Molecular data implicate bryozoans as hosts for PKX (Phylum Myxozoa) and identify a clade of bryozoan parasites within the Myxozoa

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SUMMARY

Proliferative kidney disease (PKD), a condition associated with high mortality in salmonid fish, represents an abnormal immune response to the presence of an enigmatic myxozoan, which has been designated simply as PKX organism because its generic and specific status are obscure. Phylogenetic analyses of partial sequences of the 18S rDNA of PKX and of myxozoan parasites infecting the bryozoans *Cristatella mucedo*, *Pectinatella magnifica* and *Plumatella rugosa*, including the previously named *Tetracapsula bryozoides* from *C. mucedo*, showed that these taxa represent a distinct clade that diverged early in the evolution of the Myxozoa before the radiation of the other known myxozoan genera. A common feature of the myxozoans in this clade may be the electron-dense sporoplasmosomes with a lucent bar-like structure, which occur in *T. bryozoides* and PKX but not in the myxozoans belonging to the established orders Bivalvulida and Multivalvulida. Variation of 0·5–1·1% was found among the PKX 18S rDNA sequences obtained from fish from North America and Europe. The 18S rDNA sequence for *T. bryozoides* showed that it is a distinct taxon, not closely related to PKX but some sequences from myxozoans infecting 2 of the bryozoan species were so similar to those of PKX as to be indistinguishable. Other sequences from the new myxozoans in bryozoans at first appeared distinct from PKX in a maximum likelihood tree but, when analysed further, were also found to be phylogenetically indistinguishable from PKX. We propose that at least some variants of these new myxozoans from bryozoans are able to infect and multiply in salmonid fish, in which they stimulate the immune reaction and cause PKD but are unable to form mature spores to complete their development.

Key words: PKX, salmonid PKD, new bryozoan hosts, 18S rDNA, myxozoan evolution.

INTRODUCTION

The phylum Myxozoa is an entirely endoparasitic group of organisms which produce complex multicellular spores. They are parasitic in poikilothermic vertebrates, mainly fish, in which the development culminates in myxosporean-type spores with one to several polar capsules. Some at least have an alternate host, a tubificid worm, in which an actinosporean-type spore is produced, with a triradial symmetry and three polar capsules (Wolf & Markiw, 1984).

One of the most serious diseases of salmonid fish, particularly under the intensive rearing conditions common in fish farms, is proliferative kidney disease (PKD), caused by an enigmatic myxozoan, known simply as PKX organism. A recent review of the parasite and disease has been provided by Hedrick, MacConnell & de Kinkelin (1993). So far, PKD has only been reported from the northern hemisphere but occurs widely in Europe and North America. The diseased condition represents an abnormal immune response to the presence of PKX, with the kidney and spleen being the most severely affected organs. Fish stock losses of 20–100% can be sustained. Although PKD as an identifiable disease has been known since the early part of the century, it was not until 1985 that the myxozoan characters of PKX were recognized (Kent & Hedrick, 1985*a*). The detection of immature spores with spherical polar capsules in infected fish led these authors to speculate that PKX was a species of the myxozoan genus *Sphaerospora* but this was not substantiated by sequence data of the 18S rRNA gene (Kent *et al.* 1998). Sequences for the 18S rRNA gene of PKX (Saulnier & de Kinkelin, 1997) have provided confirmation, independent of morphology, that PKX is a myxozoan.

In 1996 a myxozoan parasite, *Tetracapsula bryozoides*, that forms spores in large sacs was described from the body cavity of colonies of the freshwater bryozoan, *Cristatella mucedo*, collected from several lakes in the UK (Canning, Okamura & Curry, 1996). Recently, similar myxozoan sacs were encountered in several bryozoan species from North America

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(NA). Here we report the very close matching of 18S rDNA sequences of the myxozoan parasites of the NA bryozoans with each other and with PKX and the sequence similarity of these taxa with *T. bryozoides* indicating close phylogenetic relationship.

MATERIALS AND METHODS

Specimens of *C. mucedo* infected with *T. bryozoides* were collected at regular intervals during the summers of 1996, 1997 and 1998. Collection sites were Tufty's Corner Lake, Berkshire; Beale Bird Park Lake, Berkshire and Blenheim Lake, Oxfordshire, UK.

In 1998, several bryozoan species were collected from lakes in North America as part of a broader ecological study and, amongst these, were specimens infected with free-floating sacs in the body cavity, similar to those of *T. bryozoides* found by Okamura (1996) in *C. mucedo*. Infected colonies of *C. mucedo* were found in Big Evans Lake, Mecosta County, Michigan; *Pectinatella magnifica* from Cowan Lake, Clermont County, Ohio; and laboratory cultured *Plumatella rugosa* derived from Huffman Lake, Montgomery County, Ohio all in July 1998. Kidney samples from PKX-infected rainbow trout, *Oncorhynchus mykiss*, were obtained from Lake Quinault, Washington State, USA and from the River Avon, Hampshire, UK.

DNA extraction

Whole infected bryozoan colonies, preserved in absolute ethanol, were homogenized by hand in 1·5 ml Eppendorf tubes in 300 μl of homogenization buffer (0·1 M NaCl, 0·2 M sucrose, 0·1 M Tris–HCl (pH 9·1), 0·05 M EDTA, 0·05 M SDS). About 50 μl of Zirconium beads were added to each tube to aid disruption of spores. The homogenate was extracted, twice with an equal volume of a mixture of phenol, chloroform, and isoamyl alcohol (25 : 24 : 1) and once with an equal volume of pure chloroform. One tenth volume of 3 M sodium acetate, pH 5·6, was added and the nucleic acids precipitated with 2-propanol. Pellets were washed once with 70 % ethanol, excess liquid was removed and the pellets were resuspended in 50 μl of sterile water.

DNA from PKX in River Avon rainbow trout was extracted by a similar procedure from ca. 10 mg of infected kidney. DNA from PKX in Lake Quinault rainbow trout was extracted from ca. 10 mg of kidney using DNAsol (Life Technologies), following the manufacturer's protocol.

DNA amplification by PCR

A nested PCR strategy was employed using a variety of primer combinations. After an initial round of PCR using the 18e and 18g primers (Hillis & Dixon, 1991), 0·5 μl of untreated reaction product was used for a second round of PCR using PKX-specific primers designed from the PKX sequence in the data base (Accession number U70623) (Saulnier & de Kinkelin, 1997). Primer PKX F1 (5′-taagtacatacttcggtagag-3′) was used in combination with PKX R2 (5′-attgaataggagccgatatttctc-3′), PKX R3 (5′-tgttgccctccttagttaggtagaca-3′), PKX R4 (5′-ttctgctaagtcattcaatcggta-3′) or 18S5R (5′-caaagggcagggactaaatc-3′). Primer PKX F3 (5′-tgttgcctccttagttaggtagaca-3′) was used in combination with PKX R4 or 18S5R. Reactions were carried out in 50 μl volumes of 10 mM Tris–HCl (pH 9·0), 1·5 mM $MgCl_2$, 50 mM KCl, with 10 pmols of each primer, 0·2 mM of each dNTP and 2 units of *Taq* polymerase (Life Technologies).

The PCR temperature regime was 2 min at 92 °C, followed by 35–40 cycles of 15 s at 92 °C, 20 s at 51 °C, 30 s at 72 °C, then a 5 min incubation at 72°C. Annealing temperatures of 50 and 52 °C were also successful and were used for some reactions. Products were run on 1 % agarose gels to check the size of the amplification productions and to estimate nucleic acid concentrations.

PCR products were separated from unincorporated nucleotide and extra amplification primers with the Wizard PCR Preps purification system (Promega) following the manufacturer's protocol.

Sequencing

Primer-free amplification products were either sequenced directly or ligated into pGem-T cloning vector (Promega) without further treatment. PCR products were sequenced directly using the amplification primers as sequencing primers. Cloned fragments were sequenced using the SP6 and T7-promotor primers. Sequencing employed the ABI Prism Cycle Sequencing Dye Terminator Kit and an ABI 373 automated sequencer.

Phylogenetic analysis

Phylogenetic analyses were carried out on partial sequences obtained from PKX and from the myxozoans derived from *C. mucedo*, *P. magnifica* and *P. rugosa*. Additionally, longer sequences were analysed using PKX, the myxozoans from bryozoans and sequences obtained from the data bank for several myxozoan genera, as representative of the myxozoan orders Bivalvulida and Multivalvulida. The sequences were aligned using CLUSTAL W, version 1·74 (Thompson, Higgins & Gibson, 1994). Percentage variation in the short sequences was calculated by comparison of each sequence with that of the PKX sequence U70623 (Saulnier & de Kinkelin, 1997). Maximum likelihood (ML) and parsimony trees were constructed, using PAUP test version 4·0 b1 (Swofford, 1998) with 200 bootstrap replicates.

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RESULTS

Prevalence of myxozoans in NA bryozoans

Two infected colonies of *C. mucedo* were found amongst 30 colonies collected from Big Evans Lake. No other infected *C. mucedo* were detected although populations were sampled as part of a larger ecological study of *C. mucedo* from 16 sites in the USA and Canada (located in Ohio, Michigan, Wisconsin, Minnesota, Ontario and Manitoba), admittedly with only a few colonies collected from some of these sites (mean number of colonies collected per site = 18·4, S.D. = 3·2, range = 1–32). Six small colonies of *P. magnifica* were found in Cowan Lake out of approximately 30 colonies examined. One infected colony of *P. rugosa* was growing in laboratory culture at Wright State University. This colony had originated from Huffmann Lake, Ohio and was 1 of 2 colonies in culture at the time.

The myxozoans were present as free-floating sacs in the body cavity and were indistinguishable at the light microscopy level from stages of *T. bryozoides* in *C. mucedo* (Okamura, 1996).

18*S rDNA sequence data*

18S rDNA sequences obtained from our material have been deposited in the data bank as follows:

Tetracapsula bryozoides in *C. mucedo*: AJ133564–607 bp; AJ133565–1591 bp.

PKX in *O. mykiss* from River Avon: AJ133560–613 bp; AJ133561–746 bp; AJ133562–547 bp; AJ133563–1558 bp.

PKX in *O. mykiss* from Lake Quinault: AJ133408–584 bp; AJ133409–575 bp; AJ133407–1516 bp.

Myxozoa in *C. mucedo* from Big Evans Lake: AJ133417–431 bp; AJ133410–684 bp; AJ133411–619 bp; AJ133412–582 bp; AJ133413–1645 bp.

Myxozoa in *P. magnifica* from Cowan Lake: AJ133416–432 bp (2 sequences); AJ133414–431 bp; AJ133415–624 bp; AJ133416–432 bp.

Myxozoa in *P. rugosa*: AJ133418–617 bp.

Phylogenetic analyses

Fourteen new 18S rDNA myxozoan sequences, spanning positions 41–641, when aligned with PKX sequence U70623 (Saulnier & de Kinkelin, 1997) were analysed by maximum likelihood (ML) and parsimony, using the corresponding sequence of *T. bryozoides* as outgroup. The sequences were 431–746 bp long. Both trees converged on the same topology (Fig. 1A). When compared with U70623 the PKX sequences from the River Avon and Lake Quinault showed 0·5–1·1 % difference. Variants were also revealed in the sequences of myxozoans from North American bryozoans with 6 sequences (3 from *C. mucedo* (AJ133410, AJ133411 and AJ133412), 2 from *P. magnifica* (AJ133414, AJ133415) and 1 from *P. rugosa* (AJ133418)), forming a clade, with 97 % bootstrap support in ML. These showed about 2·1 % (1·9–2·4 %) difference from U70623. However, 2 identical sequences from *P. magnifica* (AJ133416) and 1 from *C. mucedo* (AJ133417) showed only 0·5 % difference from U70623 and clustered with the all PKX isolates from rainbow trout. The sequences of *T. bryozoides* differed from U70623 by 19·4–21 % and all 4 sequences obtained had a 9-nucleotide insert (ATGTTGCCG) between nucleotides 206 and 207 when aligned to the PKX sequence U70623. This was not present in any of the sequences from North American bryozoans.

The relationships of these variants were further investigated by conducting 3 separate parsimony analyses in which (a) PKX isolates and (b) myxozoan isolates from NA bryozoans were constrained to be monophyletic and (c) all isolates were unconstrained. Shortest trees in all analyses were of equal length and, thus, the lack of phylogenetically informative characters in the sequences rendered these isolates phylogenetically indistinguishable by parsimony analysis.

Higher level phylogenetic relationships were also examined using longer sequences of 1250 nucleotides in ML and parsimony analyses (Fig. 1B). For the analyses we included a sequence from the new myxozoan obtained from *C. mucedo* (AJ133413), our sequences for Lake Quinault PKX (AJ133407) and River Avon PKX (AJ133563), PKX (U70623; Saulnier & de Kinkelin, 1997) and *T. bryozoides* (AJ133565). Also included were sequences, obtained from the databank, representing 5 of the 6 other genera for which data are available, i.e. *Ceratomyxa* (AF001579), *Kudoa* (AF031412), *Myxobolus* (U96-492), *Myxidium* (U13829) and *Henneguya* (AF0-31411). As our previous work (Anderson, Canning & Okamura, 1998) and that of Smothers *et al*. (1994) had indicated a triploblast origin for Myxozoa, we included a data bank sequence of a representative diploblast, *Anemonia sulcata* (X53498), as outgroup. The ML and parsimony analyses were congruent, both indicating 2 major branches, one including the myxozoan taxa belonging to the 2 established orders, Bivalvulida and Multivalvulida, with 93 % bootstrap support in ML (Group 1) and the other comprised of PKX from fish and the myxozoans from Bryozoa, including *T. bryozoides*, with 100 % bootstrap support in ML (Group 2).

Resolution within Group 1 was good with strong bootstrap support for the relationships of the genera, although not all relationships were in line with the morphology-based taxonomy. Within Group 2 the long sequence for the new myxozoan from *C. mucedo* was by chance one of the more variant new sequences from bryozoans. Even so there was weak support,

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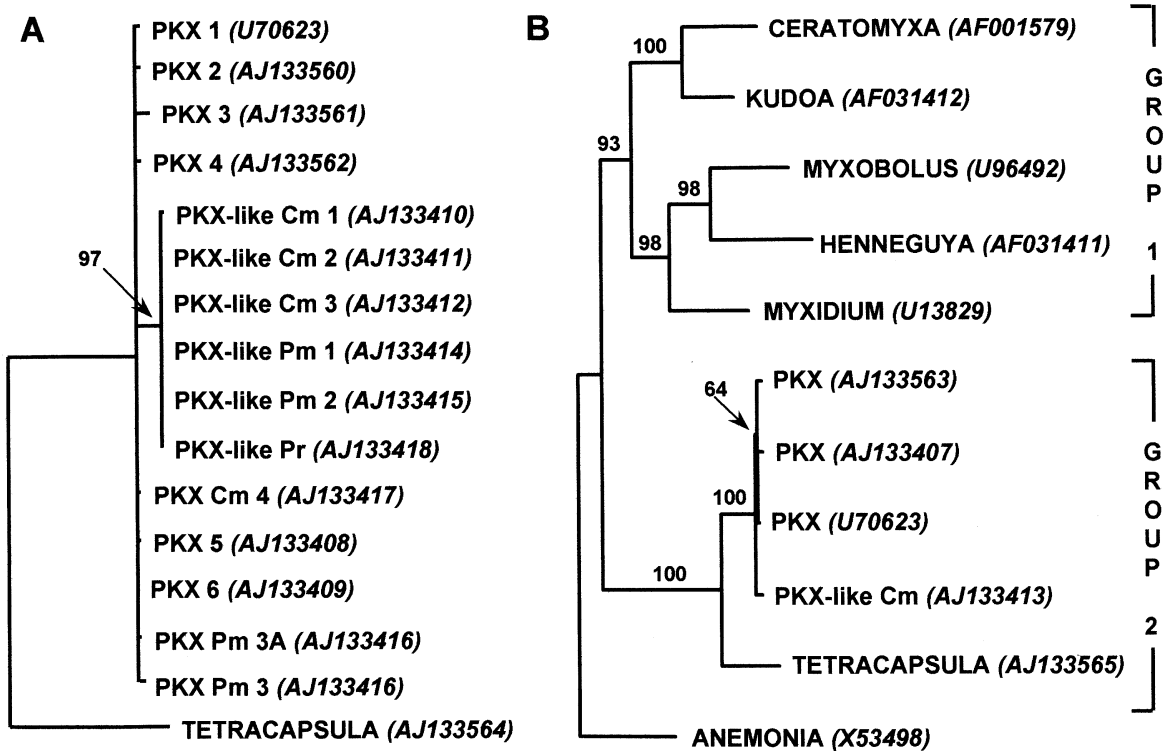

Fig. 1. Maximum likelihood (ML), 50% majority-rule consensus trees drawn from myxozoan 18S rDNA sequences. All amplification products used in the analysis were sequenced in both directions directly or after cloning. Numbers on branches are percentage maximum likelihood bootstrap values with 200 replicates and heuristic search option. Analysis assumes all sites evolve at a uniform rate. Transition:transversion ratio = 2, nucleotide frequencies empirically determined, molecular clock not enforced, gaps treated as missing data. (A) Phylogram showing results of ML analysis of 18S 5′ end, from European and North American PKX isolates from rainbow trout and PKX and PKX-like isolates from North American Bryozoa. PKX1–4 are from European rainbow trout; PKX5, 6 are from North American rainbow trout; PKX Cm4 and PKX-like Cm1–Cm3 are from *Cristatella mucedo*; PKX Pm3A, Pm3 and PKX-like Pm1, Pm2 are from *Pectinatella magnifica*; and PKX-like Pr is from *Plumatella rugosa*. Tree is rooted using *Tetracapsula bryozoides* (TETRACAPSULA) from *C. mucedo* as an outgroup since, in a higher level analysis, it was a sister group to these taxa with 100% bootstrap support (see B). PKX Pm3 and Pm3A were identical sequences. (B) Phylogram showing results of ML analysis of 1250 bp of aligned available myxozoan 18S rDNA sequences. Included are sequences from 6 of the 7 myxozoan genera, including PKX from rainbow trout (PKX), for which database information is available and new sequences, one of *T. bryozoides* (TETRACAPSULA) and the other of a PKX-like isolate from *C. mucedo* (PKX-like Cm). Regions of ambiguous alignment were excised. Tree is rooted using the diploblast cnidarian, *Anemonia sulcata*, as an outgroup.

only 64% bootstrap, for it being distinct from PKX, whereas there was 100% bootstrap support for *T. bryozoides* as a sister taxon to PKX.

DISCUSSION

The relationship of PKX and the myxozoans of Bryozoa

Previous work has established variation in the 18S rRNA gene among isolates of PKX obtained from North American and European sources, amounting to a maximum of 0·8% measured across 764 nucleotides of the gene (Kent *et al.* 1998). Our sequences for PKX cannot be compared directly with those of Kent *et al.* (1998) because their sequence information is not yet available, although the regions analysed are specified (positions 38–335; 463–700; 1436–1646 aligned with the sequence U70623 (Saulnier & de Kinkelin, 1997)). Our PKX sequences from fish differed by 0·5–1·1% compared with U70623 over 511 nucleotides spanning positions 89–600. Two myxozoan sequences from *P. magnifica* and one from *C. mucedo* fell within this range and invariably clustered with PKX in phylogenetic analyses. Furthermore, comparison of unconstrained and constrained parsimony trees indicated that all the sequences, even those giving rise to the separate clade in ML analysis, were virtually indistinguishable. At this stage we cannot determine unequivocally whether the sequence variation giving rise to a separate clade of myxozoans from North American Bryozoa is due to the existence of a very closely related taxon or to intraspecific variation in the 18S rDNA. Nevertheless we propose that at least some of the variants from *C. mucedo* and *P. magnifica*, represented by PKX Cm4 (AJ133417), PKX Pm3A and PKX Pm3 (AJ133416) are PKX and can infect

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fish and cause PKD. An alternative hypothesis to fit the data, that an extremely close relative of the myxozoans which infect Bryozoa, has made the transition to fish and no longer infects Bryozoa, is an unnecessarily devious argument and is far less likely.

Although *T. bryozoides* from *C. mucedo* was clearly different from the other myxozoans from Bryozoa and from PKX, all of these taxa belong to a single lineage, which diverged early in the evolution of the Myxozoa and are consequently likely to share morphological characteristics. We have not yet determined the genus to which the new parasites of Bryozoa belong but examination of live infected bryozoans revealed them to have the form of free-floating sacs of various sizes, which were initially thought to be *T. bryozoides*. Subsequently, the close matching of 18S rDNA sequences with PKX sequences indicated that they, and therefore PKX, are likely to be new species within the genus *Tetracapsula* or belong to a very closely related genus. A character which links *T. bryozoides* with PKX is the possession of similar organelles which were first described by Ferguson & Needham (1978). These were designated as sporoplasmosomes in *T. bryozoides*, where they occur in sporoplasms only (see Fig. 33 in Canning *et al*. 1996) and haplosporosomes in PKX where they occur in proliferative stages and not apparently in the spores (Kent & Hedrick, 1985*a*, *b*; Feist & Bucke, 1987). These organelles take the highly characteristic form of electron-dense bodies bearing a lucent bar-like inclusion, which is aligned perpendicular to the surface, when the organelles lie in a superficial position. The whole structure lies eccentrically within a membrane. Organelles with this structure have not been found in other Myxozoa and, indeed, this shared character alerted researchers to the possibility that bryozoans could harbour PKX (Feist, 1997; Kent *et al*. 1998). Another shared character may be the absence of hard spore valves. This possibility derives from our observations of the development of *T. bryozoides*, which culminates in the formation of spores typical of the myxosporean phase of Myxozoa, except that the wall is composed of 4 valvogenic cells, which surround the capsulogenic cells and sporoplasms, but appear to remain at a cellular level without secreting a hard, external component. Although PKX spores have been observed in renal tubule lumina, they also apparently lack hard valves and this has led to speculation that mature spores are produced in other fish and that salmonids are aberrant hosts for PKX (Kent & Hedrick, 1985*b*). We anticipate, on the basis of the morphology of the sac-like stages in bryozoans, that the development of PKX will follow the pattern for *T. bryozoides*.

The spores of PKX described by Kent & Hedrick (1985*a*) measured 8·5 × 5·8 μm (in sections) and had 2 adjacent spherical polar capsules formed within capsulogenic cells. *T. bryozoides* has spherical spores measuring almost 20 μm diameter with 4 apical spherical polar capsules. As our sequence data amount to strong evidence that the myxozoans newly-found in Bryozoa are PKX and are related to *T. bryozoides*, possible explanations of spore differences between fish and bryozoans are as follows. (a) The spores in salmonid fish are not those of PKX. This is unlikely because there have been several studies which link the spores to PKX, i.e. spore reactivity with a lectin and with a monoclonal antibody raised against proliferative stages of PKX (Castagnaro *et al*. 1991; Marin de Mateo *et al*. 1993). (b) The spores in salmonid fish are aberrant because PKX cannot complete its development in these hosts. (c) PKX does not belong to the genus *Tetracapsula* but to a closely related genus with 2 apical polar capsules. (d) The spore structure in the vertebrate and invertebrate hosts differs, which is quite possible in view of the plasticity of some myxozoan species which enables the parasite to alternate between myxosporean and actinosporean-type spores. This problem cannot be resolved until our ultrastructural studies are completed.

Ecological evidence also supports our hypothesis that freshwater bryozoans are hosts for PKX. This includes the timing of seasonal outbreaks of PKD and the widespread occurrence of bryozoans. PKD is known to develop when water temperatures are higher than 15 °C and freshwater bryozoans are generally restricted to warm waters, flourishing as colonies at 15–20 °C (Wood, 1991). The life-cycle of bryozoans entails hatching of colonies from small resistant stages, statoblasts, in late spring/early summer, then prolific growth throughout the summer. By autumn, having produced numerous overwintering statoblasts, colonies regress and die and the cycle begins again the following season. Bryozoans exhibit broad ecological tolerances. They are associated with a wide range of pH and most species occur in both still and running water although some species favour one or the other. Turbid water is tolerated by many species and several are also notably tolerant of contamination from sewage and industrial waste and associated low oxygen levels, while others can produce luxuriant growth in visibly eutrophic conditions. These broad tolerances can explain how PKX can develop in salmonid fisheries receiving water inputs from streams, rivers, lakes or reservoirs.

As our results strongly indicate that bryozoans act as hosts for PKX for part of its development, we can put forward several hypotheses for the completion of its life-cycle. PKX spores produced in bryozoans may lack a hard wall like those of *T. bryozoides* but be able to survive in freshwater and be infective to fish. Despite the lack of a resistant coating, spores of *T. bryozoides* are apparently tolerant of osmotic shock since they remain intact in pond water, suggesting that the valvogenic cells protecting the

sporoplasm have special surface characteristics that enable them to deal with osmotic problems. While it is possible that spores of *T. bryozoides* eventually develop resistant valves, we did not find this in any of the colonies we examined over several months. Alternatively spores produced in bryozoans may be incorporated into statoblasts, which then become free floating and could be ingested by fish. This would offer a mechanism for PKX to colonize new habitats since waterfowl carry statoblasts from site to site (J. Freeland, L. Noble & B. Okamura, unpublished data).

Another transmission mechanism may be that spores from bryozoans develop into actinosporean spores after infecting detritus-feeding worms, which occur in close association with colonies (Wood, 1991) and have been observed to prey on them (Marcus, 1934). These spores might be infective to fish. However, an actinosporean stage of PKX has not been found. The only mechanism by which infections could be transmitted from fish to bryozoans appears to necessitate the formation of spores in salmonids or other fish within which the sporoplasms are protected, but observations so far suggest that they do not complete their sporogonic phase and that infections in salmonids are incidental and represent a dead end. The unusual inflammatory response associated with PKD lends support to the notion that infection by PKX in salmonids is abnormal since such inflammatory responses are not generally observed in myxozoan infections. These obstacles for completion of the life-cycle involving salmonids point to the possibility that there may be no true hosts other than bryozoans which could become infected directly when spores are caught up in the lophophore feeding currents or when spores are incorporated into statoblasts during the development of these overwintering stages in the body cavity of bryozoans.

In the examination of higher level phylogeny the 18S rDNA sequences of the better known taxa in Group 1 provided variable support for systematics based on morphology. Whereas *Ceratomyxa* clustered with *Kudoa* with 100% bootstrap support in the molecular tree, these genera have been placed in separate orders, Bivalvulida and Multivalvulida respectively in the classical taxonomy (Lom & Dykova, 1992). This has also been found by Kent *et al*. (1998). However, the relationships of *Myxobolus*, *Myxidium* and *Henneguya* indicated by sequences, were as found by Smothers *et al*. (1994) and these upheld the systematics based on spore structure.

Although there are over 50 myxozoan genera recognized by the structure of their myxosporean type spores, 18S rDNA sequence data are known for only about 7 of these. Nevertheless, our data show that there is strong bootstrap support for a major branch of myxozoans parasitic in Bryozoa (Group 2) and indicate that there was an ancient split between these and the better known genera constituting Group 1. This suggests that bryozoans may have been the ancestral hosts of Myxozoa. Alternatively, myxozoans of vertebrates and bryozoans may have evolved for a long time as separate radiations from some ancestral type. Subsequent additional sequence data for many more myxozoan genera may alter this apparent dichotomy in the phylogeny. However, there are at least 2 characters which at present distinguish members of the bryozoan clade of myxozoans from the rest of the Myxozoa: (a) sporoplasmosomes with lucent bar-like invaginations and (b) lack of development of hard spore coats. Our results have identified bryozoans as important, but previously unrecognized hosts in which many new myxozoan species are likely to be discovered, some of which may have life-cycles linked to fish. This represents an important new avenue of investigation, particularly in marine environments where bryozoans are abundant and diverse and where there are many species of Myxozoa in fish. Further studies of these enigmatic parasites will reveal whether the life-cycles of myxozoan parasites of bryozoans incorporate other hosts and thus link the ecology of vertebrate and invertebrate stages.

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