Mycologia, 100(2), 2008, pp. 171–180.

Issued 3 June 2008

Saprolegniaceae identified on amphibian eggs throughout the Pacific Northwest, USA, by internal transcribed spacer sequences and phylogenetic analysis

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Abstract: We assessed the diversity and phylogeny of Saprolegniaceae on amphibian eggs from the Pacific Northwest, with particular focus on *Saprolegnia ferax*, a species implicated in high egg mortality. We identified isolates from eggs of six amphibians with the internal transcribed spacer (ITS) and 5.8S gene regions and BLAST of the GenBank database. We identified 68 sequences as Saprolegniaceae and 43 sequences as true fungi from at least nine genera. Our phylogenetic analysis of the Saprolegniaceae included isolates within the genera *Saprolegnia*, *Achlya* and *Leptolegnia*. Our phylogeny grouped *S. semihypogyna* with *Achlya* rather than with the *Saprolegnia* reference sequences. We found only one isolate that grouped closely with *S. ferax*, and this came from a hatchery-raised salmon (Idaho) that we sampled opportunistically. We had representatives of 7–12 species and three genera of Saprolegniaceae on our amphibian eggs. Further work on the ecological roles of different species of Saprolegniaceae is needed to clarify their potential importance in amphibian egg mortality and potential links to population declines.

Accepted for publication 8 November 2007.

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Key words: *Achlya*, amphibian decline, egg, lake, *Leptolegnia*, oomycete, *Saprolegnia ferax*, *S. semihypogyna*

INTRODUCTION

The Saprolegniaceae (Saprolegniales, Oomycota) belong to kingdom Chromista (*sensu* Cavalier-Smith 1997). These organisms often are referred to as oomycetes or ''water molds'' and can be found on decaying animal and plant debris in freshwater habitats worldwide. They can occur on adult fish and on the eggs of fish and amphibians (Hoffman 1967, Czeczuga et al 1998). The family Saprolegniaceae has 19 genera and roughly 150 species (Dick 1973).

Identification of Saprolegniaceae traditionally has relied on the observation of morphological features (Seymour 1970, but see Hulvey et al 2007). Genera of the Saprolegniaceae have been differentiated by their method of zoospore release (Seymour 1970, Daugherty et al 1998). Species identification has been more challenging because it has required presence of the sexual structures, the oogonia and antheridia. More recently molecular identification has been accomplished with selected Saprolegniaceae using the internal transcribed spacer (ITS) and 5.8S regions of ribosomal DNA (rDNA) (Molina et al 1995, Leclerc et al 2000). The most complete molecular phylogeny of this family to date identified 10 genera and 40 species through analyses of ITS and the large ribosomal subunit (LSU) (Leclerc et al 2000).

There have been relatively few published field investigations of Saprolegniaceae diversity and ecology. This is particularly true in the Pacific Northwest, USA, which is a region where *Saprolegnia* has been identified as a potential pathogen on amphibians that have experienced local declines (Kiesecker and Blaustein 1995). For example Blaustein et al (1994) suggested *Saprolegnia ferax* was responsible for mortality of nearly 95% of western toad (*Bufo boreas*) eggs at one site in Oregon. Despite these claims that *S. ferax* is a pathogenic water mold of amphibian eggs, no studies have attempted to document which species of oomycetes occur on the eggs of North American amphibians and whether egg mortality is uniquely associated with *S. ferax*.

As a result of concern over observed amphibian egg mortality and the difficulty of identifying taxa microscopically, we sought to identify oomycetes

cultured from amphibian eggs from around the Pacific Northwest with ITS and 5.8S rRNA gene sequences. We compared sequences of Saprolegniaceae cultured from amphibian eggs to reference cultures from the American Type Culture Collection (ATCC) and published sequences from GenBank. The information from this survey should be of value to mycologists and amphibian biologists who are interested in the distribution of oomycetes and their ecological relationships with amphibians.

MATERIALS AND METHODS

Collection of egg samples.—In 2001–2003 we collected egg samples (n = 200) of six pond-breeding amphibian species from the Pacific Northwest, USA (Saprolegniaceae samples TABLE I). We coded sample numbers based on physiographic provinces: IS = Snake River Plain in southern Idaho; IM = Bitterroot Mountains of Idaho and western Montana; OE = Blue Mountains of eastern Oregon; OW = Cascade Range and Willamette Basin of western Oregon; WA = Cascade Range around Mount Rainier, Washington (FIG. 1). The six amphibians included the long-toed salamander (*Ambystoma macrodactylum*), Pacific tree frog (*Pseudacris regilla*), northern red-legged frog (*Rana aurora*), Cascades frog (*R. cascadae*), Columbia spotted frog (*R. luteiventris*) and Oregon spotted frog (*R. pretiosa*). For all sampled amphibian egg masses, we collected equivalent numbers of healthy eggs and eggs that appeared diseased (with white or gray hyphal nimbus, FIG. 2) in sterile 2 oz. polypropylene containers filled with surrounding pond water. Because several *Saprolegnia* species first were isolated from salmonids, we opportunistically collected three samples from fish in southern Idaho: rainbow trout (*Oncorhynchus mykiss*) eggs from a hatchery and scale scrapings from a dead rainbow trout (*O. mykiss*) and a hatchery-raised Chinook salmon (*O. tshawytscha*).

Culturing of organisms from egg samples.—Samples were shipped in coolers and stored at 4 C at the laboratory. Because we did not want to exclude the growth of any Saprolegniaceae species, we chose to use a nonselective media for our culturing procedure. We did not sterilize egg samples because there is some indication that oomycetes can grow on the outside of eggs (e.g. Czeczuga et al 1998, Green 1999). We did not exclude any filamentous microorganisms in the culturing process so we used a nonselective media, potato-dextrose agar (PDA). PDA plates were made from 24 g of potato dextrose (Difco) and 15 g of agar (Difco) per L. We used a sterile pipette to transfer 1–2 amphibian eggs from each sample to a PDA plate. We used sterile forceps to place fish eggs and skin scrapings on PDA plates. We incubated cultures at 22 C for 24 h or until the growth of filamentous organisms was evident. To obtain a pure culture of the filamentous organism we used a sterile hypodermic needle to transfer a small amount of the cultured filamentous growth to a second PDA plate which we incubated at 22 C for 24 h. In the majority of cultures (75%) filamentous growth occurred quickly and was able to outgrow any bacteria in the culture. In cases where the filamentous growth was slower bacteria were evident on the original PDA plate. When bacteria and filamentous growth were present together in culture, successive hyphal transfers were used to obtain a pure filamentous culture.

We obtained three Saprolegniaceae isolates from the ATCC (10801 University Blvd., Manassas, Virginia 20110-2209 USA): *Saprolegnia ferax* (Gruithuisen) Thuret (ATCC 26116), *Saprolegnia parasitica* Coker (ATCC 22284) and *Achlya americana* Humphrey (ATCC 22599). We cultured and sequenced the ATCC isolates in the same manner as the egg and fish samples.

DNA extraction, PCR amplification, and sequencing.—We extracted DNA from each filamentous organism using a procedure adapted from Griffith and Shaw (1998) for DNA isolation from *Phytophthora infestans*. This process uses a modified extraction buffer (100 mM Tris-HCL, 1.4 M NaCl, 2% CTAB and 20 mM EDTA sodium salt pH 8.0) and a chloroform extraction technique. Precipitation of DNA was completed with isopropanol and centrifugation at 17 000 × *g*. We confirmed the DNA was intact by running each sample on 1.2% agarose gels. Samples were stored at 4 C until use in the polymerase chain reaction (PCR).

The ITS1 and ITS2 regions and the intermediate 5.8S ribosomal gene were amplified using the primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (White et al 1990) in a 50 nmole concentration (HPLC purified) from Operon (QIAGEN). We performed PCR reactions in a 50 μL volume using the HotStarTaq Master Mix Kit (QIAGEN) and a final 1 μM concentration of each primer. The final template DNA concentration used for each reaction was 10 ng/μL. We performed PCR amplifications with an Applied Biosystems 2400 thermocycler (Applied Biosystems). The PCR temperature profile was an initial activation at 95 C for 15 min followed by 30 cycles with target temperatures at 94 C, 54 C and 72 C each for 1 min. A final extension at 72 C for 10 min completed the run. We analyzed PCR products by electrophoresis on 1.2% agarose gels and stained the gels with ethidium bromide (50 μg/μL) to confirm the presence of the PCR product. PCR products were directly purified with the MinElute PCR Purification Kit (QIAGEN) for sequencing. Purified PCR products were sequenced with big dye terminator primers in the 3100 Genetic Analyzer (Applied Biosystems) at the Molecular Core Facility at Idaho State University at Pocatello.

Alignment and phylogenetic analysis.—We used PHRED (Ewing et al 1998a) to assess the quality of DNA base call sequences. We considered 111 sequences to be of excellent quality to be used for further analysis. We used PHRAP (PHRED II, Ewing et al 1998b) to build a consensus sequence from forward and reverse sequences identified by PHRED.

Quality sequences were identified by BLAST (Basic Local Alignment Search Tool, Altschul et al 1990) using the National Center for Biotechnology Information (NCBI) BLAST feature (http://www.ncbi.nlm.nih.gov/BLAST). We used the nucleotide-nucleotide search option, which uses a

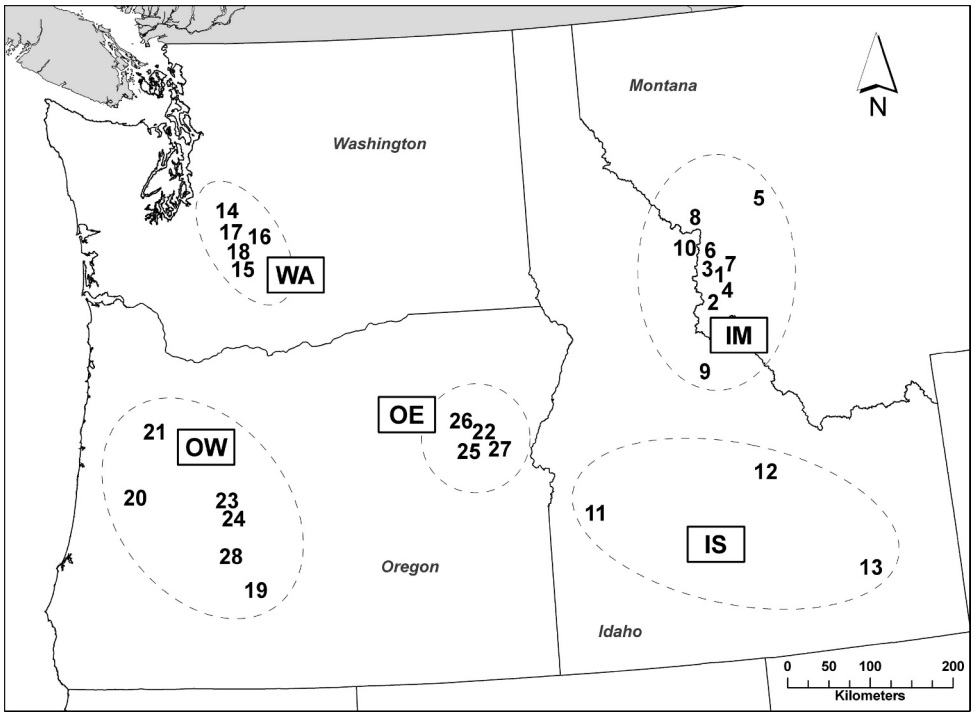

FIG. 1. Sample sites in the Pacific Northwest, USA. Site numbers correspond to TABLE I. Physiographic regions are: Idaho-Montana (IM), Idaho-South (IS), Oregon-East (OE) and Oregon-West (OW), Washington (WA).

heuristic algorithm to search for sequence homology (Altschul et al 1990).

To see the phylogenetic relationships among our Saprolegniaceae sequences, we used Geneious Pro® (version 2.5.2) to produce a multiple global alignment of 88 sequences. The sequences in our phylogeny included: (i) all samples identified by BLAST as *Saprolegnia*, *Achlya*, or *Leptolegnia* (n = 68); (ii) three ATCC type strains that were cultured and sequenced in our lab (*S. ferax* [Gruithuisen] Thuret, *S. parasitica* Coker and *A. americana* Humphrey); (iii) 16 *Saprolegnia*, *Achlya*, or *Leptolegnia* (Leclerc et al 2000) that we downloaded from the GenBank NCBI database (TABLE II); and (iv) the non-Saprolegniaceae outgroup *Phytophthora botryosa* (ITS sequence GenBank AF266784, Cooke et al 2000). We saved the alignment in the Nexus format.

FIG. 2. Oregon spotted frog (*Rana pretiosa*) egg mass with high mortality and microorganisms on embryos (center) surrounded by healthy developing egg masses from Oregon.

The sequence alignment was analyzed with both a distance and parsimony analysis using PAUP (version 4.0 beta; Swofford 2003). We performed the distance analysis using the Jukes-Cantor option with equal rates for variable sites (Jukes and Cantor 1969). A bootstrap analysis of the neighbor joining tree was done with a neighbor joining search with 1000 replicates. Groups with 70% or greater frequency were retained. The parsimony analysis was done using the heuristic search option. The maximum number of trees saved was set to 2000. The parsimony consensus tree was computed with 70% majority rule. A bootstrap analysis of the parsimony consensus tree was done using a fast heuristic search with 1000 replicates.

RESULTS

Of the 203 samples we cultured, 138 filamentous cultures grew. BLAST was able to identify sequences from 111 isolates from 28 collection sites. We identified 68 sequences as Saprolegniaceae (TABLE I). The remaining 43 sequences were true fungi: *Trichoderma* (n = 27), *Mucor* (n = 6), *Verticillium*

TABLE I. Site and host species for successful cultures of Saprolegniaceae (n = 68). Sample codes are in format 'Region-Site-Egg Sample'. Amphibians are AMMA (*Ambystoma macrodactylum*); PSRE (*Pseudacris regilla*), RAAU (*Rana aurora*), RACA (*Rana cascadae*), RALU (*Rana luteiventris*), RAPR (*Rana pretiosa*). Fish are *Oncorhynchus tshawytschsa* (Chinook Salmon) and *O. mykiss* (Rainbow Trout)

Site	Site Name	State	Sample code	Species
1	Lost Horse Creek	MT	IM-1-1	AMMA
2	LRC Frog Pond	MT	IM-2-1	RALU
3	Lost Horse Pond	MT	IM-3-6, IM-3-7	AMMA
			IM-3-5	PSRE
			IM-3-1, IM-3-2, IM-3-3, IM-3-4	RALU
4	Kramis Pond	MT	IM-4-8	AMMA
			IM-4-1, IM-4-2, IM-4-3, IM-4-4, IM-4-5	PSRE
			IM-4-6, IM-4-7	RALU
5	Jones Pond	MT	IM-5-1, IM-5-2, IM-5-3, IM-5-4	AMMA
6	Duffy Pond East	MT	IM-6-1, IM-6-2, IM-6-3, IM-6-4, IM-6-5, IM-6-6	RALU
7	Little Rock Lake	MT	IM-7-1, IM-7-2, IM-7-3	RALU
8	Granite Lake	ID	IM-8-1, IM-8-2, IM-8-3	RALU
9	Frog Pond Lake	ID	IM-9-1, IM-9-2	RALU
10	North Walton Lake	ID	IM-10-1	RALU
11	Eagle Fish Health Laboratory	ID	IS-11-1 (fish)	*Oncorhynchus tshawytschsa*
12	Fish Hatchery	ID	IS-12-1 (fish eggs)	*O. mykiss*
13	Gibson Jack Stream	ID	IS-13-1 (fish)	*O. mykiss*
14	Marjorie Lake Pond	WA	WA-14-1	RACA
15	Snow Lake	WA	WA-15-1, WA-15-2, WA-15-3, WA-15-4, WA-15-5	RACA
16	Upper Deadwood Lake	WA	WA-16-1	RACA
17	Ethel Lake	WA	WA-17-1	RACA
18	Bench Lake	WA	WA-18-1, WA-18-2	RACA
19	Jack Creek	OR	OW-19-1	RAPR
20	Half Moon Pond	OR	OW-20-1	RAAU
21	Dugout Pond	OR	OW-21-1	PSRE
			OW-21-2, OW-21-3	RAAU
22	Black Mountain Lower Pond	OR	OE-22-1, OE-22-2, OE-22-3, OE-22-4	RALU
23	Penn Lake	OR	OW-23-1	RACA
			OW-23-2, OW-23-3	RAPR
24	Muskrat Lake	OR	OW-24-1	PSRE
25	China Lake Small Pond	OR	OE-25-1	RALU
26	Camp Creek Lowest Pond	OR	OE-26-1, OE-26-2	RALU
27	Black Mountain Middle Pond	OR	OE-27-1	AMMA
			OE-27-2	RALU
28	Big Marsh	OR	OW-28-1	RAPR

(n = 3), *Penicillium* (n = 1), *Cordyceps* (n = 1), *Engyodontium* (n = 1), *Gibberella* (n = 1), *Guignardia* (n = 1), *Leptosphaeria* (n = 1) and one unidentified fungus. Because we did not sterilize our egg samples, it is possible that these true fungi were environmental contaminants that were present in the egg samples. While we considered it important to report all the organisms cultured from our amphibian eggs, we focused on the Saprolegniaceae for this study.

Of 884 total characters, 312 were parsimony informative, 411 were constant and 161 variable characters were parsimony uninformative. The neighbor joining and maximum parsimony analysis produced identical groupings of *Leptolegnia*, *Saprolegnia*, *Achlya* and *S. semihypogyna*. While the groupings were identical, the bootstrap support was higher in the branch groupings of the neighbor joining than the parsimony consensus tree. We chose to show the neighbor joining (FIG. 3) rather than the parsimony consensus tree to see the amount of evolutionary change and therefore determine how closely isolates were related within each grouping of *Saprolegnia*, *Leptolegnia*, *Achlya* or *S. semihypogyna*.

Our distance analysis resolved the *Leptolegnia* and *Saprolegnia* groups from the rest of the tree with bootstrap support of 75. The *Achlya* and *S. semihypo-*

TABLE II. Reference sequences and cultures used for phylogenetic analysis

Reference Cultures	Source and accession number
Achlya americana	GenBank AF218145
Achlya americana Humphrey ATCC 22599	our laboratory
Achlya aquatica	GenBank AF218150
Achlya colorata	GenBank AF218159
Achlya intricata	GenBank AF218148
Achlya oligacantha	GenBank AF218162
Achlya papillosa	GenBank AF218161
Achlya racemosa	GenBank AF218158
Leptolegnia CBS 177.86	GenBank AY310502
Phytophthora botryosa	GenBank AF266784
Saprolegnia anomalies	GenBank DQ322632
Saprolegnia bulbosa	GenBank AY267011
Saprolegnia diclina Humphrey ATCC 90215	GenBank AY455775
Saprolegnia ferax (Gruithuisen) Thuret ATCC 26116	our laboratory
Saprolegnia hypogyna	GenBank AY647188
Saprolegnia longicaulis	GenBank AY270032
Saprolegnia oliviae	GenBank AY270031
Saprolegnia parasitica Coker ATCC 22284	our laboratory
Saprolegnia salmonis	Genbank AY647193
Saprolegnia semihypogyna	GenBank AY647194

gyna groups are resolved with bootstrap support of 100. The *Saprolegnia* group is resolved from *Leptolegnia* with bootstrap support of 91. The consensus parsimony tree has lower bootstrap support of 73 for the resolution of the *Saprolegnia* and *Leptolegnia* groups from the tree and lower bootstrap support (86) for the resolution of the *Achlya* and *S. semihypogyna* groupings. The *Saprolegnia* group is resolved from *Leptolegnia* with bootstrap support of 71 instead of the 91 seen in the neighbor joining tree.

Our neighbor joining distance tree (FIG. 3) resolved field and reference samples into two major groups outside the *Phytophthora* outgroup. The first major group (bootstrap value 75) comprised two subgroups that further resolved into a total of six groupings. Thirteen isolates from amphibian eggs grouped strongly with the *Leptolegnia* reference sequence (bootstrap 100), and two egg samples separated from that group with strong support (bootstrap 100). A group including all *Saprolegnia* reference sequences except *S. semihypogyna* had strong support (bootstrap 91). The separation between the group including *S. parasitica* and *S. diclina* and the group including the other seven reference *Saprolegnia* had weaker support (bootstrap <70). Isolates from two Idaho-Montana amphibian egg samples grouped with *S. parasitica* ATCC 22284 (bootstrap 93) which was near but distinct from 14 amphibian samples that grouped strongly with *S. diclina* ATCC 90215 (bootstrap 100). Thirteen of the 14 isolates in the *S. diclina* grouping were from the Idaho-Montana cluster. A sample from a wild trout scraping (IS-13-1) also might be associated with the group including *S. parasitica* and *S. diclina*. The group containing *S. salmonis*, *S. hypogyna* and *S. ferax* ATCC 26116 included one field sample from a Chinook salmon scraping (IS-11-1). A grouping of *S. bulbosa*, *S. oliviae*, *S. longicaulis* and *S. anomalies* included 13 isolates from amphibian eggs and one isolate from eggs of hatchery rainbow trout (IS-12-1). With the exception of the hatchery trout, all samples in this group were collected in Oregon and Washington.

The second major group also was resolved into two subgroups. The *Achlya* group included all eight *Achlya* reference sequences and was resolved further into five groupings. Our finding of low bootstrap support (60) for the *Achlya* group concurs with other studies that imply the genus is likely to be polyphyletic (Leclerc et al 2000). Outside the three groups of *Achlya* reference samples were two groups of amphibian egg samples that were not directly affiliated with reference species. The first group (including IM-1-1, OW-20-1, OE-22-2, OW-21-1 and OE-22-3) was most closely related to the grouping of *A. colorata* and *A. racemosa*. The second group of egg samples (OW-23-3, WA-15-5 and WA-15-4) also was distinct from all our other *Achlya* sequences (bootstrap 100). Separate from the main *Achlya* group was a second subgroup including one amphibian egg sample with *S. semihypogyna* (bootstrap 99) and a distinct cluster of 12 amphibian egg isolates (bootstrap 100). All these

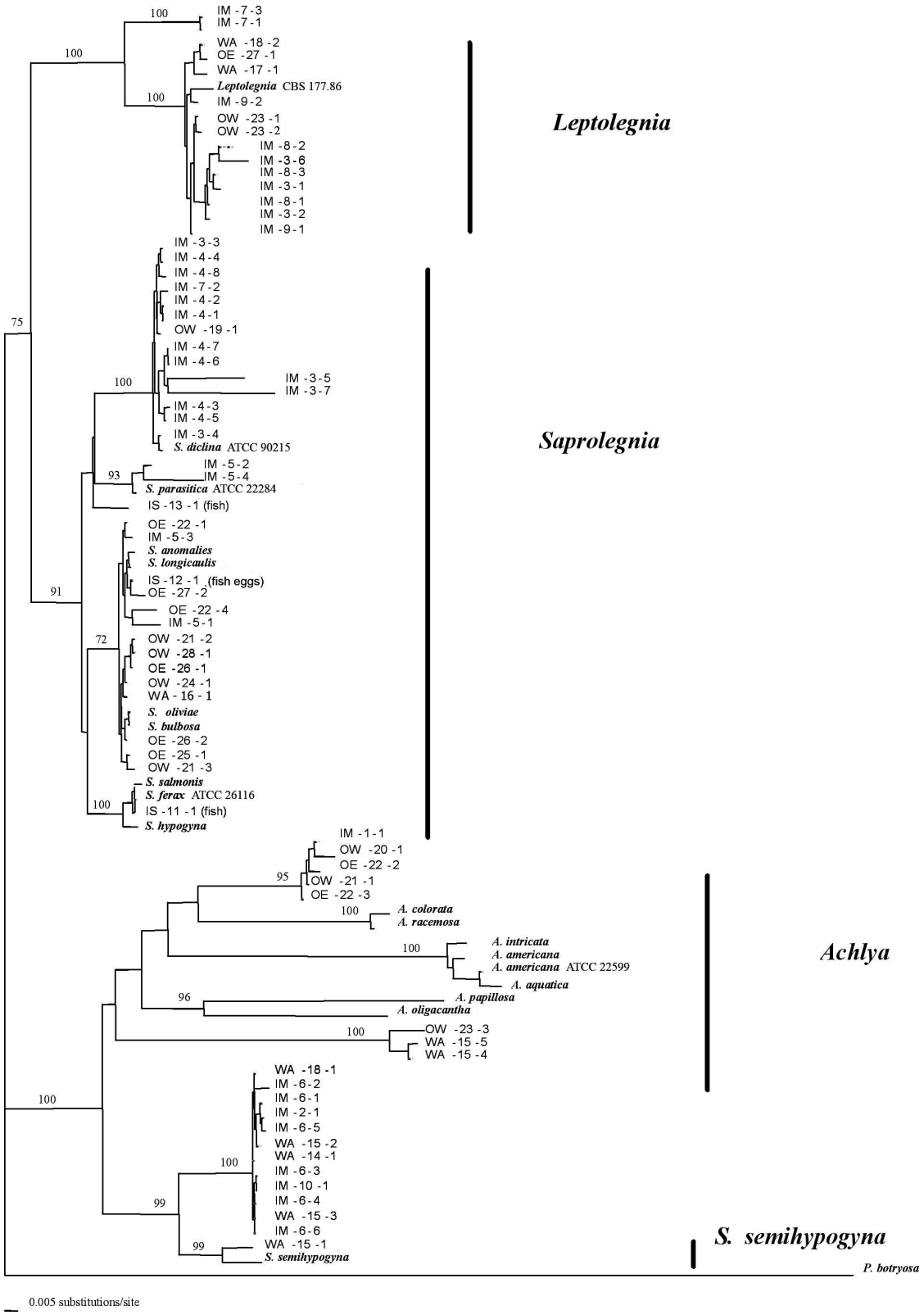

FIG. 3. Jukes-Cantor neighbor joining distance tree for Saprolegniaceae isolates from amphibian eggs (n = 65), fish scrapings or eggs (n = 3) and reference samples (n = 20). Bootstrap values <70 are not shown.

samples came from our northern sites in Washington, Montana and Idaho. The generic and specific identities of these 12 isolates are unclear.

DISCUSSION

To our knowledge this is the first broad survey of oomycetes on amphibian eggs outside work in Poland. This also appears to be one of the first studies of filamentous microorganisms on amphibian eggs that uses molecular phylogenetic techniques to circumvent some of the difficulties of oomycete identification based on unstable reproductive structures. The BLAST analysis of the NCBI database was able to identify most of our isolates to the generic level. The GenBank and ATCC reference species served as the foundation for our phylogeny. Our neighbor joining distance analysis of ITS regions successfully resolved the known *Saprolegnia*, *Achlya* and *Leptolegnia* sequences into distinct genera and species groups and helped us to see the phylogenetic relationships among these three groups.

Our sequence identification and phylogenetic analysis confirm that a diversity of Saprolegniaceae occur on amphibian eggs in the Pacific Northwest. We estimate that we had representatives of at least 7–12 species and three genera of Saprolegniaceae on our amphibian eggs. Czeczuga et al (1998) also reported a diversity of taxa based on morphological identification, including 33 species of Saprolegniaceae (14 *Achlya*, 10 *Leptolegnia*, nine *Saprolegnia*) and 18 other zoosporic fungi from Polish amphibians raised in five different water sources.

Our phylogenetic analysis is in general agreement with recent molecular work on the Saprolegniaceae (e.g. Dick et al 1999, Riethmuller et al 1999, Leclerc et al 2000). Most (78%) of our oomycete samples (68) grouped within *Saprolegnia* and *Achlya* but we also identified a subset of our amphibian egg cultures as probable members of *Leptolegnia* (n = 13) or a related but separable group (bootstrap support of 100; n = 2). Few *Leptolegnia* species are documented in GenBank, so it is possible that our samples could be further resolved with more reference species. The separation of the *Leptolegnia* group from our primary *Saprolegnia* group is strongly supported (bootstrap 100) and generally concurs with phylogenies based on 18S rDNA (Dick et al 1999) and LSU rDNA data (Leclerc et al 2000). Our *Leptolegnia* samples came from four amphibians (*A. macrodactylum*, *R. cascadae*, *R. luteiventris*, *R. pretiosa*) and from all four states. Czeczuga et al (1998) reported *L. caudata* de Bary from eggs of five amphibians when they were reared in water from five different wetlands. *Leptolegnia caudata* and other members of the genus are pathogenic on mosquito larvae (Bisht et al 1996, Scholte et al 2004). Although not sampled specifically, mosquitoes were abundant at many of our sites.

Our neighbor joining distance analysis also suggests a distinct division between most of our *Saprolegnia* samples and members of the genus *Achlya*. This generally agrees with 28S rDNA data (Riethmuller et al 1999) and the cladistic analysis of LSU rDNA data reported by Leclerc et al (2000). In contrast a neighbor joining distance tree (Leclerc et al 2000) placed some *Achlya* (including *A. racemosa*, *A. colorata*, *A. oligacantha* and *A. papillosa*) closer to *Saprolegnia* than with the main *Achlya* clade (including *A. aquatica*, *A. americana* and *A. intricata*). Both our phylogeny and analysis by Leclerc et al (2000) of the LSU identify the same three *Achlya* subgroups: (i) *A. americana*/*A. aquatica*/*A. intricata*, (ii) *A. oligacantha*/*A. papillosa* and (iii) *A. racemosa*/*A. colorata*. These *Achlya* subgroups are consistent with divisions based on oospore morphology outlined by Dick (1969). None of our eight isolates from field samples directly matched sequences of our reference *Achlya* species, so we are unsure of their species-level taxonomy. Given the paucity of field sampling for Saprolegniaceae in western North America, it is possible this lineage represents a new species. Czeczuga et al (1998) reported 15 *Achlya* species (including *A. colorata*) from amphibian eggs in Poland, but little else is published on *Achlya* on amphibians. A variety of *Achlya* (including *A. americana*, *A. colorata*, and *A. racemosa*) have been reported on fish eggs (Czeczuga and Muszynska 1997, 1999).

Most phylogenetic work suggests that the genus *Achlya* is not a monophyletic unit in its current configuration (e.g. Green and Dick 1972, Dick et al 1999, Riethmuller et al 1999, Leclerc et al 2000). Similarly Leclerc et al (2000) indicated that *Saprolegnia* might not be monophyletic, and difficulties remain in distinguishing generic affiliations of some *Achlya* and *Saprolegnia* species. Our phylogeny grouped reference sequences of *Saprolegnia* (bootstrap 91) and *Achlya* together in relatively cohesive respective groups. However our *S. semihypogyna* and an associated group of field samples resolved more closely with *Achlya* than to the main group of *Saprolegnia*. Type specimens of *S. semihypogyna* were described from Japan (Inaba and Tokumasu 2002), and ITS and 28 LSU sequences are in GenBank. We did not inspect the morphology of any of our samples, but if our phylogeny is correct the generic attribution of *S. semihypogyna* may merit reconsideration. The identity of the tightly clustered group adjoining *S. semihypogyna* (bootstrap 99) and its relationship to other *Achlya* and *Saprolegnia* is unclear.

The distribution of samples among *Saprolegnia* species in our main group was somewhat unexpected. Fourteen isolates grouped strongly with *S. diclina* ATCC 90215. The strong support for our *S. diclina* group (bootstrap 100) and our *S. parasitica* group (bootstrap 93; n = 2 egg isolates) concurs with the conclusion by Molina et al (1995) that *S. diclina* and *S. parasitica* are closely related but separate species. Our *S. diclina* group was the most geographically homogeneous of our groups with larger sample sizes: 13 of 14 samples came from the Bitterroot Mountain region of Idaho-Montana. Also consistent with Molina et al (1995) was our finding of a close relationship between *S. ferax* and *S. hypogyna*.

Although others (Blaustein et al 1994, Kiesecker and Blaustein 1995) report *S. ferax* associated with amphibian egg mortality in the Pacific Northwest, none of our isolates from amphibian eggs were grouped closely with this species. Many of our isolates from amphibians were grouped closely with *S. diclina* and other *Saprolegnia* spp. (*S. bulbosa*, *S. oliviae*, *S. anomalies* and *S. longicaulis*). The single sample that aligned near our reference *S. ferax* came from a Chinook fish scraping (IS-11-1). Our two other fish samples were grouped with *S. parasitica* (sample IS-13-1) and *S. anomalies/longicaulis* (IS-12-1). The *S. diclina/S. parasitica* complex is commonly associated with saprolegniosis in fish around the world (e.g. Beakes and Ford 1983). Of note *S. diclina* colonized dead or unfertilized perch eggs in lab trials but did not invade adjacent live perch eggs (Paxton and Willoughby 2000).

Our lack of *S. ferax* seems noteworthy because it is among the most widespread and abundant taxa of its genus (Seymour 1970, Dick 1971, Czeczuga et al 1998, Johnson et al 2002). A recent study that combined genetic and morphological data (Hulvey et al 2007) showed that species designation of *Saprolegnia* based solely upon morphological characteristics is not sufficient. Because we used sequence rather than morphology for identification, we should not have encountered this problem to the same degree that earlier observational studies may have experienced. This might explain why we found a large diversity of *Saprolegnia* species on our amphibian eggs. We suggest that future identification of field-collected *Saprolegnia* with sequence and morphological data will contribute to a new understanding of distribution and ecological function of this diverse group in aquatic systems.

The effects of colonization by Saprolegniaceae on amphibian eggs and populations remain unclear. Most of the amphibian populations we sampled do not appear to be in decline (CAP and DSP unpublished data). Similar to Czeczuga et al (1998), we cultured Saprolegniaceae from amphibian eggs that were developing normally. Many pond-breeding amphibians deposit eggs among herbaceous vegetation in shallow margins, and these littoral zones can support the highest density and diversity of oomycete propagules in ponds or lakes (O'Sullivan 1965; Dick 1971, 1976). Several amphibians with this oviposition habit have adaptations to reduce egg losses to aquatic microorganisms, such as thickened egg capsules, separating egg masses to reduce direct hyphal invasion or accelerating development in presence of oomycete hyphae (Kiesecker and Blaustein 1997, Green 1999, Gomez-Mestre et al 2006, Touchon et al 2006). Similar to saprolegniosis in fish, it is unclear how frequently different members of the Saprolegniaceae act as pathogens on healthy amphibian eggs, colonize dead eggs (Robinson et al 2003) or take advantage of another stressor or infection (Kiesecker and Blaustein 1995, Lefcort et al 1997).

To better understand the threat posed by Saprolegniaceae to pond-breeding amphibians afield, we recommend additional work to clarify the taxonomy of the colonizing microorganisms, controlled tests of pathogenicity on individual amphibian species and experimental study of mechanisms by which stressors increase susceptibility of embryos.

ACKNOWLEDGMENTS

This work was supported by the Idaho National Engineering and Environmental Laboratory (now INL) Education Outreach BBW Bechtel grant and a grant from the US Geological Survey Amphibian Research and Monitoring Initiative (ARMI). We thank V. Winston for his contributions throughout this project and G. Carroll for his thoughtful review of an earlier version of this manuscript. Samples were gathered under scientific collecting permits from the National Park Service, Washington Department of Fish and Wildlife, Oregon Department of Fish and Wildlife, Idaho Department of Fish and Game and Montana Department of Fish and Wildlife. We thank R. Hoffman (USGS FRESC), B. Samora (Mount Rainier National Park), P. Murphy (Idaho Department of Fish and Game) and B. Maxell (Montana National Heritage Program) for help identifying sampling sites. We thank S. Galvan for assembling the map and B. Cummings, J. Jones, J. Kittrell, B. McCreary, J. Oertley and C. Rombough for assistance in the field.

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