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Ribosomal DNA sequences indicate isolated populations of *Ichthyophonus hoferi* in geographic sympatry in the north-eastern Pacific Ocean

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Abstract

Infections of *Ichthyophonus hoferi*, a cosmopolitan parasite of marine fish, have recently been reported in rockfish, *Sebastes* spp., from the north-eastern Pacific. Because *I. hoferi* also infects Pacific herring, *Clupea pallasi* Valenciennes, and salmonids in this region, we wanted to determine if *Ichthyophonus* parasites from rockfishes, Pacific herring and chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), were the same. Small subunit ribosomal deoxyribonucleic acid sequence data revealed two haplotypes that were fixed among host species in geographic sympatry, one from rockfish and the other from both Pacific herring and salmon. These isolated populations of *Ichthyophonus* could be part of the same species that are ecologically separated because of host behaviours, or they could be distinct species that are host specific. Dietary patterns of the hosts indicate that ecological separation among hosts is possible, but the presence of distinct species may better explain the observed *Ichthyophonus* haplotype association with host species.

Keywords: Clupea pallasi, *Ichthyophonus hoferi*, *Oncorhynchus tshawytscha*, Pacific Ocean, *Sebastes* spp., small subunit 18S rDNA.

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Introduction

Ichthyophonus hoferi (Plehn & Mulsow) is a cosmopolitan parasite that infects most organs and tissues of many marine fish (McVicar 1999). The disease caused by *I. hoferi* is of economic significance because epizootics have resulted in mass mortality of commercial fish species such as Atlantic herring, *Clupea harengus* L. (Patterson 1996; Rahimian & Thulin 1996; Mellergaard & Spanggaard 1997). Although systemic infections are lethal, variation in pathogenic effects has been observed between isolates of *I. hoferi* and among host species (McVicar & McLay 1985; Kocan, Hershberger, Mehl, Elder, Bradley, Wildermuth & Stick 1999). For example, experimental infections with a Pacific isolate of *I. hoferi* resulted in 80% mortality of Pacific herring, *C. pallasi* Valenciennes, whereas no mortality resulted from an Atlantic isolate (Kocan *et al.* 1999). Such variation, along with the broad range of hosts from which *I. hoferi* has been reported (McVicar 1999), raises the question of whether cryptic species (genetically distinct, but morphologically indistinguishable) occur among populations of *I. hoferi*.

Molecular data provide a means to identify cryptic species, as was suggested by the description of *I. irregularis* by Rand, White, Cannone, Gutell, Murphy & Ragan (2000). Otherwise, little attention has been given to the use of this approach to understand speciation among populations of *I. hoferi*. Use of deoxyribonucleic acid (DNA) sequence data has primarily focused on the phylogenetic relationship of *I. hoferi* to other eukaryotes (Ragan, Goggins, Cawthorn, Cerenius,

Jamieson, Plourde, Rand, Soderhall & Gutell 1996; Spanggaard, Skouboe, Rossen & Taylor 1996). Several analyses using nuclear small subunit 18S ribosomal DNA (ssu-rDNA) sequences have corroborated that *I. hoferi* belongs to a clade (Mesomycetozoa) that is closely related to choanoflagellates and basal animals (Baker, Beebee & Ragan 1999; Herr, Ajello, Taylor, Arseculeratne & Mendoza 1999; Medina, Collins, Silberman & Sogin 2001). Yet, only three published ssu-rDNA sequences exist for *Ichthyophonus* spp. (Ragan *et al.* 1996; Spanggaard *et al.* 1996; Rand *et al.* 2000). Given the availability of ssu-rDNA sequences to design specific primers for *Ichthyophonus*, variation can be examined at this locus. Comparing this variation with patterns of geographic association or host specificity should prove useful in determining whether this economically important fish pathogen is composed of an assemblage of cryptic species.

Ichthyophonus was first reported in the north-eastern Pacific Ocean in 1983 (Olson 1986). It has since been associated with the mortality of Pacific herring in the waters off Alaska and Washington, USA (Marty, Freiberg, Meyers, Wilcock, Farver & Hinton 1998; Kocan *et al.* 1999). In surveys conducted by Kent, Watral, Dawe, Reno, Heidel & Jones (2001), *Ichthyophonus* infections were detected in Pacific Ocean perch, *Sebastes alutus* (Gilbert), and yellowtail rockfish, *S. flavidus* (Ayres), with a prevalence of 48 and 51%, respectively. Recent declines among populations of rockfish, *Sebastes* spp., in the north-eastern Pacific are the cause of increasing concern (Musick, Harbin, Berkeley, Burgess, Eklund, Findley, Gilmore, Golden, Ha, Huntsman, McGovern, Parker, Poss, Sala, Schmidt, Sedberry, Weeks & Wright 2000). As part of ongoing studies to ascertain the role infectious diseases may have in this decline, we are conducting experiments on the pathogenicity and routes of transmission of the *Ichthyophonus* recovered from *Sebastes* spp. Because *I. hoferi* is pathogenic for Pacific herring, an important first step is to establish if *Ichthyophonus* parasites infecting other Pacific hosts are a single species. Therefore, our goal, reported here, was to use ssu-rDNA sequence data to determine if specimens of *Ichthyophonus* from Pacific herring, rockfish and chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), were the same in the north-eastern Pacific. We also discuss future directions for studying speciation in *Ichthyophonus*.

Materials and methods

Fish were collected from the north-eastern Pacific in May to September 2000 and examined macroscopically for *Ichthyophonus* infections. Infected muscle tissue or visceral organs were then preserved in 95% ethanol for DNA analysis. Table 1 shows the locations for host collection and infected tissues used for DNA extractions.

The DNA was extracted from infected tissues using the DNeasy Tissue Kit (Qiagen Valencia, CA, USA) according to the manufacturer's protocol. Polymerase chain reaction (PCR) primers specific for the ssu-rDNA of *Ichthyophonus* were designed using available sequences from GenBank (accession number). These included *I. hoferi* (U25637) collected from a yellowtail flounder, *Limanda ferruginea* (Storer), in the Atlantic off the Nova Scotia shelf (Ragan *et al.* 1996), *I. hoferi* (U43712) from Atlantic herring, *C. harengus* L., in the North Sea and a rainbow trout, *O. mykiss* (Walbaum), presumably from Japan (Spanggaard *et al.* 1996),

Table 1 GenBank accession numbers for partial ssu-rDNA sequences of *Ichthyophonus* with hosts, geographic location and tissues from which samples were obtained

	GenBank accession numbers				
Host	Host ID no.	Location[a]	Tissue	A region	B region
Sebastes flavidus	YT 54	BC	Spleen/kidney	AF467785	AF467786
Sebastes alutus	POP 222	BC	Spleen/kidney	AF467787	AF467788
Sebastes alutus	POP 25	OR	Spleen/kidney	AF467789	AF467790
Sebastes alutus	POP 6	OR	Spleen/kidney	AF467791	AF467792
Clupea pallasi	HER 258	BC	Heart/spleen/kidney	AF467793	AF467794
Clupea pallasi	HER 134	BC	Heart/spleen/kidney	AF467795	AF467796
Clupea pallasi	HER 128	BC	Heart/spleen/kidney	AF467797	AF467798
Oncorhynchus tshawytscha	YUK 10	YR	Muscle	AF467799	AF467800
Oncorhynchus tshawytscha	YUK 5	YR	Muscle	AF467801	AF467802

[a] BC, British Columbia, Canada; OR, Oregon, USA; YR, Yukon River, Canada.

and *I. irregularis* (AF232303) from a yellowtail flounder off the Nova Scotia shelf (Rand *et al.* 2000). The forward primer GO1 (5′-CAC CTG GTT GAT CCT GCC AG-3′) (Saunders & Kraft 1994) and reverse ICH2R (5′-CGA CCA GTA AGC AAT TTC CA-3′) were used to amplify a fragment (region A) of approximately 640 base pairs (bp), excluding primers. A second fragment (region B) consisting of approximately 673 bp was amplified with ICH1F (5′-TGG AAA TTG CTT ACT GGT CG-3′) and ICH4R (5′-TGT AAC TAT TTA GTA GGT TAA-3′). In reference to *I. hoferi* (U25637) (Ragan *et al.* 1996), GO1 is located at bp 1–21, ICH2R at 661–642, ICH1F at 642–661 and ICH4R at 1355–1335.

The PCR was performed using 50 μL reactions consisting of 1X PCR buffer containing 1.5 mM $MgCl_2$ (Qiagen), 0.2 mM dNTPs, 0.5 μM each primer, 1.25 units *Taq* polymerase (Qiagen) and 3 μL of sample DNA. Quantification of parasite DNA was not possible as samples contained both host and parasite DNA. Reactions were run in a MJ Research DNA Engine 200 (MJ Research, Watertown, MA, USA). Region A was amplified with an initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C for 45 s, 57.4 °C for 60 s, 72 °C for 90 s and a single final extension at 72 °C for 7 min. Amplification of region B followed the same protocol, but the annealing temperature was 51.7 °C. The DNA extracted from uninfected brown rockfish, *S. auriculatus* Girard, was used as a control for specificity of the *Ichthyophonus* primers. The PCR products were visualized on a 1.5% agarose gel (containing 0.1 μg mL^{-1} ethidium bromide), which was run at 100 V for 1 h. PCR products were excised from the gel and purified using the Qiagen Gel Extraction Kit. Sequencing was performed in both directions using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA) and visualized on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) by Davis Sequencing (Davis, CA, USA). Sequencing primers were the same as those used in the PCR.

The A and B regions were combined for all subsequent analyses. We created a parsimony network to examine the relationships among the available *Ichthyophonus* haplotypes including those from this study. In addition, to see if a standard amount of sequence divergence would help indicate potential cryptic species of *Ichthyophonus*, we examined sequence divergence among sister species in the Mesomycetozoa. In order to do this, a maximum likelihood (ML) analysis was used to create a phylogram for the Mesomycetozoa clade. *Ichthyophonus* sequences from this study were aligned with the following species obtained from GenBank (accession number): *Acanthocoepsis unguiculata* (L10823), *Amoebidium parasiticum* (Y19155), *Anurofeca richardsi* (AF070445), *Dermocystidium salmonis* (U21337), *Dermocystidium* sp. (U21336), *Diaphanoeca grandis* (L10824), *I. hoferi* (U25637), *I. hoferi* (U43712), *I. irregularis* (AF232303), *Pseudoperkinsus tapetis* (AF192386), *Psorospermium haeckelii* (U33180), *Rhinosporidium seeberi* (AF118851), rosette agent of salmon (L29455) and *Sphaerosoma arcticum* (Y16260). ClustalW (Thompson, Higgins & Gibson 1994), followed by visual inspection, was used to align the partial ssu-rDNA sequences.

Sequence divergence and the ML analysis were computed using PAUP*4.0b8 [Swofford D.L., 2001 PAUP* phylogenetic analysis using parsimony (*and other methods) Sinauer Associates, Sunderland, MA, USA]. The choanoflagellates *A. unguiculata* and *D. grandis* were used as outgroups to root the Mesomycetozoa based on the results of previous studies (Herr *et al.* 1999; Figueras, Lorenzo, Ordas, Gouy & Novoa 2000). The nucleotide substitution model for the ML analysis was determined with hierarchical log-likelihood ratio tests as implemented in the program Modeltest 3.06 (Posada & Crandall 1998). The TrN + I + G model (Tamura & Nei 1993) was selected by Modeltest with a gamma distribution shape parameter (G) of 0.5572 and a proportion of invariable sites (I) of 0.4569. The TrN model assigns different transition rates to purines (2.0643) and pyrimidines (5.1564) and gives a third rate (1.0) to all transversions. A heuristic search with a single random stepwise addition was used for the ML analysis under the TrN + I + G model. Stability was inferred by 100 bootstrap replicates. Both uncorrected pairwise (p) distances among species and ML distances under the TrN + I + G model of sequence evolution were calculated.

Results

All sequences (Table 1) had clear chromatograms with no background signals, and forward and reverse sequencing enabled verification of bases. No PCR products were observed from the DNA of uninfected brown rockfish. Samples from Pacific

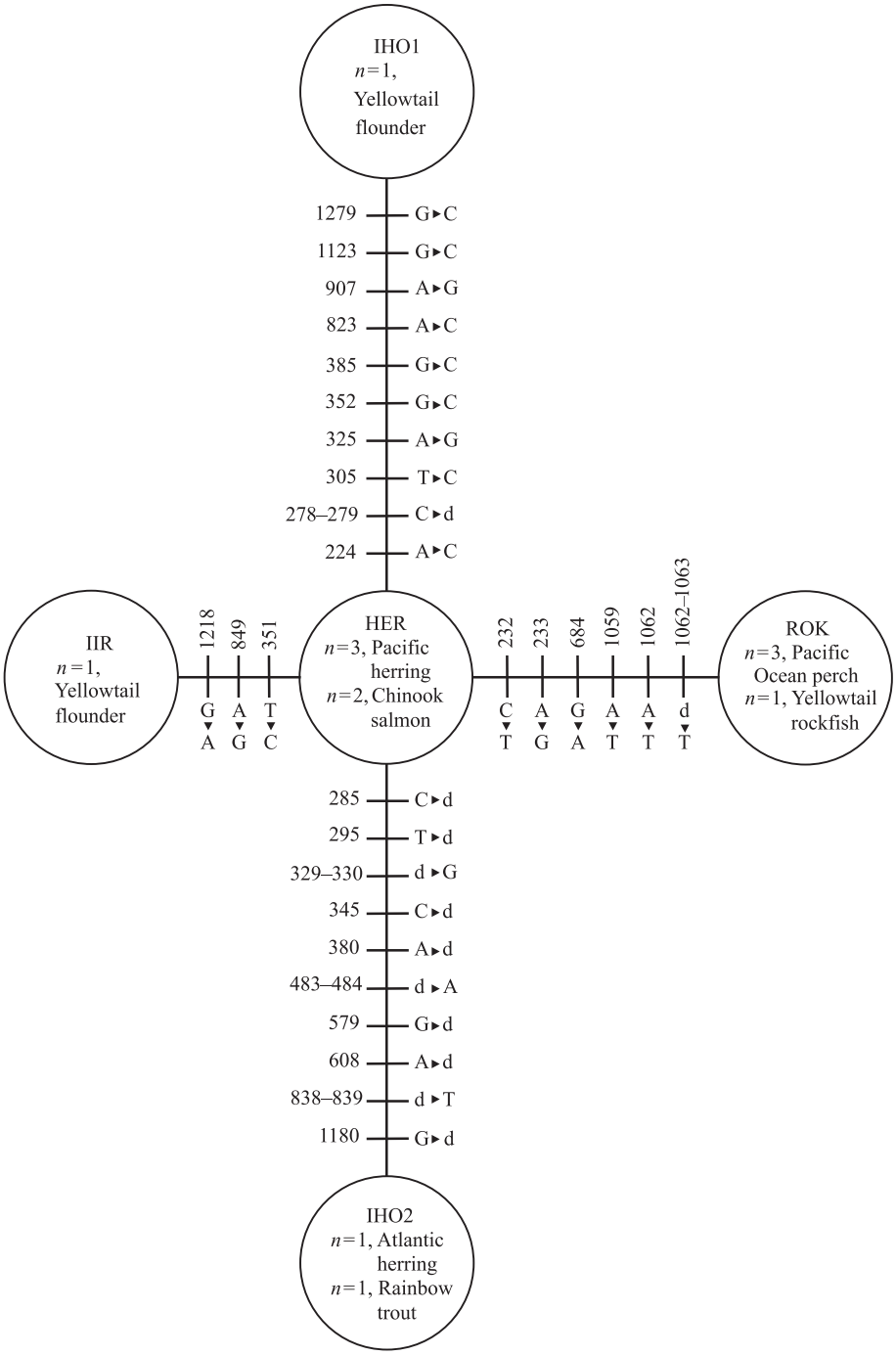

Figure 1 Parsimony network of *Ichthyophonus* ssu-rDNA haplotypes. No reversals were present. IHO1, haplotype of *I. hoferi* (Ragan *et al.* 1996); IHO2, *I. hoferi* (Spanggaard *et al.* 1996); IIR, *I. irregularis* (Rand *et al.* 2000); HER, samples from both *Clupea pallasi* and *Oncorhynchus tshawytscha* (this study, see Table 1); ROK, samples from *Sebastes* spp. (this study, see Table 1); *n*, sample size for each haplotype followed by the hosts from which samples were obtained. Numbers indicate nucleotide position in reference to IHO1. The ssu-rDNA regions analysed include bases 51–630 (region A) and 671–1314 (region B). The arrow (▶) points to the nucleotide mutation in reference to the interior HER haplotype. N▶d, deletion; d▶N, insertion.

Table 2 Distance matrix for partial ssu-rDNA sequences of *Ichthyophonus*. Above the diagonal are pairwise distances based on uncorrected (*p*) and below are pairwise distances based on the maximum likelihood model TrN + I + G

	IHO1	IHO2	IIR	HER	ROK
IHO1 (1224)[a]	–	0.0074	0.0098	0.0074	0.0114
IHO2 (1221)	0.0077	–	0.0025	0.0000	0.0041
IIR (1225)	0.0103	0.0025	–	0.0025	0.0065
HER (1225)	0.0077	0.0000	0.0025	–	0.0041
ROK (1226)	0.0122	0.0042	0.0067	0.0042	–

[a] Abbreviations are those used in Fig. 1. The number of nucleotides used in the calculations is in parentheses. Calculations exclude gapped or ambiguous characters.

herring and chinook salmon were identical in both the A (581 bp) and B (644 bp) regions. Likewise, all rockfish samples were identical in the A (581 bp) and B (645 bp) regions. There were six nucleotide differences between the Pacific herring/salmon and rockfish haplotypes of *Ichthyophonus*; two substitutions were in the A region with three substitutions and a single nucleotide indel in the B region (Fig. 1). Table 2 shows the pairwise distances for partial ssu-rDNA sequences of *Ichthyophonus*.

Alignment for the ML analysis resulted in 1253 nucleotide positions including gaps. Lengths of the partial ssu-rDNA sequence ranged from 1204 to 1241 nucleotides (excluding gaps) for the taxa used in the analysis. The tree resulting from the ML analysis showed the *Ichthyophonus* haplotypes as a polytomy (Fig. 2). Although our analysis used only a partial ssu-rDNA sequence, our data set was robust in that the relationships found among the Mesomycetozoa (Fig. 2) were consistent with studies using the entire ssu-rDNA sequence (Ragan *et al.* 1996; Spanggaard *et al.* 1996; Baker *et al.* 1999; Herr *et al.* 1999; Figueras *et al.* 2000; Rand *et al.* 2000; Ustinova, Krienitz & Huss 2000).

Discussion

Two ssu-rDNA haplotypes differing at six nucleotide positions were found among the samples of *Ichthyophonus* in the north-eastern Pacific Ocean. One haplotype was recovered from rockfish samples while the other was found in both Pacific herring and chinook salmon samples (Fig. 1). Thus, in geographic sympatry, two genotypes of *Ichthyophonus* were fixed among host species. Two hypotheses could explain these isolated populations of *Ichthyophonus*. First, these haplotypes might belong to the same species gene pool, but ecological separation of the hosts may have resulted in rare admixture of the *Ichthyophonus* haplotypes among host species. Alternatively, the haplotypes might reflect genetically distinct *Ichthyophonus* species that are host specific.

Ichthyophonus hoferi can be acquired via the ingestion of infected fish (Kocan *et al.* 1999; Jones & Dawe 2002), thus, an ecological separation via different transmission routes seems plausible in light of the dietary patterns of the host species. Pacific herring are a major part of the diet of chinook salmon (Heal 1991) and experimental infections have demonstrated that chinook salmon can acquire *Ichthyophonus* from infected herring tissue (Jones & Dawe 2002). Thus, the predator–prey interaction between chinook salmon and Pacific herring could provide a natural transmission route resulting in the identical ssu-rDNA haplotypes from these hosts. On the other hand, Pacific herring are absent from or constitute a small portion of the stomach contents of Pacific Ocean perch and yellowtail rockfish (Pereyra, Pearcy & Carvey 1969; Brodeur & Pearcy 1984). If rockfish have an alternative source of infection other than the Pacific herring/salmon route, then an association between the two ssu-rDNA haplotypes and the different host species may have resulted.

Other potential modes of transmission, however, would not suggest an ecological separation. Jones & Dawe (2002) suggest that Pacific herring can acquire *Ichthyophonus* via ingestion of infected crustaceans, which also constitute a major portion of the diet of Pacific Ocean perch and yellowtail rockfish (Brodeur & Pearcy 1984). Furthermore, infections may be acquired via direct exposure to *Ichthyophonus* spores (Kocan *et al.* 1999); therefore, any spatial overlap among hosts should increase exposure. If no ecological separation existed, admixture of the two haplotypes among the host species would be expected. Therefore, the association between the *Ichthyophonus* haplotypes and host species would best be explained by host

specificity. If hosts were exposed to both genetic variants of *Ichthyophonus*, host specificity factors could inhibit the establishment of one of the variants. A correlation between host specificity and fixed genetic differences of the parasite among host species would be indicative of separate species. Cross infection experiments are planned to determine the host specificity of the two *Ichthyophonus* haplotypes.

Other evidence supports the presence of two *Ichthyophonus* species. Kent *et al.* (2001) did not observe *Ichthyophonus* in the heart tissue of rockfish, but infections are common in the heart muscle of Pacific herring (Marty *et al.* 1998; Jones & Dawe

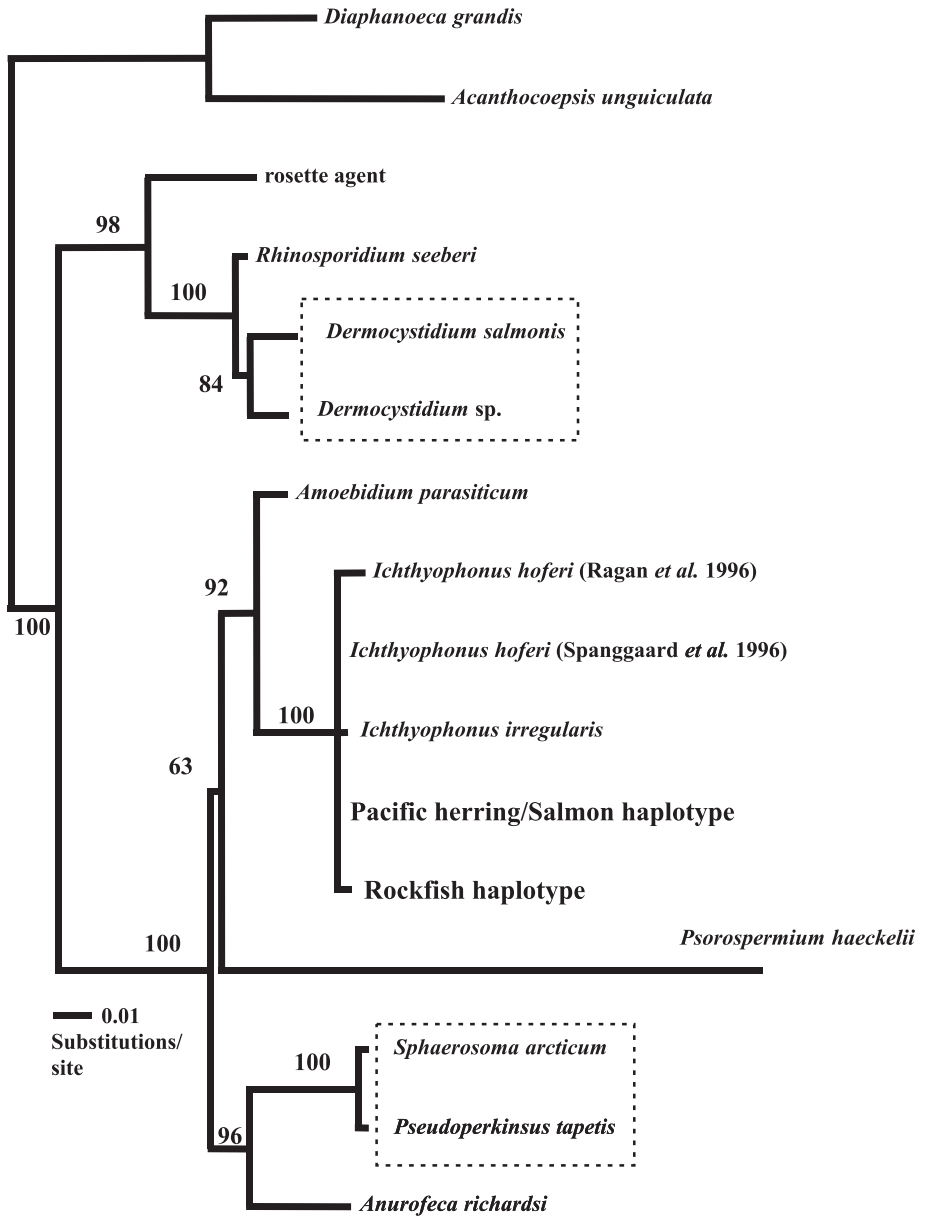

Figure 2 The ML analysis for the Mesomycetozoa clade using partial ssu-rDNA sequences under the TrN + G + I model. There is no clear indication from the ML phylogram of cryptic *Ichthyophonus* species because a standard amount of sequence divergence (substitutions per site) among sister species in the Mesomycetozoa (boxed regions) is not discernible and because of the polytomy of *Ichthyophonus* haplotypes. Branch lengths are based on the ML analysis and numbers on internal branches are percentages based on 100 bootstrap replicates.

2002). Thus, there may be specificity for site of infection. However, we are not yet inclined to call the *Ichthyophonus* samples from the Pacific herring/salmon and rockfish different species, and retain the use of *I. hoferi* when referring to all haplotypes for the following reasons. The sequences of *I. hoferi* (U25637) (Ragan *et al.* 1996) and *I. irregularis* are of a single sample. Therefore, it is not possible to determine if the autapomorphic sequence differences (unique substitutions) reflect sequencing errors, intraspecific variation, or fixed differences among hosts or geographic regions. Although *I. hoferi* isolates from an Atlantic herring and a rainbow trout were found to have an identical ssu-rDNA sequence by Spanggaard *et al.* (1996), the sequence of *I. hoferi* (U43712) is suspect in that all differences from the Pacific herring/salmon haplotype were single nucleotide indels (Fig. 1). Spanggaard *et al.* (1996) used manual sequencing; therefore, nucleotide-scoring errors may be more prominent. Additionally, the ML analysis could not resolve the phylogenetic relationships among the ssu-rDNA haplotypes of *Ichthyophonus* (Fig. 2). Therefore, objective criteria under the evolutionary species concept could not be used to delimit species (see Nadler, Adams, Lyons, DeLong & Melin 2000). For the above reasons, we also do not find molecular support for the species status of *I. irregularis* (Rand *et al.* 2000). It should be noted that we do not refute the developmental and morphological differences (Rand 1994) that Rand *et al.* (2000) also used to designate *I. irregularis* as a separate species.

Even when subjective criteria such as a genetic yardstick [but see Nadler *et al.* (2000) for problems associated with using genetic yardsticks] are examined within the Mesomycetozoa, there is no clear indication of cryptic species among isolates of *I. hoferi* (Fig. 2). For example, pairwise ML distance between the sister taxa S. *arcticum* and *P. tapetis* was 0.0068 (0.0066 for uncorrected *p*), but between the *Dermocystidium* spp. there was a distance of 0.0208 (0.0194). Pairwise ML distances among the *Ichthyophonus* isolates ranged from 0 to 0.0122 (Table 2), thus, a standard amount of sequence divergence among recognized species in the Mesomycetozoa is not discernible (see also Fig. 2).

Examination of evolutionary relationships among the Pacific and Atlantic haplotypes of *Ichthyophonus* is currently limited because of small sample sizes from the Atlantic. However, if the network in Fig. 1 is considered accurate, it is interesting that the Pacific herring/salmon haplotype falls out as the ancestor to either two or three haplotypes recovered from the Atlantic (Fig. 1). This sets the stage for a phylogeographic comparison of *I. hoferi* from the Atlantic and Pacific oceans to determine the population history and structure of *I. hoferi*. Furthermore, current phylogeographic analyses (Templeton 2001) that test for significant associations between sequence data and morphological/developmental data, such as that in Rand (1994), offer an objective means to delimit potential cryptic species among populations of *I. hoferi*. However, a more variable gene than ssu-rDNA may provide greater resolution in future phylogeographic studies.

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