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Ichthyophonus parasite phylogeny based on ITS rDNA structure prediction and alignment identifies six clades, with a single dominant marine type

Jacob L. Gregg[1,2,*], Rachel L. Powers[3], Maureen K. Purcell[3], Carolyn S. Friedman[2], Paul K. Hershberger[1]

[1]Marrowstone Marine Field Station, US Geological Survey, 616 Marrowstone Point Road, Nordland, Washington 98358, USA
[2]School of Aquatic and Fishery Sciences, University of Washington, Box 355020, Seattle, Washington 98195, USA
[3]Western Fisheries Research Center, US Geological Survey, 6505 N.E. 65th Street, Seattle, Washington 98115, USA

ABSTRACT: Despite their widespread, global impact in both wild and cultured fishes, little is known of the diversity, transmission patterns, and phylogeography of parasites generally identified as *Ichthyophonus*. This study constructed a phylogeny based on the structural alignment of internal transcribed spacer (ITS) rDNA sequences to compare *Ichthyophonus* isolates from fish hosts in the Atlantic and Pacific oceans, and several rivers and aquaculture sites in North America, Europe, and Japan. Structure of the *Ichthyophonus* ITS1–5.8S–ITS2 transcript exhibited several homologies with other eukaryotes, and 6 distinct clades were identified within *Ichthyophonus*. A single clade contained a majority (71 of 98) of parasite isolations. This ubiquitous *Ichthyophonus* type occurred in 13 marine and anadromous hosts and was associated with epizootics in Atlantic herring, Chinook salmon, and American shad. A second clade contained all isolates from aquaculture, despite great geographic separation of the freshwater hosts. Each of the 4 remaining clades contained isolates from single host species. This study is the first to evaluate the genetic relationships among *Ichthyophonus* species across a significant portion of their host and geographic range. Additionally, parasite infection prevalence is reported in 16 fish species.

KEY WORDS: *Ichthyophonus* · Parasite phylogenetics · Internal transcribed spacer · RNA secondary structure prediction · rDNA · Herring · Salmon · Halibut · Shad · Rainbow trout

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INTRODUCTION

Parasites in the genus *Ichthyophonus* impact fish populations, fisheries, and aquaculture across a wide geographic range. Infections have been reported in over 145 fish species, from the Barents Sea, AK, to the southern tip of Africa, in Atlantic and Pacific oceans, and in freshwater on 6 continents (see Table S1 in the Supplement at www.int-res.com/articles/suppl/d120p125_supp.pdf). Impacts in wild populations vary from acute, mass-mortality events, as occur in Atlantic herring *Clupea harengus* (Cox 1916, Fish 1934, Rahimian & Thulin 1996) and Chinook salmon *Oncorhynchus tshawytscha* (Kocan et al. 2004), to chronic epizootics that change population structure without causing synchronous fish kills. Evidence suggests the latter is true for populations of European plaice *Pleuronectes platessa* (McVicar 1981), Pacific herring *Clupea pallasii* (Hershberger et al. 2002, Marty et al. 2003), and American shad *Alosa sapidissima* (Hershberger et al. 2010), and likely occurs in many less-studied populations. In addition to culling individuals from commercially exploited populations, *Ichthyophonus* spp. destroy or reduce the value of fillets in haddock (McVicar 1979), Chinook salmon (Kocan et al. 2004), Atlantic mackerel

*Corresponding author: jgregg@usgs.gov

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Scomber scombrus (Sproston 1944) and walleye pollock *Gadus chalcogrammus* (White et al. 2014).

First identified as a problem in the culture of brown trout *Salmo trutta* and brook trout *Salvelinus fontinalis* in Germany at the end of the 19th century (von Hofer 1893), *Ichthyophonus* spp. have subsequently emerged, and persist today, in aquaculture around the globe. The parasites are reported in cultured salmonids (Rucker & Gustafson 1953, Ono et al. 1966, Slocombe 1980, Athanassopoulou 1992), carangids (Fujiya 1976, Egusa 1983), cichlids (El-Ghany & El-Ashram 2008, Shawer et al. 2011), cyprinids (Reichenbach-Klinke 1954, Prabhuji & Sinha 2009), sparids (Athanassopoulou 1992, Franco-Sierra et al. 1997), moronids (Sitja-Bobadilla & Alvarez-Pellitero 1990, Franco-Sierra et al. 1997) and mugilids (Paperna 1986, Franco-Sierra et al. 1997). As in wild populations, effects in aquaculture range from significant acute mortality (Rucker & Gustafson 1953) to chronic low level mortality and destruction of fillets (Erickson 1965, Miyazaki & Jo 1985, Franco-Sierra et al. 1997).

Despite their widespread impact, little is known about the diversity, transmission patterns or phylogeography of *Ichthyophonus* spp. The internal, histozoic parasites are difficult to differentiate under microscopic examination due to highly plastic morphology *in vivo* (Sproston 1944) and *in vitro* (Okamoto et al. 1985, Spanggaard et al. 1994). Interspecific transmission demonstrated in the laboratory (Gustafson & Rucker 1956, McVicar & McLay 1985) made it convenient to label them generalists, and for a century, descriptions of these parasites were lumped into a single specific name, *I. hoferi* (Plehn & Mulsow 1911), with uncertain placement within Fungi. The fungal classification was held in common use until 1996 when 2 groups of researchers used 18S rDNA sequences to determine that *Ichthyophonus* spp. are not a fungi, but rather, single-celled relatives of choanoflagellates and metazoans (Ragan et al. 1996, Spanggaard et al. 1996). Subsequently, *Ichthyophonus* was placed in a newly erected class, Mesomycetozoea, with other single-celled eukaryotes, primarily parasites of aquatic species (Mendoza et al. 2002). At lower taxonomic levels, molecular genetic studies, based on both 18S and internal transcribed spacer (ITS) rDNA regions, identified novel *Ichthyophonus* types in 5 of the 6 new hosts examined (Rand et al. 2000, Criscione et al. 2002, Hershberger et al. 2010, Rasmussen et al. 2010) with a single new species formally described (Rand et al. 2000).

The generalizations applied to *Ichthyophonus* and lack of information regarding parasite strain distribution are problematic for a parasite that is globally distributed. These information gaps severely limit our understanding of the processes that lead to epizootics. Historically, outbreaks of ichthyophoniasis were attributed to changes in host population or environment, and the role of parasite distribution, adaptation, and strain differences were generally ignored. The primary aim of this study was to gain an understanding of *Ichthyophonus* species richness by constructing a new ITS rDNA-based phylogeny using parasite isolates from several hosts and regions, and to identify any phylogeographic patterns that occur in the distribution of the parasite. Secondarily, we report infection prevalence data for several of the host species from which the parasites were isolated.

MATERIALS AND METHODS

Sample collection

During 2010 and 2011, 2215 fish from 16 known and potential host species were sampled opportunistically from academic, state, provincial, and federal fishery research surveys occurring in Canada and the USA (see Table 1). Sampling occurred along the Pacific and Atlantic Coasts of North America, off shore, and in coastal bays and rivers. Target sample number per species per location was 60 individuals, allowing for 95% confidence of detection where apparent prevalence was ≥5% (Dohoo et al. 2009). In some cases, this sample number was not met. Data are reported for all samples where n ≥ 50 and for smaller samples when parasites were detected.

Ichthyophonus infections were detected by explant culture of heart tissue, except in the case of yellowtail rockfish where liver tissue was cultured. A small piece (approx. 0.5 cm^3) of tissue was aseptically removed from each carcass and placed in a 15 ml tube containing 6 ml of Eagle's minimum essential medium (MEM), buffered to pH 7.8 with Tris, and supplemented with fetal bovine serum (5% v/v), penicillin (100 IU ml^{-1}), streptomycin (100 µg ml^{-1}), and gentamycin (100 µg ml^{-1}). Cultures were kept cool during transport to the US Geological Survey Marrowstone Marine Field Station (Nordland, WA, USA) where they were incubated at 15°C and examined microscopically (40× magnification) for the presence of *Ichthyophonus* schizonts and/or hyphae. Generally, each sample was examined twice, after 7 and 14 d incubation. However, these times were adjusted to account for duration of sampling and

transit time. Questionable cultures were examined a third time after 21 d, and medium was exchanged in tubes that became turbid due to host tissue autolysis. Prevalence of infection (%) was calculated as: 100 × number of positive cultures per sample/total number cultures in sample. A sample included all individuals of a single species from a given location.

rDNA sequencing

Sequences of the rDNA ITS region (i.e. ITS1–5.8S–ITS2) were generated for 81 *Ichthyophonus* isolates from 17 host species. These isolates came from a subset of tissue explant cultures obtained from the prevalence survey (n = 39), fresh cultures collected during ongoing epizootics (n = 13), and material that had been archived at cooperating laboratories (n = 29) (see Table 2). Host tissue not consumed by parasites was removed from cultures, and medium was exchanged every 5 to 10 d. In many cultures, hydrochloric acid, HEPES buffer (Gibco®, Life Technologies) and glucose were added to the MEM to lower pH (ca. 3.5) and stimulate parasite growth following the method of Spanggaard et al. (1994). When sufficient parasite material was present, samples were preserved in 100% ethanol. DNA was extracted from preserved cultures using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer's instructions, with the following modifications: tissue lysis buffer and Proteinase K volumes were increased 2-fold (360 µl and 40 µl, respectively), beads (lysing matrix D; MP Biomedicals) were added to the extraction tubes, and tissues were homogenized with a Fast-Prep®-24 bead mill (MP Biomedical) for 40 s prior to digestion. PCR amplification of the ITS1–5.8S–ITS2 locus was performed as previously described (Hershberger et al. 2010, Rasmussen et al. 2010) using primers that annealed on the 18S–ITS1 and ITS2–28S boundaries (Out ITS1-F: 5′-GCG GAA GGA TCA TTA CCA AAT AAC G; and Out-ITS2-R: 5′-GCC TGA GTT GAG GTC AAA TTT, respectively). Multiple clones were sequenced from each isolate to account for possible intra-isolate genetic variation. PCR products from the above reactions were purified using the QIAquick PCR purification protocol (Qiagen) and cloned with the Topo-TA Cloning Kit for Sequencing (Invitrogen). Cloned inserts were amplified by PCR, insert length was verified by gel electrophoresis, and inserts of the correct size were sequenced in the forward and reverse direction using stock vector primers (i.e. M13 Forward-21, M13 Reverse-29) at the high-throughput genomics unit at the University of Washington (htSEQ). Forward and reverse sequences from each clone were combined, chromatographs were visually inspected, and ambiguous base calls were edited manually using Sequencher Software (version 5.0, Gene Codes). A subset of clones (n = 93) was resequenced to clarify ambiguous chromatographs that followed a polyadenylation region at the 3′ end of ITS1. A forward primer that annealed at the 3′ end of the 5.8S sequence (IH.ITS_Seq_F_Int: 5′-ACA ACT TTT AAC GGT GGA TCT C) and a reverse primer that annealed in the 3′ region of ITS2 (IH.ITS_Seq_R_Int: 5′-CCG TGA ACT TCA TTT ATT CCA CAT) were used in addition to the M13 primers during this second sequencing run. These 4-sequence sets were combined and edited as described above. All newly generated clone sequences (n = 723) were combined with 137 ITS rDNA clone sequences from 13 isolates previously deposited in GenBank (Hershberger et al. 2010, Rasmussen et al. 2010). Clone sequences were aligned with Clustal and average evolutionary distance (*p*-distance) over sequence pairs within each isolate was calculated using MEGA5 (Tamura et al. 2011) (see Table 2).

Consensus ITS sequences were produced from clone sequences of each isolate by majority rule, except in cases where intra-isolate variation indicated divergent haplotypes were present in 1 isolate. Multi-haplotype isolates were identified by large intra-isolate *p*-distance (>0.01) and confirmed by estimating neighbor-joining phylogenies in MEGA5 using all the clone sequences from a given host species and consensus sequences from a 'backbone tree' made up of isolates from Chinook salmon (n = 1), Pacific herring (n = 1), Greenland turbot (n = 2), Dolly Varden (n = 1), rainbow trout (n = 3), American shad (n = 3) and yellowtail rockfish (n = 1). When all clones of an isolate formed a polytomy in 1 clade, a single majority consensus sequence was used for that isolate. When clones from an isolate diverged into more than 1 well supported clade, these sequences were grouped accordingly and multiple haplotypes from that isolate were considered in subsequent structural alignment and phylogenetic inference.

Alignment

The secondary structure of the ITS rRNA transcript was used to aid alignment of *Ichthyophonus* isolate consensus sequences. An iterative process utilizing thermodynamic optimization (Zuker & Stiegler 1981, Mathews et al. 1999) and a phylogenetic comparative

method (Noller & Woese 1981, Mai & Coleman 1997) were used to predict secondary structure. ITS consensus sequences were first aligned, with primer sequences in place, using Clustal in MEGA5, and then divided into 3 sequences corresponding to constituent molecules (i.e. ITS1, 5.8S, and ITS2) with the 5′ end of 5.8S set at 5′-UUU AGA CAA CUU UUA ACG-3′ and the 3′ end at 5′-CAU GCC UGG UUG AGU GUC-3′ following Vaughn et al. (1984) and Gottschling & Plotner (2004). Structure was predicted for the 3 molecules separately using the MFOLD web server (Zuker 2003). Default folding parameters were employed with no constraints on bases. Optimal and suboptimal structures were examined to identify locally conserved, thermodynamically stable features. Structure prediction was repeated with constraints to account for the connection of the 3 molecules, ITS2 interaction with flanking regions, and 5.8S–28S hybridization (Vaughn et al. 1984) that occurs during formation of the ribosome large subunit. The 5′ end of an *Ichthyophonus* 28S sequence (GenBank acc. no. AY026370) was joined to the 3′ end of each ITS2 sequence, and locations of 5.8S–28S hybridizations were identified by alignment of 5.8S sequences with reversed 28S transcript using Sequencher. These 5.8S regions were forced to remain unpaired during the second MFOLD run. Sequences with optimal dot-bracket structural annotation from the 3 molecules were then re-combined in the program 4SALE (Seibel et al. 2006, 2008) and the entire alignment was manually edited using the phylogenetic method described by Mai & Coleman (1997). Homology with structures from related taxa, published in the ITS2 (Koetschan et al. 2010) and RFAM (Burge et al. 2013) databases, was considered during manual editing. Structural homology across isolates was given priority over sequence alignment, while Clustal alignment was retained in large unpaired regions. The complete resulting structures were visualized in 4SALE and VARNA (Darty et al. 2009). The final structural alignment was trimmed to remove primer and 28S sequences prior to model selection.

Phylogenetic inference

To account for presumed heterogeneity of molecular evolution across regions and local structures (Brandley et al. 2005, Brown & Lemmon 2007), the data were analyzed under 4 partition strategies: complete sequence (no partitioning), partitioned by stem and loop (2 partitions), partitioned by region (i.e. ITS1, 5.8S, and ITS2; 3 partitions) and partitioned by stem and loop within regions (6 partitions). Nucleotide substitution model selection was implemented in jModelTest2 (Posada 2008, Darriba et al. 2012) for the entire structural alignment, for the individual molecules, and for paired and unpaired regions. From an initial BIONJ topology (Gascuel 1997), likelihood scores were calculated based on 24 candidate models (reviewed by Posada & Crandall 2001). Akaike's information criterion (AIC) was used to select the model that best fit sequence data (see Table S2 in the Supplement at www.int-res.com/articles/suppl/d120p125_supp.pdf) (Posada & Buckley 2004).

Gene trees were estimated using Bayesian Metropolis-coupled Markov chain Monte Carlo (MC^3) methods in MrBayes (Ronquist & Huelsenbeck 2003). Duplicate MC^3s of 2×10^7 generations were run for each partition scheme with parameter sampling every 2×10^4 generation. The first quarter of samples were discarded as 'burn-in'. Heating values for the 4 Markov chains in each run were left at default values. During the partitioned analyses a nucleotide substitution (*nst*) value was set for each partition, and rate parameters were allowed to vary among partitions. MrBayes parameters *revmat*, *tratio*, *statefreq*, *shape*, and *pinvar* were unlinked across partitions. In stem partitions the doublet model (nucmodel = doublet) was used to account for correlation of paired bases (Schöniger & von Haeseler 1994), other partitions were assigned the standard 4 by 4 model. The exponential branch length prior was decreased to 0.01 to avoid entrapment of Markov chains in unrealistic 'long tree' parameter space, which can occur in partitioned models (Brown et al. 2010, Marshall 2010). MC^3 results were visualized using the program Tracer (Rambaut & Drummond 2007) to verify a plateau in the plot of likelihood ($-\ln L$) over generation time and to check for abnormal distributions in estimated parameters. To determine which partitioning scheme best fit the data, Bayes factors were calculated for pairwise comparison of phylogenies on the estimated harmonic means of likelihoods from the *sump* command in MrBayes (Kass & Raftery 1995, Nylander et al. 2004). Differences between major clades of the final (i.e. best fit) phylogeny were characterized by calculating pairwise percent nucleotide differences for ITS1 and ITS2 sequences, and by identifying compensatory base pair changes (CBCs) present in paired regions.

In order to relate our findings to previous work based solely on 18S rDNA sequences, a subset of isolates representing distinct ITS clades was selected for 18S sequencing. Forward and reverse primers (18S-82F and 18S-1520R) from Takishita et al. (2005) were

used for PCR amplification of a region approximately 1590 bp long. The amplicon was direct sequenced in the forward and reverse directions using the above primers and a second set that annealed near its center (ICH1F and ICH4R; Criscione et al. 2002). Sequences were generated with the ABI BigDye® Terminator v1.1 Cycle Sequencing Kit and ABI 3130 Genetic Analyzer (Applied Biosystems). The 4 segments from each isolate were combined and ambiguous base calls were manually edited using the Sequencher program. These sequences were combined with GenBank sequences representing *Ichthyophonus* isolates from 7 host species (Ragan et al. 1996, Rand et al. 2000, Criscione et al. 2002, Hershberger et al. 2010). Alignment, model testing, and phylogenetic analysis followed that of ITS sequences, except that no secondary structure or partitioning were incorporated into models, and an 18S sequence from *Amoebidium parasiticum* (GenBank acc. no. Y19155) was included as an outgroup.

RESULTS

Infection prevalence

Ichthyophonus was widespread in the NE Pacific, occurring in 10 of the 13 host species surveyed from the Bering Sea to the coast of Washington (Table 1). Among positive hosts, prevalence ranged from 3.3 to 73.7%. More fish were sampled in the NW Atlantic, but from fewer host species. *Ichthyophonus* was detected in American shad and Greenland halibut, but at relatively low prevalence, 0.007 and 6.75% respectively.

ITS sequence and structure

Clone sequences produced for the *Ichthyophonus* rDNA ITS region were 645 to 684 nt long with a mean G+C content of 35.4%. Both ITS1 (220 to 235 nt) and ITS2 (263 to 288 nt) sequences varied in length by 15 nt, while 5.8S sequences (161 to 162 nt) varied by only 1 nt. Intra-isolate p-distance was of similar magnitude and small (i.e. <0.01) in all isolates except Atlantic mackerel (3 haplotypes), yellowtail rockfish (2 haplotypes), Puget Sound rockfish (2 haplotypes) and copper rockfish (1 haplotype) (Table 2).

Structures predicted for the ITS rRNA transcript of *Ichthyophonus* are relatively conserved and contained many features common among eukaryote taxa (Fig. 1). ITS1 formed an open central loop of 92 to 102 unpaired bases with 4 hairpin helices. Helices I, II, III, and IV contained 5 to 6, 9 to 10, 22 to 26, and 5 to 6 paired bases, respectively. Sequence variation in ITS1 primarily took the form of indels that occurred in unpaired regions of the central loop and in the bulged bases and terminal loop of Helix III. Substitutions did occur in the paired region of Helix I, where 2 to 5 CBCs occur between isolates. A single CBC (G-C/T-A) occurred in Helix II and 1 hemi-CBC occurred in Helix III (G-C/G-U).

The 5.8S secondary structure was completely conserved except for a single indel at the 5′ end of 2 isolates. The structure was similar to the universal model proposed by Vaughn et al. (1984). A dichotomous branched structure formed by 3 helices (Fig. 1: B, C, and D) and 2 hairpin helices (Fig. 1: E and F) extended from a 63 nt unpaired region. Three G/A transitions were present in the dichotomous helical structure, 2 occurred in bulged bases and 1 contributed to a hemi-CBC (G-U/A-U) in Helix D. One T/C transition occurred in an unpaired region. The sites of putative 28S hybridization that occurred at the 3′ end and Region A of 5.8S were 100% conserved, and showed high fidelity in both location and sequence to these sites described in other eukaryotes (Vaughn et al. 1984, Keller et al. 2009).

ITS2 formed a closed loop of 47 to 50 unpaired bases, terminating on 5′ and 3′ ends at the site of 5.8S–28S hybridization. As is typical in Eukaryota (Coleman 2007), 4 helices were present with the third being the longest. In *Ichthyophonus*, Helices I′, II′, III′, and IV′ contained 3, 15 to 22, 42 to 49, and 13 base pairings, respectively. Sequences of the central loop and the proximal paired bases were highly conserved. The majority of polymorphisms occurred in bulged bases and distal paired bases of Helices II′ and IV′. Other features conserved within Eukaryota were also present in the ITS2 structure of *Ichthyophonus*, including a pyrimidine-pyrimidine mismatch near the base of Helix II′, a UGGN motif 5′ to the apex of Helix III′, and a highly conserved segment (40 nt) on the 5′ side of Helix III′ (Schultz et al. 2005, Coleman 2007, Hamilton et al. 2010).

Phylogenetics

The final structural alignment of *Ichthyophonus* ITS sequence was 719 nt in length with primer sequences removed. MC^3 for all partitioning strategies reached stationarity by the end of burn-in. The 6-partition model resulted in the highest likelihood

Table 1. *Ichthyophonus* infection prevalence in 16 host species from the NW Atlantic and NE Pacific regions. Capture method, length (mean ± SD), weight (mean ± SD) and age data (mean [range]) of host are given when available. Prevalence (Prev.) of infection was determined by microscopic examination of explant heart cultures (see 'Materials and methods' for details). fl = fork length, tl = total length, YOY = young of the year, NAFO = North Atlantic Fisheries Organization

Species Collection locations	Capture method	Length (cm)	Weight (g)	Age (yr)	Infected/n	Prev. (%)
NE Pacific						
American shad *Alosa sapidissima*						
Puget Sound	Trawl[a]	20.8 ± 2.6 fl	104 ± 27	–	28/38	73.7
Columbia River, WA	Trap[b]	34.5 ± 4.4 fl	567 ± 243	–	17/60	28.3
Arctic cod *Boreogadus saida*						
Bering Sea	Trawl[c]	17.2 ± 1.5 tl	34 ± 9	–	0/58	0
Capelin *Mallotus villosus*						
Bering Sea	Trawl[c]	14.7 ± 1.1 tl	20 ± 5	–	0/61	0
Great sculpin *Myoxocephalus polyacanthocephalus*						
Bering Sea	Trawl[c]	52.6 ± 10.4 tl	2742 ± 1640	–	4/65	6.2
Greenland halibut *Reinhardtius hippoglossoides*						
Bering Sea	Trawl[c]	56.5 ± 20.4 tl	2617 ± 3036	–	5/28	17.9
Pacific cod *Gadus macrocephalus*						
Bering Sea	Trawl[c]	67.5 ± 8.6 tl	3509 ± 1528	–	2/56	3.6
Pacific halibut *Hippoglossus stenolepis*						
Cook Inlet	Hook & line[d]	85.8 ± 17.6 tl	–	–	30/60	50.0
Pacific herring *Clupea pallasii*						
Bering Sea	Trawl[c]	29.7 ± 1.8 tl	253 ± 52	–	0/64	0
Puget Sound	Trawl[a]	14 ± 2 fl	28 ± 14	–	45/156	28.8
Pacific staghorn sculpin *Leptocottus armatus*						
Puget Sound	Trawl[a]	18 ± 2.9 tl	91 ± 39	–	1/17	5.9
Plain sculpin *Myoxocephalus jaok*						
Bering Sea	Trawl[c]	47 ± 7.2 tl	1340 ± 594	–	4/34	11.8
Walleye pollock *Gadus chalcogrammus*						
Bering Sea	Trawl[c]	65 ± 9.7 tl	1907 ± 850	–	6/57	10.5
Yellowfin sole *Limanda aspera*						
Bering Sea	Trawl[c]	26.5 ± 6.9 tl	259 ± 166	–	0/59	0
Yellowtail rockfish *Sebastes flavidus*						
WA Coast	Hook & line[e]	–	–	–	1/30	3.3
NW Atlantic						
American shad *Alosa sapidissima*						
St. Lawrence River, QC	Gillnet[f]	49.8 ± 2.3 fl	1612 ± 238	–	5/60	8.3
Merrimack River, MA	Trap[g]	43.5 ± 4.4 fl	1195 ± 392	–	1/60	1.7
Connecticut River, MA	Trap[g]	48.2 ± 5 tl	1154 ± 413	–	0/60	0
Connecticut River, CT	Gillnet[h]	45.6 ± 3 fl	–	–	1/65	1.5
Nanticoke River, MD&DE	Electro[i,j]	44.8 ± 3.8 tl	991 ± 309	4.4 [3–6]	0/55	0
Patuxent River, MD	Electro[i]	44.2 ± 3.6 tl	–	4.1 [3–7]	0/60	0
Potomac River, DC	Gillnet[k]	46.9 ± 2.4 tl	1192 ± 235	–	0/53	0
Rappahannock River, VA	Gillnet[l]	47.5 ± 2.4 tl	1350 ± 219	5.5 [4–7]	0/60	0
York River, VA	Gillnet[l]	48.1 ± 2.4 tl	1377 ± 237	5.6 [4–9]	0/60	0
James River, VA	Gillnet[l]	48.1 ± 2.1 tl	1369 ± 176	5.4 [4–7]	0/60	0
Albemarle Sound, NC	Gillnet[m]	45.8 ± 4.5 tl	1116 ± 373	–	0/53	0
Roanoke River, NC	Electro[n]	44.4 ± 4.7 tl	686 ± 237	–	0/60	0
Tar River, NC	Electro[n]	45.1 ± 4.1 tl	820 ± 267	–	0/61	0
Cape Fear River, NC	Electro[n]	45.3 ± 3.9 tl	999 ± 355	–	1/60	1.7
Santee River, SC	Electro/Trap[o]	49.3 ± 2.9 tl	1400 ± 259	4.1 [3–5]	0/60	0
Savannah River, GA	Electro[p]	46.7 ± 3.8 tl	999 ± 290	5.4 [4–7]	0/58	0
Altamaha River, GA	Gillnet[p]	45.9 ± 3.1 tl	1143 ± 276	5.1 [4–7]	0/60	0
St. Johns River, FL	Electro[q]	42.7 ± 4.7 tl	752 ± 268	4.4 [2–7]	0/72	0
Bay anchovy *Anchoa mitchilli*						
Chesapeake Bay	Trawl[l]	–	1	YOY	0/60	0
Croaker *Micropogonias undulatus*						
Chesapeake Bay	Gillnet[l]	24.2 ± 3 tl	200 ± 89	–	0/60	0
Greenland halibut *Reinhardtius hippoglossoides*						
Davis Strait, NAFO area 0B	Trawl[r]	49.9 ± 7.2 tl	–	–	4/59	6.8
Newfoundland, NAFO area 3K	Trawl[r]	43.1 ± 8 tl	739 ± 450	–	4/60	6.7
Spot *Leiostomus xanthurus*						
Chesapeake Bay	Gillnet/Trawl[l]	14.7 ± 5.4 tl	68 ± 64	–	0/56	0

[a]Washington Department of Fish and Wildlife, [b]US Fish and Wildlife Service, [c]US National Marine Fisheries Service, [d]Alaska Department of Fish and Game, [e]US Geological Survey, [f]Ministry of Natural Resources and Wildlife Quebec, [g]Massachusetts Division of Fisheries and Wildlife, [h]Connecticut Department of Energy and Environmental Protection, [i]Maryland Department of Natural Resources, [j]Delaware Department of Natural Resources and Environmental Control, [k]District Department of Ecology, [l]Virginia Institute of Marine Science, [m]North Carolina Department of Environment and Natural Resources, [n]North Carolina Wildlife Resource Commission, [o]South Carolina Department of Natural Resources, [p]Georgia Department of Natural Resources, [q]Florida Fish and Wildlife Conservation Commission, [r]Department of Fisheries and Oceans Canada

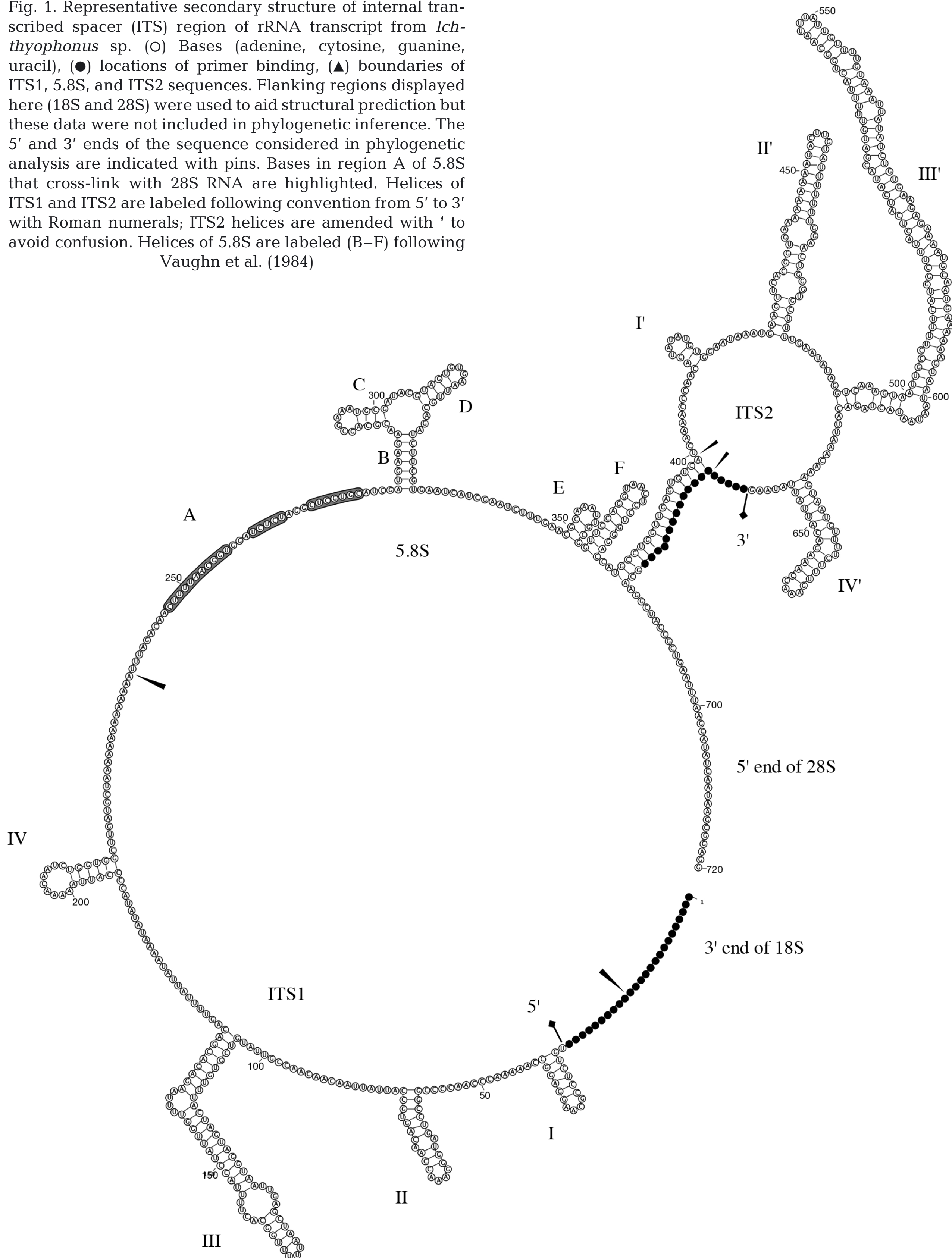

Fig. 1. Representative secondary structure of internal transcribed spacer (ITS) region of rRNA transcript from *Ichthyophonus* sp. (○) Bases (adenine, cytosine, guanine, uracil), (●) locations of primer binding, (▲) boundaries of ITS1, 5.8S, and ITS2 sequences. Flanking regions displayed here (18S and 28S) were used to aid structural prediction but these data were not included in phylogenetic inference. The 5′ and 3′ ends of the sequence considered in phylogenetic analysis are indicated with pins. Bases in region A of 5.8S that cross-link with 28S RNA are highlighted. Helices of ITS1 and ITS2 are labeled following convention from 5′ to 3′ with Roman numerals; ITS2 helices are amended with ′ to avoid confusion. Helices of 5.8S are labeled (B–F) following Vaughn et al. (1984)

Table 2. Summary data for 94 *Ichthyophonus* isolates used in phylogenetic comparison. Novel isolates were obtained from the prevalence survey of this study, and from material archived by several cooperating laboratories (see footnotes). Number of clone sequences is indicated. Isolates with clone sequences previously deposited in GenBank have the prefix 'gb' on the isolate ID. Average evolutionary divergence over sequence pairs within isolates (*p*-distance) was calculated using MEGA5 (see 'Materials and methods'), with SE estimated from 500 bootstrap samples

Host species	Collection location	Year	No. of	*p*-distance		GenBank acc. no(s).	
Isolate ID			clones	Mean	SE	ITS	18S
American shad							
IA285	Cape Fear River, North Carolina, USA	2011	15	0.001	0.0004	KF987552–KF987566	
IA013	Columbia River, Oregon, USA[a]	2005	8	0.004	0.0014	KF987093–KF987100	
gb IA014	Columbia River, Oregon, USA	2005	12	0.006	0.0017	GQ402860–GQ402871	GQ370770, GQ370791
gb IA052	Columbia River, Oregon, USA	2007	13	0.004	0.0015	GQ402887–GQ402899	GQ370774, GQ370795
IA327	Columbia River, Oregon, USA	2011	8	0.003	0.0014	KF987730–KF987737	
IA328	Columbia River, Oregon, USA	2011	7	0.005	0.0018	KF987738–KF987744	
IA295	Connecticut River, Connecticut, USA	2011	8	0.003	0.0013	KF987639–KF987646	
IA296	Merrimack River, Massachusetts, USA	2011	6	0.002	0.0011	KF987647–KF987652	
gb IA065	Merrimack River, Massachusetts, USA	2008	6	0.003	0.0013	GU059890–92, GU059901–03	GQ370782, GQ370803
gb IA066	Merrimack River, Massachusetts, USA	2008	8	0.002	0.0011	GU059893–GU059900	GQ370783, GQ370804
gb IA067	Merrimack River, Massachusetts, USA	2008	14	0.005	0.0014	GQ402900–06, GU146052–58	GQ370781, GQ370802
IA007	Puget Sound, Washington, USA[a]	2005	8	0.007	0.0020	KF987085–KF987092	
IA239	Puget Sound, Washington, USA	2010	8	0.002	0.0014	KF987364–KF987371	
IA240	Puget Sound, Washington, USA	2010	8	0.004	0.0017	KF987372–KF987379	
IA241	Puget Sound, Washington, USA	2010	14	0.002	0.0013	KF987380–KF987393	
IA242	Puget Sound, Washington, USA	2010	7	0.003	0.0016	KF987394–KF987400	
IA243	Puget Sound, Washington, USA	2010	8	0.002	0.0014	KF987401–KF987408	
IA244	Puget Sound, Washington, USA	2010	8	0.003	0.0015	KF987409–KF987416	
IA290	St. Lawrence River, Quebec, Canada	2011	16	0.000	0.0003	KF987575–KF987590	
IA291	St. Lawrence River, Quebec, Canada	2011	16	0.000	0.0003	KF987591–KF587606	
IA292	St. Lawrence River, Quebec, Canada	2011	8	0.001	0.0007	KF987607–KF987614	
IA293	St. Lawrence River, Quebec, Canada	2011	8	0.002	0.0012	KF987615–KF987622	
IA294	St. Lawrence River, Quebec, Canada	2011	16	0.003	0.0008	KF987623–KF987638	
Atlantic herring							
IA303	Atlantic Ocean, Iceland[b]	2011	8	0.003	0.0014	KF987692–KF987699	
IA304	Atlantic Ocean, Iceland[b]	2011	7	0.004	0.0017	Kf987700–KF987706	
IA307	Atlantic Ocean, Iceland[b]	2011	7	0.005	0.0019	KF987707–KF987713	
IA308	Atlantic Ocean, Iceland[b]	2011	8	0.004	0.0014	KF987714–Kf987721	
Atlantic mackerel							
IA288	Atlantic Ocean, Algarve, Portugal[c]	2011	8[h]	0.036	0.0047	KF987567–KF987574	
Atlantic salmon							
gb 9-27	Connecticut River, Massachusetts, USA	2008	7	0.003	0.0015	GU059874–GU059880	
IA106	Connecticut River, Massachusetts, USA[d]	2009	8	0.003	0.0016	KF987164–KF987171	
IA108	Connecticut River, Massachusetts, USA[d]	2009	9	0.003	0.0013	KF987172–KF987180	
gb 9-80	Merrimack River, Massachusetts, USA	2008	9	0.007	0.0019	GU059881–GU059889	
IA246	Merrimack River, New Hampshire, USA[d]	2009	8	0.003	0.0013	KF987417–KF987424	
IA247	Merrimack River, New Hampshire, USA[d]	2009	16	0.002	0.0010	KF987425–KF987440	
IA248	Merrimack River, New Hampshire, USA[d]	2009	8	0.003	0.0016	KF987441–KF987448	
Chinook salmon							
IA171	Chena River, Alaska, USA[e]	2004	9	0.002	0.0011	KF987232–KF987240	
IA189	Chena River, Alaska, USA[e]	2006	9	0.002	0.0010	KF987267–KF987275	
IA169	Salcha River, Alaska, USA[e]	2004	9	0.003	0.0015	KF987214–KF987222	
IA170	Salcha River, Alaska, USA[e]	2004	9	0.003	0.0015	KF987223–KF987231	
IA190	Salcha River, Alaska, USA[e]	2006	9	0.002	0.0014	KF987276–KF987284	
IA192	Salcha River, Alaska, USA[e]	2006	9	0.002	0.0014	KF987285–KF987293	
IA177	Yukon River, Alaska, USA[e]	2004	8	0.004	0.0017	KF987241–KF987248	
IA185	Yukon River, Alaska, USA[e]	2006	9	0.003	0.0016	KF987249–KF987257	
IA187	Yukon River, Alaska, USA[e]	2006	9	0.003	0.0014	KF987258–KF987266	
Copper rockfish							
gb IA011	Puget Sound, Washington, USA	2006	5	0.011	0.0027	GQ402855–GQ402859	GQ370769, GQ370790
Dolly Varden							
IA264	Aquaculture, Yamanashi Pref., Japan[f]	2002	8	0.002	0.0010	KF987486–KF987493	
Great sculpin							
IA298	Bering Sea, Alaska, USA	2011	8	0.000	0.0000	KF987660–KF987667	
IA300	Bering Sea, Alaska, USA	2011	8	0.002	0.0015	KF987676–KF987683	

Table 2 (continued)

Host species Isolate ID	Collection location	Year	No. of clones	*p*-distance Mean	SE	GenBank acc. no(s). ITS	18S
Greenland halibut							
IA260	Atlantic Ocean, Canada, NAFO area 3K	2010	8	0.002	0.0010	KF987467–KF987474	
IA262	Atlantic Ocean, Canada, NAFO area 3K	2010	11	0.003	0.0012	KF987475–KF987485	
IA339	Bering Sea, Alaska, USA	2011	8	0.004	0.0014	KF987759–KF987766	
IA340	Bering Sea, Alaska, USA	2011	11	0.003	0.0014	KF987767–KF987777	
IA377	Davis Strait, Canada, NAFO area 0B	2011	8	0.003	0.0016	KF987800–KF987807	
Pacific cod							
IA338	Bering Sea, Alaska, USA	2011	7	0.002	0.0010	KF987752–KF987758	
Pacific halibut							
IA251	Cook Inlet, Alaska, USA	2010	10	0.002	0.0012	KF987449–KF987458	
IA253	Cook Inlet, Alaska, USA	2010	8	0.003	0.0014	KF987459–KF987466	
IA363	Pacific Ocean, Oregon, USA	2011	7	0.002	0.0012	KF987786–KF987792	
IA364	Pacific Ocean, Oregon, USA	2011	7	0.005	0.0019	KF987793–KF987799	
IA316	Prince William Sound, Alaska, USA	2011	8	0.003	0.0016	KF987722–KF987729	
Pacific herring							
IA147	Lynn Canal, Alaska, USA[e]	2007	8	0.004	0.0018	KF987181–KF987188	
IA148	Lynn Canal, Alaska, USA[e]	2007	7	0.003	0.0012	KF987189–KF987195	
IA084	Prince William Sound, Alaska, USA[a]	2009	8	0.004	0.0017	KF987114–KF987121	
IA085	Prince William Sound, Alaska, USA[a]	2009	8	0.004	0.0014	KF987122–KF987129	
IA086	Prince William Sound, Alaska, USA[a]	2009	9	0.002	0.0009	KF987130–KF987138	
IA150	Prince William Sound, Alaska, USA[e]	2009	9	0.004	0.0016	KF987196–KF987204	
gb IA002	Puget Sound, Washington, USA	2005	12	0.005	0.0012	GQ402831–GQ402842	GQ370767, GQ370788
IA233	Puget Sound, Washington, USA	2010	9	0.003	0.0016	KF987308–KF987316	
IA234	Puget Sound, Washington, USA	2010	9	0.002	0.0013	KF987317–KF987325	
IA235	Puget Sound, Washington, USA	2010	9	0.003	0.0015	KF987326–KF987334	
IA236	Puget Sound, Washington, USA	2010	6	0.001	0.0009	KF987335–KF987340	
IA237	Puget Sound, Washington, USA	2010	15	0.002	0.0014	KF987341–KF987355	
IA238	Puget Sound, Washington, USA	2010	8	0.003	0.0016	KF987356–KF987363	
gb IA051	Sitka Sound, Alaska, USA	2007	15	0.007	0.0016	GQ402872–GQ402886	GQ370773, GQ370794
IA151	Sitka Sound, Alaska, USA[e]	2008	9	0.002	0.0011	KF987205–KF987213	
IA099	Sitka Sound, Alaska, USA[a]	2009	7	0.003	0.0016	KF987139–KF987145	
IA100	Sitka Sound, Alaska, USA[a]	2009	9	0.002	0.0012	KF987146–KF987154	
IA101	Sitka Sound, Alaska, USA[a]	2009	9	0.002	0.0014	KF987155–KF987163	
Pacific staghorn sculpin							
IA220	Puget Sound, Washington, USA	2010	14	0.002	0.0014	KF987294–KF987307	
Plain sculpin							
IA347	Bering Sea, Alaska, USA	2011	8	0.002	0.0011	KF987778–KF987785	
Puget Sound rockfish							
IA023	San Juan Channel, Washington, USA[a]	2005	13[h]	0.017	0.0035	KF987101–KF987113	
Rainbow trout							
IA273	Aquaculture, Epirus Periphery, Greece[g]	2011	8	0.001	0.0009	KF987494–KF987501	
IA276	Aquaculture, Epirus Periphery, Greece[g]	2011	7	0.002	0.0012	KF987502–KF987508	
IA278	Aquaculture, Epirus Periphery, Greece[g]	2011	11	0.002	0.0012	KF987509–KF987519	
IA281	Aquaculture, Epirus Periphery, Greece[g]	2011	7	0.003	0.0013	KF987520–KF987526	
IA282	Aquaculture, Epirus Periphery, Greece[g]	2011	10	0.002	0.0013	KF987527–KF987536	
IA283	Aquaculture, Epirus Periphery, Greece[g]	2011	7	0.003	0.0015	KF987537–KF987543	
IA284	Aquaculture, Epirus Periphery, Greece[g]	2011	8	0.001	0.0010	KF987544–KF987551	
gb RBT11	Aquaculture, Idaho, USA	2008	10	0.005	0.0015	GQ402928–GQ402937	GQ370776, GQ370801
gb RBT12	Aquaculture, Idaho, USA	2008	10	0.003	0.0012	GQ402938–GQ402947	GQ370777, GQ370797
gb RBT13	Aquaculture, Idaho, USA	2008	16	0.003	0.0012	GQ402948–GQ402963	GQ370778, GQ370798
Walleye pollock							
IA299	Bering Sea, Alaska, USA	2011	8	0.002	0.0015	KF987668–KF987675	
IA301	Bering Sea, Alaska, USA	2011	8	0.002	0.0015	KF987684–KF987691	
IA337	Bering Sea, Alaska, USA	2011	7	0.002	0.0013	KF987745–KF987751	
Yellowtail rockfish							
IA297	Pacific Ocean, Washington, USA	2011	7[h]	0.029	0.0036	KF987653–KF987659	

[a]Archived material from the US Geological Survey, Marrowstone Marine Field Station, Nordland WA, USA
[b]Fresh isolates from epizootic occurring in Iceland herring stocks
[c]Fresh isolates from mackerel fishery collected at Oceanario de Lisboa, Lisbon, Portugal
[d]Archived material form the US Fish and Wildlife Service, Northeast Fish Health Center, Lamar PA, USA
[e]Archived material from the Alaska Department of Fish and Game, Fish Health Laboratory
[f]Archived material from the Nippon Veterinary and Life Sciences University, Yamanashi Prefecture, Japan
[g]Fresh isolated from epizootic occurring in Greek trout farms
[h]Isolate contained divergent haplotypes that were considered separately in phylogeny

and Bayes factors indicated there was very strong support (2lnBayesFactor > 10) for selection of the 6-partition model over all other partition schemes (see Table S3 in the Supplement at www.int-res.com/articles/suppl/d120p125_supp.pdf).

The *Ichthyophonus* phylogeny based on rDNA ITS data under a 6-partition model contained 6 major divisions with limited lower-order structure (Fig. 2). The majority of the isolates (71 of 98 isolates) were nearly identical, producing a large polytomy (Fig. 2: Clade D) that included parasite material from 13 NE Pacific and 4 Atlantic hosts sampled across a wide geographic range. A well-supported (posterior probability = 0.987) daughter clade (Fig. 2: Clade E) within this polytomy included 8 isolates exclusively from American shad in the Merrimack and St. Lawrence Rivers. A single clade (Fig. 2: Clade C) included all the isolates derived from freshwater aquaculture hosts, rainbow trout and Dolly Varden, and included haplotypes from Puget Sound rockfish and Atlantic mackerel. Clades D and C formed an unresolved tricotomy with isolates from Greenland halibut captured in the NW Atlantic (Fig. 2: Clade B). The 2 remaining clades contained more divergent ITS isolates: Clade A included haplotypes from yellowtail rockfish and Atlantic mackerel and Clade F contained a single isolate from an American shad collected in the St. Lawrence Waterway. Mean pairwise percent nucleotide differences between the major clades varied from 0.4 to 10.6 for ITS1 and from 0.6 to 11.7 for ITS2 (Table 3).

The *Ichthyophonus* phylogeny based on 18S sequences (Fig. 3) had limited structure, but effectively related some of our isolates to those of other researchers. The 18S sequence from the unique American shad isolate, IA293, was identical to that of *I. irregularis* described by Rand et al. (2000). The partial 18S sequence of isolate IA297 from yellowtail rockfish was identical to sequences previously isolated from yellowtail rockfish and Pacific ocean perch *Sebastes alutus* (Criscione et al. 2002). Further, these 3 isolates grouped with an IA288 haplotype isolated from Atlantic mackerel (Clade A). Freshwater isolates in Clade C formed a separate clade in the 18S phylogeny. This is contrary to the results presented by Rasmussen et al. (2010), where 18S sequences were identical between freshwater and marine types. Here we amplified and sequenced a larger section of 18S rDNA than that used by Criscione et al. (2002) and Rasmussen et al. (2010), and discovered 4 transitions that occur in a span of 14 nt near the 3′ end of the 18S sequence.

DISCUSSION

The most prominent feature of the ITS rDNA phylogeny of *Ichthyophonus* is the large polytomy containing a majority of isolates from both the Atlantic and Pacific Oceans. (Fig. 2: Clade D). This ubiquitous marine form was widely distributed in pelagic (herring, mackerel), semi-pelagic (walleye pollock, Pacific cod), benthic (copper rockfish, Greenland halibut, Pacific halibut, cottid sp.) and anadromous (Atlantic salmon, Chinook salmon, American shad) hosts, thus confirming, for at least 1 species in the genus, the long held supposition of low host specificity that was attached to *I. hoferi* (McVicar 1999). This parasite type is also the only one we can associate with severe epizootics in wild hosts. Isolates obtained during epizootics in Chinook salmon from the Yukon River (Kocan et al. 2004), American shad from the Columbia River (Hershberger et al. 2010) and Atlantic herring from Iceland (Óskarsson & Pálsson 2011) are all contained in Clade D (Fig. 2).

The predominance of a single *Ichthyophonus* type with nearly identical ITS sequences across a large host and geographic range suggests broad scale transmission processes with little allopatry imposed by different host species, or conversely, that the ITS locus is not variable enough to differentiate phylogenetic structure resulting from host or geographic separation of *Ichthyophonus* stains. Waterborne transmission has been demonstrated among rainbow trout in freshwater (Yokota et al. 2008), but to date we have been unable to replicate this phenomenon in a common marine host, Pacific herring (Gregg et al. 2012). At higher trophic levels, it is clear that *Ichthyophonus* spp. can be acquired through consumption of infected prey fishes (Gustafson & Rucker 1956, McVicar & McLay 1985, Jones & Dawe 2002). These infections may be dead ends in many predatory species, and selection on a parasite life history that is closed in 1, or a few, forage species could result in the fairly uniform parasite distribution we encountered. In the Bering Sea, White et al. (2014) proposed that walleye pollock could be a source of *Ichthyophonus* infection for many other fish species. At many life history stages, walleye pollock are forage for other species, and may even transmit *Ichthyophonus* into the Bering Sea food chain as infected fisheries offal. In this scenario, a selection process that homogenized the *Ichthyophonus* population in pollock, could result in the uniform genetic distribution in other fishes in the Bering Sea. We isolated *Ichthyophonus* from 5 new hosts in the Bering Sea, from walleye pollock, and from Chinook salmon

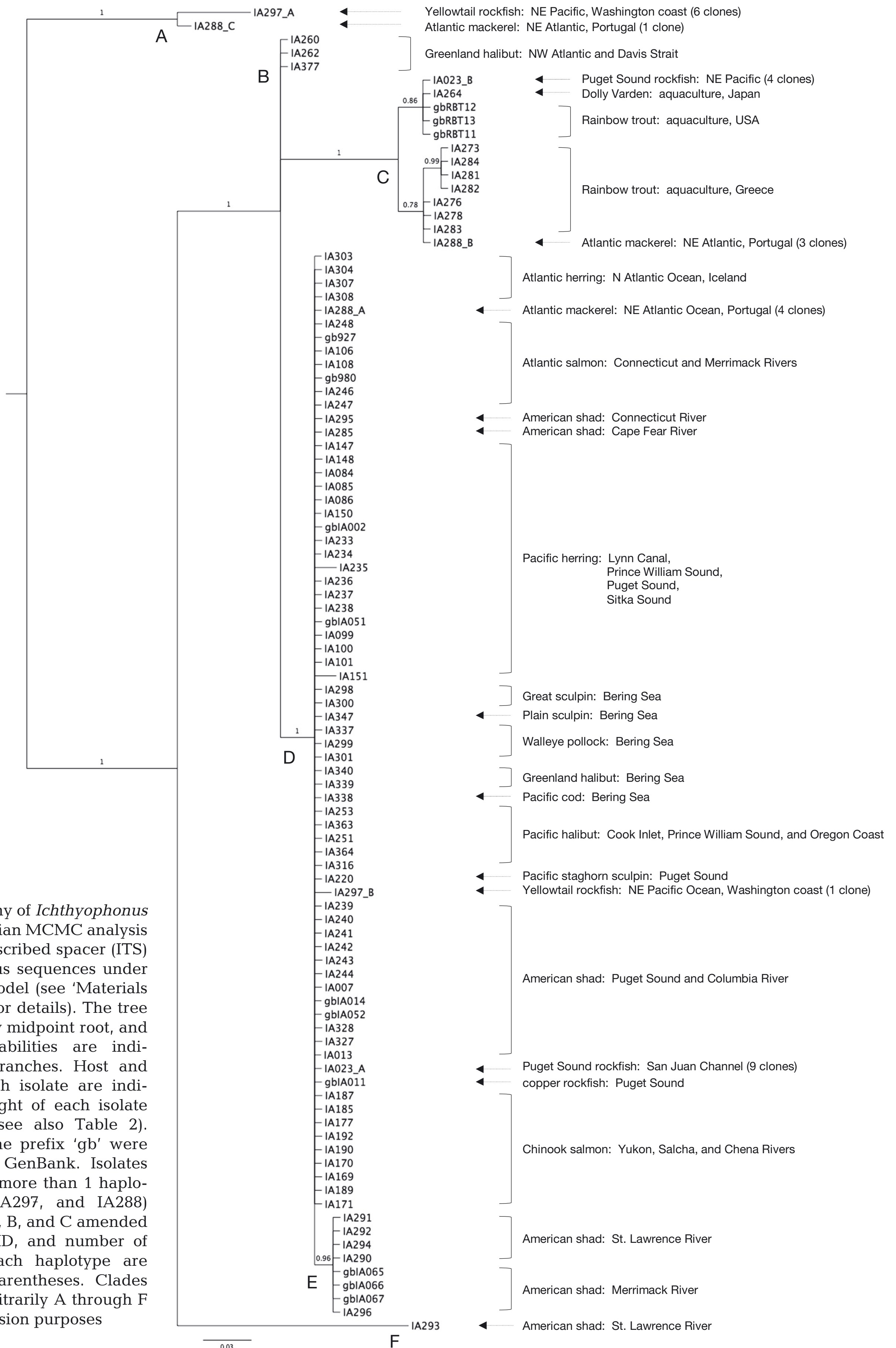

Fig. 2. Phylogeny of *Ichthyophonus* based on Bayesian MCMC analysis of internal transcribed spacer (ITS) rDNA consensus sequences under a 6-partition model (see 'Materials and methods' for details). The tree has an arbitrary midpoint root, and posterior probabilities are indicated above branches. Host and location of each isolate are indicated to the right of each isolate identification (see also Table 2). Isolates with the prefix 'gb' were obtained from GenBank. Isolates that contained more than 1 haplotype (IA023, IA297, and IA288) have suffixes A, B, and C amended to the isolate ID, and number of clones with each haplotype are indicated in parentheses. Clades are labeled arbitrarily A through F for discussion purposes

Table 3. Mean pairwise nucleotide differences (%) between major clades of the *Ichthyophonus* phylogeny (Fig. 2). Percent difference calculated separately for internal transcribed spacer 1 (ITS1) and ITS2 regions. Length of ITS1 alignment = 245 nt. Length of the ITS2 alignment = 311 nt

	ITS1 % diff./ITS2 % diff.				
	Clade F	Clade E	Clade D	Clade C	Clade B
Clade A	8.0/11.7	6.5/9.1	6.5/9.6	7.3/10	6.5/8.8
Clade B	9.4/2.6	0.8/1.0	0.4/0.9	0.8/4.3	
Clade C	10.2/6.1	1.6/5.2	1.2/4.9		
Clade D	9.8/3.0	0.4/0.6			
Clade E	10.6/3.5				

in the Yukon River watershed (presumably infected in the Bering Sea). The consensus ITS sequences of all these isolates were nearly identical.

In contrast to the ubiquitous marine form, the 4 other *Ichthyophonus* types detected in marine and anadromous fishes had narrow host distributions. Two were found in American shad (Clades E and F), a single species was encountered in Greenland turbot in the NW Atlantic (Clade B), and 2 sister-isolates (Clade A) occurred in Atlantic mackerel (IA288_C) and yellowtail rockfish (IA297_A). Due to the relatively small sample size within hosts, it was not possible to determine whether these forms are specialists in the hosts encountered or if they are multi-host parasites that occur less frequently than the ubiquitous marine type. Regions of identical 18S DNA sequence, though not conclusive, suggest that isolate IA293, from yellowtail rockfish, may also be capable of infecting a sympatric congener, Pacific Ocean perch (Criscione et al. 2002), and that IA293 isolated from American shad may be a species previously described in yellowtail flounder (Rand et al. 2000).

Despite great geographic separation of the isolates (i.e. Greece, North America, and Japan), parasites

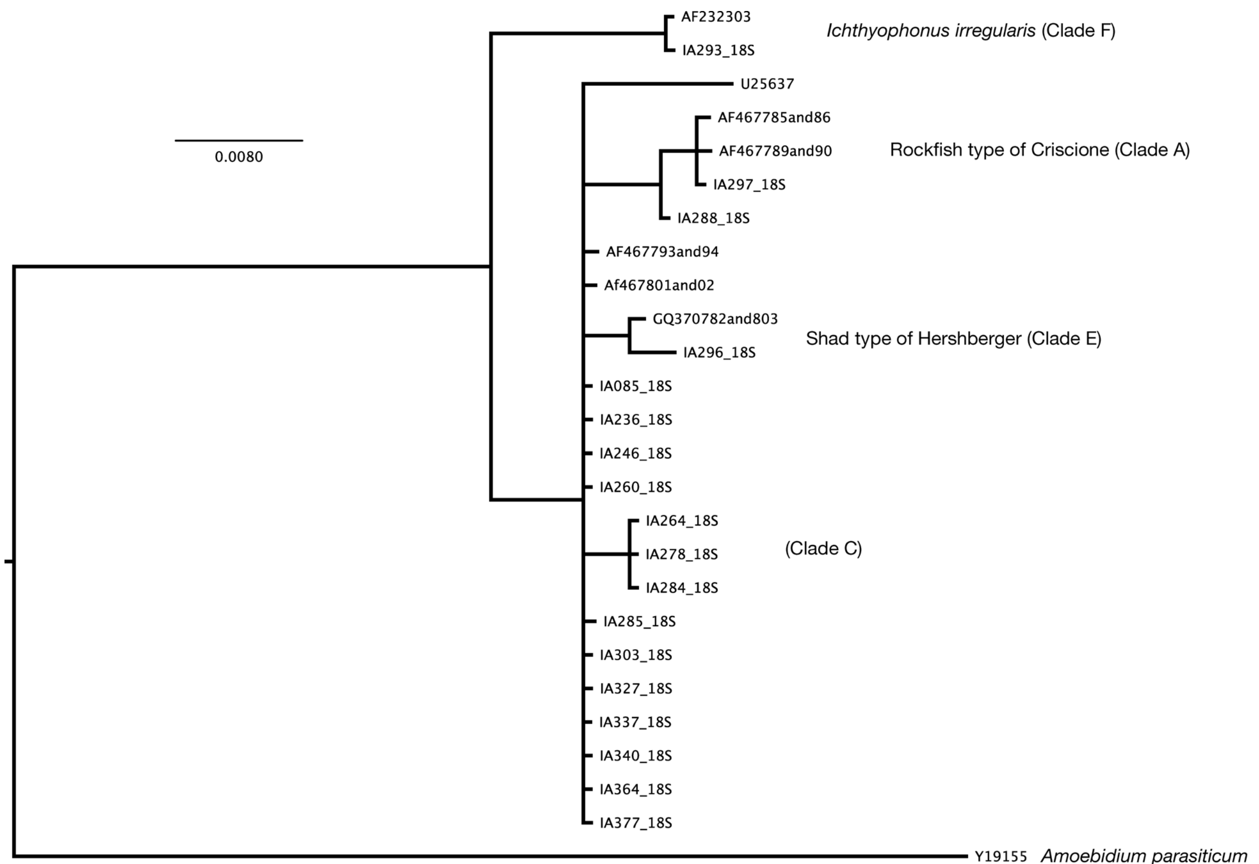

Fig. 3. Phylogeny of *Ichthyophonus* based on Bayesian MCMC analysis of 18S rDNA sequences (see 'Materials and methods' for details). Sequences were generated for a subset of samples used in internal transcribed spacer (ITS) phylogeny and selected from GenBank based on the publications of Ragan et al. (1996), Rand et al. (2000), Criscione et al. (2002), and Hershberger et al. (2010). Clade designations reference the ITS phylogeny presented in Fig. 2. *Amoebidium parasiticum* is used as the outgroup

from freshwater aquaculture hosts fell into a single clade (Fig. 2: Clade C). This ITS genotype was first identified in rainbow trout from North America by Rasmussen et al. (2010). Clade C haplotypes also occurred in the minority of clones from 2 marine isolates that contained variable ITS sequences, a Puget Sound rockfish from the inland marine waters of Washington State and an Atlantic mackerel captured near Portugal. We do not know if freshwater aquaculture infections originate in wild sympatric hosts, are introduced from a marine source, or are the legacy of an historic introduction and have been perpetuated by the global movement of animals and animal products. Some evidence for local introduction existed in the structure of Clade C, where a well-supported dichotomy mimicked the Pacific versus Atlantic distribution of the isolates. A daughter clade containing rainbow trout from western North America, Dolly Varden from Japan, and the haplotype from Puget Sound rockfish formed a sister group to that containing rainbow trout from Greece and the haplotype isolated from Atlantic mackerel. Anecdotal reports attribute *Ichthyophonus* spp. epizootics in aquaculture to the feeding of unpasteurized tissues of wild freshwater (Erickson 1965) or marine (Rucker & Gustafson 1953, Slocombe 1980, Egusa 1983, Athanassopoulou 1992) hosts. There were no isolates from wild freshwater hosts in this study; however, if *Ichthyophonus* spp. were introduced to aquaculture from marine sources, the absence of the ubiquitous marine species in the fish farms is notable, and may indicate rapid adaptation from standing genetic diversity. Improved facultative survival of the Clade C type has been demonstrated in freshwater (Hershberger et al. 2008) and may explain the absence of other forms in our aquaculture samples. A more focused study with extensive sampling in and around aquaculture facilities is necessary to determine the processes that establish and maintain *Ichthyophonus* spp. in fish farms.

The 6 main clades identified in the ITS rDNA phylogeny of *Ichthyophonus* may represent separate species. Mean pairwise nuleotide differences for ITS1 and/or ITS2 (Table 3) among Clades A, C, and F, and between these clades and all others, were greater than the intraspecific variation that occurs within 75 to 77% of fungal species (Nilsson et al. 2008). However, there are no consensus methods for delimiting species within *Ichthyophonus*, primarily because only 1 modern species description has been published.

Rand (1994) provided a thorough morphological and histochemical description of an irregular form of *Ichthyophonus* that was detected during a large scale survey of yellowtail flounder in the North Atlantic. Sequences of 18S rDNA from this irregular form were different from the 2 *Ichthyophonus* 18S sequences available at the time (Ragan et al. 1996, Spanggaard et al. 1996), and *I. irregularis* was established (Rand et al. 2000). While Rand et al. (2000) provided a good model for the description of novel species within the group, it is likely that the 18S locus is not variable enough to delimit species (Rasmussen et al. 2010), and as more genotypes are described, a new set of lineage-relevant phenotypic characteristics may replace the histochemical and morphological characteristics they used. We feel that species delimitation should be confirmed by multiple genetic markers to insure that the process occurs in a robust phylogenetic framework (Boenigk et al. 2012). As a practical matter, the recondite life history of *Ichthyophonus*, coupled with the varied morphology that can occur in culture and in host tissues, may result in a species identification process that relies heavily on molecular markers and less so on morphological characteristics.

We cannot determine if the present taxonomic units include the species originally described by Plehn & Mulsow (1911). However, their material was isolated from farmed rainbow trout in freshwater, suggesting that the specific name *I. hoferi*, if retained, be used solely for the ITS genotypes of Clade C. Sequences of the 18S rDNA gene indicated that our Clade F likely corresponds to *I. irregularis* (Rand et al. 2000) and this relationship can be confirmed by sequencing ITS rDNA from the archived holotype. Neither formal description nor specific epithet exists for the remaining taxonomic units presented here (i.e. Clades A, B, D, and E).

A limitation of this study is the inability to determine if the multi-haplotype isolates detected in yellowtail rockfish (IA297), Puget Sound rockfish (IA023), and Atlantic mackerel (IA288) resulted from heterozygous parasites or co-infections. Hundreds of copies of the ribosome coding sequence can occur at more than 1 location in the genome, opening the possibility of polyzygous individuals. However, the arrangement of these genes in tandem repeats and concerted evolution at rDNA multi-locus genes has a strong homogenizing effect (Ganley & Kobayashi 2007) that can maintain homogeneity through biased gene conversion during clonal reproduction (Hillis et al. 1991). Variation was low among clones of the other 91 isolates we sequenced; therefore, we hypothesize that these multiple sequences were the result of co-infections rather than heterozygosity.

This hypothesis will need to be confirmed by sequencing hundreds of clones from single spore isolations of these mixed haplotype isolates.

The prevalence and genetic distribution of *Ichthyophonus* spp. isolated from American shad in their native range were markedly different from those in the NE Pacific, where shad are an introduced species. Infections were detected in only 8 of 1077 adult shad returning to rivers from the NW Atlantic, whereas 73.7% (28/38) of juveniles captured in the Puget Sound were infected and 28.3% (17/60) of adults returning to the Columbia River were infected. Three *Ichthyophonus* types were detected in the 11 isolates sequenced from the Atlantic region, while only the ubiquitous marine type was detected in the 12 isolates sequenced from the Pacific region. Based on a smaller sample, Hershberger et al. (2010) posited that American shad on the east and west coasts of North America carried different *Ichthyophonus* types, but our data show that the form present in the Columbia River also occurs in the NW Atlantic. Infections in the NE Pacific, while more numerous, seem to represent a subset of the ITS genotypes present in the NW Atlantic. We detected the highest *Ichthyophonus* genetic diversity in the area where the first large *Ichthyophonus* epizootics were reported (i.e. Gulf of St. Lawrence and Gulf Maine) (Cox 1916, Fish 1934, Sindermann 1958).

CONCLUSION

We have identified 6 taxonomic units within *Ichthyophonus* that may represent different species. However, it appears that the majority of global impacts in wild fish populations result from a single taxon. This ubiquitous marine form, which lacks formal description, is the only one that we can associate with epizootics in wild fishes, as all isolates from the Atlantic herring epizootic near Iceland (Óskarsson & Pálsson 2011), the American shad epizootic in the Columbia River (Hershberger et al. 2010), and the Chinook salmon epizootic in the Yukon River (Kocan et al. 2004) were of this type. Similarly, a single form that is adapted to low salinity environments appears to be widespread in salmonid culture.

The broad host and geographic distribution of these 2 ITS types will confound efforts to use this marker to infer inter-host transmission patterns. More variable molecular markers should be developed to elucidate the transmission dynamics of *Ichthyophonus* spp. in systems were they significantly affect fishery production and aquaculture. While this study represents the first broad examination of species richness within *Ichthyophonus,* only about 10% of nearly 150 reported host species were sampled, and these samples were geographically focused around North America. Further sampling in marine and freshwater environments, especially those of the Eastern Hemisphere, would significantly improve our understanding of *Ichthyophonus* global diversity.

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