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The phylogeography of salmonid proliferative kidney disease in Europe and North America

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Salmonid proliferative kidney disease (PKD) is caused by the myxozoan *Tetracapsuloides bryosalmonae*. Given the serious and apparently growing impact of PKD on farmed and wild salmonids, we undertook a phylogeographic study to gain insights into the history of genealogical lineages of *T. bryosalmonae* in Europe and North America, and to determine if the global expansion of rainbow trout farming has spread the disease. Phylogenetic analyses of internal transcribed spacer 1 sequences revealed a clade composed of all North American sequences plus a subset of Italian and French sequences. High genetic diversity in North America and the absence of genotypes diagnostic of the North American clade in the rest of Europe imply that southern Europe was colonized by immigration from North America; however, sequence divergence suggests that this colonization substantially pre-dated fisheries activities. Furthermore, the lack of southern European lineages in the rest of Europe, despite widespread rainbow trout farming, indicates that *T. bryosalmonae* is not transported through fisheries activities. This result strikingly contrasts with the commonness of fisheries-related introductions of other pathogens and parasites and indicates that fishes may be dead-end hosts. Our results also demonstrate that European strains of *T. bryosalmonae* infect and induce PKD in rainbow trout introduced to Europe.

Keywords: salmonid proliferative kidney disease; internal transcribed spacer 1 sequences; phylogeography; bryozoans; aquaculture

1. INTRODUCTION

The combination of overfishing and changing attitudes about consuming healthy food has contributed to the recent rapid expansion of the aquaculture industry worldwide. The total global production of farmed aquatic meat has increased 15-fold from 1970 to 2000, and aquaculture now dominates all other animal food-producing sectors in terms of growth (Tacon 2003). Coincident with such expansion have been disease problems in farms and hatcheries (Subasinghe *et al.* 2000) and growing concerns about human-mediated transport of pathogens and parasites (Harvell *et al.* 1999; Daszak *et al.* 2000). For example, transfer of infected stocks can spread disease and cause mass mortality as has occurred through oyster shipments to new regions in the shellfish industry (Farley 1992). Diseases have also been introduced through food used in the industry. Salmonid whirling disease is believed to have been introduced to the USA initially through importation of frozen infected fishes from Europe (Hoffman 1990). The disease has gone on to spread across much of North America, presumably through the subsequent transfer of live trout from infected hatcheries and by movement of infected fishes in streams (Hoffman 1990). It now poses a severe threat to the sport fishing industry in the intermountain region of western North America (Hedrick *et al.* 1998). Fisheries activities probably also account for the occurrence and spread of whirling disease in Asia, Australia, New Zealand and South Africa (Hoffman 1990).

Proliferative kidney disease (PKD) is another devastating disease of salmonids which, like whirling disease, is a result of infection by a myxozoan parasite. Although the organism causing PKD was recognized for many decades, it was only determined to be a myxozoan in the 1980s (Kent & Hedrick 1985). The recent discovery, based on molecular evidence, that freshwater bryozoans are hosts of the causative agent of PKD (Anderson *et al.* 1999), simultaneously allowed the parasite to be described as *Tetracapsula bryosalmonae* (Canning *et al.* 1999) (now *Tetracapsuloides bryosalmonae* (Canning *et al.* 2000)) and finally identified the long-sought source of PKD, which is not transmitted from fish to fish. These studies revealed that *T. bryosalmonae* develops into freely circulating sacs in the body cavity of freshwater bryozoan hosts and that mature sacs become filled with infective spores. Subsequent transmission studies confirmed that PKD results when infective spores are released from freshwater bryozoans and invade fishes (Feist *et al.* 2001).

PKD has been documented in a range of salmonids, and causes particular economic loss to rainbow trout (*Oncorhynchus mykiss*) fish farms in the UK and Europe and to salmon hatcheries in North America (Hedrick *et al.* 1993). PKD is also a disease of wild and feral fishes (MacConnell & Peterson 1992; Feist *et al.* 2002; Wahli *et al.* 2002) and appears to be on the increase, possibly as a consequence of eutrophication or increasing temperatures (El-Matbouli & Hoffman 2002; Wahli *et al.* 2002). The disease is manifested as a massive immune response to the presence of *T. bryosalmonae*, but the temperature dependence of the fishes' immune systems means that the disease does not develop until temperatures exceed 15 °C, although fishes can become infected at lower temperatures (Gay *et al.* 2001). The complete life cycle of *T. bryosalmonae* is unresolved. The magnitude of the immune response and apparent lack of spore maturation have long been cited as evidence that salmonids may be accidental hosts; however, it is now clear that spore-like

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stages are released in the urine of some salmonids (Hedrick *et al.* 2004). The functionality of these spore-like stages remains equivocal. First, they are relatively rare in fishes and the numbers released in urine are low (Hedrick *et al.* 2004). Second, transmission studies have either provided negative evidence for transmission of *T. bryosalmonae* from fishes to bryozoans (Tops *et al.* 2004) or have not controlled for contamination (Morris *et al.* 2002).

Tetracapsuloides bryosalmonae parasitizes a broad range of freshwater bryozoan hosts, some of which occur throughout the holarctic (Bushnell 1973; Okamura & Wood 2002; Wood 2002). This widespread distribution of bryozoan hosts and their seasonal growth to form dense stands of intertwined colonies in late spring or early summer help to explain the distribution and timing of PKD outbreaks in salmonid fish (Anderson *et al.* 1999; Okamura & Wood 2002). In addition, recent genetic studies providing evidence for ongoing gene flow among bryozoan populations across northwest Europe, point to migratory waterfowl as agents of occasional long-distance dissemination of bryozoan dispersive stages (statoblasts) (Freeland *et al.* 2000*a*). The widespread distribution of PKD could thus be a function of the distribution of bryozoans, and the dispersal of statoblasts may allow *T. bryosalmonae* to colonize new host populations. Further evidence for waterfowl-mediated dispersal of bryozoans includes the presence of intact bryozoan statoblasts in waterfowl guts and faeces (Figuerola *et al.* 2003), viability of at least some statoblasts that have been excreted by waterfowl (Charalambidou *et al.* 2003), and the narrow intercontinental ranges of several bryozoan species in accord with migratory flyways (Wood 2002). However, it is not yet known whether *T. bryosalmonae* is able to infect statoblasts. Fisheries activities represent an alternative or additional potential mechanism for the spread of PKD. In particular, the transport of rainbow trout, which are native to North America, may have resulted in broad dissemination of the disease when fishes were introduced to Europe, much as has occurred with salmonid whirling disease. Such a fisheries-based route of disease spread would, of course, depend on successful transmission of parasites from salmonids, either directly or indirectly, into local bryozoan hosts.

In view of the serious and apparently growing impact of PKD in farmed and wild salmonids (Hedrick *et al.* 1993; Burkhardt-Holm 2002; Feist *et al.* 2002; Wahli *et al.* 2002) we conducted a phylogeographic investigation of *T. bryosalmonae* from sites in Europe and North America. We were particularly interested to determine (i) whether the phylogeographic pattern might provide insights into the history of genealogical lineages of *T. bryosalmonae* in Europe and North America; (ii) if there is evidence that farmed rainbow trout are infected by native strains of *T. bryosalmonae*; and (iii) if there is evidence of recent introduction as a result of activities associated with the fisheries industry.

2. MATERIAL AND METHODS

(a) *Study material*

Samples of kidney material from fishes apparently infected with PKD were taken from a range of salmonids throughout Europe and North America (see table 1). Most samples were collected from fish farms and hatcheries during PKD outbreaks, but some wild fishes were also collected. In one example, infected bryozoans were collected from the vicinity of the hatcheries and farms. The low prevalence of *T. bryosalmonae* infections in many bryozoan populations, as well as cryptic parasitic stages that cannot be readily identified in bryozoan hosts, precluded collecting further infected bryozoan material for the study (Okamura *et al.* 2001; Tops & Okamura 2003). All tissue was fixed in absolute ethanol and stored at −20 °C for later molecular work. The study contributed 119 new sequences which have been deposited with the European Molecular Biology Laboratory (EMBL) data bank (accession numbers provided in table 1).

(b) *Molecular analysis*

Host (fish or bryozoan) and parasite DNA were extracted together from the tissue samples. All kidney tissue samples were extracted using a 5% chelex protocol: 20–40 mg of tissue was chopped into small fragments (*ca.* 2 mm × 2 mm) and incubated in 200 µl of 5% chelex solution at 56 °C for 3 h; vortexed for 30 s; incubated at 95 °C for 15 min; vortexed for a further 30 s, and PCR carried out on 1 µl of the supernatant. Bryozoan samples were extracted using a modified hexadecyltrimethylammonium bromide (CTAB) protocol (Tops & Okamura 2003).

Primers for the internal transcribed spacer 1 (ITS-1) rDNA region of *T. bryosalmonae* were designed. Forward primers were based on a published sequence for the 3′ end of the 18S region of *T. bryosalmonae* (U70623 from Saulnier *et al.* (1999)). The use of universal 5.8S rDNA primers (Hillis & Dixon 1991) as reverse primers was explored extensively, but these produced only fragments of host sequence, i.e. the partial 18S and ITS-1 of the bryozoan or fish host (depending on the material from which the DNA sample was extracted) and not fragments of the parasite. Eventually we were able to generate a putative *T. bryosalmonae* sequence based on a primer designed from conserved sequences in the 5.8S from sequences that aligned with the 3′ end of the 18S of *T. bryosalmonae* in a BLAST search. This sequence was confirmed as being *T. bryosalmonae* as the first 114 bp exhibited 100% homology with the 3′ end of the published 18S sequence for *T. bryosalmonae* (accession number U76023), and a maximum of 55.8% and 58.4% homology to the published 18S sequence for one of the fish hosts (*Oncorhyncus mykiss*: accession number AF308735) and a relative of the freshwater bryozoan host *Fredericella sultana* (*Cristatella mucedo*: accession number AF025947), respectively. Subsequent PCR of fish kidney and bryozoan material, using these primers, yielded multiple bands on 1.5% low melting point agarose electrophoretic gels, containing 0.2 mg ml^{-1} of ethidium bromide. These gels were visualized on an ultraviolet transilluminator, and the bands were carefully excised from the gels, cloned and sequenced. All sequences were subjected to BLAST searches to identify the sequences most likely to be *T. bryosalmonae*. *Tetracapsuloides bryosalmonae* specific forward (ITS1-SpecA 5′-GAATGACTTAGCGAGAACTTGGTGGTA-3′; ITS1-SpecB 5′-GCTTTTAAATTCCCAAAAGAGTTTATTAGC-3′; ITS1-SpecC 5′-GAGTTTATTAGCTACAAACTTGTTTGAGC-3′; ITS1-SpecD 5′-AGCTACAAACTTGTTTGAGCTTGGTCG-3′) and reverse (*5.8mh* 5′-CGCAGCAAGCTGCGTTCTTCATCGA-3′) primers were then designed from the sequences generated. These primers produced a fragment of 569–604 bp extending from the 18S through the ITS-1 and terminating in the 5.8S.

Table 1. Details of *Tetracapsuloides bryosalmonae* material used in phylogenetic analyses including the site codes (see figure 1), group identity (EU, European group; NA, North American clade; see figure 1) (and the number of sequences of each group and the accession numbers at the site), fish and bryozoan hosts (and type of host population followed by the date the material was collected), and location of sites (and the associated geographical coordinates). Atlantic salmon (*Salmo salar*); rainbow trout (*Oncorhynchus mykiss*); brown trout (*Salmo trutta*); chinook salmon (*Oncorhynchus tshawytscha*); coho salmon (*Oncorhynchus kisutch*); cutthroat trout (*Oncorhynchus clarki*); and bryozoan (*Fredericella sultana*).

site code	group identity (number of sequences) (accession numbers)	host species (population, date collected)	location
Scot 1	EU (3) (AJ639958–AJ639960)	*Salmo salar* (farmed, 08/01)	North Uist, Scotland (57°34′ N, 07°13′ W)
Engl 1	EU (5) (AJ639965–AJ639969)	*Oncorhynchus mykiss* (farmed, 11/00)	Berkshire, England (51°24′ N, 01°08′ W)
Engl 2	EU (5) (AJ639978–AJ639982)	*O. mykiss* (farmed, 08/00)	Hampshire, England (51°06′ N, 01°14′ W)
Engl 3	EU (5) (AJ639970–AJ639974)	*Salmo trutta* (wild, 01/01)	Hampshire, England (51°04′ N, 01°31′ W)
Engl 4	EU (3) (AJ639975–AJ639977)	*S. trutta* (wild, 01/01)	Hampshire, England (51°03′ N, 01°19′ W)
Engl 5	EU (4) (AJ639961–AJ639964)	*O. mykiss* (farmed, 08/99)	Hampshire, England (50°59′ N, 01°30′ W)
Engl 6	EU (12) (AJ639983–AJ639994)	*Fredericella sultana* (wild, 05–06/03)	Dorset, England (50°47′ N, 02°28′ W)
Fran 1	EU (9) (AJ640007–AJ640015)	*O. mykiss* (farmed, 06/00)	Seine-et-Marne, France (48°18′ N, 02°05′ E)
Fran 2	EU (7) (AJ640000–AJ640006)	*O. mykiss* (farmed, 06/00)	Finistère, France (48°39′ N, 04°03′ W)
Fran 3	EU (5), NA (1) (AJ639995–AJ639999; AJ640034)	*O. mykiss* (farmed, 06/00)	Charente-Maritime, France (46°06′ N, 00°06′ W)
Germ 1	EU (3) (AJ640016–AJ640018)	*O. mykiss* (farmed, 01/03)	Bavaria, Germany (48°05′ N, 10°49′ E)
Switz 1	EU (1) (AJ240021)	*S. trutta* (wild, 10/02)	Luzern, Switzerland (47°08′ N, 08°25′ E)
Switz 2	EU (2) (AJ240019; AJ240020)	*S. trutta* (wild, 07/02)	Thurgau, Switzerland (47°30′ N, 09°22′ E)
Ital 1	EU (3), NA (3) (AJ640030–AJ640032; AJ240044, AJ240047; AJ240051)	*O. mykiss* (farmed, 08/01)	Friuli-Venezia Giulia, Italy (45°59′ N, 13°02′ E)
Ital 2	NA (3) (AJ240038; AJ240039; AJ240041)	*O. mykiss* (farmed, 08/01)	Friuli-Venezia Giulia, Italy (45°51′ N, 13°21′ E)
Ital 3	EU (1), NA (5) (AJ240033; AJ240035; AJ240042; AJ240048; AJ240050; AJ240052)	*O. mykiss* (farmed, 08/01)	Friuli-Venezia Giulia, Italy (45°51′ N, 13°19′ E)
Ital 4	EU (3), NA (3) (AJ640022–AJ640024; AJ640036; AJ640043; AJ640045)	*O. mykiss* (farmed, 07/01)	Lombardia, Italy (45°27′ N, 09°53′ E)
Ital 5	EU (4), NA (2) (AJ640025–AJ640028; AJ640037; AJ640040)	*O. mykiss* (farmed, 07/01)	Lombardia, Italy (45°23′ N, 08°50′ E)
Ital 6	EU (1), NA (2) (AJ240029; AJ240046; AJ240049)	*O. mykiss* (farmed, 07/01)	Lombardia, Italy (45°16′ N, 10°41′ E)
USA 1	NA (3) (AJ240068–AJ240070)	*O. mykiss* (hatchery, 08/02)	California, USA (38°38′ N, 121°14′ W)
USA 2	NA (3) (AJ240065–AJ240067)	*O. tshawytscha* (hatchery, 05/02)	California, USA (39°31′ N, 120°22′ W)
USA 3	NA (12) (AJ240053–AJ240064)	*O. clarki* (feral, 08/00)	Montana, USA (45°36′ N, 111°35′ W)
USA 4	NA (4) (AJ240071–AJ240074)	*O. mykiss* (hatchery, 07/02)	Washington, USA (46°59′ N, 123°45′ W)
Can 1	NA (2) (AJ240075; AJ240076)	*O. kisutch* (hatchery, 07/02)	British Columbia, Canada (49°40′ N, 125°07′ W)

All fish kidney and bryozoan extracts were screened with existing diagnostic primers (primers PKX5f and PKX6r from Kent *et al.* (1998)) to independently establish whether the sample was taken from an individual infected with *T. bryosalmonae*. Positive samples were selected for PCR with *T. bryosalmonae* specific ITS-1 primers. Reagents and reaction concentrations were 1 × Promega PCR buffer A (containing 50 mM Tris-HCl (pH 9.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol and 1% Triton), dNTPs (0.2 mM), primers (0.5 μM each), Promega *Taq* DNA polymerase (0.5 units), 2 μl of template DNA (1 : 10 dilution in double-distilled H_2O of extraction stock), and double-distilled H_2O up to a reaction volume of 20 μl; the thermocycler regime consisted of an initial denaturing step of 95 °C for 5 min, then 35 cycles of 95 °C for 1 min, 50 °C

for 1 min and 72 °C for 1 min, this concluded with a final extension step of 72 °C for 5 min.

As parasite DNA was extracted from individual organisms within the host tissue material, the possibility of DNA from multiple strains of parasite being present in any single sample could not be discounted. Consequently, any product of a single PCR reaction may contain several different sequences, therefore it was imperative that any product being sequenced was isolated from others in the reaction. To this end, all sequencing was performed on cloned products. Cloning was achieved using the Promega pGEM-T Easy vector system into Promega JM109 ultra-competent *Escherichia coli* cells. The plasmids and inserts were harvested using Promega Wizard *Plus* SV Minipreps DNA Purification System. They were then submitted to MWG Biotech (http://www.mwg-biotech.com/html/i_custom/i_custom.shtml) and sequenced with forward and reverse M13 Universal primers using their 'Value Read' service.

Recent studies (e.g. Vogler & DeSalle 1994; Harris & Crandall 2000) have suggested that, as a result of its multicopy nature, intragenomic variation may occur in the ITS-1 region of a number of species. To establish the background level of such intragenomic variation of the ITS-1 region of *T. bryosalmonae*, 13 single *T. bryosalmonae* sacs were isolated from individual bryozoan hosts collected from the River Cerne, Dorset (see table 1) on three separate occasions in the summer of 2003. The DNA from each sac was extracted separately using the modified CTAB protocol (Tops & Okamura 2003). Following PCR and cloning, multiple colonies were lifted from a single agar plate (five per sac for the first three sacs, and 10 per sac from the 10 subsequent sacs) and sequenced. All sequences obtained from the same sacs were compared with each other to assess the presence and extent of intra-genomic variation. Intra-genomic ITS-1 variation was compared with inter-genomic variation to determine the extent to which the former might be expected to influence interpretation of results.

(c) *Phylogenetic analyses*

All sequences were aligned in BioEdit (Hall 1999) using ClustalW (Thompson *et al.* 1994) then corrected by eye. Indel sites, which formed a large proportion of the phylogenetically descriptive data, were largely in the form of microsatellite repeat units. Weighting was performed by reducing each repeat unit to an arbitrary binary character representing a single mutational event. Sequences were then analysed in three formats: (i) a raw aligned sequence; (ii) a hybrid aligned sequence plus binary indel data; and (iii) binary indel data alone. Unrooted maximum-likelihood, quartet-puzzling trees were constructed for the raw aligned sequences and the hybrid aligned sequence plus binary indel data using TreePuzzle v. 5.0 (Strimmer & von Haeseler 1996) employing an HKY matrix with four gamma-distributed categories. The bootstrap support values were calculated for 1000 replications. Trees were also constructed with binary indel data using the recently developed software MultiBayes (Pagel & Meade 2004): a Markov chain Monte Carlo-based program (available at http://www.rubic.reading.ac.uk/meade/Mark), which samples the universe of trees in proportion to their likelihood (Larget & Simon 1999). For this analysis the chain was run for 600 000 iterations with a burn-in of 100 000. One hundred trees were sampled from the chain at 5000 apart, giving an autocorrelation of 0.1. The general time reversible (GTR) model of sequence evolution was used with gamma rate heterogeneity with four categories. We explored the possibility of rooting our trees, but found that the ITS-1 sequence for *Buddenbrockia plumatellae*, the known sister taxon of *T. bryosalmonae*, was so divergent that sequences could not be aligned along the majority of their length. The rapid rate of evolution of the ITS-1 within the Myxozoa thus precludes an appropriate root.

We employed two approaches to investigate levels of genetic variation within groups of interest. Percentage divergence was calculated as the percentage of polymorphic sites among sequences within groups. The variation within groups of interest was also measured by calculating the mean and variance from sequence identity matrices (calculated using the 'alignment' > 'similarity identity matrix' option within BioEdit). These analyses used only the raw aligned sequence data.

3. RESULTS

Analyses by both TreePuzzle and MultiBayes gave results which identified a distinct clade of sequences largely from North America, which we refer to as the NA clade. The results of the maximum-likelihood analysis of the hybrid dataset are shown in figure 1. The NA clade also contained a subset of Italian sequences and one French sequence. The remaining sequences represented the majority of European samples and we will collectively refer to these as the EU group. Within this group there were several well-supported groups which were poorly resolved with regard to geographical location but which, apart from one, showed low levels of divergence. The remaining European sequences were unresolved. The Italian and French sequences in the NA clade derive from geographical locations which also have representatives from the EU group (table 1). As analyses were forced to be conducted on unrooted trees, we cannot infer which groups are more basal and which are more derived.

Despite the tight clustering exhibited by the North American samples, the genetic diversity was much greater than observed in the EU group or in the European samples belonging to the NA clade. This is exemplified by both the greater level of divergence (percentage of polymorphic sites), the range of divergence and the respective variances based on sequence identity matrices of pairwise comparisons (see table 2).

There were minor levels of variation in ITS-1 sequences obtained from isolated sacs of *T. bryosalmonae* indicating a low level of intra-genomic variation among multiple copies. Eight out of the 10 sacs showed some evidence of sequence differentiation at ITS-1 loci. The intra-genomic variation (mean of 0.26%; s.d. of 0.07%) was an order of magnitude lower than inter-genomic variation between sacs (mean of 4.80%; s.d. of 1.24%). We therefore conclude that undetected intra-genomic variation at ITS-1 loci will not compromise the interpretation of our results. The presence of intra-genomic variation in ITS-1 sequences illustrates that concerted evolution has not completely homogenized the multiple copies of ITS-1 repeats in *T. bryosalmonae*.

4. DISCUSSION

(a) *Patterns of genetic divergence and infection of fishes*

Our data provide evidence that *T. bryosalmonae* has been present in both North America and Europe for a

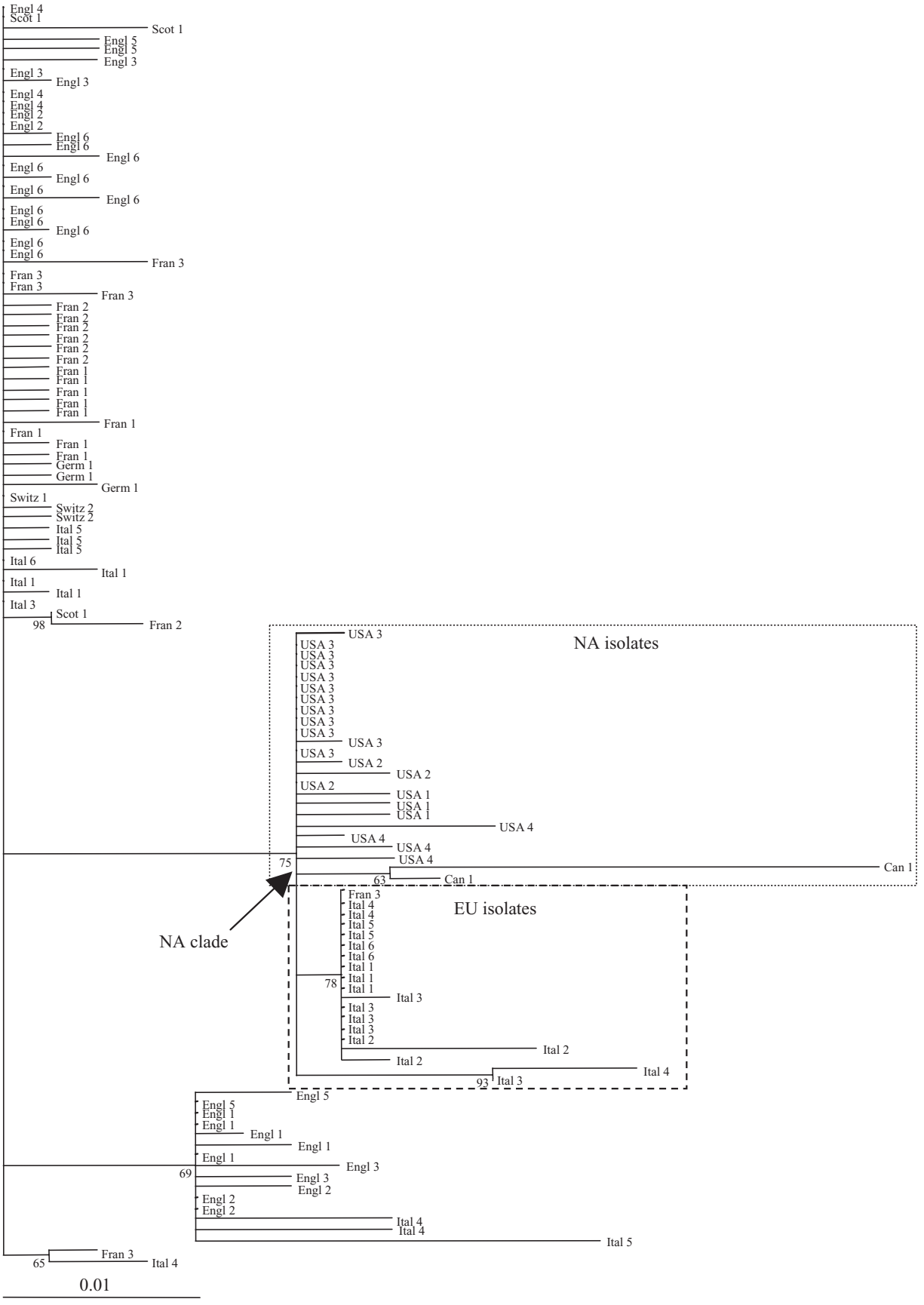

Figure 1. An unrooted maximum-likelihood tree of ITS-1 sequence data from *Tetracapsuloides bryosalmonae*, constructed using the program TreePuzzle v. 5.0 (Strimmer & von Haeseler 1996). Boxed data show the sequences associating with the North American group of genotypes (the NA clade); the box labelled 'NA isolates' (dotted line) shows sequences obtained from North America, and the box labelled 'EU isolates' (dashed line) shows the European members (from Italy and France) of the NA clade. All unboxed sequences comprise the EU group. For the site code key see table 1. The scale bar represents nucleotide substitutions per site.

Table 2. The sample size, percentage divergence (based on number of polymorphic sites across all sequences), range in percentage sequence divergence (based on pairwise comparisons) and variance based on pairwise comparisons for isolates from the EU group, from North America and from the European sites (Italian and French) in the NA clade.

group	*n*	percentage of polymorphic sites	range in percentage divergence	variance
North American samples	24	18.3	0.2–12.2	0.00135
EU group	76	12.8	1.1–2.4	0.00014
European samples from NA clade	19	4.3	0.2–4.6	0.00014

period of time sufficient to promote differences in ITS-1 sequences between the majority of isolates from the two continents. The higher genetic divergence observed among the North American ITS-1 isolates relative to that in the EU group (see table 2) could be explained by a longer time period during which these isolates have been evolving in North America; differential patterns of retraction into glacial refugia and postglacial expansions undergone by populations on the two continents; differential patterns of lineage extinction; or all of these. This greater range of sequence variation in North America is striking, especially given the smaller number of sequences obtained in the study, and suggests the possibility that *T. bryosalmonae* originated in North America, but further investigation using different genetic markers would be required for any strong inference of origin. However, our results do answer one question of specific interest: European strains of *T. bryosalmonae* are capable of infecting and inducing PKD in rainbow trout introduced to Europe. This is further supported by identification of ITS-1 sequences diagnostic of the EU group from both infected wild brown trout (*Salmo trutta*) and farmed rainbow trout.

(b) *Evidence that expansion of aquaculture has not spread proliferative kidney disease*

A particularly striking result of our study is the similarity of some of the Italian and French isolates to those from North America. Given the greater genetic diversity within North America and the absence of genotypes diagnostic of the NA clade in the rest of Europe, the best explanation for this result is colonization of southern Europe by *T. bryosalmonae* from North America after divergence of the NA clade. An alternative explanation is the colonization of North America from Europe, but this is unsupported by the relatively lower genetic diversity in Europe.

The inferred direction of colonization from North America to Europe is of interest given the introduction of rainbow trout to Europe from North America through the aquaculture industry. Perhaps, as in whirling disease, the introduction of diseased fishes from North America has entailed parasite transport, but there are several lines of evidence which suggest that this has not been the case. Most important is that there is no support for introduction to other European regions, despite extensive rainbow trout farming throughout Europe. In this regard, the spread of salmonid whirling disease across North America is informative. The myxozoan causative agent of whirling disease, *Myxobolus cerebralis*, was introduced to a rainbow trout farm in Pennsylvania from Europe in the 1950s and has rapidly spread to farmed and wild fish populations in some 22 states (Gilbert & Granath 2003). This recent introduction and spread is characterized by almost completely identical ITS-1 sequences for European and North American isolates of *M. cerebralis* (Whipps *et al.* 2004). This is not the case for *T. bryosalmonae*: the Italian and French sequences that group with the NA clade differ from North American sequences (the range in percentage divergence is 1.9–11.9%) and furthermore, show a range of variation among themselves (0–4.6% divergence). It is possible that this sequence variation in *T. bryosalmonae* resulted from the recent introduction of multiple genotypes to a few sites in southern Europe, but this explanation leaves unresolved the lack of subsequent spread across Europe and the absence of such sequences in North American material. These considerations suggest that an introduction is likely to have pre-dated the recent importation of rainbow trout and also that the EU isolates in the NA clade and those in the EU group have undergone similarly long periods of time diverging within Europe (e.g. see similar variance values in table 2).

(c) *Mechanisms of introduction and regional patterns of diversity*

Given the foregoing arguments, the best explanation for our results is that *T. bryosalmonae* was introduced to Europe from North America through transcontinental dispersal which greatly predated aquaculture activities. Because the unprotected soft spores produced by *T. bryosalmonae* are viable for a period of between 12 and 24 h after they are released into water (De Kinkelin *et al.* 2002), colonization of distant sites by *T. bryosalmonae* must be achieved by immigration of infected hosts. Possible modes of such colonization are (i) long-distance transport of infected bryozoan statoblasts by waterfowl, and (ii) introduction through infected fishes. The latter could theoretically have been achieved by infected anadromous salmonids that went off course. This, however, would require that parasite stages which developed within salmonids were successfully transmitted to new hosts: a result which has so far eluded demonstration (Tops *et al.* 2004) and is not supported by our results (see above). Conversely, there is a growing body of evidence for waterfowl-mediated transport of bryozoan statoblasts including: gene flow along annual migratory routes of waterfowl (Freeland *et al.* 2000*a*); the presence of statoblasts in bird guts and faeces (Figuerola *et al.* 2003); and viability of statoblasts collected from waterfowl faeces (Charalambidou *et al.* 2003). Furthermore, a phylogeographic study of mtDNA sequences (Freeland *et al.* 2000*b*) indicates that a freshwater bryozoan host of *T. bryosalmonae* (Anderson *et al.* 1999) colonized Europe from North America. Finally,

there is a long history of stray migratory North American birds appearing in Europe (Alerstam 1990).

Within Europe, southern regions appear to harbour the highest levels of genetic diversity of *T. bryosalmonae*, because representatives of both the EU group and the NA clade are present. This genetic diversity in southern Europe could be attributed to the Iberian Peninsula acting as a southern refugial area during periods of glaciation (Hewitt 2000), but this does not provide a full explanation. The North American type strains in southern Europe have apparently not spread into Europe, apart from one site in France. This suggests that the introduced strain and its variants were pre-adapted or became adapted to southern European conditions or that they are currently largely excluded from other parts of Europe as a result of, for example, competition.

(d) *Conclusions*

This study is, to our knowledge, the first phylogeographic investigation of myxozoans belonging to the Class Malacosporea and one of only a handful of phylogeographic studies of parasitic metazoans. For schistosomes, both human and non-human vectors of dispersal have been implicated. *Schistosoma mansoni* was apparently introduced to South America via the African slave trade (Després *et al.* 1993), while the early radiation of *Schistosoma* has been attributed to climate-driven dispersal of small mammals (Attwood *et al.* 2002). We hypothesize that waterfowl acted as disease vectors, introducing *T. bryosalmonae* from North America to southern Europe prior to recent fisheries activities. Our results may therefore provide a historical example of birds acting as vectors of disease as they clearly do at present, e.g. in the case of West Nile virus (Rappole & Hubálek 2003). The contributions to regional levels of genetic diversity, effected by such introductions of parasites, have far-reaching implications including potential explanations for geographical variation in virulence and a broader genetic basis for accommodation to environmental change.

Our lack of evidence for the introduction and subsequent spread of *T. bryosalmonae* via fisheries or other human-mediated activities is striking given the growing list of such introductions of other pathogens and parasites (Harvell *et al.* 1999; Daszak *et al.* 2000), and the widespread farming of rainbow trout. Indeed, there is even evidence for fisheries' related transport of non-parasitic taxa which, all else being equal, might less likely be introduced than parasitic taxa because they are not intimately associated with fishes. For example, the cladoceran *Daphnia lumholtzi* may well have been introduced to North America along with stock fishes from Africa (Sorensen & Sterner 1992). The lack of spread of *T. bryosalmonae* through fisheries activities would be readily explained if fishes are dead-end hosts, and could thus represent an important clue regarding the life history of *T. bryosalmonae*. By contrast, the use of both salmonid fish and tubificid worms as obligate hosts in the life cycle of *M. cerebralis* has promoted the successful introduction and rapid spread of whirling disease in North America. Further clarification of the life cycle of *T. bryosalmonae* and investigations based on independent genetic markers will help to explain the phylogeography of PKD.

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