

NOTE

Sequence analysis of the internal transcribed spacer (ITS) region reveals a novel clade of *Ichthyophonus* sp. from rainbow trout

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ABSTRACT: The mesomycetozoean parasite *Ichthyophonus hoferi* is most commonly associated with marine fish hosts but also occurs in some components of the freshwater rainbow trout *Oncorhynchus mykiss* aquaculture industry in Idaho, USA. It is not certain how the parasite was introduced into rainbow trout culture, but it might have been associated with the historical practice of feeding raw, ground common carp *Cyprinus carpio* that were caught by commercial fisherman. Here, we report a major genetic division between west coast freshwater and marine isolates of *Ichthyophonus hoferi*. Sequence differences were not detected in 2 regions of the highly conserved small subunit (18S) rDNA gene; however, nucleotide variation was seen in internal transcribed spacer loci (ITS1 and ITS2), both within and among the isolates. Intra-isolate variation ranged from 2.4 to 7.6 nucleotides over a region consisting of ~740 bp. Majority consensus sequences from marine/anadromous hosts differed in only 0 to 3 nucleotides (99.6 to 100% nucleotide identity), while those derived from freshwater rainbow trout had no nucleotide substitutions relative to each other. However, the consensus sequences between isolates from freshwater rainbow trout and those from marine/anadromous hosts differed in 13 to 16 nucleotides (97.8 to 98.2% nucleotide identity).

KEY WORDS: *Ichthyophonus hoferi* · Rainbow trout · Ribosomal gene complex · ITS1 · ITS2

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INTRODUCTION

Taxonomic uncertainties have accompanied the identification of *Ichthyophonus hoferi* since its original description by B. von Hofer in 1893 (reviewed by McVicar 1999). More recently, sequence analysis of the small subunit (SSU, also termed 18S) region of the ribosomal RNA gene complex (rDNA) confirmed that *Ichthyophonus* belongs to a primitive protistan group that is related to the common ancestor of animals and fungi (Wainright et al. 1993). This novel group initially consisted of *Dermocystidium*, the rosette agent, *Ichthyophonus* and *Psorospermium*, giving rise to the

acronym 'DRIP clade' (Ragan et al. 1996) that was later reorganized into the newly created class Mesomycetozoa (Herr et al. 1999).

A lack of distinguishing characteristics in the original species description (von Hofer 1893), combined with a high degree of morphological plasticity in life history stages (Okamoto et al. 1985), likely resulted in the grouping of several closely related organisms into a species assemblage that is collectively referred to as *Ichthyophonus hoferi* (Alderman 1982, Spanggaard et al. 1996). Phylogenetic studies using 18S rDNA sequences of *I. hoferi* have revealed only 5 SSU rDNA haplotypes worldwide (Criscione et al. 2002). In the NE

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Pacific, 2 SSU haplotypes that share 99.5% nucleotide similarity have been reported, with sequences from coastal rockfishes *Sebastes* spp. differing from those of sympatric Pacific herring *Clupea pallasii*, Chinook salmon *Oncorhynchus tshawytscha* (Criscione et al. 2002) and Puget Sound rockfish *Sebastes emphaeus* (Halos et al. 2005). Additionally, a phenotypic difference in salinity tolerance has been reported among *I. hoferi* isolates from freshwater rainbow trout *O. mykiss* and marine fish, although they share 100% SSU rDNA nucleotide identity (Hershberger et al. 2008). To avoid the confusion surrounding the *I. hoferi* species complex in this paper, the organism will hereafter be referred to generically as *Ichthyophonus*.

The rDNA complex of eukaryotes consists of a tandem set of genes and spacers, including the 18S rRNA, an internal transcribed spacer 1 (ITS1), the 5.8S rRNA, a second internal transcribed spacer (ITS2), the 28S rRNA, and a second external transcribed spacer (ETS2) (Cooper 2000). The goal of this study was to use the rDNA sequences of the less conserved ITS1 and ITS2 regions to assess potential genetic differences among *Ichthyophonus* isolates that were derived from a range of marine, anadromous and freshwater fish species in the NE Pacific. The isolates that were used included representatives of the most common SSU rDNA sequence types that were observed in the NE Pacific (Criscione et al. 2002, Halos et al. 2005, Hershberger et al. 2008).

MATERIALS AND METHODS

***Ichthyophonus* isolates.** *Ichthyophonus* was isolated and cultivated from heart tissue explants of 4 host species (Table 1) as previously described (Hershberger et al. 2008). Briefly, heart tissue was aseptically removed and placed in an isolation medium consisting of Eagle's MEM supplemented with foetal bovine serum (5% v/v), penicillin (100 IU ml⁻¹), streptomycin (100 µg ml⁻¹) and gentamycin (100 µg ml⁻¹), and buffered to pH 7.8 with 1 M Tris (MEM, pH 7.8). Cultures were incubated at 15°C for ~14 d, after which excess host tissue was removed. To generate sufficient quantities of *Ichthyophonus* for nucleic acid extraction, parasite growth was stimulated by passing the isolates into a hyphenation medium consisting of MEM that was adjusted to pH 3.5 and supplemented with 1% glucose (Spanggaard et al. 1994). Resporulation was then stimulated by returning the isolates to pH 7.8 for several days before genomic DNA was isolated. The rainbow trout RBT2 sample (Table 1) was not cultured prior to molecular analysis.

Isolation of genomic DNA. Genomic DNA was isolated from cultured *Ichthyophonus* or from infected heart tissue using a DNeasy blood and tissue kit (Qiagen) following the manufacturer's recommendations

Table 1. Details of the *Ichthyophonus* isolate and characteristics of the ITS rDNA gene regions. ITS PCR clones: single band of the ITS rDNA gene regions (ITS-5.8S-ITS2) was PCR amplified and cloned, and multiple PCR clones were sequenced per isolate. Size of ITS region: average number of nucleotides in the ITS1-5.8S-ITS2 rDNA gene regions for an individual isolate. ITS intra-isolate variation: average number of nucleotide changes and the p-distances among the PCR clones for each isolate spanning the ITS1-5.8S-ITS2 regions

Isolate ID	Host species	Location	Year	18S GenBank no. Region A/Region B	ITS GenBank no. (PCR clones)	No. of ITS PCR clones	Size of ITS region (bp)	ITS intra-isolate variation (p-distances)
IA2	Pacific herring	Puget Sound, WA	2005	GQ370767/GQ370788	GQ402831–GQ402842	12	727	4.6 (0.006)
IA113	Pacific herring	Puget Sound, WA	2008	GQ370766/GQ370787	GQ402907–GQ402916	10	730	3.8 (0.005)
IA51	Pacific herring	Sitka, AK	2007	GQ370773/GQ370794	GQ402872–GQ402886	15	727	5.9 (0.008)
IA6	American shad	Puget Sound, WA	2005	GQ370768/GQ370789	GQ402843–GQ402854	12	727	5.7 (0.008)
IA52	American shad	Columbia River, WA	2007	GQ370774/GQ370795	GQ402887–GQ402899	13	731	3.5 (0.005)
IA14	American shad	Columbia River, WA	2005	GQ370770/GQ370791	GQ402860–GQ402871	12	733	4.8 (0.006)
IA11	Copper rockfish	Puget Sound, WA	2006	GQ370769/GQ370790	GQ402855–GQ402859	5	729	7.6 (0.010)
RBT48	Rainbow trout	Hagerman Valley, ID	2007	GQ370772/GQ370793	GQ402995–GQ403008	14	738	3.8 (0.005)
RBT27	Rainbow trout	Hagerman Valley, ID	2007	GQ370771/GQ370792	GQ402982–GQ402994	13	739	3.6 (0.005)
RBT11	Rainbow trout	Hagerman Valley, ID	2008	GQ370776/GQ370801	GQ402928–GQ402937	10	739	3.7 (0.005)
RBT12	Rainbow trout	Hagerman Valley, ID	2008	GQ370777/GQ370797	GQ402938–GQ402947	10	740	2.5 (0.003)
RBT13	Rainbow trout	Hagerman Valley, ID	2008	GQ370778/GQ370798	GQ402948–GQ402963	16	738	2.4 (0.003)
RBT15	Rainbow trout	Hagerman Valley, ID	2008	GQ370779/GQ370799	GQ402964–GQ402969	6	740	5.1 (0.007)
RBT16	Rainbow trout	Hagerman Valley, ID	2008	GQ370780/GQ370800	GQ402970–GQ402981	12	738	3.2 (0.004)
RBT2 ^a	Rainbow trout	Hagerman Valley, ID	2008	GQ370775/GQ370796	GQ402917–GQ402927	11	738	4.1 (0.006)

^a*Ichthyophonus* was not cultured from this sample, and molecular analysis was performed directly on the infected tissue

for tissue extraction, with several modifications. *Ichthyophonus* spores or hyphae were initially placed in a tube containing beads (lysing matrix D; Bio 101) and incubated with 360 μ l of tissue lysis buffer and 40 μ l of proteinase K (Qiagen) at 55°C for 2 h before being homogenized in the Fast Prep FP120 instrument (Bio 101) for 40 s. The samples were centrifuged at 7200 \times *g* for 1 min, an additional 40 μ l of proteinase K was added, and the homogenates were incubated overnight at 55°C. The *Ichthyophonus* homogenates were then subjected to a second round of bead homogenization, and the samples were extracted with no further modifications to the manufacturer's protocol.

PCR amplification, cloning, and sequencing of SSU (18S) and ITS (ITS1-5.8S-ITS2) rDNA regions. PCR amplification and sequencing of 2 segments (A and B) of the SSU rDNA were performed as previously described (Criscione et al. 2002, Halos et al. 2005). Briefly, region A primers amplify a 640 bp segment, while region B primers amplify a 673 bp segment. Segments were direct sequenced in the forward and reverse directions using the ABI Big Dye Terminator v1.1 and an ABI 3700 automated sequencer (Applied Biosystems).

The region spanning ITS1, 5.8S and ITS2 was amplified using the following primers: 5'-GTT TCC GTA GGT GAA CCT GC-3' and 5'-TCC TCC GCT TAT TRA TAT GC-3'. DNA amplification was achieved using the GoTaq PCR enzyme and buffer system (Promega) with 1 cycle of 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, 62°C for 45 s, and 72°C for 1.5 min. PCR products were cloned using the pGEM-T easy vector system (Promega) or Topo-TA cloning kit for sequencing (Invitrogen), to assess intra-isolate sequence variation. Clone insert sizes were assessed by PCR amplification using vector primers. Multiple clones of PCR products from each isolate containing inserts of expected size were sequenced using the high-throughput genomic unit at the University of Washington (Seattle, WA, USA). A total of 5 to 16 clones were sequenced per isolate (Table 1).

Sequence and phylogenetic analyses. Sequence chromatograms were visually assessed and edited using the Sequencher v4.5 software (Gene Codes). Phylogenetic comparisons were performed after the sequences had been aligned using ClustalX (Thompson et al. 1997), with default parameter settings including the pairwise and multiple alignment gap-opening/extension penalties of 10/0.10 and 3/1.8, respectively. Phylogenetic analyses were performed using either the majority consensus sequences for each isolate (derived from all PCR clones for each isolate) or all individual PCR clone sequences. Alignments were examined by eye and no adjustments were deemed necessary. The ITS1-5.8S-ITS2 sequence from a related mesomycetozoean, *Amoebidium parasiticum* (AY388646), was used

as an outgroup species. Phylogenetic inferences were made using the neighbor-joining and maximum parsimony methods in the MEGA v4.0 software package (Tamura et al. 2007). Tree topologies were validated by bootstrapping 1000 \times .

RESULTS

All isolates that were used in this study were identical across the A and B regions of the SSU rDNA (Table 1), and the sequences were identical to those of *Ichthyophonus hoferi* (e.g. AF467798 and AF467797).

Primers that were designed to amplify the ITS1-5.8S-ITS2 region of *Ichthyophonus hoferi* produced an amplicon of ~740 bp. Sequencing of multiple clones for each isolate showed that intra-isolate variation ranged from 2.4 to 7.6 nucleotides in the region examined (p-distances range: 0.003 to 0.010) (Table 1). The isolate from copper rockfish *Sebastes caurinus* (IA11) had the highest level of intra-specific variation (7.6 changes out of 729 nucleotides). Majority consensus sequences were derived from all the clones for each isolate. Consensus sequences from the 6 isolates from herring or shad had no nucleotide substitutions relative to each other, and those from the 8 isolates from trout had no nucleotide substitutions relative to each other. The majority consensus sequence from the single rockfish isolate differed from those of herring/shad isolates by 3 nucleotides and from those of trout isolates by 16 nucleotides (Table 2). The consensus sequences from herring/shad differed from those of trout by 13 nucleotides.

The ITS2 region had the greatest mean number of nucleotide differences (8.7 changes out of 311 nucle-

Table 2. Pairwise differences among representative *Ichthyophonus* isolates for the ITS rDNA regions (ITS1-5.8S-ITS2). The p-distance is shown above the diagonal, while the number of nucleotide differences (out of 637 aligned nucleotides) is shown below the diagonal. IA2 (herring) is shown as the representative for IA2, IA113 (herring), IA51 (herring), IA6 (shad), IA52 (shad), and IA14 (shad), whose majority consensus sequences had no nucleotide substitutions relative to each other. RBT2 is shown as the representative for all isolates from rainbow trout (RBT2, RBT48, RBT27, RBT11, RBT12, RBT13, RBT15, and RBT16), whose majority sequences had no nucleotide sequences relative to each other

	IA2 (herring)	IA11 (rockfish)	RBT2 (trout)	<i>Amoebidium parasiticum</i>
IA2 (herring)	–	0.005	0.020	0.190
IA11 (rockfish)	3	–	0.025	0.190
RBT2 (trout)	13	16	–	0.193
<i>A. parasiticum</i>	121	119	123	–

otides) among the consensus sequences from herring (represented by IA2), rockfish (IA11) and rainbow trout (represented by RBT2). The ITS1 region also varied among these 3 representative isolates (2.7 changes out of 230 nucleotides). The rainbow trout 5.8S rDNA sequence had a single nucleotide change (out of 109 nucleotides) relative to the herring/rockfish consensus sequences.

Phylogenetic analysis using the entire ITS region consensus sequence for each isolate (Table 1) supports a genetic division between freshwater and marine isolates of *Ichthyophonus*. Neighbor-joining and maximum parsimony analyses gave similar topologies (Fig. 1). All isolates that were derived from either marine or anadromous host species clustered to the exclusion of isolates from freshwater rainbow trout. Phylogenetic analysis using all PCR clone sequences, rather than the consensus sequences, gave a similar topology (data not shown).

DISCUSSION

We demonstrate that genetic variation in the ITS regions can be informative in distinguishing among isolates of *Ichthyophonus* that have 100% sequence identity in the more highly conserved SSU rDNA region, and that phylogenetic analysis of these ITS sequences supports the presence of a major division between *Ichthyophonus* isolates from rainbow trout in Idaho and those from marine and anadromous hosts in the NE Pacific (Fig. 1). The genetic difference between the 2 clades appears to mirror phenotypic differences

between representative isolates that include differences in stability in freshwater and seawater (Hershberger et al. 2008). Because rDNA regions exist as multi-copy genes, we expected some level of sequence variation among clones that were generated from individual isolates. However, the *Ichthyophonus* isolates that were used in this study did not originate from single cell cultures; thus, we cannot exclude the possibility that some of the sequence variation among the clones of a given isolate was due to genetic differences between individual cells within that isolate. Nevertheless, the level of ITS sequence variation between marine and freshwater clades was substantially greater than that among clones from individual cultures (Table 1).

Ichthyophonus is generally considered to be a pathogen of wild, marine fish (McVicar 1999), and reports in fish from the eastern North Pacific began surfacing in the late 1970s and early 1980s (reviewed by Hershberger et al. 2002). The pathogen is currently ubiquitous in many marine hosts throughout the NE Pacific, where it has been implicated as a population-limiting factor (Marty et al. 1998, Hershberger et al. 2002). *Ichthyophonus* was first reported in freshwater rainbow trout from intensive culture facilities in Washington State during an epizootic in 1952 (Rucker & Gustafson 1953). Early epizootics in Idaho trout culture were linked to the practice of feeding raw tissues from common carp *Cyprinus carpio* (Erickson 1965). Epizootics due to *Ichthyophonus* ceased when this practice was stopped, and there were no reports of the parasite for ~40 yr. It is not clear whether *Ichthyophonus* remained endemic in the trout industry or if the con-

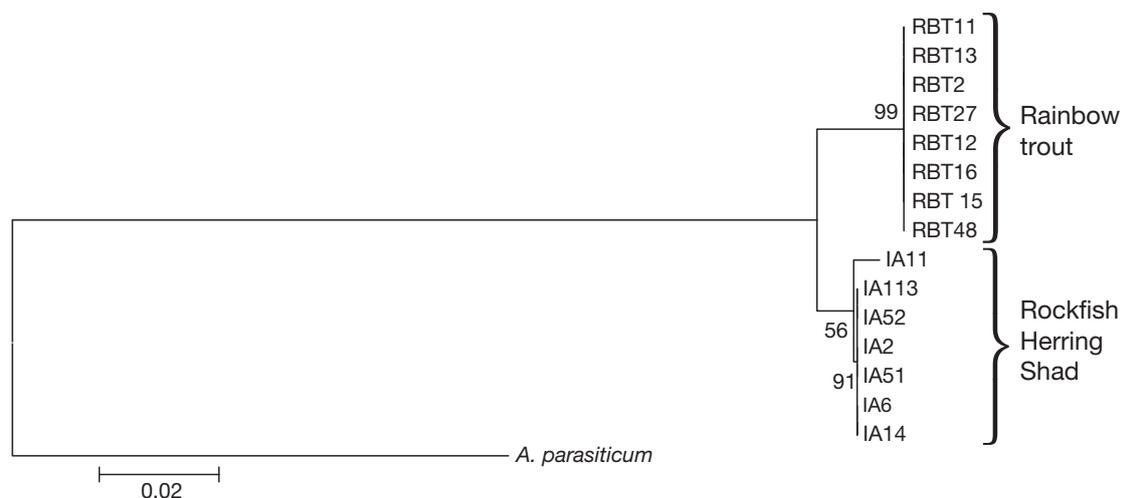


Fig. 1. Evolutionary relationships of *Ichthyophonus* isolates based on the majority consensus sequence of the ITS region (ITS1-5.8S-ITS2) as inferred using the neighbour-joining method (1000 bootstrap iterations). A similar tree topology was obtained using the maximum parsimony method (not shown). Isolates are derived from rainbow trout (RBT-11, 13, 2, 27, 12, 16, 15 and 48), Pacific herring (IA2, IA113 and IA51), American shad (IA6, IA52 and IA14), and copper rockfish (IA11). *Amoebidium parasiticum* (AY388646) was used as an outgroup

temporary isolates represent a new introduction. Typing of contemporary and historical archived samples from a larger host and geographical range will be needed to determine if the unique ITS lineage that is identified in rainbow trout reflects (1) divergence from the common Pacific northwest marine type within the aquaculture setting, (2) a unique parasite source (e.g. North American carp), or (3) both.

It is unlikely that a single species occupies the broad host and geographical ranges reported for *Ichthyophonus hoferi* that include a near-circumpolar distribution in more than 80 host species, including freshwater and marine elasmobranch and teleost fish, amphibians, reptiles, and fish-eating birds (reviewed by McVicar 1999). Morphological (Rand 1994) and SSU rDNA sequence differences in *I. hoferi* isolates from fish in the Atlantic Ocean provided initial evidence for speciation within the genus (Rand et al. 2000), and it is likely that additional variation will be found on a larger biogeographical scale. In addition to identifying genetic separation between freshwater and marine isolates of *Ichthyophonus* in the Pacific northwest, preliminary evaluations of the ITS regions also indicate genetic separation between marine isolates in the Atlantic and Pacific Oceans (P. K. Hershberger & M. K. Purcell unpubl. data). In the future, sequence analysis of ITS or other informative regions of the *Ichthyophonus* genome may assist in the understanding of phenotypic and epidemiological differences among isolates (e.g. host-specific virulence, route of infection, mechanisms of transmission and features of the life cycle), including the identification of alternate hosts.

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