-Matbouli, Hoffman & Mandok 1995). Due to the small size, number and the cryptic nature of myxozoan early developmental stages, very little is known about the infective primary cell and its migration through the different tissues in other species. Using general histological techniques, the detection of early myxozoan developmental stages and their differentiation from host cells proved difficult. The use of lectins and antibodies (Bartholomew, Smith, Rohovel & Fryer 1989, Adams, Richards & Marin de Mateo 1992, Marin de Mateo, Adams, Richards, Castagnaro & Hedrick 1993) showed the inherent limitations of their use for diagnosis of myxozoan developmental stages due to physical, chemical and immunological changes during the parasite development in the fish host. To date, the most promising approach is once again the use of DNA based methods. *In situ* hybridisation (ISH) aims to detect parasite DNA or RNA in sections of host tissues. This procedure combines the specificity and sensitivity of the PCR detection technique with the ability to examine the parasites in their biological context. With regard to the myxozoans, this method has so far only been used to detect the causative agent of whirling disease, *Myxobolus cerebralis*, at an early subclinical stage (Antonio, Andree, McDowell & Hedrick 1998), and to identify the portal of entry of the PKX organism, *Tetracapsuloides bryosalmonae* into the fish host (Morris, Adams & Richards 1999, Morris, Adams & Richards 2000, Longshaw, Le Deuff, Harris & Feist 2002).

1.6 Background to the Present Study

Sphaerospora truttae Fischer-Scherl, El-Matbouli & Hoffmann, 1986 was first described from brown trout, *Salmo trutta* L. in Germany. After its identification as a serious pathogen in Atlantic salmon *Salmo salar* in Scottish smolt farms comprehensive studies were conducted on the life cycle, development and epidemiology of this parasite in the new host Atlantic salmon, *Salmo salar*. The pathology of *S. truttae* was primarily related to extrasporogonic stages, which are abundant in high numbers during the summer months and which may result in mortalities at this time (McGeorge 1994, McGeorge, Sommerville & Wootten 1994, McGeorge, Sommerville & Wootten 1996a, McGeorge, Sommerville & Wootten 1996b). Using conventional histological and imprint techniques, developmental stages of *S. truttae* were detected for the first time 2-4 weeks after infection, while proliferative stages in the interstitial tissue of the kidney were found after 6 weeks. Nothing was known regarding the infection process or development up to 2 weeks.

McGeorge, Sommerville & Wootten (1997) and Özer (1999) investigated the actinosporean fauna of the oligochaete population in the outflow of a salmon farm with annually reoccurring infections of *S. truttae*. In subsequent infection studies under laboratory conditions the life cycle of *S. truttae* was apparently completed by relating an actinosporean of the echinactinomyxon type to the known myxosporean spore (Özer & Wootten 2000).

As a result of the comprehensive investigations over the last 10 years, a large database is available for *S. truttae* and the parasite is an ideal myxozoan model organism for further studies. Due to the invaluable contributions molecular biology has made to today's state of knowledge of the Myxozoa, the aim of this study was to take a

molecular based approach in order to unveil aspects of the life cycle of *S. truttae* which have so far remained unknown using conventional histological techniques. The following objectives were targeted in the investigation:

1. Identification and occurrence of the different *S. truttae* life cycle stages by detection of the parasite in its vertebrate and invertebrate host and in the environment by specific and sensitive PCR amplification employing oligonucleotides designed on the basis of the 18S rDNA sequence.
2. Detection of the infection locus and the early development and migration of *S. truttae* in the vertebrate host using an *in situ* hybridisation (ISH) protocol specifically designed for *S. truttae*.
3. Study of alternate invertebrate hosts in an *S. truttae* enzooic habitat and characterisation of both actinosporeans and myxosporeans sharing the habitat with *S. truttae*.
4. Evaluation of relationships between *S. truttae* and the myxozoans co-occurring in the same habitat using molecular phylogeny.

CHAPTER 2

General Materials and Methods

2 General Materials and Methods

2.1 Sampling Sites

Figure 2.1.A Map of Scotland showing the location of the *Sphaerospora truttae* infected salmon hatchery on the North coast.

The major sampling site used in this study was an *S.* truttae-infected Atlantic salmon hatchery and its supplying river system, situated on the North coast of Scotland (site A, 58° 27.0′ N and 4° 45.8′ W, Figure 2.1.A & 2.1.B). In the hatchery of the farm, salmon fry are reared in river water, which is heated to 6-8°C using conventional boilers, from January to March. Thereafter the fish are transferred into 5 m diameter tanks, which are supplied by river water of ambient temperature (varying between 18°C in August and 1°C in January).

Figure 2.1.B General view of the hatchery (H) and 5 m diameter tanks on the salmon farm.

The parr are kept under these conditions over the summer and the following winter. After up to 16 months on site the fish are sold as smolts and transferred to sea cages. The farm has a history of annually recurring *S. truttae* infections with 100% prevalence in the farmed fish. Despite the high prevalence on this site, only an insignificant number of mortalities can be related to this parasite. This study site has been used in previous investigations (McGeorge 1994, McGeorge *et al.* 1996b, McGeorge *et al.* 1997, Özer 1999, Özer & Wootten 2000, Özer, Wootten & Shinn 2002a, Özer, Wootten & Shinn 2002b).

The river supplying the farm is named Amhainnan Stratha Bhig (Figure 2.1.C) and it receives water from several small tributaries running off high land. Approximately 3km downstream from the origin of Amhainnan Stratha Bhig, a waterfall possibly prevents the migration of fish. Below the waterfall, five different points were chosen for the collection of water and invertebrate samples (summarised in Figure 2.1.C). In the upstream area, the river is shallow and meanders with sandbanks and big single boulders in the riverbed (sample point 1). After a large bend in a northerly direction the sediment of the riverbed becomes coarser with an average gravel size of around 4 cm diameter and remains like that downstream until the river meets the sea. On this stretch, the outflow of Loch Bad na h-Achlaise (sample point 2), which lays to the west of Amhainnan Stratha Bhig, meets the main river two kilometres before the salmon farm. Two wide bends in the river (sample point 3) contain deposits of fine sediment (sand/mud). Here, spawning of Atlantic salmon is repeatedly observed. Below these spawning points the river does not show major bends and its sediment is coarse (average gravel size 4-5 cm diameter, sample point 4). The inflow of the salmon farm

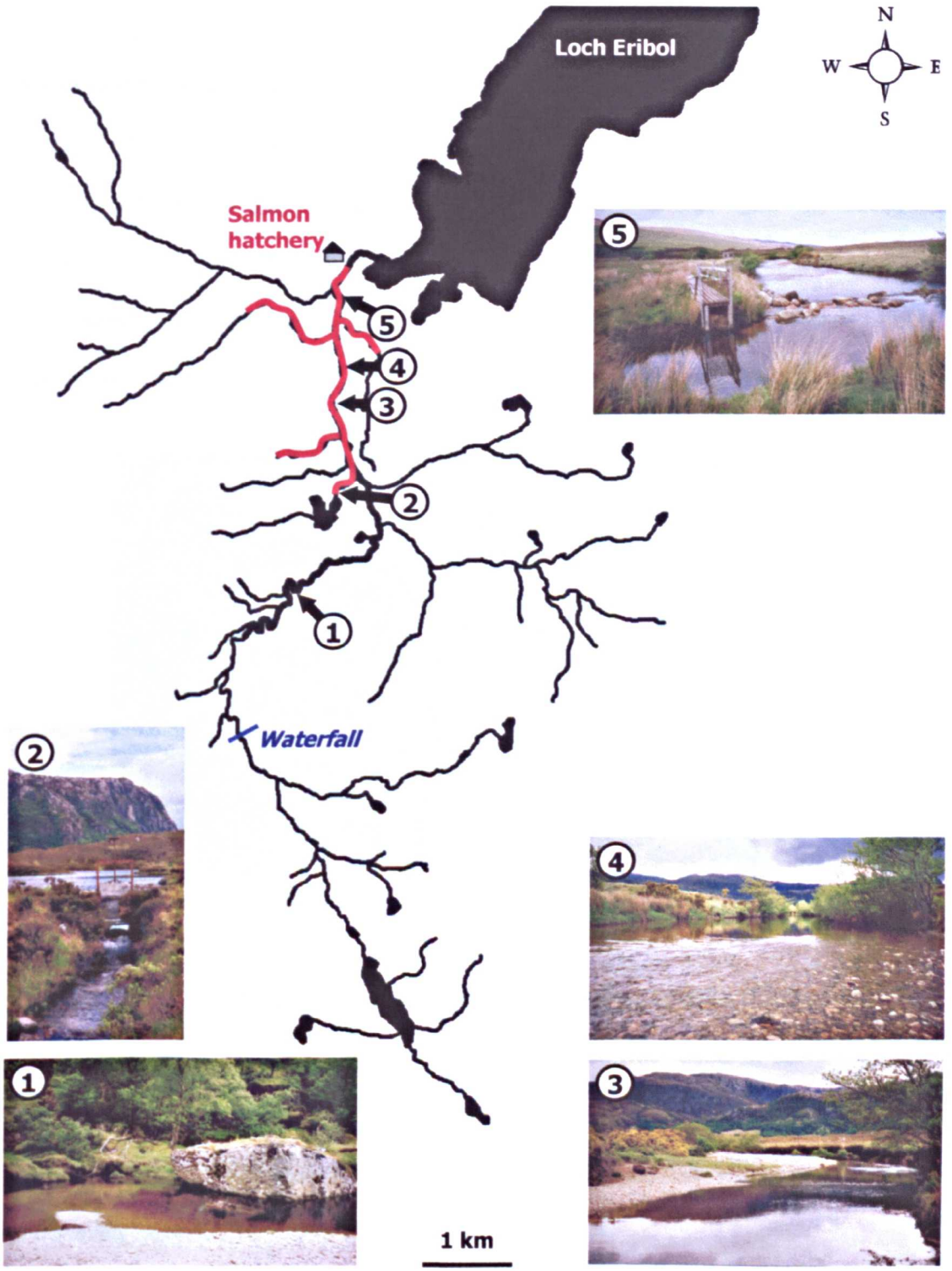


Figure 2.1.C Main sampling site on the North coast of Scotland: Outline of the river Amhainnan Stratha Bhig with its tributaries and adjacent lochs; numbers indicate sampling sites and relate to the pictures of the sampling locations for water and invertebrates; river stretches marked in **red** indicate the sites where electrofishing was conducted.

lies approximately 800m upstream from the farm in an artificial rock pool with deeper water (approximately 1m depth, sample point 5). At the downstream end of the farm, a wooden planked outflow canal leads into a gravely settlement pond of approximately 40 cm depth (Figure 2.1.D), in which uneaten food and fish faeces are sedimented out prior to discharge of the effluent water into the river. One kilometre downstream from the outflow of the farm, Amhainnan Stratha Bhig enters Loch Eribol, a large sea loch.

Figure 2.1.D Settlement pond at the end of the outflow canal of the salmon hatchery.

The farm and supplying river on the North coast represents the main sampling site of this study, however, in autumn 2002, *S. truttae* was found to occur with 40% prevalence in salmonids from a fish farm near Stirling (56° 4.2′ N and 3° 57.0° W). Following this finding, environmental samples of oligochaetes and bryozoans were additionally collected from this site.

For use in infection studies, *S. truttae*-free salmon fry were obtained from a hatchery situated on a highland stream just before it empties at the north-eastern coast

of Loch Fyne (56° 13.9' N and 4° 59.2' W). At this site, the incoming water is filtered through a 10 μm filter and subsequently ozonated before entering the hatchery.

2.2 Sampling Procedures on Site

2.2.1 Sampling Dates

Atlantic salmon and oligochaetes were collected throughout the whole year at the site on the North coast. According to the seasonality of the *S. truttae* infection, salmon parr and smolts were collected between November and April each year to obtain mature spores for infection studies and for DNA analysis. Salmon fry and parr were sampled between May and September in order to study the early infection process and the progression of the presporogonic development of *S. truttae* in the farm environment. The site in Stirling was visited on six occasions, and *S. truttae*-free salmon fry were obtained on three occasions from the site at Loch Fyne. All sampling dates are summarised in Table 2.2.1

Table 2.2.1 Sampling dates on the different sites and material collected.

Location	Date	Material collected
North coast	16.11.2000	0+ farmed salmon parr, oligochaetes (farm)
North coast	30.01.2001	1 year old farmed salmon parr, oligochaetes (farm)
North coast	30.04.2001	1+ farmed salmon smolts, oligochaetes (farm)
North coast	31.07.2001	0+ farmed salmon parr, oligochaetes (farm)
North coast	07.11.2001	1+ farmed salmon smolts
North coast	04.04.2002	1+ farmed salmon smolts, oligochaetes (farm)
North coast	10.06.2002	0+ farmed salmon fry, oligochaetes (farm)
North coast	14./15.07.2002	0+ farmed salmon parr, oligochaetes (farm), water
North coast	01./02.10.2002	Salmon, brown trout, eels, stickleback (river), oligochaetes and div. Other invertebrates (river)
North coast	01./02.03.2003	1+ farmed salmon smolts, oligochaetes (river & farm), div. other invertebrates (river), water
North coast	21./22.05.2003	0+ farmed salmon fry, oligochaetes (river & farm), water
North coast	07./08.07.2003	Oligochaetes (river & farm), water
Stirling	05.06.2002	0+ farmed salmon fry
Stirling	18.10.2002	0+ farmed salmon parr, 0+ farmed brown trout
Stirling	31.03.2003	1+ farmed brown trout

Table 2.2.1 continued.

Location	Date	Material collected
Stirling	09.05.2003	0+ farmed salmon
Stirling	03.07.2003	Oligochaetes (river)
Stirling	05.08.2003	Bryozoa (river)
Loch Fyne	25.01.2001	0+ farmed salmon fry
Loch Fyne	14.05.2001	0+ farmed salmon fry
Loch Fyne	17.03.2003	0+ farmed salmon fry

2.2.2 Farmed Fish

On the farm sites, fish were sampled by hand netting from randomly chosen tanks. All fish were transported to the Institute of Aquaculture alive in plastic bags containing oxygenated local water. In the laboratory, they were transferred to flow-through tanks of 40 L to 180 L volume, which were supplied with aerated, chlorine-filtered mains water at ambient temperature, where the fish were kept until examination or until used for infection studies. All fish were sacrificed by an overdose of chlorobutanol (1, 1, 1–trichloro-2-methyl-2-propanol hydrate).

2.2.3 Oligochaetes from the Farm

The settlement pond at the *S. truttae*-infected farm (described in 2.1) is a highly eutrophic habitat with a very high density of oligochaetes. The sediment containing the oligochaetes was collected at different times of the year (see Table 2.2.1) to a depth of approximately 20 cm using a spade. Subsequently, the sediment was transferred into buckets containing a small amount of local water. The buckets were put into plastic bags and transported to the Institute of Aquaculture, where the sediment was topped up with de-chlorinated mains water and supplied with an airline. The sediment samples were then kept at constant 10°C until examination or use in infection studies.

2.2.4 Water and Invertebrates from the River System

At the 5 sampling points described in Section 2.1, water was collected using a 20 µm nylon plankton-net with 35 cm diameter at the inflow and a 100 ml plastic pot at the end. The net was fixed to plants and exposed for 20 min at each site. On every sampling day (summarised in Table 2.2.1), the water was filtered between 14:00 and 18:00. One plankton-net was left in the stream over night, and a second one was exposed to the water flow for 24 hours. Every time a net was taken out of the river, the sides of the net were thoroughly rinsed with a wash bottle, so that any material adhering to the filter mesh was washed into the collection pot. The collected material was transferred to 300 ml plastic bottles and, after transport to the Institute of Aquaculture, kept in the refrigerator at 4°C until further investigation.

Invertebrate samples were obtained using a 30 x 20 cm kick net with a 250 µm mesh. In order to force the invertebrates out of the fine sediment, the surface of the riverbed in front of the net was slightly pressurised by stepping on it. At sampling sites characterised by coarse sediment (sample points 2 and 4, Figure 2.1.C), the gravel in front of the net was removed stone by stone and the invertebrates drifted into the net with the water current. The invertebrates were then rinsed from the net and collected in plastic bottles.

Additionally, in the sandy areas (sample points 1 and 3, Figure 2.1.C), sediments were collected from the top 20 cm of substrate using a spade. The invertebrates were then elutriated from the sand by hand-stirring approximately 1 L of sediment in a bucket full of river water and filtering the water containing the elutriated organisms immediately through a cascade of sieves (1 mm, 500 µm and 100 µm). The invertebrates were then rinsed from the sieves, collected in a tray and transported to

the Institute in plastic bottles. Due to the depth of the inflow pond of the farm, sample point 5 (Figure 2.1.C) was only sampled using the kick net.

2.2.5 Fish from the River

On the 2nd October 2002 electro-fishing was conducted in Amhainnan Stratha Bhig by using a portable electro-fishing unit configured to deliver 4 Amperes of pulsed DC current and 600 Volts. Several areas of the main river as well as the stream which connects the river with Loch Bad na h-Achlaise together with 3 of the tributaries running off high land to the west and to the east (Figure 2.1.C) were sampled.

Fish exposed to the electric field showed anaesthesia for a few seconds. During this period the fish were captured with a net and transferred into buckets, where all fish recovered within less than a minute. The water in the buckets was replaced by fresh stream water every 30 min while sampling the river. Later the fish were sorted according to size and species and then transported to the Institute of Aquaculture in plastic bags containing aerated river water. At the Institute, fish were kept in flow-through tanks of 40 L to 180 L volume, which were supplied with aerated, de-chlorinated mains water at ambient temperature. Within the following 3 weeks, fish were sacrificed by an overdose of chlorobutanol (1, 1, 1–trichloro-2-methyl-2-propanol hydrate) and examined.

2.3 Examination and Culture of Live Material

2.3.1 Oligochaetes and Other Invertebrates

Oligochaetes from the outflow of the *S. truttae*-infected farm were examined for the prevalence of actinosporean infections and patterns of spore release. Thus the

oligochaetes were separated from the substrate by elutriation, in a similar way to that described in 2.2.4: Approximately 1 L of substrate containing the worms was placed in an elevated, slightly inclined bucket with a notch cut. Tap water was directed down the inside of the bucket with a hose to create a strong, swirling current. Unattended elutriation (30 min) lifted the oligochaetes to the surface where they spilled into a cascade of sieves (1 mm, 500 μm and 100 μm) placed under the bucket's notch. The samples were then rinsed from the sieves and collected in a tray. Subsequently, bigger worms (Lumbriculidae) were transferred into 24 cell well plates (5 individuals per well), smaller worms (Tubificidae, Enchytraeidae and Naididae) were kept in 96 cell well plates (3 individuals per well for Tubificidae and Enchytraeidae, and 10 individuals per well for Naididae). The oligochaetes were kept for 2 to a maximum of 10 days in these wells at ambient temperature and light conditions outdoors. Every second day, the water and the faecal pellets in the wells were scrutinised for actinosporean spores, using an inverted microscope on phase contrast at x 150 magnification. The water in the wells was changed after each spore check. If spores were detected in one of the wells the worms were anaesthetised with clove oil (4 drops of clove oil diluted in 1 ml of absolute ethanol before adding 99 ml of de-chlorinated tap water). The worms were then screened microscopically for sporogonic stages in the gut epithelium by placing them into a frame made of several layers of salar tape mounted on a slide with a cover slip loosely on top (x 100-200 magnification). The infected worms were isolated into a well with fresh water and usually released spores for several days after recovery from anaesthesia. Oligochaetes collected from the river sediment were treated in the same way.

In June 2002, 500 individual oligochaetes were randomly taken from the mixed sample from the outflow of the salmon hatchery in order to identify the species diversity present. Some species with easy recognisable characteristics were identified live, while all other oligochaetes were fixed in 70% ethanol and dehydrated in 95% ethanol before being incubated for 10 min in a few drops of Euparal (Flatters & Garnett Ltd.) on a glass slide. Thereafter the worms were coverslipped and dried in an oven at 60°C overnight. The oligochaetes were identified using the key of Brinkhurst (1971). Worms in cell well plates which were found to release actinosporeans, were identified in the same way after spores had been collected for several days.

Invertebrates other than oligochaetes were kept in small containers with up to 200 ml of freshwater for 10 days under natural light and temperature conditions outdoors. After this period, the water was filtered through a 5 µm nylon mesh and the material on the filter was re-suspended in a small amount of water in order to examine it for actinosporean type spores under the microscope (x 200). Thereafter, the invertebrates were viewed under a dissection microscope and identified at least to family level using the key of Jessup, Markowitz & Stribling (1999). Subsequently, they were submitted to PCR or ISH analysis, respectively.

Several attempts were made to culture oligochaetes in family cohorts in order to obtain a new (presumably uninfected) generation of worms for infection studies. Thus, oligochaetes were kept in aquaria at temperatures between 10°C and 20°C, and their biomass growth over time was determined (see Chapter 5, Section 5.2.2.1).

2.4 Post Mortem Techniques

Unless stated differently in parentheses, all standard buffers and solutions for use in molecular biology studies were made up according to the descriptions of Sambrook, Fritsch & Maniatis (1989).

2.4.1 DNA Extraction

Whole genomic DNA was extracted from isolated myxosporean and actinosporean spores as well as from different organs of fish and whole or parts of invertebrates. Whenever possible, subsamples were fixed in 10% buffered formalin for histological processing.

For DNA extraction actinosporean spores were collected in a small amount of water from the cell wells containing infected worms. After centrifugation in a 1.5 ml Eppendorf tube (5 min at 2000g) the supernatant water was removed and the spore pellet resuspended in 400 µl of TNES urea DNA extraction buffer (50 mM TRIS-HCl (pH 8), 100 mM EDTA (pH 8), 100 mM NaCl, 1% SDS and 5M urea). Whole infected and uninfected organs or organ parts collected from fish infected with myxosporean spores were also introduced into TNES urea. All samples were kept in the urea-stabilised buffer at 4°C until DNA extraction (storage up to a maximum of two months). For extraction, the samples were digested overnight with 100 µg ml^{-1} Proteinase K in a water bath at 55°C. The next day, each sample was incubated with 50 µg ml^{-1} of DNAse free RNAse at 37°C for 1 hour.

Generally, DNA was extracted from the cell digests using a Phenol-Chloroform protocol: Four hundred µl of phenol were added to each tube. The samples were then shaken vigorously for a few seconds and afterwards gently inverted for 10 min.

Subsequently, 400 µl of chloroform was added, and the samples were repeatedly shaken vigorously for a few seconds and then inverted for 10 min. The phases were then separated by centrifugation for 5 min at 15 000 g. Subsequently, 300 µl of the top aqueous layer was removed to a new 1.5 ml Eppendorf, taking care not to disturb the material at the interface. DNA was precipitated from the aqueous solution by adding 3 volumes (900 µl) of 92% ethanol, shaking vigorously for a few seconds and leaving the samples for 3 min on the bench. In most cases, a cloudy DNA precipitate became visible. The samples were then centrifuged for 10 min at 15 000 g to pellet the DNA. The pellet was washed with 1 ml of 70% ethanol, the alcohol was decanted off and the samples were left to dry for 3-5 min in a vacuum centrifuge. The DNA pellet was left to re-suspend in 40-250 µl of nanopure water overnight in the fridge before 4 µl of each sample was electrophoresed in a 0.75% agarose gel (made up in 0.5 x TAE buffer containing ethidium bromide) for 30 min at 80 Volts in order to determine the amount and quality of the extracted DNA under UV light.

2.4.2 PCR and DNA Sequencing

The polymerase chain reaction (PCR) was used for the amplification of different fragments of the small subunit (18S) ribosomal DNA gene. Generally, PCRs were performed in 10 µl volumes with 0.5 units of TITANIUM Taq DNA polymerase and the related 10 x buffer containing 1.5 mM $MgCl_2$ (BD Biosciences Clonetech), 0.2 mM of each dNTP, 12.5 pmole of each primer, and 1 µl of template. Denaturation of DNA (95°C for 3 min) was followed by 35 cycles of amplification (95°C denaturation for 50 sec, 58-65°C annealing for 50 sec, and 70°C extension for 1 min 20 sec) and ended by

a 4 min final extension (70°C). The optimal and specific annealing temperature for each primer pair was determined in a temperature gradient (50°C-70°C). The PCR products obtained were electrophoresed in agarose gels (see 2.4.1).

Products to be sequenced were amplified in 30 μl PCR reactions containing the same concentration of ingredients. Five μl of each PCR product were run on either side of three lanes in which the remainder of the sample was electrophoresed. The gel was then sliced and only the lanes containing the subsamples were exposed to UV light in order to prevent the splicing of the DNA used for sequencing. After the marking of the amplified band size in the lanes with the subsamples the whole gel was re-arranged and the sample for sequencing was excised from the gel without exposure to UV. The gel slice containing the PCR product for sequencing was collected in a 1.5 ml Eppendorf tube and the remainder of the gel was checked for the precision of cutting under UV. Subsequently all PCR products for sequencing were purified using GFX PCR DNA and gel band purification spin columns (Amersham Pharmacia Biotech Inc.). The purified products were then used in sequencing terminator reactions applying the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) in a 1:4 dilution in Tris-HCl buffer (pH 9.0). Twenty μl reactions were prepared using 8 μl of the diluted sequencing kit, 5 pmole (1 μl) of primer and approximately 50 ng (5 μl) of DNA template in nanopure water. The reactions were run on a thermocycler (25 cycles of 10 sec at 95°C, 20 sec at 60°C and 1 min at 50°C) and thereafter precipitated with a mixture of 50 μl (2.5 volumes) of 95% ethanol and 2 μl of sodium acetate. The resultant DNA product was sequenced in an ABI PRISM 377 DNA sequencer (PE Applied Biosystems).

Whenever possible, 3 replicate samples of each species/spore type were sequenced. The different primers used for PCR and sequencing are listed in the relevant chapters (Chapters 3 and 6).

2.4.3 Sequence Alignments and Primer Design

The obtained sequences were submitted to a BLAST (Basic Local Alignment Search Tool) on GenBank™ (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov), and new sequences identified as a result of this study were submitted to GenBank™ and accession numbers were obtained.

Sequences were corrected and assembled using the BioEdit program (Tom Hall, http://www.mbio.ncsu.edu/BioEdit/bioedit.html). With the aid of the computer program CLUSTAL X v1.18 (Thompson, Higgins & Gibson 1994, Thompson, Gibson, Plewniak, Jeanmougin & Higgins 1997), the obtained sequences were aligned with numerous other 18S rDNA sequences obtained from GenBank™. The PrimerSelect (DNAstar Inc.) software was used in order to design genus/species-specific oligonucleotides in those areas of the 18S gene which showed high sequence variability. Generally, emphasis was placed on designing primers at similarly high T_m (=melting temperature, i.e. temperature at which 50% of a given oligonucleotide is hybridised to its complementary strand) in order to guarantee stringent conditions when using them in the same PCR reaction. The PrimerSelect software was also used to analyse the primer sequences for strong secondary structure and dimer formation with themselves or with their corresponding reverse PCR primer or other primers used in the same PCR reaction. Furthermore, potential oligonucleotide sequences were sent to the BLAST server as queries to discard primers that have non-target gene homologies.

Suitable oligonucleotides were commercially synthesised by MWG-Biotech AG and tested for their specificity using PCR. For use in the *in situ* hybridisation, primers were 5' biotin-labelled by the same company.

2.4.4 Phylogenetic Analysis

Following sequence alignment using CLUSTAL X the alignment was manually corrected to eliminate minor inconsistencies between different taxa and with reference to known elements of secondary structure (Van de Peer & De Wachter 1997). Neighbour-joining analyses (Saitou & Nei 1987) were performed using the Phylogeny Inference Package (PHYLIP version 3.5c, Felsenstein 1993), with distances calculated using the Kimura two-parameter model. Parsimony analyses were performed using the PAUP 3.1.1 program (Swofford 1993), using the branch-and-bound algorithm. Maximum-likelihood analyses were performed using the PHYLIP package, using a transition:transversion ratio of 2:1, empirical base frequencies, one rate class for nucleotide substitutions across sites, and global branch re-arrangements. The robustness of the positioning of each taxon on the final cladogram was assessed using bootstrapping with 100 replicates.

2.4.5 General Histology and in Situ Hybridisation Technique

Whole fish (fry), different fish tissues and several invertebrate species were prepared for light microscopic histological studies and *in situ* hybridisation. Specimens were fixed in 4% neutral buffered formalin for 24 hours to 1 week. Whole fry were fixed with their body cavity opened ventrally. Prior to further processing, fry were decapitated, the heads were sagittally halved and the remainder of the body was transversally trisected (1st plane: head-kidney, liver, stomach, 2nd plane: trunk kidney,

spleen, gut, 3rd plane: caudal peduncle). This corresponds to the planes of cutting of the fry samples on the microtome, while organs from bigger fish were usually cut in the plane of maximum surface area obtainable and invertebrates were always sectioned sagittally.

All samples were dehydrated through an ethanol series (1 x 80% and 3 x 100% methylated spirits for 90 min each, and 2 x 100% ethanol for 90 min each) and transferred into paraffin (2 x chloroform for 50 min each, and 3 x paraffin for 90 min each) in an automatic tissue processor. For general histology, 4 μm sections were cut from the paraffin embedded specimens. The sections were stretched in a water bath at 45°C and floated onto glass slides. Slides were dried overnight in the oven at 60°C prior to staining using Hematoxylin & Eosin (H&E) according to standard methodology (Pearse 1968-1972).

For *in situ* hybridisation, 7 μm sections were cut and mounted on 3-(aminopropyl)triethoxysilane (APES) coated slides in order to ensure adhesion of the tissues and preservation of the morphology during tissue digestion in the ISH protocol. The ISH protocol designed in this study is based on that of Morris, Adams & Richards (1999). Several steps of the protocol were modified and optimised as appropriate for the probes and tissues used, and the incubation times, especially for the steps which damage tissue, were shortened as far as possible in order to maintain the tissue structure (see Chapter 4, Section 4.2.1).

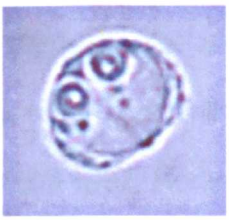
Photographs of hybridised and stained sections were taken using an Olympus BH-2 compound microscope with an attached digital camera (Axio Cam MRC, Carl Zeiss Inc.).

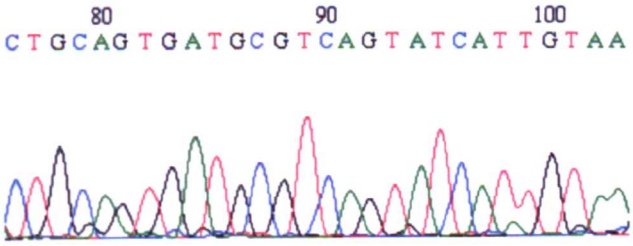
2.4.6 Scanning Electron Microscopy

Specimens to be examined with the scanning electron microscope were pre-fixed at 4°C for 1 hour in 1% glutaraldeyde in 0.1 M sodium cacodylate. This initial fixation was followed by 2-3 days of immersion in 3% glutaraldehyde in 0.1M sodium cacodylate buffer at 4°C followed by rinsing in 0.1 M cacodylate buffer. Specimens were then post-fixed in 1% osmium tetroxide in 1% borax solution for 2 hours before being dehydrated through an ethanol series (30% ethanol, 60% ethanol, 90% ethanol, 2 x 100% ethanol, for 30 min each). Subsequently, the specimens were transferred to a 1:1 mix of 100% ethanol and hexamethyldisilanzane (HMDS) for 30 min before incubation in pure HMDS for another 30 min. The samples were then air-dried at room temperature in the fume cupboard before being mounted on aluminium stubs followed by sputter coating with gold at 40 mA for 90 sec using an Edwards S150B sputter coater. Examination of specimens was undertaken using a Philips 500 scanning electron microscope operating at 15 kV.

CHAPTER 3

Molecular Characterisation and Specific Identification of *Sphaerospora truttae* Fischer-Scherl, El-Matbouli and Hoffmann, 1986





3 Molecular Characterisation and Specific Identification of *Sphaerospora truttae* Fischer-Scherl, El-Matbouli and Hoffmann, 1986

3.1 Introduction

3.1.1 Diagnosis of Myxozoan Infections

Presumptive diagnosis of *Sphaerospora truttae* relies on the detection of the characteristic mature spores in the intratubular space. Using conventional imprint and histological techniques (e.g. Giemsa, H&E), large developmental stages of *S. truttae* can be detected in the blood and the interstitial tissue of the kidney 2-4 weeks after infection (McGeorge 1994). These stages do not allow species identification as other myxosporeans of the same genus and potentially of other genera produce pre-sporogonic blood stages (Lom & Dyková 1992, Moran, Margolis, Webster & Kent 1999). The diagnosis of the earliest developmental stages has not been possible, probably due to the small size, low number and cryptic nature of myxozoans in their early infection state in fish. These stages are generally difficult to detect and to differentiate from host cells using conventional techniques.

The first non-morphological approach to overcome this myxozoan problem was the development of immunological or lectin-based histochemical assays (Bartholomew *et al.* 1989, Adams *et al.* 1992, Marin de Mateo *et al.* 1993). However, antibodies and lectins were shown to have limited diagnostic usefulness because major antigens and lectin-binding sites can change dramatically during parasite ontogeny, and they can be

shared by different host cell types and by other parasite species (Lumsden 1986, Jacobson & Doyle 1996). Due to the consistent structure of most genes, diagnostic methods based on the detection of parasite DNA circumvent most of these problems.

3.1.2 The Nuclear 18S Ribosomal DNA Cistron

The quality of a diagnostic assay allowing the detection of parasite DNA is highly dependent not only on the optimisation of the methodology applied (i.e. PCR, *in situ* hybridisation) but primarily on the choice of the identifiable gene. The importance of specific identification as a means of differentiation of pathological organisms from closely related (non pathological) species cannot be underestimated. The optimal gene thus contains variable regions which differ interspecifically even between closely related species. Additionally, the early detection of an infection before its escalation into a disease is desirable. In molecular biological measures this is expressed in the detection of low numbers of the identifiable gene. The optimal gene thus occurs in multiple identical copies per cell.

Within eukaryotic genomes, ribosomal DNA (rDNA) is universally distributed, functionally equivalent, and organised in long arrays of tandem repeats separated by intergenic spacers (IGS), often distributed over different chromosomes (Gerbi 1985). Between the three ribosomal genes (18S, 5.8S and 28S) lie two internal transcribed spacers (ITS1 and ITS2). The total number of cistronal repeats per genome varies from several hundred in mammals and insects to several thousands in plants. This represents one percent or more of the genome (Long & Dawid 1980).

The most studied eukaryotic ribosomal DNA is the small subunit gene (18S rDNA), which shows interspersion patterns of conserved and non-conserved regions of

the sequence. The islands of highly conserved sequences within the 18S genes are very useful for constructing "universal" primers that can be used for sequencing rDNA from different species, in order to amplify and sequence intermediate regions of specific interest, i.e. highly variable regions. The areas of high variety in the 18S rDNA gene can then be used to develop specific molecular diagnostic tests.

Despite its many advantageous characteristics, some important problems are related to the small subunit ribosomal gene. One of these problems is that the 18S rDNA is among the slowest evolving sequences found throughout living organisms. It has therefore been very useful for examining ancient evolutionary events but its potential in differentiating between closely related species has to be questioned although Kent, Hervio, Docker & Devlin (1996) consider a "fast molecular clock" mode of evolution in the case of the myxozoan 18S gene, finding significant differences in the 18S sequences of presumably closely related species.

3.1.3 The Myxozoan Molecular Database

Despite the concerns related to the 18S rDNA gene, only the availability of homologous sequences of the same gene from related species allows the development of specific diagnostic probes or phylogenetic analysis on the basis of divergent sequence fragments. In the case of myxozoans, this is the database of 18S ribosomal DNA sequences, which has been growing constantly since the first submission by Smothers *et al.* (1994). Today, 136 different myxozoan sequences, belonging to 12 myxosporean genera, to 7 actinosporean collective groups and to the malacasporean order are available on GenBank™ and in published reports. As a result of the awareness of the problems related to the 18S gene, efforts are now being made to acquire

sequences for other genes. Recently, sequences coding for the beta-actin gene and different protease genes have been submitted to GenBank™ (12 entries). Furthermore, several sequences of the more variable 28S large ribosomal subunit of different *Kudoa* species were submitted. However, the most comprehensive collective sampling to date is represented by the 18S rDNA sequences.

Sequences of this gene have been successfully used for the confirmation of the actinosporean and myxosporean life cycle stages of *Myxobolus cerebralis* (Andree *et al.* 1997), *Ceratomyxa shasta* (Bartholomew *et al.* 1997), *Henneguya ictaluri* (Lin *et al.* 1999) and *Thelohanellus hovorkai* (Anderson *et al.* 2000). Furthermore, *Tetracapsuloides bryosalmonae* spores from the bryozoan host (homology of these spores to the actinosporean stages of other freshwater myxozoans is questioned) were proven to be molecularly identical with the known PKX stages in salmonids (Anderson *et al.* 1999). To date, 20 other actinosporean stages have been identified as myxosporean counterparts by experimental infection but without molecular confirmation of the life cycle. This includes *S. truttae*, which apparently develops into *Echinactinomyxon* type 5 (Özer & Wootten 2000, Figure 3.1.3).

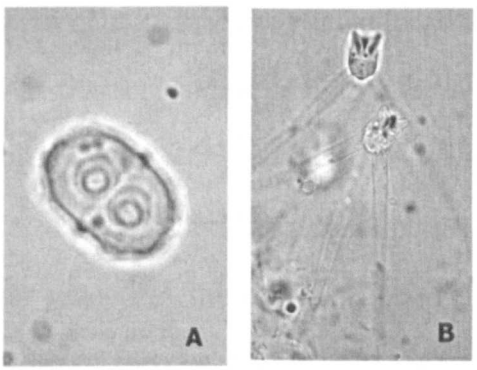


Figure 3.1.3 Myxosporean (A) and proposed actinosporean (B) stage of *Sphaerospora truttae* as determined by infection studies.

Beside their successful use in the confirmation of myxozoan life cycles, 18S rDNA sequences have also been employed for the design of DNA probes in the interspersed islands of less conserved sequences within the 18S gene, in order to develop specific diagnostic assays for *Ceratomyxa shasta* (Bartholomew *et al.* 1995, Palenzuela *et al.*

1999), *Myxobolus cerebralis* (Andree *et al.* 1998, Baldwin & Myklebust 2002), *Kudoa thyrsites* (Hervio *et al.* 1997), *Kudoa amamiensis* (Yokoyama *et al.* 2000), *Henneguya ictaluri* (Whitaker, Pote, Khoo, Shivaji & Hanson 2001) and *Tetracapsuloides bryosalmonae* (Saulnier & de Kinkelin 1997, Kent *et al.* 1998). However, it is important to validate the specificity of these assays with regard to other closely related or co-current species, as differentiation between pathogenic and non-pathogenic myxozoans is vital.

3.1.4 Objectives

This part of the study was aimed at the isolation of *S. truttae* from its vertebrate and invertebrate hosts in order to enable molecular characterisation of *S. truttae* by sequencing its 18S rDNA and to confirm the molecular identity of the myxosporean with the actinosporean stage *Echinactinomyxon* type 5. Based on the alignment of the obtained *S. truttae* sequence with other myxozoan sequences the design of primers specific for *S. truttae* was targeted. These primers would then be applied in a nested PCR assay aiming at the detection and identification of *S. truttae* in genomic DNA extractions. Finally, the PCR assay would be validated for its specificity by testing for cross-reactions with various other myxozoan species and for its sensitivity by amplifying a known concentration of *S. truttae* spores.

3.2 Materials and Methods

3.2.1 Molecular Characterisation of Echinactinomyxon type 5, the Proposed Actinosporean Stage of Sphaerospora truttae

Echinactinomyxon type 5 spores were collected from the water in cell wells containing the oligochaete host *Lumbriculus variegatus.* These oligochaetes had been sampled in the outflow pond of the salmon hatchery on the North coast, on 30.04.2001. Three samples from three individual worms were processed. Genomic DNA was extracted from all samples and whole 18S rDNA was amplified by PCR using universal 18S primers 18e and 18g (Table 3.2.1). In order to optimise the PCR reaction on the basis of different actinosporean types, DNA was additionally extracted from *Echinactinomyxon* type 1 spores collected from an infected specimen of *L. variegatus.* Using genomic DNA from *Echinactinomyxon* type 5 and *Echinactinomyxon* type 1, the optimal annealing temperature of the primers 18e and 18g was determined in a temperature gradient PCR (50° to 70°C).

The 18S rDNA amplicon obtained from *Echinactinomyxon* type 5 was sequenced using the PCR primers (18e and 18g) and two additional nested universal 18S primers 3LIN and 10LIN (Table 3.2.1). From the four overlapping partial sequences, a consensus sequence was obtained which was submitted to the BLAST server. The closest matches (≥80% sequence identity), all other *Sphaerospora* species available on GenBank™ (*Sphaerospora molnari* Lom, Dykova & Grupcheva, 1983, Accession number AF378345; *Sphaerospora dicentrarchi* Sitjà-Bobadilla & Alvarez-Pellitero, 1992, AF201373; *Sphaerospora oncorhynchi* Kent, Whitaker & Margolis, 1993, AY278564) as well as 18S sequences from the oligochaete host (*Lumbriculus variegatus*, AF209457)

and the presumed fish host (*Salmo salar*, AJ427629) were used in an alignment in order to design four specific DNA probes for *Echinactinomyxon* type 5.

Table 3.2.1 Primers utilised for the amplification and sequencing of the 18S rDNA of the actinosporean *Echinactinomyxon* type 5 (Dir=priming direction with F=forward and R=reverse, Locus=Location of the primer on a 2000bp myxozoan model 18S gene, T_m=melting temperature of the primer).

Name	Dir	Sequence	Locus	T_m	Reference
18e	F	5'-TGGTTGATCCTGCCAGT-3'	1	52.8°C	Hillis & Dixon 1991
18g	R	5'-GGTAGTAGCGACGGGCGGTGTG-3'	2000	67.7°C	Hillis & Dixon 1991
3LIN	F	5'-GCGGTAATTCCAGCTCCA-3'	570	56.0°C	Lin *et al.* 1999
10LIN	R	5'-CACTCCACGAACTAAGAA-3'	1410	51.4°C	Lin *et al.* 1999

3.2.2 Isolation and Molecular Characterisation of the Myxosporean Stage of Sphaerospora truttae

Spore Isolation

PCR amplification of *S. truttae* from infected kidneys using the universal 18S rDNA primers 18e and 18g (see 3.2.1) resulted in consistent amplification of host DNA only. Therefore, spores had to be isolated from the tissue of the fish host in order to obtain DNA from the myxosporean stage. In contrast to myxosporean species which produce spores in vast numbers inside large cysts, the collection of spores of *S. truttae* which develop asynchronously inside bisporous pseudoplasmodia scattered in the renal tubules, proved difficult. Thus, different methods for the isolation of mature *S. truttae* spores from the kidney of Atlantic salmon were tested. The efficacy of spore recovery and purity from homogenised kidney tissues was checked after isolation by: (1) centrifugation, (2) modified colloidal silica (Percoll™) gradient, and (3) a two-phase system of polyethylene glycol (PEG) and dextran.

The kidneys of 20 salmon smolts (sampling date 07.11.2001) were homogenised with a piston in a small amount of distilled water. Subsequently the homogenate was washed and pelleted (8 min at 1000g) in a conical 15 ml polypropylene tube before filtration of the re-suspended pellet through a 50 µm nylon mesh. The solution was then left to stand and disintegrate for 2 hours at 15°C before being centrifuged again for 8 min at 1000 g. The supernatant was discarded and the pellet containing host cells and spores was re-suspended in 3 ml of distilled water. The average number of spores obtained by haemocytometer counts (2 x large square) showed that 700 000 spores ml^{-1} were present amongst the host cells. 100 µl of this solution (equalling 70 000 spores) was used for each of the spore isolation methods:

1. Centrifugation: Several centrifugation speeds and times were tested in order to sediment the spores out of the kidney homogenate. In 1.5 ml Eppendorf tubes, 100 µl portions of the homogenate were mixed with 1 ml of distilled water and subsequently centrifuged for 1, 5 and 10 min at 200g, 500g and 1000g. 5 µl of the sediment from the bottom of the Eppendorf tube were pipetted onto a glass slide, coverslipped and the number of *S. truttae* spores counted.

2. Modified colloidal silica (Percoll™) gradient: Density centrifugation of *S. truttae* spores was attempted in 3 different, non-continuous gradients of Percoll™ (stock density 1.13 g ml^{-1}, Sigma-Aldrich Co.) in distilled water. 100 µl of the homogenate was mixed with 1 ml of distilled water and layered on top of each gradient. The first gradient consisted of 5 layers (2 ml each) of 30%, 50%, 60%, 80% and 100% Percoll™, the second and the third gradient consisted of 2 layers each (2.5 ml per layer) of 15% and 75% Percoll™ and of 15% and 30% Percoll™, respectively. All gradients were centrifuged for 30 min at 1500g. All interphases and pellets were

collected, washed with distilled water and centrifuged (8 min at 1000g). 5 µl of pellet were pipetted onto a glass slide, coverslipped and the number of *S. truttae* spores counted.

3. Two-phase system of polyethylene glycol (PEG) and dextran: The aqueous two-polymer phase systems were prepared from 20% stock solutions of PEG 8000 (Sigma-Aldrich Co.) and dextran (Sigma-Aldrich Co.) in distilled water. Ten ml systems with final concentrations of 4% PEG/8% Dextran, 4% PEG/5% Dextran, and an isopycnic mixture of 4% PEG/4% Dextran were each prepared with 100 µl of the kidney homogenate diluted in the suitable amount of water to produce a total volume of 10 ml (Table 3.2.2.A).

Having added all the ingredients, the tubes were gently mixed by inversion 30 times. The phase systems were then allowed to separate for 30 min at room temperature. After separation, the top phase (containing the spores) was drawn off, diluted with an equal volume of distilled water, and the spores were collected by centrifugation (1000g for 8 min). 5 µl of the pellet from the bottom of each tube was pipetted onto a glass slide, coverslipped and the number of *S. truttae* spores determined.

Table 3.2.2.A Volumes of ingredients used to prepare differently proportioned separation systems from 20% stock solutions of PEG/Dextran.

	PEG	Dextran	Sample & distilled H_2O
System 1	4% (2 ml)	8% (4 ml)	100 µl & 3.9 ml
System 2	4% (2 ml)	5% (2.5 ml)	100 µl & 5.4 ml
System 3	4% (2 ml)	4% (2 ml)	100 µl & 5.9 ml

DNA extraction, 18S Amplification and Sequencing

DNA was extracted from the samples of *S. truttae* spores separated by (1) 5 min centrifugation at 200g, (2) 15%/30% Percoll™ and (3) 4%/5% PEG/Dextran (see above). Each sample was pelleted a second time (8 min at 1000g) and, after the removal of the supernatant, TNES urea was added and DNA was extracted. The 18S rDNA was amplified using primers 18e and 18g (see Table 3.2.1). These PCR primers, primers 3LIN and 10LIN (see Table 3.2.1), as well as several additional primers (Table 3.2.2.B), were used to sequence the complete 18S of the *S. truttae* myxosporean stage.

Table 3.2.2.B Primers utilised for the sequencing of the 18S rDNA of *Sphaerospora truttae* myxosporean spores (Dir=direction of priming with F=forward, R=reverse, Locus=Location on a 2000bp myxozoan model 18S gene, T_m=melting temperature of the primer).

Name	Dir	Sequence	Locus	T_m	Reference
Myxgp2f	F	5'-TGGATAACCGTGGGAAA-3'	130	61.3°C	Kent *et al.* 1998
Act1R	R	5'-AATTTCACCTCTCGCTGCCA-3'	1060	57.3°C	Hallett *et al.* 2002
PKX1032	R	5'-CGCTCCTCCAACTTTCGTTC-3'	950	59.4°C	Saulnier & de Kinkelin 1997
Act3F	F	3'-CATGGAACGAACAAT-5'	860	42.4°C	Hallett *et al.* 2002
MX3	R	3'-CCAGGACATCTTAGGGCATCACAGA-5'	1720	64.6°C	Antonio *et al.* 1998

A consensus sequence was obtained by aligning the partial sequences obtained. Due to the unusual length of this sequence in comparison with other myxozoan sequences from GenBank™ together with the results of the BLAST (no close myxozoan matches), the sequence was submitted to a preliminary phylogenetic analysis including 18S sequences of Porifera, Ctenophora, Cnidaria, Bilateria and Myxozoa, with Fungi and Choanozoa as outgroups, in order to confirm the myxozoan identity of the *S. truttae* sequence obtained (species included in the analysis are given in Table 3.2.2.C).

After alignment of the *S. truttae* myxosporean consensus sequence with the 10 closest myxozoan matches from BLAST, with all other *Sphaerospora* species available on GenBank™ (*S. molnari,* Accession number AF378345; *S. dicentrarchi,* AF201373; *S. oncorhynchi,* AY278564) as well as with the 18S sequence from the fish host (*Salmo salar,* AJ427629) four specific DNA probes were designed in the variable regions of the 18S gene of *S. truttae.*

Table 3.2.2.C List of 18S rDNA sequences used in the analysis to confirm the myxozoan identity of the sequence obtained from *Sphaerospora truttae* myxosporean spores.

Species	**Length (bp)**	**GenBank™ acc. no.**
Outgroup I: Fungi:		
Boletus satanas Lenz, 1831	1759	M94337
Neocallimastix frontalis Braune, 1913	1736	X80341
Saccharomyces cerevisiae (Hansen, 1883)	1798	J01353
Outgroup II: Choanozoa:		
Dermocystidium salmonis Davis, 1947	1780	U21337
Ichthyophonus hoferi (Plehn & Muslow, 1911)	1808	U25673
Psorospermium haeckelii Haeckel, 1857	1792	U33180
Porifera:		
Clathrina cerebrum Haeckel,1872 (Calcispongea)	1796	U42452
Sycon calcaravis Hozawa, 1929 (Calcispongea)	1681	D15066
Axinella polypoides Schmidt,1862 (Silicispongea)	1813	U43190
Tetilla japonica Lampe, 1886 (Silicispongea)	1716	D15067
Ctenophora:		
Beroe cucumis (Fabricius,1780)	1676	D15068
Cnidaria:		
Anemonia sulcata Pennant, 1766 (Anthozoa)	1799	X53498
Haliclystus sp. (Scyphozoa)	1799	AF099103
Hydra littoralis (Robertson 1997) (Hydrozoa)	1716	U32392
Polypodium hydriforme Ussov, 1885 (Hydrozoa)	1790	U37526
Myxozoa:		
Buddenbrockia plumatellae Schröder, 1910	1784	AY074915
Ceratomyxa shasta Noble, 1950.	1643	AF001579
Myxidium sp.	1879	U13829
Myxobolus sp.	1903	U13830
Bilateria:		
Chaetonotus sp. (Gastrotricha)	1814	AJ001735
Stenostomum leucops (Duges, 1828) (Platyhelminthes)	1654	D85095
Stylaria sp (Oligochaeta)	1821	U95946

3.2.3 Development and Validation of a Single Round Nested PCR Assay for Specific Identification of Sphaerospora truttae

The four primers designed on basis of the 18S rDNA sequence obtained from *S. truttae* myxosporean spores were used in a single nested PCR assay. After determination of the optimal annealing temperature in a temperature gradient PCR, the assay was tested on *S. truttae* infected and uninfected salmon kidney. Thereafter, cross-reactivity and detection limits of the PCR assay were determined.

Testing for Cross-reactivity

Tissues of several fish species containing other myxozoan species were collected in order to test the specificity of the newly developed nested PCR assay. The myxozoans were selected to represent species (1) of the same genus (*Sphaerospora*), (2) of the same organ localization (urinary system) and (3) parasitising the same host family (Salmonidae). The myxozoan species chosen and their hosts are given in Table 3.2.3 (sample source see Chapter 5, Section 5.3.1.1). Genomic DNA for each species was isolated from infected tissues, uninfected salmon kidneys served as negative control.

Table 3.2.3 Myxozoan species (including their hosts and organ localisation) used in the specificity testing of the newly designed nested PCR assay for *Sphaerospora truttae.*

Myxozoan	Host	Localisation
Sphaerospora renicola Dyková & Lom, 1982	*Cyprinus carpio* L.	Renal tubules
Sphaerospora elegans Thélohan, 1892	*Gasterosteus aculeatus* L.	Renal tubules
***Chloromyxum* sp.**	*Salmo salar* L.	Renal tubules
Tetracapsuloides bryosalmonae Canning *et al.*, 2002	*Salmo salar* L.	Renal tubules
Myxobilatus gasterostei Davis, 1944	*Gasterosteus aculeatus* L.	Renal tubules
Hoferellus gilsoni Debaisieux, 1925	*Anguilla anguilla* (L.)	Urinary bladder
Myxidium giardi Cépède, 1906	*Anguilla anguilla* (L.)	Kidney
Myxidium truttae Léger, 1930	*Salmo salar* L.	Gall bladder
Chloromyxum truttae Léger, 1906	*Salmo salar* L.	Gall bladder
Myxobolus cerebralis Hofer, 1903	*Oncorhynchus mykiss* (Walbaum)	Cartilage

Determination of Limits of Detection

A 4%/5% PEG/dextran separation isolate (for methodology see 3.2.2) of *S. truttae* myxosporean spores from 10 salmon smolts was used in order to determine the limits of detection of the newly designed PCR assay. The concentration of spores was counted in a haemocytometer chamber and calculated as 217 000 spores ml^{-1}. 1 ml of this solution was diluted with 1170 µl of distilled water to achieve a standard of 100 000 spores ml^{-1}. The spores in 1 ml of this solution were pelleted in a 1.5 ml Eppendorf at 1000g (8 min), the supernatant was removed, 400 µl of TNES urea were added and DNA was extracted, precipitated and dried. The dry DNA pellet was re-suspended in 100 µl of parasite-free genomic salmon DNA in nanopure water (equalling a concentration of 1000 spores μl^{-1} host DNA solution). 1:10, 1:100 and 1:1000 dilutions of this stock were prepared and PCRs were conducted using the volumes equivalent to 1000 (1µl of original solution), 100 (1µl of 1:10), 10 (1µl of 1:100), 5 (5µl of 1:1000) and 1 (1µl of 1:1000) *S. truttae* spores.

Early Detection Study

In order to test the single round nested PCR for its ability to detect early developmental stages of *S. truttae* in the blood of infected salmon, 9 salmon fry (0.8 g) were exposed to an *S. truttae* infective environment and one fish was sampled each day for 9 days after initial exposure. Fish were killed by an overdose of chlorobutanol (1, 1, 1–trichloro-2-methyl-2-propanol hydrate) and after a transverse cut in the area of the peduncle a blood sample of 5 µl volume was taken from the caudal vein using a heparinised pipette tip. The blood was transferred to a 1.5 ml Eppendorf tube

containing 300 µl of TNES urea, and DNA was extracted and amplified applying the nested PCR assay designed for *S. truttae*.

Parallel to the blood samples taken for DNA extraction, one blood smear was prepared from each of the 9 fish, in order to compare the molecular assay with the ability to recognize the presence of *S. truttae* using conventional light microscope techniques. The blood smears were air dried and immediately fixed in methanol for a few seconds before being stained with a rapid conventional blood stain containing methylene blue and eosin (Lamb Stain-Quick staining kit, Raymond A Lamb Ltd.). The stained blood preparations were viewed under x 400, and the whole smear was thoroughly and systematically checked for the presence of blood stages.

3.3 Results

3.3.1 Molecular Characterisation of Echinactinomyxon type 5

Despite their large difference in T_m (=melting temperature, i.e. temperature at which 50% of a given oligonucleotide is hybridized to its complementary strand) the universal primers 18e and 18g proved to amplify 18S rDNA successfully when used in PCR. The temperature gradient PCRs using samples of *Echinactinomyxon* type 5 and *Echinactinomyxon* type 1 showed that the optimal annealing temperature for this primer pair lies around 65°C (Figure 3.3.1.A), and results in a myxozoan PCR product of approximately 1900 bp. In contrast to *Echinactinomyxon* type 1, a double band was amplified from all three replicate samples of *Echinactinomyxon* type 5. For sequencing, the two obtained bands were left to separate by running the samples over the full length of the agarose gel (12 cm). Both bands were cut from the gel, purified and

sequenced. The upper band was shown to be of myxozoan identity when submitted to BLAST, whereas the lower band appeared to have a ciliate origin. The partial sequences obtained from the upper band produced a consensus sequence of 1983 bp for *Echinactinomyxon* type 5 (GenBank™ accession number AJ417562). The results of the BLAST search of the 1983 bp fragment placed *Echinactinomyxon* type 5 clearly within the myxozoan group and showed that it is very closely related to *Myxobolus portucalensis* Saraiva & Molnar, 1990, a myxosporean found on the fins of the eel, *Anguilla anguilla* (L.). *Echinactinomyxon* type 5 shares 92.8% identical base pairs with this species and more than 80% sequence identity with other species of the genus *Myxobolus*, whereas the *Sphaerospora* species available on GenBank share only 62.7 to 74% identical base pairs with *Echinactinomyxon* type 5 (Table 3.3.1).

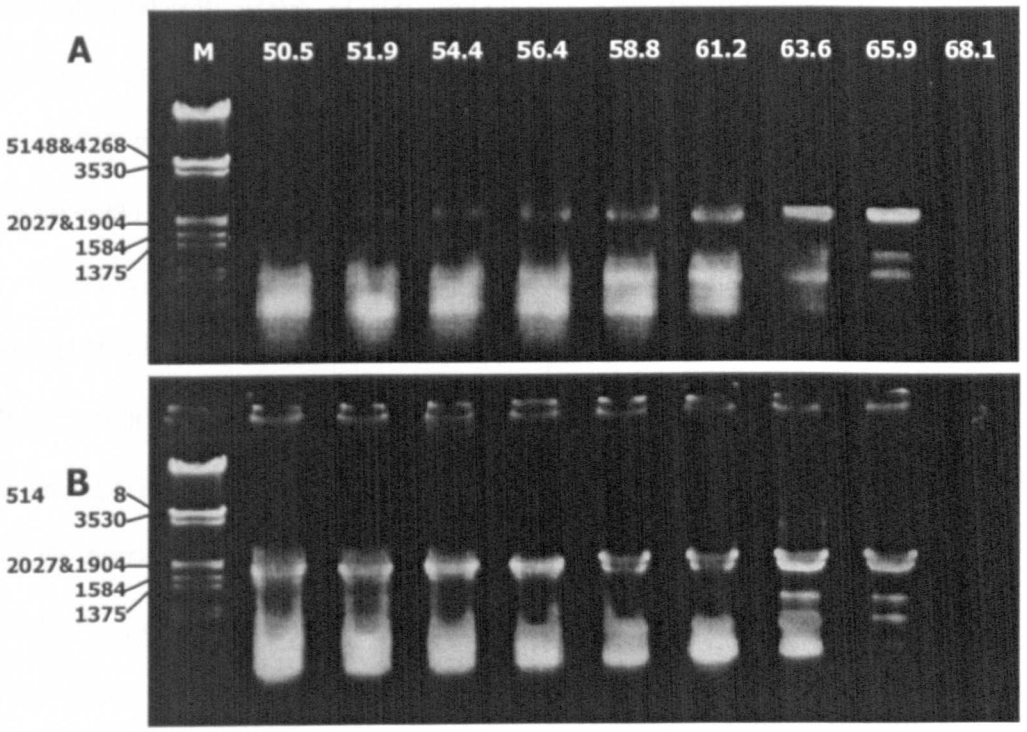


Figure 3.3.1.A Temperature gradient PCR (temperatures given on top) for primers 18e and 18g using genomic DNA extracted from (**A**) *Echinactinomyxon* type 1 and (**B**) *Echinactinomyxon* type 5. Two distinct bands are visible in all samples of *Echinactinomyxon* type 5; **M**=Marker showing fragment sizes (in basepairs).

Table 3.3.1 Sequence identities of *Echinactinomyxon* type 5 with its closest myxozoan relatives (≥80% sequence identity) as well as with all available species of the genus *Sphaerospora;* 1650 bp (844 bp in case of *S. dicentrarchi*) used for comparison.

Name	GenBank Acc. No.	% Sequence identity
Myxobolus portucalensis Saraiva & Molnár, 1990	AF085182	92.8
Myxobolus cultus Yokoyama, Ogawa & Wakabayashi, 1995	AB121146	81.2
Myxobolus lentisuturalis Dyková, Fiala & Nie, 2002	AY119688	80.8
Myxobolus sp Kent et al., 2001	AF378343	80.2
Sphaerospora molnari Lom et al., 1983	AF378345	74.0
Sphaerospora oncorhynchi *Kent et al., 1993*	AF201373	73.0
Sphaerospora dicentrarchi Sitjà-Bobadilla & Alvarez-Pellitero, 1992	AY278564	62.7

Alignment of the *Echinactinomyxon* type 5 sequence with these myxozoan sequences and with those of the oligochaete host and the supposed teleost host, identified variable regions which differed considerably between all sequences but the very similar ones of *Echinactinomyxon* type 5 and *Myxobolus portucalensis.* In four variable areas, four non-complementary primers were designed (Figure 3.3.1.B).

The designed primers amplified *Echinactinomyxon* type 5 successfully from DNA samples of infected *Lumbriculus variegatus* when used in two subsequent rounds of PCR (1st round using primers E5OF & E5OR, 2nd round using nested primers E5IF & E5 IR), as well as in a single round nested PCR (all primers in one reaction) (Figure 3.3.1.C). According to the nature of a PCR reaction containing the two forward and the two reverse primers, the 258 bp amplicon as the smallest of the 4 amplicons produced in the course of the reaction was consistently favoured. A temperature gradient identified the optimal annealing temperature of the designed primers as 61°C.

It was impossible to get the desired amplicon from kidney samples of *S. truttae*-infected salmon parr harbouring sporogonic stages of the parasite (Figure 3.3.1.C).

Name	Sequence	T_m	Position
E5OF	5'-CAGCCACTGTATGGATGGGTA-3'	59.8°C	222
E5OR	5'-AGGGATGCTGTGCGCCTATTC-3'	61.8°C	1573
E5IF	5'-GTTCTACCGTGGACGCATTCAA -3'	60.3°C	717
E5IR	5'-CATGGGATTAGCACACTCAGC-3'	59.8°C	953

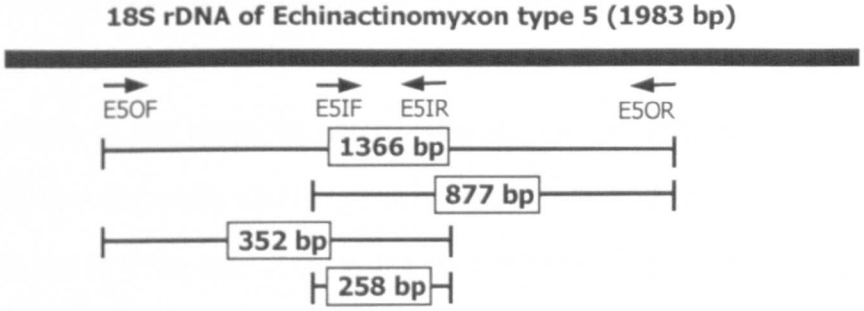


Figure 3.3.1.B Primers designed for *Echinactinomyxon* type 5 (sequences, T_m=melting temperature, position relative to the *Echinactinomyxon* type 5 18S rDNA) and fragment sizes of the 4 different PCR products possible in a reaction containing all 4 primers.

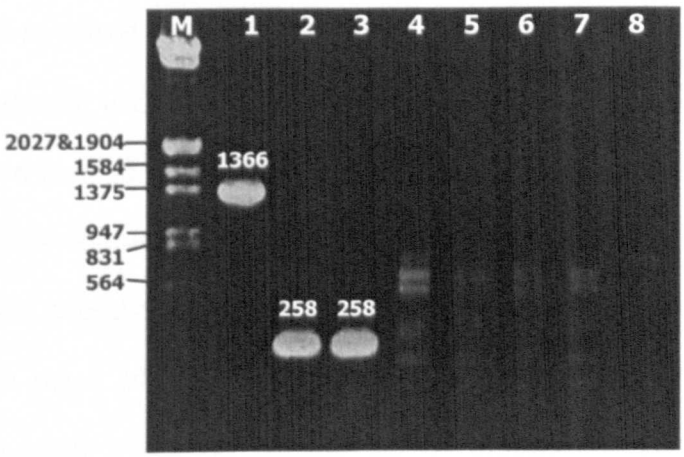


Figure 3.3.1.C PCR amplicons produced using the primers designed for *Echinactinomyxon* type 5. Lanes: **M**=Marker showing fragment sizes (in basepairs); **1-3=** *Echinactinomyxon* type 5, using (**1**) primers E5OF&E5OR, (**2**) primers E5IF&E5IR, (**3**) all 4 primers; **4-8**=*Sphaerospora truttae* positive kidney samples from 4 individual salmon parr.

3.3.2 Isolation and Molecular Characterisation of the Sphaerospora truttae Myxosporean Stage

Spore Isolation

Of the three methods used to purify *S. truttae* myxosporean spores from infected kidney tissues, the two-phase system of polyethylene glycol (PEG) and dextran proved to be the most effective. Simple centrifugation allowed the recovery of spores even at low speed (200g) when conducted for 10 min. When higher speeds (500g and 1000g) were used a higher number of spores were recovered but at the same time the amount of tissue debris in the pellet increased. However, in all centrifugation samples the pelleted spores were mixed with large amounts of melanin granules, and due to their inhibition of the PCR reaction of the extracted DNA it was not possible to amplify 18S rDNA from these samples. In contrast to the simple centrifugation method, density gradient centrifugation (Percoll™) allowed better separation of spores from host tissue and its melanin, but a considerable "loss" of spores in the different Percoll™ layers was noted. The spores were disseminated over the 40-60% Percoll™ layers, whereas the tissue debris remained in or on top of layers containing ≥30% Percoll™. The number of recovered spores was low (10-32% of the amount recovered using the simple centrifugation method at 1000g) but the purity of the sample allowed amplification in PCR. Reduction of the number of layers to only two layers improved the recovery of spores. In the two-phase systems of PEG and dextran, spores always partitioned into the PEG-rich upper phase, whereas tissue debris partitioned into the interface and dextran-rich lower phase. In contrast to the system containing 4% PEG and 5% dextran, the system containing 4% PEG and 8% dextran as well as the isopycnic mixture separated very slowly. The isopycnic mixture did not seem to demix completely

even when left to separate for another 30 min. Spore recovery was high in all systems but the mixture of 4% PEG and 5% dextran showed optically and numerically the best result. The results of all spore separation trials are summarized in Figure 3.3.1.D.

Centrifugation	1 min	5 min	10 min
200g	13 spores	253 spores	362 spores
500g	199 spores	409 spores	553 spores
1000g	326 spores	575 spores	599 spores

Percoll™	
30%/50%	73 spores
50%/60%	94 spores
60%/80%	17 spores
80%/100%	0 spores
Bottom	0 spores
15%/75%	256 spores
Bottom	1 spore
15%/30%	22 spores
bottom	198 spores

PEG/Dextran	
4%/8%	409 spores*
4%/5%	512 spores
4%/4%	461 spores*

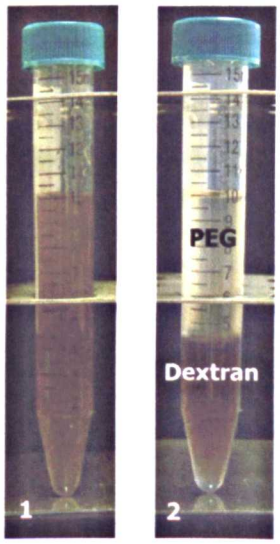


Figure 3.3.1.D Summary of the spore separation trials by centrifugation, Percoll™ gradients and PEG/Dextran methods. Spore numbers obtained by collection of spores from pellet (centrifugation)/interphase (Percoll)/PEG phase (PEG/Dextran). Some cell debris from host tissue present; More cell debris than spores; Red numbers indicated the best result of each method; *Slow or incomplete separation of phases; Vials **1 & 2**: 4% PEG/5% dextran system (**1**) after mixing and (**2**) after 30 min of separation.

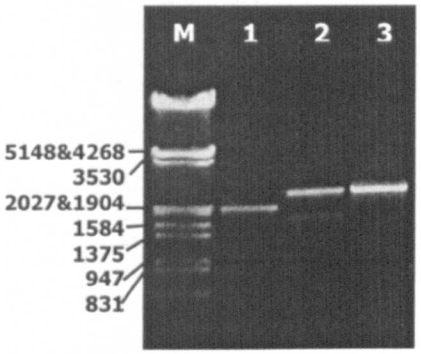


Figure 3.3.2.E 18S rDNA amplicons of (**1**) *Echinactinomyxon* type 5 and (**2&3**) *Sphaerospora truttae* spores; (**2**) Spores isolated by Percoll™ and (**3**) by PEG/Dextran; **M**=Marker showing fragment sizes (in basepairs).

Molecular Characterisation

DNA was extracted from the samples of *Sphaerospora truttae* spores which had been separated by (1) 5 min centrifugation at 200g, (2) 15%/30% Percoll™ and (3) 4%/5% PEG/Dextran (see above). PCR amplification of 18S rDNA was successful only from the samples lacking large quantities of melanin (Percoll™ and PEG/Dextran methods). The 18S amplicon produced from the *S. truttae* myxosporean spores was considerably longer than that obtained from *Echinactinomyxon* type 5 actinosporean spores (Figure 3.3.2.E). In the sequencing reactions, primer LIN3 repeatedly failed to sequence the *S. truttae* PCR product, but the partial sequences obtained from all other primers were sufficient to produce a consensus sequence of 2541 bp for the *S. truttae* myxosporean (GenBank™ Accession number AJ581915). This sequence shared less than 50% of identical basepairs with the sequence obtained from *Echinactinomyxon* type 5, as well as with the closest myxozoan BLAST matches (*Aurantiactinomyxon* sp. Hallett, *Myxobolus bononiense* Caffara et al., unpublished, *Myxobolus intimus* Zaika, 1965 and *Myxobolus obesus* Gurley, 1893) and with all other *Sphaerospora* species (Table 3.3.2).

Table 3.3.2 Sequence identities of *Sphaerospora truttae* with the closest myxozoan BLAST matches, with all available species of the genus *Sphaerospora* and with *Echinactinomyxon* type 5.

Name	GenBank Acc No	% Sequence identity
Echinactinomyxon type 5	AJ417562	48.9
***Aurantiactinomyxon* sp.** Hallett 2002	AF487455	50.0
Myxobolus bononiese Caffara et al., unpubl.	AY278563	47.3
Myxobolus intimus Zaika, 1965	AY325285	46.7
Myxobolus obesus Gurley, 1893	AY325286	46.4
Sphaerospora molnari Lom et al., 1983	AF378345	46.5
Sphaerospora oncorhynchi *Kent et al., 1993*	AF201373	48.6
Sphaerospora dicentratchi Sitjà-Bobadilla & Alvarez-Pellitero, 1992	AY278564	43.9

Due to the unusual length of the sequence of *S. truttae* in comparison with other myxozoan sequences from GenBank™ (2541 bp in contrast to 1800-2100 bp) and the results of the BLAST search, which did not show similarities of *S. truttae* with other myxozoans, the sequence was submitted to a preliminary phylogenetic analysis including Porifera, Ctenophora, Cnidaria, Bilateria and Myxozoa, with Fungi and Choanozoa as an outgroup, which confirmed its myxozoan identity (Figure 3.3.2.F). *S. truttae* grouped within the Myxozoa which, together with the aberrant cnidarian fish parasite *Polypodium hydriforme,* form a sister group to the cnidarian core group (see also Chapter 6, Section 6.1.1).

The alignment of the *S. truttae* sequence with the sequences of the myxozoans from Table 3.3.2 and with the sequence from the fish host showed that the extraordinary length of the *S. truttae* 18S gene is caused by extremely long variable

regions. This improved the conditions for specific primer design in these regions. Four *S. truttae* specific oligonucleotides were designed (Figure 3.3.2.G).

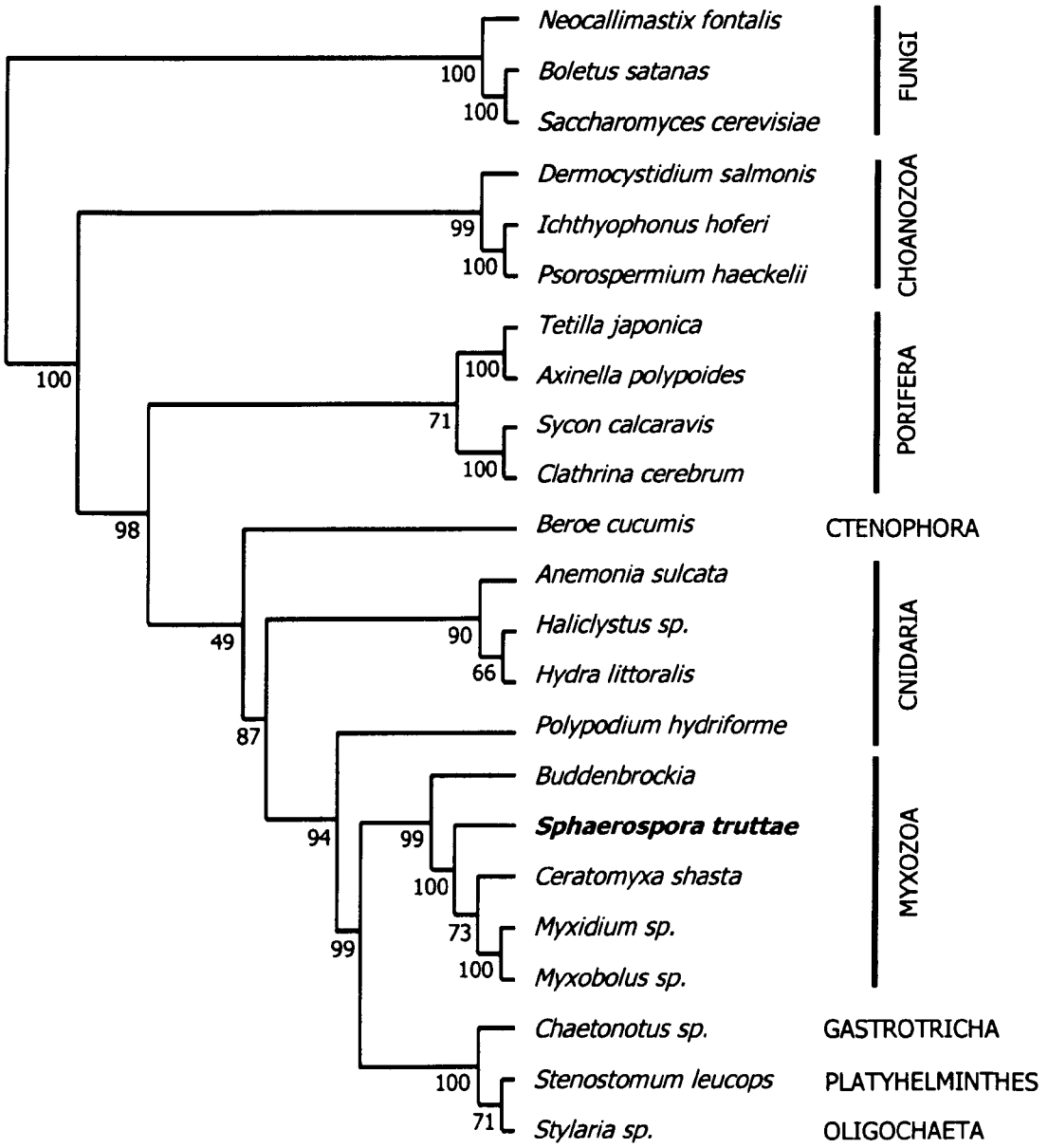


Figure 3.3.2.F Maximum likelihood tree showing the membership of *Sphaerospora truttae* in the myxozoan group (100 % bootstrap support) when analysed with different species of Porifera, Ctenophora, Cnidaria, Bilateria and Myxozoa, using Fungi and Choanozoa as an outgroup.

Name	Sequence	T_m	Position
StrOF	5'-TCTCGGGGTAGCGTGCATTTATTC-3'	62.7°C	158
StrOR	5'-AGACCTTCCGCACAGCCAACAGTA-3'	64.4°C	711
StrIF	5'-GATCGGTCTCAGCCCTTC -3'	58.8°C	1239
StrIR	5'-GGACACCCACTACACCCATCT-3'	61.8°C	2011

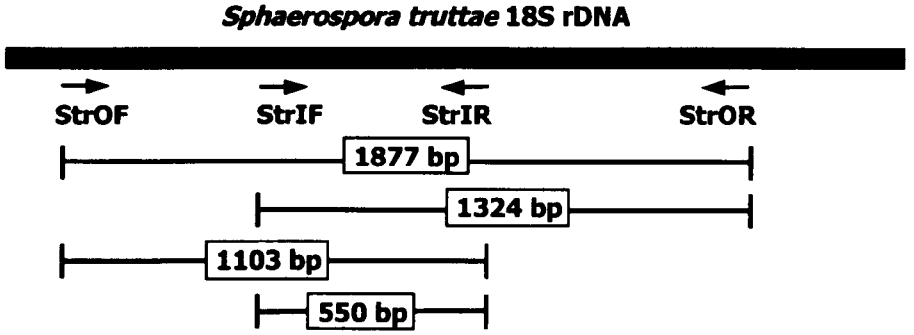


Figure 3.3.2.G Primers designed for *Sphaerospora truttae*: Sequences, T_m (melting temperature), position relative to the *Sphaerospora truttae* 18S rDNA and fragment sizes of the 4 different PCR products possible in a reaction containing all 4 primers.

3.3.3 Development and Validation of a Single Round Nested PCR Assay for Specific Identification of Sphaerospora truttae

Single Round Nested PCR Amplification

Using the 4 primers designed for *S. truttae* in a single round nested PCR assay showed that the PCR reaction favoured the amplification of the smallest PCR product, i.e. the nested PCR product of 550 bp. This amplicon was present in all kidney samples containing sporogonic stages of *S. truttae* but not in parasite-free control fish from Loch Fyne (Figure 3.3.3.A). The specific optimal annealing temperature of the reaction was determined as 63°C in a 50°C-70°C temperature gradient PCR.

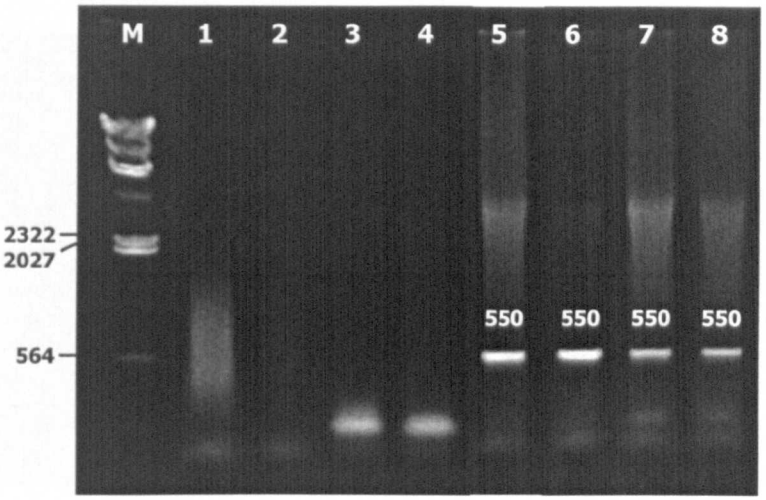


Figure 3.3.3.A Nested PCR assay using the primers designed for *Sphaerospora truttae*. **M**=Marker showing fragment sizes (in basepairs); **1-4**=uninfected salmon kidneys of 4 individual fish; **5-8**=*S. truttae* infected salmon, (**5-6**) kidney extracts, (**7-8**) blood extracts;

Specificity Testing

The cross-reactivity trial of the nested PCR assay designed for *Sphaerospora truttae* was tested with genomic DNA from *Sphaerospora renicola*, *Sphaerospora elegans*, *Chloromyxum* sp., *Tetracapsuloides bryosalmonae*, *Myxobilatus gasterostei*, *Hoferellus gilsoni*, *Myxidium giardi*, *Myxidium truttae*, *Chloromyxum truttae* and *Myxobolus cerebralis* and demonstrated that only *S. truttae* yielded the 550 bp PCR product (Figure 3.3.3.B). *Tetracapsuloides bryosalmonae* cross-linked weakly with two of the primers (identified as StrOF and StrIR) by producing small amounts of an approximately 1300 bp product (Figure 3.3.3.B). However, due to the weakness of the signal and its size, this does not interfere with the correct identification of *S. truttae*.

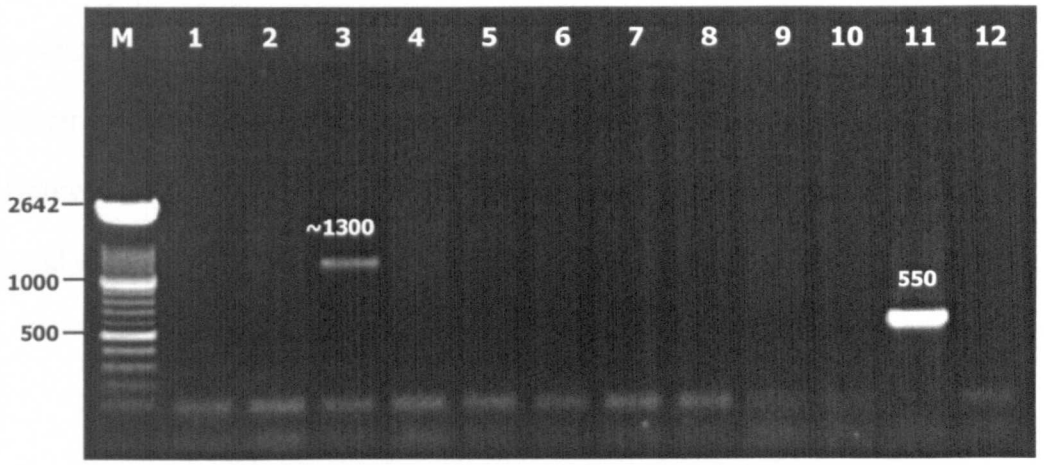


Figure 3.3.3.B Specificity of the nested PCR assay for *Sphaerospora truttae*: **M**=Marker showing fragment sizes (in basepairs); **1**=*Sphaerospora renicola;* **2**=*Sphaerospora elegans*; **3**=*Tetracapsuloides bryosalmonae*; **4**=*Chloromyxum* sp; **5**= *Myxobilatus gasterostei*; **6**=*Hoferellus gilsoni*; **7**=*Myxidium giardi*; **8**=*Myxidium truttae*; **9**=*Chloromyxum truttae*; **10**=*Myxobolus cerebralis*; **11**=*Sphaerospora truttae*; **12**=uninfected salmon kidney; Only *S. truttae* (**11**) exhibits the 550bp amplicon; *Tetracapsuloides bryoslamonae* (**3**) shows a weak, non-specific band at approximately 1300bp;

Limits of Detection

Isolated myxosporean spores of *Sphaerospora truttae* mixed with host DNA were used to examine the limits of detection of the nested PCR assay. Samples containing 1000, 100, 10, 5 and 1 spore in a 10 ml PCR reaction of 35 cycles tested positive. DNA from a single spore (i.e. in this case 6 parasite cells) was sufficient to be detected by the nested PCR assay (Figure 3.3.3.C).

Early Detection Study

Nine *S. truttae* free salmon fry were exposed to an *S. truttae* infective environment. On day one to day 7 after initial exposure, as well as two weeks and four weeks after initial exposure, 1 fish was sacrificed and a blood sample was analysed using the new PCR assay and light microscopy of stained blood smears. DNA

extractions from the salmon fry tested positive for the first time on day 3 after initial exposure when tested by PCR (Figure 3.3.3.D). DNA extractions from every fry examined at all following time points showed that *S. truttae* was present in the blood (Figure 3.3.3.D). In contrast to the PCR results, only 2 blood smears (fry sampled after 2 and after 4 weeks) were found to contain proliferative blood stages of *S. truttae.*

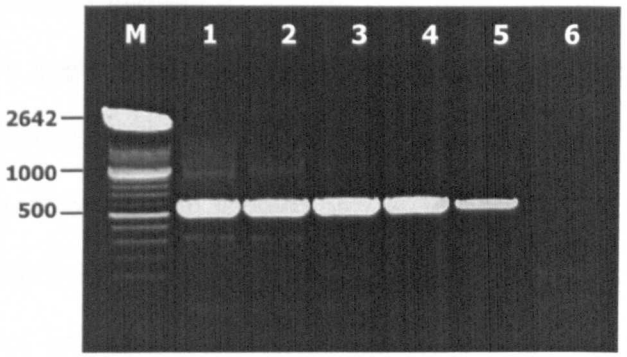


Figure 3.3.3.C Limits of detection of the nested PCR assay for *Sphaerospora truttae* using different concentrations of mature myxosporean spores in extractions mixed with host DNA: **M**=Marker showing fragment sizes (in basepairs); **1**=1000 spores; **2**=100 spores; **3**=10 spores; **4**=5 spores; **5**=1 spore; **6**=uninfected salmon kidney.

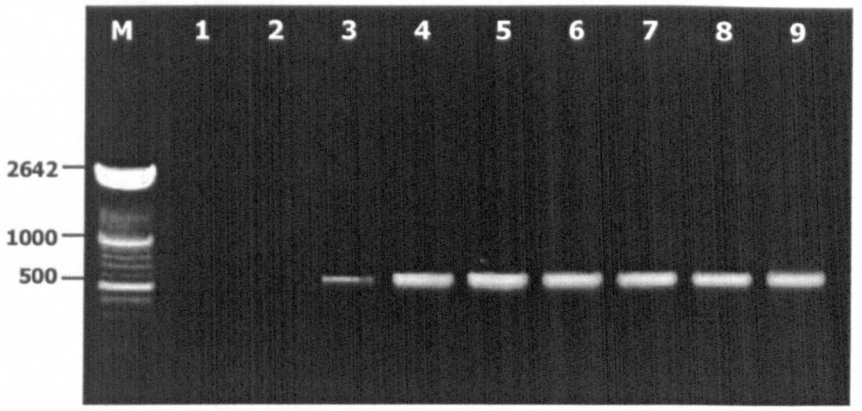


Figure 3.3.3.D Early detection of *Sphaerospora truttae* developmental stages in the blood: **M**=Marker showing fragment sizes (in basepairs); Lanes **1-9**: Blood samples taken on day 1-7, two weeks and one month after initial exposure of salmon fry to an infective *S. truttae* environment.

3.4 Discussion

3.4.1 Molecular Non-Homogeneity of Sphaerospora truttae and Echinactinomyxon type 5

The characterisation of *Sphaerospora truttae* was initially attempted by sequencing the 18S rDNA of the proposed actinosporean stage, i.e. *Echinactinomyxon* type 5. The consequent inability to amplify *S. truttae* from infected salmon kidneys using the primers designed for *Echinactinomyxon* type 5 led to the isolation and molecular characterisation of the *S. truttae* myxosporean stage. Sequences obtained from the *S. truttae* myxosporean showed only 48.9% sequence identity with the ones obtained for *Echinactinomyxon* type 5, and it can therefore be stated that these two myxozoans are not alternate life cycle stages of one myxozoan but represent two different species. This finding means that the life cycle of *S. truttae* outside the fish host is unknown, and raises the general problem of identifying alternate stages in myxozoan life cycles using non-molecular versus molecular techniques.

Echinactinomyxon type 5 had been related to the *S. truttae* myxosporean stage using infection studies (Özer 1999): Three infection trials were carried out using salmon fry reared on bore hole water as receptor fish for *Echinactinomyxon* type 5 spores released from the oligochaete host *Lumbriculus variegatus*. Two of these three attempts resulted in the production of mature *S. truttae* spores in some receptor fish 3.5-4.5 months after infection. As the present molecular study contradicts the species identity of *Echinactinomyxon* type 5 and the myxosporean *S. truttae* the following considerations try to identify the cause of the anomaly:

In the infection study, receptor fish might have been already infected with *S. truttae*. This seems unlikely since they were reared on bore hole water. However, it

cannot be excluded that the inflow canal leading from the bore hole to the farm or parts of the farm itself are populated by the unknown alternate hosts. The salmon fry used in the experiments were challenged with several types of actinosporeans but only those challenged with *Echinactinomyxon* type 5 developed an *S. truttae* infection. It is suggested that unfavourable conditions, like the challenge with a specific actinosporean, in susceptible fish might stress the fish immune system enough to allow an already established *S. truttae* infection to proliferate. However, in the current study, receptor fish from a different source were used and tested *S. truttae* free by the nested PCR assay designed on the basis of the *S. truttae* myxosporean stage. All attempts of infecting these fish with freshly shed *Echinactinomyxon* type 5 spores failed (see Chapter 5, Section 5.3.3.1). Published reports show that infection studies involving myxozoans generally produce strongly varying results and raise questions about optimal conditions for infections (see Chapter 5, Section 5.1.2). Due to the commonly encountered problems in infection studies, life cycles are now being described with the support of molecular data on the different life cycle stages (Andree *et al.* 1997, Bartholomew *et al.* 1997, Anderson *et al.* 1999, Lin *et al.* 1999, Anderson *et al.* 2000).

The genetic heterogeneity of *Echinactinomyxon* type 5 and *S. truttae*, as shown in the current study, allows the molecular characterisation of two different myxozoans. The sequence obtained from *Echinactinomyxon* type 5 proved a close relationship with some representatives of the genus *Myxobolus,* identifying *Myxobolus portucalensis* as the closest relative sequenced so far. With 92.8 % identical base pairs over a 1650 bp fragment and far-ranging similarities in the variable regions of the 18S gene it cannot be guaranteed that the primers specifically designed for *Echinactinomyxon* type 5 are able to differentiate between this myxozoan and *Myxobolus portucalensis*. However,

Myxobolus portucalensis is a parasite occurring on the skin of the eel *Anguilla anguilla* (L.) (Saraiva & Molnar 1990). The next closest relatives of *Echinactinomyxon* type 5 show around 80% sequence identity, and they belong to the same genus: e.g. *Myxobolus cultus* which inhabits the cartilage of goldfish *Carassius auratus* (L.) (Yokoyama *et al.* 1995), or *Myxobolus lentisuturalis* which occurs in the muscle of the Prussian carp *Carassius gibelio* Bloch (Dyková *et al.* 2002). The close molecular relationship of *Echinactinomyxon* type 5 with these myxosporean species suggests that this actinosporean might represent the alternate stage of a myxosporean of the *Myxobolus* type (see also Chapter 6). However, despite lacking evidence for this assumption the results show that other myxosporean genera are more distantly related and other *Sphaerospora* species share only 67.9-74% sequence identity with *Echinactinomyxon* type 5.

For the current study the molecular study of *S. truttae* had to be based on the sequence obtained from the myxosporean stage. The extraordinary length of this sequence (2541 bp in contrast to the average myxozoan with 1800-2100 bp) and far-reaching differences in the variable regions of the 18S gene mean it lies so far from the myxozoan core group that a phylogenetic analysis had to be conducted in order to ensure its myxozoan identity. Thereby, *S. truttae* was placed into the myxozoan group with 100% bootstrap support.

3.4.2 PEG / Dextran - A New Myxosporean Spore Isolation Method

In order to obtain the 18S rDNA sequence of the *S. truttae* myxosporean the parasite had to be isolated from its host as PCR amplification from extractions of

infected kidneys using general primers 18e and 18g consistently resulted in amplification of host DNA only.

In contrast to some other myxosporean spores, which are formed in vast numbers inside large cysts, the collection of spores developing asynchronously inside bisporous pseudoplasmodia, which lie scattered in the renal tubules, is more difficult. Thus, methods based on centrifugation, Percoll™ gradients and polyethylene glycol (PEG)/dextran separation aiming at the isolation of mature *S. truttae* spores from the kidney of Atlantic salmon were tested. The results showed that centrifugation only cannot be recommended for isolation of myxosporean spores as a considerable amount of tissue debris and melanin remain in the spore pellet. The melanin granules liberated in the process of homogenising kidney tissue were shown to inhibit PCR after DNA extraction. Melanin has been shown to bind to the DNA polymerase and functions as a PCR inhibitor at concentrations of less than 200 ng ml^{-1} (Eckhart, Bach, Ban & Tschachler 2000). Both Percoll™ and PEG/dextran separation were able to avoid this problem. Due to density differences between the *S. truttae* spores, which was presumably related to different states of maturity, the spores disseminated over a wide range of Percoll™ layers below 80%. Inevitably, this resulted in a major loss of spores which was diminished by decreasing the number of layers. Optimally, one or two layers (15-30% Percoll™) which allow the spores to pass through and pellet but which causes the tissue debris to remain on top of the silica gel should be used. Percoll™ has previously been used for the isolation of myxosporean spores of *Ceratomyxa shasta* (Fryer 1987), *Myxobolus cerebralis* (Hamilton & Canning 1988) and *Kudoa thyrsites* (Chase, Dawson-Coates, Haddow, Stewart, Haines, Whitaker, Kent, Olafson & Pearson 2001). In all these myxosporean infections large numbers of spores were produced in

the infected organs. Hamilton & Canning (1988) isolated up to 510 000 *M. cerebralis* spores ml^{-1} from a single 7-month-old trout, whereas in this study the initial concentration of *S. truttae* spores in the kidney homogenate of one salmon parr was only 35 000 ml^{-1}. In comparison with the Percoll™ method the PEG/dextran separation tested in this study isolated a much higher (approximately double) number of spores. As a result, this method is especially useful in infections where only a limited number of spores are present.

The aqueous bi-phase system of polyethylene glycol (PEG) and dextran has been well studied for the use of separation of labile biomolecules, e.g. enzymes from broken crude cell material. In this system, PEG forms the upper, more hydrophobic phase in the presence of dextran. The method offers mild conditions due to the low interfacial tension between the phases (i.e. about 400-fold less than that between water and an immiscible organic solvent) allowing small droplet size, large interfacial areas and efficient mixing under very gentle stirring and rapid partition. The polymers also have a stabilising influence on most molecules. This suggests that it might be possible to use the spores after separation for various other purposes (e.g. immunological studies).

In the case of *S. truttae,* the spores partitioned consistently into the PEG-rich upper phase, whereas tissue debris partitioned into the interface and dextran-rich lower phase. The system containing 4% PEG and 5% dextran separated rapidly and showed optically and numerically the best result. It isolated twice as many spores as the Percoll™ gradients and can therefore be highly recommended for the isolation of myxosporean spores.

3.4.3 A Nested PCR Assay for Identification of Sphaerospora truttae

Presumptive diagnosis of *Sphaerospora truttae* has so far relied on the recognition of proliferative parasite stages in blood smear preparations and the identification of mature spores in the kidney tubules using imprint and histological techniques. In Atlantic salmon and brown trout from different locations in Scotland, *S. truttae* was found to occur in mixed infections with another myxozoan of the genus *Chloromyxum*, and occasionally with the PKX organism *Tetracapsuloides bryosalmonae* (personal observation). All of these species settle in the interstitial tissue as vegetative, cryptic stages prior to their intratubular occurrence. In order to enable early recognition and prevent morphological misidentification of *S. truttae* with these or other species, 4 specific oligonucleotides were designed on the basis of the 18S rDNA sequence obtained from the myxosporean spores. The extraordinary length of the variable regions of this gene in *S. truttae* in comparison with other myxozoans facilitated the design of specific oligonucleotides as other species showed far-reaching deletions in these areas. In addition, the primers were designed for use at a relatively high annealing temperature (specifically evaluated as 63°C), which further reduces the possibility for non-specific cross-linking of the oligonucleotides in a PCR reaction. As a result the nested PCR assay proved to be very specific for *S. truttae*. Only one of ten myxozoans used in the specificity tests produced any PCR product at all. This species, *Tetracapsuloides bryosalmonae,* shares the renal interstitial and intratubular location with *S. truttae* in both salmon and brown trout. As a result of cross-linking of two of the primers, a weak band of approximately 1300 bp was produced in the PCR reaction containing *T. bryosalmonae*. However, due to the different size of amplicons produced by *T. bryosalmonae* (1300 bp) and *S. truttae* (550 bp) misidentification cannot occur.

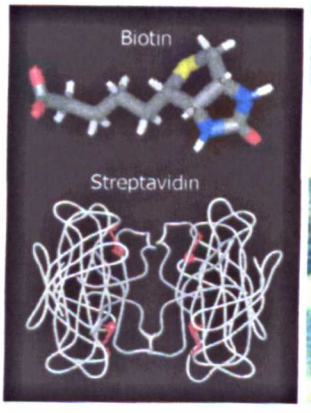
The convincing specificity of the PCR for *S. truttae* was accompanied by a high sensitivity of the assay. Presumably, this is related to the multiple copy gene character of the ribosomal DNA. Copy numbers between 39 and 19300 in eukaryotic cells (Prokopowich, Gregory & Crease 2003) make the 18S rDNA an ideal target for the detection of low-level parasitic infections. Due to the application of a nested PCR reaction, the sensitivity of the assay is increased further by multiple re-amplification of *S. truttae* amplicons produced in the course of the reaction. As a result the assay proved to be sensitive enough to detect a single spore in a 10 µl reaction (35 cycles). Specific PCR assays have been developed for other commercially important myxozoans, and the detection limit for PKX was reported to be 5 cells (Saulnier & de Kinkelin 1997) whereas the nested PCR test designed for *Myxobolus cerebralis* (Antonio *et al.* 1998) achieved a threshold of detection of the equivalent of a single parasite cell using 2 PCR rounds of 35 cycles each. In the case of *S. truttae,* the external and the nested primer pairs were specifically designed to work at the same temperature, to not cross-react and therefore can be applied in a single round PCR of 35 cycles, saving time and material costs. The lowest number of parasite cells tested was 6 (equalling one spore) but the amount of amplified DNA product resulted in a strongly visible band suggesting that lower levels might well be achievable. However, due to the equally high copy number of the 18S gene in different myxozoan species, the detection threshold in nested PCR assays should be similarly low for all species and differences are probably rather a reflection of the quality of the DNA extraction technique.

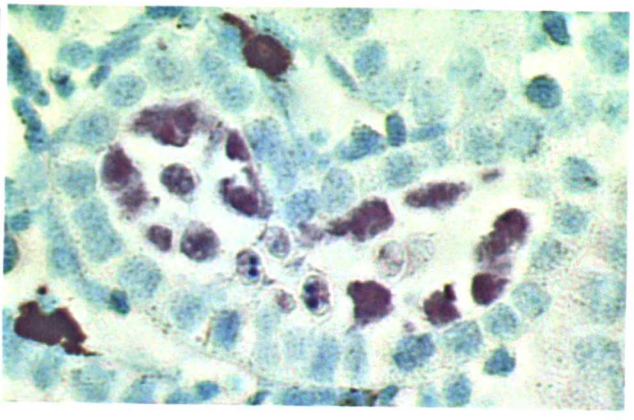
In order to test the ability of the PCR assay to detect early developmental stages, salmon fry were exposed to a natural infective environment. It is known that *S. truttae* proliferates in the blood in the form of multicellular stages before sporogony takes

place (McGeorge *et al.* 1996a). It has not previously been possible to confirm the presence of blood stages using light microscopy before the parasite had proliferated in the blood for at least 2 weeks. Using PCR, blood samples tested positive for *S. truttae* from day 3 after infection onwards. Thus, compared with conventional imprint and histological methods, the PCR assay allows earlier detection of *S. truttae* and additionally specifically identifies the proliferative stages, as blood stages of other myxozoans would otherwise be morphologically indistinguishable.

CHAPTER 4

Detection of the Spatio-Temporal Migration of *Sphaerospora truttae* in the Atlantic Salmon Using *in Situ* Hybridisation





4 Detection of the Spatio-Temporal Migration of *Sphaerospora truttae* in the Atlantic Salmon Using *in Situ* Hybridisation

4.1 Introduction

4.1.1 Detection of the Early Development of Myxozoans

Little is known about the early development of myxozoans in their fish hosts. To date, the complete details of development have been resolved only in the case of *Myxobolus cerebralis* (El-Matbouli *et al.* 1995). Comprehensive descriptions of presporogonic stages exist only for two further pathogenic species, which occur in high numbers in their fish hosts, i.e. *Sphaerospora renicola* (Csaba 1976, Lom, Dyková & Lhotáková 1982, Lom, Dyková & Pavlásková 1983, Dyková, Lom & Körting 1990) and *Tetracapsuloides bryosalmonae,* the PKX myxosporean (Kent & Hedrick 1986, Kent, Khattra, Hedrick & Devlin 2000). In the case of *Sphaerospora truttae,* the detection of presporogonic stages in blood and kidney imprints of Atlantic salmon has hitherto only been possible 2-4 weeks after exposure of the fish to *S. truttae* enzootic water. The newly developed PCR assay (Chapter 3, Section 3.3.3) improved the early detection of an *S. truttae* infection (3 days p. i.) and allows also specific identification of the myxozoan. However, it does not provide information about the localisation and morphology of the earliest stages of development in the fish host.

The presumed small number, size, and cryptic nature of the earliest stages of myxosporeans makes them difficult to detect and to differentiate from host cells using conventional histological techniques. Diagnostic tools applying lectins (Castagnaro, Marin de Mateo, Ghittino & Hedrick 1991, Hedrick, Marin de Mateo, Castagnaro, Monge,

& de Kinkelin 1992, Marin de Mateo, McGeorge, Morris & Kent 1996, Marin de Mateo, Bovo, Comuzzi & Adams 1997, Munoz, Palenzuela, Alvarez-Pellitero & Sitja-Bobadilla 1999a) and antibodies (Adams *et al.* 1992, Bartholomew *et al.* 1989, Marin de Mateo *et al.* 1993, Markiw 1989, Morris, Adams & Richards 1997, Munoz, Sitja-Bobadilla & Alvarez-Pellitero 1998, Munoz, Palenzuela, Sitja-Bobadilla & Alvarez-Pellitero 1999b, Chase *et al.* 2001, Saulnier & de Kinkelin 1996) have therefore received special attention. However, both lectins and antibodies, show inherent limitations in their use for clinical diagnosis as they may exhibit stage-specific affinities (Bartholomew *et al.* 1989, Marin de Mateo *et al.* 1996, Morris *et al.* 1997, Saulnier & de Kinkelin 1996) and may show cross-reactivity with host tissues and with other parasites (Marin de Mateo *et al.* 1996, Munoz *et al.* 1999a). Thus, the most promising approach in the consideration of physical, chemical and immunological changes during parasite development in the fish host is the use of DNA based methods as most genes have a consistent structure throughout the ontology of an organism.

4.1.2 The In Situ Hybridisation Technique

In situ hybridisation (ISH), aims to detect parasite DNA or RNA in sections of host tissues by hybridisation of specifically designed nucleic acid probe sequences to complementary DNA/RNA sequences. The procedure combines the specificity and sensitivity of the PCR detection technique with the ability to examine the parasite in its biological context.

The ISH histochemistry involves several steps which are known to be important for the outcome of a protocol. An overview of the reagents and procedures in an ISH using a biotin-streptavidin sandwich detection method is given in Table 4.1.2. For a

specific target sequence to be detected, individual protocols have to be elaborated in order to optimise the specific signal from the target DNA/RNA studied.

With regard to the myxozoans, ISH has so far been successfully used only to detect the causative agent of whirling disease, *Myxobolus cerebralis*, at an early subclinical stage (Antonio *et al.* 1998), and to identify the portal of entry of the PKX organism, *Tetracapsuloides bryosalmonae* into the fish host (Morris *et al.* 1999, Morris *et al.* 2000, Longshaw *et al.* 2002). These initial ISH studies on myxozoans proved that *in situ* hybridisation is a powerful tool to visualise cryptic presporogonic stages of myxozoans and that the full potential of the technique to investigate myxozoan pathways in their hosts is yet to be tapped.

4.1.3 Objectives

The present chapter describes the development of a rapid and optimised *in situ* hybridisation protocol for *Sphaerospora truttae* employing the primers specifically designed for this species (see Chapter 3, Section 3.3.2). Subsequently, it was of particular interest to use the ISH technique as a tool in order to determine, in an experimental infection study, the entry locus as well as the spatio-temporal occurrence of *S. truttae* developmental stages in the different organs of the Atlantic salmon before spore maturation in the target organ, the kidney. Furthermore, quantification of the parasite stages detected by ISH would help to gain a greater understanding of the processes of migration and proliferation of the parasite in different organs.

Table 4.1.2 A generalised ISH protocol using a biotin-streptavidin detection method: the different steps and their functions, reagents applied and their usual concentration (summarised from Southern & Herrington 1998)

Treatment	Reagents	Concentration	Function
1. Rapid fixation	Buffered formalin	10%	Prevents loss of nucleic acids and maintains tissue morphology
2. Slide adhesive	Aminopropyltriethoxysilane (APES)	2%	Minimization of section loss
3. Permeabilisation	Proteinase K	<15μg ml^{-1} (RNA) <500μg ml^{-1} (DNA)	Removal of cross-links between nucleic acids and cellular proteins to allow access of the probe to the target sequence
4. Post-fixation	Paraformaldehyde	0.4%	Post-fixation of DNA/RNA in the digested tissues
5. Acetylation	Acetic anhydride	0.25%	Blocking of non-specific probe interaction
6. Blocking of non-specific enzyme	Hydrogen peroxide	10%	If peroxidase is used in the detection system, the activity of peroxidase naturally present in the tissues has to be blocked
7. Pre-hybridisation	Hybridisation buffer		Equilibration of tissues in the high salt concentration of the hybridisation mix
8. Hybridisation	Hybridisation buffer + probe	1-2ng μl^{-1} probe	Hybrid formation between probe and target sequences
9. Post-hybridisation washes	Sodium saline citrate (SSC)	2 x SSC followed by 0.1 x SSC	High salt concentration removes unbound probes and lower ionic strength wash removes mis-matched hybrids
10. Detection (direct)	Peroxidase or alkaline phosphatase conjugated to streptavidin	1/50-1/1000	Binding of an enzyme-linked, high affinity reporter molecule (streptavidin) to the primer label (biotin)
11. Visualisation	Substrate (e.g. Fast Red, NBT/BCIP)		Visualisation of the enzyme coupled to the streptavidin-biotin complex, using a chromogen substrate
12. Counterstaining	Cytoplasmatic stain		Visualisation of background tissue, enhancement of contrast of chromogen substrate

Hybridisation buffer components	Concentration	Function
Formamide	50%	Destabilizes hydrogen bonding between probe and target sequences
Sodium saline citrate (SSC)	2 x SSC	Enhances hybrid stability
Dextran sulphate	10%	Inert polymer which, by molecular exclusion, concentrates the probe
Sonicated salmon/herring sperm DNA	100 μg ml^{-1}	Blocks non-specific binding of DNA probes
Sonicated yeast tRNA	1 mg ml^{-1}	Blocks non-specific binding of RNA probes
Bovine serum albumine (BSA)	0.02-0.5%	Reduces non-specific binding of the probe
Ficoll and polyvinylpyrrolidine	0.02-0.5% each	Reduces non-specific binding of the probe
Tris buffer	0.25 M	Provides a neutral pH
EDTA	5 mM	Inhibits nucleases
Sodium dodecyl sulphate (SDS)	1%	Reduces background staining

4.2 Materials and Methods

4.2.1 Development of the In Situ Hybridisation Technique

For the detection of *Sphaerospora truttae* developmental stages in the Atlantic salmon, an ISH protocol based on a biotin-streptavidin sandwich detection system was used, a method which had been successful in visualising developmental stages of *Tetracapsuloides bryosalmonae* (Morris *et al.* 2000). A non-specific primer (PKX1032, see Chapter 3, Table 3.2.2.B) which had originally been developed for *T. bryosalmonae* but which was shown to cross-link with *S. truttae* by Morris *et al.* (2000) was used in the initial ISH trials. Later, in order to develop a specific ISH protocol for *S. truttae*, the primers specifically designed for *S. truttae* (Chapter 3, Figure 3.3.2.G) were tested for their ability to form hybrids with complementary sequences on the sections. All primers were synthesised and 5' biotin-labelled by MWG Biotech AG.

For the development and optimisation of the ISH technique the kidney of one Atlantic salmon was used (collected on the North coast, on 16.11.2000). Fresh squash preparations of this kidney were found to contain high numbers of intratubular sporogonic stages and mature spores of *S. truttae*. A large piece of kidney was dissected, fixed and processed for histology as described in 2.4.5. Six µm sections were cut and mounted on 3-(aminopropyl)triethoxysilane (APES) coated slides to avoid detachment of the tissues during the tissue digestion in the ISH.

In order to optimise the procedure and the incubation times while maintaining the tissue structure and shortening the protocol, the ingredients and steps of the ISH protocol were varied on serial sections of the kidney:

After de-paraffination and re-hydration of the sections in a graded ethanol series they were equilibrated in tris-buffered saline (TBS, pH 8).

Permeabilisation

Serial sections were permeabilised with 100 µg ml^{-1} of proteinase K (Sigma-Aldrich Co.) in TBS (pH 8) for 30 min, 20 min or 10 min at 37°C. Following incubation with the enzyme, sections were washed for 5 min in PBS.

Post-fixation

After proteinase treatment the sections were post-fixed for 15 min with 0.4 % paraformaldehyde in PBS and washed in distilled water. In order to test the influence of post-fixation on the retention of DNA in the cells, 5 sections were not fixed but directly transferred into distilled water.

Blocking of Non-specific Peroxidase Activity

Serial sections were exposed to 10% H_2O_2 in methanol for 30 min, 20 min or 10 min. Thereafter the H_2O_2 was washed off with distilled water. For comparison, non-specific peroxidase activity was left unblocked on 5 sections.

Pre-Hybridisation/Dehydration

Five sections were pre-hybridised for 1 hour in hybridisation buffer (no primers added) whereas all subsequent sections were not pre-hybridised but dried at 45°C in an oven for 10 min.

Hybridisation Buffer

The hybridisation mix applied contained 4 x SSC in TBS (pH 7.6), 0.5% Ficoll, 0.5% polyvinylpyrrolidine, 0.5% BSA, and 0%, 10%, 20% or 50% of formamide. The mixtures were kept frozen at -70°C. Before use for ISH, 100 $\mu g\ ml^{-1}$ of calf thymus DNA and 1.5 $ng\ \mu l^{-1}$ of biotin labelled probe was added to the hybridisation mixtures. All primers (PKX1032 as well as STROF, STROR, STRIF, STRIR, Chapter 3, Figure 3.3.2.G) were tested individually on serial sections.

Hybridisation Procedure

Sections were framed with a GeneFrame and equilibrated in the hybridisation mixture. Sealed with a cover slip the sections were then denatured for 4 min at 95°C. Subsequently, the probes were left to hybridise to complementary sequences on serial sections for one hour, two hours and overnight at 37, 45, 50 or 55°C.

Washing Steps

The incubation was followed by a non-stringent wash for 5 min in 2 x SSC in order to remove the cover slip and unbound probes. A high-stringent wash for 45, 30 or 15 min in 0.1 x SSC containing 0.1% TWEEN20 (Sigma-Aldrich, Co.) was conducted at an elevated temperature (45°C) in order to ensure the specificity of binding of the probes to the target DNA.

Enzyme Linking, Substrate Binding and Counterstaining

The hybridisation signals were detected by incubation with horseradish peroxidase-labelled streptavidin using Ready to Drop-On Mix (Vector Laboratories) for

40, 20 or 10 min. This step was followed by two thorough washes in PBS for 5 min each. Binding of the enzyme was visualised using the VIP chromogen substrate (Vector Laboratories). The substrate reaction was observed under the microscope and was stopped as soon as background staining became visible (usually after 15 min). 0.5% methyl green (Vector Laboratories) was applied for 1 min in order to counterstain the sections. After washing in distilled water and rapid dehydration in 95% and 100% alcohol the sections were transferred into xylene and mounted in Pertex.

4.2.2 Specificity Testing of the ISH Method

In order to test the specificity of the DNA probes in the ISH protocol, sections from other *Sphaerospora* species as well as from other myxosporean kidney parasites belonging to different genera, were tested for cross-reactivity (Table 4.2.2).

Table 4.2.2 Myxosporean species used for specificity testing of the ISH protocol designed for *Sphaerospora truttae.*

Myxosporean	Host	Tissue localisation
Sphaerospora renicola Dyková & Lom, 1982	*Cyprinus carpio* L.	Kidney tubules
Sphaerospora galinae Evlanov, 1981	*Tinca tinca* L.	Kidney tubules
Sphaerospora elegans Thélohan, 1892	*Gasterosteus aculeatus* L.	Kidney tubules
Sphaerospora molnari Lom et al., 1983	*Cyprinus carpio* L.	Gill filaments
Myxobilatus gasterostei Davis, 1944	*Gasterosteus aculeatus* L.	Kidney tubules
Hoferellus gilsoni (Debasieux, 1925)	*Anguilla anguilla* (L.)	Urinary bladder
***Chloromyxum* sp**	*Salmo salar* L.	Kidney tubules
Tetracapsuloides bryosalmonae Canning et al., 2002	*Salmo salar* L.	Kidney (tubules & interstitial tissue)

4.2.3 Experimental Infection Study

Due to the lack of knowledge of the alternate actinosporean stage of *S. truttae*, gravel sediment and small rocks from the outflow pond of the fish farm on site A (North coast) was used for experimental infection of salmon fry (for further infection trials see Chapter 5, Section 5.2.3). The sediment was collected on the 30.04.2001, approximately 6-8 weeks before first signs of infection are detectable in fish from this site by conventional histological techniques (McGeorge *et al.* 1994). The sediment contained various invertebrates and a high density of oligochaetes (see Chapter 5, Table 5.3.1.2.A) from which 21 different actinosporean spore types have been recorded (Özer *et al.* 2002, see also Chapter 5, Section 5.3.1.2). At the Institute, 23 L of sediment from the hatchery outflow were placed in a 180 L flow through tank. The tank was supplied with de-chlorinated tap water, aerated and maintained at a temperature of 14±3°C. After 6 weeks of acclimatisation (on 15.06.2001), 80 naïve salmon fry with an average length of 3.5 cm were introduced into the tank, separated from the sediment by a wire mesh (day 0). These receptor fish were obtained from site C (Loch Fyne) at 14.05.2001, and they were found to be *S. truttae* free, microscopically and when amplified in PCR (20 fry tested). Thereafter, 2 fish were removed from the tank daily for 14 days, followed by 1 fish daily for the remainder of the experiment (46 days). A control tank of the same dimensions harboured 10 salmon fry and no sediment. One fish from the control tank was sacrificed every week and examined for myxosporean parasites.

4.2.4 Evaluation of Parasite Numbers During the Development of Sphaerospora truttae in the Fish Host

In order to study the proliferation processes of *S. truttae* in different host organs, the parasites present at different stages of development within the fish were quantified in the kidney, spleen and liver of 12 individual fish (days 15 to 26 post infection). The organs were manually outlined on digital images of sections and their surface areas were calculated (Zeiss KS300, v 3.0). The number of parasites within the tissues and the vascular system was then counted on a sufficient number of randomly selected sections to examine 1 mm^2 of each organ. From these data the proportion of parasites in the different organs and the related vascular system as a percentage of the total number counted per fish was calculated. This enabled comparisons of the organs between different fish independent from the intensity of infection, which varied between individuals.

In order to test for the statistical significance of differences between parasite numbers in the tissues, a two-way ANOVA (using GLM function) was conducted using SPSS v. 11 for Windows™.

4.3 Results

4.3.1 Development and Optimisation of the ISH Technique

The influence of different concentrations of the reagents in the ISH protocol and the variation of incubation times on the success of the protocol is summarised in Table 4.3.1.A. It was found that the omission of the post-fixation step following tissue permeabilisation with proteinase K did not cause loss of DNA/signal. Drying of the tissues in an oven prior to hybridisation was found to have the same effect as conducting the widely used but time-consuming pre-hybridisation step. The hybridisation temperature lowering reagent, formamide, was excluded from the hybridisation mix due to undesirable background effects in relation with its use. However, hybrid-formation was found to be activated sufficiently at 45 °C without the addition of formamide. A strong hybridisation signal was detected after two hours of incubation with the hybridisation mix and longer incubation did not improve the intensity of the signal. The combination of maximum signal and minimum incubation time in each step resulted in the development of a rapid (approximately 5 hours) ISH protocol with maximum tissue preservation (Table 4.3.1.B.).

The primers PKX1032, STIR and STOR showed strong hybrid-forming reactivity whereas STOF produced only a weak signal and STIF did not react under the given conditions. Successful binding of the probes to *S. truttae* target sequences and therefore presence of the parasite, was observed as a purple signal in greenish blue stained target tissues. The protocol used for specificity testing and the subsequent study of the presporogonic development of *S. truttae* in the Atlantic salmon employed the *S. truttae* specific primers STIR and STOR.

Table 4.3.1.A Results obtained by the variation of reagent concentrations and incubation times in the ISH protocol. - no signal, + weak signal, ++ moderate signal, +++ very strong signal, BS background staining, SBS strong background staining.

Treatment	Concentration//Duration	Result
Permeabilisation with proteinase K	100 μg ml^{-1}//30 min	+++
	100 μg ml^{-1}//20 min	+
	100 μg ml^{-1}//10 min	-
Post-fixation in 0.4% paraformaldehyde	Fixed	+++
	Non-fixed	+++
Blocking of non-specific peroxidase with 10% H_2O_2	30 min	+++
	20 min	+++
	10 min	++/BS
	No blocking	SBS
Pre-hybridisation	1 hour	++
	Drying	+++
	None	+
Formamide in hybridisation buffer	0%	+++
	10%	+++/BS
	20%	+++/BS
	50%	+++/BS
Hybridisation temperature (using formamide-free buffer)	37°C	-
	45°C	+++
	50°C	+
	55°C	-
Hybridisation time	1 hour	++
	2 hours	+++
	overnight	+++
Post-hybridisation wash in 0.1% SSC	45 min	+++
	30 min	+++
	15 min	+++/BS
Incubation with streptavidin-peroxidase	40 min	+++
	20 min	+++
	10 min	+

Table 4.3.1.B Summary of the optimised ISH procedure developed for *Sphaerospora truttae.*

Treatment	**Duration**
Dehydration through alcohol series and equilibration in TBS (pH 8)	10 min
Tissue permeabilisation with 100 μg ml^{-1} proteinase K in TBS (pH 8) at 37 °C	30 min
PBS wash	5 min
Blocking of non-specific peroxidase with 10% H2O2	20 min
Water wash	5 min
Drying	10 min
45°C incubation in hybridisation buffer (4 x SSC in TBS, 0.5% ficoll, 0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin, 100μg ml^{-1} calf thymus DNA, 1.5 ng μl^{-1} of biotin-labelled probe)	2 hours
Low stringent wash in 2 x SSC	5 min
High stringent wash at 45 °C in 0.1 x SSC containing 0.1% TWEEN20	10 min
Incubation with streptavidin-peroxidase	20 min
Washing 2 x in PBS	10 min
Substrate (Vector VIP)	15 min
Water wash	5 min
Counterstaining (Methyl green)	5 min
Water wash	5 min
Dehydration through alcohol, xylene, mounting in pertex	10 min
Total time	**4 hrs 45 min**

4.3.2 Specificity of the Protocol

Using the *S. truttae*-specific primers STIR and STOR, the ISH protocol proved to be specific for *S. truttae* and, under the given conditions of stringency, did not bind to *Sphaerospora renicola, S. galinae, S. elegans, S. molnari, Myxobilatus gasterostei, Hoferellus gilsoni, Chloromyxum* sp. or *Tetracapsuloides bryosalmonae.*

4.3.3 Entry Locus and Spatio-Temporal Occurrence of Sphaerospora truttae in the Host Tissues

The ISH protocol developed proved to be a sensitive tool for the rapid, specific, high contrast detection of *Sphaerospora truttae* developmental stages down to the single cell level. The development of *S. truttae* within the fish host from the initial infection to the development of mature spores was observed. The complete development of *S. truttae* in the Atlantic salmon was found to require approximately 40 days at 14°C and is summarised in Figure 4.3.3.A.

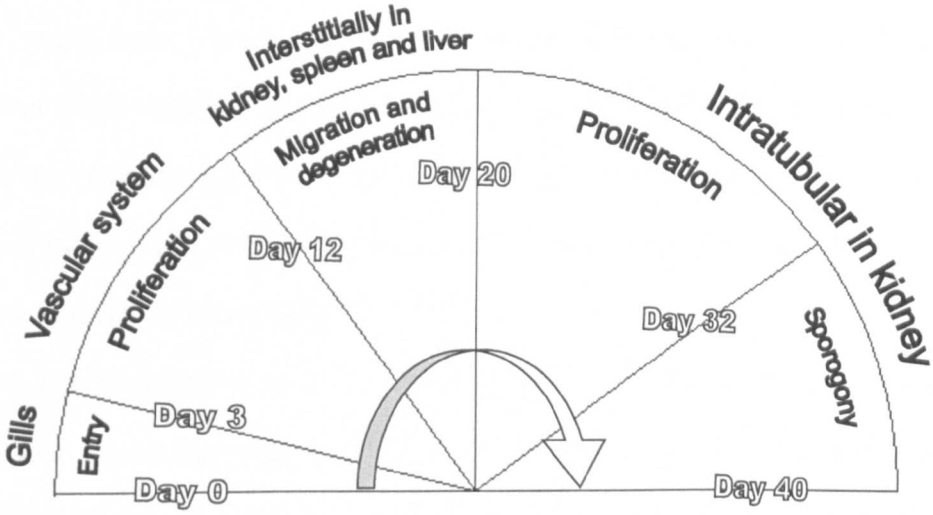


Figure 4.3.3.A Schematic summary of the developmental cycle of *Sphaerospora truttae* in the Atlantic salmon, *Salmo salar*.

Figure 4.3.3.B (1-20) illustrates the spatio-temporal sequence of life stages of *S. truttae* as observed in the tissues of the Atlantic salmon using ISH. On day 1 following initial exposure of salmon fry to the presumed waterborne infective stage of *S. truttae*, ISH identified the epithelium of the secondary gill lamellae as the predominant entry locus of the parasite (Figure 4.3.3.B-1&2). To a much lesser extent, *S. truttae* was also

found to invade its host through the epithelium of the body surface and fins. Penetration via the gut epithelium was never observed. The stages entering the fish appeared as multicellular units consisting of up to 8 cells each with a diameter of 1.8-2.5 μm. The initial epithelial penetration of *S. truttae* was not observed and is assumed to be very rapid. However, on day 1, hybridisation signals were frequently found within mucous cells of the gill epithelium (Figure 4.3.3.B-3&4). These mucous cells usually contained a single (rarely two) spherical structure 2.5 μm in diameter. On the same day, multicellular parasites were also recognisable at the base of the secondary lamellae where these, now migratory stages entered the vascular system through the blood vessel walls (Figure 4.3.3.B-5). On day 3, *S. truttae* was first observed in the lumen of blood vessels (Figure 4.3.3.B-6). At this time, most parasites were seen either within the epithelial layer of the gills (possibly representing new entrants) or within the vascular system, but a small number were also found inside lacunae of the cartilaginous tissue of the gill lamellae (Figure 4.3.3.B-7).

Rapid proliferation of the parasite within the vascular system was observed resulting in increasing numbers of stages of variable size and cell number ranging from 2 to approximately 120 (Figure 4.3.3.B-8). These stages were readily visible in all blood rich organs, i.e. gills, heart, spleen, kidney and liver of the infected salmon fry. Occasionally, large stages with more than 30 cells were observed to obstruct capillaries, e.g. in the secondary gill lamellae (Figure 4.3.3.B-9). Occasionally, the cytoplasm of single erythrocytes in various organs exhibited a strong ISH signal accompanied by a granular structure in the otherwise smooth cytoplasm of the affected erythrocytes (Figure 4.3.3.B-10 & 11). These signals could indicate the presence of intracellular stages of *S. truttae* as they were absent in the organs of fish from the control group.

Only the large extracellular blood stages were found to accumulate in capillaries in the spleen, liver and kidney (Figure 4.3.3.B-12). Attachment of the parasites to the blood vessel walls was followed by their penetration (Figure 4.3.3.B-13) and entry into the surrounding tissue, observed for the first time on day 12 after infection. Intercellular occurrence was not restricted to the target organ, the kidney, but was also observed in spleen and liver (Figure 4.3.3.B-14). There was no obvious site preference of *S. truttae* for the organ of sporogony, the kidney (see Section 4.3.4).

Incidental degeneration of the intercellular *S. truttae* stages was a common feature in all organs including kidney. Disintegration of the multicellular structure (Figure 4.3.3.B-15) was followed by engulfment of the parasites by macrophages (Figure 4.3.3.B-16), observed for the first time on day 16. In spleen and liver all parasites died and only a weak ISH signal within the macrophage cytoplasm indicated their former presence. Further development of *S. truttae* took place exclusively in the kidney. From day 20 onwards, the multicellular intercellular stages were observed to form plasmodia-like extensions which established and maximised surface area contact with the renal tubules (Figure 4.3.3.B-17) and finally penetrated them (Figure 4.3.3.B-18). From day 20 onwards, individual multicellular parasites were found in the tubular lumina. After the entry of individual parasite stages, the number of *S. truttae* stages in infected tubules increased quickly, gradually filling the tubules and slightly dilating them. This was followed by the transition into sporogony (Figure 4.3.3.B-19). Immature, developing spores inside disporous pseudoplasmodia were first detected on day 32. Mature spores with readily distinguishable polar filaments inside the polar capsules and spore valves exhibiting surface ridges were only present from day 40

onwards. Sporogony was also frequently found to take place inside glomeruli (Figure 4.3.3.B-20).

Blood stages and histozoic stages were found together with sporogonic stages in the renal tubules until the end of the experiment on day 60. Continuous re-infection of fry due to continual exposure to the infected sediment in the tank cannot be excluded. The development of spores was observed to be non-synchronous, thus undifferentiated early sporogonic stages were present alongside mature spores.

Fish from the uninfected control group never showed binding of the labelled probes to cells in the tissues.

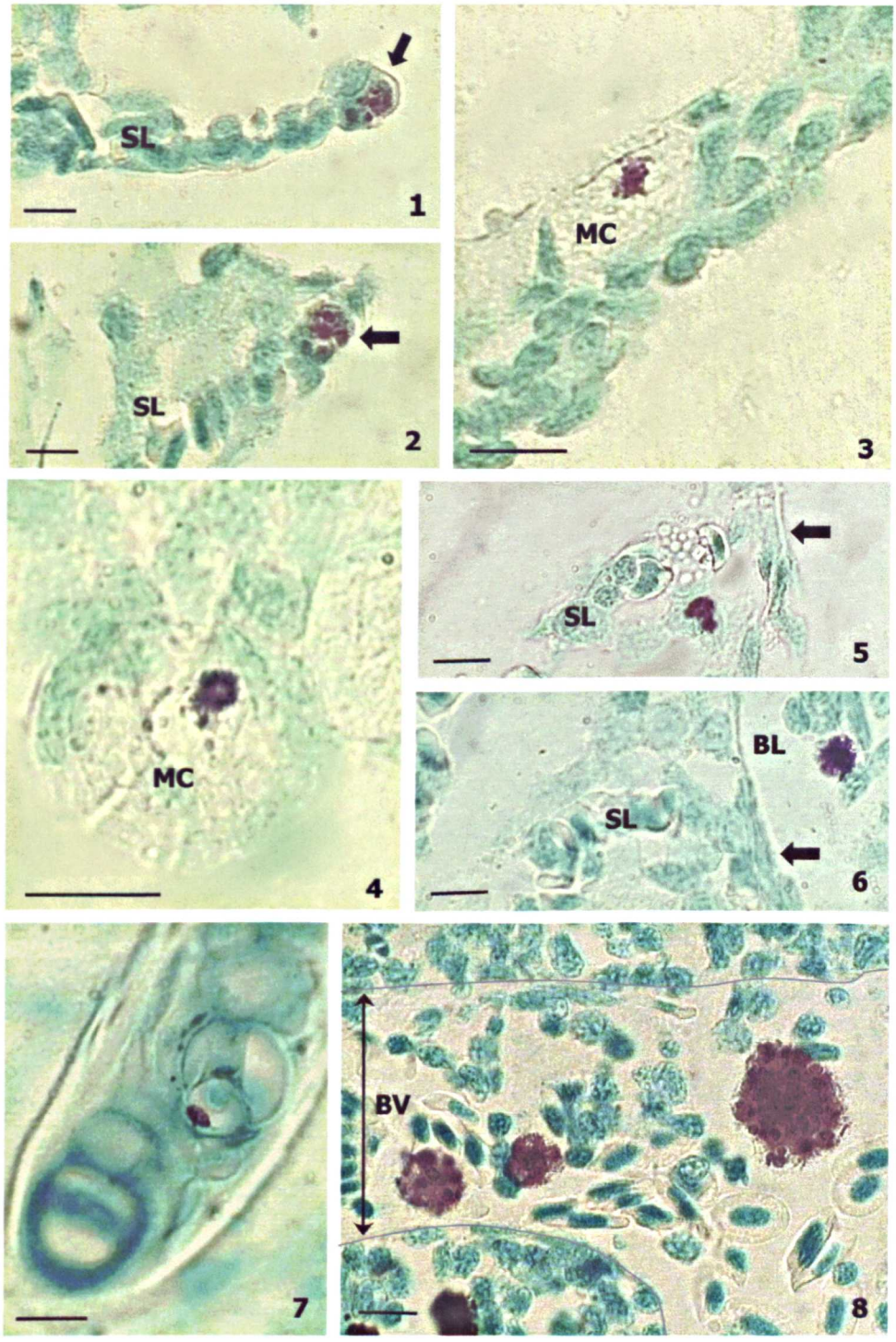


Figure 4.3.3.B *In situ* hybridisation showing the development and migration of *Sphaerospora truttae* in its host, *Salmo salar* (**1-8**); (**1 & 2**) Entry of the parasite (arrows) through the secondary lamellae of the gills (SL); (**3 & 4**) Single *S. truttae* cells inside mucous cells (MC) in the gills; (**5 & 6**) Migration from the secondary lamellae (SL) through blood vessel wall (arrows) into the vascular system (BL=blood vessel lumen); (**7**) Parasite inside a lacuna of the gill cartilage; (**8**) Blood stages of different size inside a vessel (BV) in the kidney; bars: 10 μm.

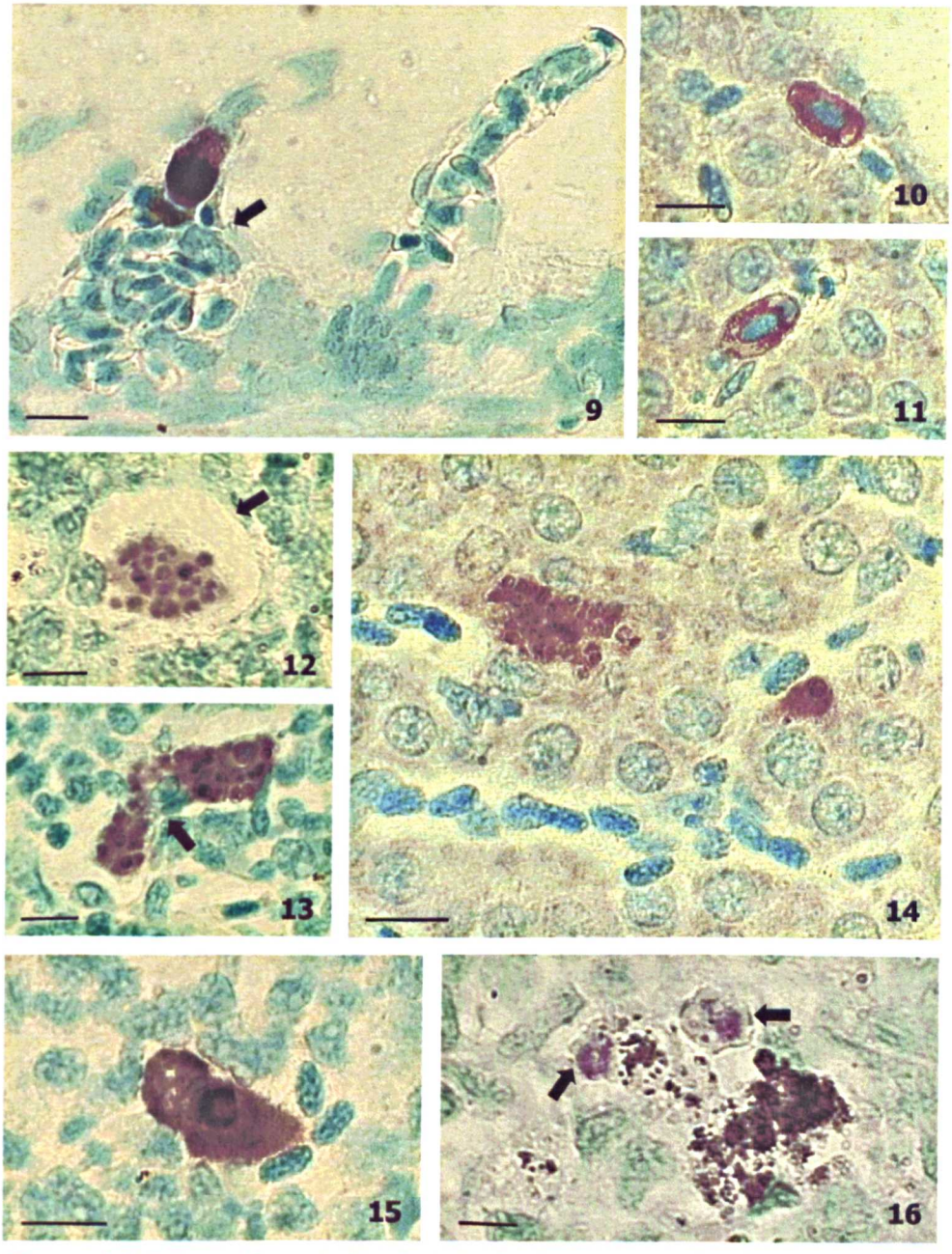


Figure 4.3.3.B continued. *In situ* hybridisation showing the development and migration of *Sphaerospora truttae* in its host, *Salmo salar* (**9-16**); (**9**) Large parasite causing congestion of blood (arrow) in lamella; (**10 & 11**) Possible intracellular occurrence of *S. truttae* within erythrocytes; (**12**) Multicellular parasite inside a capillary in the kidney (arrow indicating capillary wall); (**13**) *S. truttae* leaving the vascular system by breaking through the vessel epithelium (arrow) and entering the interstitial tissue of the kidney; (**14**) Multicellular interstitial stages in the liver; (**15**) Degenerating stage showing loss of structure; (**16**) Remains of *S. truttae* inside melanomacrophages (arrows); bars: 10 μm.

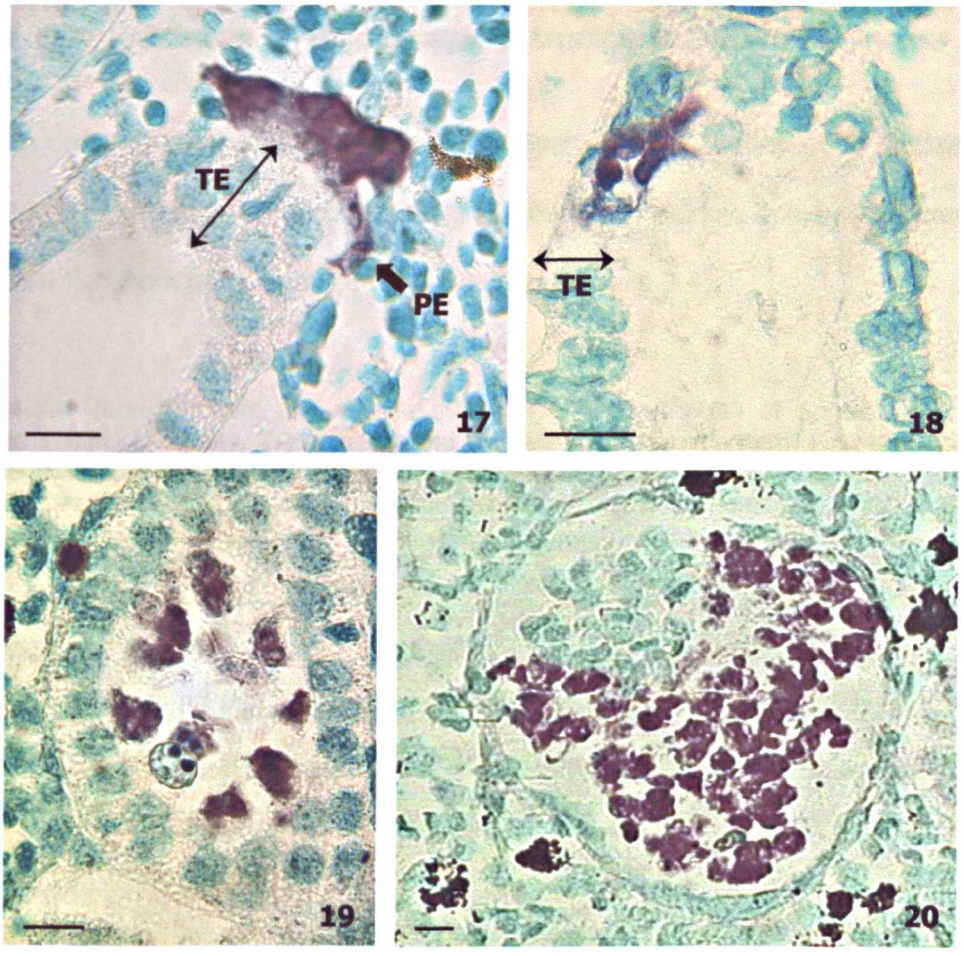


Figure 4.3.3.B continued. *In situ* hybridisation showing the development and migration of *Sphaerospora truttae* in its host, *Salmo salar* (**17-20**); (**17**) Multicellular stage forming plasmodia-like extensions (PE) maximising surface contact with a tubular epithelium (TE); (**18**) Multicellular stage of *S. truttae* penetrating tubular epithelium (TE); (**19**) Intratubular sporogonic stages and mature spore; (**20**) Sporogonic stages inside a glomerulus; bars: 10 μm.

4.3.4 Quantification of Sphaerospora truttae in Different Tissues

In order to gain information on the invasion and distribution of *S. truttae* in the different host organs, the parasites were quantified in kidney, spleen and liver of 12 individual fish from days 15 to 26 post infection (Table 4.3.4).

Table 4.3.4 Total numbers and relative percentages (parentheses) of *Sphaerospora truttae* stages in 1 mm^2 of kidney, liver, spleen and the related vascular system. First three columns: percentages related to parasites occurring in the tissue versus the vascular system of each organ; fourth column: percentages related to distribution of parasites over the different organs.

	Kidney		Spleen		Liver		Organ totals		
	Tissue	Blood	Tissue	Blood	Tissue	Blood	Kidney	Spleen	Liver
Fish1 (Day15)	2 (40.0%)	3 (60.0%)	1 (25.0%)	3 (75.0%)	3 (60.0%)	2 (40.0%)	5 (35.7%)	4 (28.6%)	5 (35.7%)
Fish2 (Day16)	6 (46.2%)	7 (53.8%)	5 (55.6%)	4 (44.4%)	2 (28.6%)	5 (71.4%)	13 (44.8%)	9 (31.0%)	7 (24.2%)
Fish3 (Day17)	5 (45.5%)	6 (54.5%)	4 (36.4%)	7 (63.6%)	4 (57.1%)	3 (42.9%)	11 (37.9%)	11 (37.9%)	7 (24.2%)
Fish4 (Day18)	4 (57.1%)	3 (42.9%)	2 (28.6%)	5 (71.4%)	3 (42.9%)	4 (57.1%)	7 (33.3%)	7 (33.3%)	7 (33.3%)
Fish5 (Day19)	8 (40.0%)	12 (60.0%)	11 (52.4%)	10 (47.6%)	5 (50.0%)	5 (50.0%)	20 (39.2%)	21 (41.2%)	10 (19.6%)
Fish6 (Day20)	3 (60.0%)	2 (40.0%)	2 (40.0%)	3 (60.0%)	1 (25.0%)	3 (75.0%)	5 (35.7%)	5 (35.7%)	4 (28.6%)
Fish7 (Day21)	7 (53.8%)	6 (46.2%)	5 (50.0%)	5 (50.0%)	5 (45.5%)	6 (54.5%)	13 (38.2%)	10 (29.4%)	11 (32.4%)
Fish8 (Day22)	1 (33.3%)	2 (66.7%)	1 (25.0%)	3 (75.0%)	0 (0%)	2 (100%)	3 (33.3%)	4 (44.4%)	2 (22.2%)
Fish9 (Day23)	10 (50.0%)	10 (50.0%)	9 (56.3%)	7 (43.7%)	5 (41.7%)	7 (58.3%)	20 (41.7%)	16 (33.3%)	12 (25.0%)
Fish10 (Day24)	4 (36.4%)	7 (63.6%)	5 (45.5%)	6 (54.5%)	4 (57.1%)	3 (42.9%)	11 (37.9%)	11 (37.9%)	7 (24.1%)
Fish11 (Day25)	8 (50.0%)	8 (50.0%)	8 (53.3%)	7 (46.7%)	7 (58.3%)	5 (41.7%)	16 (37.2%)	15 (34.9%)	12 (27.9%)
Fish12 (Day26)	3 (37.5%)	5 (62.5%)	5 (55.6%)	4 (44.4%)	4 (50.0%)	4 (50.0%)	8 (32.0%)	9 (36.0%)	8 (32.0%)
TotalNo	**61**	**71**	**58**	**64**	**43**	**49**	**132**	**122**	**92**
Mean%	**45.8%**	**54.2%**	**43.6%**	**56.4%**	**43.0%**	**57.0%**	**37.3%**	**35.3%**	**27.4%**
±SD	±8.6%	±8.6%	±12.2%	±12.2%	±17.6%	±17.6%	±3.6%	±4.7%	±5.0%

The number of *S. truttae* stages found in the same organ of different fish varied considerably whereas the relative percentage of stages in the different organs of

individual fish showed a small degree of variation. Thus relative percentages were chosen for the illustration of the relationships in Figure 4.3.4. The statistical analysis, however, was based on absolute numbers. The graph illustrates that there is no clear site preference for the organ of sporogony, the kidney. Of all the histozoic stages encountered, the overall proportion in the kidney (37.3±3.6%) was not much higher than the proportion in the spleen (35.3±4.7%) or in the liver (27.4±5%), and statistical differences between the organs were not significant (Wilks' Lambda; F=1.576, df=2, p=0.222). The comparison of parasite numbers in the tissues with the numbers in the vascular system of the same organ showed that in individual fish, the number of *S. truttae* stages in the tissues was generally lower than in the blood vessels (Wilks' Lambda; F=5.061, df=33, p=0.031, Figure 4.3.4). This showed that the *S. truttae* stages did not increase in number once they became histozooic.

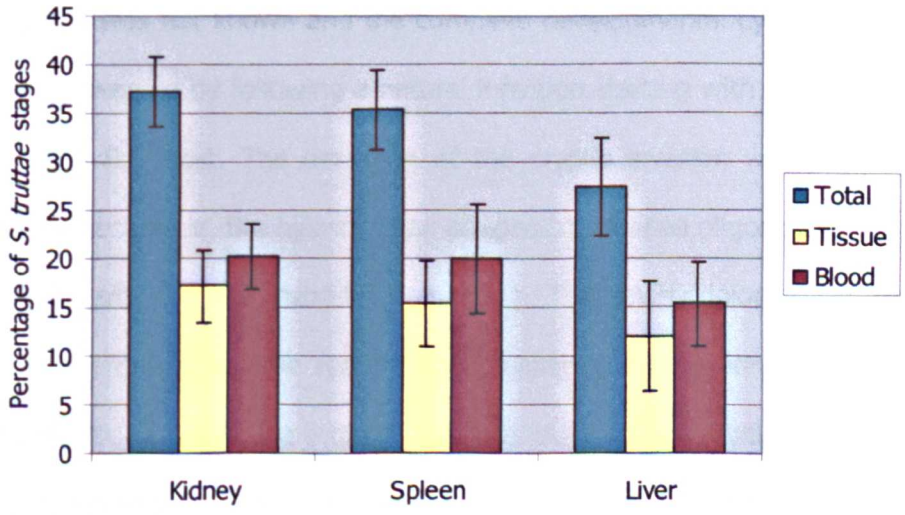


Figure 4.3.4 Graph demonstrating the relative percentage of *Sphaerospora truttae* stages (± standard deviation) in the different organs invaded. Total percentage per organ is made up by percentages in the tissue and in the blood of each organ.

4.4 Discussion

4.4.1 Potential of the ISH Technique with Regard to Myxozoans

Due to their small number, size and cryptic nature, early myxosporean developmental stages are generally extremely difficult to detect and to differentiate from host cells using conventional histological techniques. Only the ability to produce and enrich actinosporean spores experimentally by production in known oligochaete hosts made it possible to achieve the artificial massive invasion of actinosporeans into fish and thus to detect their portals of entry (El-Matbouli *et al.* 1995, El-Matbouli *et al.* 1999a, Yokoyama & Urawa 1997, Belem & Pote 2001) and migration routes within the fish, which have to date only been completely elucidated for *Myxobolus cerebralis* (El-Matbouli *et al.* 1995).

In contrast to these investigations, in this study, the infective alternate actinosporean was not known and the complete developmental cycle of *Sphaerospora truttae* was observed by following a natural infection starting with only a few parasites entering each fish host. The detection of the cryptic invasion was possible using a sophisticated technique, the hybridisation of specific, labelled oligonucleotides to target DNA/RNA *in situ* (*in situ* hybridisation, ISH). This methodology was successfully modulated in order to achieve specific identification and detection of *S. truttae* in the host tissues. ISH proved to be a highly valuable tool for the investigation of the spatio-temporal occurrence of a myxozoan during its development in the fish host. It allowed the visualisation of low numbers of developmental stages of *S. truttae* down to single cell level. It is to date probably the only methodology which has the potential to answer questions on pathways of myxozoans of which the infective actinosporean stage is

unknown. Due to the specificity of the designed DNA probes this technique also provides confirmatory identification of the parasite before the development of identifiable spore stages.

The ISH technique has only been applied occasionally to myxozoans (Antonio *et al.* 1998, Morris *et al.* 1999, Morris *et al.* 2000, Longshaw *et al.* 2002) and only Morris *et al.* (2000) used the technique for the purpose of identifying the entry locus of *Tetracapsuloides bryosalmonae* into rainbow trout. The present study is the first to detect the complete sequence of developmental stages of a myxozoan in its fish host, using ISH. Furthermore, in comparison with the other ISH protocols used for myxozoans, the one developed for *S. truttae* uses shorter processing times and it also reduces the use of reagents to a minimum, making the successful technique a time- and cost-efficient procedure.

It is assumed that the successful design of this protocol was strongly aided by the fact that the 18S rDNA is an ideal gene for *in situ* hybridisation as it occurs in multiple copies per cell and it is thus much easier to visualise than other target sequences, e.g. scarce numbers of relatively unstable mRNA copies. As hybridisation signals were also found in the cytoplasm of the target cells it is assumed that in addition to the genomic 18S rDNA copies, rRNA is detected. This explains also why the reverse primers produced a much better signal in the tissue sections than the foreward primers which can only hybridise to the nuclear rDNA copies.

Cross-linking of the labelled primers to other myxozoans was not observed under the given conditions of stringency which shows that *in situ* species identification is possible despite the use of the 18S rDNA, a slowly evolving gene (see Chapter 3, Section 3.1.2).

4.4.2 Myxozoan Entry Into Fish

The skin, fins, gills and buccal cavity of rainbow trout, *Oncorhynchus mykiss,* have been demonstrated as the portals of entry for the triactinomyxon spores of *M. cerebralis* by El-Matbouli *et al.* (1995). Their scanning electron microscopic study revealed that, as early as 1 min post exposure to fish, the polar filaments of the triactinomyxon discharge and the sporoplasms penetrate through the opening of the mucous cells of the epidermis (El-Matbouli *et al.* 1999a). In contrast, the aurantiactinomyxon spores of *Thelohanellus hovorkai* labelled with a fluorescent dye by Yokoyama & Urawa (1997) were observed to invade carp mainly via the gill filaments. The aurantiactinomyxon spores of the proliferative gill disease organism, *Henneguya ictaluri,* utilizes predominantly the gastric mucosa but also the skin and buccal cavity of channel catfish *Ictalurus punctatus,* as the portal of entry as shown by Belem & Pote (2001). Similar to *Thelohanellus hovorkai,* the gills appear to be the predominant entry site of *S. truttae* into the Atlantic salmon. Only occasionally were single parasites observed in the body epidermis. Yokoyama & Urawa (1997) suggested that small actinosporean stages (e.g. aurantiactinomyxon) invade the fish host through the gills whereas large actinospores (e.g. triactinomyxon and raabeia) attach mainly to the skin. It is possible that morphologically different spores show passive selection of the entry locus. Some actinospore morphotypes might attach more easily in particular areas, due to reduced water flow rates, e.g. in the buccal cavity.

The initial entry of *S. truttae* into salmon was not observed and it is presumed to be very rapid. However, the presence of hybridisation signals for *S. truttae* within the mucous cells suggests an entry through the secretory openings of these cells (as in the case of *M. cerebralis*, El-Matbouli *et al.* 1999a) and perhaps suggests chemotactic

orientation of the spores towards the acidic mucus. Differences in the composition of mucus released in the intestine and the skin of eels can initiate a remarkably different intensity of chemotactic response in *Vibrio anguillarum* (O'Toole, Lundberg, Fredriksson, Jansson, Nilsson & Wolf-Watz 1999). Therefore, active selection of an entry site following chemical clues cannot be excluded in myxozoans. Comparison of the reaction of actinospores exposed to mucus from intestine, skin and gills might help to elucidate this question.

Whatever the mechanism of entry site selection, *S. truttae* targets entry of the Atlantic salmon at a site with a well-established vascular system close to the outer surface of the host, which must increase the possibility of the parasite reaching the blood and decrease the possibility of encountering other tissues which represent a dead end (e.g. cartilage).

4.4.3 Comparison of Myxozoan Migration Routes in Fish Hosts

There is little available information on the early development of other myxozoan species in their fish hosts. The complete details of development have been resolved only for *M. cerebralis* (El-Matbouli *et al.* 1995). During the first 60 min following penetration, the actinosporean sporoplasms of *M. cerebralis* were shown to remain compact and to migrate intercellularly in the epidermis. After 60 min, the sporoplasm-enveloping cell disintegrated and each sporoplasm cell was shown to enter an epidermal cell. Within the host cell, a series of rapid synchronous mitotic divisions took place resulting in a primary cell containing a number of secondary cells. The morphology of these intra-epithelial cell-in-cell stages is comparable with the morphology of the blood stages of *S. truttae,* which were detected two days after exposure to the infective environment. In contrast to the epithelial stages of *M.*

cerebralis, the typical blood stages of *S. truttae* do not occur within host cells. In the case of *M. cerebralis,* every sporoplasm cell of the actinosporean was shown to undergo a cycle of intracellular proliferation (El-Matbouli *et al.* 1995) and intracellular development of plasmodia giving rise to mature spores has been reported for several other myxosporeans (Lom & Dyková 1992). Following the assumption that nutrients have to be available in order to proliferate or grow rapidly, the localisation within a host cell might improve the availability of these substances in comparison with their presence in the intercellular space. It is also suggested that the intracellular development of early parasite stages improves their survival, as they seem to be protected from recognition by the immune system of the host. Thus, by increase of parasite numbers in a protected space more parasites are available to reach the target organ. The ISH signals detected within the cytoplasm of erythrocytes of *S. truttae* infected fish, but not in the control group, suggest a stage of intracellular development also for *S. truttae.* However, the importance of the intra-erythrocytic stages is questionable as these stages were extremely rare in comparison with the number of other blood stages. Similar to *M. cerebralis,* they could represent stages which evade detection by immunocompetent host cells. Further studies on the ultrastructural morphology of the possibly intracellular *S. truttae* stage could shed more light on their significance for the parasites' further development in the fish host. This would be facilitated once high numbers of infective actinosporean stages of *S. truttae* can be experimentally produced and used for targeted infection studies, potentially resulting in higher numbers of infected erythrocytes.

Myxobolus cerebralis continues its development by migrating in the intercellular space of the nervous tissue to the target site, the cartilage. The nervous tissue offers

the same kind of protection from the host's immune system as the intracellular location but, due to its extracellular occurrence, the parasite can actively move towards its final destination. In contrast, the blood stages of *S. truttae* are constantly exposed to the cellular defence mechanisms of the fish host. Despite this exposure, the vascular system seems to be a successful pathway for some myxozoans, as blood stages have been described for other *Sphaerospora* species (Lom *et al.* 1983, Lom, Pavlásková & Dyková 1985, Molnar 1988, Baska & Molnar 1988, Dyková *et al.* 1990, Supermattaya, Fischer-Scherl, Hoffmann & Boonyaratpalin 1993) and their presence has also been suggested for *Kudoa thyrsites* (Moran *et al.* 1999) after the successful transmission of the parasite to uninfected fish by injection of blood from infected fish. The success of blood stages in the development of myxozoans could be explained by the blood representing a rapid and energy-efficient transport medium which allows the parasite to reach the target organ. In the case of *S. truttae,* a minimum of ten days residence in the vascular system was observed, which is perhaps necessary for the rapid proliferation, observed here, to take place.

It might be expected that the exit of *S. truttae* from the vascular system would target the invasion of a suitable site for spore production and spore release from the host. Despite the importance of such a site, the rather non-specific "selection" of kidney, spleen and liver observed in *S. truttae* is possibly induced by some very general cues, e.g. changes in pH or blood oxygen levels in these organs. The number of parasites entering the kidney was only slightly greater than those entering the spleen and the liver, with no statistical difference detectable. This suggests that there is no specific recognition of the target organ at this stage.

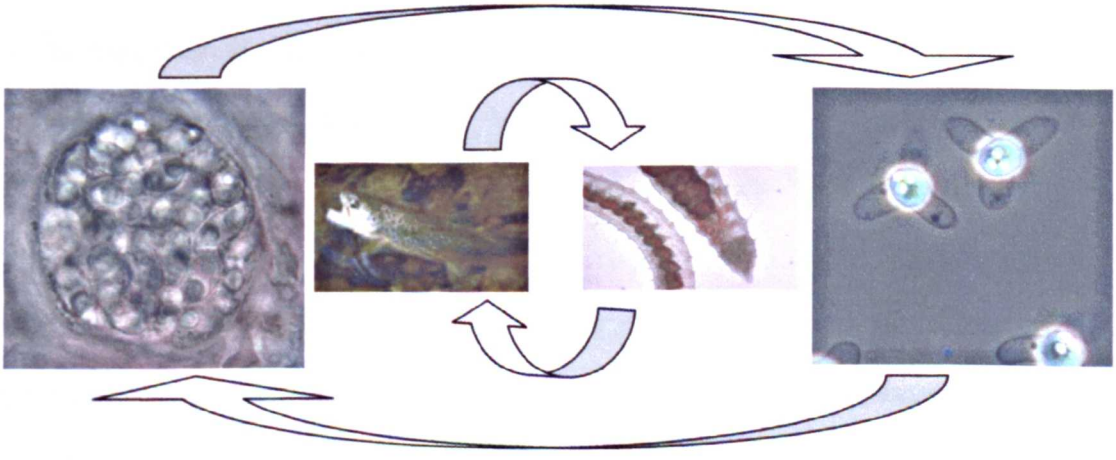
The number of *S. truttae* stages in the tissues tended to be slightly lower than in the vascular system in each organ. This suggests that the proliferation observed in the blood does not continue once the intercellular location is reached. Given the commonly observed mortality and breakdown of the intercellular stages, the survival and further development of *S. truttae* seems to be very dependent on the number of parasites produced in the vascular system. It remains unclear why "histozoic" stages break down in all organs but this observation suggests that many either survive only for a short period in the tissues or are detected and destroyed by the cellular immune system of the fish host. However, further successful development is restricted to the kidney tubules. The intercellular stages in liver and spleen do not develop further but die and become engulfed by macrophages. This ingestion might serve as a basis for the immunity to renewed infection with *S. truttae* which was described by McGeorge *et al.* (1994).

Further development of *S. truttae* was bound to the intratubular location. Single, isolated multicellular parasite stages entered the renal tubules from day 25 onwards. These appeared to undergo further cycles of proliferation for at least 10 days before they switched into sporogony, resulting in the production of high numbers of spores. Proliferation within renal tubules is suggested by the fact that initially small numbers of tubules are found containing single parasites. Subsequently the number of infected tubules did not appear to increase much, but the number of parasites per tubule increased considerably. Due to the asynchronous development of the disporous pseudoplasmodia mature spores are then released over several months (McGeorge 1994).

The intra-piscine development of *S. truttae* was found to be completed in 40 days. In contrast, at the same temperature (14°C), *M. cerebralis* was shown to take 80 days from the entry of the fish host to sporogony in the target organ (El-Matbouli *et al.* 1995). The time difference in the development of these two myxozoan species can probably be explained by the different migration routes. *M. cerebralis* migrates slowly but actively along the nerve fibres into the central nervous system and finally into the cartilage of the skull and the spine. *S. truttae* reaches its target organ rapidly but passively by using the vascular system as a means of transport followed by a short migration from the capillaries in the kidney to the renal tubules. Despite the time advantage for *S. truttae,* the development of this species seems less sophisticated than that of *M. cerebralis. S. truttae* proliferates in the blood without protection from the immune system of the fish and subsequently invades various organs (spleen, kidney and liver) although sporogony can only take place in the kidney. Similarly, *Tetracapsuloides bryosalmonae* was shown to invade the same host organs but to form spores only in the kidney (Kent & Hedrick 1986, Clifton-Hadley, Bucke & Richards 1987). In contrast, the route of *M. cerebralis* to the organ of sporogenesis, the cartilage, was demonstrated to be very targeted and direct and no parasite stages were found in other tissues than the epidermis, the dermis, the nervous tissue and the cartilage. Additionally, most of the development of *M. cerebralis* appears to be protected from the immunocompetent cells of the host. The development of *M. cerebralis* thus seems more evolved than that of *S. truttae* as perhaps indicated by the phylogenetic position of the two species. *S. truttae* appears to be a very "old" myxozoan in a basal phylogenetic position whereas *M. cerebralis* is a member of the most recent clade of freshwater myxozoans (see Chapter 6).

CHAPTER 5

The Community of Myxozoans in a *Sphaerospora truttae*-Enzooic Habitat And Infection Studies Involving Potential Invertebrate Hosts



5 The Community of Myxozoans in a *Sphaerospora truttae*-Enzooic Habitat and Infection Studies Involving Potential Invertebrate Hosts

5.1 Introduction

5.1.1 Myxozoan Life-Cycles

Following the revolutionary discovery by Wolf & Markiw (1984) that a single myxozoan life cycle exists in which there is alternation between myxosporean and actinosporean stages, scientific interest in actinosporeans and their hosts increased markedly. To date, 26 actinosporeans from invertebrate hosts have been linked with myxosporeans infecting fish (Table 5.1.1). All but one recent report (Køie *et al.* 2004) describe myxozoan life cycles from the freshwater environment and the main focus has been on the genus *Myxobolus.* The connection between the myxosporean genus *Myxobolus* and the actinosporean type triactinomyxon is the most common finding. Other corresponding patterns of myxosporean and actinosporean morphotypes are not predictable. Cladistic analyses using the two different morphotypes showed that the actinosporean morphology has a lack of taxonomic congruity (Xiao & Desser 2000).

In the freshwater environment, all actinosporeans which have been related to myxosporeans are produced in aquatic oligochaetes, except for *Ceratomyxa shasta,* the myxosporean causing intestinal dysfunction in salmonids, which is the only freshwater species which has been shown to require a freshwater polychaete (*Manayunkia speciosa* Leidy, 1858) as an alternate host in its life cycle (Bartholomew *et al.* 1997). The first myxozoan life cycle from the marine environment has been elucidated only

recently. *Ellipsomyxa gobii* Køie, 2003 from the common goby *Pomatoschistus microps* (Krøyer, 1838) has been found to develop within seawater polychaetes of the genus *Nereis* (Køie *et al.* 2004).

A separate class of myxozoans, the Malacasporea (including the proliferative kidney disease (PKD) organism *Tetracapsuloides bryosalmonae*) utilize bryozoans as alternate hosts (Anderson *et al.* 1999, Okamura, Anderson, Longshaw, Feist & Canning 2001, Okamura & Wood 2002). However, although the infection of salmonids with *T. bryosalmonae* spores from bryozoan hosts has been successful (Feist, Longshaw, Canning & Okamura 2001, Longshaw *et al.* 2002) infection from fish to bryozoans has not been achieved with certainty (Morris, Morris & Adams 2002b), Okamura & Tops, unpublished observations), leaving the life cycle of this species incomplete. Furthermore, it is not well understood whether the relationship between the two stages in bryozoan and salmonid hosts is biologically equivalent to that between myxosporean and actinosporean life cycle stages of myxozoans.

Although an indirect life cycle is considered a general rule in myxozoans, direct transmission from fish to fish has been successful for two intestinal myxozoans, *Enteromyxum leei* and *E. scophthalmi* (Diamant 1997, Redondo, Palenzuela, Riaza, Macias & Alvarez-Pellitero 2002). This does not exclude the presence of an alternate invertebrate host for these species.

Table 5.1.1 Elucidated myxozoan life-cyles: Related myxosporeans and actinosporeans and their vertebrate and invertebrate hosts. * Indicate species of which myxosporean and actinosporean stages have been related on a molecular basis (18S rDNA).

Myxosporean	Vertebrate host	Actinosporean type	Invertebrate host	Reference
Myxobolus cerebralis * Hofer, 1903	*Oncorhynchus mykiss* (Walbaum)	*Triactinomyxon*	*Tubifex tubifex* L.	Wolf & Markiw 1984
Myxobolus cotti El-Matbouli & Hoffmann, 1987	*Cottus gobio* L.	*Triactinomyxon*	*Tubifex tubifex* L.	El-Matbouli & Hoffmann 1989
Myxobolus pavlovskii (Akhmerov, 1954)	*Hypothalmichthys molitrix* (Valenciennes)	*Hexactinomyxon*	*Tubifex tubifex* L.	Ruidisch *et al.* 1991
Myxobolus arcticus Pugachev & Khokhlov, 1979 (Canada)	*Oncorhynchus nerka* (Walbaum)	*Triactinomyxon*	*Stylodrilus heringianus* Claparède	Kent *et al.* 1993
Myxobolus arcticus Pugachev & Khokhlov, 1979 (Japan)	*Oncorhynchus masu* (Brevoort)	*Triactinomyxon*	*Lumbriculus variegatus* (Müller)	Urawa 1994
Myxobolus carassii Klokačeva, 1914	*Leuciscus idus* L.	*Triactinomyxon*	*Tubifex tubifex* L.	El-Matbouli & Hoffmann 1993
Myxobolus cultus Yokoyama, Ogawa & Wakabayashi, 1995	*Carassius auratus* (L.)	*Raabeia*	*Branchiura sowerbyi* Beddard	Yokoyama *et al.* 1995
Myxobolus hungaricus (Molnár & Baska, 1999)	*Abramis brama* L.	*Triactinomyxon*	*Tubifex tubifex* L.	El-Mansy & Molnar 1997a
Myxobolus drjagini Akhmerov, 1954	*Hypothalmichthys molitrix* (Valenciennes)	*Triactinomyxon*	*Tubifex tubifex* L.	El-Mansy & Molnar 1997b
Myxobolus portucalensis Saraiva & Molnár, 1990	*Anguilla anguilla* (L.)	*Triactinomyxon*	*Tubifex tubifex* L.	El-Mansy *et al.* 1998a
Myxobolus dispar Thélohan, 1895	*Cyprinus carpio* L.	*Raabeia*	*Tubifex tubifex* L.	Molnar *et al.* 1999a
Myxobolus bramae Reuss, 1906	*Abramis brama* L.	*Triactinomyxon*	*Tubifex tubifex* L.	Eszterbauer *et al.* 2000
Myxobolus pseudodispar Gorbunova, 1936	*Rutilus rutilus* L.	*Triactinomyxon*	*Tubifex tubifex* L.	Szekely *et al.* 1999

Table 5.1.1 continued.

Myxosporean	Vertebrate host	Actinosporean type	Invertebrate host	Reference
Henneguya exilis * Kudo, 1929	*Ictalurus punctatus* (Rafinesque)	*Aurantiactinomyxon ictaluri*	*Dero digitata* (Müller)	Lin *et al.* 1999
Henneguya ictaluri * Pote, Hanson & Shivaji, 2000	*Ictalurus punctatus* (Rafinesque)	*Aurantiactinomyxon*	*Dero digitata* (Müller)	Styer *et al.* 1991, Pote *et al.* 2000
Hoferellus carassii Achmerov, 1960 (Germany)	*Carassius auratus* (L.)	*Aurantiactinomyxon*	*Tubifex tubifex* L. *Tubifex ignotum* (Štolc) *Limnodrillus hoffmeisteri* Claparède	El-Matbouli *et al.* 1992
Hoferellus carassii Achmerov, 1960 (Japan)	*Carassius auratus* (L.)	*Neoactinomyxum*	*Branchiura sowerbyi* Beddard	Yokoyama *et al.* 1993a
Hoferellus cyprini (Doflein, 1898)	*Cyprinus carpio* L.	*Guyenotia*	*Nais sp.*	Grossheider & Körting 1992
Thelohanellus hovorkai * Achmerov, 1960	*Cyprinus carpio* L.	*Aurantiactinomyxon*	*Branchiura sowerbyi* Beddard	Yokoyama 1997
Thelohanellus nikolskii Achmerov, 1955	*Cyprinus carpio* L.	*Aurantiactinomyxon*	*Tubifex tubifex* L.	Szekely *et al.* 1998
Sphaerospora renicola Dyková & Lom, 1982	*Cyprinus carpio* L.	*Neoactinomyxum*	*Branchiura sowerbyi* Beddard	Molnar *et al.* 1999b
Sphaerospora truttae Fischer-Scherl, El-Matbouli & Hoffmann, 1986	*Salmo salar* L.	*Echinactinomyxon*	*Lumbriculus variegatus* (Müller)	Özer & Wootten 2000
Ceratomyxa shasta * Noble, 1950	*Oncorhynchus mykiss* (Walbaum)	*Tetractinomyxon*	*Manayunkia speciosa* Leidy	Bartholomew *et al.* 1997
Zschokkella sp.	*Carassius auratus* (L.)	*Echinactinomyxon*	*Branchiura sowerbyi* Beddard	Yokoyama *et al.* 1993a
Zschokkella nova Klokacewa, 1914	*Carassius auratus* (L.)	*Siedleckiella*	*Tubifex tubifex* L.	Uspenskaya 1995
Myxidium giardi Cépède, 1906	*Anguilla anguilla* (L.)	*Aurantiactinomyxon*	*Tubifex sp.*	Benajiba & Marques 1993

Table 5.1.1 continued.

Myxosporean	Vertebrate host	Actinosporean type	Invertebrate host	Reference
Chloromyxum truttae Legér, 1906	*Salmo salar* L.	*Aurantiactinomyxon*	*Tubifex tubifex* L.	Özer 1999
Tetracapsuloides bryosamonae Canning *et al.*, 2002 (PKX) *	Fam. Salmonidae	*Tetracapsuloides bryosalmonae* Canning et al., 2002	*Cristatella mucedo* Cuvier, *Pectinatella magnifica* (Leidy), *Plumatella rugosa* Cuvier	Anderson *et al.* 1999
Ellipsomyxa gobii * Køie 2003	*Pomatoschistus microps* (Krøyer)	*Tetractinomyxon*	*Nereis diversicolor* OF Müller, *Nereis succinea* Frey & Leuckart	Køie *et al.* 2004

5.1.2 Problems Related to Finding Myxosporean Counterparts

Of the 26 myxozoan life cycles so far resolved (see Table 5.1.1) 12 have been described for species of the entirely histozoic genus *Myxobolus*. Although a few representatives of other myxosporean genera have elucidated life cycles, several genera are still missing, especially from the marine environment. One of the factors impeding the search for alternate hosts is the identification of the microhabitat of the specific invertebrate host in an extensive pond or river system in the freshwater environment and in the, even larger, oceanic system. The low prevalence of actinosporean infections in the specific host further impedes their identification. Although infections in oligochaetes may persist for a lifetime (Gilbert & Granath 2001) they usually occur with prevalences <1% (Lom *et al.* 1997).

In order to increase actinosporean production in invertebrates and thus identify actinosporean counterparts, oligochaetes have been experimentally exposed to high numbers of myxosporean spores which generally results in a higher prevalence in the invertebrate host than in natural infections (Table 5.1.2). However, there is a high degree of variability in the success of experimental infection studies, and a number of influential factors have been identified: Stevens, Kerans, Lemmon & Rasmussen (2001) and Beauchamp, Gay, Kelly, El-Matbouli, Kathman, Nehring & Hedrick (2002) recently demonstrated that susceptibility to *M. cerebralis* differs among strains of *Tubifex tubifex* and that some strains cannot be infected at all. However, in successfully infected oligochaetes, a high, temperature dependent, variability in the developmental time of different myxozoan species was found, ranging between 60 and 217 days (Table 5.1.2), and development of *M. cerebralis* in *Tubifex tubifex* was found to stop

completely at temperatures higher than 25°C (Hedrick, El-Matbouli, Adkison & MacConnell 1998, El-Matbouli, McDowell, Antonio, Andree & Hedrick 1999b). Furthermore, seasonal cycles of development have been identified for some species (e.g. McGeorge *et al.* 1996a, Gilbert & Granath 2001, Allen & Bergersen 2002, Oumouna, Hallett, Hoffmann & El-Matbouli 2003).

Apart from the factors which might influence the outcome of an experimental infection study negatively, cryptic stages which can occur naturally in the experimental hosts (see Chapter 4, Section 4.3.3) may result in spore formation and might be mistaken for the outcome of an experimental infection. Unfortunately, it is difficult to obtain specific myxozoan-free oligochaetes and fish for infection studies. Thus, infection studies alone do not seem to reliably confirm myxozoan life cycles and may result in misidentifications as in the case of *Sphaerospora truttae,* which was falsely related to *Echinactinomyxon* type 5 (see Chapter 3, Section 3.3.1 & 3.3.2).

In only 7 myxozoans have both life cycle stages been characterised on the basis of DNA sequences, thus proving that the two morphologically different spore stages have homologous genotypes and thus belong to one single species (Andree *et al.* 1997, Bartholomew *et al.* 1997, Lin *et al.* 1999, Anderson *et al.* 1999, Anderson *et al.* 2000, Pote *et al.* 2000, Køie *et al.* 2004, Table 5.1.1, marked with *). The molecular characterisation of myxosporean and actinosporean alternate life cycle stages substantially supports experimental infection studies involving vertebrate and invertebrate hosts, and editors of scientific journals now commonly demand sequence data from myxosporean and actinosporean stages for publications on new myxozoan life cycles. However, although DNA sequences can confirm the identity of two spore

stages, infection studies are necessary to investigate whether these two stages can complete the life cycle of a myxozoan species.

Table 5.1.2 Successful experimental production of actinosporean counterparts of known myxosporeans: Prevalence, developmental time (Dev. time) in the oligochaete host and temperature (Temp.) for actinosporean production.

Myxosporean	Actinosporean	Prevalence (%)	Dev. time (days)	Temp. (°C)	Reference
Myxidium giardi	*Aurantiactinomyxon*	-	77	-	Benajiba & Marques 1993
Myxobolus arcticus	*Triactinomyxon*	-	87-95	12-20	Kent *et al.* 1993
Myxobolus bramae	*Triactinomyxon*	5	70-81	20-30	Eszterbauer *et al.* 2000
Myxobolus carassii	*Triactinomyxon*	-	91	13-14	El-Matbouli & Hoffmann 1993
Myxobolus cerebralis	*Triactinomyxon*	20	104	12.5	Markiw 1986
Myxobolus cerebralis	*Triactinomyxon*	-	94	16-17	El-Matbouli & Hoffmann 1989
Myxobolus cotti	*Triactinomyxon*	-	125	16-17	El-Matbouli & Hoffmann 1989
Myxobolus dispar	*Raabeia*	99.2	217	-	Molnar *et al.* 1999b
Myxobolus drjagini	*Triactinomyxon*	9.8	91	18-22	El-Mansy & Molnar 1997b
Myxobolus hungaricus	*Triactinomyxon*	43.3	102	18-22	El-Mansy & Molnar 1997a
Myxobolus macrocapsularis	*Triactinomyxon*	10-18	66-99	-	Szekely, Racz, Molnar & Eszterbauer 2002a
Myxobolus pavlovskii	*Hexactinomyxon*	-	93	15-16	Ruidisch *et al.* 1991
Myxobolus portucalensis	*Triactinomyxon*	52.5	91	18-22	El-Mansy *et al.* 1998a
Myxobolus pseudodispar	*Triactinomyxon*	-	76	-	Szekely *et al.* 1999
Thelohanellus hovorkai	*Aurantiactinomyxon*	19.4	90	20	Yokoyama 1997
Thelohanellus hovorkai	*Aurantiactinomyxon*	16.7	104	18-22	Szekely *et al.* 1998
Thelohanellus nikolskii	*Aurantiactinomyxon*	12.5	60	22-24	Szekely *et al.* 1998
Sphaerospora renicola	*Neoactinomyxum*	37	98	18-22	Molnar *et al.* 1999a
Hoferellus carassii	*Aurantiactinomyxon*	-	90	-	El-Matbouli *et al.* 1992
Hoferellus carassii	*Neoactinomyxum*	8.5-27.5	90-120	-	Yokoyama *et al.* 1993a
Zschokkella nova	*Siedleckiella*	10	98-101	18	Uspenskaya 1995

5.1.3 Invertebrate Hosts, Their Habitats and Feeding Behaviour

Oligochaetes are the most commonly found group of invertebrate hosts for myxozoans. Beside a number of freshwater species, marine oligochaetes have recently been identified as hosts for actinosporean types (Hallett & Lester 1999, Hallett, Erseus & Lester 1999, Hallett, Erseus, O'Donoghue & Lester 2001). This sediment bound annelid class is common in most freshwater and marine habitats and three families, Tubificidae, Naididae and Lumbriculidae, have so far been found to be involved in myxozoan life cycles. Invasion by myxozoans seems to be bound to the feeding process as most actinosporeans have been reported to infect the gut epithelium of the oligochaetes (Kent *et al.* 2001). Oligochaetes are generally "conveyor belt" feeders, continuously ingesting whole sediment, perhaps selected for particle size and organic content. Myxosporean spores are of suitable size to be ingested with the sediment and consequently invade the gut epithelium of the worms. Oligochaetes are traditionally thought to feed with the anterior end down in the sediment, leaving the posterior end protruding into the water column for defaecation and respiration (Brinkhurst 1996). This behaviour and the morphology of the actinosporean spores, which usually show characteristic hydrostatic appendages, enable their release into the open water body, the habitat of the alternate fish host.

Other invertebrates inhabiting the same habitat and showing a similar unselective feeding behaviour have the potential to function as hosts for myxozoans and should be included in studies on alternate hosts. A report from 1912 describes the presence of actinosporeans in a marine sipunculid (Ikeda 1912). Furthermore, 4 actinosporean types have been described from sedentary as well as errant polychaetes (Bartholomew *et al.* 1997, Køie 2000). In contrast to the widespread habitat of soft to coarse

sediments which most oligochaetes and *Nereis* spp. inhabit, the filter-feeding polychaete *Manayunkia speciosa,* which becomes infected with *Ceratomyxa shasta*, occurs in a very restricted ecological niche, i.e. on the surface of freshwater mussels *Margaritifera margaritifera* L. (Bartholomew *et al.* 1997). The myxozoan class Malacasporea uses Bryozoa as alternate hosts which settle hard bottom surfaces (Anderson *et al.* 1999, Okamura *et al.* 2001, Okamura, Curry, Wood & Canning 2002, Canning, Tops, Curry, Wood & Okamura 2002, Morris *et al.* 2002b, Okamura & Wood 2002).

Two reports from other groups complete the invertebrate host spectrum of myxozans elucidated so far: *Chloromyxum diploxys* Thélohan, 1895 in the lepidopteran *Tortrix viridana* L. is probably a myxozoan, although unlikely to be a species of the genus *Chloromyxum* (cited by Canning & Okamura 2004). As myxozoan life-cycles are generally related to aquatic habitats, it is unclear where the lepidopteran became infected as larvae and adults of this species inhabit trees and not the aquatic environment. However, insect larvae of several different families are known to inhabit the sediments of freshwater environments but their parasitology has been little studied. Finally, trematodes have twice been found to be infected with myxosporean-type spores, apparently in the absence of myxozoan infections in the fish harbouring the flatworms. Both of these myxozoan species, *Fabespora vermicola* Overstreet, 1976 (Overstreet 1976) and *Fabespora* sp. (Siau, Gasc & Maillard 1981) were parasites of trematodes in fish belonging to the family Sparidae.

5.1.4 Vertebrate Hosts

With 1400 myxosporean species described from teleosts, fish represent the most important group of vertebrate hosts for myxozoans (Lom & Noble 1984). However, 20 species have been reported to infect amphibians and reptiles (Lom 1990, Upton *et al.* 1992, Upton *et al.* 1995, Hill *et al.* 1997), one report demonstrated the presence of a myxozoan-like parasite in the brain of a mole *Talpa europea* L. (Friedrich *et al.* 2000), and a recent paper described anatid ducks as vertebrate hosts for myxozoans (Lowenstine *et al.* 2002).

With the exception of the mole, in which no mature spores were found at any time of the year, all vertebrate as well as invertebrate hosts for myxozoans reported so far inhabit the aquatic environment at least during some period of their life. Thus it can be concluded that the survival and propagation of actinosporean and myxosporean spores as well as the infection process of vertebrate and invertebrate hosts is bound to the characteristics of aquatic habitats.

5.1.5 Habitat Studies

Research on myxozoans has been focused mainly on teleosts as the economically most important hosts, but since the discovery of the involvement of *T. tubifex* in the life cycle of *M. cerebralis* (Wolf & Markiw 1984) interest in oligochaetes has increased. However, habitat studies including both vertebrate and invertebrate hosts are rare and restricted to artificial systems, i.e. ponds of fish farms. The importance of the myxozoan fauna in streams and rivers feeding fish farms should not be underestimated as native fish can harbour myxosporean infections and actinosporean spores from the

alternate invertebrate hosts might be introduced into the farmed environment with the incoming water and cause pathology in farmed fish.

5.1.6 Objectives

The aim of this study was to investigate the vertebrate and invertebrate fauna of a *Sphaerospora truttae* enzooic freshwater habitat for the presence of myxosporean and actinosporean types sharing the habitat with *S. truttae*. The survey aimed to provide an overview of the myxozoans characterising the specific habitat of a typical oligotrophic highland stream and it would also target the identification of an alternate host for *S. truttae* by employing the PCR assay specifically developed for *S. truttae* and by conducting infection studies with potential invertebrate hosts and actinosporean stages collected in the same freshwater habitat.

5.2 Materials and Methods

The following section (5.2.1) was aimed at determining which vertebrates and invertebrates are present in the investigated highland river and which of these serve as hosts for myxozoans. It also includes the screening of water for the presence of myxozoans in the water column. The subsequent sections (5.2.2 & 5.2.3) were designed to determine the involvement of the invertebrates and their actinosporeans in the life cycle of *S. truttae,* using infection studies and the *S. truttae* specific PCR assay designed in Chapter 3 (Section 3.3.3). Section 5.2.2 concentrates on the culture of parasite-free invertebrates in the laboratory and the infection of invertebrates with *S. truttae* myxosporean spores, whereas section 5.2.3 focuses on the infection of the vertebrate host, the Atlantic salmon with actinosporean stages. In order to test for the

possibility of direct transmission *S. truttae* myxosporean spores were also exposed to salmon.

5.2.1 Identification of the Myxozoans and Their Hosts in a Scottish Highland Stream

5.2.1.1 Vertebrate Sampling and Screening

On the 2nd October 2002, electrofishing was carried out in the river on the North coast of Scotland (electro-fishing area and procedure see Chapter 2, Section 2.2.5). In order to obtain a representative sample of the fish population in the river, 100 fish were caught, belonging to the families Salmonidae, Anguillidae and Gasterosteidae. Squash preparations of skin, muscle, gills, brain, heart, liver, gall bladder, spleen, kidney and intestine of all fish were scrutinized for the presence of myxosporean spores. The prevalence of the myxosporeans encountered was determined and the different spore types were measured on fresh squash preparations and identified according to Lom & Dyková (1992) and Shul'man (1966) followed by a comparison with the original or re-description of each species (see 5.3.1.1)

On the day of electrofishing (2nd October 2002) 6 frogs, *Rana temporaria* L., were captured at the river and its tributaries. Squash preparations of skin, brain, lungs, intestine, gonads, fat body, liver, kidney and cloaca were examined for the presence of myxosporean spores.

5.2.1.2 Invertebrate Sampling and Screening

Sediment containing predominantly oligochaetes was collected from the outflow of the Atlantic salmon farm on the North coast of Scotland. Between October 2002 and

July 2003, oligochaetes and diverse other invertebrates were sampled at different sites on the river feeding the fish farm (for sampling sites, dates and procedures see Chapter 2).

A total of 13309 oligochaetes (204 from the river) and 326 other invertebrates (296 from the river) were elutriated from the sediments. 5304 oligochaetes (including those from the river) and all other invertebrates were maintained in cell well plates or small containers up to a maximum of 10 days and checked for the release of actinosporean spore stages (methodology see Chapter 2, Section 2.3.1) before they were submitted for DNA extraction and PCR amplification (methodologies see Chapter 2, Sections 2.4.1 & 2.4.2). When spores were detected they were measured and identified (Özer *et al.* 2002a, Janiszewska 1957, Marques 1984).

A sub-sample of 240 oligochaetes from the outflow of the fish farm and all oligochaetes sampled in the river were identified to species level following the key of Brinkhurst & Jamieson (1971). All other invertebrates collected were identified to family level using the key of Jessup *et al.* (1999), before DNA extraction.

All invertebrates (oligochaetes and others) were submitted to DNA extraction and PCR in order to screen for (cryptic) myxozoan infections and to determine the infection prevalence in the invertebrate hosts. Therefore, primers 18e and 18g were used in a non-specific PCR reaction at an annealing temperature of 65°C in order to amplify any 18S rDNA present (see 3.2.1). Subsequently, the obtained PCR product was diluted 1:4 in nanopure water and two myxozoan-specific primers, Myxgp2f and Act1R (see Table 3.2.2.B), were employed in a nested PCR reaction at an annealing temperature of 58°C. The invertebrates and all actinosporeans found were submitted to the *S. truttae* specific

PCR assay (Chapter 3, Section 3.3.3) in order to test for the involvement of the invertebrates and the actinosporeans in the life cycle of *S. truttae.*

Unless too small, non-oligochaete invertebrates were halved and one half was used for PCR whereas the other half was conserved in formalin for use in ISH (methodology see Table 4.3.1.B) in case of a positive PCR result. Serial sections of 30 whole chironomids from the outflow pond of the farm were also submitted to ISH.

5.2.1.3 Occurrence of Myxozoans in the Water Column

In order to test for the presence of myxozoans in the water, river water was filtered with a 20 µm plankton-net on 4 occasions between the months of March and July (sampling dates, sites and methodology see Chapter 2, Table 2.2.1 and section 2.2.4). A small portion of each sample was examined microscopically (x100-x200), another portion was centrifuged and fixed in 96% alcohol, and the largest portion of each sample was left to settle and was subsequently separated into the upper, relatively transparent water phase and the brown and organically enriched bottom phase. Both phases were filtered through a 5 µm screen, and the material on the filter was centrifuged for 5 min at 2000g and the supernatant removed. The pellet obtained from the bottom phase was split into 10 Eppendorf tubes whereas the top layer was split into 2 Eppendorf tubes. In order to test for PCR inhibition, two of the samples (one from the top and one from the bottom layer) were spiked with approximately 50 actinosporeans of *Echinactinomyxon* type 1 obtained from infected *Lumbriculus variegatus.* All samples were dissolved in TNES urea and DNA was extracted (see Chapter 2). The DNA obtained was used directly and in dilutions of 1:10, 1:100 and

1:1000 in the nested PCR assay for myxozoans (described above) as well as in the *S. truttae* specific PCR assay (see Chapter 3).

5.2.2 Culture of Invertebrate Hosts and Exposure to Sphaerospora truttae

5.2.2.1 Invertebrate Culture

In order to obtain parasite-free oligochaetes for infection with *S. truttae* myxosporean spores, cultures of the oligochaetes present in the outflow of the fish farm on the North coast were set up in the laboratory. For this purpose, sediment containing oligochaetes was collected in November 2000. As for screening, the oligochaetes were elutriated from the sediment and then separated into 4 culture groups: (1) mixed (natural) population of oligochaetes, (2) tubificid population, (3) population of *Lumbriculus variegatus,* (4) naidid population.

The sediment from which the oligochaetes had been elutriated was autoclaved and washed with tap water and 1 L was placed into each of 10 aquaria of 6 L volume. Three aquaria each were stocked with oligochaetes from culture groups 1-3. A total biomass of 10 g of oligochaetes was added to each aquarium. One aquarium of each group was kept in water baths at 10°C, 15°C and 20°C. Due to the very small size of the naidids only one aquarium containing a non-determined amount of worms was maintained at 15°C. The setup of aquaria for culture and their subsequent use for infection studies is summarised in Figure 5.2.2.1. All aquaria were equipped with 4 liters of 5 µm filtered local stream water in order to provide the natural microbial climate required for the digestion processes of the oligochaetes. Continuous aeration was achieved by placing an airstone inside the sediment.

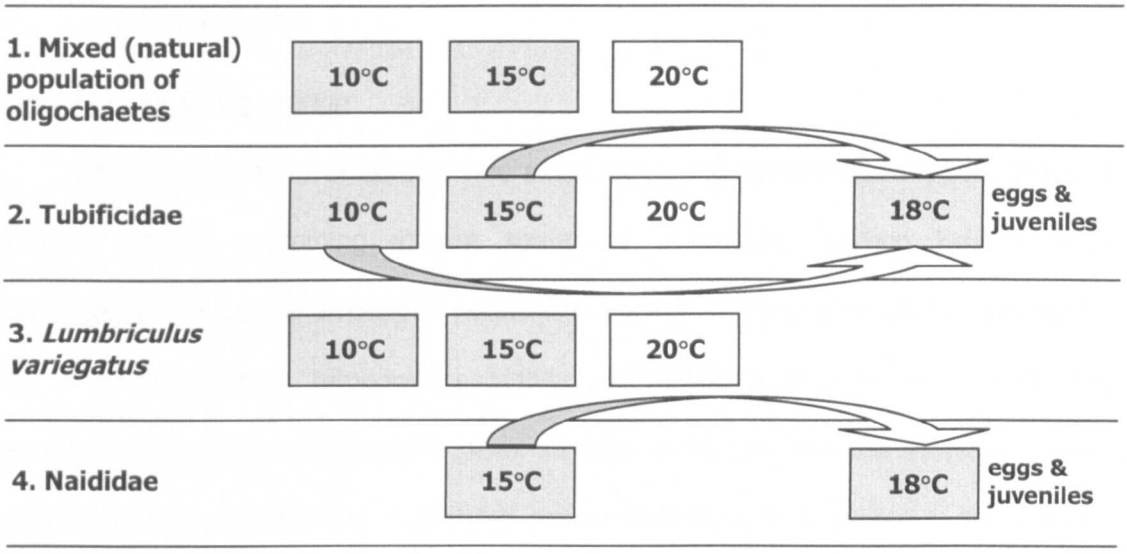


Figure 5.2.2.1 Summary of the culture trials using oligochaetes. Every aquarium was equipped with 10 g of oligochaetes and population growth was checked every 20 days for 80 days. All culture groups were maintained at 10, 15 and 20°C and the eggs and juveniles of the tubificids were separated into one aquarium at 18°C. [ ] =Oligochaetes from these aquaria were used for infection experiments after the culture trials (see 5.2.2.2).

Every 20 days over an 80 day period the worms of culture groups 1-3 were elutriated, their biomass was determined, and they were observed under the dissection microscope. The naidids were also elutriated and observed without weighing. Cocoons containing eggs and juvenile worms were separated from the tubificid and naidid cultures and transferred to new aquaria with autoclaved sediment. After the weighing, the oligochaetes were returned to their aquaria and the habitat in the aquaria was organically enriched with a mixture of cow dung and ground fish food, in order to provide nutrients for the worms. At the same time, approximately a third of the water was replaced by freshly filtered stream water. All aquaria were exposed to an 18 hr light : 6 hr dark photoperiod which represents natural late spring / early summer light conditions in Scotland.

5.2.2.2 Exposure of Invertebrates to Sphaerospora truttae Spores from Kidney Homogenates

Spore Origin & Preparation

Infection of the invertebrates was attempted using homogenised kidney tissue of Atlantic salmon containing mature spores of *S. truttae.* Salmon kidneys were homogenised in a small amount of water. The homogenate was then left to disintegrate for 2 hours at room temperature before centrifugation at 800g for 5 min. The supernatant was discarded and the pellet containing the concentrated spores and some cell debris was resuspended in distilled water. The number of spores in the solution obtained was counted in a haemocytometer.

Spore Viability

In order to ensure that viable (and potentially infective) spores are used in the infection studies, the viability of the spores was tested using an aliquot of the spore suspension stained with propidium iodine (PI) and fluorescein diacetate (FDA), as described by Yokoyama, Danjo, Ogawa & Wakabayashi (1997): Stock solutions of of PI (0.02 mg ml^{-1} dissolved in distilled water) and FDA (5 mg ml^{-1} dissolved in acetone) were stored at -20°C in 1 ml aliquots. Immediately before use, 20 µl of FDA stock solution was diluted with 4.2 ml of distilled water. Twenty-five microlitres of freshly diluted FDA solution, 25 µl of PI solution and 50 µl of spore suspension were combined in a microtube, mixed with a vortex and then left undisturbed in the dark at 4°C overnight. On the following day, the stained spore supension was viewed at x 400 in the fluorescent microscope and the proportion of dead (red) and viable (green) spores was determined in 100 spores.

Oligochaete Origin & Preparation

The oligochaetes from the culture trials (see 5.2.2.1, all 10°C and 15°C cultures) and the presumed parasite-free offspring (eggs and juveniles) separated from the tubificid and naidid cultures (see 5.2.2.1) were used in the infection trials. Additionally, 6 earthworms *Eiseniella tetraedra,* 50 chironomid larvae (sampled in the river on the North coast) and a population of tubificids obtained from a pet shop in Vienna (Austria) were exposed to *S. truttae* spores.

One litre glass beakers were prepared for the infection experiments. Four hundred ml of autoclaved sediment was placed into each beaker before the experimental oligochaetes or chironomids were added. All beakers were topped up with 5 µm–filtered stream water and equipped with an airline. A second beaker containing the same invertebrate populations as used for the infection experiments was maintained under the same conditions but was not exposed to kidney homogenates and served as a control group.

In order to ensure the uptake of the spores with the food particles, some oligochaetes from the cultures and some from Vienna were kept in another sediment type of considerably smaller particle size than the gravel from the North coast, i.e. autoclaved mud, obtained from the River Forth in Stirling.

Infection

Infection of oligochaetes and chironomids with kidney homogenates was attempted at constant temperatures (large waterbaths) of 8°C, 10°C and 15 °C or at ambient temperatures (outdoors). The light regime was set to a 18 hr light : 6 hr dark photoperiod. Variable numbers of oligochaetes (100-1000) were added to the 1 L

beakers, resulting in an exposure to 16550-217000 *S. truttae* myxosporean spores per worm. Some oligochaetes were repeatedly exposed. When the spore suspension was pipetted into the beakers, the water was agitated briefly in order to ensure mixing. A second beaker maintained in the same way as the experimental one was used as control for each trial. Table 5.2.2.2 summarises all invertebrate infection studies.

Screening

In order to check the experimental invertebrates for actinosporean release, the water in the beakers was 5 µm-filtered and the organic matter on the filter was collected in a small amount of water and viewed on a glass slide under the microscope (x100 and x200 magnification). At each sample date, 40 oligochaetes were transferred into cell well plates containing de-chlorinated tap water and were checked for the release of actinosporeans every second day for one week. Once the *S. truttae* specific PCR assay had been developed, these oligochaetes were additionally screened by PCR. In order to avoid false positive PCR signals caused by intestinal passage but not infection with *S. truttae*, DNA was only extracted from the invertebrates after a 1 week dwelling time in spore-free water.

The experimental chironomids were monitored in cell well plates in the same way as the oligochaetes and serial sections of all experimental chironomids were used for ISH after termination of the infection period (day 123).

Table 5.2.2.2 Summary of the experimental infection trials using oligochaetes and chironomids. Invertebrates: *L. variegatus = Lumbriculus variegatus,* Oligochaetes (mixed) = natural mixed population present in the outflow pond of the fish farm on the North coast. Invert No = Number of invertebrates used in the infection study. Temp. = Temperature throughout the experiment. Spore No = number of spores used for infection. Check (days p.i.) = number of days p.i. after which the water in the beakers was checked for the presence of actinosporeans by filtration through a 5µm mesh, W = weekly, PCR = *Sphaerospora truttae* specific PCR of 100 worms per date.

Invertebrate	Invert No	Sediment type	Temp.	Infection date	Spore No	Check (days p.i.)
L. variegatus	600	gravel	8-13°C	01.03.2001	34 550	84, 96,123
L. variegatus	100	gravel	10-19°C	04.05.2001	16 550	W day 45-154
L. variegatus	300	gravel	8°C	15.05.2001	186 200	W day 40-160
L. variegatus	100	gravel	11-19°C	15.05.2001	93 100	W day 40-160
L. variegatus	200	gravel	15°C	15.05.2001	93 100	W day 40-160
L. variegatus	200	gravel	20°C	15.05.2001	93 100	W day 40-160
Oligochaetes (Mixed)	500	gravel	8°C	15.05.2001	186 200	W day 40-160
Oligochaetes (Mixed)	400	gravel	10°C	15.05.2001	93 100	W day 40-160
Oligochaetes (Mixed)	400	gravel	15°C	15.05.2001	93 100	W day 40-160
Oligochaetes (Mixed)	400	gravel	20°C	15.05.2001	93 100	W day 40-160
Tubificidae	100	gravel	11-19°C	15.05.2001	93 100	W day 40-160
Tubificidae JUVENILES	200	gravel	11-19°C	15.05.2001	93 100	W day 40-160
Naididae	1000	gravel	11-19°C	15.05.2001	186 200	W day 40-160
Naididae JUVENILES	200	gravel	11-19°C	15.05.2001	93 100	W day 40-160
Tubificidae	500	gravel	9-17°C	29.04.2002 05.05.2002 14.05.2002 21.05.2002	217 000 153 000 90 000 203 000	W day 60-200 (PCR day 21, 40)
Tubificidae-Vienna	500	gravel	9-19°C	29.04.2002 05.05.2002 14.05.2002 21.05.2002	217 000 153 000 90 000 203 000	W day 60-200
Tubificidae	500	mud	9-19°C	29.04.2002 05.05.2002 14.05.2002 21.05.2002	217 000 153 000 90 000 203 000	W day 60-200 (PCR day 21, 40)
Tubificidae-Vienna	500	mud	9-19°C	29.04.2002 05.05.2002 14.05.2002 21.05.2002	217 000 153 000 90 000 203 000	W day 60-200
Oligochaetes (Mixed population)	500	gravel	9-19°C	29.04.2002 05.05.2002 14.05.2002 21.05.2002	217 000 153 000 90 000 203 000	W day 40-160 (PCR day 60)
Eiseniella tetraedra	6	gravel	9-20°C	18.04.2003	128 000	W day 40-123
Chironomidae	25	gravel	10°C	18.04.2003	128 000	W day 40-123
Chironomidae	25	gravel	13°C	18.04.2003	128 000	W day 40-123

5.2.2.3 Exposure of Invertebrates to Sphaerospora truttae by Co-Habitation With Infected Atlantic Salmon

Twenty litres of sediment each containing oligochaetes and other invertebrates was collected in the outflow of the fish farm on the North coast of Scotland on the 04.04.2002 and were placed into two 180 litre flow-through tanks. One tank served as a control tank so that natural infections in the oligochaetes would not be mistaken for the outcome of an infection due to co-habitation. On the 09.04.2002, 20 Atlantic salmon smolts (1+) from the fish farm on the North coast location (sampled on the 04.04.2002) were introduced into the second tank. Smear preparations of the kidneys of 5 smolts from the same population tested positive for the presence of mature *S. truttae* spores and the farm has a known history of annually recurring *S. truttae* infections with 100% prevalence (McGeorge 1994, Özer 1999, personal observation). The smolts were left to shed spores in the co-habitation tank for approximately two months (until 14.06.2002). Thereafter, the fish were examined for the presence of *S. truttae* spores and all were found uninfected.

From day 21 of co-habitation onwards until 31.07.2002 (for 3 months), 50 oligochaetes from each tank were separated into cell well plates each week and tested for the release of actinosporean stages. After one week in the cell well plates, the oligochaetes were submitted to PCR screening using the *S. truttae* specific PCR assay (see Chapter 3, Section 3.3.3).

5.2.3 Exposure of Atlantic Salmon to Actinosporeans and Sphaerospora truttae Myxosporean Spores

5.2.3.1 Exposure to Actinosporean Spores

Atlantic salmon fry were exposed to five different types of actinosporeans released from the oligochaetes inhabiting the outflow pond of the fish farm on the North coast, i.e. *Echinactinomyxon* type 1, *Echinactinomyxon* type 5, *Raabeia* type 4, *Synactinomyon* type 1 and *Synactinomyxon* type 3 (description of spore types see 5.3.1.2 and Özer *et al.* 2002a). For 5 subsequent days starting on the 19.06.2002, fresh actinosporean spores were collected and pipetted into 5 different aquaria each containing 30 Atlantic salmon fry of 0.5 g body weight and obtained from a fish farm in Stirling (see Chapter 2, Section 2.1., on 05.06.2002). These fry were considered to be myxosporean-free.

The actinosporeans were counted when collected from the wells with the worms and a volume equivalent to 10000 spores per fish was added to each aquarium daily for 5 days. During this period, the fry were maintained in 2 litres of aerated de-chlorinated mains water, which was replaced by a larger volume of water on day 6. The water temperature throughout the experiment was 14±3°C. An additional thirty fry were kept as a control group under the same conditions as the experimental fish.

In order to determine whether and at which loci different actinosporeans enter the Atlantic salmon, two fry each were removed 1, 3, 6, 10 and 20 minutes after first exposure to each actinosporean type, euthanised with 0.1 g l^{-1} of chlorobutanol and fixed for SEM and general histology (see Chapter 2, Sections 2.4.5 & 2.4.6). The remainder of the fish were sampled 3 months after first exposure to the actinosporeans

and squash preparations of all organs (stated in 5.2.4) were scrutinised for the presence of myxosporean infections.

In a second experiment, 10 fry were euthanased and immediately after death, fins and gills were removed and exposed to a solution containing fresh actinosporeans of the same types as listed above in a 1.5 ml Eppendorf tube which was agitated for 5 min before observation under the microscope and subsequent fixation for SEM and general histology. Mucus was collected from the freshly euthanased fish and the reaction of the actinosporean types to the salmon mucus was observed under the microscope (x400) by adding a 1:1 mixture of mucus in water to spores on a glass slide.

5.2.3.2 Co-Habitation of Atlantic Salmon with Sediment Containing Various Invertebrates

Section 4.2.3 (Chapter 4) describes the experimental design for infecting Atlantic salmon fry by co-habitation with sediments collected in the outflow pond of the salmon hatchery on the North coast. In order to test whether this experimental infection could be repeated, the experiment was conducted a second time using the same setup with sediment collected in the same location on 02.03.2003 and 50 myxozoan-free Atlantic salmon fry from Loch Fyne, collected and introduced into the experimental tank on 17.03.2003. Three fry were tested weekly for 3 months for the presence of *S. truttae* using the *S. truttae* specific PCR assay (Chapter 3, Section 3.3.3) and *ISH* protocol (Chapter 4, Section 4.3.1)

5.2.3.3 Exposure to Sphaerospora truttae Myxosporean Spores

In order to rule out a direct life cycle for *S. truttae,* 20 myxozoan-free Atlantic salmon fry from Loch Fyne (14.05.2001) were exposed to kidney homogenates containing myxosporean spores of *S. truttae* obtained from 8 infected salmon smolts from the North coast (30.04.2001). The homogenates were produced and the spores tested for viability as described in 5.2.2.2.

The experimental fry were kept in an aerated 6 litre aquarium at 15°C and were not fed for 3 days prior to the experiment to ensure uptake of the homogenised tissue with added food pellets to allow for a potential entry of the parasite via the intestinal epithelium. The water in the aquarium was changed once a week and replaced by de-chlorinated tap water. A control group of 20 fry from the same population as the experimental fish was kept under the same conditions. Ten fish from the experimental and control group were sacrificed 2 months p.i. and the remainder of the fish were sacrificed after 3 months. Squash preparations of all organs were examined for the presence of myxosporeans.

5.3 Results

5.3.1 The Myxozoan Fauna and Their Hosts in a Scottish Highland Stream Habitat

5.3.1.1 Vertebrate Hosts and Their Myxosporean Infections

Teleosts

Electrofishing in the oligotrophic river feeding the fish farm on the North coast showed that the river was populated mainly by salmonids which share the habitat with eels and sticklebacks (Table 5.3.1.1.A).

Table 5.3.1.1.A Fish species caught by electrofishing in the river on the North coast.

Fish Family	Species	No. of fish caught/examined
Salmonidae	*Salmo salar* L.	49
Salmonidae	*Salmo trutta* L.	29
Anguillidae	*Anguilla anguilla* (L.)	19
Gasterosteidae	*Gasterosteus aculeatus* L.	3

Nine different myxosporean species belonging to 6 genera were detected in the organs of the examined fish. Most myxosporean species were found in the urinary system (kidney, renal tubules and urinary bladder) of the different fish species. Only two species, *Chloromyxum truttae* Léger, 1906 and *Myxidium truttae* Léger, 1930 occurred in the epithelium of the gall bladder and their spores were found floating in the bile.

Atlantic salmon as well as brown trout were infected with *Sphaerospora truttae*. Only juvenile fish (all ≤7cm) harboured *S. truttae* spores, which corresponds to findings from farmed fish which are known to acquire immunity after their first exposure to *S.*

truttae (McGeorge *et al.* 1996a). Frequently, *S. truttae* was detected in mixed infections with a *Chloromyxum* species, which also develops spores intratubularly. The occurrence of early sporogonic stages in the tubuli of small fish sometimes obscured the differentiation between the two species in a squash preparation. A second mixed infection of two intratubular kidney myxosporeans, *Sphaerospora elegans* and *Myxobilatus gasterostei* was found in one of the sticklebacks.

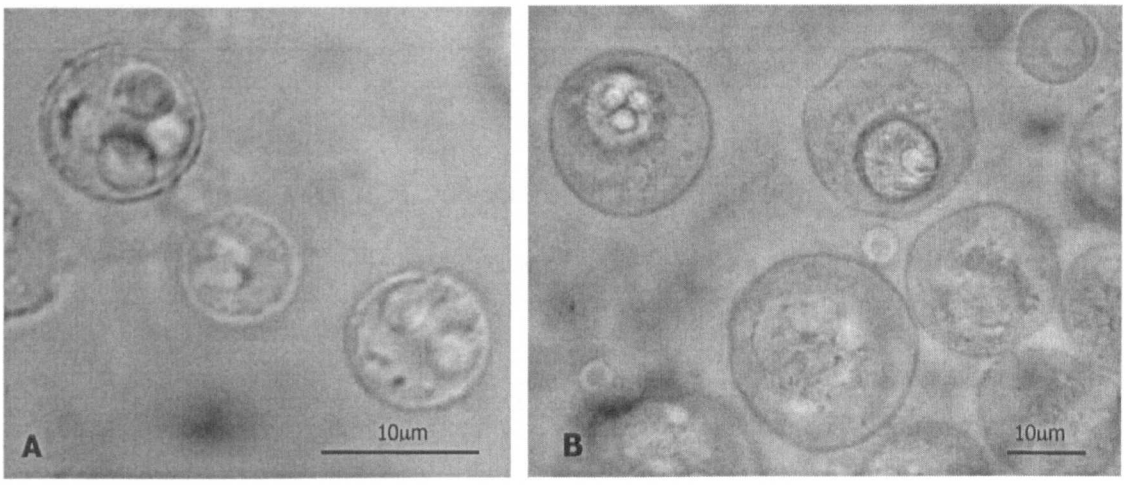
The tissue localisation and prevalence of each species as determined by smear preparation of the organs is summarized in Table 5.3.1.1.B, followed by measurements of the spores obtained in this study in comparison with the measurements in published records.

Table 5.3.1.1.B Myxosporeans found in the fish of the river investigated on the North coast and their infection prevalence in the host species. * Excludes 2 *Salmo salar* and 8 *Salmo trutta* containing intratubular developmental stages but lacking mature spores for species identification

Myxosporean	Host	Infection prevalence
Chloromyxum truttae Léger, 1906	*Salmo salar* L.	18%
	Salmo trutta L.	10%
Myxidium truttae Léger, 1930	*Salmo trutta* L.	17%
Sphaerospora truttae Fischer-Scherl, El-Matbouli & Hoffmann, 1986	*Salmo salar* L.	14%
	Salmo trutta L.	10% *
Sphaerospora elegans Thélohan 1892	*Gasterosteus aculeatus* L.	67%
Myxobilatus gasterostei Davis, 1944	*Gasterosteus aculeatus* L.	67%
***Chloromyxum* sp.**	*Salmo salar* L.	86% *
	Salmo trutta L.	21% *
Myxidium giardi Cépède, 1906	*Anguilla anguilla* (L.)	26%
***Zschokkella* sp.**	*Anguilla anguilla* (L.)	11%
Hoferellus gilsoni (Debaisieux, 1925)	*Anguilla anguilla* (L.)	26%
Myxobolus portucalensis Saraiva & Molnar, 1990	*Anguilla anguilla* (L.)	11%

Description of Myxosporeans Encountered

Chloromyxum truttae Léger, 1906



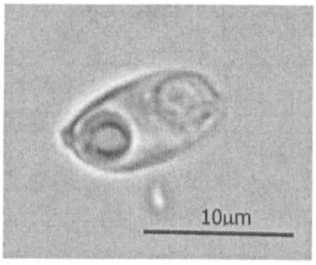
- **Host:** *Salmo salar* L., *Salmo trutta* L.
- **Host tissue localisation:** Epithelium of gall bladder, bile
- **Reference:** Alvarez-Pellitero, Pereira-Bueno & Gonzales-Lanza 1982

Description: Spores (A) – Almost spherical with numerous surface ridges which form concentric circles in front view. Four pyriform polar capsules in subapical position, two slightly smaller than the others. Trophozoites (B) – Spherical trophozoites, early stages with refractile vesicles in the endoplasm, which disappear when spores are formed; trophozoites develop 1-5 spores and are 30-40 µm in diameter.

- **Spore measurements:**

	Alvarez-Pellitero et al. 1982	**this study**
Spore:		
Length	9.7 (8.2-10.5) µm	9.65 (8.3-10.12) µm
Width	9.4 (7.9-10.5) µm	9.38 (8.03-10.0) µm
Thickness	9.3 (8.2-10.0) µm	9.3 (8.13-9.78) µm
Polar capsules:		
Length x width (smaller pair)	3.4 (3.0-3.8) x 2.6 (2.0-3.0) µm	3.2 (3.0-3.58) x 2.59 (2.1-2.8) µm
Length x width (larger pair)	3.9 (3.7-4.2) x 2.9 (2.4-3.8) µm	3.8 (3.67-4.1) x 2.81 (2.38-3.2) µm

Myxidium truttae Léger, 1930



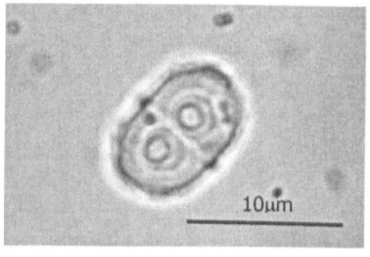
- **Host:** *Salmo trutta* L.
- **Host tissue localisation:** Epithelium of gall bladder, bile.
- **Reference:** Lom & Dyková 1992

Description: Spores – Ellipsoid shape, surface with ridges, spores are pointed at both ends and slightly S-shaped. Trophozoites – Large plasmodia with up to 420 µm diameter; thousands of spores matured within these large units.

- **Spore measurements:**

	Lom & Dyková 1992	**this study**
Spore:		
Length	11.5 µm	11.2 (10.6-11.92) µm
Width	8 µm	7.5 (7-8.1) µm
Thickness		
Polar capsules:		
Length x width	4.3 x 2.5 µm	4.0 (3.5-4.43) x 2.9 (2.64-3.1) µm

Sphaerospora truttae Fischer-Scherl, El-Matbouli & Hoffmann 1986



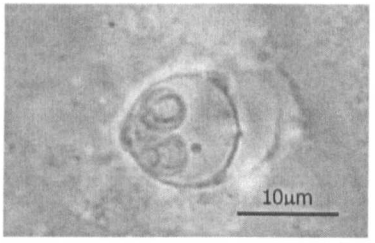
- **Host:** *Salmo salar* L., *Salmo trutta* L.
- **Host tissue localisation:** Renal tubules and sometimes Bowman's capsules
- **Reference:** Fischer-Scherl *et al.*1986; McGeorge *et al.* 1994

Description: Spores – Broadly ellipsoid spores with finely obliquely ridged surface, polar capsules spherical. Pseudoplasmodia – Consistently bisporous. Pre-sporogonic development see Chapter 4, Section 4.3.3.

- **Spore measurements:**

	Fischer-Scherl et al. 1986	McGeorge et al. 1994	this study
Spore:			
Length	6.84 (6.58-8.68) µm	7.4 (6.5-8.4) µm	7.5 (6.61-8.3) µm
Width	8.81 (8.22-10.11) µm	9.9 (8.8-11.2) µm	9.78 (8.75-10.8) µm
Polar capsules:			
Diameter	-	2.4 (1.9-3.3) µm	2.5 (2.0-3.1) µm

Sphaerospora elegans Thélohan, 1892



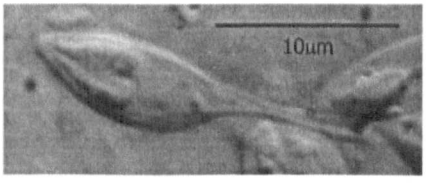
- **Host:** *Gasterosteus aculeatus* L.
- **Host tissue localisation:** Renal tubules
- **Reference:** Feist, Chilmonczyk & Pike 1991

Description: Spores – Pitted surface, subspherical in sutural view, slightly flattened posteriorly, anterior thickening of valves along the suture, 2 "horn-like" protuberances on the posterior portion of the valves equidistant from sutural line; polar capsules spherical. Pseudoplasmodia – Consistently bisporous.

- **Spore measurements:**

	Feist et al. 1991	this study
Spore:		
Length	10.2 (7.8-11.6) µm	11.0 (8.1-11.5) µm
Width	10.1 (7.8-11.6) µm	10.6 (7.5-11.1) µm
Polar capsules:		
Diameter	3.9 (3.65-4.1) µm	4 (3.75-4.2) µm

Myxobilatus gasterostei Davis, 1944



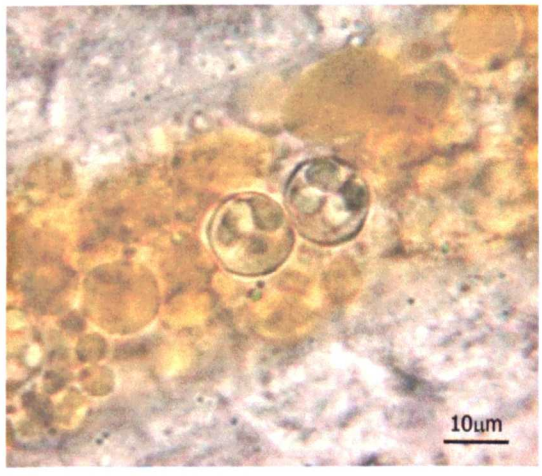
- **Host:** *Gasterosteus aculeatus* L.
- **Host tissue localisation:** Renal tubules
- **Reference:** Sultana 1994

Description: Spores – Asymmetrical, spindle shaped in sutural view and one side slightly flattened in valvular view; surface ridges present, running parallel to the suture; posterior end with forked caudal process; polar capsules are pyriform and mostly of equal length. Plasmodia – Mono-, di- and polysporous with a maximum of 8 spores.

- **Spore measurements:**

	Sultana 1994	**This study**
Spore body:		
Length	10.5 (10.2-12.6) μm	10.2 (10.0012.33) μm
Width	5 (4.2-6.3) μm	5.1 (4.5-6.02) μm
Thickness	5.2 (4.2-6.8) μm	5.2 (4.65-6.3) μm
Caudal process:		
Length	20.32 (10.5-26.25) μm	19.8 (15.9-24.35) μm
Polar capsules:		
Length x width	5.4 (4.2-7.3) x 3 (1.5-3.1) μm	5.2 (4.75-6.6) x 2.9 (2.0-3.23) μm

***Chloromyxum* sp**



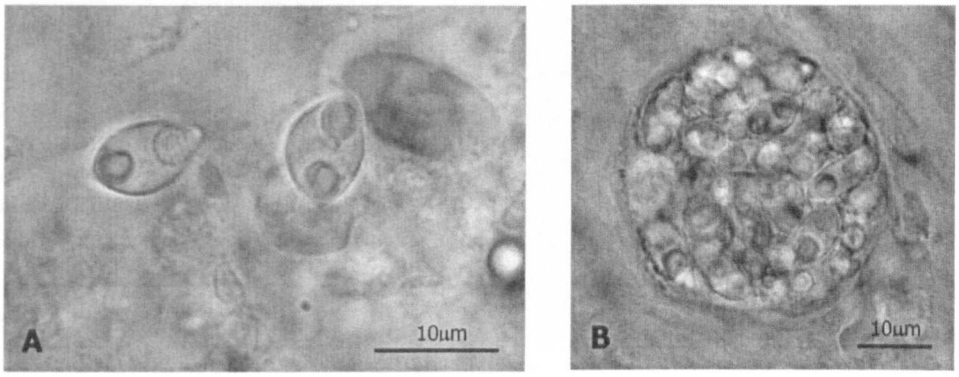
- **Host:** *Salmo salar* L., *Salmo trutta* L.
- **Host tissue localisation:** Renal tubules
- **Reference:** Sedlaczek 1991

Description: Spores – Spherical, slightly tapering at anterior pole, surface with fine ridges, 4 pyriform polar capsules with a larger pair in the sutural plane and a smaller pair in right angle to it. Pseudoplasmodia – Mainly bisporous. Pseudoplasmodia are typically accompanied by extracellular material containing yellow pigment in the intertubular space.

- **Spore measurements:**

	Sedlaczek 1991	this study
Spore:		
Length	7-7.8 µm	8.39 (7.2-8.58) µm
Width	7-7.8 µm	8 (7.56-8.3) µm
Polar capsules:		
Length x width (smaller pair)	3.9 x 3.1 µm	3.65 (3.45-3.8) x 2.9 (2.73-3.1) µm
Lenth x width (larger pair)	3.1 x 2.3 µm	3 (2.78-3.3) x 2.2 (2.98-2.42) µm

Myxidium giardi Cépède, 1906



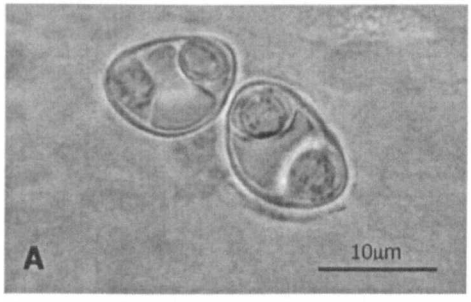
- **Host:** *Anguilla anguilla* (L.)
- **Host tissue localisation:** Connective tissue?; always present in kidney, occurrence in other organs varies, most frequently in the gills and the peritoneum.
- **Reference:** Copland 1981

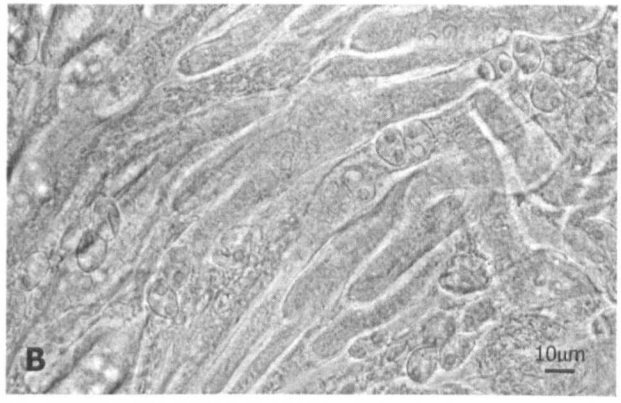
Description: Spores (A) – Spindle-shaped, rather wide, surface with longitudinal ridges, 2 subspherical polar capsules. Plasmodia (B) – spherical units of different sizes, largest plasmodia up to 2mm diameter producing thousands of spores.

- **Spore measurements:**

	Copland 1981	this study
Spore:		
Length	9.4 (7.2-11.8) µm	9 (8.1-10.24) µm
Width	4.8 (4.5-7.2) µm	4.4 (4-5.05) µm
Thickness	5.9 (4.5-7.2) µm	5.5 (5-6.1) µm
Polar capsules:		
Length x width	3.2 (2.3-4.5) x 3.1 (2.3-4.5) µm	3 (2.6-3.41) x 2.8 (2.4-3.3) µm

***Zschokkella* sp.**





- **Host:** *Anguilla anguilla* (L.)
- **Host tissue localisation:** Urinary bladder.

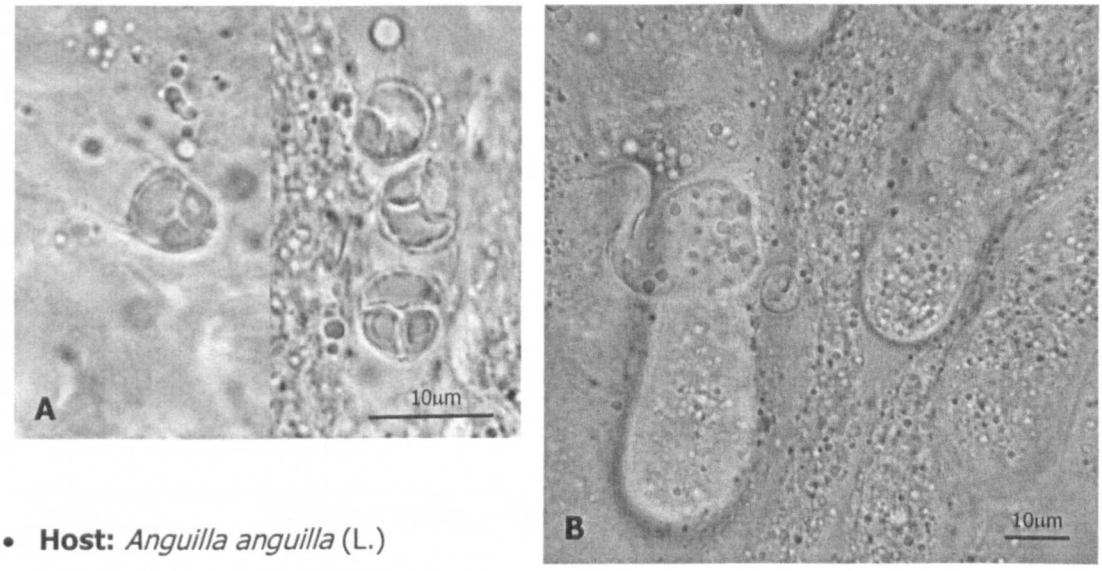
Description: Spores (A) – Laterally widened on one side whereas the other side appears flat; the two polar capsules are situated at opposite ends of the spore; polar capsules subspherical. Plasmodia (B) – Predominantly elongated, usually 140 µm long and very slim (15 µm wide), attached side by side to the bladder epithelium and containing refractile granules; spores develop in disporous units within the plasmodia.

This species is potentially synonymus with *Zschokkella stettinensis* Wierzbicka, 1987. Wierzbicka's drawings of the spores are very similar but measurements are only given from stained smears so that shrinking of the spores cannot be excluded.

- **Spore measurements:**

	Zschokkella stetteniensis (stained preparations) **Wierzbicka 1987**	***Zschokkella* sp. this study**
Spore:		
Length	10.53 (9.6-11.6) μm	12.41 (11.46-12.9) μm
Width	8.62 (7.2-10.0) μm	9.42 (8.83-10.1) μm
Thickness	7.6-8.0 μm	8.80 (8.21-9.13) μm
Polar capsules:		
Length x width	3.22 (2.8-4.0) x 3.22 (2.8-4.0) μm	3.91 (3.2-4.43) x 3.63 (3.11-4) μm

Hoferellus gilsoni (Debaisieux, 1925)



- **Host:** *Anguilla anguilla* (L.)
- **Host tissue localisation:** Urinary bladder.
- **Reference:** Lom, Molnar & Dyková 1986

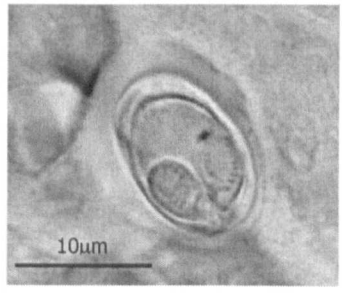
Description: Spores (A) – Subspherical, posterior side flattened with a prominent brush border; surface finely striated; pyriform polar capsules. Plasmodia (B) – Elongated plasmodia (up to 200 μm long) but wider than in case of *Zschokkella* sp. (30μm); plasmodia side by side in the infected epithelium; spores develop in disporous units within the plasmodia.

- **Spore measurements:**

	Lom et al. 1986	this study
Spore:		
Length	7.0 µm	7.0 (6.5-7.6) µm
Width	7.0 µm	6.8 (6.41-7.3) µm
Brush border		4.0 (3.78-5.2) µm
Polar capsules:		
Length x width	3.4 x 3.4 µm	3.5 (3.1-3.79) x 3.2 (2.87-3.5) µm

Myxobolus portucalensis Saraiva & Molnar, 1990

- **Host:** *Anguilla anguilla* (L.)
- **Host tissue localisation:** Gills
- **Reference:** Saraiva & Molnar 1990



Description: Spores – Elliptical in shape with anterior and posterior ends similarly rounded, sutural markings (8-10) around the edge of the spore; polar capsules pyriform with a polar filament in 10 coils; thick mucous envelope surrounding each spore. Single spores were detected in smear preparations of the gill, plasmodia were not found.

- **Spore measurements:**

	Saraiva & Molnar 1990	this study
Spore:		
Length	12.6 (11.25-15) µm	12.8 (11.5-18.81) µm
Width	8.9 (7.5-10) µm	8.5 (7.71-9.98) µm
Thickness	6.8 (5.63-7.5) µm	6.7 (5.5-7.27) µm
Polar capsules:		
Length x width	5.8 (3.75-7.5) x 3.0 (2.5-3.75) µm	5.5 (4.4-6.63) x 3.2 (2.8-3.6) µm

Frogs

Myxosporean spores were not detected in any of the organ squash preparations of the 6 specimens of *Rana temporaria* which were examined.

5.3.1.2 Invertebrate Hosts and Their Myxozoan Infections

Oligochaete Population

In the outflow pond of the fish farm on the North coast, oligochaetes were present at very high density whereas large quantities of sediment from the river had to be washed in order to elutriate single oligochaetes. The species community in the outflow pond was estimated from a total of 240 individual worms and was found to consist of a high variety of species, listed in Table 5.3.1.2.A. In the river, oligochaetes were exclusively found at sampling points with a gravel size below 3 cm diameter (sample points 1 and 2, Chapter 2, Figure 2.1.C). Only 4 species were found in the river (total of 204 individuals, Table 5.3.1.2.A).

Table 5.3.1.2.A Species constituting the oligochaete population in the outflow pond of the salmon farm and in the river feeding the farm. * Worm species which were not present amongst the number of specimens identified but which were found during the study period.

Oligochaete		Percentage of population	
Family	**Species**	**Outflow**	**River**
Lumbriculidae	*Lumbriculus variegatus* Claparède	32%	81%
Lumbriculidae	*Stylodrilus heringianus* Claparède	-	1%
Lumbricidae	*Eiseniella tetraedra* (Savigny)	<1%*	16%
Naididae	*Uncinais uncinata* (Ørstedt)	46%	-
Naididae	*Pristina idrensis* Sperber	4%	-
Naididae	*Chaetogaster langi* Bretscher	2%	-
Naididae	*Vejdovskella comata* Vejdovsky	1%	-
Tubificidae	*Tubifex tubifex* (Müller)	9%	-
Tubificidae	*Tubifex ignotus* (Stolč)	1%	-
Tubificidae	*Limnodrillus hoffmeisteri* Claparède	5%	-
Enchytraeidae	Undetermined	<1%*	2%

Oligochaete Infection Status

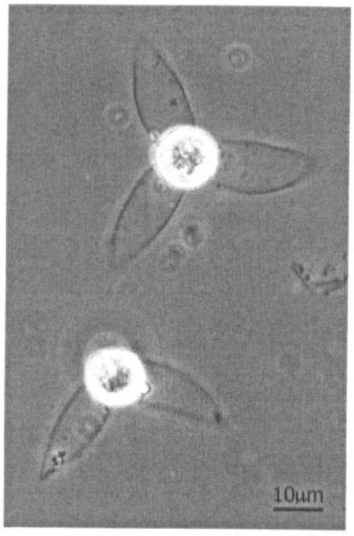
Twelve actinosporean types belonging to the collective groups raabeia, echinactinomyxon, aurantiactinomyxon, neoactinomyxum and synactinomyxon were released from the 5304 oligochaetes monitored microscopically for actinosporean production in cell well plates. Ten types occurred in the outflow settlement pond of the hatchery and were previously described by Özer *et al.* (2002a). The other two forms, *Neoactinomyxum eiseniellae* Omieres & Frezil, 1969 and the 'petite forme' of *Aurantiactinomyxon pavinsis* Marques, 1984 were only released from one oligochaete specimen each, both found in the river. The number of *Eiseniella tetraedra* Savigny, the host of *Neoactinomyxum eiseniellae* was considerably higher in the stream than in the outflow area of the farm and the presence of *Stylodrilus heringianus* Claparède, the host of *Aurantiactinomyxon pavinsis* was restricted to the river habitat (see Table 5.3.1.A). None of the naidid species were found to release actinosporeans and they never tested positive for myxozoans in PCR. None of the actinosporeans detected were amplified in the PCR assay developed for *S. truttae*.

The overall infection prevalence of the 5304 worms kept in cell wells was 0.9% (2.2% excluding the Naididae) in the outflow pond and 1.3% in the river (no Naididae found). In contrast, the overall infection prevalence determined by PCR employing general myxozoan primers was 12.0% (300 specimens analysed). Prevalences of actinosporean release of all actinosporean types from their invertebrate hosts are given in Table 5.3.1.2.B, followed by a description of each type found in this study and measurements of the spores in comparison with measurements given in the literature.

Table 5.3.1.2.B Actinosporean types released from the oligochaetes of the investigated habitats. Infection prevalence in the host species as recorded in the original description and in this study, (O) in the outflow pond of the salmon hatchery and (R) in the river. * Prevalence data based on only 3 worms found.

Actinosporean	Host	Infection prevalence previously recorded	Infection prevalence in this study
***Aurantiactinomyxon* type 1** Özer 2002a	*Tubifex tubifex* L.	0.09%	0.3% (O)
***Aurantiactinomyxon* type 3** Özer 2002a	*Tubifex tubifex* L.	0.1%	0.2% (O)
Aurantiactinomyxon pavinsis ('petite form') Marques, 1984	*Stylodrilus heringianus* Claparède	0.02%	33.3% (R)*
***Echinactinomyxon* type 1** Negredo & Mulcahy 2001, Özer 2002a	*Lumbriculus variegatus* (Müller)	0.9% (Özer 2002a)	1.6% (O) 0.6% (R)
***Echinactinomyxon* type 5** Özer 2002a	*Lumbriculus variegatus* (Müller)	0.14%	0.3% (O)
Echinactinomyxon radiatum Janiszewska, 1957 (= E. type 4, Özer 2002a)	*Tubifex tubifex* L.	0.01% (Özer 2002a)	0.02% (O)
***Raabeia* type 1** Özer 2002a	*Tubifex tubifex* L.	0.007%	0.0005% (O)
***Raabeia* type 3** Özer 2002a	*Tubifex ignotus* (Stolc)	0.01%	0.0005% (O)
***Raabeia* type 4** Özer 2002a	*Tubifex tubifex* L.	0.41%	0.92% (O)
***Synactinomyxon* type 1** Özer 2002a	*Tubifex tubifex* L.	0.65%	2.0% (O)
Synactinomyxon longicauda Marques & Ormieres, 1982, (= S. type 3, Özer 2002a)	*Tubifex tubifex* L.	0.04% (Özer 2002a)	0.4% (O)
Neoactinomyxum eiseniellae Ormieres & Frezil, 1969	*Eiseniella tetraedra* (Savigny)	0.5-5%	0% (O) 3.1% (R)

Description of Actinosporean Types Encountered

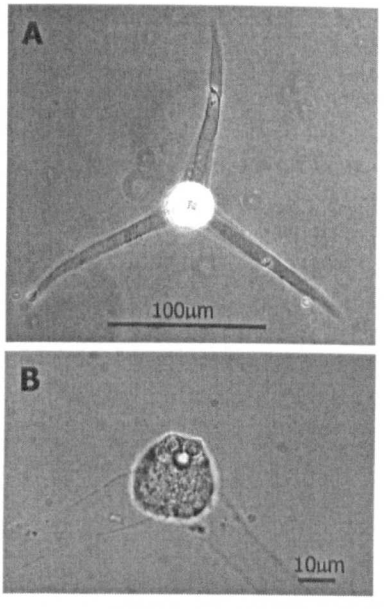


***Aurantiactinomyxon* type 1**

- **Host:** *Tubifex tubifex* L.
- **Reference:** Özer *et al.* 2002a
- **Description:** Single spores, 3 leaf-like processes of equal length with pointed ends, narrowing at the point of insertion into the spore body, spore body spherical, 3 polar capsules.
- **Spore measurements:**

	Özer *et al.* 2002a	this study
Diameter of spore body	14.4 (12-15) µm	14.2 (12.8-15.21) µm
Secondary cells	64-128	32
Length x width of polar capsules	2.7 (2-3) x 2.7 (2-3) µm	2.6 (2.1-3) x 2.5 (2.0-2.98) µm
Length of processes	32 (31-36) µm	33 (30.2-35.6) µm

***Aurantiactinomyxon* type 3**

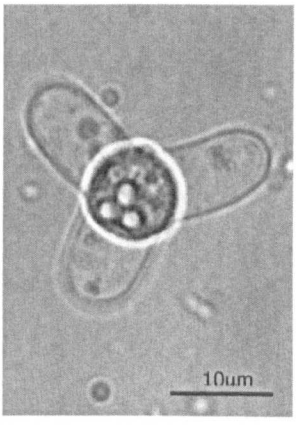


- **Host:** *Tubifex tubifex* L.
- **Reference:** Özer*et al.* 2002a
- **Description:** Single spores, 3 very elongated straight processes of equal length, pointed at the distal end, spore body spherical in apical view (A) but widened at the posterior end in lateral view (B), 3 polar capsules.

- **Spore measurements:**

	Özer *et al.* 2002a	this study
Length x width of spore body	24 (23.4-24.9) x 21.8 (20.3-23.4)µm	21.1 (20.3-22.6) x 19.3 (18.67-20) µm
Secondary cells	32	32
Length x width of polar capsules	4 x 3.2 µm	4 (3.4-4.5) x 3.5 (3.02-4.2) µm
Length of processes	114.5 (101.4-124.8) µm	114 (105-122.3) µm

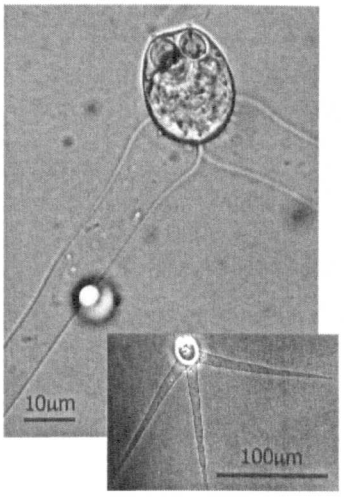
Aurantiactinomyxon pavinsis 'petite forme' Marques, 1984



- **Host:** *Stylodrilus heringianus* Claparède
- **Reference:** Marques 1984
- **Description:** Very small single spores, 3 leaf-like processes of equal length, pointed at the the distal end (visible only in lateral view), spore body spherical, 3 polar capsules.
- **Spore measurements:**

	Marques 1984	this study
Diameter of spore body	8 µm	9 (8.5-12.3) µm
Secondary cells	16	16
Length x width of polar capsules	-	2.8 (2.1-3.2) x 2.3 (1.7-2.81) µm
Length of processes	10-12 µm	10 (8.56-12.8) µm

***Echinactinomyxon* type 1**

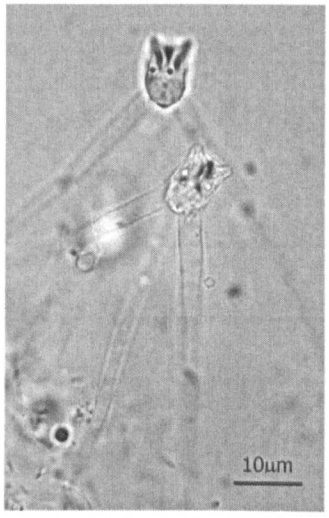


- **Host:** *Lumbriculus variegatus* (Müller)
- **Reference:** Negredo & Mulcahy 2001, Özer *et al.* 2002a
- **Description:** Single spores, typical barrel-shaped spore body, straight processes of equal length, widened at the point of attachment to the spore body, distally tapered to a point, 3 polar capsules.

- **Spore measurements:**

	Negredo & Mulcahy 2001	Özer *et al.* 2002a	this study
Length x	22.4 (18.2-23.4) x	22.4 (20.8-23.4)x	22.0 (20.9-23.5)x
Width of spore body	19.9 (14.7-20.8) µm	18.3 (18.2-19.7) µm	19.3 µm
Secondary cells	16	64	32
Length x	7.3 (5.7-7.8) x	7.8 x 5.8 µm	5.8 (5.3-6.1) x
Width of polar capsules	5.8 (5.2-6.5) µm		5.1 (4.7-5.77) µm
Length of processes	126 (117-143) µm	114.9 (103.9-124.7) µm	123 (115-138) µm

***Echinactinomyxon* type 5**



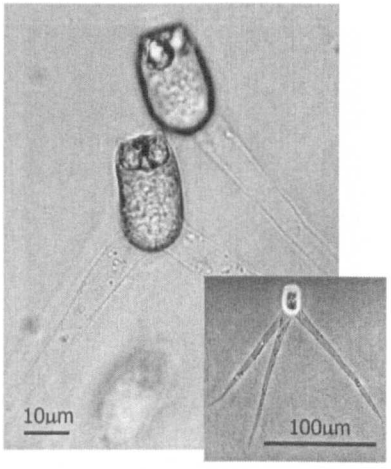
- **Host:** *Lumbriculus variegatus* (Müller)
- **Reference:** Özer & Wootten 2000, Özer *et al.* 2002a
- **Description:** Tiny individual spores, 3 straight elongated processes of equal length and with pointed ends, spore body elongated, 3 dominant pyriform and elongated polar capsules.
- Measurements:

	Özer *et al.* 2002a	this study
Length x	11.2 (10-12) x	11 (10.3-12) x
Width of spore body	8.1 (7-9) µm	8.1 (7.41-8.77) µm
Secondary cells	8	8
Length x width of polar capsules	5 x 2 µm	4.9 (4.43-5.21) x 2.2 (1.9-2.5) µm
Length of processes	58 (38-75) µm	60 (39.2-68.2) µm

***Echinactinomyxon radiatum* Janiszewska, 1957**

- **Host:** *Tubifex tubifex* L.
- **Reference:** Janiszewska 1957, Özer *et al.* 2002a (= E. type 4)

- **Description:** Single spores, elongated spore Body with straight, parallel sides, caudal processes thin and straight but curve slightly upwards distally, processes of equal length, pointed at the ends, 3 polar capsules.

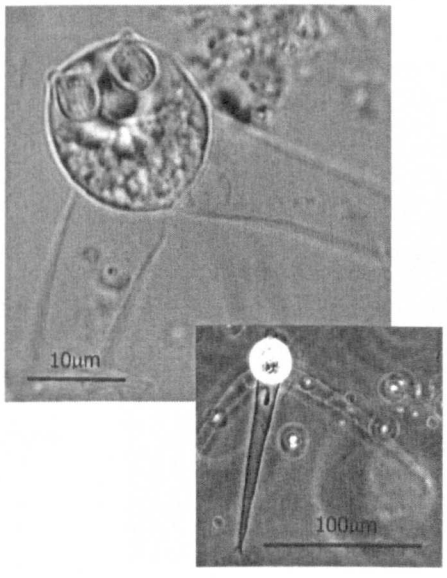


- **Spore measurements:**

	Janiszewska 1957	**Özer *et al.* 2002a**	**this study**
Length x	25-30 µm (length)	27.3 (24.9-28.4) x 15.5	26 (25.1-27.8) x
Width of spore body		(14-16.8) µm	15.2 (14.5-16) µm
Secondary cells	32	128	32
Length x	5 µm (length)	6.5 x	6.6 (6.1-7) x
Width of polar capsules		4.5 µm	4.7 (4.2-5.1) µm
Length of processes	100-125 µm	122.2 (106.8-135.7) µm	123 µm

***Raabeia* type 1**

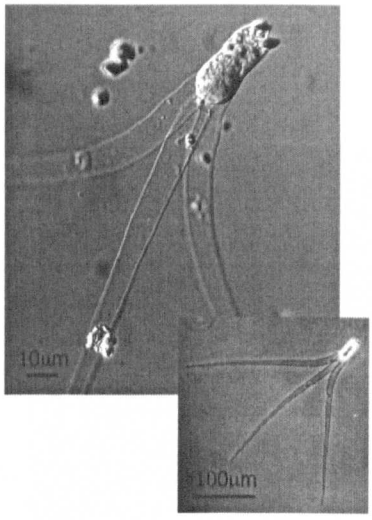
- **Host:** *Tubifex tubifex* L.
- **Reference:** Özer *et al.* 2002a
- **Description:** Individual spores, 3 straight processes of equal length, curving slightly towards inwards, 4 (sometimes only 2) small branches at each end, spore body spherical, 3 polar capsules.



- **Spore measurements:**

	Özer *et al.* 2002a	**this study**
Length x	18.1 (16-19) x	17.7 (16.1-18.89) x
Width of spore body	15.7 (14-18) µm	15.0 (10.1-16.2) µm
Secondary cells	-	32
Length x width of polar capsules	5 x 4 µm	7 (6.4-7.6) x 5.5 (5.0-5.89) µm
Length of processes	94.5 (85-103) µm	84 (82-99.2) µm

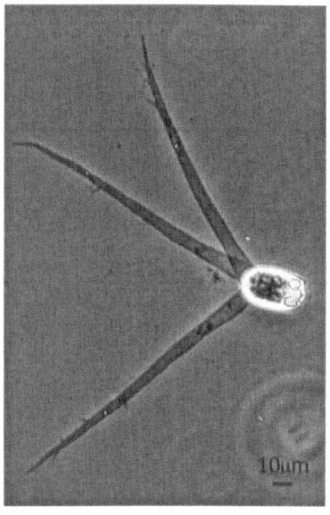
Raabeia type 3



- **Host:** *Tubifex ignotus* (Stolc)
- **Reference:** Özer *et al.* 2002a
- **Description:** Single spores, elongated spore body, processes run almost parallel to each other before they curve upwards approximately 20 µm below the spore body, processes of equal length, rounded at the ends, 3 polar capsules.
- **Spore measurements:**

	Özer *et al.* 2002a	this study
Length x	33.9 (31.3-37.5) x	32.6 (31.5-35.1) x
width of spore body	12.8 (12.5-14.0) µm	12.2 (11.8-13) µm
Secondary cells	16	32
Length x width of polar capsules	6.4 x 4.3 µm	6.3 (5.8-6.7) x 4.2 (3.9-4.45) µm
Length of processes	228.3 (212.5-243.8) µm	230 µm

Raabeia type 4

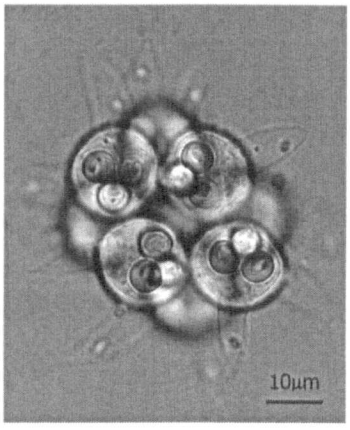


- **Host:** *Tubifex tubifex* L.
- **Reference:** Özer *et al.* 2002a
- **Description:** Individual spores, 3 straight elongated processes of equal length and with fine branches on the distal two third of the processes, pointed ends, spore body ovoid, 3 polar capsules.
- **Spore measurements:**

	Özer *et al.* 2002a	this study
Length x	29.6 (28.3-31.2) x	23.7 (22.9-25) x
width of spore body	16.5 (14.8-18.2) µm	20.1 (18.9-22.1) µm
Secondary cells	32	32
Length x width of polar capsules	8 x 5 µm	6.3 (5.82-6.7) x 6.4 (5.9-6.8) µm
Length of processes	142.7 (125-164) µm	144 (130-162.2) µm

***Synactinomyxon* type 1**

- **Host:** *Tubifex tubifex* L.
- **Reference:** Özer *et al.* 2002a
- **Description:** 8 spores arranged in a star-like structure, spherical spore body, one short and one longer process per spore with the shorter ones linked to form a syn-organism, processes distally rounded, 3 large polar capsules.

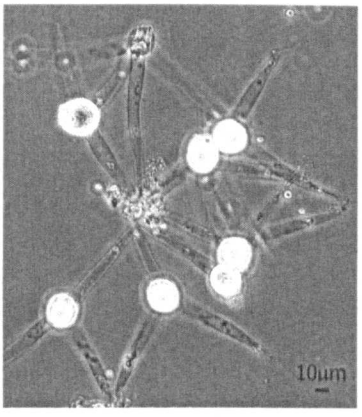


- **Spore measurements:**

	Özer *et al.* 2002a	this study
Diameter of spore body	17 (14.0-19.2) µm	16.4 (14.6-18.81) µm
Secondary cells	32	32
Length x width of polar capsules	5 x 4 µm	5.8 (5.1-6.2) x 4.4 (3.8-4.5) µm
Length of short/long process	5.3 (3.2-7.4)/18 (15.0-21) µm	5.4 (3.9-7)/18.1 (16.7-19) µm

Synactinomyxon longicauda Marques & Ormieres, 1982

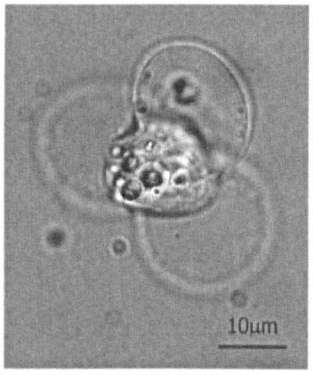
- **Host:** *Tubifex tubifex* L.
- **Reference:** Marques 1984, Özer *et al.* 2002a (=type 3)
- **Description:** 8 spores of echinactinomyxon type joined at the distal end of one of the three processes of each spore; processes of equal length, widened proximal to the spore body, spore body subspherical, 3 polar capsules.



- **Spore measurements:**

	Marques 1984	Özer *et al.* 2002a	this study
Length x width of spore body	22-25 µm	25.6 (21.8-26.5) x 21.8 (18.7-23.4) µm	25.9 (24.2-26.8) x 22 (21-22.7)µm
Secondary cells	16	16	16
Length x width of polar capsules	7 µm (length)	5 x 4 µm	5.1 x 3.9 µm
Length of processes	80 µm	74 (71.8-78) µm	76 (70.4-77.9) µm

Neoactinomyxum eiseniellae (Ormieres & Frezil, 1969)



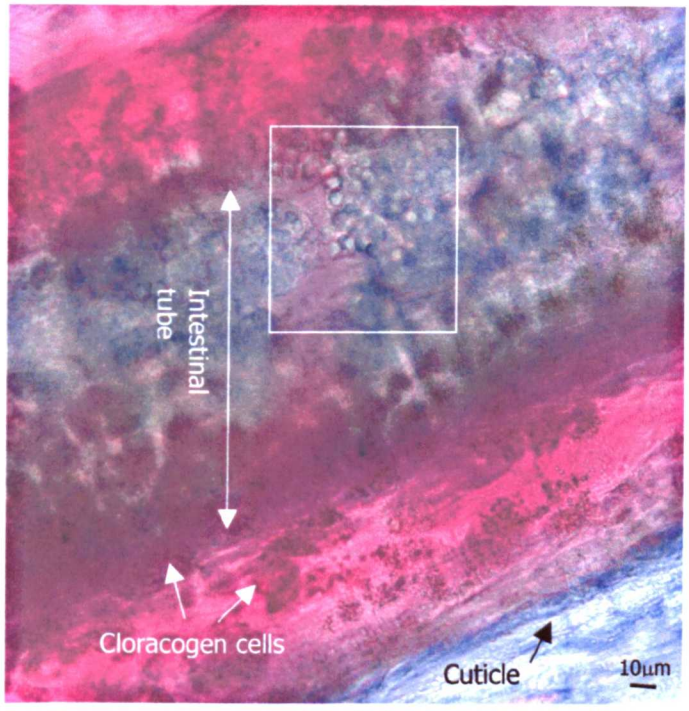
- **Host:** *Eiseniella tetraedra* (Savigny)
- **Reference:** Marques 1984
- **Description:** Small individual spores, 3 leaf-like, round processes, subspherical spore body, 3 small polar capsules.
- **Spore measurements:**

	Marques 1984	**this study**
Diameter of spore body	Sporoplasm 6-8 µm	14.3 (12.2-15) µm
Secondary cells	32	32
Diameter of polar capsules	2.5 µm	2.5 (2-2.97) µm
Diameter of processes	11-14 µm	12 (10.6-13) µm

Observation of Myxozoan Infections in Oligochaetes

Observation of anaesthetised infected oligochaetes under the microscope showed that the different actinosporean types all developed within the gut epithelium of the worms. Infected worms exhibited spherical pansporocysts within which 8 spores were found to develop (Figure 5.3.1.2.A). Most infected oligochaetes harboured pansporocysts in the anterior third of the body but in heavily infected specimens maturing actinosporean stages were present along the whole intestine.

Individual oligochaetes were found to release spores for a period of 6-13 days. Thereafter, spherical developmental stages were sometimes still visible in the oligochaetes but spore development was no longer observed.



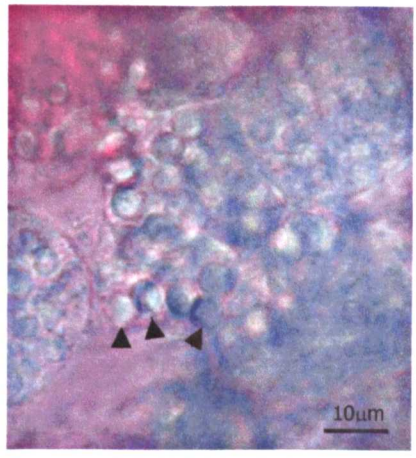


Figure 5.3.1.2.A Actinosporean infection in *Tubifex tubifex.* Left: pansporocysts of *Synactinomyxon* type 1 localised in the gut epithelium. Right: magnification of one pansporocyst with developing spores (arrows indicate polar capsules).

Other Invertebrates

In contrast to the outflow pond of the hatchery, which was dominated by oligochaetes, the sediments of the river were dominated by insect larvae. A total of 296 subadult insects belonging to 5 different orders and 14 different families (Table 5.3.1.2.C) were sampled in the river system and observed under the microscope before their examination by PCR.

During the microscopical examination, large cysts were found in the coelom and the gut of 4 tipulids and 5 chironomids. In squash preparations these cysts were identified as xenomas filled with microsporidian spores. Another chironomid specimen harboured a parasitic nematode in its coelom. Myxozoan pansporocysts as seen in infected oligochaetes were not detected in any of the insect larvae.

Table 5.3.1.2.C Insect larvae collected in the river system.

Order	Family	No. of individuals
Coleoptera	Gyrinidae	8
Coleoptera	Elmidae	12
Coleoptera	Staphylinidae	21
Diptera	Chironomidae	56
Diptera	Culicidae	8
Diptera	Empididae	21
Diptera	Tipulidae	32
Ephemeroptera	Baetidae	30
Plecoptera	Leuctridae	15
Plecoptera	Nemouridae	23
Plecoptera	Perlidae	14
Plecoptera	Perlodidae	16
Trichoptera	Polycentropodidae	19
Trichoptera	Sericostomatidae	21

Of the 296 insect larvae tested for the presence of myxozoans by PCR, two individual chironomids were found to be PCR positive (Figure 5.3.1.2.B). The myxozoans within these chironomids were identified as *Sphaerospora truttae* and *Chloromyxum* sp. by specific PCR (*S. truttae*) and DNA sequencing (both species). The chironomids harbouring the myxozoans had been halved before submission to DNA extraction and PCR analysis, and the second half of the body was used to produce serial histological sections for ISH. Additionally, serial sections of 30 whole chironomids collected in the outflow pond of the farm were submitted to ISH. Neither the 30 whole chironomids nor the two PCR positive specimens were found to contain myxozoan developmental stages when screened using ISH. However, in the histological sections, the intestines of all chironomids were found to contain large amounts of food, despite the 10 day dwell time in water in the cell-well plates prior to processing for PCR.

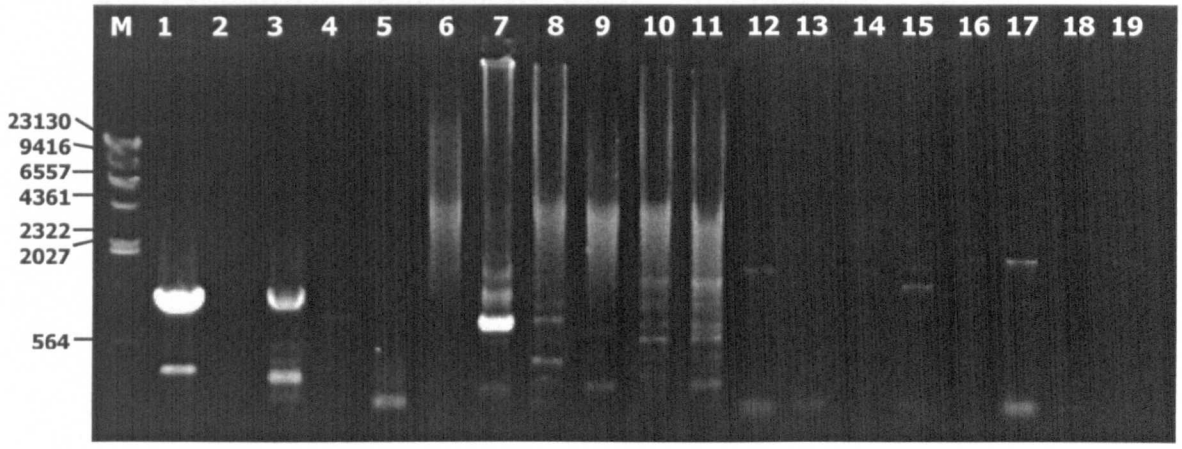


Figure 5.3.1.2.B Nested, myxozoan-specific PCR of some insect larvae collected in the river system. **M**=Marker showing fragment sizes (in basepairs); **1** *S. truttae* positive control (infected salmon kidney); **2-8** Chironomidae with **3** positive for *S. truttae* (same amplicon size as control) and **7** positive for *Chloromyxum* sp. (slightly smaller amplicon); **9-11** Baetidae; **12-19** Tipulidae.

5.3.1.3 River Water Analysis

Microscopic examinations of the water samples showed that large numbers of algae were present, especially in the bottom layer of the water samples which had been left to settle. Using microscopy, actinosporean spores were not detected in the water samples. Using PCR, it was impossible to amplify 18S rDNA from the water samples, even when they were diluted 1:10, 1:100 and 1:1000 in order to dilute PCR inhibitors present. None of the samples spiked with *Echinactinomyxon* type 1 spores produced the expected amplicon in the myxozoan specific PCR reaction.

5.3.2 Culture of Invertebrate Hosts and Exposure to Sphaerospora truttae

5.3.2.1 Oligoculture

All four oligochaete culture groups (mixed population, tubificids, naidids and *Lumbriculus variegatus*) were successfully maintained under laboratory conditions. Figure 5.3.2.1.A shows the change in biomass in cultures of the natural mixed population, the tubuficids and *Lumbriculus variegatus* at three different temperatures (10°C, 15°C and 20°C) over an 80 day period, starting with a biomass of 10 g each.

The optimal temperature for maximum population growth in the culture groups differed considerably although all oligochaetes share the same habitat. The tubificids showed the highest population growth rate at relatively high temperatures (20°C) with a doubling of their biomass over an 80 day period. In contrast, *Lumbriculus variegatus* preferred temperatures around 10-15 degrees (70-80% increase of biomass) and did not tolerate higher temperatures. At 20°C, the biomass of the *Lumbriculus variegatus* population was shown to decrease by 40%. The natural, mixed population was shown to increase its density best at 15 °C, resulting in an 85% increase over an 80 day period.

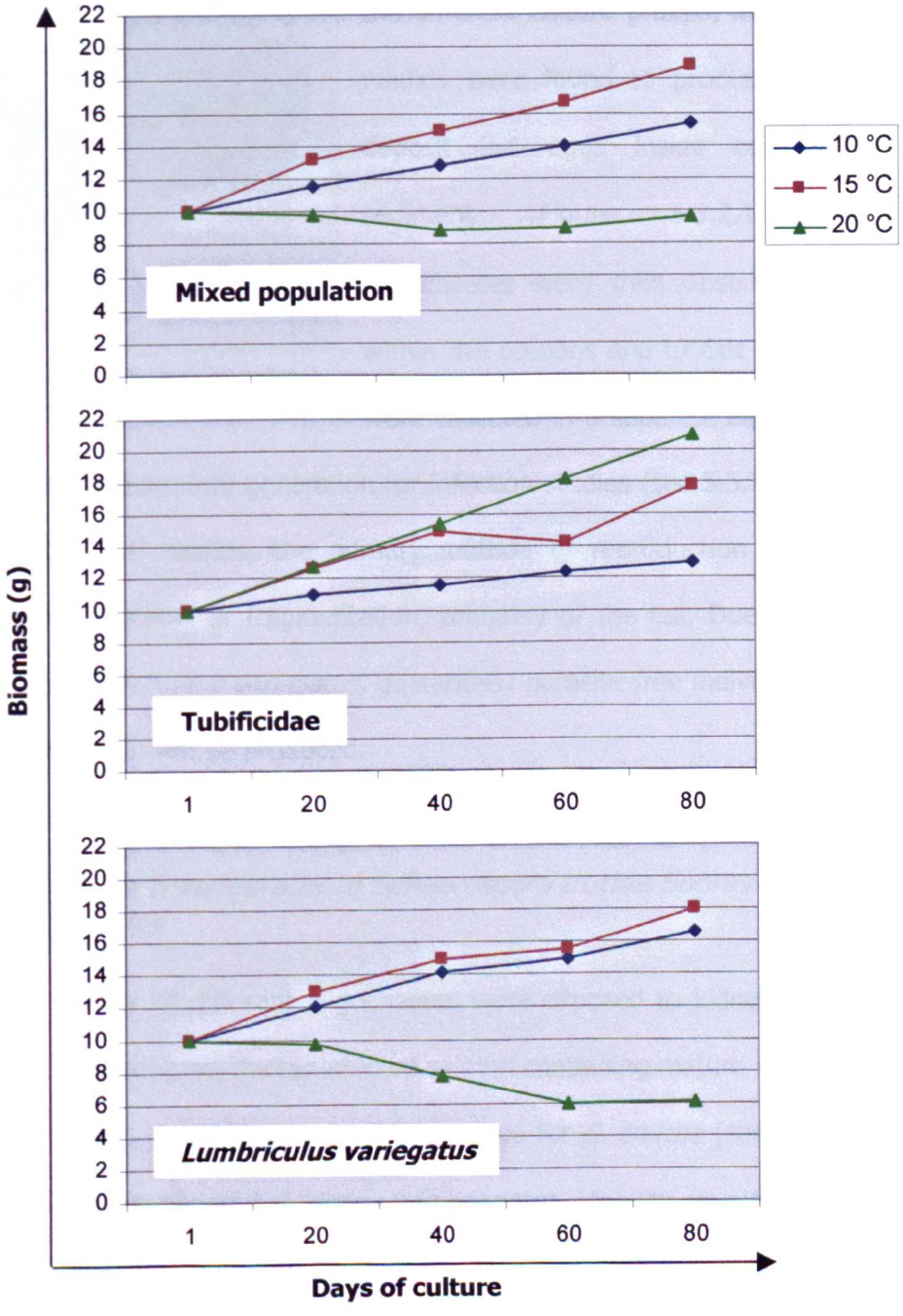


Figure 5.3.2.1.A Changes in biomass of different oligochaete populations kept at three different temperatures under laboratory conditions.

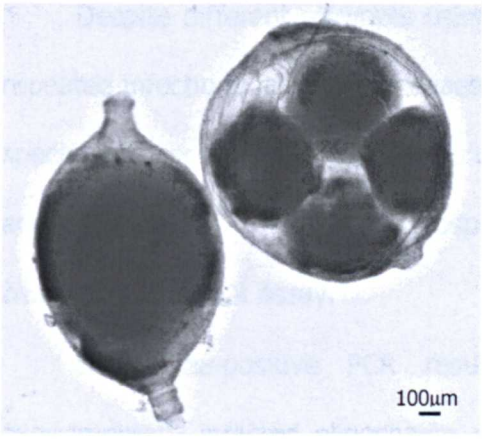


Figure 5.3.2.1.B Cocoons containing eggs found in the culture of tubificids.

During the microscopical observation of the different culture groups, the tubificids and naidids were found to produce eggs and to deposit 2-4 eggs inside cocoons in the sediment (Figure 5.3.2.1.B). Juvenile oligochaetes were then observed to develop within the cocoons and to exit them. The eggs were collected in a separate aquarium in order to breed a new, parasite-free generation for infection studies (see 5.3.2.2). In contrast to the tubificids and naidids, the primary method of reproduction in *Lumbriculus variegatus* was architomy or fragmentation, primarily of the tail. Due to the asexual reproduction of *Lumbriculus variegatus,* guaranteed parasite-free individuals for use in infection studies could not be produced.

5.3.2.2 Exposure of Invertebrates to Sphaerospora truttae Spores From Kidney Homogenates

A large number of different oligochaetes were exposed to kidney homogenates obtained from *Sphaerospora truttae* infected salmon containing mature spores. Because a chironomid species was found to be PCR positive for *S. truttae* (see 5.3.1.2) some chironomids were also exposed to kidney homogenates. Viability staining of the kidney homogenates showed that an average of 84±6% of the spores used for the infection were viable. The compact appearance of their valves and the fully differentiated polar capsules also indicated that most of them were mature.

Despite different attempts using two types of substrate, different temperatures, repeated infection, various oligochaete species and the use of adults as well as juvenile specimens, none of the oligochaetes was found to develop an increased number of one actinosporean type, and no actinosporeans were produced which amplified in the *S. truttae* specific PCR assay.

S. truttae-positive PCR results were repeatedly found to occur in all experimentally infected oligochaete groups if DNA was extracted from worms which were directly taken from the infectious sediment and not kept in de-chlorinated tap water for a week in order to empty the gut contents. However, in the latter case, none of the oligochaetes was found to be PCR positive for *S. truttae.*

In experimental and control aquaria, tubificids released spores of *Synactinomyxon* type 1, *Synactinomyxon longicauda, Aurantiactinomyxon* type 1, *Aurantiactinomyxon* type 3 and *Raabeia* type 4. Only the juvenile tubificids which had been separated from the adults did not release actinosporeans. *Lumbriculus variegatus* produced *Echinactinomyxon* type 1 and *Echinactinomyxon* type 5 in experimental and control tanks. Neither adults nor juveniles of the naidid family released actinosporeans. Although not quantified, the number of actinosporeans in the water was very low in the 8°C aquaria when compared with those kept at 10°C, 15°C and 20°C.

The chironomids exposed to *S. truttae* kidney homogenates did not release actinosporean stages and hybridisation signals were not detected in any of the 50 chironomids examined using the *S. truttae* specific ISH protocol.

5.3.2.3 Exposure of Invertebrates to Sphaerospora truttae-Infected Atlantic Salmon by Co-Habitation

The oligochaetes co-habited for two months with *Sphaerospora truttae*-shedding salmon smolts released the same actinosporean types (*Synactinomyxon* type 1, *Synactinomyxon longicauda, Aurantiactinomyxon* type 1, *Aurantiactinomyxon* type 3, *Raabeia* type 4, *Echinactinomyxon* type 1 and *Echinactinomyxon* type 5) as those in the control tank, which did not hold fish. None of the 650 oligochaetes from the co-habitation tank or the control tank tested positive for *S. truttae* using the specific PCR assay when checked over the 3 month period.

5.3.3 Exposure of Atlantic Salmon to Actinosporeans and Sphaerospora truttae Myxosporean Spores

5.3.3.1 Exposure to Actinosporean Spores

Atlantic salmon fry were exposed to five different actinosporean types, i.e. *Echinactinomyxon* type 1, *Echinactinomyxon* type 5, *Raabeia* type 4, *Synactinomyon* type 1 and *Synactinomyxon* type 3. Skin and gills of the infected fish collected 1, 3, 6, 10 and 20 min after exposure were not found to contain any sporoplasms when examined histologically. Furthermore, actinosporeans were not found attached to the skin or the gills when examined using SEM. The examination of squash preparations of the different organs, 3 months after initial exposure to the actinosporeans, showed that 100% of all experimentally infected fry and 100% of the control group were infected with *Chloromyxum* sp. Within the renal tubules, sporogonic stages and mature spores as well as yellow pigment, typically generated in relation to this specific myxozoan infection was found.

In the second experiment, where fins and gills were exposed to the actinosporeans, spores were readily visible in the mucus on the surface of the organs, when observed microscopically immediately after bathing in the concentrated spore solution. However, after fixation for SEM and histology the actinosporeans were no longer visible on the organ surfaces and sporoplasms were not detected in histological sections.

All spores reacted to the exposure to salmon mucus by extrusion of polar filaments, and the fastest release of polar filaments was observed in *Synactinomyxon* type 1.

5.3.3.2 Co-Habitation of Atlantic Salmon with Sediment Containing Various Invertebrates

Co-habitation of Atlantic salmon fry from Loch Fyne with sediments collected in the outflow pond of the fish farm on the North coast resulted in 100% infection with *S. truttae* in the fry in 2001 (see Chapter 4, Section 4.3.3). However, when the experiment was repeated in 2003, none of the salmon tested positive for *S. truttae* using PCR and ISH.

5.3.3.3 Exposure of Atlantic Salmon to Sphaerospora truttae Myxosporean Spores

Atlantic salmon fry from Loch Fyne were experimentally fed with, and exposed to ,homogenised *S. truttae* infected kidneys. These fish did not develop an *S. truttae* infection 2 and 3 months after exposure and were free of myxosporean infections in all organs when examined using smear preparations.

5.4 Discussion

5.4.1 The Community of Myxosporeans and Actinosporeans in a Scottish Highland River Habitat

Data on communities of actinosporeans and myxosporeans in the same habitat are rare. Most of these studies focus on ponds where myxosporeans are pathogenic to cultured fish species (Burtle, Harrison & Styer 1991, Pote & Waterstrat 1993, Yokoyama *et al.* 1993b, Grossheider & Körting 1993, Oumouna *et al.* 2003), and they do not take into consideration the involvement of myxozoan infections in the natural river system from which the inflow water originates. Only a small number of papers report the myxozoan fauna in natural habitats. Four publications from a lacustrine habitat in the Algonquin Park in Ontario, Canada provide information about the actinosporeans (Xiao & Desser 1998a, Xiao & Desser 1998b, Xiao & Desser 1998c) as well as the myxosporeans of the genus *Myxobolus* (Salim & Desser 2000). Negredo & Mulcahy (2001) described the actinosporean fauna in the Irish Cloonee river system and O'Flynn (1998) reported the myxosporeans occurring in the same water.

The present synopsis of vertebrate and invertebrate hosts and their myxozoan infections in a Scottish highland river is unique and is a substantial contribution to our understanding of the ecology of myxosporean and actinosporean types in such a habitat. Furthermore, the comparison with the morphotypes detected in this study with those from the Clonee river and from the lacustrine habitat in Canada might offer some clues about the functionality of the design of the different myxozoan life cycle stages found in the different habitats.

The river system on the North coast of Scotland is typical for the Scottish Highlands and is characterised by acidic and oligotrophic water. Despite the oligotrophy

and the sparse populations of oligochaetes, the four fish species present in the habitat harboured 10 different myxosporean species. Assuming that all myxosporeans have an alternate actinosporean stage and thus an alternate host, the system based on the propagation of spores in the water column shows a remarkable success rate regarding the infection of the specific vertebrate host. Due to the heavy proliferation observed in the *Sphaerospora truttae* infection in the Atlantic salmon (Chapter 4, Section 4.3.3) it is assumed that, in some species, possibly only one or very few actinosporean spores have to enter the vertebrate host in order to produce masses of myxosporean spores. Similarly, catfish *Ictalurus punctatus* exposed to small numbers of the aurantiactinomyxon stage of *Henneguya ictaluri* have been reported to develop a massive infection resulting in clinical proliferative gill disease (Pote, personal communication).

In contrast to the myxosporean fauna described from the lake in Canada (Salim & Desser 2000) which is dominated by histozoic *Myxobolus* species, the myxosporeans found in the present riverine habitat were, with the exception of *Myxobolus portucalensis* and to a certain extent *Myxidium giardi*, exclusively coelozoic. Apart from two myxosporeans infecting the gall bladder epithelium, most of the myxosporean species detected in the present study inhabit the excretory epithelia of the urinary system, i.e sporogony takes place in the epithelium of the urinary bladder or attached to the epithelium of the renal tubules. The epithelia of the gall bladder and the excretory system, provide a microvilli-rich surface suitable for the attachment of developmental myxosporean stages. In the urinary system, the preferred localisation in eels was the bladder whereas in salmonids it seemed to be the renal tubules. It is unclear if myxozoan parasites have the ability to differentiate between tubular and

bladder epithelium or if the different localisation might simply be ascribed to the considerably smaller number of tubules (and thus surface available for attachment) detected in the kidney smears from the eels in comparison with those obtained from the salmonids. However, kidney tubules from all eels were free from myxosporean sporogonic stages.

Myxidium giardi differs from all other myxozoans found in the urinary system due to its location, which is not the excretory epithelium, but probably connective tissue areas in the kidney, and the development of trophozoites (cysts) instead of pansporoblasts. Although the kidney was identified as the primary location in the infected eels, skin, gills, and viscera are known host organs of *M giardi*, and the species can also be coelozoic in the gall bladder and the urinary tract (Lom & Dyková 1992, Copland 1981, Copland 1983). The use of connective tissue as target organ would explain the potential of the parasite to sporogenate in other locations than the kidney, as connective tissue is readily available in most organs. However, *M. giardi* seems to primarily infect the kidney as confirmed by Benajiba & Marques (1993).

Myxozoan parasites infecting the urinary system and the gall bladder have so far been found to develop mainly into actinosporean stages which lack long floating appendages (aurantiactinomyxon and neoactinomyxum types, see Table 5.1.1). The occurrence of these small spore stages might be related to a riverine habitat which provides a relatively strong water current preventing spores devoid of large floating appendages from settling on the bottom. Xiao & Desser (1998a) found 23 of 25 actinosporean forms to have large floating appendages in the lacustrine habitat investigated in Canada, which supports this hypothesis. Furthermore, Salim & Desser (2000) found the myxosporean fauna in the investigated lake to be represented by a

large number of *Myxobolus* species, and *Myxobolus* species have so far been related exclusively to large actinosporean stages of the triactinomyxon and raabeia types (see Table 5.1.1.). Negredo & Mulcahy (2001), Szekely, Urawa & Yokoyama (2002b), Özer *et al.* (2002a) and the present study investigated relatively fast flowing riverine habitats and identified small as well as large actinosporean stages. However, it is hypothesised that, for the myxosporeans present in the habitat studied here, the small actinosporeans found are more likely to be involved in their life cycles.

The Scottish highland river habitat is very similar in its oligotrophy, latitude and geography to the Irish Cloonee River system in Ireland. The Cloonee contains a similar fish fauna consisting of eels, brown trout, salmon and minnows, and two myxosporeans (*M. giardi* and *Chloromyxum* sp.) found in this study, were also present in the Irish system together with *Myxidium rhodei* and *Myxobolus* sp. (O'Flynn 1998). The oligochaete fauna in the Cloonee (Negredo & Mulcahy 2001) differs to some degree, but overlaps in species constitution with the present study. As a result, similarities can also be found in the actinosporean fauna: Similar dimensions of the actinosporean types indicate that *Echinactinomyxon* type 1 of Negredo & Mulcahy (2001) is identical with *Echinactinomyxon* type 1 from this study. *Echinactinomyxon radiatum,* which was originally described from the river Oder in Poland (Janiszewska 1957) was found in both studies and was also found in the lacustrine Canadian habitat (Xiao & Desser 1998b) and a trout farm in Germany (Oumouna *et al.* 2003). This type, together with *Neoactinomyxum eiseniellae, Synactinomyxon longicauda* and *Aurantiactinomyxon pavinsis,* which were found in rivers in France and Germany (Ormieres & Frezil 1969, Marques & Ormieres 1982, Marques 1984, Oumouna *et al.* 2003) appear to have a wider geographic distribution.

Özer *et al.* (2002a) found 21 actinosporean types in the outflow pond of the investigated farm on the North coast of Scotland. Only 10 of these types were detected in the course of the present study, possibly for the following reasons: apart from two types, all the actinosporeans found by Özer, but not in this study, occurred with low prevalences (0.001-0.05%) in the oligochaete hosts. Furthermore, the release of most types has been related to a seasonal increase in water temperature (Özer *et al.* 2002b). There might be a very narrow window of release of some species and the sampling in this study, unlike that of Özer, was not done continuously every month. In the case of *Myxobolus cerebralis* for example, the optimal triactinomyxon release temperature has been reported between to be 10°C and 15°C, whereas at lower temperature, minimal releases were detected and at higher temperatures the production of actinosporeans was arrested completely (Hedrick & El-Matbouli 2002). Additionally, the identification of actinosporean types according to the descriptions of Özer (1999, 2002b) was not always conclusive. There are differences between the photographs taken and the drawings produced for some actinosporean types, probably due to the difficulties related to projecting the three-dimensional structure of the spore onto a two-dimensional illustration. Furthermore, the number of secondary cells is difficult to count and the numbers obtained in this study did not always match Özer's description but were usually in accordance with other available descriptions (Marques 1984, Janiszewska 1957). However, the consistency of secondary cell number has not been assessed so far.

In the river feeding the fish farm investigated, two additonal actinosporean types, *Aurantiactinomyxon pavinsis* and *Neoactinomyxum eiseniellae* were found, which were not described by Özer (2002a), and were found to occur exclusively in the river. Their

hosts, the lumbriculid *Stylodrilus heringianus* and the earthworm *Eiseniella tetraedra* were absent or occurred in considerably lower numbers in the outflow pond of the farm than in the river. According to Brinkhurst (1971) *Stylodrilus heringianus* is very susceptible to organic pollution and this species might therefore avoid the eutrophic sediment of the outflow pond.

Individual prevalences of the actinosporean infections were an average of 34% higher than in the study conducted by Özer (1999, 2002b) for all spore types except two very rare types, i.e. *Raabeia* type 1 and *Raabeia* type 3, which were only released by one individual worm. The detection of generally higher individual prevalences may be ascribed to the method used in this study, namely, while Özer (1999, 2002b) determined the infection of an oligochaete specimen at a given moment in time, in the present study worms were monitored for 7-10 days. Negredo and Mulcahy (2001) kept their oligochaetes for approximately 2 months in cell well plates and found prevalences between 0.8 and 23.8% for different actinosporean types. Thus it can be concluded that longer monitoring periods improve actinosporean recovery. The overall prevalence observed in the outflow pond in this study was 0.9%, considerably lower than observed previously (2.9%, Özer *et al.* 2002b). This is explained by the oligochaete species composition found. Özer (1999) found the Naididae to represent only 0.8% of the oligochaete population in contrast to 53% in the current study. By exclusion of this family of oligochaetes from the analysis, the overall prevalence is 2.2% and thus similar to Özer's data (2.9%). It was 0.9% higher than in the natural river habitat (1.3%). Similar low prevalences of actinosporean infection in the wild have been found previously (usually 1-3%, Lom *et al.* 1997). However, using PCR, the actinosporean infection prevalence determined was more than 5 times higher (12%) than estimated

by the cell well method. This shows that due to to the presence of cryptic stages, the true prevalence of actinosporean infections in their oligochaete hosts is probably always underestimated. As most actinosporeans require developmental times of at least 90 days (see Table 5.1.2) the worms would have to be monitored over a period of at least three months in order to obtain a more accurate estimation of infection using the cell well method. Additionally, other factors, e.g. temperature, rearing substate and diel light cycle can influence the release of actinosporeans, as shown in the case of *Myxobolus cerebralis* (Arndt, Wagner, Cannon & Smith 2002).

5.4.2 Experimental Infection Studies and Specific PCR Assay to Determine the Involvement of Invertebrates and Their Actinosporeans in the Life Cycle of Sphaerospora truttae

In order to determine if the oligochaetes in the habitat investigated are involved in the life cycle of *Sphaerospora truttae*, cultures were set up to produce a new, myxozoan-free generation of the oligochaetes occurring in the habitat. Although an increase in biomass due to reproduction was possible in all oligochaete cultures established, only the tubificids and the naidids were found to reproduce sexually. In contrast to their parents, the new generation of tubificids was found to be free from infection by actinosporeans, suggesting that infections are not transferred from one generation to the next. The generation of worms collected in the natural habitat was found to release spores for more than one year. Gilbert & Granath (2001) reported that infections can persist for a lifetime, which is a highly variable timespan, depending on the oligochaete species from 70 days (Poddubnaya 1984) to 4 to 6 years (Matsumoto & Jammoto 1966). As a result, actinosporeans were frequently found in the aquaria

where oligochaetes had been experimentally infected with homogenised kidney tissue harbouring *S. truttae* myxosporeans and also in their control groups. An increased production of actinosporeans in experimental infections is common (see Table 5.1.2) but was not observed in any of the trials conducted in this study, despite various experiments using different oligochaete species, soil types and temperatures. It was shown that 84.6% of the myxosporean spores used for the infection studies were viable and it is assumed that a large percentage of these spores were mature and infective, especially when spores were added to the oligochaete cultures repeatedly over a long period. It is furthermore assumed that the unselective uptake of *S. truttae* myxosporean spores by the oligochaetes was successful as they proved to be PCR positive if taken from the infectious sediment, rinsed and used for DNA extraction. However, an infection did not develop within these worms as shown by the unsucessful amplification of *S. truttae* by PCR after the oligochaetes had been kept in de-chlorinated tap water for a week in order to empty their gut contents. It thus may be concluded that none of the oligochaete species used for the infection studies represents the natural alternate host of *S. truttae.* However, the presence of an obligate alternate host is assumed, as direct transmission was not successful in this or a previous study (McGeorge 1994). Of all other invertebrates found to share the sedimentary habitat with the oligochaetes, only 2 individual chironomid larvae tested PCR positive for myxozoans. *S. truttae* and *Chloromyxum* sp. were sequenced from these chironomids, but using the *S. truttae* specific ISH an infection could not be detected in serial sections of the PCR positive specimen, or of numerous other chironomids from the river or after experimental infection of chironomid larvae. Due to the presence of organic matter in the guts of all chironomids sectioned, it is suggested that the myxozoans sequenced

from these larvae do not develop within the chironomids but were present in the gut as a result of the uptake and the retention of food particles together with myxosporean spores or were trapped in cryptic spaces, e.g. joints of the exoskelton of these invertebrates.

In the infection studies, Atlantic salmon fry were exposed to 4 actinosporean spore types with long floating appendages (*Echinactinomyxon* type 1, *Echinactinomyon* type 5, *Raabeia* type 4, *Synactinomyxon* type 3) and one small type (*Synactinomyxon* type 1). Since all spore types reacted to salmon mucus by the extrusion of their polar filaments it is suggested that this process represents a relatively unspecific reaction to the low pH, acidic mucus. Polar capsule release has been shown to be triggered artificially by various chemicals and, invariably, by a saturated aqueous solution of urea (Lom & Dyková 1992). Thus, attachment to the exposed epithelia of the salmon fry is assumed for all actinosporean types used in the infection studies, however, the entry of sporoplasms might be a more specific process as it was not observed in this study. It is thus assumed that none of the spore types used in the infection studies represent alternate life cycle stages of the myxosporeans found in the Atlantic salmon in this study.

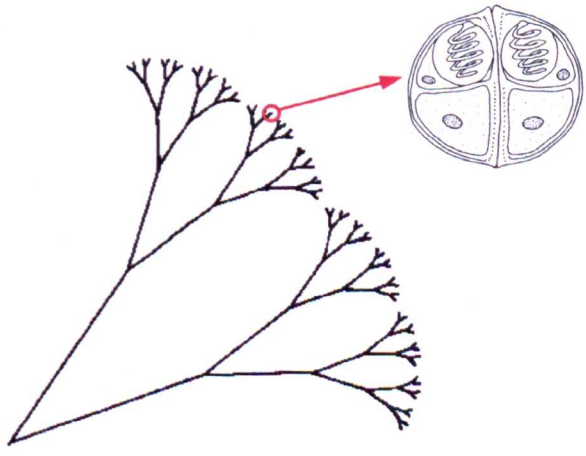
In the infection studies, all Atlantic salmon fry from the site near Stirling were found to be infected with *Chloromyxum* sp. in the kidney. No fry obtained from the site at loch Fyne developed myxosporean infections. The *Chloromyxum* sp. infection was found in experimental as well as control fry, possibly a result of infective actinosporean stages entering the hatchery environment via the incoming water. It is unclear if the established infection and its challenge to the immune system of these fry had any influence on the entry of other actinosporean stages infective to salmon. As a result,

based on the infection studies only, the involvement of *Echinactinomyxon* type 1, *Echinactinomyon* type 5, *Raabeia* type 4, *Synactinomyxon* type 3 or *Synactinomyxon* type 1 in the life cycle of the myxosporean parasites detected in the Atlantic salmon cannot be excluded with certainty.

Although none of the oligochaetes and actinosporeans found in this study tested positive for an *S. truttae* infection using PCR, Atlantic salmon were successfully infected with *S. truttae* by co-habitation with sediments from the outflow pond of the fish farm on the North coast in 2001, without being able to repeat the experiment in 2003. This strongly suggests that an alternate invertebrate host for *S. truttae* exists, but it might occur at low densities or in a specific microhabitat which was present in the co-habitation tank in 2001 but not in 2003. It is suggested that different ecological niches, e.g. rock surfaces or pieces of wood are included in future studies aiming at the identification of the alternate host of *S. truttae.* Although it was impossible to determine the actinosporean stage of *S. truttae*, some of the 12 actinosporeans found might represent alternate life cycle stages for the 9 other myxosporeans identified in the habitat.

CHAPTER 6

Molecular Phylogeny



6 Molecular Phylogeny

6.1 Introduction

6.1.1 Phylogenetic Placement and Origins of the Myxozoa

Despite their multicellular character, the Myxozoa were generally considered a protist taxon until the 1990s. Stolc (1899) was the first to claim that the Myxozoa should be ascribed to the Metazoa due to their multicellularity. Weill (1938) reaffirmed this hypothesis and suggested a close relationship to the cnidarians due to the identical discharge properties of myxozoan polar capsules and cnidarian nematocysts. Furthermore, remarkable similarities in the development of the narcomedusan *Polypodium hydriforme*, a cnidarian parasite of sturgeon, and the myxozoans in fish led Weill (1938) to suggest that they were closely related. When molecular sequence data became available it was shown that the myxozoans are closely related to the cnidarians, as a sister group to *P. hydriforme* (Siddall *et al.* 1995, Siddall & Whiting 1999), precisely as predicted by Weill (1938). The molecular data were substantially supported by ultrastructural studies which proved metazoan affinities by the presence of desmosomes, tight junctions and collagen production (Siddall *et al.* 1995). With regard to cnidarian origins, Siddall *et al.* (1995) provided ultrastructural characterization of the development of myxozoan polar capsules and found this indistinguishable from that seen in narcomedusan nematocysts, which was confirmed by Lom & Dyková (1997). However, Zrzavý, Mihulka, Kepka, Bezdek & Tietz (1998) found that the inclusion of the Myxozoa in the Cnidaria is strongly dependent on the presence of *P. hydriforme* in the analysis. Using 18S molecular data, several authors showed affinities of the myxozoans with triploblast (bilaterian) organisms (Smothers *et al.* 1994,

Katayama, Wada, Furuya, Satoh & Yamamoto 1995, Schlegel *et al.* 1996, Cavalier-Smith, Allsopp, Chao, Boury-Esnault & Vacelet 1996, Hanelt, van Schyndel, Adema, Lewis & Loker 1996, Anderson, Canning & Okamura 1998, Kim, Kim & Cunningham 1999). With regard to the 18S rDNA database, the outcome of the analyses of the origins of the Myxozoa (cnidarian or bilaterian) seems to be influenced by the methodologies used to assess phylogenetic optimality criteria. Maximum parsimony algorithms do not account for multiple substitutions or unequal rates of change in a DNA sequence and the analysis might be influenced by long-branch attraction. Thus Zrzavý & Hypša (2003) conducted an study, in which they identified and neutralised long-branch attractions of the taxa involved according to the "long-branch extraction" method of Siddall & Whiting (1999). The authors found that under all conditions the analysis supported a basal branching pattern of Placozoa-Cnidaria versus *Polypodium,* the Myxozoa and the bilateria. This idea was also supported by Anderson *et al.* (1998), who analysed Hox genes from *Buddenbrockia plumatellae* (then *Tetracapsula bryozoides*) and *Myxidium lieberkuehni.* The results of this study showed that the myxozoan Hox genes can be clearly aligned to genes in bilaterians but not in cnidarians.

In conclusion, it is suggested that the *Polypodium,* the unusual cnidarian fish parasite, and the myxozoans form a bridge between the more primitive diploblast organisms and the bilaterians. Furthermore, since closer relatives have not been identified so far, *Polypodium hydriforme* is commonly accepted as an outgroup in phylogenetic analysis of the myxozoans although its long-branch status might add "noise" to pylogenetic analyses.

6.1.2 Relationships Among the Myxozoa

Based solely on morphological studies, Shul'man (1966) suggested that the first myxozoans were coelozoic, inhabiting the gall bladder and later the urinary bladder of marine teleost fish. Myxozoans later evolved to infect other tissues, with some forms becoming histozoic. Shul'man (1966) also suggested that the frist myxozoans were bipolarid (e.g. *Myxidium, Sphaeromyxa*) and that in freshwater they gave rise to the platysporid types (e.g. *Myxobolus* and *Henneguya*). Spore morphology has often been used as the sole criterion for higher taxonomy of the myxozoans and their separation into orders and genera. When 18S rDNA sequences were first applied to the taxonomy of myxosporeans, discrepancies soon arose between the traditional taxonomic scheme based on spore morphology and the findings from molecular data (Kent *et al.* 1994, Smothers *et al.* 1994, Siddall *et al.* 1995, Schlegel *et al.* 1996). Currently, 18S rDNA sequence data are available for 136 myxozoan taxa. Most sequences are available for the genus *Myxobolus* (35 species) followed by the genus *Kudoa* (27 species). However, sequences are still unavailable for several genera (e.g. *Hoferellus*), and for others (e.g. *Myxidium, Sphaerospora*) only a few sequences are available. The sequences published so far have been used to create phylogenies to obtain information about the relationships amongst the myxozoans and about general trends in myxozoan evolution. The most comprehensive study to date was presented by Kent *et al.* (2001) who analysed 18S sequences of 59 different myxozoan taxa. The phylogram presented by these authors (Figure 6.1.2) shows the general trends for the clustering within the phylum Myxozoa and led them to the following conclusions:

1. The Malacosporea branch off early in myoxozoan evolution before the radiation of all other marine and freshwater species.
2. Generally marine taxa (e.g. *Kudoa, Ceratomyxa*) branch earlier in the evolution than freshwater species.
3. Spore morphology is not a criterion for clustering of taxa and an overall lack of generic monophyly is apparent.
4. Actinosporean morphology offers little information for determining the morphology of myxosporean counterparts.

Subsequent additions of new sequences have produced phylogenetic trees which were in general accordance with the system of Kent *et al.* (2002) (Palenzuela *et al.* 2002, Molnar *et al.* 2002, Dyková *et al.* 2002, Negredo *et al.* 2003, Whipps, Grossel, Adlard, Yokoyama, Bryant, Munday & Kent 2004).

18S rDNA sequences have also been successfully used for the confirmation of alternate life cycle stages of *Myxobolus cerebralis* (Andree *et al.* 1997), *Ceratomyxa shasta* (Bartholomew *et al.* 1997), *Tetracapsuloides bryosalmonae* (Anderson *et al.* 1999), *Henneguya ictaluri* (Lin *et al.* 1999), *Thelohanellus hovorkai* (Anderson *et al.* 2000), and most recently for *Ellipsomyxa gobii* (Køie *et al.* 2004), the first marine myxozoan whose life cycle has been elucidated.

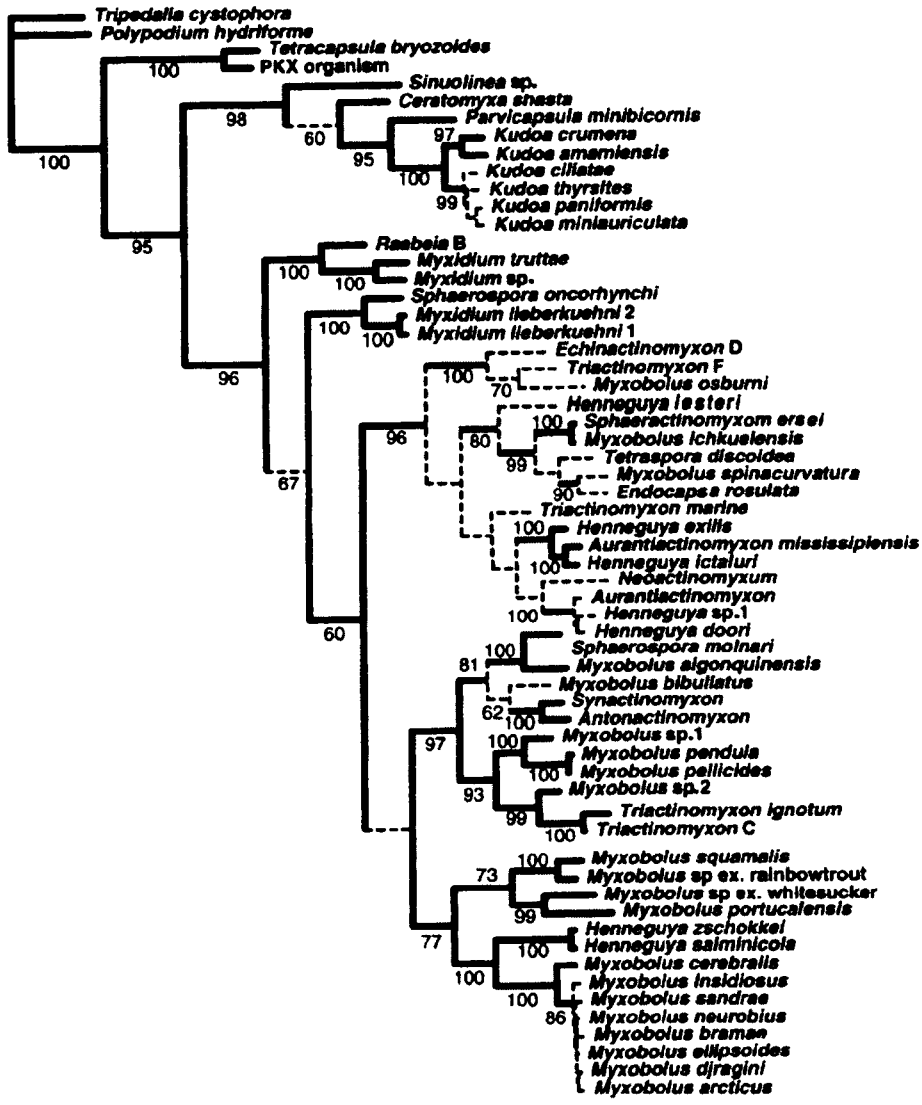


Figure 6.1.2 Phylogenetic organisation of the myxozoan taxa to date (from Kent et al. 2001).

6.1.3 Objectives

Twenty of the 22 myxozoan taxa found in the riverine habitat on the North coast of Scotland (Chapter 5, Sections 5.3.1.1 & 5.3.1.2) have not previously been characterised on a molecular basis. The myxosporeans belong to genera which are poorly represented (*Myxidium, Chloromyxum, Sphaerospora, Zschokkella*) or missing (*Hoferellus, Myxobilatus*) from the myxozoan molecular database. Thus the current

chapter was aimed at the molecular characterisation of the myxozoan community found in the study site, on the basis of the 18S rDNA, in order to investigate the following relationships:

1. Sequence identities between myxosporeans from the fish population and actinosporeans from the oligochaetes allowing the identification of alternate myxosporean-actinosporean life cycle stages.
2. Special emphasis was put on determining relationships between *Sphaerospora truttae* and the other myxozoans as none of the actinosporeans detected were found to represent alternate life cycle stages of *S. truttae,* using PCR and infection studies.
3. Sequence similarities between the myxozoans from the river system and other taxa published on GenBank.
4. Phylogenetic positioning of these new myxozoan sequences within the phylum.

6.2 Materials & Methods

6.2.1 Myxozoan Samples for Sequencing

Fish organs containing mature spores of *Chloromyxum truttae, Myxidium truttae, Myxidium giardi, Sphaerospora elegans, Myxobilatus gasterostei, Chloromyxum* sp., *Zschokkella* sp. and *Hoferellus gilsoni* (see Chapter 5, Section 5.3.1.1) were used for sequencing of the 18S rDNA of the myxosporeans in the habitat on the North coast of Scotland. Samples of each myxosporean were taken from three individual fish in order to obtain replicate sequences. *Sphaerospora elegans* and *Myxobilatus gasterostei* were sequenced from one stickleback each from the river on the North coast, and 2 further samples of these species were obtained from Airthrey Loch (Stirling University), where sticklebacks commonly harbour infections of these species (Sultana 1994). *Zschokkella* sp. was only found in 2 eels and thus only sequenced in two replicates, and *Myxobolus portucalensis* was not sequenced as only single spores were found in two fish. The sequence of *S. truttae* had been obtained previously (Chapter 3, Section 3.3.2).

Spores of *Aurantiactinomyxon* type 1, *Aurantiactinomyxon* type 3, *Aurantiactinomyxon pavinsis* ('petite form'), *Echinactinomyxon* type 1, *Echinactinomyxon radiatum, Raabeia* type 1, *Raabeia* type 3, *Raabeia* type 4, *Synactinomyxon* type 1, *Synactinomyxon longicauda* and *Neoactinomyxum eiseniellae* (see Chapter 5, Section 5.3.1.2) were collected from cell wells containing the oligochaete hosts. Whenever possible, three replicate samples of each type from different worm specimens were submitted to sequencing. *Neoactinomyxum eiseniellae, Aurantiactinomyxon pavinsis, Raabeia* type 1 and type 3 were only detected in, and

sequenced from, a single worm each. *Echinactinomyxon* type 5 had been sequenced previously (see Chapter 3, Section 3.3.1)

6.2.2 Amplification and Sequencing of Myxozoan 18S rDNA

From the DNA extracted from these samples, DNA was amplified, purified and sequenced according to the methodolody given in Chapter 2, Section 2.4.2. 18S rDNA was amplified using the universal eukaryotic 18S primers 18e and 18g (Chapter 3, Table 3.2.1). Subsequently, the 18S PCR products were diluted 1:4 in nanopure water for use in a nested PCR employing the more specific myxozoan primers, Myxgp2f and Act1R (Chapter 3, Table 3.2.2.B), and resulting in an approximately 900 bp nested PCR product. This is the same PCR assay as used for the screening of invertebrates (Chapter 5, Section 5.2.1.2). The PCR products obtained were excised from agarose gels and purified for sequencing using spin columns. Primers Myxgp2f, Act1R and LIN3 (Chapter 3, Tables 3.2.1 and 3.2.2.B) were used for cycle sequencing of the 18S fragments.

For a more detailed investigation of closely related species a second 18S fragment was produced in another nested PCR reaction. In this reaction, other published myxozoan primers, Myx1F (Table 6.2.2) and MX3 (Chapter 3, Table 3.2.2.B) were employed at an annealing temperature of 66.5°C, and the products were sequenced using myxozoan primers Act3F (Chapter 3, Table 3.2.2.B), MX3 and the general primer LIN10 (Chapter 3, Table 3.2.1). Joining the Act3F/MX3 fragments with the correlating Myxgp2f/Act1R fragments produced consensus sequences of approximately 1550 bp. Due to the non-specificity of the primer MX3 and the low level infection with *Chloromyxum* sp. in the kidney tubules, this species could only be

amplified and sequenced using the more specific reverse primer MyxUrinR (Table 6.2.2) in combination with Myxgp2F. All sequences were submitted to GenBank.

Table 6.2.2 Additional primers utilised for the amplification of the approx. 1550 bp fragment of the 18S rDNA (F=forward primer, R=reverse primer, Locus= Location of the primer on a 2000bp myxozoan model 18S gene, T_m=melting temperature of the primer).

Name	Dir	Sequence	Locus	T_m	Reference
Myx1F	F	3'- GTGAGACTGCGGACGGCTCAG-5'	80	65.7°C	Hallett *et al.* 2002
MyxUrinR	R	5'-TGGTTGTCTTTTCATAGCACATT-3'	1780	55.3°C	This study

6.2.3 Phylogenetic Analysis

In order to relate the obtained myxozoan sequences to each other and other myxozoan species from GenBank, phylogenetic analyses were carried out. Due to the time-consuming nature of analyses using phylogenetic algorithms, the obtained sequences were aligned with sequences selected from GenBank according to the following criteria: 1. closest relatives (determined on a BLAST search), 2. representatives of all myxosporean genera and 3. a selected variety of actinosporean types (summarised in Table 6.2.3). Sequences were aligned and phylogenetic analyses were performed using neighbour-joining, parsimony and maximum likelihood methods (see Chapter 2, Section 2.4.4). *Polypodium hydriforme* was used as outgroup (Siddall *et al.* 1995, Siddall & Whiting 1999, and recently reviewed in Kent *et al.* 2001, see 6.1.1).

Due to the unexpected position of two freshwater myxozoans from this study (*S. truttae* and *S. elegans*) within the marine clade of myxozoans, they were submitted to a separate analysis including most of the published marine species in order to determine their position more accurately. The myxozoans used for this analysis are included in Table 6.2.3.

Table 6.2.3 Myxozoan species and their 18S rDNA sequences used in the phylogenetic analyses.

Species/Type	Length (bp)	GenBank™ acc. no.
Polypodium hydriforme Ussov, 1885	1790	U37526
Buddenbrockia plumatellae Schröder, 1910	1784	AY074915
Tetracapsuloides bryosalmonae Canning et al. 2002	1803	U70623
Ceratomyxa labracis Sitjá-Bobadilla & Alvarez-Pellitero, 1993	1763	AF411472
Ceratomyxa shasta Noble, 1950	1643	AF001579
Ceratomyxa sparusaurati Sitjá-Bobadilla et al. 1995	1741	AF411471
Chloromyxum cyprini Fujita, 1927	2126	AY604198
Chloromyxum legeri Tourraine, 1931	2042	AY604197
Chloromyxum leydigi Mingazzini, 1890	1868	AY604199
Ellipsomyxa gobii Køie, 2003	1697	AY505126
Enteromyum scophthalmi Palenzuela, Redondo & Alvarez-Pellitero, 2001	1589	AF411335
Enteromyxum leei (Diamant, Lom et Dyková, 1994)	1609	AF411334
Henneguya doori Guilford, 1963	2020	U37549
Henneguya ictaluri Pote et al., 2000	2083	AF195510
Henneguya lesteri Hallett & Diamant, 2001	1966	AF306794
Henneguya salminicola Ward, 1919	1950	AF031411
Kudoa amamiensis Egusa & Nakajima, 1980	1739	AY152748
Kudoa crumena Iversen & Van Meter, 1967	1682	AF378347
Kudoa dianae Dykova et al., 2002	1568	AF414692
Kudoa hypoepicardialis Blaylock, Bullard & Whipps, 2004	1721	AY302722
Kudoa miniauriculata Whitaker et al., 1996	1563	AF034639
Kudoa minithyrsites Whipps et al., 2003	1683	AY152749
Kudoa ovivora Swearer & Robertson 1999	1570	AY152750
Kudoa paniformis Kabata & Whitaker, 1981	1562	AF034640
Kudoa permulticapsula Whipps et al., 2003	1679	AY078429
Kudoa quadricornis Whipps et al., 2003	1564	AY078428
Kudoa shiomitsui	1719	AY302724
***Kudoa* sp.** CMW	1564	AY302723
Kudoa thyrsites Gilchrist, 1924	1812	AY078430
Myxidium lieberkuehni Bütschli, 1882	2085	X76639
Myxidium sp.	1879	U13829
Myxobolus algonquinensis Xiao & Desser, 1997	1988	AF378335
Myxobolus arcticus Pugachev & Khokhlov, 1979	1609	AF085176
Myxobolus bramae Reuss, 1906	1608	AF085177
Myxobolus cerebralis Hofer, 1903	1937	U96492
Myxobolus ellipsoides Thélohan, 1982	1611	AF085178
Myxobolus neurobius Schuberg & Schröder, 1905	1611	AF085180
Myxobolus portucalensis Saraiva & Molnar, 1990	1656	AF085182
Parvicapsula minibicornis Kent et al., 1997	1831	AF201375
Parvicapsula pseudobranchiola Karlsbakk, Sæther, Høstlund, Fjellsøy & Nylund, 2002	1663	AY308481
Pentacapsula neurophila Grossel, Dyková, Handlinger & Munday, 2003	1552	AY172511
***Raabeia* sp.** KAB	2005	AF378352
Septemcapsula yasunagai Hsieh and Chen, 1984	1679	AY302741
Sphaerospora molnari Lom, Dyková & Grupcheva 1983	1879	AF378345
Sphaerospora oncorhynchi Kent, Whitaker & Margolis, 1993	1909	AF201373
***Unicapsula* sp.** CMW	1683	AY302725
Zschokkella mugilis Sitjá-Bobadilla & Alvarez-Pellitero, 1993	1632	AF411336
Aurantiactinomyxon CN	1946	AF483598
Aurantiactinomyxon KAB	2058	AF378356
Aurantiactinomyxon mississippiensis	2087	AF021878
Aurantiactinomyxon SLH	1690	AF487455
Neoactinomyxum KAB	2020	AF378353
Triactinomyxon	1751	AF306792
***Triactinomyxon* F** Kent	2020	AF378351
Triactinomyxon ignotum Stolc, 1899	2020	AF378349
Triactinomyxon SLH	1554	AY162270

6.3 Results

6.3.1 Sequences Obtained From the Myxozoan Community and Their Cladistic Analysis

Twenty-one new myxozoan sequence entries were submitted to GenBank, of which only *Myxidium truttae* had been sequenced before (origin: Canada, Kent *et al.* 2000). The genera *Chloromyxum, Hoferellus* and *Myxobilatus* were entered to GenBank for the first time. The size of the 18S rDNA fragments sequenced and submitted to GenBank as well as the accession numbers obtained are given in Table 6.3.1.A.

Analysis of the approximately 900 bp fragment amplified using primers Myxgp2f and Act1R was found sufficient to provide interspecies information. The percentage of identical base pairs for this fragment is given in Table 6.3.1.B. Analysis of the larger fragment (1550 bp) produced using primers Myx1F and MX3 did not show major differences in the percentage of identical base pairs between the various species (average delta of 0.2% and a maximum delta of 2.9%, Table 6.3.1.C). However, the inclusion of more sequences and the long variable region located at the 3' end of the Myx1F/MX3R fragment improved the support for positioning of the taxa in the phylogenetic analysis (higher bootstrap values). As a result, the 1550 bp fragment was sequenced and analysed for all myxosporean species (except for *Myxidium giardi* and *Sphaerospora elegans* which failed to be amplified with the primers applied), and for those actinosporean types which were found to have molecular identity with myxosporeans.

Three myxosporeans were found to share 100% identical base pairs with actinosporean types. It is assumed that the latter represent alternate stages in the life cycle of these species: *Myxidium truttae* was related to *Raabeia* type3 (1479 identical

base pairs), *Chloromyxum truttae* to *Aurantiactinomyxon pavinsis* (1567 identical base pairs) and *Chloromyxum* sp. to *Neoactinomyxum eiseniellae* (1253 identical base pairs).

Table 6.3.1.A Summary of the 18S rDNA fragments sequenced in this study and their GenBank™ accession numbers (sequences of *S. truttae* and *Echinactinomyxon* type 5 from Chapter 3 included).

Name	18S rDNA fragment	GenBank Acc. No.
Myxosporea		
Chloromyxum truttae	1572 bp	AJ581916
Myxidium truttae	1492 bp	AJ582061
Sphaerospora truttae	2541 bp	AJ581915
Sphaerospora elegans	1384 bp	AJ609590
Myxobilatus gasterostei	1561 bp	AJ582063
Chloromyxum sp.	1253 bp	AJ581917
Myxidium giardi	910 bp	AJ582213
Zschokkella sp.	1610 bp	AJ581918
Hoferellus gilsoni	1558 bp	AJ582062
Actinosporea		
Aurantiactinomyxon type 1	909 bp	AJ582004
Aurantiactinomyxon type 3	931 bp	AJ582005
Aurantiactinomyxon pavinsis	1567 bp	AJ582006
Echinactinomyxon type 1	917 bp	AJ582000
Echinactinomyxon type 5	1983 bp	AJ417562
Echinactinomyxon radiatum	899 bp	AJ582001
Raabeia type 1	933 bp	AJ582008
Raabeia type 3	1541 bp	AJ582009
Raabeia type 4	934 bp	AJ582010
Synactinomyxon type 1	931 bp	AJ582002
Synactinomyxon longicauda	934 bp	AJ582003
Neoactinomyxum eiseniellae	1570 bp	AJ582007

Table 6.3.1.B Percentage of identical base pairs of the sequences obtained from the myxosporean and actinosporean forms, comparing an approximately 900 bp fragment of 18S rDNA. Identities >80% in bold. (C tru=*Chloromyxum truttae / Aurantiactinomyxon pavinsis*, M tru=*Myxidium truttae / Raabeia* type 3, S tru=*Sphaerospora truttae*, S ele=*Sphaerospora elegans*, M ga=*Myxobilatus gasterostei*, C sp=*Chloromyxum* sp. / *Neoactinomyxum eiseniellae*, M gi=*Myxidium giardi*, Zsch=*Zschokkella* sp., H gil=*Hoferellus gilsoni*, A=*Aurantiactinomyxon*, E=*Echinactinomyxon* with ra=*radiatum*, R=*Raabeia*, S=*Synactinomyxon* with lo=*longicauda*). * Indicate myxosporeans which show 100% sequence identity with their actinosporean counterparts, therefore the actinosporeans are not listed separately.

	C tru*	**M tru***	**S tru**	**S ele**	**M ga**	**C sp***	**M gi**	**Zsch**	**H gil**	**A 1**	**A 3**	**E 1**	**E 5**	**E ra**	**R 1**	**R 4**	**S 1**	**S lo**
C tru*	100.0	75.0	40.5	40.6	71.0	70.5	70.4	70.4	68.6	65.9	66.7	65.8	70.4	67.6	70.3	66.8	66.8	65.5
M tru*	---	100.0	41.9	40.5	67.2	68.4	68.4	68.4	67.7	64.8	65.8	64.0	68.7	66.0	68.1	66.0	65.6	65.1
S tru	---	---	100.0	64.2	41.7	43.2	43.1	43.6	41.3	42.6	42.5	42.1	39.7	41.8	40.1	42.9	42.5	42.1
S ele	---	---	---	100.0	40.6	40.1	40.0	40.5	40.6	41.3	40.7	40.7	40.6	41.0	40.2	41.0	41.7	41.0
M ga	---	---	---	---	100.0	**80.7**	**80.4**	**81.2**	**81.8**	68.7	70.5	67.6	73.3	72.3	72.3	70.5	70.0	68.8
C sp*	---	---	---	---	---	100.0	**98.9**	**98.1**	**82.9**	68.9	70.3	68.1	73.0	75.9	73.5	70.3	69.7	69.6
M gi	---	---	---	---	---	---	100.0	**97.7**	**83.1**	69.1	70.5	68.8	73.0	75.7	73.5	70.5	69.9	69.8
Zsch	---	---	---	---	---	---	---	100.0	**82.7**	68.9	70.5	68.3	72.8	75.9	73.3	70.6	69.6	69.6
H gil	---	---	---	---	---	---	---	---	100.0	67.6	69.3	67.6	70.6	73.6	70.7	69.6	68.6	69.4
A 1	---	---	---	---	---	---	---	---	---	100.0	**91.9**	**89.6**	65.3	66.9	66.2	**91.9**	**91.0**	**86.3**
A 3	---	---	---	---	---	---	---	---	---	---	100.0	**92.0**	66.7	69.2	66.7	**98.6**	**93.1**	**88.3**
E 1	---	---	---	---	---	---	---	---	---	---	---	100.0	65.0	68.3	66.3	**91.9**	**91.8**	**85.3**
E 5	---	---	---	---	---	---	---	---	---	---	---	---	100.0	69.2	**91.9**	66.7	66.7	65.5
E ra	---	---	---	---	---	---	---	---	---	---	---	---	---	100.0	67.8	69.1	69.3	67.9
R 1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	100.0	67.1	67.4	65.4
R 4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	100.0	**93.1**	**88.2**
S 1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	100.0	**88.0**
S lo	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	100.0

Table 6.3.1.C Percentage of identical base pairs of the myxozoans sequenced from analysis of an approximately 1550 bp 18S rDNA fragment (showing the difference to the analysis of the 900 bp fragment in parenthesis). Species abbreviations as in Table 6.3.1.B.

	C tru	**M tru**	**S tru**	**M ga**	**C sp**	**Zsch**	**H gil**
C tru	100.0	76.5 (+1.5)	43.4 (+2.9)	70.4 (-0.6)	69.7 (-0.8)	69.7 (-0.7)	68.8 (+0.2)
M tru	---	100.0	44.4 (+2.5)	68.2 (+1.0)	68.4 (±0.0)	68.5 (+0.1)	68.3 (+0.6)
S tru	---	---	100.0	43.8 (+2.1)	44.0 (+0.8)	43.9 (+0.3)	43.1 (+0.2)
M ga	---	---	---	100.0	79.9 (-0.8)	80.4 (-0.8)	82.3 (+0.5)
C sp	---	---	---	---	100.0	96.0 (-2.1)	81.7 (-1.2)
Zsch	---	---	---	---	---	100.0	81.8 (-0.9)
H gil	---	---	---	---	---	---	100.0

Figure 6.3.1 shows cladograms, which are the result of the maximum parsimony and the maximum likelihood analysis of the taxa from the river system:

Sphaerospora truttae and *Sphaerospora elegans* share less than 45% of identical base pairs with any other myxozoan sequenced in this study due to the long inserts in the variable 18S regions (Table 6.3.1.B & C). Excluding these regions from the analysis results in approximately 70% sequence similarity of *S. truttae* and *S. elegans* with the other myxozoans. In the phylograms, *S. truttae* and *S. elegans* occupy the most basal position in the tree, close to the malacosporeans and separated from all other species, a position which is well supported by bootstrap values. This position is maintained also when the long variable regions are excluded from the phylogenetic analysis.

The next diverging branch of the tree is formed by the gall bladder parasite *Chloromyxum truttae* (well supported by bootstrap values), which is the closest relative of the other gall bladder parasite *Myxidium truttae* (75% identical base pairs). *M. truttae* shares fewer base pairs with the other representative of the genus, the kidney myxosporean *Myxidium giardi* (68.4% identical base pairs). However, the positioning of

C. truttae close to *M. truttae* is questionable as the maximum parsimony analysis, in contrast to the maximum likelihood analysis, hosts *C. truttae* in another branch and bootstrap support for the positioning of *C. truttae* was weak in both analyses.

The actinosporean types *Echinactinomyxon* type 5 and *Raabeia* type 1 are closely related (91.9% identical base pairs) and are separated from the rest of the actinosporeans with an indefinite position in both analyses. Apart from these two representatives and *Echinactinomyxon radiatum,* all actinosporeans form a cluster of very closely related species (between 85.3% and 98.6% sequence identity), which forms a sister group of the largest myxosporean clade of this cladogram in both the maximum parsimony as well as the maximum likelihood analyses, despite little bootstrap support.

This myxosporean lineage shows a very high percentage of sequence identity (98%) between the species *Zschokkella* sp., *Myxidium giardi* and *Chloromyxum* sp. and >80% between these species and *Myxobilatus gasterostei* as well as *Hoferellus gilsoni.* This strongly suggests a close relationship of the five different species from the same organ localisation (urinary system) independent of their spore morphology (five genera) and host species (three different families).

Echinactinomyxon radiatum joins this group of urinary system myxosporeans as the only actinosporean member and shares approximately 76% sequence identity.

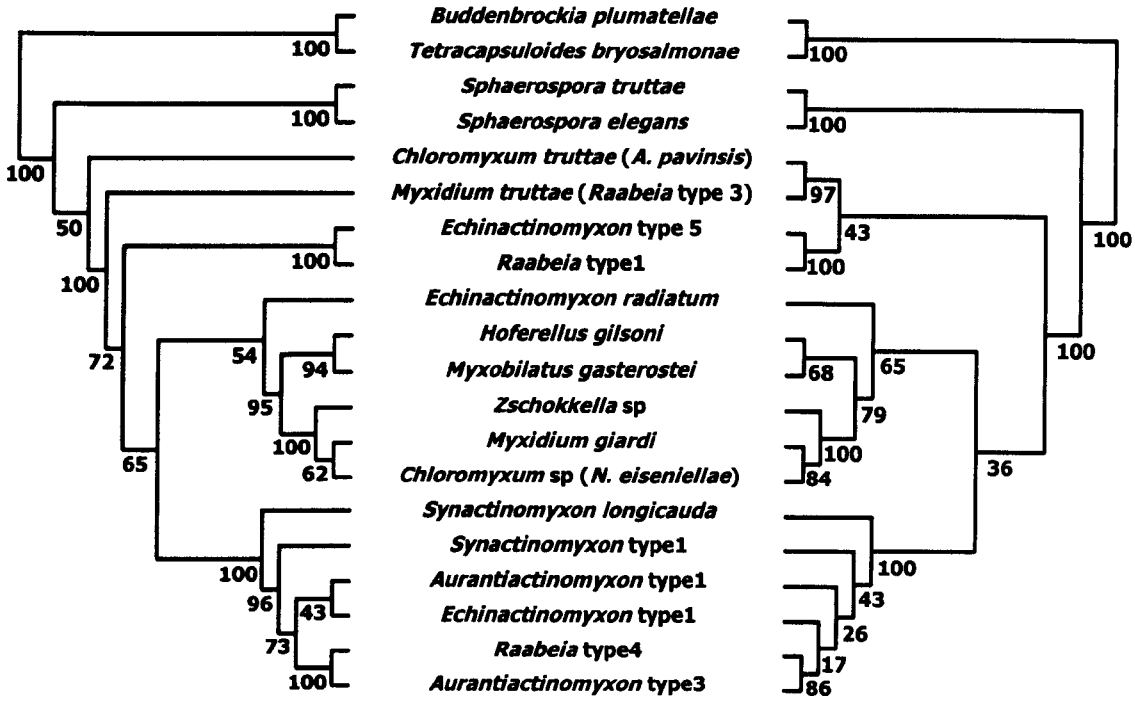


Figure 6.3.1 Maximum parsimony tree (left) and maximum likelihood tree (right) of the 18S rDNA of the myxozoans sequenced, rooted at *Tetracapsuloides bryosalmonae* (U70623) and *Buddenbrockia plumatellae* (AY074915). Transversion/transition ratio 1:2, bootstrap values shown represent percentage of 100 bootstrap replicates.

6.3.2 Phylogenetic Analysis of the Species From the River System With Other Myxozoan Taxa

The sequences obtained in this study were submitted to a BLAST search on GenBank and then aligned with the closest matches as well as a random selection of representatives of all known genera.

Due to the addition of related species, the subsequent phylogenetic analysis resulted in an improvement of the positioning of some of the species sequenced in the myxozoan phylogenetic system. The result of the maximum parsimony analysis is shown in Figure 6.3.2.

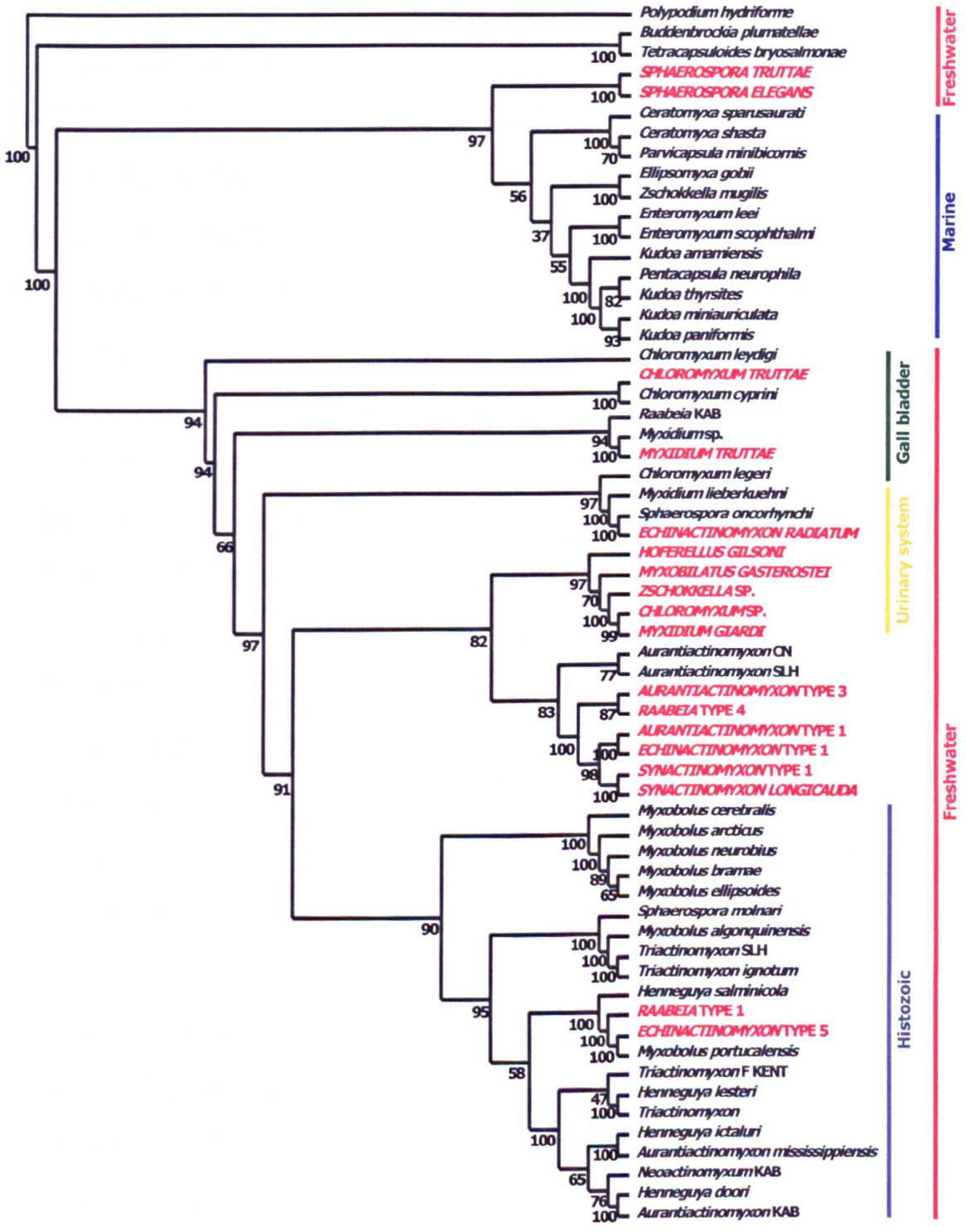


Fig 6.3.2 Maximum parsimony tree of the 18S rDNA of the myxozoans sequenced and other myxozoan 18S rDNA sequences obtained from GenBank™, rooted at *Polypodium hydriforme*. Transversion/transition ratio 1:2, bootstrap values shown represent percentage of 100 bootstrap replicates; freshwater/marine habitat indicated as well as host tissue localisation of myxosporeans from the freshwater environment. Red=Sequenced obtained in this study.

Using *Polypodium hydriforme* as the outgroup, the tetracapsulid malacasporeans form the most basal lineage in the myxozoan system. Applying the most parsimonious analysis under the given conditions, a basal marine clade diverges before the origins of a principally freshwater cluster. *Sphaerospora truttae* and *S. elegans* cluster together at the basis of this marine clade. *C. sparausaurati* is the closest relative to *S. truttae* but yet shares only 51% of identical base pairs over a 2400 (*S. truttae*)/1720 (*C. sparusaurati*) bp fragment. Another freshwater parasite, *Ceratomyxa shasta,* occurs in the same, principally marine, group.

In their recent review of the Myxozoa, Kent *et al.* (2001) showed that the freshwater clade branches into three well-supported subclades. In this analysis the same subclades were found but additional ones were identified. These were three clades harbouring *Chloromyxum* species and one clade represented by the myxozoans found in the urinary system. The present phylogram shows that the first three freshwater clades host only gall bladder parasites, the fourth clade is shared by a gall bladder myxozoan and two species from the urinary system, the fifth contains only myxozoans inhabiting the urinary system, and the two most recent branches host exclusively histozoic species.

The first freshwater branch, which diverges after the basal marine species, is strongly supported by bootstrap values and contains *Chloromyxum leydigi* Mingazzini, 1890, a gall bladder parasite from the marine environment. In the next clade, also well supported, the freshwater gall bladder parasites *Chloromyxum truttae* and *Chloromyxum cyprini* Fujita, 1927 from the silver carp, *Hypophthalmichthys molitrix* (Valenciennes) cluster together, sharing 85% of identical base pairs. The uncertain phylogenetic relationship determined between *C. truttae* and *Myxidium truttae* in the

analysis of the river myxozoans only was not improved by the addition of other species from GenBank. The relationship between *C. truttae / C. cyprini* and the next clade, which hosts *M. truttae*, is not clear as bootstrap support is weak and sequence identity of the species of these two clades is around 75%. However, *M. truttae* is closely related to another *Myxidium* sp. from the gall bladder of *Cottus bairdii* Girard, 1850. The sequence obtained for *M. truttae* in this study differs by 1% from the sequence of *M. truttae* from *Oncorhynchus mykiss* in Canada (AF201374). *Raabeia* type 3 as the alternate life cycle stage of *M. truttae,* together with another *Raabeia* type from *Lumbriculus hoffmeisteri* L., completes this closely related group sharing around 90% sequence identity.

The next clade in the phylogram hosts *Chloromyxum legeri* from the gall bladder of the silver carp, *Myxidium lieberkuehni* from the urinary bladder of the eel, *Sphaerospora oncorhynchi* from the renal tubules of *Oncorhynchus nerka* (Walbaum) and *Echinactinomyxon radiatum.* The latter species shows a very close relationship with *S. oncorhynchi* (94.4% identical base pairs) and forms a sister group with 100% bootstrap support.

The myxosporeans from the urinary system sequenced in this study (*Myxobilatus gasterostei, Hoferellus gilsoni, Zschokkella* sp, *Chloromyxum* sp and *Myxidium giardi*) occur next to this group, and their clustering, independent from spore morphology and host species (Figure 6.3.1), is maintained when analysed with other myxozoan sequences from GenBank. The large actinosporean sister group from the river system is also maintained and joined by two further actinosporeans of the aurantiactinomyxon type (AF487455 and AF483598), but no myxosporeans.

The most recent freshwater lineage is represented by exclusively histozoic species, predominantly of the genera *Myxobolus* and *Henneguya*. A gill parasitic species of *Sphaerospora*, i.e. *S. molnari* also clusters within this clade. *Echinactinomyxon* type 5 and *Raabeia* type 1 cluster within this clade, and their position is strongly supported by the formation of a sister group of *Myxobolus portucalensis* from *Anguilla anguilla* (sequence identity with *Echinactinomyxon* type 5 92.8%) with 100% bootstrap support.

6.3.3 Positioning of Sphaerospora truttae

With 2541 bp, the 18S rDNA sequence of *S. truttae* represents not only the longest myxozoan sequence encountered so far but also one of the longest metazoan sequences present on GenBank. The alignment of the complete 18S rDNA sequence of *S. truttae* with the other myxozoan species showed that it exhibits extraordinarily long inserts in the variable regions of the 18S rDNA and its alignment and exact phylogenetic positioning is problematic. However, its positioning close to the basal marine species is well supported by bootstrap values and is independent from the exclusion of the variable regions (and thus the long inserts) from the analysis. The results of an analysis focusing on the myxozoan species from the marine environment are shown in Figure 6.3.3.

S. elegans from stickleback, for which only 1384 base pairs were sequenced, shares 64.2% of identical base pairs with *S. truttae* and its variable inserts are slightly shorter than in *S. truttae* but longer than in other myxozoan sequences. As the closest relative of *S. truttae* and sharing several of these unusual sequence characters with the latter species, the sequence of *S. elegans* is supportive in the analysis.

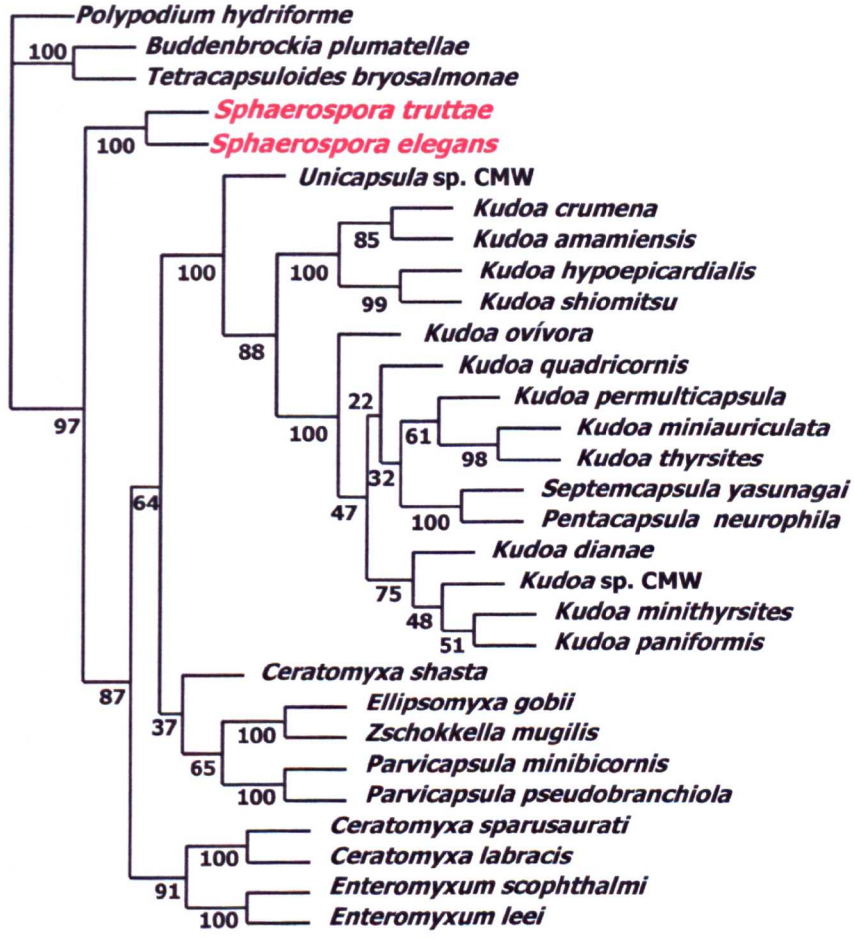


Figure 6.3.3 Maximum parsimony tree of the 18S rDNA of marine myxozoans showing the basal position of *Sphaerospora truttae* and *Sphaerospora elegans*. Tree is rooted at *Polypodium hydriforme*. Transversion/transition ratio 1:2, bootstrap values shown represent percentage of 100 bootstrap replicates.

With 97% bootstrap support, *S. truttae* and *S. elegans* occur in a separate clade which diverges after the malacasporeans. The *Sphaerospora* species thus occupy a position basal the marine species, which branch into three further clades, two closely related ones hosting *Ceratomyxa, Enteromyxum, Ellipsomyxa, Zschokkella* and *Parvicapsula* species, and one which contains the monophyletic group of *Kudoa* spp. with some species which show variability in the number of polar capsules.

6.4 Discussion

In this chapter, a molecular inventory was provided of the myxozoans present in a typical Scottish highland river habitat, including the outflow area of the adjacent salmon hatchery on the North coast of Scotland. The comprehensive collection of new 18S rDNA sequence data from 9 myxosporeans and 12 actinosporeans allowed a phylogenetic analysis *sui generis* of the relationships between the myxozoan of the community and with other myxozoan sequences available on GenBank™.

The general structure of the phylogram obtained in this study is consistent with previous analyses (Kent *et al.* 2001, Palenzuela *et al.* 2002, Molnar *et al.* 2002, Dyková *et al.* 2002, Negredo *et al.* 2003). However, the addition of the new sequences from this study identifies the basal origin of two species (*Sphaerospora truttae* and *S. elegans*) which form a sister group of the generally marine species and it strongly aids the understanding of the cladistic arrangement within the more recent clade of freshwater taxa due to the addition of new species belonging to poorly represented genera or genera so far unavailable on the molecular database:

Kent *et al.* (2001) identified three well-supported freshwater clades, which are maintained in the present analysis but three further sub-clades are added. Two of the new branches in the freshwater tree were also identified by Dyková *et al.* (2004), who submitted 3 *Chloromyxum* sequences which strongly improve the positioning of the obtained *Chloromyxum truttae* sequence but also aid the interpretation of the general arrangement within the freshwater myxozoans. The most basal clades of the freshwater species were found to be represented by *Chloromyxum* and *Myxidium* species which exclusively infect the gall bladder epithelium, the "middle field" hosted various genera which inhabit the excretory system and the phylogenetically youngest

groups are exclusively histozoic species, mainly represented by the genera *Myxobolus* and *Henneguya.* This suggests that the first myxozoans to invade the freshwater environment were gall bladder parasites before they settled in the urinary system and most recently became histozoic. This general organization of the freshwater myxozoans and the fact that five myxozoans (*M. gasterostei, H. gilsoni, Zschokkella* sp., *Chloromyxum* sp and *M. giardi*) sequenced from the excretory system, belong to five different genera and three different host families but show an extraordinarily close relationship with each other (up to 98.9 % sequence identity), suggests that tissue tropism represents a strongly determining factor in myxozoan phylogeny. It also implies that spore morphology is of inferior importance in terms of taxonomy although it is the criterion most often employed for assigning species (Lom 1987, Moser & Kent 1994, Lom *et al.* 1997).

Support for an arrangement of species by tissue location can also be found in the freshwater lineage consisting of the histozoic genera *Myxobolus* and *Henneguya.* As the largest group with numerous sequences published, most *Myxobolus* and *Henneguya* species are parasites of skin, gills, muscle and the nervous system. (Andree, Szekely, Molnar, Gresoviac & Hedrick 1999) reported the clustering of 10 *Myxobolus* species from different regions in the USA and from Hungary according to tissue location. In the present analysis, the gill parasite *Sphaerospora molnari* clusters clearly within the *Myxobolus-Henneguya* group representing a sister taxon of *Myxobolus algonquinensis* Xiao & Desser, 1997. Both of these myxosporeans mature in the epithelium and share the same tissue tropism although their spores are morphologically different. A more comprehensive resolution of this group can be found in Dyková *et al.* (2002), confirming these findings.

However, although relatedness according to localisation was the dominant finding in the present analysis of freshwater myxosporeans, tissue tropism, although strongly influential, is probably not the only factor driving myxozoan evolution.

The gall bladder myxozoans diverged early in evolution into four distinctive clades, sometimes only sharing 65% of identical base pairs with each other in contrast to the radiation of the histozoic species which took place more recently and resulted in higher sequence identities. *Myxidium truttae* clusters with another *Myxidium* species from the gall bladder of *Cottus bairdi* and a *Raabeia* type, which most probably represents an alternate stage of another *Myxidium* gall bladder parasite similar to *Raabeia* type 3 which was found to be identical with *M. truttae*. The *Chloromyxum* species sequenced from gall bladders cluster separately from the *Myxidium* branch in 3 different clades. It is thus suggested that the gall bladder as a host organ was invaded by several pathways at the beginning of the conquest of the freshwater environment by myxozoans. Supporting this hypothesis is the finding that the actinosporean stage related to *C. truttae* is a very small type, i.e. *Aurantiactinomyxon pavinsis* (approximately 20 μm spore diameter), whereas the life cycle of *M. truttae* involves *Raabeia* type 3, a spore type with large (230μm) floating appendages. As both species target the gall bladder epithelium of the same host (*Salmo trutta*) it is suggested that *M. truttae* and *C. truttae* have developed different strategies to infect the host. The variety of functional designs of actinosporean spores is important in the context of floating behaviour in the water column and for attachment to the fish, but only the sporoplasms finally enter the host. Yokoyama & Urawa (1997) found that small actinosporean stages (e.g. aurantiactinomyxon) invade the fish host through the gills

whereas large actinosporeans (e.g. triactinomyxon and raabeia) attach mainly to the skin.

Chloromyxum legeri from the gall bladder of the silver carp clusters with *Myxidium lieberkühni* and *Sphaerospora oncorhynchi.* This topology is supported by 97% bootstrap support despite a sequence similarity of only 72%. This arrangement does not necessarily contradict a clustering according to tissue localisation as there are minor anatomical and physiological differences between a gall bladder epithelium and e.g. a urinary bladder epithelium. However, there is far less sequence similarity between *C. legeri* and *M. lieberkühni* or *S. oncorhynchi* (72%) than between the two urinary parasites (86%), and topologies might change when new sequences are added to GenBank.

The two *Sphaerospora* species sequenced in this study occupy a distinctively different position from all other myxosporeans and actinosporeans sequenced, despite the homology of tissue tropism with five of the other myxosporeans found. The extraordinary long inserts in the 18S rDNA of these species complicate alignments and exact phylogenetic positioning, but *S. truttae* shows clear relatedness to *S. elegans* from stickleback and both species occupy a position basal to the marine myxozoan species, with highest sequence identity with a marine species, *C. sparusaurati* (51%).

Interestingly, *S. truttae* and *S. elegans* as well as the freshwater parasite *C. shasta,* which clusters within the marine myxozoans have hosts which migrate to and from the marine environments (anadromous migration of salmonids and occurrence of stickleback in freshwater as well as in coastal marine waters, Bell & Foster 1994). Whereas *C. shasta* was most likely introduced into the freshwater habitat from the

marine environment, the origin of *S. truttae* and *S. elegans* seems to be the freshwater habitat with closest relation to species which conquered the marine environment.

The genus *Sphaerospora*, with 2 further species sequenced from freshwater (*S. oncorhynchi* which clusters with *Myxidium lieberkuehni* and *S. molnari* which is a representative of the histozoic clade), thus presents a strongly polyphyletic group. This poses the question why this spore morphology developed on several occasions in myxozoan evolution. Due to their spherical design *Sphaerospora* spores sink relatively rapidly and are likely to be taken up by non-selective feeding alternate hosts, such as oligochaetes. The spherical shape might be a very successful spore design, developed on several occasions during myxozoan evolution. On the other hand, the appendages of the spores of, e.g. the genus *Henneguya,* might alter their sinking behaviour in the water column or have an influence on the uptake by the host. One would assume that they cannot be swallowed as easily as spherical spores, but would favour e.g. filter feeding hosts, as the appendages allow the spore to get caught in a filter apparatus. However, the only alternate host for *Henneguya* spp identified so far is the oligochaete *Dero digitata* (Styer *et al.* 1991, Lin *et al.* 1999, Pote *et al.* 2000). In common with most oligochaetes, *D. digitata* feeds with its head buried in the sediment whereas the posterior end is moved in the water column for aeration (Brinkhurst 1971). The posterior end of the worm exhibits delicate gill filaments which could function as a catching apparatus for myxosporean spores which have appendages. Unfortunately, little is known about the entry of myxosporeans into their worm hosts. However, it is assumed that, presumably as a result of some functionality, caudal appendages arose on multiple occasions in myxozoan evolution. The assumption that spore morphology is of functional rather than of taxonomic importance supports the idea that most

myxosporean genera are polyphyletic (Figure 3, and review by Kent *et al.* 2001) and that, analogous to the myxosporeans, most actinosporean phenotypes are spread over the freshwater clades without any identifiable pattern.

The extraordinary phylogenetic position of *S. truttae* close to the marine myxozoans also allows a new hypothesis regarding the alternate host in the life cycle of *S. truttae.* The life cycles of two members of the generally marine group have so far been elucidated. One of them is a freshwater parasite (*Ceratomyxa shasta*, the only freshwater parasite nesting within the marine group) of the otherwise entirely marine genus *Ceratomyxa,* the other one is *Ellipsomyxa gobii,* a typical marine species from the gall bladder of the common goby *Pomatoschistus microps. C. shasta* infects *Manayunkia speciosa* (Bartholomew *et al.* 1997) and *E. gobii* utilises two *Nereis* species as alternate hosts (Køie *et al.* 2004); all of these hosts are representatives of the class Polychaeta. Due to its closer molecular relationship with these marine species than with any freshwater myxozoans, it is suggested that *S. truttae* might also utilise a polychaete rather than an oligochaete as an alternate host.

Of the actinosporeans sequenced in this study, six species with different phenotypes (*Synactinomyxon longicauda, Synactinomyxon* type 1, *Raabeia* type 4, *Echinactinomyxon* type1, *Aurantiactinomyxon* type 1 and *Aurantiactinomyxon* type 3) were very closely related (85.3-98.6% identical base pairs) and they clustered together with two other aurantiactinomyxon types (AF487455 and AF483598) as a sister group to the myxosporean species from the urinary system. Due to the complete lack of myxosporeans in this sister group and the frequent occurrence of the latter in oligochaetes it may be questioned whether these actinosporeans have myxosporean counterparts at all. As the only sexual process (gametogony) in the myxozoan life cycle

described so far takes place in the oligochaete host (El-Matbouli & Hoffmann, 1998), the worm *per definitionem* has to be regarded as the definitive host, and perhaps some myxozoans might not employ an obligatory alternate fish host. In all three actinosporean types which were related to myxosporean counterparts in this study, only one worm was found infected with each type.

The actinosporean *Echinactinomyxon* type 5 was previously believed to represent the alternate stage of *S. truttae* (Özer & Wootten 2000). As shown previously (Chapter 3, Section 3.3.1 and 3.3.2) *Echinactinomyxon* type 5 shares only 48.9% of identical base pairs with *S. truttae*. In contrast, *Echinactinomyxon* type 5 together with *Raabeia* type 1 form a closely related sister group of *Myxobolus portucalensis*, with 100% bootstrap support. It is suggested that these two actinosporean types are part of life cycles including histozoic myxosporean stages, most likely *Myxobolus* or *Henneguya* types. Similarly, *Echinactinomyxon radiatum* with 94.4% sequence identity with *S. oncorhynchi* might represent the alternate stage of a myxosporean inhabiting the urinary system.

Due to the identity of 18S rDNA sequences obtained for three myxosporeans with those for three actinosporean types it is assumed that the different life cycle stages of each identical pair belong to individual myxozoan species. As a result, the actinosporean names should be suppressed and become junior synonyms of the named myxosporeans as proposed by Kent *et al.* (1994) although this contradicts the International Code of Zoological Nomenclature in case of *Neoactinomyxon eiseniellae* (the first name of genus or species has priority): *Neoactinomyxum eiseniellae* Marques, 1984 becomes a junior synonym of *Chloromyxum* sp., *Aurantiactinomyxon pavinsis*

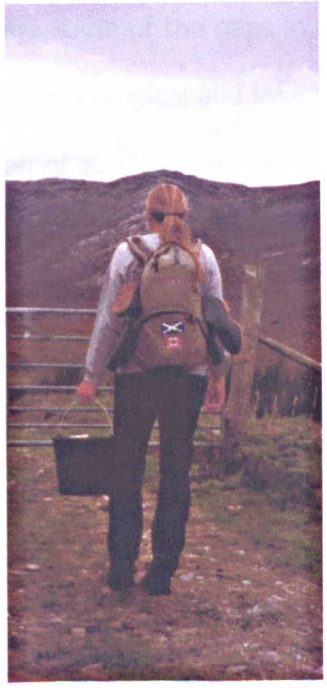
Marques, 1984 a junior synonym of *Chloromyxum truttae* Léger, 1906 and *Raabeia* type 3 a junior synonym of *Myxidium truttae* Léger, 1906.

The use of the 18S ribosomal gene as molecular "chronometer" can be problematic (see Chapter 3, Section 3.1.2, and also Hillis & Dixon 1991). The gene has a very conservative character, but Kent *et al.* (1996) mentions a "fast clock" in the evolution of myxozoan 18S, which helps to explain the high degree of variability among the species. In this study, two *Sphaerospora* species shared less than 52% of identical base pairs with all other myxozoans sequenced so far. In contrast, some species of different genera (phenotypes) were found to exhibit up to 98.6% sequence identity and they therefore must have diverged into different species very recently. It cannot be excluded that different phenotypes of the same species exist in different fish hosts, as myxozoan species do develop more than one spore type in their life cycle (myxosporean and actinosporean) and additionally, a single actinosporean genotype was recently found to develop two different phenotypes in the same oligochaete host (Hallett *et al.* 2002), possibly designed for different fish hosts. It is furthermore difficult to distinguish between different species and different geographic genotypes of one single species. For example, the present *M. truttae* sequence was shown to be only 99% identical with *M. truttae* from Canada and suggests that this species has been developing in geographical isolation for a very long time so that genotypically different subspecies have emerged. There is a need for other myxozoan genes to be analysed in order to verify or refute observations of relatedness made on the basis of the 18S gene.

A comprehensive data set including more taxa and other genes is desirable to confirm the interpretation of these results and to evaluate the importance of other factors such as geographic distribution or alternate hosts.

CHAPTER 7

Conclusions, Questions & Suggestions



7 Conclusions, Questions & Suggestions

This study investigated *Sphaerospora truttae* and other myxozoans co-occurring in the same habitat using molecular methods. This represents a different approach to previous studies on *S. truttae* which investigated the seasonality, epizootiology and potential alternate host using conventional morphological techniques and infection studies. The results from the present study elucidate so far unknown myxozoan life cycle stages and shed more light on the phylogeny of the phylum Myxozoa. It is also demonstrated that the molecular techniques applied in this study represent powerful tools which substantially aid in filling some of the gaps in the traditional knowledge of the myxozoans, which is based on morphological and infection studies.

The molecular characterisation of *S. truttae* on the basis of its 18S rDNA showed that this parasite is a very unusual myxozoan with extraordinary long inserts in the variable regions of the 18S rDNA sequence, resulting in the longest 18S sequence recorded amongst the myxozoans so far and one of the longest metazoan sequences. It is suggested that further information about this unusual 18S sequence could be gained from the analysis of its secondary structure. However, according to the present state of analysis, *S. truttae* is placed at the basis of a clade of generally marine genera of myxozoans. *Sphaerospora elegans*, another freshwater myxozoan found in the same habitat, shares most of the unusual sequence characters with *S. truttae,* and might be helpful if included in future studies.

In this study, 18S rDNA sequences showed that the specific actinosporean stage (*Echinactinomyxon* type 5) which had previously been related to *S. truttae* using infection studies (Özer & Wootten 2000), is in fact a different myxozoan species which

shows less than 50% sequence identity with *S. truttae.* This demonstrates that infection studies alone can result in false identification of infective actinosporean stages, probably due to the presence of cryptic infections in the form of early developmental stages in the receptor fish. It also underlines the importance of confirming alternate actinosporean and myxosporean stages on a molecular basis.

Comparing 18S rDNA sequences obtained in this study, 3 pairs of genetically identical actinosporean-myxosporean counterparts were found, identifying the alternate actinosporean stages of *Myxidium truttae, Chloromyxum truttae* and *Chloromyxum* sp. This is a low number of myxosporean-actinosporean pairs identified considering the number of myxosporeans (10) and actinosporeans (12) discovered in the habitat. Furthermore, despite 100% infection prevalence of *S. truttae* in the farm, the alternate actinosporean stage of *S. truttae* was not found, and worldwide only 25 myxozoan life cycles have been elucidated in the course of 20 years of research since the discovery of the first myxozoan life cycle by Wolf and Markiw in 1984. Low prevalences of actinosporean infections in the oligochaetes do not entirely explain the problems related to the elucidation of myxozoan life cycles. It might be speculated that some myxozoans have a direct life cycle. Despite numerous attempts, direct transmission of myxozoans has so far only been found in *Enteromyxum* spp. which excrete myxosporean developmental stages from infected intestines of fish, and these are taken up and infect receptor fish (Diamant 1997, Redondo *et al.* 2002). However, whereas direct transmission of myxosporeans seems rather rare, it might well be possible for some actinosporeans. Six actinosporeans sequenced in this study occurred with relatively high prevalence and formed a phylogenetic clade which is devoid of myxosporeans. It was shown that sexually produced offspring of oligochaetes are

myxozoan-free and they could be used as receptor worms in infection studies aiming at the clarification of the question whether direct actinosporean transmission of these spore types is possible.

Another explanation for the low number of myxosporean-actinosporean matches is the potential involvement of other vertebrate and invertebrate hosts in myxozoan life cycles. The number of non-oligochaete invertebrates tested for myxozoan infections was low and should be expanded in future studies. Although only a few papers report myxozoans in invertebrates other than oligochaetes, bryozoa, polychaetes, sipunculids, lepidoptera and trematodes have been identified as hosts for myxozoans (see Chapter 5, Section 5.1.3) and the species-rich invertebrate life in the investigated river offers a great potential. On the other hand, vertebrate hosts other than teleosts might play a role in the life cycle of some of the actinosporeans found. Myxosporeans were not detected in the 6 frogs screened but Morelle (1929) found the same species (*Rana temporaria*) infected with a kidney myxosporean of the genus *Leptotheca* Thélohan, 1895. Mature spores in frogs have been reported to be present for a very brief period only (2 weeks; Fiala, personal communication). Thus other vertebrate hosts (amphibians, birds) should be included in surveys and screening of vertebrate and invertebrate hosts should be done throughout the year as seasonality plays an important role in numerous myxozoan infections.

In order to identify small windows of spore release and investigate the seasonality of different myxozoan species it would also be desirable to be able to test for their presence in the water column. Due to the presence of PCR inhibitors, it was not possible to amplify DNA from filtered water extracts. Special kits which are

designed for extractions from soil or water rich in suspended solids might be able to remove PCR inhibitors and allow the detection of myxozoans in water samples.

The successful relation of two of the three myxosporeans to their actinosporean counterparts in this study was based on the investigation of the natural river habitat rather than the artificial, organically enriched sediments of the fish farm area. Most actinosporean research has been focussed on such artificial habitats. Two oligochaete species which preferred the oligotrophic habitat of the highland stream to the outflow pond of the farm were found to be involved in the life cycle of two myxosporeans.

The molecular identity of the actinosporean and myxosporean stages detected in the study classifies them as alternate life cycle stages of one myxozoan species, but only the combination of infection studies and molecular identification of all stages allows the elucidation of the complete life cycle of a myxozoan species. However, molecular identification of the two life cycle stages is an invaluable basis for future studies on different aspects of the life cycles of these three myxozoans.

In the case of *S. truttae* the spatio-temporal development in the fish host, the Atlantic salmon, was studied using another molecular technique, *in situ* hybridisation (ISH). This highly sophisticated technique combines the specificity and sensitivity of a PCR detection with the possibility to observe the parasite in its biological context. A rapid protocol was developed, which allows the specific detection of *S. truttae* in morphologically well preserved host tissue. ISH was able to determine the gills as the predominant portal of entry of *S. truttae* into the fish, despite the small number of invasive sporoplasms occurring in the "natural" infection studied. This outlines the advantage of this unique approach as the complete developmental cycle in the fish has been elucidated for only one other species (*Myxobolus cerebralis*), for which the

actinosporean stage is well known and the study of its development in the host fish was based on a targeted infection using 10 000 infective stages per fish, which had been produced in an artificial system.

The large *M. cerebralis* triactinomyxon stage was found to enter the host mainly via skin and fins but also through the gills and buccal cavity (El-Matbouli & Hoffmann 1998), whereas the small aurantiactinomyxon spores of *Thelohanellus hovorkai* were observed to invade carp predominantly via the gill filaments (Yokoyama & Urawa 1997), as in *S. truttae.* This might indicate that the *S. truttae* actinosporean stage is also small. It is suggested that infection with small spore stages which are devoid of large floatation appendages is dependent on water currents in both freshwater and marine environments, and *S. truttae* has exclusively been reported from riverine habitats. The phylogenetically most recent freshwater myxozoans (histozoic species mainly of the genera *Myxobolus* and *Henneguya*) all develop large triactinomyxon or raabeia stages, perhaps indicating that in order to allow for the conquest of less turbulent (e.g. lacustrine) freshwater environments, evolutionary pressure lead to the development of appendages in order to be able to float in the standing water column and thus be able to infect non-benthic fish hosts.

Differences between phylogenetically more basal myxozoans and those which have evolved more recently also exist regarding their migration routes within the fish host, although, due to lack of data, analysis can only be based on a few species. In this study, *S. truttae* was found to use the blood stream as a rapid and direct route to the target organ. The presence of blood stages has also been reported for other *Sphaerospora* species (Lom & Dyková 1992) and is suggested for the marine myxozoan *Kudoa thyrsites* after the successful transmission of the parasite to uninfected fish by

intraperitoneal injection of blood from infected fish (Moran *et al.* 1999). In contrast, *M. cerebralis* migrates along the nervous tissue into the brain and from there into the cartilage (El-Matbouli & Hoffmann 1998). In comparison to the blood stages, this developmental route represents a more sophisticated type of migration as it escapes the host immune system and does not depend on an energy consuming, extensive proliferation in the blood in order to reach the target organ, as seen in *S. truttae.* Furthermore, the host tissue finding mechanism in *M. cerebralis* appears far more targeted than in *S. truttae,* where different non-target organs were found to be invaded to the same extent as the target organ. In order to close the large gap in the knowledge of migration routes within fish hosts, it would be important to conduct similar spatio-temporal development studies with other myxozoan species. The present study showed that ISH is, due to its sensitivity and specificity, currently the best methodology to investigate myxozoan pathways within their hosts.

For *S. truttae,* a highly specific, nested PCR assay was designed, and several thousand oligochaetes and other sedimentary invertebrates were screened but thus failed to identify the alternate invertebrate host of *S. truttae.* Only the investigation of phylogenetic relationships between *S. truttae* and the other myxozoans suggested an explanation for the failure to find the alternate host of *S. truttae. M. truttae, C. truttae* and *Chloromyxum* sp. are all members of the more recently evolved freshwater group in which oligochaetes have so far been identified as the only invertebrate host group, whereas *S. truttae* is located at the base of a generally marine group of myxozoan parasites of which only two members have known alternate hosts, both belonging to the class Polychaeta. The freshwater parasite *Ceratomyxa shasta* is one of these species and thus it was shown that, even in freshwater, this annelid group can serve as

an alternate host for myxozoans. All actinosporean spore types so far identified from polychaetes belong to the tetractinomyxon type (summarized by Køie 2002). This is a minute spore type and it would fit into the hypothesis posted earlier that the actinospore of *S. truttae* might be very small.

Polychaetes are a typically marine class of invertebrates which are rare in the freshwater environment and none were detected in the sediment samples in the course of this study, but they might be present in specific microhabitats. In the case of *Manayunkia speciosa,* the host of *C. shasta,* the polychaete was confined to the surface of the freshwater mussels *Margaritifera margaritifera* and of rocks (Bartholomew *et al.* 1997). If a polychaete inhabiting a similar habitat was involved in the life cycle of *S. truttae,* it would explain the unsuccessful screening of oligochaetes and other sedimentary invertebrates, as well as the unsuccessful infection studies when fish were exposed to oligochaetes in autoclaved sediment. However, Atlantic salmon were successfully infected once when fish were co-habited with sediment containing gravel and stones of various sizes and their invertebrate fauna. As errant, sedimentary polychaetes would not have escaped recognition in the screening of sediments in this study, it is suggested that in future investigations, rock surfaces, tree roots etc. should be screened for tube dwelling polychaetes as the next step towards the identification of the alternate host of *S. truttae.* Due to the position of *S. truttae* in a clade next to the most basal myxozoans, the malacosporeans, the possibility of bryozoans as hosts for this species should be borne in mind.

All other myxozoans found in the *S. truttae* enzooic habitat were found to be representatives of typical freshwater clades, and their sequences contributed considerably to the understanding of the clustering of the species inhabiting the

freshwater environment, as they were all representatives of genera which were absent or only scarcely represented on the genetic database. Due to this valuable collection of sequences, it is now possible to identify an evolutionary pathway for the freshwater myxozoans. This identifies parasites of the gall bladder epithelium as the initial immigrants into freshwater, from where they settled the excretory epithelia and finally became histozoic. This allows the interpretation of important differences in the development of different freshwater species as evolutionary adaptations. The more sophisticated pathway of *M. cerebralis* in comparison with *S. truttae* has already been mentioned. Furthermore, the pathology of freshwater myxozoans is related mainly to histozoic species (e.g. *Myxobolus cerebralis, Henneguya ictaluri, Thelohanellus hovorkai*), possibly due to the loss of the natural exit routes which exist for gall bladder, renal or urinary bladder parasites, and thus the dependency of histozoic species on the death of the host.

Another important finding was that spore morphology does not seem to play an important role in the evolution of the freshwater species despite its importance in the taxonomic classification system of the myxozoans. Features other than spore morphology are difficult to identify so that it may be inappropriate to change the nomenclature based solely on molecular data. However, it is suggested that molecular data should be included in all new descriptions of myxosporeans, as well as actinosporeans, as these data provide valuable information on the relationships between myxozoan species and therefore their identification.

Recently, studies on myxozoans have made rapid progress, resulting in more than 60 related publications annually. Two major findings have been responsible for the significant advance in our knowledege of the myxozoans. One of these is the discovery

of the life cycle of *M. cerebralis* in 1984 and the other is the elucidation of the taxonomic position of the Myxozoa using molecular systematics. In this study, molecular methods were chosen to describe myxozoan taxa, to identify the infection route of *S. truttae* in the fish host and to determine relationships and the phylogenetic coherence of the different taxa of a myxozoan community. The information gained by the use of these molecular methods is invaluable. However, molecular methods can only complete our understanding in combination with other techniques. Future approaches to close some of the numerous gaps in our knowledge of the myxozoans should aim at the following areas:

- Investigate vertebrate and invertebrate hosts other than teleosts and oligochaetes for their potential to serve as hosts for myxozoans.
- Comparing portals of entry and routes of infection in the vertebrate hosts, investigating myxozoans from along the phylogenetic tree, comparing e.g. typically marine with coelozoic freshwater species. Routes of infection could be investigated using ISH, followed by the study of the cellular development using ultrastructure.
- Host tissue localisation has been identified as an important factor in the myxozoan evolution but other factors, e.g. habitat, geographic distribution or alternate hosts are yet to be investigated.
- Other myxozoan genes have to be analysed in order to verify or refute observations of relatedness made on the basis of a single gene (18S rDNA).
- As there is a lack of taxonomic congruity between myxosporean and actinosporean morphology, but also an independence of myxosporean spore morphology from the degree of molecular relatedness, the functionality of myxsporean and actinosporean

spores in connection with the environment and the host to be infected should be analysed.

- Study of the infection process (does sexual reproduction in the invertebrate host occur in other species than *M. cerebralis*?) and the biology of the invertebrate hosts, e.g. life-span, reproduction, feeding habits and defence mechanisms, and investigations of the influence of anthropogenic activities in streams and rivers on the populations of invertebrate hosts are required.
- Finally, the identification of the alternate host of *S. truttae* would aid in the understanding of the (post-malacosporean) origins of the myxozoans.

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Tracing the route of *Sphaerospora truttae* from the entry locus to the target organ of the host, *Salmo salar* L., using an optimized and specific *in situ* hybridization technique

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Abstract

Sphaerospora truttae is an important pathogen of Atlantic salmon parr in Scottish aquaculture. To trace the early development of *S. truttae* and to overcome the common problem of detecting low numbers of cryptic, early myxosporean stages, a DNA-based approach was applied in this study. Specific primers were designed for *S. truttae* from Atlantic salmon, based on 18S rDNA sequences, obtained from isolated myxosporean spores. These were 5′ biotin-labelled and used in an optimized and rapid *in situ* hybridization (ISH) protocol, which provided a strong and specific signal of the parasite within host tissue sections and, at the same time, minimized structural damage to tissues due to processing. This methodology provided a reliable tool enabling the detection of *S. truttae* stages down to single cell level. Using ISH the epithelium of the gills was identified as the predominant entry locus of the parasite. By 3 days after infection *S. truttae* had penetrated the vascular epithelia and thereafter proliferated in the blood for at least 10 days before exiting the vascular system through capillary walls. From day 12 post-infection onwards, the kidney, as well as the spleen and the liver, were invaded. Numbers of *S. truttae* invading the kidney (37.3%) differed little from numbers found in the spleen (35.3%) and the liver (27.4%). The latter organs represented a dead end in the development of *S. truttae* as all stages in these organs degenerated and sporogony was found to take place exclusively inside the renal tubules. Early sporogonic stages were found from day 25 post-infection but mature spores only developed after at least 15 days of proliferation within the tubules.

Keywords: in situ hybridization, invasion, migration, Myxozoa, *Salmo salar*, *Sphaerospora truttae*.

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Introduction

Sphaerospora truttae was first described by Fischer-Scherl, El-Matbouli & Hoffmann (1986) from brown trout, *Salmo trutta* L., in Germany and has been a significant pathogen in the culture of Atlantic salmon, *Salmo salar* L., in Scotland. Extensive studies have been conducted on the morphology of presporogonic and sporogonic stages of *S. truttae*, and on the parasite's biology and seasonal dynamics (McGeorge, Sommerville & Wootten 1994, 1996a,b; McGeorge 1994). Infections can be detected in salmon fry 2 weeks after exposure to *S. truttae* enzootic water. At this point extrasporogonic stages are numerous and large enough to be detected in stained smears of blood and kidney.

The presumed small number, size and cryptic nature of the earliest stages of myxosporeans makes them difficult to detect and to differentiate from host cells using conventional histological techniques. Therefore, interest has increased in the use of DNA-based approaches for the diagnosis of these early parasite stages. One of these approaches employs *in situ* hybridization (ISH), the binding of labelled nucleic acid probes to complementary sequences in infected tissue sections. With the use of specific nucleic acid probes this method combines the specificity and sensitivity of DNA detection techniques with the ability to examine the parasite *in situ*. With regard to the myxozoans, this method has so far only been used to detect the causative

agent of whirling disease, *Myxobolus cerebralis*, at an early subclinical stage (Antonio, Andree, McDowell & Hedrick 1998), and to identify the portal of entry of the PKX organism, *Tetracapsuloides bryosalmonae* into the fish host (Morris, Adams & Richards 1999; Longshaw, Le Deuff, Harris & Feist 2002).

The present study aimed to investigate the entry locus as well as the spatio-temporal occurrence of *S. truttae* in the different organs of the Atlantic salmon up to the point of spore maturation in the target organ, the kidney. A rapid and optimized, biotin-based ISH protocol was applied using primers designed specifically on the basis of the 18S rDNA sequence of *S. truttae*.

Materials and methods

Source of material

Sediment was collected from the outflow area of a salmon hatchery on the north coast of Scotland. This site shows annually recurring *S. truttae* infections with 100% prevalence in salmon parr and was used in previous studies (McGeorge, Sommerville & Wootten 1997; Özer & Wootten 2000; Özer, Wootten & Shinn 2002). The sediment in the outflow area of the farm contains a high density of invertebrates and several species of oligochaetes, i.e. *Lumbriculus variegatus* (Müller), *Limnodrillus hoffmeisteri* Claparède, *Tubifex* spp., *Nais* spp., *Unicinais unicinata* (Ørsted), *Vejdovskyella comata* (Vejdovsky), *Pristina idrensis* Sperber, *Chaetogaster* sp., *Eiseniella tetraedra* L. and one undetermined representative of the family Enchytraeidae (unpublished observations). *Lumbriculus variegatus* and the tubificid species at this site were found to produce 21 different actinosporean spore types (Özer *et al.* 2002).

Ten salmon parr from the same hatchery containing mature myxosporean spores of *S. truttae* were used for spore isolation, DNA extraction and sequencing of *S. truttae*.

Naïve salmon fry employed in the experimental infection study were derived from a different farm site, which utilizes ozonated water in the hatchery and is known to be *S. truttae* free.

Experimental exposure of salmon fry

In May 2001, which is the beginning of the period when 0 + salmon become infected with *S. truttae* at the salmon hatchery described above, 23 L of sediment from the hatchery outflow and containing those oligochaetes listed above, were placed in a 180-L flow through tank. The identity of the actinosporean stages released by the oligochaetes was not determined. The tank was supplied with de-chlorinated tap water, aerated and maintained at a temperature of 14 ± 3 °C. In June 2001, 80 naïve salmon fry with an average length of 3.5 cm were introduced into the tank, separated from the sediment by a wire mesh (day 0). Thereafter, two fish were removed from the tank daily for 14 days, followed by one fish daily for the remainder of the experiment (46 days). A control tank of the same dimensions harboured 10 salmon fry and no sediment. One fish from the control tank was killed every week and examined for myxosporean parasites.

Tissue fixation and histological preparation

Fish were killed by an overdose of chlorobutanol (1,1,1-trichloro-2-methyl-2-propanol hydrate) and fixed whole in 4% neutral-buffered formalin for 24–48 h with their peritoneal cavity opened ventrally. Prior to further processing the fish were decapitated, the heads were sagittally halved and the remainder of the body was transversally trisected (first plane: head-kidney, liver, stomach; second plane: trunk kidney, spleen, gut; third plane: caudal peduncle). The samples were then dehydrated through an ethanol series and transferred into paraffin according to standard procedures. Six-micrometer sections were cut and mounted on 3-(aminopropyl)triethoxysilane (APES) coated slides and stored until used for ISH.

Design of specific oligonucleotide probes

Atlantic salmon kidneys containing mature *S. truttae* spores were homogenized in distilled water and filtered through a 50-μm nylon mesh. Thereafter spores were separated from the host cells using a two-phase system of 4.8% dextran and 3.76% polyethylene glycol (MW 6000). To achieve this, 4.8 mL of the filtrate containing *S. truttae* spores was mixed with 2.4 mL of a 20% dextran solution and 1.88 mL of a 20% polyethylene glycol solution in a 10-mL tube. After several inversions the two phases were left to separate for approximately 30 min. The spores settled in the upper polyethylene glycol layer whereas the tissue debris remained in the lower dextran phase. The spore-containing polyethylene glycol phase was washed twice with distilled water and centrifuged at 2000 ***g*** for 10 min.

The spore pellet was dissolved in DNA extraction buffer [50 mM Tris–HCl (pH 8), 100 mM EDTA (pH 8), 100 mM NaCl and 1% SDS]. Subsequently, the sample was digested overnight with 100 μg mL^{-1} proteinase K (Sigma, Dorset, UK) at 55 °C, and DNA was extracted using the Miniprep Express Matrix (Bio 101 Inc., La Jolla, CA, USA). The extracted DNA was resuspended in nanopure water and used for PCR.

Universal primers 18e and 18g (Hillis & Dixon 1991; Table 1) were used for the amplification of the small subunit (18S) ribosomal DNA. The PCRs were performed in 25 μL volumes with 1 unit of TITANIUM *Taq* DNA polymerase and the related 10× buffer containing 1.5 mM $MgCl_2$ (BD Biosciences Clonetech, Oxford, UK), 0.2 mM of each dNTP, 12.5 pmol of each primer, and 1 μL of template. Denaturation of DNA (95 °C for 3 min) was followed by 35 cycles of amplification (95 °C for 50 s, 65 °C for 50 s, and 72 °C for 1 min 10 s) and ended by a 7-min extension (72 °C). The approximately 2500-bp PCR product obtained was excised from a 0.75% agarose gel and purified using GFX PCR DNA and gel band purification spin columns (Amersham Pharmacia Biotech Inc., Little Chalfont, UK). Primers 18e, 18g, Act 1, 10 and 1032 (Table 1) were used for sequencing of the clean PCR product. The product was sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK), applied as a 1:4 dilution in Tris–HCl buffer (pH 9.0) in the terminator reactions. Twenty-microlitre reactions were prepared, run on a thermocycler and precipitated according to the manufacturer's instructions. The resultant DNA product was sequenced in an ABI PRISM 377 DNA sequencer (Applied Biosystems). On the basis of the obtained sequences (GenBank accession number AJ581915) and their alignment (BioEdit v 5.0.3) with numerous other myxozoan sequences from GenBank, four primers were designed in variable regions of the 18S rDNA. These oligonucleotides proved to be specific for *S. truttae* not only when used in a nested PCR for numerous specimens of different myxozoan genera, but also when applied to other species of *Sphaerospora* (Holzer, unpublished data). Two of these primers (StrIR and StrOR, Table 1) proved to be highly reactive for hybridization and were therefore used for the ISH. To test the specificity of the designed probes, sections of tissues infected with *S. renicola* (from *Cyprinus carpio* L.), *S. galinae* [from *Tinca tinca* (L.)], *S. elegans* (from *Gasterosteus aculeatus* L.) and *S. molnari* (from *C. carpio*) were used as controls for the ISH. The primers were synthesized and 5′ biotin-labelled by MWG Biotech AG (Ebersberg, Germany).

ISH technique

The ISH protocol used in this study is based on that of Morris *et al.* (1999). Several steps of the protocol were modified and optimized as appropriate for the probes and tissues used, and the incubation times, especially for the steps which damage tissue, were shortened as far as possible in order to maintain the tissue structure.

Sections were deparaffinized and rehydrated in a graded ethanol series and then equilibrated in tris-buffered saline (TBS, pH 8). For optimal probe penetration to target sequences, sections were permeabilized with 100 μg mL^{-1} of proteinase K in TBS (pH 8) for 25 min at 37 °C. Following incubation with the enzyme, sections were washed for 5 min in phosphate-buffered saline (PBS, pH 7.4) and post-fixed for 15 min with 0.4% paraformaldehyde in PBS. After a distilled water wash, sections were exposed to 10% H_2O_2 in methanol for 10 min in order to prevent nonspecific peroxidase binding. The H_2O_2 was washed off with distilled water and sections were subsequently dried at 45 °C, thus omitting a time-consuming prehybridization step. Thereafter the sections were encircled with a PAP-pen and equilibrated in

Table 1 Primers used for PCR, DNA sequencing and *in situ* hybridization

Name	Sequence	Reference
18e (F)	5′-TGGTTGATCCTGCCAGT-3′	Hillis & Dixon (1991)
18g (R)	5′-GGTAGTAGCGACGGGCGGTGTG-3′	Hillis & Dixon (1991)
Act 1 (R)	5′-AATTTCACCTCTCGCTGCCA-3′	Hallett & Diamant (2001)
10 (R)	5′-CACTCCACGAACTAAGAA-3′	Lin, Hanson & Pote (1999)
1032 (R)	5′-CGCTCCTCCAACTTTCGTTC-3′	Saulnier & DeKinkelin (1997)
StrIR	5′-GGACACCCACTACACCCATCT-3′	This study
StrOR	5′-AGACCTTCCGCACAGCCAACAGTA-3′	This study

hybridization buffer consisting of 4× saline-sodium citrate buffer (SSC) in TBS containing 0.5% Ficoll, 0.5% polyvinylopyrrolidone, 0.5% bovine serum albumin, 100 μg mL^{-1} calf thymus DNA, and 1.5 ng μL^{-1} of each oligonucleotide probe. Formamide was not included in the hybridization buffer because trials including formamide showed an undesirable increase in background signal. Sealed with a cover slip the sections were then denatured for 4 min at 95 °C, and the probes were left to hybridize to complementary sequences for 1 h at 45 °C. The incubation was followed by a nonstringent wash in 2x SSC in order to remove the cover slip and unbound probes. A high-stringent wash of 0.1% SSC containing 0.1% TWEEN20 at elevated temperature (45 °C) then ensured the specificity of binding of the probes to the target DNA only. The hybridization signals were detected by incubation with 1/100 horseradish peroxidase-labelled streptavidin (SAPU, Scottish Antibody Production Unit, Edinburgh, UK) for 20 min. This step was followed by three thorough washes in PBS for 5 min each and the final visualization of the signals with the substrate (VIP chromogen; Vector Laboratories, Peterborough, UK), resulting in a purple signal; 0.5% methyl green was applied for 5 min in order to counterstain the sections. After rapid dehydration in 95 and 100% alcohol and transfer into xylene, sections were mounted in Pertex.

Parasite numbers

In order to study the proliferation processes of *S. truttae* in different host organs, the parasites present within the fish at different stages of development were quantified in kidney, spleen and liver of 12 individual fish (days 15–26 post-infection). The organs were manually outlined on digital images of sections and their surface areas were calculated (Zeiss KS300, v 3.0; Imaging Associates Limited, Thame, UK). The number of parasites within the tissues and the vascular system was then counted on a sufficient number of sections to examine 1 mm^2 of each organ. From these data the proportion of parasites in the different organs and the related vascular system as a percentage of the total number counted per fish was calculated. This enabled comparisons of the organs between different fish independent from the intensity of infection, which varied between individuals.

Results

Outcome and specificity of the ISH protocol

The ISH protocol used here proved to be a sensitive tool for the rapid, specific, high contrast detection of *S. truttae* developmental stages down to the single cell level. Successful binding of the probes to *S. truttae* target sequences and therefore presence of the parasite, was observed as a purple signal in greenish blue-stained target tissues, which were morphologically well preserved using the ISH method developed here.

Furthermore, the ISH protocol proved to be specific for *S. truttae* and, under the given conditions of stringency, did not bind to *S. renicola*, *S. galinae*, *S. elegans* and *S. molnari*. Fish from the uninfected control group never showed binding of the labelled probes to cells in the tissues.

No other myxosporean stages were observed in the experimental fish and no other myxosporean species have been observed in salmon from the hatchery over a period of 10 years.

Entry locus and spatio-temporal occurrence of *S. truttae* in host tissues

Using ISH, the development of *S. truttae* within the fish host from the initial infection to the development of mature spores was observed. The migration and development of *S. truttae* in the Atlantic salmon requires approximately 40 days at 14 °C and is summarized in Fig. 1.

On day 1 following initial exposure of salmon fry to the presumed waterborne infective stage of *S. truttae*, ISH identified the epithelium of the secondary gill lamellae as the predominant entry locus of the parasite (Fig. 2a,b). To a much lesser extent, *S. truttae* was also found to invade its host through the epithelium of the body surface and fins. Penetration via the gut epithelium was never observed. The stages entering the fish appeared as multicellular units consisting of up to eight cells each with a diameter of 1.8–2.5 μm. The initial epithelial penetration of *S. truttae* was not observed and is assumed to be very rapid. However, on day 1 hybridization signals were frequently found within mucous cells of the gill epithelium (Fig. 2c,d). These mucous cells usually contained a single (rarely two) spherical structure 2.5 μm in diameter. This could represent a polar capsule, which is left behind as the sporoplasm of the presumed infective spore enters the fish host. On the same day,

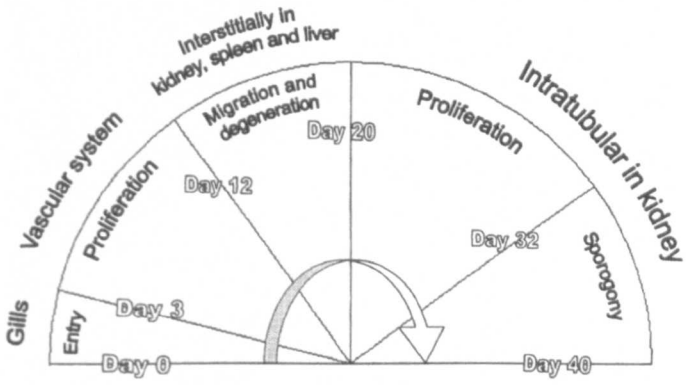


Figure 1 Schematic summary of the developmental cycle of *Sphaerospora truttae* in the Atlantic salmon, *Salmo salar*.

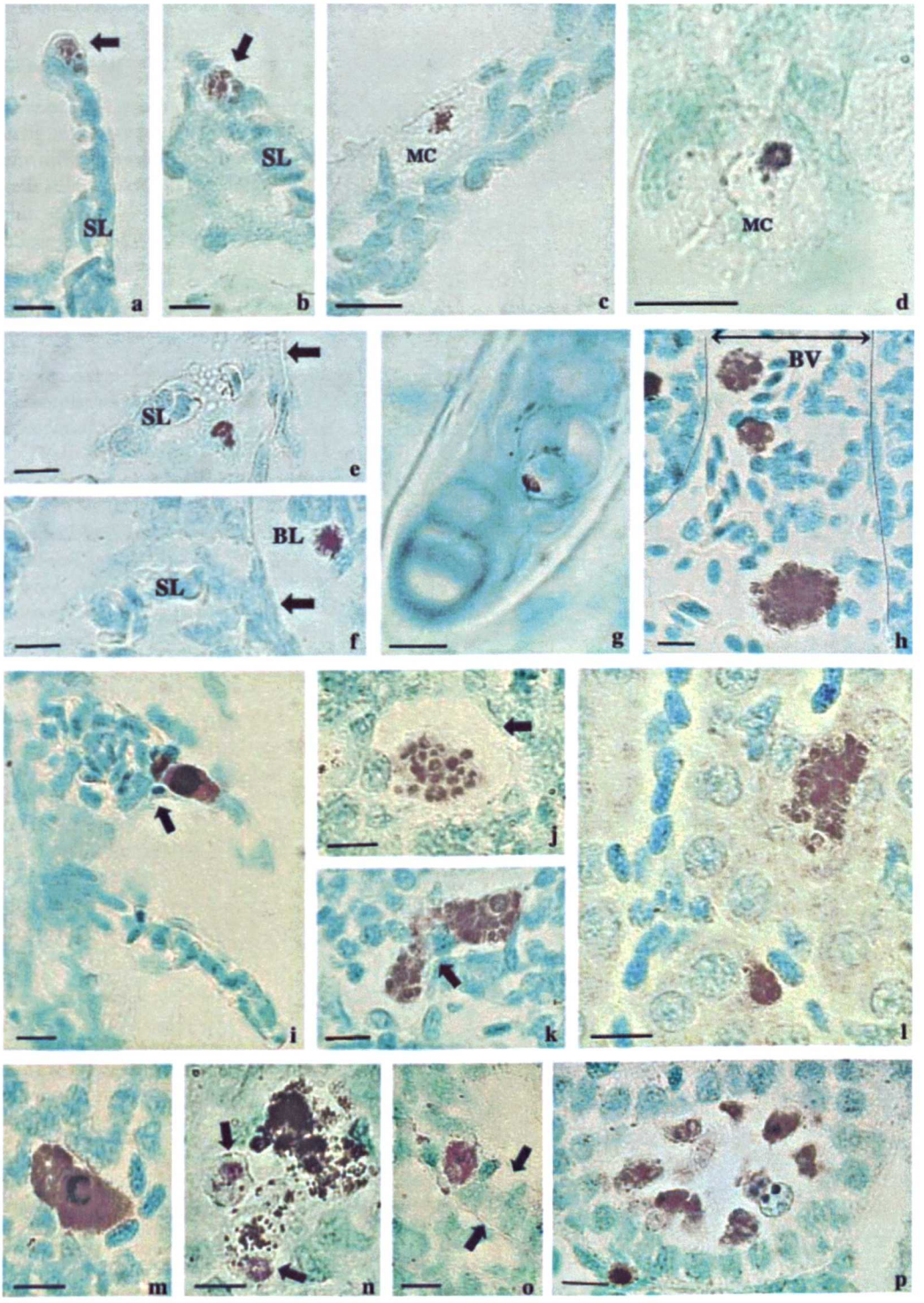
multicellular parasites were also recognizable at the base of the secondary lamellae where these, now migratory stages entered the vascular system through the blood vessel walls (Fig. 2e). On day 3, *S. truttae* was first observed in the lumen of blood vessels (Fig. 2f). At this time most parasites were seen either within the epithelial layer of the gills (possibly representing new entrants) or within the vascular system, but a small number were also found inside lacunae of the cartilaginous tissue of the gill lamellae (Fig. 2g).

Rapid proliferation of the parasite within the vascular system was observed resulting in increasing numbers of stages of variable size and cell numbers ranging from 2 to approximately 120 (Fig. 2h). These stages were readily visible in all blood rich organs, i.e. gills, heart, spleen, kidney and liver of the infected salmon fry. Occasionally, large stages with more than 30 cells were observed to obstruct capillaries, e.g. in the secondary gill lamellae (Fig. 2i).

The large multicellular blood stages accumulated in the capillaries in spleen, liver and kidney (Fig. 2j). Attachment of the parasites to the vessel walls was followed by their penetration (Fig. 2k) and the entry into the surrounding tissue, observed for the first time on day 12. Intercellular occurrence was not restricted to the target organ, the kidney, but was also observed in spleen and liver (Fig. 2l). During this histozoic occurrence of *S. truttae*, the parasites did not show evidence of proliferation. In individual fish, parasite numbers in the tissues were generally lower than in blood vessels from the same organ (Table 2). Surprisingly, site preference of *S. truttae* for the organ of sporogony, the kidney, was not obvious. Of all the histozoic stages encountered in 12 individual fish, the overall proportion in the kidney (37.3 ± 3.6%) was significantly but marginally higher than the proportion in the liver (27.4 ± 5%), but not significantly different from the proportion in the spleen (35.3 ± 4.7%) (Table 2).

Incidental degeneration of the intercellular *S. truttae* stages was a common feature in all organs including kidney. Disintegration of the multicellular structure (Fig. 2m) was followed by engulfment of the parasites by macrophages (Fig. 2n), observed for the first time on day 16. In spleen and liver all parasites died and only a weak ISH signal within the macrophage cytoplasm indicated their former presence. Further development of *S. truttae* took place exclusively in the kidney. From day 20 onwards, intercellular stages were observed to penetrate the renal tubules (Fig. 2o) and occasionally also the glomeruli. Individual multicellular parasites were then found in the tubular lumina. Thereafter, the number of *S. truttae* stages in infected tubules increased quickly, gradually filling the tubules and slightly dilating them. This was followed by the transition into sporogony (Fig. 2p). Immature, developing spores inside disporous pseudoplasmodia were first detected on day 32. Mature spores with readily distinguishable polar filaments inside the polar capsules and spore valves exhibiting surface ridges were only present from day 40 onwards.

Blood and histozoic stages were found together with sporogonic stages in the renal tubules until the end of the experiment on day 60. Continuous re-infection of fry due to continual exposure to the infected sediment in the tank cannot be excluded. The development of spores was observed to be nonsynchronous, thus undifferentiated early sporogonic stages were present alongside mature spores.



Discussion

Due to their small number, size and cryptic nature, early myxosporean developmental stages are generally difficult to detect and to differentiate from host cells using conventional histological techniques. In this study, the hybridization of specific labelled oligonucleotides to target DNA *in situ* in a biotin-based ISH protocol was successfully modulated in order to achieve specific identification and detection of *S. truttae* in host tissues and to elucidate the entry and early development of *S. truttae* in its host, *Salmo salar*. The rapid, high-contrast method demonstrated the complete spatio-temporal development of the parasite in the fish host.

There is little available information on the early development of other myxozoan species in their fish hosts. The complete details of development have been resolved only for *M. cerebralis* (El-Matbouli & Hoffmann 1998). In the case of *M. cerebralis*, the related triactinomyxon form actinospore was observed to enter rainbow trout over the entire epidermis, the buccal cavity, and the respiratory epithelial cells of the gills (El-Matbouli, Hoffmann, Schoel, McDowell & Hedrick 1999). Other studies on the entry of *Henneguya ictaluri* (Belem & Pote 2001) and *T. bryosalmonae* (Morris, Adams & Richards 2000; Longshaw *et al.* 2002) also suggest a nonspecific route of entry of these myxozoans into their fish hosts.

The gills appear to be the predominant entry site of *S. truttae* into the Atlantic salmon. Only occasionally were single parasites observed in the body epidermis. Yokoyama & Urawa (1997) suggested that small

Table 2 Total numbers and relative percentages (parenthesis) of *Sphaerospora truttae* in 1 mm^2 of kidney, liver, spleen and the related vascular system

	Kidney[a]		Spleen[a]		Liver[a]		Organ totals[b]			
	Tissue	Blood	Tissue	Blood	Tissue	Blood	Kidney	Spleen	Liver	
Fish 1 (day 15)	2 (40.0)	3 (60.0)	1 (25.0)	3 (75.0)	3 (60.0)	2 (40.0)	5 (35.7)	4 (28.6)	5 (35.7)	
Fish 2 (day 16)	6 (46.2)	7 (53.8)	5 (55.6)	4 (44.4)	2 (28.6)	5 (71.4)	13 (44.8)	9 (31.0)	7 (24.2)	
Fish 3 (day 17)	5 (45.5)	6 (54.5)	4 (36.4)	7 (63.6)	4 (57.1)	3 (42.9)	11 (37.9)	11 (37.9)	7 (24.2)	
Fish 4 (day 18)	4 (57.1)	3 (42.9)	2 (28.6)	5 (71.4)	3 (42.9)	4 (57.1)	7 (33.3)	7 (33.3)	7 (33.3)	
Fish 5 (day 19)	8 (40.0)	12 (60.0)	11 (52.4)	10 (47.6)	5 (50.0)	5 (50.0)	20 (39.2)	21 (41.2)	10 (19.6)	
Fish 6 (day 20)	3 (60.0)	2 (40.0)	2 (40.0)	3 (60.0)	1 (25.0)	3 (75.0)	5 (35.7)	5 (35.7)	4 (28.6)	
Fish 7 (day 21)	7 (53.8)	6 (46.2)	5 (50.0)	5 (50.0)	5 (45.5)	6 (54.5)	13 (38.2)	10 (29.4)	11 (32.4)	
Fish 8 (day 22)	1 (33.3)	2 (66.7)	1 (25.0)	3 (75.0)	0 (0)	2 (100)	3 (33.3)	4 (44.4)	2 (22.2)	
Fish 9 (day 23)	10 (50.0)	10 (50.0)	9 (56.3)	7 (43.7)	5 (41.7)	7 (58.3)	20 (41.7)	16 (33.3)	12 (25.0)	
Fish 10 (day 24)	4 (36.4)	7 (63.6)	5 (45.5)	6 (54.5)	4 (57.1)	3 (42.9)	11 (37.9)	11 (37.9)	7 (24.1)	
Fish 11 (day 25)	8 (50.0)	8 (50.0)	8 (53.3)	7 (46.7)	7 (58.3)	5 (41.7)	16 (37.2)	15 (34.9)	12 (27.9)	
Fish 12 (day 26)	3 (37.5)	5 (62.5)	5 (55.6)	4 (44.4)	4 (50.0)	4 (50.0)	8 (32.0)	9 (36.0)	8 (32.0)	
Total (average% ± SD)	61 (45.8 ± 8.6)	71 (54.2 ± 8.6)	58 (43.6 ± 12.2)	64 (56.4 ± 12.2)	43 (43.0 ± 17.6)	49 (57.0 ± 17.6)	132 (37.3 ± 3.6)	122 (35.3 ± 4.7)	92 (27.4 ± 5.0)	

[a] Percentages related to parasites occurring in the tissue vs. the vascular system of each organ.
[b] Percentages related to distribution of parasites over the different organs.

Figure 2 *In situ* hybridization showing the spatio-temporal migration of *Sphaerospora truttae* in its host, *Salmo salar*: parasite stages in purple, background green-blue. (a, b) Entry of the parasite (arrows) through the secondary lamellae of the gills (SL). (c, d) Single *S. truttae* cells inside mucous cells (MC) in the gills. (e, f) Migration from the secondary lamellae (SL) through blood vessel wall (arrows) into the vascular system (BL). (g) Parasite inside a lacuna of the gill cartilage. (h) Blood stages of different size inside a vessel (BV) in the kidney. (i) Large parasite stage causing congestion of blood (arrow) in lamellae. (j) Multicellular parasite inside a capillary in the kidney. (k) *Sphaerospora truttae* leaving the vascular system by breaking through the vessel epithelium (arrow) and entering the interstitial tissue of the kidney. (l) Multicellular interstitial stages in the liver. (m) Degenerating stage of *S. truttae* showing loss of structure. (n) Remains of *S. truttae* inside melanomacrophages (arrows). (o) Migration through epithelium (arrows) of renal tubule. (p) Sporogonic stages and a mature spore inside a renal tubule. All bars = 10 μm.

actinosporean stages (e.g. aurantiactinomyxon) invade the fish host through the gills whereas large actinospores (e.g. triactinomyxon and raabeia) attach mainly to the skin. Özer & Wootten (2000) suggested the actinosporean stage of *S. truttae* was an echinactinomyxon type. Sequencing data suggest that this is incorrect (unpublished data). However, it is possible that morphologically different spores show passive selection of the entry locus due to variations in water flow, e.g. through the buccal cavity, which might allow some actinospore morphotypes to attach more easily in particular areas. The presence of hybridization signals for *S. truttae* within the mucous cells suggest an entry through the secretory openings of these cells (as in case of *M. cerebralis*, El-Matbouli *et al.* 1999) and perhaps suggests chemotactic orientation of the spores towards the acidic mucus. Differences in the composition of mucus released in the intestine and the skin of eels can initiate a remarkably different intensity of chemotactic response in *Vibrio anguillarum* (O'Toole, Lundberg, Fredriksson, Jansson, Nilsson & Wolf-Watz 1999). Therefore, active selection of an entry site following chemical cues cannot be excluded in myxozoans. Comparison of the reaction of actinospores exposed to mucus from intestine, skin and gills might elucidate this question.

Whatever the mechanism, *S. truttae* targets entry of the Atlantic salmon at a site with a well-established vascular system close to the outer surface of the host, which must increase the possibility of the parasite reaching the blood and decrease the possibility of encountering other tissues which represent a dead end (e.g. the cartilage).

Two days after exposure to the infective stage ISH successfully detected *S. truttae* intravascularly in the salmon. The occurrence of blood stages has so far only been reported from the genus *Sphaerospora* among the myxozoans (McGeorge 1994). There is then a minimum of 10 days residence in the vascular system, which is perhaps necessary for the uptake of nutrients from the blood in order for the rapid proliferation, observed here, to take place.

It might be expected that exit of *S. truttae* from the vascular system would target the invasion of a suitable site for spore production and spore release from the host. Despite the importance of such a site, the rather unspecific 'selection' of kidney, spleen and liver observed is possibly induced by some very general cues, e.g. changes in pH or blood oxygen levels in these organs.

The number of parasites entering the kidney was only slightly greater than those entering the spleen and the liver, suggesting there is no specific recognition of the target organ at this stage. The number of parasites in the tissues tended to be slightly lower than in the vascular system in each organ. The proliferation observed in the blood does not continue once the intercellular location is reached. Given the commonly observed mortality and breakdown of the intercellular stages, the survival and further development of *S. truttae* seems to be very dependent on the number of parasites produced in the vascular system. It remains unclear why 'histozoic' stages break down in all organs but it is suggested that they survive only for a short period in the tissues, and further development is restricted to the kidney tubules. The intercellular stages in liver and spleen do not continue their development but die and become engulfed by macrophages. This ingestion might serve as a basis for the immunity to renewed infection with *S. truttae*, described by McGeorge (1994).

Further development of *S. truttae* is bound to the intratubular location. Single parasite stages enter renal tubules from day 25 onwards and appear to undergo further cycles of proliferation for at least 10 days before they switch into sporogony, resulting in the production of high numbers of spores. Proliferation within renal tubules is suggested by the fact that initially small numbers of tubules are found containing single parasites. Subsequently the number of infected tubules does not appear to increase much, but the number of parasites per tubule does increase considerably. Due to the asynchronous development of the disporous pseudoplasmodia mature spores are released over several months (McGeorge 1994).

In this study, ISH proved to be a highly valuable tool for the investigation of the complete spatio-temporal migration of *S. truttae* during its development in the fish host. Furthermore, ISH allowed detection of small numbers of *S. truttae* in a low-level infection, without targeted exposure to massive numbers of infective spore stages. Thus ISH has a high potential to identify myxozoan pathways without knowing the actinosporean involved in the life cycle of the investigated species. Due to the specificity of the designed DNA probes this technique also provides confirmatory identification of the parasite before the development of identifiable spore stages.

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Molecular relationships and phylogeny in a community of myxosporeans and actinosporeans based on their 18S rDNA sequences☆

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Abstract

The community of myxosporeans and actinosporeans inhabiting a typical Scottish highland stream and the outflow area of an adjacent salmon hatchery was analysed on the basis of their 18S rDNA sequences. Nine myxosporeans belonging to the genera *Sphaerospora*, *Chloromyxum*, *Zschokkella*, *Myxidium*, *Hoferellus* and *Myxobilatus* were identified from mature spores in different organs of the fish species present. Twelve actinosporean types belonging to the collective groups of neoactinomyxum, aurantiactinomyxon, raabeia, echinactinomyxon and synactinomyxon were found to be released from oligochaete worms collected from sediments. Twenty of the 21 sequences obtained from these myxozoans are new entries to the myxozoan database, and the genera *Chloromyxum*, *Hoferellus* and *Myxobilatus* were entered for the first time. Study of the molecular relationships between the different taxa and with other myxozoan sequences available showed that the myxosporeans inhabiting the urinary system clearly cluster together, independently of host species or spore morphology. However, the sequences of the two *Sphaerospora* species encountered show considerable differences from other members of this group and all other freshwater myxosporeans, and they were found to occupy an ancestral marine position. Three actinosporeans, i.e. *Neoactinomyxum eiseniellae*, *Aurantiactinomyxon pavinsis* and *Raabeia* 'type 3' were found to represent alternate life cycle stages of *Chloromyxum* sp., *Chloromyxum truttae* and *Myxidium truttae*, respectively (approximately 1400 identical base pairs each). Three other actinosporeans encountered (two echinactinomyxon and one raabeia type) showed over 92% sequence identity with myxosporeans from GenBank™, whereas all other actinosporeans formed a closely related group devoid of any known myxosporeans.

Keywords: Myxosporea; Actinosporea; 18S rDNA; Phylogeny; Evolution; Tissue tropism

1. Introduction

Since the discovery of the alternate actinosporean triactinomyxon stage in the life cycle of the myxosporean *Myxobolus cerebralis* (Wolf and Markiw, 1984), actinosporeans and their annelid hosts have been demonstrated to be involved in the life cycle of 25 further myxosporeans (review by Kent et al., 2001; Køie et al., 2004). With the growing interest in the actinosporeans as infective stages to fish, several studies have been conducted focussing mainly on the actinosporean fauna in ponds where myxosporeans are pathogenic to cultured fish species (Burtle et al., 1991; Pote and Waterstrat, 1993; Yokoyama et al., 1993; Grossheider and Körting, 1993; Özer et al., 2002a,b; Oumouna et al., 2003). A smaller number of papers also report oligochaetes and their actinosporeans in natural habitats (Xiao and Desser, 1998a,b; Negredo and Mulcahy, 2001; Szekely et al., 2003).

The alternate actinosporean and myxosporean life cycle stage of a myxozoan species can be identified either by controlled infection studies, which include both hosts or by analysis of DNA sequence data obtained from both life cycle stages. Several research groups have now recommended the inclusion of DNA sequences in the identification of actinosporeans (e.g. El-Mansy et al., 1998;

☆ Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers AJ581916, AJ582061, AJ609590, AJ582063, AJ581917, AJ582213, AJ581918, AJ582062, AJ582004, AJ582005, AJ582006, AJ582000, AJ417562, AJ582001, AJ582008, AJ582009, AJ582010, AJ582002, AJ582003, AJ582007.

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Hallett et al., 1999; Negredo et al., 2003), and small subunit ribosomal (18S rDNA) sequences have been successfully used for the confirmation of the different life cycle stages of *M. cerebralis* (Andree et al., 1997), *Ceratomyxa shasta* (Bartholomew et al., 1997), *Tetracapsuloides bryosalmonae* (Anderson et al., 1999), *Henneguya ictaluri* (Lin et al., 1999), *Thelohanellus hovorkai* (Anderson et al., 2000) and most recently for *Ellipsomyxa gobii* (Køie et al., 2004), the first marine myxozoan whose life cycle has been elucidated.

The introduction of molecular data also enabled the study of phylogenetic relationships between different myxozoan taxa. Spore morphology is often used as a sole criterion for higher taxonomy of the myxosporeans and their separation into orders and genera. When 18S rDNA sequences were first applied to the taxonomy of myxosporeans, discrepancies soon arose between the traditional taxonomic scheme based on spore morphology and the findings from molecular data (Kent et al., 1994; Smothers et al., 1994; Siddall et al., 1995; Schlegel et al., 1996). Recent molecular studies employing increasing numbers of species agree on the paraphyly of almost all of the traditional myxozoan 'genera' (Kent et al., 2001; Palenzuela et al., 2002; Molnar et al., 2002; Dyková et al., 2002; Negredo et al., 2003; Whipps et al., 2003). However, despite the growing data set, sequences for some genera (e.g. *Chloromyxum, Hoferellus*) are still unavailable and only a few representatives of others (e.g. *Myxidium, Sphaerospora*) are available.

In the current study, the community of myxosporeans and actinosporeans sharing the natural habitat of a typical Scottish highland stream and the outflow area of an adjacent salmon hatchery was analysed to provide a comprehensive inventory of the myxozoans present and their molecular characterisation on the basis of the 18S rDNA. At the same time the molecular relationships between the myxosporeans and the actinosporeans encountered were determined and compared with other published myxozoan sequences. This is the first study investigating both actinosporean and myxosporean life stages from the same habitat analysing their molecular phylogeny on the basis of a collective data set of 18S rDNA sequences.

2. Materials and methods

2.1. Location

The study site is a typical, acidic, oligotrophic highland freshwater system consisting of small tributaries and lochs, which flow into a medium-sized river named Amhainnan Stratha Bhig (58° 27′ N and 4° 45′ W; Fig. 1). This river, which runs into a sea loch (Loch Eribol) on the Northwest coast of Scotland represents a natural spawning ground for Atlantic salmon. A small, commercial salmon hatchery is located at the lower third of the river (Fig. 1). This farm has annually recurring infections of *Sphaerospora truttae* with 100% prevalence in parr and its eutrophic and oligochaete enriched outflow settlement pond was used in previous studies (McGeorge et al., 1997; Özer and Wootten, 2000; Özer et al., 2002a,b.

2.2. Oligochaete sampling

Oligochaetes were sampled from sandbanks and gravel deposits with up to an average particle diameter of up to 3 cm at various sites in the lower two thirds of the main river (Fig. 1) and from the farm outflow between November 2000 and July 2003. Sediment was collected from completely submerged substrate as well as from the water–land-interphase. The sediment from the river and the outflow of the farm was collected with a spade and placed in a 10 l bucket. The bucket was filled with stream water and the contents were stirred by hand. Oligochaetes were thus elutriated from the sediment and the eluate was passed through a cascade of sieves (1 mm, 500 and 100 μm). The oligochaetes were then collected by rinsing them from the sieves.

After transport to the laboratory, the worms were transferred into 24 and 96 cell well plates, depending on their size, as described by Yokoyama et al. (1991). The oligochaetes were kept in the wells 7–10 days at ambient temperature and light conditions outside. Every second day the water and the faecal pellets in the wells were scrutinised for actinosporean spores, using an inverted microscope on phase contrast at ×150. When spores were detected they were measured, identified (Janiszewska, 1957, Marques, 1984) and for sequencing, three samples of the same spore type were collected from three individual worms (a single sample was used for sequencing in those cases where a spore type was found only once). For DNA extraction, the spores were then pelleted by centrifugation (2000×*g*). The oligochaete hosts were identified according to the methods and key of Brinkhurst (1971).

2.3. Fish sampling

In order to obtain a representative sample of the fish population in the river, 100 fish were caught by electrofishing in the lower third of the river in October 2002. Several areas of the main river, as well as a stream which connects the river to an adjacent loch, and three tributaries were fished (Fig. 1). The fish were sorted according to size and species and then transported to the Institute where they were kept in flow-through tanks supplied with aerated, dechlorinated mains water at ambient temperature. Within the following 2 weeks, the fish were sacrificed by an overdose of chlorobuthanol (1,1,1-trichloro-2-methyl-2-propanol hydrate) and squash preparations of skin, gills, muscle, brain, heart, liver, gall bladder, spleen, kidney, intestine and gonads of each fish were examined for the presence of myxosporeans. The prevalence of each species found was determined, their spore types were measured

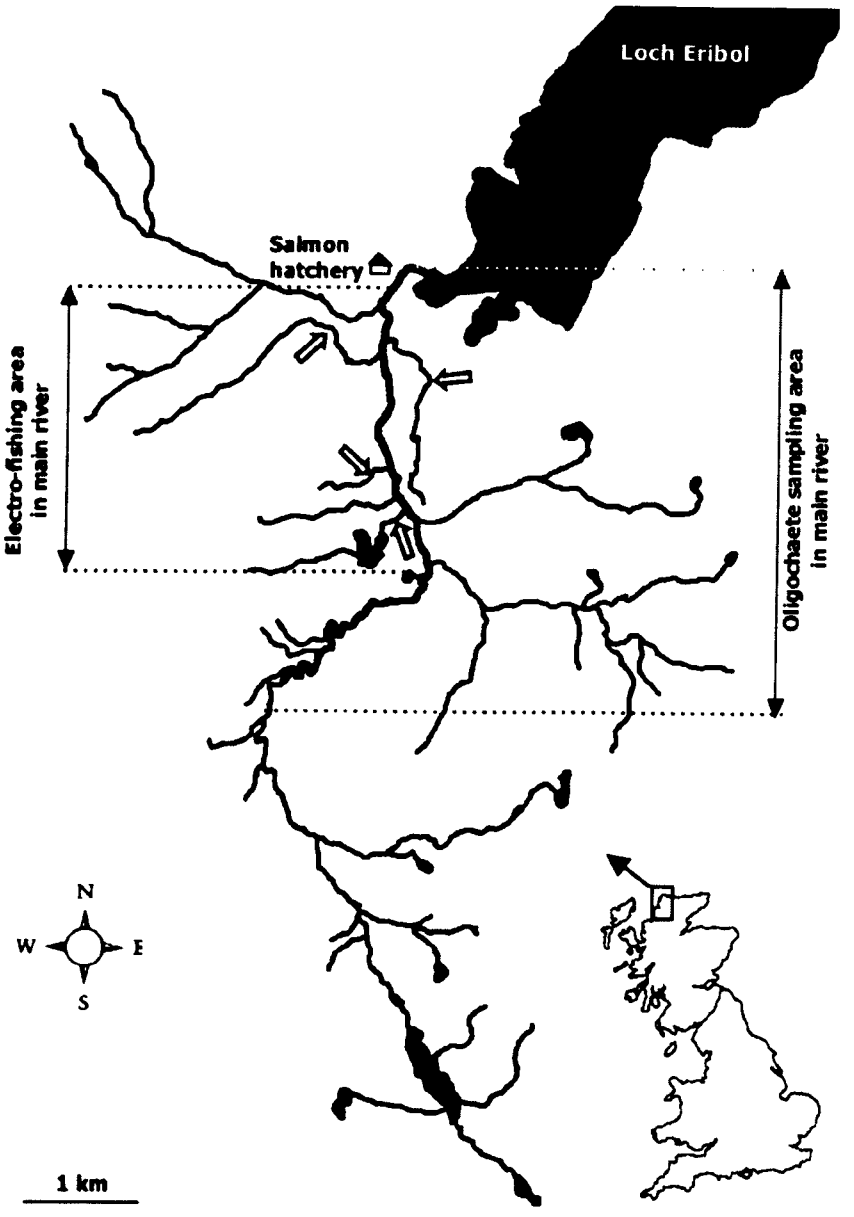

Fig. 1. Location and outline of the river system sampled on the North coast of Scotland. Location of the salmon farm and sampling areas for fish and oligochaetes in the main river are indicated. Arrows indicate the tributaries which were also sampled for fish.

and identified, and three samples of organs infected with the same species but from different fish were submitted to sequencing.

2.4. DNA extraction, PCR amplification and sequencing

The actinosporean pellets and infected organs from the fish were dissolved in 300 µl of TNES urea DNA extraction and conservation buffer (10 mM Tris–HCL (pH 8), 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea). The samples were digested with 100 µg/ml Proteinase K overnight at 55 °C. Thereafter, DNA was extracted by adding 300 µl of phenol and later 300 µl of chloroform, and mixing well by overend turning. The phases were then separated by centrifugation at 15,000×*g* for 5 min; the aqueous top layer was removed to a fresh tube and DNA was precipitated by mixing the sample with 3 volumes (900 µl) of 92% ethanol. After pelleting (20 min at 15,000×*g*), the DNA was washed with 1 ml of 70% ethanol, dried for 3–5 min in a vacuum centrifuge and left to re-suspend in 50–500 µl of RNAse/DNAse-free water overnight in a fridge.

From the obtained DNA samples 18S rDNA was amplified using universal eukaryotic 18S primers, 18e and 18g (Hillis and Dixon, 1991). PCRs were performed in 30 µl

volumes with 1.5 units of TITANIUM Taq DNA polymerase and the related 10×buffer containing 1.5 mM $MgCl_2$ (BD Biosciences Clonetech), 0.2 mM of each dNTP, 15 pmol of each primer, and 2 μl (10–150 ng) of template. Denaturation of DNA (95 °C for 2 min) was followed by 35 cycles of amplification (95 °C for 50 s, 65 °C for 50 s, and 70 °C for 1 min 20 s) and ended by a 4 min extension (70 °C). The 18S PCR products were diluted 1:6 in nanopure water for use in a nested PCR employing more specific myxozoan primers, Myxgp2F (Kent et al., 1998) and Act1R (Hallett et al., 2002) PCR ingredients and cycles were the same as for the first PCR with an annealing temperature of 58 °C instead of 65 °C. The approximately 900 bp nested PCR product obtained was excised from a 1% agarose gel in Tris-acetate-EDTA (TAE) and purified for sequencing using GFX PCR DNA and gel band purification spin columns (Amersham Pharmacia Biotech Inc.). Primers Myxgp2F, Act1R and LIN3F (Lin et al., 1999) were used for cycle sequencing of the 18S fragments in an ABI PRISM 377 DNA sequencer using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

For a more detailed investigation of some closely related species a second 18S fragment was produced in another nested PCR reaction. In this reaction, primers Myx1F (Hallett et al., 2002) and MX3 (Andree et al., 1998) were employed at an annealing temperature of 66.5 °C, and the products were sequenced using primers Act3F (Hallett et al., 2002), MX3 and LIN10 (Lin et al., 1999). Joining the Act3/MX3 fragments with the correlating Myxgp2F/Act1R fragments produced consensus sequences of approximately 1550 bp. Due to the non-specificity of the primer MX3 and the low level infection with *Chloromyxum* sp. in the kidney tubules, this species could only be amplified and sequenced using the more specific reverse primer MyxUrinR (this study) in combination with Myxgp2F. Due to its proposed identity with *S. truttae* (Özer and Wootten, 2000) the complete 18S rDNA gene of echinactinomyxon 'type 5' was sequenced (18e/18g fragment). All sequences were submitted to GenBank™. Primer sequences and their location and references are summarised in Table 1.

Table 1
Primers used for PCR and sequencing and their approximate location on a 2000 bp myxozoan model 18S gene

Name	Sequence	Location	Reference
18e	5′-TGG TTG ATC CTG CCA GT-3′	1	Hillis and Dixon (1991)
18g	5′-GGT AGT AGC GAC GGG CGG TGT G-3′	2000	Hillis and Dixon (1991)
Myxgp2F	5′-TGG ATA ACC GTG GGA AA-3′	130	Kent et al. (1998)
Act1R	5′-AAT TTC ACC TCT CGC TGC CA-3′	1060	Hallett et al. (2002)
Myx1F	5′- GTG AGA CTG CGG ACG GCT CAG-3′	80	Hallett et al. (2002)
Act3F	5′-CAT GGA ACG AAC AAT-3′	900	Hallett et al. (2002)
MX3	5′-CCA GGA CAT CTT AGG GCA TCA CAG A-3′	1770	Andree et al. (1998)
3LINF	5′-GCG GTA ATT CCA GCT CCA-3′	590	Lin et al. (1999)
10LINR	5′-CAC TCC ACG AAC TAA GAA-3′	1450	Lin et al. (1999)
MyxUr-inR	5′-TGG TTG TCT TTT CAT AGC ACA TT-3′	1780	This study

2.5. Sequence alignment and phylogenetic analysis

Alignments of the newly obtained sequences with each other and with other myxozoan sequences from GenBank™ were performed using CLUSTAL X v1.18 (Thompson et al., 1994; Thompson et al., 1997). The alignment was then manually corrected to eliminate minor inconsistencies between different taxa and with reference to known elements of secondary structure (Van De Peer and De Wachter, 1997). Parsimony analysis as well as maximum-likelihood analysis were performed using the Phylogeny Inference Package (PHYLIP version 3.5c; Felsenstein, 1993, University of Washington, Seattle), applying a transition:transversion ratio of 2:1, empirical base frequencies, one rate class for nucleotide substitutions across sites, and global branch rearrangements. The robustness of the positioning of the taxa on the final cladogram was assessed using bootstrapping with 100 replicates.

Polypodium hydriforme was used as outgroup assuming a cnidarian origin of the myxozoans (Siddall et al., 1995; Siddall and Whiting, 1999).

3. Results

3.1. Myxosporean fauna

The proportions of fish caught in the river were 49% Atlantic salmon *Salmo salar* L., 29% brown trout *Salmo trutta* L., 19% eel *Anguilla anguilla* L. and 3% stickleback *Gasterosteus aculeatus* L. Nine different myxosporean species belonging to six different genera were found in the examined fish. Their tissue localisation, prevalence and the measurements of the spores, together with the length of the sequenced 18S rDNA fragment and the GenBank™ accession number are given in Table 2.

Most myxosporean species were found in the urinary system (kidney, renal tubules and urinary bladder) of the different fish species. Only two species, *Chloromyxum truttae* and *Myxidium truttae*, occurred in the epithelium of the gall bladder with their spores floating in the bile.

The kidneys of Atlantic salmon and brown trout were infected with *S. truttae*. Only juvenile fish (≤7 cm) were infected with *S. truttae*. Frequently, *S. truttae* was detected in mixed infections with a *Chloromyxum* species, which also

Table 2
Myxosporean species found in the fish from the investigated river system

Myxosporean	Reference for identification	Host	Prevalence % (total nr)	Host tissue	Spore dimensions[a] (μm)	18S rDNA fragment (bp)	GenBank Acc. No.
Chloromyxum truttae (Léger, 1906)	Alvarez-Pellitero et al. (1982)	*Salmo salar*	18 (9/49)	Gall bladder epithelium	S (L9.65×W9.38)	1572	AJ581916
		Salmo trutta	10 (3/29)		PC (L_S3.2×W_S2.59, L_L3.8×W_L2.81)		
Myxidium truttae (Léger, 1930)	Lom and Dykova (1992)	*Salmo trutta*	17 (5/29)	Gall bladder epithelium	S (L11.2×W7.5)	1492	AJ582061
					PC (L4.0×W2.9)		
Sphaerospora truttae (Fischer-Scherl et al., 1986)	Fischer-Scherl et al. (1986), McGeorge et al. (1994)	*Salmo salar*	14 (7/49)[b]	Kidney tubules	S (L7.4×W9.9)	2541	AJ581915
		Salmo trutta	10 (3/29)[b]		PC (D2.4)		
Sphaerospora elegans (Thélohan, 1892)	Feist et al. (1991)	*Gasterosteus aculeatus*	67 (2/3)	Kidney tubules	S (L11.0×W10.6)	1384	AJ609590
					PC (D4.0)		
Myxobilatus gasterostei (Parisi, 1912)	Feist et al. (1991)	*Gasterosteus aculeatus*	67 (2/3)	Kidney tubules	S (10.2×W5.1, C19.8)	1561	AJ582063
					PC (L5.4×W2.9)		
Chloromyxum sp.	Sedlaczek (1991)	*Salmo salar*	86 (42/49)[b]	Kidney tubules	S (L8.39×W8)	1253	AJ581917
		Salmo trutta	21 (6/29)[b]		PC (L_L3.65×W_L2.9, L_S3.0×W_S2.2)		
Myxidium giardi (Cépede, 1906)	Ventura and Paperna (1985)	*Anguilla anguilla*	26 (5/19)	Kidney	S (L9.0×W5.4)	910	AJ582213
					PC (L3.5×W2.2)		
Zschokkella sp.	-	*Anguilla anguilla*	11 (2/19)	Urinary bladder epithelium	S (12.41×9.42)	1610	AJ581918
					PC (L3.91×W3.63)		
Hoferellus gilsoni (Debaisieux, 1925)	Lom et al. (1986)	*Anguilla anguilla*	26 (5/19)	Urinary bladder epithelium	S (L7.0×W6.8, B4.0)	1558	AJ582062

[a] S, spore; PC, polar capsule; L, length; W, width; D, diameter (if spherical); subscript $_S$ and $_L$, dimensions of smaller ($_S$) and larger ($_L$) pair of polar capsules; C, length of caudal appendices; B, length of brush border.
[b] Excluding two *Salmo salar* and eight *Salmo trutta* containing intratubular developmental stages but lacking mature spores for species identification.

develops spores intratubularly. The occurrence of early sporogonic stages in the tubuli of small fish sometimes prevented differentiation between the two species.

A mixed infection of two intratubular kidney myxosporeans, *Sphaerospora elegans* and *Myxobilatus gasterostei*, was found in one of the sticklebacks. A species of *Zschokkella* was found in the urinary bladder epithelium of two eels. The size and shape of the spores did not match published descriptions of *Zschokkella* species. *Hoferellus gilsoni* was also found exclusively in the urinary bladder epithelium, whereas *Myxidium giardi* was restricted to the kidney of infected eels.

3.2. Actinosporean fauna

The following species of oligochaetes were found in the river and the outflow area of the salmon farm: *Lumbriculus variegatus* (Müller), *Stylodrilus heringianus* Claparède, *Limnodrillus hoffmeisteri* Claparède, *Tubifex* spp, *Nais* spp., *Unicinais unicinata* (Ørsted), *Vejdovskyella comata* (Vejdovsky), *Pristina idrensis* Sperber, *Chaetogaster* sp., *Eiseniella tetraedra* L., and an undetermined representative of the family Enchytraeidae (500 individuals identified). Twelve actinosporean types belonging to the collective groups raabeia, echinactinomyxon, aurantiactinomyxon, neoactinomyxum and synactinomyxon were released from the oligochaetes. Ten of these were previously described by Özer et al. (2002a). Two other forms, *Neoactinomyxum eiseniellae* and the 'petite forme' of *Aurantiactinomyxon pavinsis* (Marquez, 1984; PhD thesis, Universite des Sciences et Techniques de Languedoc, Montpellier) were only released from one oligochaete specimen each, sampled from the river. All actinosporean types, their hosts, their prevalence and measurements, together with the length of

Table 3
Actinosporean types released from oligochaetes

Actinosporean	Reference for identification	Host	Prevalence (%)[a]	Spore dimensions[b] (μm)	18S rDNA fragment (bp)	GenBank Acc. No.
Aurantiactinomyxon 'type 1'	Özer et al. (2002a,b)	*Tubifex tubifex*	0.3 (2000)	SB (D14.2) F33 PC (2.6×2.5)	909	AJ582004
Aurantiactinomyxon 'type 3'	Özer et al. (2002a,b)	*Tubifex tubifex*	0.2 (2000)	SB (L21.1×W19.3) F114 PC (L4.0×W3.5)	931	AJ582005
Aurantiactinomyxon pavinsis ('petite form') Marquez 1984	Marques (1984)[c]	*Stylodrilus heringianus*	33.3 (3)	SB (D9) F10 PC (L2.8×W2.3)	1567	AJ582006
Echinactinomyxon 'type 1'	Özer et al. (2002a,b)	*Lumbriculus variegates*	1.5 (2000)	SB (L22.0×19.3) F116 PC (L5.8×W5.1)	917	AJ582000
Echinactinomyxon 'type 5'	Özer et al. (2002a,b)	*Lumbriculus variegatus*	0.3 (2000)	SB (L11.0×W8.1) F60 PC (L4.9×W2.2)	1983	AJ417562
Echinactinomyxon radiatum Janiszewska, 1957	Marques (1984)[c], Özer et al. (2002a,b) (E. 'type 4')	*Tubifex tubifex*	0.02 (2000)	SB (L26.0×W15.2) F123 PC (L6.6×W4.7)	899	AJ582001
Raabeia 'type 1'	Özer et al. (2002a,b)	*Tubifex tubifex*	0.0005 (2000)	SB (L17.7×W15.0) F84 PC (L7.0×W5.5)	933	AJ582008
Raabeia 'type 3'	Özer et al. (2002a,b)	*Tubifex tubifex*	0.0005 (2000)	SB (L32.6×W12.2) F230 PC (L6.3×W4.2)	1541	AJ582009
Raabeia 'type 4'	Özer et al. (2002a,b)	*Tubifex tubifex*	0.92 (2000)	SB (L29.6×W16.5) F144 PC (L6.3×W6.4)	934	AJ582010
Synactinomyxon 'type 1'	Özer et al. (2002a,b)	*Tubifex tubifex*	2.0 (2000)	SB (D16.4) F_S18.1, F_L5.4 PC (L5.8×W4.4)	931	AJ582002
Synactinomyxon longicauda Marques and Ormieres (1982)	Marques (1984),[c] Özer et al. (2002a,b) (S. 'type 3')	*Tubifex tubifex*	0.4 (2000)	SB (L25.9×W22.0) F76 PC (L5.1×W3.9)	934	AJ582003
Neoactinomyxum eiseniellae Ormieres and Frezil 1969	Marques (1984)[c]	*Eiseniella tetraedra*	2.5 (40)	SB (D14.3) F12	1570	AJ582007

[a] In parentheses: total number of worms checked.
[b] SB, spore body; F, length of floating appendices with F_S, length of shorter floating appendix and F_L, length of longer floating appendix; PC, polar capsule; L, length; W, width; D, diameter (if spherical).
[c] PhD thesis, Universite des Sciences et Techniques de Languedoc, Montpellier.

the sequenced 18S rDNA fragment and the GenBank™ accession number are given in Table 3.

3.3. Sequences, alignments and phylogenetic analysis

Twenty-one new myxozoan sequence entries were submitted to GenBank™, of which only *M. truttae* had been previously sequenced (origin: Canada, Kent et al., 2000). Sequences for the genera *Chloromyxum*, *Hoferellus* and *Myxobilatus* were entered to GenBank™ for the first time.

Analysis of the approximately 900 bp fragment amplified using primers Myxgp2F and Act1R was found sufficient to provide interspecies information. The percentage of identical base pairs for this fragment is given in Table 4. Analysis of the larger fragment (1550 bp) produced using primers Myx1F and MX3 did not show major differences in the percentage of identical base pairs between the various species (average delta of 0.2% and a maximum delta of 2.9%). However, the inclusion of more base pairs and the long variable region located at the 3′ end of the Myx1F/MX3 fragment improved the support for positioning of the taxa in the cladogram (higher bootstrap values). As a result, the 1550 bp fragment was sequenced and analysed for all myxosporean species (except for *M. giardi* and *S. elegans* which failed to be amplified with the primers applied),

Table 4
Percentage of identical base pairs of the sequences obtained from the myxosporean and actinosporean forms, comparing an approximately 900 bp fragment of 18S rDNA

	C tru[a]	M tru[a]	S tru	S ele	M ga	C sp[a]	M gi	Zsch	H gil	A 1	A 3	E 1	E 5	E ra	R 1	R 4	S 1	S lo
C tru[a]	100.0	75.0	40.5	40.6	71.0	70.5	70.4	70.4	68.6	65.9	66.7	65.8	70.4	67.6	70.3	66.8	66.8	65.5
M tru[a]	–	100.0	41.9	40.5	67.2	68.4	68.4	68.4	67.7	64.8	65.8	64.0	68.7	66.0	68.1	66.0	65.6	65.1
S tru	–	–	100.0	64.2	41.7	43.2	43.1	43.6	41.3	42.6	42.5	42.1	39.7	41.8	40.1	42.9	42.5	42.1
S ele	–	–	–	100.0	40.6	40.1	40.0	40.5	40.6	41.3	40.7	40.7	40.6	41.0	40.2	41.0	41.7	41.0
M ga	–	–	–	–	100.0	**80.7**	**80.4**	**81.2**	**81.8**	68.7	70.5	67.6	73.3	72.3	72.3	70.5	70.0	68.8
C sp[a]	–	–	–	–	–	100.0	**98.9**	**98.1**	**82.9**	68.9	70.3	68.1	73.0	75.9	73.5	70.3	69.7	69.6
M gi	–	–	–	–	–	–	100.0	**97.7**	**83.1**	69.1	70.5	68.8	73.0	75.7	73.5	70.5	69.9	69.8
Zsch	–	–	–	–	–	–	–	100.0	**82.7**	68.9	70.5	68.3	72.8	75.9	73.3	70.6	69.6	69.6
H gil	–	–	–	–	–	–	–	–	100.0	67.6	69.3	67.6	70.6	73.6	70.7	69.6	68.6	69.4
A 1	–	–	–	–	–	–	–	–	–	100.0	**91.9**	**89.6**	65.3	66.9	66.2	**91.9**	**91.0**	**86.3**
A 3	–	–	–	–	–	–	–	–	–	–	100.0	**92.0**	66.7	69.2	66.7	**98.6**	**93.1**	**88.3**
E 1	–	–	–	–	–	–	–	–	–	–	–	100.0	65.0	68.3	66.3	**91.9**	**91.8**	**85.3**
E 5	–	–	–	–	–	–	–	–	–	–	–	–	100.0	69.2	**91.9**	66.7	66.7	65.5
E ra	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0	67.8	69.1	69.3	67.9
R 1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0	67.1	67.4	65.4
R 4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0	**93.1**	**88.2**
S 1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0	**88.0**
S lo	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0

and for all actinosporean types which were found to be identical with myxosporeans.

In the analysis, three myxosporeans were found to share 100% identical base pairs with actinosporean types recovered. It is assumed that they represent alternate stages in the life cycle of these species: *M. truttae* shares 1479 identical base pairs with *Raabeia* 'type3', *C. truttae* shares 1567 identical base pairs with *A. pavinsis* and *Chloromyxum* sp. shares 1253 identical base pairs with *N. eiseniellae*.

The complete 18S rDNA sequence of *S. truttae* used for this analysis was obtained from salmon from the same location as a basis for another study (Holzer et al., 2003).

3.4. Relationships between the myxozoans from the river system (Fig. 2)

The analysis of the taxa from the river system showed that *S. truttae*, together with *S. elegans*, share less than 45% of identical base pairs with any other myxozoans sequenced in this study (Table 4). Phylogenetically these two species occupy the most ancestral position in the tree close to the malacosporean ancestors and separated from all other species, which is well supported by bootstrap values.

The next diverging branch of the tree is formed by the gall bladder parasite *M. truttae* (well supported by bootstrap values), which is more closely related to the other gall bladder parasite *C. truttae* (75% identical base pairs) than to the other representative of the genus, the kidney myxosporean *M. giardi* (68.4% base pairs identical). However, the positioning of *C. truttae* close to *M. truttae* is questionable as the maximum parsimony analysis, in contrast to the maximum likelihood analysis, places *C. truttae* in another branch and bootstrap support for the positioning of *C. truttae* was weak in both analyses.

The actinosporean types *Echinactinomyxon* 'type 5' and *Raabeia* 'type 1' are closely related (91.9% identical base pairs), are separated from the rest of the actinosporeans and have an indefinite position in both analyses. Apart from these two representatives and *Echinactinomyxon radiatum*, all actinosporeans form a cluster of very closely related species (between 85.3 and 98.6% sequence identity) which is separated from the largest myxosporean clade of this cladogram by both the maximum parsimony as well as the maximum likelihood analysis, despite little bootstrap support. This myxosporean lineage shows a very high percentage of sequence identity (98%) between *Zschokkella* sp., *M. giardi* and *Chloromyxum* sp and >80% between these species and *M. gasterostei*, as well as *H. gilsoni*. This shows the close relatedness of five different species from the same organ localisation (urinary system) independently of their spore morphology (five genera) and host species (three different families). *Echinactinomyxon radiatum* joins this group of urinary system myxosporeans as the only actinosporean member, with approximately 76% sequence identity.

3.5. Relationships between the species from the river system and other myxozoan taxa (Fig. 3)

Phylogenetic analysis of the sequence data obtained in this study in relation with other myxozoan sequences available from GenBank™ improved the positioning of some species in the myxozoan phylogenetic system.

Using the narcomedusan fish parasite *P. hydriforme* as outgroup, the tetracapsulid malacasporeans form the most ancestral lineage in the myxozoan system. Applying the most parsimonious analysis under the given conditions, *Sinuolinea* sp. as the most ancestral marine species clustered with the malacosporeans. Successively, two ancestral marine clades diverge before the origins of a principally freshwater cluster. One of these contains the intestinal parasites *Enteromyxum leei* and *Enteromyxum scophthalmi*, the second one all other marine species (incomplete set of the *Kudoa* species analysed in this study, for further resolution of this group see Whipps et al., 2003). *Sphaerospora truttae* and *S. elegans* cluster within the marine clade, most closely related to *Ceratomyxa sparusaurati* with which, however, *S. truttae* shares only 51% of identical base pairs when aligned with the 1741 bp sequence available for *C. sparusaurati*. Another freshwater parasite, *C. shasta* occurs in the same group.

In accordance with the recent review of Myxozoa (Kent et al., 2001), the subsequent freshwater clade of myxozoans branches into three well-supported subclades. The first freshwater subclade, which diverges after the ancestral marine species, is strongly supported by bootstrap values and contains the gall bladder parasites *M. truttae* and another *Myxidium* sp. (from *Cottus bairdii* Girard). The sequence obtained for *M. truttae* in this study differs by 1% from the sequence of *M. truttae* from *Oncorhynchus mykiss* (Walbaum) in Canada (AF201374). Together with *Raabeia* 'type 3' as the alternate life cycle stage of *M. truttae* another *Raabeia* type (AF378352) from *Limnodrillus hoffmeisteri* L. completes this group.

The uncertain position of *C. truttae* is maintained when other myxozoans are included in the analysis. Despite *M. truttae* as its closest match (75% sequence identity) the most parsimonious analysis places *C. truttae* in an indefinite position (bootstrap values) within a clade of myxosporeans parasites which otherwise all inhabit the urinary system.

However, the clustering of the myxosporeans from the urinary system (Fig. 2), independent of their spore morphology and host species, is maintained in this tree, and all other myxosporean freshwater species which inhabit this organ system and which are available on GenBank™ (i.e. *Myxidium lieberkühni* from the urinary bladder of pike *Esox lucius* L. and *Sphaerospora oncorhynchi* from the renal tubules of *Oncorhynchus nerka* (Walbaum)) grouped within the same branch. The position of *E. radiatum*, which joined the 'urinary group' in the analysis of the river myxozoans (Fig. 2) is confirmed by forming a sister group relationship with *S. oncorhynchi* (94.4% identical base pairs) with 100% bootstrap support.

Two further aurantiactinomyxon type actinosporeans (AF487455 and AF483598) group within the actinosporean

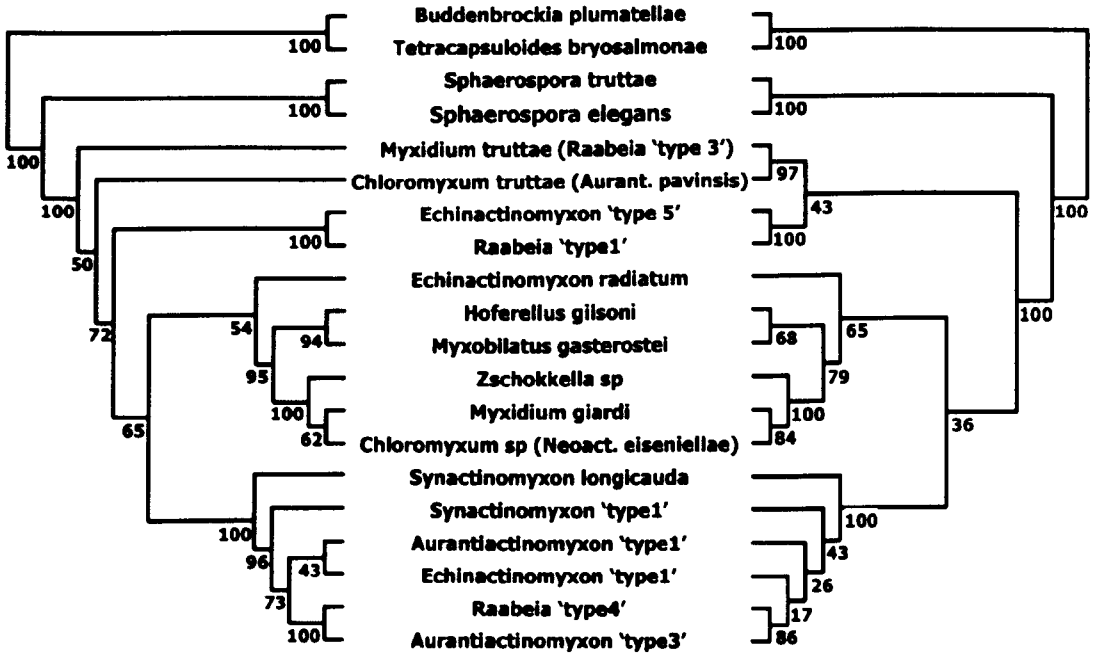

Fig. 2. Maximum parsimony tree (left) and maximum likelihood tree (right) of the 18S ribosomal gene sequences of the myxozoans encountered, rooted at *Tetracapsuloides bryosalmonae* (U70623) and *Buddenbrockia plumatellae* (AY074915). Transversion/transition ratio 1:2, bootstrap values shown represent percentage of 100 bootstrap replicates.

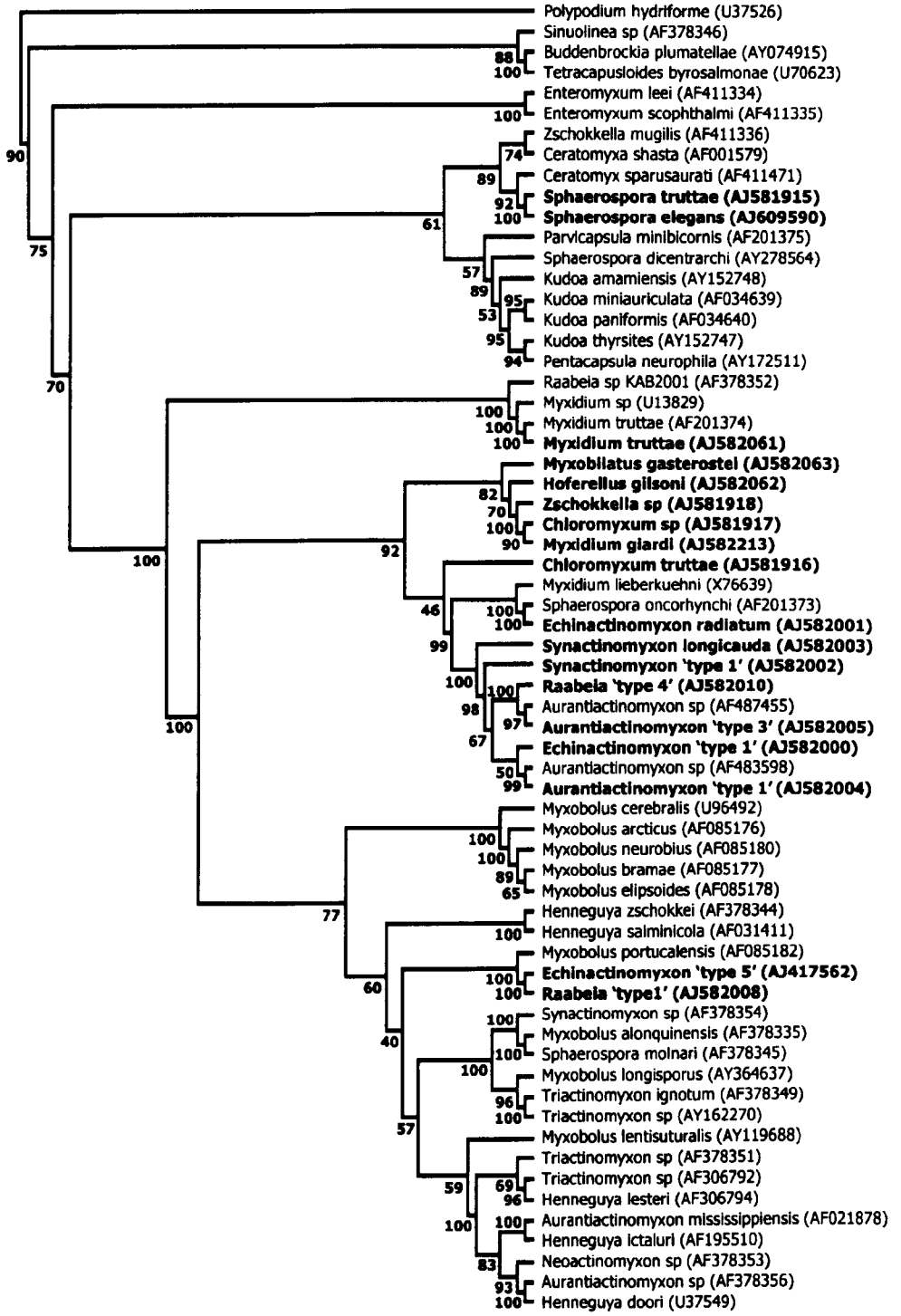

Fig. 3. Maximum parsimony tree of the 18S ribosomal gene sequences of the myxozoans found (bold) in context with other myxozoan species from GenBank, rooted at *Polypodium hydriforme*. GenBank accession numbers are in parenthesis; transversion/transition ratio 1:2, bootstrap values shown represent percentage of 100 bootstrap replicates.

cluster from the river system. This actinosporean group merges with *S. oncorhynchi*, *M. lieberkühni* and on a wider scale also with *M. gasterostei*, *H. gilsoni*, *Zschokkella* sp, *Chloromyxum* sp and *M. giardi*.

The most recent freshwater lineage is represented by the species *Myxobolus* and *Henneguya* (set incomplete; recent discrimination of all species by Dyková et al., 2002), most of them parasites of the skin, gills and nervous system.

A gill parasitic species of *Sphaerospora*, i.e. *S. molnari* also clusters within this clade. The position of *Echinactinomyxon* 'type 5' and *Raabeia* 'type 1' within this clade is strongly supported by the formation of a sister group of *Myxobolus portucalensis* from *A. anguilla* (sequence identity with *Echinactinomyxon* 'type 5' 92.8%) with 100% bootstrap support.

4. Discussion

Since the mid-1990 s the myxozoan database of 18S sequences has been growing. Kent et al. (2001) presented a comprehensive phylogram of myxozoan species involving most of the sequences published up to 2001, and subsequent additions of new species produced phylogenetic trees which were in general accordance with this system (Palenzuela et al., 2002; Molnar et al., 2002; Dyková et al., 2002; Negredo et al., 2003; Whipps et al., 2003). The general structure of the phylogram obtained in this study is consistent with these previous analyses. However, the addition of the new sequences from this study identifies the ancestral marine origin of two freshwater species and it strongly aids the understanding of the cladistic arrangement within the more recent clade of freshwater taxa due to the addition of new species belonging to poorly represented genera or genera so far unavailable on the molecular database:

In accordance with Kent et al. (2001), in this study the freshwater clade of myxosporeans branches into three well-supported sub-clades. One of these sub-clades has until now been represented by only two species, *S. oncorhynchi* and *M. lieberkühni*.. In this analysis the sequences obtained from *M. gasterostei*, *H. gilsoni*, *Zschokkella* sp., *Chloromyxum* sp., and *M. giardi*, which show an extraordinarily close relationship to each other (up to 98.9% sequence identity) were found to form a sister group of *S. oncorhynchi* and *M. lieberkühni*. All of these species with the exemption of *M. giardi* show sporogony in or attached to the excretory epithelium of the urinary system and cluster within the same sub-clade independently from spore morphology (six different genera) and host species (four different families). This suggests that tissue tropism represents a strongly determining factor in myxozoan phylogeny. It also implies that spore morphology is of inferior importance in terms of taxonomy although it is the criterion most often employed for assigning species (Lom, 1987; Moser and Kent, 1994; Lom et al., 1997).

Support for an arrangement of species by tissue location can also be found in the freshwater lineage consisting of the 'genera' *Myxobolus* and *Henneguya*. As the largest group with numerous sequences published, most *Myxobolus* and *Henneguya* species are parasites of skin, gills, muscle and the nervous system. Andree et al. (1999) reported the clustering of 10 *Myxobolus* species from different regions in the USA and from Hungary according to tissue location. In the present analysis, the gill parasite *Sphaerospora molnari* clusters clearly within the *Myxobolus-Henneguya* group representing a sister taxon of *Myxobolus algonquinensis*. Both of these myxosporeans mature in the epithelium and share the same tissue tropism although their spores are morphologically different. A more comprehensive resolution of this group can be found in Dyková et al. (2002), confirming these findings.

However, although relatedness according to localisation was the dominant finding in the present analysis of the three sub-clades of freshwater myxosporeans, tissue tropism, though strongly influential, is probably not the only factor driving myxozoan evolution. Although the two myxosporeans found sporogenating in the gall bladder epithelium (*M. truttae* and *C. truttae*) show the closest sequence similarity with each other, they do not cluster together in the cladistic analysis. *Myxidium truttae* has a very defined position in the first freshwater sub-clade which diverges after the deep division of the freshwater myxozoans from the group of ancestral marine genera. *Myxidium truttae* clusters with another *Myxidium* species from the gall bladder of *Cottus bairdi* and a *Raabeia* type, which most likely represents an alternate stage of another *Myxidium* gall bladder parasite similar to *Raabeia* 'type 3' which we found to be identical with *M. truttae*.. Although certainly not a member of this clade, the position of *C. truttae* remains unclear. With about 73% sequence identity with some of the urinary parasites sequenced, *C. truttae* is located at an indefinite position (46% bootstrap support) within the urinary clade. Due to the lack of other published *Chloromyxum* sequences the interpretation of the position of *C. truttae* in the system is problematic. However, it is suggested that *C. truttae* is a representative of a second group of bile parasites, which developed later in the evolution of the myxozoans. Supporting this hypothesis is the controversial finding that the actinosporean stage related to *C. truttae* is a very small type, i.e. *A. pavinsis* (approximately 20 μm spore diameter) whereas the life cycle of *M. truttae* involves *Raabeia* 'type 3', a spore type with large (230 μm) appendices. As both species target the gall bladder epithelium of the same host (*S. trutta*) it is suggested that the *M. truttae* and *C. truttae* have developed different strategies to reach this organ. The variety of functional designs of actinosporean spores is important in the context of floating behaviour in the water column and for the attachment to the fish, but only the sporoplasms finally enter the host. Yokoyama and Urawa (1997) found that small actinosporean stages (e.g. aurantiactinomyxon) invade the fish host through the gills whereas large actinosporeans (e.g. triactinomyxon and raabeia) attach mainly to the skin.

The two *Sphaerospora* species sequenced in this study occupy a distinctively different position from all other myxosporeans and actinosporeans sequenced, despite the homology of tissue tropism with five of the other myxosporeans found. The extraordinary long inserts in the 18S rDNA of *S. truttae* complicate alignments and the exact

phylogenetic positioning of this species, but *S. truttae* shows clear relatedness to *S. elegans* from stickleback. Both species occupy a position within the ancestral marine myxozoan species, according to the present analysis most closely related to *C. sparusaurati*. Despite confirmation of this position with a relative high percentage of bootstrap replicates (92% for assignment to a sister group of *C. sparusaurati*), sequence identity between *S. truttae* and *C. sparusaurati* is only 51%. Analyses using longer 18S rDNA fragments and other phylogenetic algorithms (neighbourhood-joining, maximum likelihood) ascribes *S. truttae* to the same nearest neighbours but result in varying percentages of bootstrap replicates (89–65%, Holzer et al., unpublished). This demonstrates that caution is necessary in drawing firm conclusions concerning the positioning of *S. truttae* and *S. elegans* within the marine clade, and this matter is currently subject of a more detailed investigation. However, it is suggested that *S. truttae* and *S. elegans* have a similar position within the marine group as the freshwater parasite *C. shasta:* they are all myxozoans with marine origin which were possibly introduced into freshwater due to the migration of their fish hosts between the freshwater and the marine environments (anadromous migration of salmonids and occurrence of stickleback in freshwater as well as in costal marine waters, Bell and Foster, 1994). *Sphaerospora truttae* and *S. elegans* presumably left the marine environment a very long time ago and earlier in the evolution of Myxozoa than *C. shasta* as they share only 51% of identical base pairs with their recent marine relatives in contrast to 74% sequence identity of *C. shasta* with *C. sparusaurati*.

Sphaerospora dicentrarchi occurs in the same group of marine myxozoans but shows less relatedness with *S. truttae* and *S. elegans* than other marine species. The 'genus' *Sphaerospora*, with 5 species sequenced so far, thus presents itself as a strongly polyphyletic group. This poses the question why this spore morphology developed on several occasions in the myxozoan evolution. Due to their spherical design *Sphaerospora* spores sink relatively rapidly and are likely to be taken up by non-selective feeding alternate hosts, such as oligochaetes. The spherical shape might be a very successful spore design, developed on several occasions during myxozoan evolution. The assumption that spore morphology is of functional rather than of taxonomic importance supports the idea that most myxosporean genera are paraphyletic (Fig. 3, and review by Kent et al., 2001) and that, analogous to the myxosporeans, most actinosporean phenotypes are spread over the freshwater clades without any identifiable pattern.

Of the actinosporeans sequenced in this study, six species with different phenotypes (*Synactinomyxon longicauda*, *Synactinomyxon* 'type 1', *Raabeia* 'type 4', *Echinactinomyon* 'type1', *Aurantiactinomyxon* 'type 1' and *Aurantiactinomyxon* 'type 3') were very closely related (85.3–98.6% identical base pairs) and they clustered together with two other aurantiactinomyxon types (AF487455 and AF483598) as a sister group to the myxosporean species from the urinary system. Due to the complete lack of myxosporeans in this sister group and frequent occurrence of the latter in oligochaetes it may be questioned if the host belongs to another vertebrate group (e.g. amphibians) or whether these actinosporeans have myxosporean counterparts at all. As the only sexual process (gametogony) in the myxozoan life cycle described so far takes place in the oligochaete host (El-Matbouli and Hoffmann, 1998), the worm per definitionem has to be regarded as the definitive host, and perhaps some myxozoans might not employ an obligatory alternate fish host.

The actinosporean *Echinactinomyxon* 'type 5' was believed to represent the alternate stage of *S. truttae* (Özer and Wootten, 2000).Sequence analysis shows that *Echinactinomyxon* 'type 5' shares only 48.9% of identical base pairs with *S. truttae*. In contrast, *Echinactinomyxon* 'type 5' together with *Raabeia* 'type 1' form a closely related sister group of *M. portucalensis*, with 100% bootstrap support. It is suggested that these two actinosporean types are part of life cycles including myxosporean stages of the *Myxobolus* or *Henneguya* type. Similarly, *E. radiatum* with 94.4% sequence identity with *S. oncorhynchi* might represent the alternate stage of a myxosporean inhabiting the urinary system.

Due to the identity of 18S rDNA sequences obtained for three myxosporeans with those obtained for three actinosporean types it is assumed that the different life cycle stages of each identical pair belong to individual myxozoan species. As a result, the actinosporean names should be suppressed and become junior synonyms of the named myxosporeans (International Code of Zoological Nomenclature and Kent et al., 1994): *N. eiseniellae* Marques 1984 becomes a junior synonym of *Chloromyxum* sp., *A. pavinsis* Marques 1984 a junior synonym of *C. truttae* Léger, 1906 and Raabeia 'type 3' a junior synonym of *M. truttae* Léger, 1906.

The use of the 18S ribosomal gene as molecular 'chronometer' can be problematic and is discussed elsewhere (Hillis and Dixon, 1991). The gene has a very conservative character, but Kent et al. (1996) mentions a 'fast clock' in the evolution of the myxozoan 18S, which helps to explain the high degree of variability among the species. In this study, two *Sphaerospora* species were encountered which share less than 52% of identical base pairs with all other myxozoans sequenced so far. In contrast, some species of different genera (phenotypes) were found to exhibit up to 98.6% sequence identity and they therefore must have diverged into different species very recently. It cannot be excluded that different phenotypes of the same species exist in different fish hosts, as myxozoan species do develop more than one spore type in their life cycle (myxosporean and actinosporean) and additionally, a single actinosporean genotype was recently found to develop two different phenotypes in the same oligochaete host (Hallett et al., 2002), possibly designed for different fish hosts. It is furthermore difficult to distinguish between different species

and different geographic genotypes of one single species. For example, the present *M. truttae* sequence was shown to be only 99% identical with *M. truttae* from Canada and suggests that this species has been developing in geographical isolation for a very long time so that genotypically different subspecies have emerged. There is a need for other myxozoan genes to be analysed in order to verify or refute observations of relatedness made on the basis of the 18S gene. A comprehensive data set including more taxa and other genes is desirable to confirm the interpretation of these results and to evaluate the importance of other factors such as geographic distribution or alternate hosts.

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