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DNA BARCODING AS A MEANS TO IDENTIFY ORGANISMS ASSOCIATED WITH AMPHIBIAN EGGS

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***Abstract.*—Water molds, primarily in the genus *Saprolegnia,* have been implicated in large-scale mortality of amphibian eggs under a variety of environmental conditions. Although a number of water mold species infect amphibian eggs, the pathogens identified from die-offs or utilized in ecological studies often remain unidentified or identified as only one of two species (*S. ferax* and *S. parasitica*). Lack of adequate identification makes it difficult to assess factors of the host-parasite interaction that contribute to saprolegniasis in amphibians. We obtained isolates from three species of amphibian eggs and used traditional morphological characteristics and a DNA barcoding procedure to evaluate both types of identification. Traditional morphological identifications performed poorly and resulted in a number of apparent misidentifications and the failure to identify morphologically cryptic species associated with amphibian eggs. In addition, a large number of apparent species synonyms are in use. While DNA barcoding did not allow firm assignment of species names, the approach adequately assigned isolates to well-supported groupings. This phylogenetic approach should allow the eventual assignment of appropriate binomial names as we accumulate a larger more reliable database. These data also suggest that these pathogens require precise identification because we isolated multiple species of water molds from all three amphibian species. Further, we isolated some pathogens from only one of the three amphibian species. We recommend that future studies on amphibian saprolegniasis include DNA barcoding information for the pathogens to facilitate studies of host specificity and pathogenicity.**

Key Words.—Achlya, DNA barcoding, internal transcribed spacer, Pacific Northwest, rDNA, *Saprolegnia*, saprolegniasis

INTRODUCTION

Members of the Saprolegniaceae (Stramenopila, Oomycota) are fungus-like protists (commonly referred to as water molds) that have been linked to infections and mortality in amphibians (Banks and Beebee 1988; Blaustein et al. 1994; Green 1999; Berger et al. 2001) and fish (Scott 1964). *Saprolegnia* infections are common in amphibian eggs throughout the world (Beebee 1996). They are linked to large-scale mortality of amphibian eggs in the Pacific Northwest (Blaustein et al. 1994). *Saprolegnia* infections may interact with other factors to cause amphibian declines (Kiesecker et al. 2001a; Blaustein and Kiesecker 2002; Blaustein et al. 2003). Possible contributing factors to *Saprolegnia* related mortality include UV-B radiation (Kiesecker and Blaustein 1995), temperature (Banks and Beebee 1988; Sagvik et al. in press), pollutants (Lefcort et al. 1997; Romansic et al. 2006), species and genotype of amphibians (Sagvik et al. in press), egg-laying behavior (Kiesecker and Blaustein 1997; Green 1999), and hatching plasticity (Touchon et al. 2006). However, the pathogen most often responsible for mortality is identified only as *Saprolegnia* sp. (e.g., Banks and Beebee 1988; Robinson et al. 2003; Romansic et al. 2006), *S. ferax* (e.g., Kiesecker and Blaustein 1997; Kiesecker and Blaustein 1999; Kiesecker et al. 2001b) or *S. parasitica* (e.g., Lefcort et al. 1997). However, a number of morphologically similar species of water molds occur on amphibian eggs (Czeczuga et al. 1998). The lack of precise identification in previous work makes it impossible to assess the relative pathogenicity or host specificity of various species of water molds under differing environmental conditions.

The accurate identification of species is a cornerstone of biological research, but accurate identification of microorganisms is difficult due to the required taxonomic expertise. The limited number of morphological characteristics and the large number of undescribed species make identifications challenging. As much as 90% of all the fungi and fungus-like protists, including water molds, remain undescribed (Hawksworth 1991). Morphological identification of members of the Saprolegniaceae depends on the sexual reproductive anatomy (Coker 1923; Seymour 1970; Johnson, Jr., et al. 2002); however, strains from amphibians are often sexually sterile (see Table 1). Further, traditional species diagnosis of these organisms is based on relatively few and often phenotypically plastic characters (Willoughby 1978) or characters that overlap between described species (Diéguez-Uribeondo et al. 2007; Hulvey et al. 2007). This led to extensive lists of synonyms for most species and persistent difficulties in diagnosis (Johnson, Jr., et al. 2002; Seymour 1970). Recent molecular studies suggest that the use of traditional morphological characteristics may

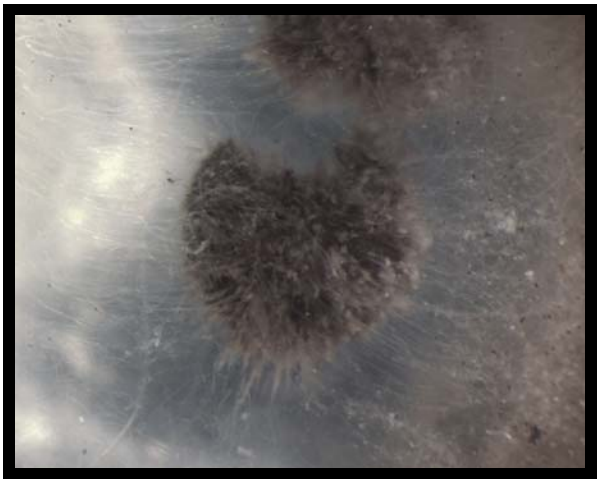

FIGURE 1. Infected *Bufo boreas* egg showing hyphae of water molds, 64 × magnification.

not adequately identify phylogenetic species, further complicating identification (Diéguez-Uribeondo et al. 2007; Hulvey et al. 2007).

DNA barcoding is a means of rapidly identifying species using short, standardized sequences of DNA coupled with phylogenetic analyses (Hebert et al. 2003a). This approach has several advantages for the identification of microorganisms because it does not require specialized taxonomic expertise. Isolates lacking sexual structures or DNA isolated from mixed samples soils can be identified by this approach. It also detects previously undescribed and morphologically cryptic species. Most tests of DNA barcoding use sequences of the mitochondrial cytochrome c oxidase subunit I (CO1) gene to identify species of animals (Hebert et al. 2003b; Hebert et al. 2004b; Witt et al. 2006; Wiemers and Fiedler 2007). Few studies of fungi and fungus-like organisms employ CO1 (Seifert et al. 2007). Therefore, primers for CO1 are not available for most groups and the number of identified sequences for comparison is small (Seifert et al. 2007). However, many use sequences of the Internal Transcribed Spacer region (ITS) of the nuclear ribosomal DNA (rDNA) for phylogenetic studies and many exist in public databases (Kõljalg et al. 2005). ITS has many short insertions/deletions that can complicate alignment and analysis (Seifert et al. 2007); however, it is the most widely available and utilized sequence for studies of fungal systematics, ecology, and DNA barcoding (Horton and Bruns 2001; Diéguez-Uribeondo et al. 2007; Summerbell et al. 2007).

In this study, we made isolates of members of the Saprolegniaceae from a number of infected amphibian eggs to compare the effectiveness of morphological and DNA barcode identifications. In addition, the study investigated the feasibility of a DNA barcode based system for the identification of water molds associated with amphibians.

MATERIALS AND METHODS

We collected diseased eggs from one Western Toad (*Bufo boreas*) clutch on 27 April 2007, three Pacific Treefrog (*Pseudacris regilla*) clutches on 4 May 2007, and one Northwestern Salamander (*Ambystoma gracile*) clutch on 4 May 2007 from Swamp Lake (T21N, R12E, Section 14, SE ¼), Kittitas County, Washington, USA and stored the samples at 4°C prior to making the isolates. We placed diseased eggs in sterile Petri dishes and examined them at 100 × magnification using a Leica MZ12 dissecting microscope (Fig. 1). We excised individual hyphal tips with a sharpened stainless steel microprobe and transferred them to Difco Corn Meal Agar (CMA) containing 200 mg/L penicillin G and 100 mg/L streptomycin sulfate. Repeated subculturing of individual hyphal tips yielded axenic cultures (Table 1). We obtained several additional isolates from organic debris, seeds, and other materials found associated with the amphibian embryos (unpubl. data). We deposited vouchers of all isolates in the culture collection at the Central Washington University Herbarium (ELRG). To examine isolate morphology, we transferred a small (~5) mm block of CMA from the margin of an actively growing colony to a sterile Petri dish along with enough sterile filtered pond water (Fuller 1978) to completely cover the agar block. We incubated replicates of each isolate at 7°C and 20°C (Willoughby 1978), and after approximately one week of growth added one sterile radish seed (*Raphanus sativus* L.) near the margin of the colony (Steciow 2003). We periodically examined isolates with a dissecting microscope over 5-10 weeks for the development of sexual and asexual structures. We used a Leica DMLB research microscope with differential interference contrast optics (DIC) to examine cultures. We identified sexually reproducing isolates using available keys and species descriptions (Johnson, Jr., 1956; Seymour 1970; Johnson, Jr., et al. 2002).

We obtained cultures for DNA extraction by transferring a single hyphal tip from the margin of an actively growing colony on CMA to a sterile 1.5 mL centrifuge tube containing 1.0 mL of sterile Cantino's Peptone, Yeast-extract, Glucose broth (Fuller 1978). After 12-24 hours at 25°C, we centrifuged the tubes at 5,000 r.p.m. for five minutes and discarded the supernatant. We extracted DNA from the pellet using a Chelex-100 based method (Walsh et al. 1991). We added 200 µL of 10% w/v chelex-100 in sterile distilled water to the pellet, briefly vortex mixed the contents, and incubated the samples at 90°C for 20 minutes with frequent mixing. We briefly chilled the samples on ice and then centrifuged the sample tubes at 5,000 r.p.m. for

TABLE 1. Isolates used in this study. Amphibian Species is the species of amphibian egg from which we obtained the isolate. Sexual reproduction refers to the production of sexual structures in culture. Isolates codes indicate the identification code for the isolates in the Central Washington University Culture Collection. Isolates with the same code under Merged were combined into a single sequence. Group designations I-VI follow Diéguez-Uribeondo et al. 2007, additional clade designations are from this study.

Genbank Accession No.	Species Identification	Amphibian Species	Sexual Reproduction	Isolates	Merged	Group
EU124762	*Achlya stellata* de Bary = *Newbya stellata* (de Bary) M. A. Spencer & M. W. Dick	*Pseudacris regilla*	Fertile	H2E2		XI
Not Sequenced	*Saprolegnia anisospora*	*Bufo boreas*	Sterile	BBE02, BBE04, and BBE07		VIII
Not Sequenced	*S. anisospora*	*Bufo boreas*	Fertile	BBE03, BBE06, BBE09, BBE11, BBE12, BBE13, BBE14, and BBE19		VIII
EU124746	*S. anisospora*	*Bufo boreas*	Fertile	BBE05	8	VIII
EU124747	*S. anisospora*	*Bufo boreas*	Fertile	BBE10	8	VIII
EU124748	*S. anisospora*	*Bufo boreas*	Sterile	BBE16	8	VIII
EU124765	*S. diclina*	*Bufo boreas*	Sterile	BBE01	3b	III
EU124764	*S. diclina*	*Bufo boreas*	Fertile	BBE18	3b	III
EU124763	*S. ferax*	*Ambystoma gracille*	Fertile	AE3		II
EU124766	*S.* cf *kauffmaniana*	*Pseudacris regilla*	Sterile	H1E2		VII
Not sequenced	*S.* cf *kauffmaniana*	*Pseudacris regilla*	Sterile	H1E3		VII
EU124759	*S.* sp. 1	*Bufo boreas*	Sterile	BBE08	9	IX
EU124749	*S.* sp. 1	*Bufo boreas*	Fertile	BBE15	9	IX
EU124753	*S.* sp. 1	*Bufo boreas*	Sterile	BBE20	9	IX
EU124754	*S.* sp. 1	*Pseudacris regilla*	Sterile	H1E1	9	IX
EU124758	*S.* sp. 1	*Pseudacris regilla*	Sterile	H1E4		IX
EU124750	*S.* sp. 1	*Pseudacris regilla*	Sterile	H2E1	9	IX
EU124751	*S.* sp. 1	*Pseudacris regilla*	Sterile	H2E3	9	IX
EU124756	*S.* sp. 1	*Pseudacris regilla*	Sterile	H3E1	9	IX
EU124755	*S.* sp. 1	*Pseudacris regilla*	Sterile	H3E2	9	IX
EU124760	*S.* sp. 1	*Pseudacris regilla*	Sterile	H3E3	9	IX
EU124757	*S.* sp. 1	*Pseudacris regilla*	Sterile	H3E5	9	IX
EU124752	*S.* sp. 1	*Ambystoma gracille*	Sterile	AE1	9	IX
EU124761	*S.* sp. 2	*Bufo boreas*	Fertile	BBE17		VII

five minutes. All subsequent PCR amplifications used the supernatant from this procedure.

We PCR-amplified the ITS region using the primers ITS1 and ITS4 (White et al. 1990). PCR mixtures contained 2.5 mM magnesium chloride, 0.4 µM each primer, 0.2 mM each dNTPs, 2.5 units Taq DNA polymerase, and 10 µL of sample DNA with standard reaction conditions (White et al. 1990). To quickly screen isolates and sort them into operational taxonomic units (OTUs), we digested PCR products using the restriction enzymes RsaI and AluI by adding five units of each enzyme directly to 20 µL of PCR product in the PCR buffer, and then incubating the samples at 37°C for 4 hours. We separated restriction fragments by electrophoresis in gels containing 2% agarose and run in 1× TBE for 90 minutes at 100 volts. We stained the DNA with 1.0 µg/mL ethidium bromide, and visualized gels using a Molecular Imager FX (Bio-Rad Laboratories, Hercules, California, USA). We estimated lengths of restriction fragments by comparing them to known size standards using the software package ImageJ (National Institutes of Health. 1997-2007. ImageJ. Available from, http://rsb.info.nih.gov/ij/ [Accessed 17 February 2008]) and the molecular weight macro package (PHASE GmbH. 1997-2007. Available from http://www.phase-hl.com/ImageJ/index.htm [Accessed 17 February 2008). We sorted isolates into OTUs based on possession of a unique restriction fragment pattern and utilized at least two individuals of each distinct OTU (if available) for DNA sequencing. The High-Throughput Genomics Unit (Seattle, Washington, USA) purified and sequenced undigested amplification products in both directions using the primers ITS1 and ITS4. We assembled sequence contigs using the computer package STADEN (Staden et al. 2000),

Table 2. GenBank accession numbers for sequences included in this study. Isolate identification codes are provided if available. Instances of taxonomic changes between the names applied in GenBank and those applied in the taxonomic literature are indicated. Isolates with the same code under Merged were combined into a single sequence. Group designations I-VI follow Diéguez-Uribeondo et al. 2007, additional group designations are from this study. Samples indicated with an * were isolated from fish or amphibian eggs (or larvae).

GenBank	Species	Strain ID	Merged	Group
AF218159	*Achlya colorata* Pringsh.			
AF218162	*A. oligacantha* de Bary = *Newbya oligacantha* (de Bary) M. A. Spencer (Spencer et al. 2002)			XI
AF218161	*A. papillosa* Humphrey			XI
AF218158	*A. racemosa* Hildebr.		13	
AF218160	*A. radiosa* Maurizio			
AB219373	*A. treleaseana* (Humphrey) Kauffman = *A. androgyna* (Archer) Johnson and Seymour (Johnson Jr. et al. 2005)			XI
AY310501	*Aphanomyces astaci* Schikora			OG
AY647192	*Aph. frigidophilus* Kitanch. & Hatai			OG
AY310498	*Aph. stellatus* de Bary	CBS 578.67	13	
AB219374	*Aplanes androgynus* (Archer) Humphrey = *Achlya androgyna* (Archer) Johnson and Seymour (Johnson Jr. et al. 2005)			XI
AB219375	*Aplanopsis terrestris* Höhnk			
AM228851	*Leptolegnia* sp.	SAP248	11	VIb
AY310502	*Leptolegnia* sp.	CBS 177.86		VII
DQ393553	*Protoachlya paradoxa* Coker	CBS 158.45		
AB219381	*Pythiopsis cymosa* de Bary			
AB219382	*Pythiopsis humphreyana* Coker			VIII
AB219384	*Saprolegnia anisospora* de Bary			VIII
DQ322632	*S. anomalies* Gandhe & Kurne		2a	II
EF064134	*S. anomalies*	WD1D	2a	II
AB219385	*S. australis* Elliot			IV
AM228817	*S. australis*	SAP202	5	V
AM228818*	*S. australis*	SAP203		III
AM228819*	*S. australis*	SAP204	5	V
AM228837	*S. australis*	SAP222		IV
AY647195	*S. australis*			III
EF126324	*S. bulbosa*	WD20A	2b	II
AB219386	*S. diclina* Humphrey		3a	III
AM228738	*S. diclina*	SAP90		III
AM228811	*S. diclina*	SAP175	5	V
AM228844	*S. diclina*	SAP229	3a	III
AM228848*	S. diclina	SAP243	3a	III
AM228849*	*S. diclina*	SAP244		III
AM228850	*S. diclina*	SAP132	2a	II
AY455775	*S. diclina*		3a	III
AB219376	*S. eccentrica* (Coker) R. L. Seymour			VIII
AB219387	*S. ferax* (Gruith.) Thuret		2b	II
AB219379	*S. torulosa* de Bary			VIb
AB219397	*S. turfosa* (Minden) Gäumann			
AB219380	*S. unispora* (Coker & Couch) Seymour		12	
AB219398	*Scoliolegnia asterophora* (de Bary) M. W. Dick			X

Continued on next page

manually corrected base calls (when possible), and removed primer sequences.

We identified similar sequences from GenBank using BLASTN (Altschul et al. 1990; see Table 2 for a list of sequences used in subsequent analyses). We selected representative sequences for taxa that have large numbers of available sequences and the other previously identified groups (Diéguez-Uribeondo et al. 2007). We selected two species of *Aphanomyces*, *A. astaci*, and *A. frigidophilus* as outgroups (Leclerc et al. 2000). We initially aligned sequences using the program CLUSTALW (Thompson et al. 1994) and subsequently manually adjusted them to minimize the number informative base changes. To simplify data analyses, we merged identical sequences. In addition, if two sequences had zero base differences after adjustment for gaps and ambiguities, we merged them into a single sequence using the program Mesquite (Maddison, W. P. and Maddison, D. R. 2007. Mesquite: a modular system for evolutionary analysis. Version 2.01. Available from http://mesquiteproject.org/ [Accessed 17 February 2008]; see Tables 1 and 2).

We conducted phylogenetic analyses using a variety of strategies. We conducted parsimony searches using

TABLE 2 *Continued from previous page*. GenBank accession numbers for sequences included in this study. Isolate identification codes are provided if available. Instances of taxonomic changes between the names applied in GenBank and those applied in the taxonomic literature are indicated. Isolates with the same code under Merged were combined into a single sequence. Group designations I-VI follow Diéguez-Uribeondo et al. 2007, additional group designations are from this study. Samples indicated with an * were isolated from fish or amphibian eggs (or larvae).

GenBank	Species	Strain ID	Merged	Group
AM228790	*S. ferax*	SAP159	2a	II
AM228845	*S. ferax*	SAP230	2a	II
AM228782	*S.* cf. *ferax*	SAP151		III
AB219388	*S. furcata* Maurizio			VIc
AB219389	*S. hypogyna* (Pringsh.) de Bary		1a	Ia
AM228724	*S. hypogyna*	SAP24	1b	I
AM228815*	*S. hypogyna*	SAP196	1a	Ia
AY647188	*S. hypogyna*			Ia
AB219391	*S. litoralis* Coker			VIc
AM228824	*S. litoralis*	SAP209		VIa
AM228847	*S. litoralis*	SAP232		VIc
AY310503	*S. litoralis*			II
AY270032	*S. longicaulis* Steciow		2a	II
AB219390	*S. mixta* de Bary = *S. ferax* (Seymour 1970)		2a	II
EF126339	*S. mixta*	WD8F	2a	II
AB219377	*S. monilifera* de Bary = *S. torulosa* de Bary (Johnson Jr. et al. 2002)			VIb
AB219392	*S. monoica* Pringsheim = *S. ferax* (Seymour 1970)			VIa
AY270031	*S. oliviae* Steciow		2b	II
AB219393	*S. parasitica* Coker		1b	I
AY310504	*S. parasitica*	CBS 540.67		XI
AY455771	*S. parasitica*	ATCC 90213	1b	I
AY455776	*S. parasitica*	NJM9880	1b	I
AY455777	*S. parasitica*	NJM0129		I
AB219394	*S. polymorpha* Willoughby			IV
AB219399	*S. salmonis* Hussein & Hatai		1b	I
AY647193	*S. salmonis*		1b	I
AB219395	*S. semihypogyna* S. Inaba & Tokum.			VIc
AY647194	*S. semihypogyna*			VIc
DQ393513	*S.* sp. UNCW217	UNCW217		VII
DQ393514	*S.* sp. UNCW218	UNCW218		VII
DQ393515	*S.* sp. UNCW219	UNCW219		VII
DQ393517	*S.* sp. UNCW250	UNCW250		VII
DQ393539	*S.* sp. UNCW290	UNCW290		VII
AB219378	*S. subterranea* (Dissmann) Seymour = *S. itoana* (Nagai) Seymour (Johnson Jr. et al. 2005)		12	
AB219396	*S. terrestris* Cookson		11	VIb

PAUP* (Sinauer Associates, Inc., Sunderland, Massachusetts, USA) with the gaps treated as missing data, 10 random addition sequences, TBR branch swapping, and retention of all the best trees. We estimated the robustness of tree branches using bootstrap re-sampling as implemented in PAUP* with 10,000 replicates and the following conditions: one random addition sequence in each replicate with MAXTREE set to 10,000 and NNI branch swapping. We estimated the best-fit likelihood model for the dataset using MrModeltest version 2.2 (Nylander, J.A.A. 2006. Available from http://www.abc.se/~nylander/ [Accessed 17 February 2008]) and conducted maximum likelihood heuristic searches using the best-fit likelihood model, the trees obtained from parsimony analysis as starting trees, and TBR branch swapping.

We estimated Bayesian posterior probabilities for branch support using MRBAYES (Ronquist and Huelsenbeck 2003). We used the following settings: number of generations = 1,000,000, sampling every 100th generation, burnin = first 100,000 generation, number of chain = 4, and heating = default. We examined sequence variation for intraspecific and interspecific comparisons using Kimura 2-Parameter (K2P) distances determined with PAUP* and constructed neighbor-joining trees using the distance matrix.

RESULTS

Sequences obtained in this study varied from 674 to 698 bp excluding the primer sequences. This variation in sequence size resembles that of other publicly available sequences of this region for these organisms. The sequences were shorter than the CO1 gene sequences (1,584-22,006 bp) found in the true fungal genus *Penicillium* (Seifert et al. 2007). Alignments indicated that there were numerous short insertion/deletion events that complicated alignment

across the entire dataset. However, these short insertion/deletion events frequently occurred in the most variable and diagnostically useful regions of the sequence, so we used the entire alignment for this analysis. The dataset included 303 parsimony informative characters and 50 variable characters that were not parsimony informative. Kimura two-parameter percent differences ranged from 0.0 to 32.2% with a mean of 16.4%, which closely resemble the values obtained for use of the CO1 gene in *Penicillium* (Seifert et al. 2007).

Phylogenetic analysis resulted in highly similar trees or sets of trees regardless of inference method employed and the treatment of insertion/deletions. We recovered the same well-supported terminal clades regardless of methodology. Parsimony analysis resulted in 1,770 trees with 1,248 steps. Equivalent arrangements of short terminal branches led to the large number of trees. Maximum likelihood analyses resulted in a single tree (Fig. 2). The tree was equivalent to one of the trees found in both the parsimony (C.I. = 0.45, H.I. = 0.55, R.I. = 0.81) and Bayesian analyses. The tree was also consistent with a simple neighbor-joining analysis using 232 sequences from publicly available databases and those generated in our laboratory (unpubl. data).

Using named species (based on morphology), the average intraspecific distance was 2.5% (K2P). However, this value is problematic because a number of named species, such as *S. diclina* and *S. ferax*, occurred in several distinct clades. To minimize this artificial inflation of intraspecific differences, we used terminal clades that lacked internal resolution with strong bootstrap support as proxies for species. This method reduced the average "intraspecific" variation to 0.2% within a clade. Interspecific variation was larger, but also somewhat problematic because several terminal clades contain multiple named species, such as the clade that contains isolates identified as *S. ferax*, *S. diclina*, *S. anomalies*, *S. longicaulis*, *S. mixta*., *S. bulbosa* and *S. oliviae*. However, only 1% of the total number of interspecific comparisons showed less than 2% K2P genetic distance, and a number of these cases may represent either misidentifications or synonyms.

These analyses recovered a number of distinct clades containing isolates from amphibian eggs that will be discussed below in the context of the clades of *Saprolegnia* previously described (Diéguez-Uribeondo et al. 2007). These terminal clades seldom correspond to currently recognized morphospecies.

Clades I, IV, V, and group VI were previously described (Diéguez-Uribeondo et al. 2007). These analyses found strong support for clades I, IV, and V. Clade I includes mainly isolates that occur as parasites on living adult fish including isolates typically assigned to the species *S. parasitica* and isolates in this clade have a number of unique adaptations for this type of parasitism (Diéguez-Uribeondo et al. 2007). Group VI was paraphyletic in these analyses and includes taxa occurring split among several other groups. No isolates from amphibian eggs corresponded to any of these groups.

Clade II corresponded to *S. ferax* and some members of *S. diclina* (Type 2) along with isolates assigned to a number of other named species (Diéguez-Uribeondo et al. 2007). A single isolate (AE3) from *A. gracilie* was a member of this clade. The isolate was morphologically consistent with *S. ferax*. Additional isolates derived from vegetation in this study had identical restriction fragment patterns and very similar morphology (unpubl. data).

Clade III is one of two clades previously identified to contain isolates corresponding to *S diclina sensu stricto* and including isolates from the Natterjack Toad (*Bufo calamita*) and the Western Spadefoot (*Pelobates cultripes*), but also containing isolates assigned to the species *S. australis* (Diéguez-Uribeondo et al. 2007). We obtained two isolates from *B. boreas* (BBE01 and BBE18) that also belong in this clade. One of these isolates, BBE01, was sexually sterile, but the other isolate, BBE18, was fertile and produced sexual structures consistent with assignment to the species *S. diclina sensu stricto*.

Clade VII included a number of unidentified isolates and an isolate identified as *S. diclina* that were part of a study on species boundaries in *Saprolegnia* (Hulvey et al. 2007). This clade also included a single isolate identified as *Leptolegnia* sp. and an isolate from *P. regilla*. Another isolate from *P. regilla* probably belonged to this clade based on the restriction fragment pattern. Neither of the isolates from amphibian eggs produced sexual structures in culture, however two additional isolates from vegetation with identical ITS sequences or restriction fragment patterns (unpubl. data) produced sexual structures. Morphologically, these isolates closely resembled the description of *S. kauffmaniana* (Pieters 1915), but he incompletely described this species and it may be a synonym of *S. parasitica* or *S. diclina* (Seymour 1970; Johnson, Jr., et al. 2002).

Clade VIII contained isolates from *B. boreas* eggs and sequences from GenBank that corresponded to the species *S. anisospora*, *S. eccentrica*, and *Pythiopsis humphreyana*. All of the non-sterile isolates in this study corresponded morphologically to *S. anisopora*, and not to *S. eccentrica* or *P. humphreyana*.

Clade IX contained isolates from all three amphibian species (*Saprolegnia* sp. 1) and a single isolate identified as *S. parasitica* (CBS540.67). Isolates from this clade often failed to reproduce sexually, but most produced

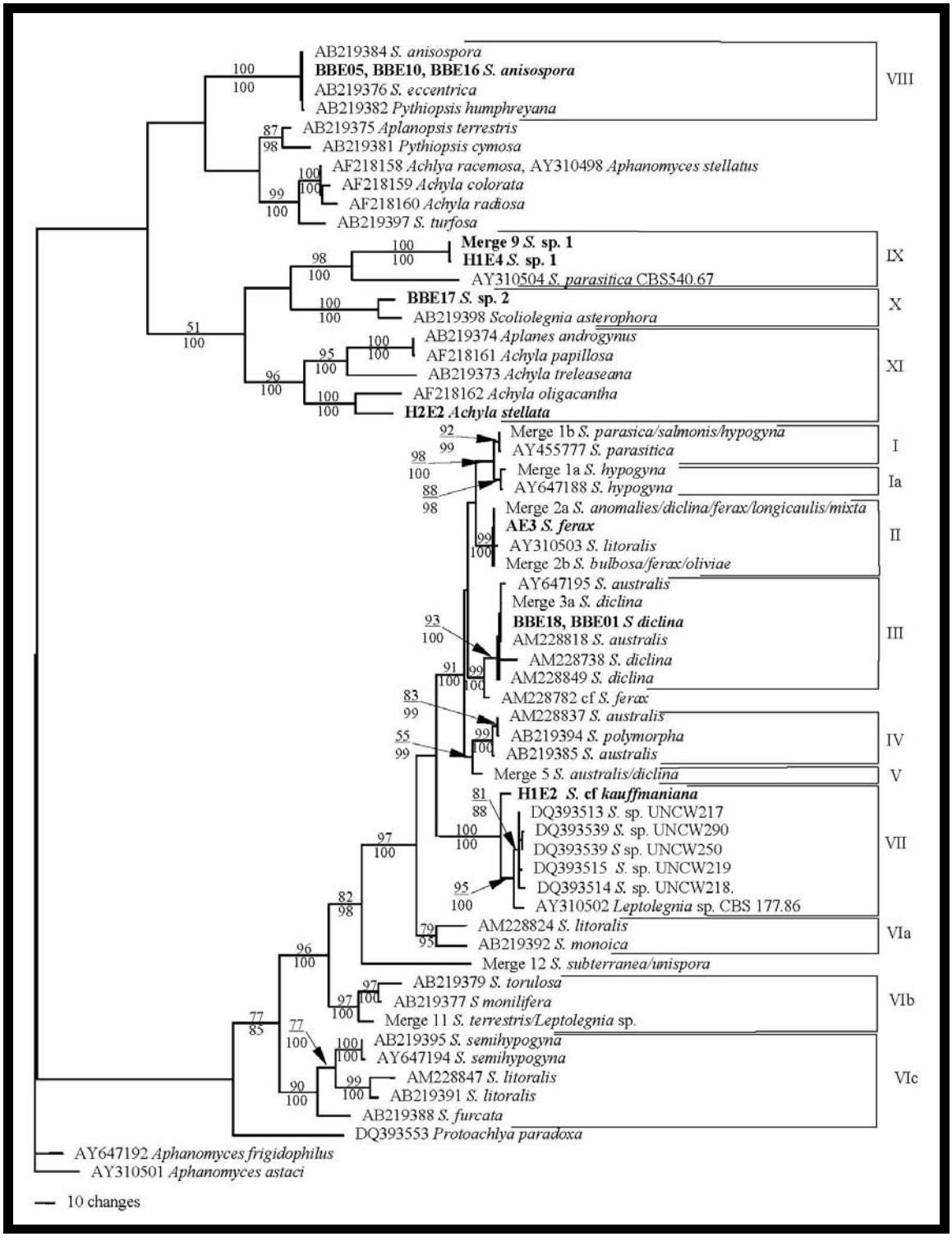

FIGURE 2. Phylogenetic tree from maximum-likelihood analysis. Parsimony bootstrap values are given above branches and Bayesian posterior probabilities are given below branches. Support values are included only in cases where the bootstrap value was greater than 70% or the posterior probability was greater than 90%. Clades are discussed in the text.

asexual reproductive structures. The zoosporangia were typical *Saprolegnia* type zoosporangia with saprolegnioid zoospore discharge. A single isolate, BBE15, produced sexual reproductive structures and corresponded most closely to the morphological description of *S. diclina*. The isolate was not morphologically distinct for any of the characteristics usually assessed as part of the traditional taxonomy from the isolate BBE18 found in Clade III.

Clade X contained a single isolate from *B. boreas* eggs (BBE17) and a GenBank sequence identified as *Scoliolegnia asterophora*. Isolate BBE17 was fertile, but lacked any of the distinguishing characteristics typical of the genus *Scoliolegnia* such as an ornamented and unpitted oogonium, 1-2 oospores per oogonium, or flaccid hyphae (Steciow et al. 2005), and it should therefore be considered a species of *Saprolegnia*. Due to the abundance of oogonia either lacking an antheridial apparatus or having monoclinous or hypogynous antheridia, traditional morphological taxonomy assigned the isolate to *S. ferax* despite having relatively few (1-8) oospores per oogonium (Seymour 1970; Johnson, Jr., et al. 2002).

Clade XI contained a single isolate from *P. regilla* eggs (H2E2) and several species of the genus *Achlya* and *Aplanes androgyna* (see Table 2). Morphologically the isolate corresponded most closely to the species *Achlya stellata*.

DISCUSSION

Analysis of the phylogeny and diversity of organisms recovered from amphibian eggs points to several important conclusions about this disease. We recovered multiple species of water molds from the eggs of all three species of amphibians, which is consistent with previous research in Europe (Czeczuga et al. 1998). This included three species (*S. anisospora*, *S. diclina*, and *S. ferax*) that parasitize fish eggs (Johnson, Jr., et al. 2002). This suggests that these species (and potentially the others) also parasitize amphibian eggs and that saprolegniasis is a complex syndrome simultaneously involving multiple pathogens. Despite the limited sampling in this study, it is worth noting that *S. anisospora* was the most commonly isolated species occurring on *B. boreas* eggs, but it was not isolated from either of the other two species. This suggests that there might be some host specificity involved. Fish eggs have defenses against water molds (Paxton and Willoughby 2000), and *Saprolegnia* isolates invade killed amphibian eggs more easily than living eggs (Robinson et al. 2003), which suggests that amphibians also have defenses against water mold infection. *Saprolegnia* strains also have different levels of virulence against the eggs of different species of amphibians (Robinson et al. 2003). These data suggest a need for further investigations into host specificity of water molds associated with amphibians.

A DNA barcoding system requires that target DNA sequences vary less within species than between species and that there is a well-populated database of identified sequences available. Examination of these data indicates that ITS is a suitable region for DNA barcoding based on the first set of criteria, but suitability based on the second set of criteria is somewhat questionable. Sequence variation was generally very low within terminal clades, and higher between them, so identification of most species should be possible. This low intraspecific variation suggests species of *Saprolegnia* may be primarily clonal despite the apparently functioning sexual apparatus (Diéguez-Uribeondo et al. 2007). These analyses generally place sequences of isolates from amphibian eggs within clades that have strong bootstrap support. Sometimes, these data combined with morphological characters allowed us to assign a species name. However, the analyses also indicated that the traditional morphospecies *S. ferax* and *S. diclina* may contain multiple cryptic phylogenetic species and that DNA barcoding provides insight into this problem. Because the database of identified sequences contains isolates with taxonomic issues such as synonymous names, cryptic species, and probable misidentifications (see below), it was difficult to assign species names to new isolates using a barcoding procedure.

One of the main difficulties with any DNA-based identification procedure is the quality of the comparative data on which identifications depend. Recent studies suggest that up to 20% of the sequences in public databases such as GenBank may be misidentified (Bridge et al. 2003). While a common source of error is simple mislabeling or misidentification (Bridge et al. 2003), another important source of error is disagreement among taxonomists or the use of plesiomorphic or homoplastic characters in the delimitation of species based on morphology (Vilgalys 2003; Diéguez-Uribeondo et al. 2007). In this dataset there are a number of probable examples of both types of misidentification. This necessitates a concerted effort to obtain good quality sequence data for type specimens in herbaria and culture collections that can provide better resolution of both misidentifications and taxonomic issues (Nilsson et al. 2006).

These data support previous conclusions that sexual reproductive anatomy does not reflect the apparent phylogenetic relationships between isolates based on nuclear ribosomal RNA genes and that morphological identification of water mold isolates may be problematic (Diéguez-Uribeondo et al. 2007; Hulvey et al. 2007). This problem results in the same species name being assigned to isolates in different clades (e.g., six named species occur in multiple, separate, well-supported

clades); as well as, clades that contain a large number of different morphospecies (e.g., 11 clades contain more than one named species with less than 2% pair-wise sequence distance (K2P) between isolates). Within the fungi, traditional morphological species concepts routinely underestimate the number of reproductively isolated or phylogenetically distinct lineages (Taylor et al. 2000), but because single gene trees may also differ from species trees, gene trees from multiple independent genes are preferred for estimating organismal phylogeny (Avise and Ball 1990; Taylor et al. 2000). A number of authors have recommended an operational species concept using multiple gene phylogenies as best option for a species concept in this type of organism (O'Donnell et al. 1998; Geiser et al. 1998; Taylor et al. 2000). The Genealogical Concordance Species Concept (Avise and Ball 1990; Taylor et al. 2000) recognizes species by the concordance between the phylogenies of multiple independent genes and may be the best available operational species concept for groups such as this. However, this type species concept requires large amounts of data to identify species boundaries, which are currently unavailable for this group, and will necessitate a major systematic revision of the group. This level of information will also provide the opportunity to evaluate fully the effectiveness any DNA barcoding procedure using a single gene to attempt to recover the organismal phylogeny.

A number of previous studies have designated short sequences that are unique to particular species within larger DNA barcode sequences (e.g., Summerbell et al. 2005; El Karkouri et al. 2007). These microcodes reduce the DNA barcode to just a few nucleotides that are useful in developing oligonucleotide probes or PCR primers for individual species, but these microcode sequences encode less phylogenetic information than a full-length sequence of the ITS (Summerbell et al. 2005). Because both this study and previous studies are still uncovering new clades, which potentially represent new species (Diéguez-Uribeondo et al. 2007; Hulvey et al. 2007), we do not recommend the designation of microcode sequences, which may become obsolete as new clades are discovered. Instead, we recommend the use of the full-length ITS sequences as DNA barcodes in order to maximize information content and identification precision while the diversity of these organisms is still being explored.

The ITS region offers several advantages over the CO1 region for barcoding of fungus-like organisms. The ITS region has fairly well populated database, despite its limitations, and primers are available that work on nearly all taxa. The primers used for this study were originally designed for the true fungi (White et al. 1990) and were effective in all strains of water mold tested. The small number of identified sequences in public databases, the presence of introns in fungal mitochondrial genomes, and the large number of primers required to amplify different groups of organisms hampered recent efforts to adopt the CO1 region for use in fungal bar coding (Seifert et al. 2007; John W. Taylor, pers. comm.). Primers for the CO1 gene must be specifically designed for the group of organisms under study and relatively closely related organisms may require a fairly large number of primers (Ward et al. 2005). On the other hand, primers are available for the rDNA region, including the ITS, that will amplify nearly all organisms even previously undescribed groups (Schadt et al. 2003). Despite these limitations, barcoding using CO1 should be evaluated for the water molds since the sequences of the CO1 for the few members of the Oomycota that are available lack introns and would be much easier to align and analyze (Seifert et al. 2007). CO1 barcodes combined with the ITS barcodes and other gene sequences provide a multiple gene phylogeny upon which to evaluate the species boundaries in clades that contain multiple named species, but only a single ITS barcode.

Finally, we recommend that ITS DNA barcodes should be obtained for as many amphibian pathogens as possible including those utilized in both previous and new ecological studies. These data are necessary because the phylogenetic relationships between and the taxonomic status of described species are unclear (Diéguez-Uribeondo et al. 2007; Hulvey et al. 2007), and a DNA barcode provides identification information even in situations where the taxonomy is unstable or incompletely known (Hebert et al. 2004a). Future publications should reference both a species name (based on a traditional morphological identification); as well as, a GenBank accession number for the barcode sequence. These data will provide the information necessary to identify most species of parasite and eventually assign appropriate species names. Even in cases of terminal clades containing multiple named species and possibly multiple phylogenetic species but a single barcode sequence, an ITS barcode would allow identification to a small group of closely related and genetically similar species. Even this somewhat ambiguous identification would be better than a condition where strains are only identified to genus or assigned to morphospecies that contain a number of distinct phylogenetic species. Adoption of a DNA barcoding system would allow the construction of a database of ecological information and pathogen identifications that would, in turn, provide the community with a better foundation for studies of pathogenicity and host specificity for all species of these pathogens and amphibians. This could then allow for a better understanding of disease process, ecology, and ultimately amphibian decline and conservation issues.

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